Biophysics of recellularized porcine cardiac extracellular matrix for myocardial tissue engineering

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NANYANG
TECHNOLOGICAL
UNIVERSITY

**BIOPHYSICS OF
RECELLULARIZED PORCINE CARDIAC
EXTRACELLULAR MATRIX
FOR MYOCARDIAL TISSUE ENGINEERING**

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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2015

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RECELLULARIZED PORCINE CARDIAC
EXTRACELLULAR MATRIX
FOR MYOCARDIAL TISSUE ENGINEERING

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Nomenclature

t: Time

σ : Stress

ϵ : Strain

E: Elastic modulus

η : Viscosity modulus

T_t : Transition temperature

T_m : Melting temperature

p: Significance value under statistical t-test or ANOVA

n: Sample size for statistics

S.D.: Standard deviation

Abbreviations

ECM: Extracellular matrix

pcECM: porcine cardiac extracellular matrix

MTE: Myocardial tissue engineering

CHD: Coronary heart diseases

LV: Left ventricle

CM: Cardiomyocytes

MSC: mesenchymal stem cells

hBM-MSC: Human bone marrow mesenchymal stem cells

EC: endothelial cells

FB: fibroblasts

SMC: Smooth muscle cells

TC Plate: Tissue Culture Plate

α MEM: Minimum Essential Medium Eagle Culture Media (Alpha Modification)

FBS: Fetal Bovine Serum

ABAM: Antibiotic Antimycotic

bFGF: Basic human Fibroblast Growth Factor

PBS: Phosphate Buffered Saline

FITC-BSA: Bovine serum albumin labeled with fluorescein isothiocyanate

PFA: Paraformaldehyde, reagent grade, crystalline

Dil: 1, 1-diotadecyl-3, 3, 3, 3-tetramethylindocarbocyanine perchlorate

PTA: Phosphotungstic acid

DDW: Double distilled water

FTIR- ATR: Fourier transform infrared spectroscopy analysis-Attenuated total reflection

SEM: Scanning Electron Microscopy

TGA: Thermogravimetric Analysis

DSC: Differential Scanning Calorimetry

SRT: Single relaxation time

MRT: Multiple relaxation time

PC: Polycarbonate

PE: Polyethylene

Stat: Reseeded construct in static culture

Dynm: Reseeded construct subjected to dynamic culture right after cell attachment

Stat-Dynm: Reseeded construct subjected to dynamic culture after a period of static culture

ANOVA: Analysis of variance

Abstract

Myocardial Infarction (MI) causes severe damage to cardiac muscles and remains the leading cause for congestive heart failure. Donations' shortage for heart transplantations and limited benefits from conventional medications and surgeries have motivated researchers to find alternative ways to restore heart function post-MI, where a large scale of myocardium is scarred and no longer contractile.

Myocardial Tissue Engineering (MTE) using scaffolds with or without cell seeding offers potential for *in-vitro* engineering of a myocardium-substitute construct for transplantation. Yet, many potential scaffold materials studied fail to give a physiological-relevant thickness as compared to native myocardium in their intact form (10-15mm). Most of them also fail to provide matched modulus and flexibility that may hinder the proper mechanical functioning of the construct. Moreover, they are mostly lacking the optimal infrastructure resembling that of native myocardium to support desired cell engraftment.

Our lab has developed and patented the decellularization of porcine left ventricle wall, yielding acellular porcine cardiac extracellular matrices (ECM), which are of surgical applicable transplant dimensions. These acellular cardiac matrices retained major ECM proteins and preserved inherent vasculature to address the above-mentioned limitations of existing biomaterials.

In this PhD research, the ECM's potential as a scaffold for cardiac restoration therapy was further investigated from the material engineering point of view. We aim to experimentally show that improved biophysical functionality of reseeded ECM-based cardio-mimetic constructs can be achieved through optimized culturing methods; and to

rationalize the results with computational modeling and material biophysical properties correlations.

Characterisation on mechanical, thermal, surface conformational, protein adsorption kinetic, and electrical properties was performed. Strong focus was put on the biomechanical properties as it crucially determines a scaffold's functional compatibility with the host tissue upon implantation. Sample mounting, experimental methods (in both uniaxial and biaxial tensile settings) and computational viscoelastic analytic tool were particularly developed for this purpose to test our unique thick and soft ECM.

To provide a basis for evaluation, native adult porcine myocardium, which possesses the closest-to-human anatomy, was thoroughly characterized in the same fashion. This allowed us to identify the similarities and discrepancies between ECM and native myocardium. The effect of recellularization towards a more native-mimicking construct was then evaluated in terms of cell proliferation and the recovery of the native-like biophysical properties. Four different seeding methods and three different culturing regimes in static and dynamic conditions were employed.

The results from this research thesis supports our hypothesis that the acellular ECM scaffold alone, and more so when reseeded with human bone-marrow derived mesenchymal stem cells (MSC), possesses biophysical properties that are close to those of the native myocardium. Its potential to be a suitable cardio-mimetic construct for myocardial tissue engineering was reaffirmed, supporting our vision to step forward in putting the ECM scaffold as an off-the-shelf biomedical product for diseased myocardium replacement and cardiac regeneration therapy.

Chapter 1. Introduction

This chapter introduces the background of this research project motivated by the problems associated with heart diseases termed myocardial infarction (MI), commonly known as heart attack. Its pathology and current treatments are covered in section 1.1 that led us to the idea of building an artificial myocardium-substitute to replace the diseased tissue. Facts revolving the myocardial tissue are presented in section 1.2 for a better understanding of its physiological properties. Section 1.3 explains the current strategies in the evolving research field of Myocardial Tissue Engineering (MTE), which utilizes a combination of cells, biomaterials and biomimetic platform to build artificial myocardium-substitute. A new class of biomaterial named 'porcine cardiac extracellular matrix (pcECM)' is introduced including the one that has been developed in our lab in section 1.3.4. Finally, section 1.4 discusses the knowledge gaps to be addressed about our ECM in order to develop it towards native-mimicking properties; and shape the scope of this project to be stated in details in Chapter 2.

1.1 Motivation

This research project was motivated by the many socio-economic and patho-physiologic problems associated with heart disease, especially following myocardial infarction (MI), commonly known as heart attack. Following MI, heart muscles are impaired. In this introductory section, general statistics on heart diseases globally, in the United States, and specifically in Singapore are presented. The pathology and current treatments to MI are covered. Limitations of the current treatments are then highlighted, substantiating the need to engineer an artificial myocardium-substitute to replace the impaired tissue.

1.1.1 Myocardial Infarction

Despite advancement in diagnosis and treatment, cardiovascular diseases (CVD) remain a major cause of mortality (31%) and morbidity (10% of global disease burden) worldwide.^[1] Among all kinds of CVD, coronary heart diseases (CHD) leads to the largest portion of deaths (40%) and disability (41%). The 2011 update report from the American Heart Association shows that CHD account for one of every six deaths in the United States, with the most prevalent type being myocardial infarction (MI), which is leading cause for heart failure and death. Half of MI survivors experience recurrent attacks that often lead to the complication of progressive heart failure, associating with a large sum of medical care of 177.5 billion USD annually.^[2-4]

While developed countries like the U.S. has observed huge decline (-27.8% over last decade) in the mortality rate from CHD, it is projected by the World Health Organization that developing countries in Asia will accommodate the largest increases, with the estimation of more than 60% of the CHD-related global burden.^[5,6] Being one of the more developed Asian countries, Singapore was associated with 18.7% of mortality from CHD

and 3.7% of hospital admissions due to CHD causes in 2010, one of the highest rates in the Asia-Pacific region ^[5,6]

1.1.2 Pathology

Owing to different risk factors, fatty deposits (e.g. cholesterol) accumulate inside the lumen of coronary arteries and atherosclerotic plaques are formed that could enlarge and bulge over time, narrowing and hardening the vessel lumen.^[1,2,4,7] In the case of MI, the unstable plaque suddenly ruptures and triggers the formation of thrombi (blood clots) that block the blood supply to cardiac muscle and cardiomyocytes, eventually leading to their death from ischemia and hypoxia. ^[3,4,7-12]

Left ventricle is more susceptible to damage due to MI among different parts of the heart, owing to its high logical demands.^[13] MIs that occur in the left ventricle are possibly followed by ventricular remodeling in which infarct expands and collagenous fibrous scar forms, resulting in ventricle dilation reaching a spherical shape with decreased efficiency in electrical pulses transmission and contraction.^[8,14-16] Unfortunately, CMs and resident cardiac stem cells lack sufficient abilities to regenerate the myocardium to remedy the cell and tissue loss,^[3,17] thus the impairment of the heart muscle wall often lead to heart failure progressively.^[3,7-12]

1.1.3 Current treatments and limitations

Patient care qualities of most HF cases have been improved by conventional treatments including pharmacological therapies, cardiac surgical procedures, and implantable medical devices. However, not only that they come at high cost, they lack the ability to induce myocardium regeneration and thus offer limited benefits for the severe CHF patient group, just slightly prolonging life expectancy by 2-3 years.^[1-3,9,12,18-19]

The definite treatment for end-stage CHF remains heart transplantation, which is still limited due to donor shortage and immune rejection.^[3,9,12,19] Surgical ventricular reconstruction (SVR/Dor procedure) is another option for severe CHF patients.^[9,19,20] During surgery, an incision is made into the LV to excise the infarct, followed by sutures closing. If the removed infarct size is too large, an endocardial or pericardial restrainer of Dacron or Teflon may be placed to re-establish ventricular wall continuity.^[20,21] Although this technique helps to reshape the LV, the weakened tissue is still not repaired and its functionality is not restored.^[19,20]

Stem cell therapy has been a recent clinical approach in which HF patients receive intracoronary transplantation of autologous bone-marrow-derived mesenchymal stem cells by standard cardiac catheterization.^[7,12,22] Most patients experienced reduction in re-infarction and improved ventricular function,^[7,12] yet the therapeutic outcomes are often minimized owing to the limited retention and survival of injected cells (< 10%), mainly because of cell leakage during implantation, and the inability of the inflamed myocardium to provide required nutrition to retained cells.^[3,4,12,23]

In conclusion, to effectively treat post-MI CHF, a myocardium-substitute should be fabricated to replace the diseased transmural infarct and foster tissue regeneration. The use of stem cells may be necessary for tissue repairing and the substitute should ideally resemble the native myocardium for optimal integration and functionality.

1.2 Myocardial Tissue

Thorough understanding of the myocardial tissue is required in order to develop tissue replacements trying to resemble the native myocardium as close as possible. The

following sections cover the working physiology; structure and composition; mechanical and electrical properties of a healthy left ventricular myocardium.

1.2.1 Physiology

Left ventricular (LV) myocardium is the muscle tissue responsible for contracting and pumping oxygenated blood to the whole human body. An illustration of the ventricular wall cross section is presented in Fig.1 showing the three major layers of the heart muscle namely pericardium, myocardium and endocardium.^[24]

Figure 1 Schematic of the ventricular wall cross-section.

For a 70 kg adult, it beats 60-70 times to pump about 5 liters of blood each minute.^[12,25,26]

Owing to the intense workload, the myocardium has a high metabolic demand for oxygen of about 27.6 nmol per mg of protein per min^[4] to be diffused throughout the tissue by a dense supporting vasculature, with capillary diameter of about 7 μ m and inter-capillary distances of about 20 μ m.^[11,18,26-28]

1.2.2 Structure and Composition

A human adult LV myocardium is a three-dimensional collagen based extracellular matrix (ECM) with approximate thickness of 15 mm and area of 50 cm², densely populated with cardiac cells (2×10^8 /cm³).^[3,11,26,28] Collagen I and III are the major collagen types, which form the structural continuum and provide rigidity and elasticity respectively to the ECM. Collagens IV, V, VI, VIII and elastin as well as adhesive proteins including fibronectin and laminin; together with hydrated proteoglycans and glycosaminoglycan are also identified within the ECM.^[3,12]

Cardiomyocytes (CMs), the actual contracting cardiac muscle cells, are the major cellular components (30% of the total cell number) in the myocardium.^[11,12] They averagely contract more than three billion times in a human's life span. They are made up of contractile fibril units called sarcomeres which consist of muscle protein filaments, myosin and actin; elongated and aligned into myofibers, which are striated in parallel with changing spatial orientations across the thickness of ventricle wall.^[4,11,12,29]

Apart from CMs, the rest 70% of cell numbers comprise of fibroblasts (FBs), smooth muscle cells (SMCs), endothelial cells (ECs), neural cells and leukocytes.^[11,12] For instance, FBs fill the gap among CMs to help transmitting mechanical signals while SMCs and ECs are mainly the vasculature components^[3,11,26].

1.2.3 Mechanical properties

A healthy myocardium is a soft and flexible material with reported moduli of 0.001-0.5 MPa, based on varied samples selections and testing methods.^[12,30-33] During systole, it deforms to a longitudinal stain of 10-15%^[3,11,34,35] to generate a contractile force of 2-4 mN/mm²^[3,28]. Alternatively, twitch force of 0.4-0.8 mN and tension of 0.1-0.3 mN

according to Vunjak-Novakovic *et al.* [4] Breaking strain of about 100% has been reported for rat myocardium.[3]

High mechanical stability and fatigue resistance are also important features of cardiac muscle, allowing it to bear the non-stop cyclic loads and withstand the systolic pressure. [3,11,34,35] Like many other biological tissues, it also possesses complicated viscoelasticity and anisotropic properties. These characteristics give significant impact on the mechanical functionality which is crucial for the proper heart functioning. [22,30,36,37]

1.2.4 Electrical properties

Electrical waves generated by specialized CMs located at the sinoatrial node are spread rapidly over the myocardium along membranes of adjacent CMs by intracellular connections named gap junctions.[4,22] Electrical impulses are propagated almost instantaneously at speed of approximately 25 cm/s^[28] and rate of 60-100 beats per minute (bpm) [4,11,22,28] with characteristic anisotropic conduction velocities which are higher in the direction coming from the node than the transverse direction.[22] Physiological voltage across myocardium has been reported in the range of 3-8V with the resistivity reported to be in the range of 200-600 Ωcm depending on the temperature & applied frequency setting in experiments.[38-42]

1.3 Myocardial Tissue Engineering (MTE)

The idea of fabricating myocardium-substitutes has given rise to the field of myocardial tissue engineering (MTE) which involves the use of cells and biomaterial scaffolds for the regeneration of heart tissue.[12,16,22,28,43] Different MTE approaches include injection of cells alone^[44]; injection of biomaterials alone^[45]; injection of both cells and biomaterials

(*in situ* MTE)^[46,47]; biomaterial wrap (left ventricle restraint)^[48,49]; and cell-seeded patch implantation (*in vitro* MTE)^[35,49-53]. Among these, *in vitro* MTE utilizing cell-seeded scaffolds represents the best prospect for creating implantable 3D constructs with controlled functionality. The construct should not only fill the tissue voids after infarct excision, but also provide mechanical support to the weakened myocardium. Moreover, by optimizing the construct culture environment, ingrowth of cells on the biomaterials can be facilitated, and such constructs possess high potential to promote reestablishment of healthy myocardium structure. ^[3,11,12,53]

This section covers the different cell sources and biomaterials that have been used in MTE; as well as biomimetic culture platforms which recapitulate *in-vivo* environment to give signals for tissue's growth and function.

1.3.1 Cell sources

Given that the cardiomyocytes (CMs), being terminally differentiated cells, have limited endogenous reproductive and regenerative capability, the efficacy of exogenous cell sources to proliferate and repopulate the MTE scaffolds; and their potential to become contractile cells are extensively explored. ^[17,54-57]

Human-body derived stem cells remain the top studied groups for the possibility of obtaining patient autologous cells, expanding *in vitro*, recellularizing the scaffolds and differentiate into CM-like cells. ^[3,58-60] there are several studies on embryonic stem cells (ESCs)^[61,62] and inducible pluripotent stem cells (iPSCs)^[63] as effective cell sources for CM and MTE due to their pluripotency. Yet, ESCs are still facing numerous ethical and practical concerns, including possibility of teratoma formation post implantations, while iPSCs are still a new cell programming technique in their infancy.^[60,64-67] Therefore, both cell types

are still under extensive examination prior to any possible FDA approval for clinical application.

Among the multipotent cell sources, human mesenchymal stem cells (hMSCs), most commonly derived from bone marrow or adipose tissues, have been of particular interest for their multipotency; ease of isolation and *in vitro* scalability to eventually provide patient-specific tissue constructs.^[12,18,54,66,68] They also possess cardiomyogenic differentiation capability into CM-like cells with some cardiac-markers expression through 5-azacytidine treatment by Yang *et al.*^[69] Furthermore, their paracrine actions and induced myocardium regeneration ability has also been demonstrated by intracoronary bone-marrow MSCs based therapy in phase-2 human clinical trials as mentioned earlier in section 1.1.3, showing their safety and feasibility in managing MI patients.

1.3.2 Current biomaterials used in MTE

A great variety of biomaterials have been studied for their performances as *in vitro* MTE scaffold, including synthetic biodegradable polymers such as Poly lactic acid (PLA)^[70], Polyglycolic acid (PGA)^[71], Polycaprolactone (PCL)^[72] and Polyurethane (PU)^[49,51]; and natural materials such as collagen^[47,73,74], gelatin^[75] and fibrin^[76]. For instances, Eschenhagen *et al.* created an epicardial heart patch by seeding embryonic chick heart cells on collagen.^[73] Xiang *et al.* used a collagen scaffolds as a delivery matrix for BM-MSCs and grafted it onto myocardial infarcts in a rat model.^[74] Caspi *et al.* formed a contracting engineered PLA-based cardiac tissue with human ESCs cocultured with CMs, ECs and FBs.^[70] Although these materials are widely available, well characterized and allow high extent of control over the materials' properties, most of them are yet to provide the complex extracellular skeleton and biological activity as of the native myocardium tissue. Hence, they lack the capability to support cell loading, survival and proliferation; as well

as fail to match the complicated mechanical characteristics with the native myocardium, which they are to replace. [3,11,12,18,22,77]

1.3.3 Cardiac extracellular matrix

Recent years, several research groups removed immunogenic cellular components from myocardium of rats or pigs to obtain the left-behind acellular cardiac extracellular matrix (ECM) as scaffolds. [58,78-80,86,115,119] Different decellularization protocols have been used and studied on several kinds of natural extracellular matrices by the different research groups. For instance, Ott et. al. used a retrograde coronary kind of perfusion to wash their rat heart with 10 μ M adenosine containing heparinized PBS, 1% SDS in deionized water, 1% Triton-X100 in deionized water, followed by antibiotic-containing PBS [78]. Wainwright et. al. decellularized intact adult porcine hearts using pulsatile retrograde aortic perfusion of several kinds of detergent and solutions including enzymatic, nonionic, ionic, acid, hypotonic and hypertonic [79]. Schulte et. al. studied the combinatorial effects of sodium dodecyl sulfate (SDS) and sodium hydroxide (NaOH) on the perfusion of decellularizing both the porcine myocardial extracellular matrix (ECM) and the vascular ECM [115]. Wang et. al. decellularized their 2-mm thick porcine myocardial sections with 0.1% sodium dodecyl sulfate [119].

The decellularized ECMs are highly origin-specific as they are secreted, modified and degraded by the resident cells at the site. Thus, decellularized myocardium appears to be the best “nature-made platforms” for optimal extracellular milieu with inherent bioactivity to instruct cell behaviors and induce regeneration. [4,18,77]

Although the advanced approach of using the origin-specific decellularized ECM scaffolds has shown superior native-tissue-mimicking capability over the general MTE biomaterials in current use, there are yet few hurdles to overcome. The first issue concerns whether

the decellularization protocol can effectively remove antigenic components that might trigger immunologic response.^[21,86,115] Secondly, the decellularization might lead to adverse alternation to the inherent composition, material properties and biological activity of the ECM.^[21,77,81] And lastly, the creation of a thick intact 3D myocardium-like structure remains challenging due to the maximum depth of oxygen diffusion (100-200 μm)^[4,11,26,28,115], hence viability of reseeded cells could only be maintained within a thin layer, far behind the clinically meaningful thickness for human LV transmural wall (10-15mm).

1.3.4 The pcECM

To address the above-stated challenges, our group has succeeded in decellularizing large and full thickness LV slabs of porcine origin yielding a 7-10mm thick porcine cardiac extracellular matrix (pcECM) of surface area 50cm². Swine samples were chosen because pigs and humans systems are close enough that their dimensions are relevant and the ECM extracted from swine organs is compatible with human body's cells.^[77,82-84] The decellularization method of thick ECM slabs is a modified version from the generation of thin ECM slabs (3mm) that had been WIPO patented by our Principal Investigator^[85], and previously published by Eitan *et al.*^[80] The modification was done in our lab in Technion Israel Institute of Technology by Dr. Udi Sarig. A paper was published in Tissue Engineering Part A. Briefly, the decellularization procedure consists of washing cycles including alternating hyper/hypotonic NaCl solutions, enzymatic agent Trypsin and non-ionic detergent tert-octylphenylpolyoxyethylen (commonly known as Triton-X-100) pumped through the left anterior descending coronary artery and perfused to the rest of the slab.

^[86] (*Scholarly Work in Appendix 22*)

Fig.2A shows an image of the dissected intact porcine left ventricle wall, whereas Fig. 2B shows an image of our decellularized left ventricle wall , i.e. thick porcine cardiac extracellular matrix.

Figure 2 Materials of interest in this thesis.

Native porcine left ventricle wall (A) and our thick ECM (B)

Importantly, the decellularized pcECM preserve largely their inherent vasculature upon decellularization.^[86,87] Therefore, by using the same perfusion method in the setting of a bioreactor, it is possible for oxygenated medium to supply the bulk as well as the reseeded cellular components. This could possibly give rise to a well-fed construct, which other scaffold materials without vascularisation could hardly provide so.

Proteomic study showed that the pcECM maintained important matrix proteins including collagen I, III, V, VI and fibronectin.^[80,86] Human umbilical vein endothelial cells HUVECs were seeded onto the lumen of vessels were found to form a monolayer lining to restore vasculature.^[87] In addition, they were non-immunogenic and supportive for the culture of ovine cardiac fibroblasts, rat cardiomyocytes and rat bone-marrow-derived mesenchymal stem cells. ^[80,86]

1.3.5 Biomimetic culture platforms

A bioreactor is a system that provides a controllable culture environment for cell and tissue growth. They provided controlled temperature, humidity, oxygen content and pH as an incubator. The advantages of bioreactor culture over static culture would be the provision of dynamic flow of culture medium, regulative factors and metabolite to resemble the *in-vivo* habitat of the native tissues. Enhanced oxygen and nutrients supply to the whole bulk of the scaffolds could be achieved, in particular the perfusion-type bioreactor.^[11,26,57] They also allow continuous monitoring of cell metabolism and proliferation. For the case of MTE, culturing of a highly recellularized thick tissue construct can hardly be achieved without the use of bioreactors.^[3,26,88]

More sophisticated designs of bioreactors with the introduction of electromechanical stimulation have been of increasing interest over recent years.^[26,89-91] Zimmermann *et al.* subjected rat neonatal CMs-seeded collagen I + Matrigel constructs (5mm) to uniaxial and cyclic stretch which resulted in morphological features and contractile properties comparable to native rat myocardium.^[35,92] Akhyari *et al.* also applied a mechanical stretch regime on gelatin scaffold seeded with human cardiac cells which led to increased cell growth and improved distribution compared to static samples.^[90] Applying electrical pacing signals could induce synchronous contractions.^[3,93-95] Radisic *et al.* and Tandon *et al.* stimulated rat neonatal CMs-seeded collagen constructs by applying pulsatile electrical fields, resulted in improved conductive and contractile properties probably due to establishment of intracellular gap junctions.^[13,93,96,97] Ott *et al.* stimulated their reseeded-decellularized rat heart and observed stroke work at day 8.^[98]

1.4 Remaining knowledge gaps and challenges

While our thick decellularized pcECM represents a good potential candidate for myocardial engineering, a few knowledge gaps and some challenges have to be overcome before their translation into clinical applications:

1. Mechanical properties of pcECM should be thoroughly evaluated
2. Surface and bulk biophysical properties of pcECM should be understood
3. Potential of human stem cells expansion on pcECM should be explored
4. Bioreactor should be used to enhance recellularization effectiveness on pcECM
5. Effect of recellularization on pcECM biomechanical and biophysical properties should be studied

The following sections 1.5-1.7 consist of in-depth literature reviews and experimental trials to address the above issues systematically, in the sequence of (Section 1.5) construct mechanics; (Section 1.6) construct surface and bulk biophysical properties; and (Section 1.7) Recellularization of construct. These sections shape the scope of this PhD project as detailed in Chapter 2, and highlight the rationale and significance of this thesis work.

1.5 Strategies addressing gap 1-construct mechanics

Mechanical performance is a crucial property of any scaffold intended to be implanted *in-vivo*. To properly function, scaffolds should have a guaranteed strength and mechanical stability; and a temporary structural and mechanical equivalence with the human tissue at the site of implantation.^[3,11,43,99,100] While much effort in the field of MTE has been directed to scaffold fabrication and the study of molecular cell biology, little focus has been put on assessing the mechanical properties of the engineered constructs, which

actually play a big role in tissue-implant interaction for any meaningful in-vivo study or clinical trial.^[101-103]

Soft biological tissues are complicated to characterize due to the difficulties in handling, mounting and clamping the thick slippery specimen. It is even more challenging for the case of thick tissues or construct like our ECM and its native counterpart. This might explain why there were very few attempts to test native porcine/human myocardium in full thickness, including its pericardium and endocardium. As discussed in section 1.2.3, even in existing literature, reported measurement can be differing in magnitudes due to variations in sample source and experimental set-up, making the results not comparable.

There is therefore a need to standardize methods that allow tissue engineers to test the mechanical properties of the soft biomaterials in full thickness on commonly-available tensile equipment in material science laboratories. A practical and clinically relevant thick sample mounting methods, a thorough mechanical testing assay, a non-biased analytic modeling tool that are user-friendly would be helpful to all tissue engineers when soft and thick biological tissues or scaffolds are concerned. The following sections consist of in-depth literature reviews and experimental trials on substrate biomechanics-related matters to address the four above-mentioned issues systematically, in the sequence of: 1.5.1) testing equipment; 1.5.2) sample mounting; 1.5.3) mechanical testing assay; 1.5.4) analytic modeling tool.

1.5.1 Testing equipment

In this section, we compare several commonly-available mechanical testing equipment in materials sciences laboratories. Numerous experimental trials were performed and got insight on the applicability of equipment and methods for our soft and thick biological materials.

Rheology is an emerging field of biological material characterization, most commonly tested on gels and pastes. It quantifies the viscoelasticity of materials over a range of time and deformation in order to attain short- and long-term tissue modulus.^[104] For instance, Yeung *et al.*^[105] tested their polyacrylamide gels on an RFS II fluid spectrometer [Rheometrics Inc.] and measured the dynamic shear moduli by placing 500 μ L of sample between two 25-mm stainless steel parallel plates. We tested our ECM using a rheometer with parallel plates [Anton Paar Rheometer] to perform oscillatory shear or stress-relaxation. Unfortunately, due to the gel like texture of the ECM, even with the use of a plastic column to confine the ECM in place, the two parallel plates failed to firmly press the wet ECM and perform oscillatory twist.

Atomic force microscopy (AFM) and nano-indentation are methods to obtain topographical imaging and at the same time micro-scale mechanical properties of materials or single molecule. They utilize an indenting tip that comes with different sizes and shapes, generally named as probe or cantilever by researchers, to vertically deform the substrate material. The force measured by the tip, after calibrating against the tip shape, tip stiffness, indentation and indentation depth will yield the moduli of the materials tested.^[106-109] These two methods have been of increasing interest in their biological applications due to their capability in producing precise mechanical measurements on biological samples including biomolecules, gels and even cells.^[108-110] The drawback of these methods is that the elasticity measured is of nano or at-most micro scale on surface of substrate. When it comes to thicker and larger samples, they can serve as complementary characterizations to conventional measurements, for instance, tensile testers.^[110]

Tensile stretching tests remain the most straightforward mechanical characterization method on the macro-mechanical properties of materials ranging from metals, ceramics, polymers, especially for large and thick specimens. Common testing assays include cyclic loading, stress relaxation and tensile failure. Uniaxial, bi-axial and even multi-axial testers are commercially available or are being custom-made by research groups. [80,111-114,119]

Hence, tensile testing seems to be the most relevant and applicable methods to characterize the macro-mechanical properties of our soft and thick biological material. Moreover, uniaxial testing alone is not sufficient to study the biomaterials, which possess anisotropic nonlinear behavior, and is loaded in all directions in physiological conditions.^[112,116-120] Hence, a biaxial testing method might need to be custom-developed to accommodate our thick and soft tissues.

1.5.2 Sample mounting

There are numerous custom designed sample mounting methods being developed by research groups to test their biological tissues^[112,121-123]. We tested a number of gripping methods on our available tensile testing machines, including screw side action grips, pneumatic action grips, the use of sand paper, and the use of cardboard. Unfortunately, most of them failed to impose sufficient gripping force to prevent the wet and thick specimens from slippage (Fig. 3A-B). In another case, some of the forced attachment method introduced intense local stress at the gripping region. The compression and alteration of the intact thickness, width and length of the samples may cause premature deformation and damage to specimen (Fig. 3C).

Figure 3 Examples of failed sample gripping.

Failed conventional screw side action grips and pneumatic action grips (A, B)
Forced gripping (C)

In conclusion, a practical and application-relevant sample mounting method should be developed and tested on our thick ECM-based construct, and such method may also benefit the testing of other soft biological materials.

1.5.3 Mechanical testing assay

The most commonly-obtained and compared mechanical measurements is the Young's modulus.^[124] Yet apart from modulus, it is also essential to study the viscoelasticity, anisotropy, toughness and failure behavior in order to reveal a complete mechanical profile of a biomaterial^[104,112,118,125,126]. Hence, one should also study the energy dissipation, stress relaxation, and stress-strain behavior of the biomaterials. Moreover, pre-cycling should be done prior to each test, in order to get reliable and repeatable results on viscoelastic biological materials to allow reorientation of macromolecular structures back to their natural alignment.^[86,124,127]

Nevertheless, mechanical tests are usually destructive. Seeing as tissue engineered biological constructs are costly and require investment of long preparation periods and labor, we believe that a thorough mechanical testing assay should allow researchers to

obtain the most information about the specimens' biomechanics tested within preferably one continuous experiment.

1.5.4 Mechanical analytic tools

Viscoelastic materials, including most biological materials, undergo stress relaxation where they relieve stresses in a nonlinear and non-Hookean fashion under constant strain. While obtaining large amounts of data on the stress–strain behavior of such biomaterials is straightforward, analyzing it in a uniform, comparable, and user-independent manner is more challenging.^[104,124]

Despite significant advancements in highly correlative mechanistic models ^[128-136] for stress–strain analysis, most literatures still rely on reporting the Young's modulus as the primary mechanical measure for biological tissues. However, as tissues are subjected to many non-elastic deformation modes and their responses may differ significantly from ideal spring behavior, measuring only this characteristic can be misleading.^[124] In order to design appropriate tissue replacements, an effort must be made to measure and match the viscoelastic properties of tissues more comprehensively.

Therefore, we aimed to develop a non-biased analytic modeling tool, which may allow researchers to utilize the most commonly used standard equipment in material science laboratories for reliable tensile and viscoelastic properties, without the hassle for extensive programme or sophisticated mechanistic model development.

In our opinion, an ideal viscoelastic model for comparison of biomaterials should not be too complicated to the extent that it may hinder its ease of usage, its users' understanding and the results yielded from it. At the same time, the model has to provide sufficiently high correlation to experimental data.

Single-relaxation time (SRT) models have been identified provide more information regarding material viscosity and elasticity in comparison to the ideal spring elastic model.^[124] The two most basic single-relaxation time (SRT) models are the Maxwell and Kelvin-Voigt models (Fig. 4), which are simple serial or parallel combinations of springs and dampers^[137].

Figure 4 Schematic illustration of Maxwell and Kelvin-Voigt viscoelastic model.

E corresponds to the spring elastic modulus while η corresponds to the dashpot/damper viscosity modulus.

We also studied the Zener model, which can be represented as a spring serially connected to a Kelvin-Voigt solid^[124] as illustrated in Fig. 5.

Figure 5 Schematic illustration of the Zener viscoelastic solid model.

The elastic moduli E_1 and E_2 correspond to the spring components while η corresponds to the viscosity modulus of the dashpot/damper.

Fig. 6 shows the typical mechanical behaviors of viscoelastic solids under tensile-relaxation test that.^[124] This kind of SRT models, by setting varying boundary conditions, may accommodate several mechanical testing modes or response regions, which are

depicted in a single time-continuous stress–strain curve. Such combinations of several modes and/or regions are not only common in many experiments but also better resemble the physiological conditions in which these biomaterials operate.^[123,124,138]

Figure 6 Typical mechanical behaviors of viscoelastic solids under tensile-relaxation.

Exhibiting two modes: tensile mode defined as the linear stress strain region (constant strain-rate); and stress relaxation mode defined as the period from where the material is held at a constant strain to allow force decay (constant strain).

SRT models are expected to correlate to a large extent with empirical data and may provide better assessments of viscoelastic properties than Young's modulus alone, with an advantage of avoiding the information redundancy commonly incurred by those highly correlative mechanistic models such as multiple relaxation time models.^[111,139,140]

SRT models may also facilitate the analysis of soft biomaterials as a bundle of strings instead of monolithic solids^[124]. Each string is having the same elastic moduli and viscosity but with different failure points, thereby enabling the modeling of such solids beyond their apparent linear viscoelastic region (LV region) and into their failure region as illustrated in Fig. 7.^[124] Such analysis, producing new measures for elasticity and integrity

failure, and providing better measures of toughness and strength, is crucial for evaluating and comparing biomaterials under harsh conditions and determining their applicability for regenerative medicine.

Figure 7 Typical mechanical behaviors of viscoelastic solids under tensile-failure.

Elastic limit is defined as the end of linear viscoelastic region (LV region) for Zener and Maxwell models, followed by a non-elastic region after the elasticity-loss. Integrity limit is defined as the maximum stress reached before rupture in the failure region for all three models.

In common analysis, normalized data (e.g., moduli, compliance) can be used in simple models for comparing the viscoelastic behavior of different samples. Such data, readily attained for many synthetic materials, is much harder to come by when dealing with soft amorphous and heterogeneous biological tissues exhibiting complex behaviors under different testing modes. In cases when several mechanical testing modes or response regions (under a single stress-strain curve) are being simultaneously analyzed, their solutions frequently require researchers to resort to complex and elaborate models and mathematical transformations, possibly further obscuring their physical meaning. Additionally, the full potential of these models is seldom exploited, as they require the use

of advanced mathematics, and considerable programming abilities as well as computational capacities, which exceed that of common spreadsheet software and users.

[111,139,140]

Accordingly, due to the lack of efficient but simple tools to implement these models, most data collected from stress–strain experiments remain un-analyzed and the significance of the measured properties of tested biomaterials is not thoroughly interpreted. Hence, we set forth to develop an efficient algorithm accompanied by an easy-to-use MATLAB® code aimed at elucidating the viscoelastic parameters and determining the failure profiles of materials tested under experiments spanning various mechanical testing modes and/or stress–strain response regions.

1.6 Strategies addressing gap 2-construct surface and bulk properties

Given that there are very few publications, which characterize decellularized biological tissues as well as native myocardial tissues, several critical surface and bulk properties should be studied in order to reveal a complete material profile of our novel pcECM. It is also important to compare our pcECM to native in order to evaluate the possible alternation of properties caused by the decellularization process.

1.6.1 Surface properties

Surface characteristics of substrates have been substantially discussed in literatures for their role in regulating cell attachment, proliferation and even cell fate determination. For instance, topography^[141-143], pore size and roughness^[60,144-147], chemical composition and functional groups^[21,105,148,149], and protein adsorption kinetics^[144,145,150-152] govern the interaction of living cells with surfaces. These properties could be evaluated qualitatively

by microscopy, and quantitatively by following protein adsorption kinetics and by spectroscopy. In our opinion, FTIR stands out from other relevant methods such as X-ray crystallography, nuclear magnetic resonance spectroscopy and Raman spectroscopy for its simple sample preparation, amenability for wide range of proteins, and straightforward interpretation of spectra. Since biological samples are mostly in wet conditions, most FTIR system also allows subtraction of the omnipresent water absorption by mathematical approaches. Garidel *et al.* published a technical article discussing the superior capability of infrared spectroscopy analysis in mid-IR range on proteins.^[153]

1.6.2 Bulk properties

The mechanical properties of collagen-based materials depend critically on certain bulk material properties such as their hydration level and the thermal stability.^[21,154,155] For instance, water content influences the structure, mechanical behaviors and chemical stability of the hydrated scaffolds;^[156-159] while thermal stability reflects the compositions and structures of proteins in the materials and their denaturation behaviors.^[160-164] Having collagen being the dominant protein composes our ECM, their bulk biophysical properties should be quantitatively measured in absolute temperature-controlled equipment such as scanning calorimetry, and thermogravimetric analysis. Transition temperature, melting temperature, degradation profiles and hydration content of the materials should be correlated to the mechanical properties of the material. In addition, as discussed in section 1.2.4 about the electrical properties of native myocardium, it might be also interesting to characterize the ECM's inherent conductivity.

1.7 Strategies addressing gap 3 to 5-recellularization of construct

The majority of the remaining challenges in MTE remain in the context of construct recellularization to a native-resembling form for possible implantation and integration with patient host cardiac tissues. Therefore, in order to put forward our ECM construct as a potential biomedical implant device, we have to optimize the seeding methods of selected cell sources and the construct culturing methods. As discussed in section 1.3.1, the potential of human stem cells to expand on the construct should be studied for the goal of obtaining clinically meaningful cell number, in particular human bone-marrow derived mesenchymal stem cells (MSC) being the CM model cells. The effect of recellularization on construct biophysical properties can then be studied in details compared with their unseeded counterparts and native porcine myocardium to evaluate the capability of the reseeded scaffold in cell-interacting and material properties discrepancies bridging. The use of a bioreactor has shown to improve the culture of the reseeded construct^[11,26,57] as discussed in section 1.3.5 and thus should also be addressed in this thesis in terms of cell proliferation and the recovery of material properties towards a more native-resembling biomaterial.

Chapter 2. Scope of Project

This chapter addresses the method of research used in this thesis work termed the PEL model, which combines presuppositions (P), evidence (E), logic (L) to support scientific knowledge advances ^[165]. Section 2.1 states the hypothesis, which is the presupposition (P) at the beginning of the project. Section 2.1 states the overall goal to define what evidence (E) and logic (L) were being attempted to obtain in this thesis work. Three aims, which were pursued in order to achieve the overall goal, are listed in section 2.3 to define the scope of work on what was, and what was not, being attempted in this project.

2.1 Hypothesis

We hypothesize that an acellular porcine cardiac extracellular matrix scaffold, when reseeded with human bone-marrow-derived mesenchymal stem cells and then cultured under optimized dynamic culture condition, can possess biophysics comparable to native myocardium in terms of mechanical, surface conformational, and thermal properties, and hence be a suitable cardio-mimetic construct for myocardial tissue engineering.

2.2 Goal

The overall goal of this project is to develop appropriate approaches to experimentally show improved biophysical functionality (in particular mechanical properties) of the reseeded ECM-based cardio-mimetic constructs through optimized culturing methods; and to rationalize the results with computational modeling and correlation among the biophysical properties, in comparison to acellular ECM and native porcine myocardium.

2.3 Aims

Aim 1: To study the mechanical properties of the ECM in comparison to native myocardium

This aim addresses the knowledge gaps and challenges stated in section 1.4.1 and 1.4.2. Comprehensive mechanical characterization was conducted on our unique ECM with our customized sample mounting methods and testing protocols, both uniaxially and biaxially. Detailed comparison with native porcine myocardium was performed. A computational analytic tool was developed for modeling experimental results and rationalizes the viscoelastic behaviors of the materials.

Aim 2: To study the surface and bulk properties of the ECM in comparison to native myocardium, and correlate the results to the established mechanical properties obtained in Aim 1.

This aim addresses the knowledge gaps and challenges stated in section 1.4.3. In order to reveal a complete material profile of our unique ECM, several crucial surface and bulk properties were investigated. The adsorption kinetics of a model serum protein onto the ECM was followed. Surface functional groups were revealed by Fourier transform infrared spectroscopy analysis (FTIR). Structure visualization was conducted by scanning electron microscopy (SEM) with the use of WETSEM™ technology. Hydration content was evaluated by thermo galvanometric analysis (TGA). Thermal property was studied by differential scanning calorimetry (DSC).

Aim 3: To evaluate the effect of cell seeding and culture methods on the material properties of recellularized ECM construct, in comparison to acellular ECM and native myocardium as tested in Aim 1 and 2

This aim addresses the knowledge gaps and challenges stated in section 1.4.3. The ECM was recellularized by human bone-marrow derived mesenchymal stem cells (MSCs) with different seeding and culturing methods, both statically and dynamically in our custom-developed bioreactor. The impact of cells on ECM towards a native-myocardium-mimetic construct was studied in terms of cell viability and proliferation, as well the material properties concerned in Aim 1 and 2. Finally, an electrical characterization was performed to obtain preliminary insight of the effect of cell presence on the conductivity of the pcECM construct.

Chapter 3. Materials and Methods

This chapter details the materials, reagents and equipment used; as well as the experimental methods and computational methods employed in this thesis work. The orders of appearance of sections follow the chronological sequences of the experiments performed. Section 3.1 and 3.2 covers our materials under investigation: ECM and native tissue respectively. Section 3.3-3.5 details the experimental methods and computational methods employed for Aim 1 to study the mechanical properties. Section 3.6 details all the equipment used and experimental/analytical methods employed for Aim 2 to study the surface and bulk biophysical properties. Section 3.7-3.9 details the recellularization strategies used to repopulate the acellular pcECM with human bone marrow derived mesenchymal stem cells (MSCs). The recellularized samples were tested as described in sections 3.3-3.6 for comparison to address Aim 3, except the biaxial mechanical testing was conducted with a more advanced set up and analytic method developed in collaboration with Israel Ben Gurion University as detailed in section 3.10. Finally section 3.11 states the statistical analysis performed in this thesis work.

3.1 ECM

As previously introduced in section 1.3.4, decellularized porcine cardiac extracellular matrix (ECM) were prepared in the lab supervised by our Principal Investigator at Technion-Israel Institute of Technology; and then shipped to Singapore in 0.1% Sodium-Azide in phosphate buffered saline for subsequent testing. Briefly, the decellularization procedure consists of washing cycles including alternating hyper/hypotonic NaCl solutions, enzymatic agent Trypsin and non-ionic detergent tert-octylphenylpolyoxyethylen (commonly known as Triton-X-100) pumped through the left anterior descending coronary artery and perfused to the rest of the slab. ^[86] Different sizes of samples were cut to fit into different characterization equipment as detailed in each section of methods. For mechanical tensile testing, two sizes of cuboidal strips from the thick ECM slabs were cut, namely, the “large ECM” of 30mm (w) x 70mm (l) x 10mm (t) and the “small ECM” of 30mm (w) x 30mm (l) x 10mm (t). (Fig. 8) The ECM slab was placed on a tissue culture plate (TC plate) and a grid paper of the dimension required was placed onto the epicardium of the ECM for a more accurate size cutting using surgical blades. Precise measurement was done by Vernier caliper (to 0.05 of a millimeter) before testing; and input for data processing.

Figure 8 ECM specimens.

Large ECM specimen (left) and three small ECM specimens (right)

3.2 Native tissue

Porcine whole hearts were collected right after harvest at a local abattoir [Primary Industries Pte. Ltd., Singapore]. CO₂ stunning system was employed as execution method before slaughtering. All hogs (mixed male and female, 5 months old) were from a pig farm located in P. Bulan, Indonesia. Left ventricular walls were dissected (*Dissection method in Appendix 11*) and stored in sterile phosphate buffered saline (PBS) supplemented with antimycotic antibiotics solutions (ABAM) [Sigma-Aldrich, St-Louis, MO, USA] at 4°C temporarily before experiments that were carried out within 1-2 days after the harvest. Large specimen of native tissues refers to 30mm (w) x 70mm (l) x 15mm (t) and small ECM specimen refers to 30mm (w) x 30mm (l) x 15mm (t). (Fig. 9) Precise measurement was done by caliper before testing; and input for data processing.

Figure 9 Native porcine heart specimens.

(A) Native porcine whole heart. (B) Large native specimen. (C) Small specimen.

3.3 Uniaxial mechanical test

Instron 5567 Universal Testing Instrument [Instron, Norwood, MA] with 500N load cell were used. Device control and data acquisition were performed with BlueHill® Software. Specimens (n=5 for each group) were mounted in the orientation where their fiber-preferred direction was aligned with the stretching Y-axis direction.

3.3.1 Sample mounting

Inspired by the surgical ventricular reconstructions procedure and the Dor suturing practices, we tested the method of suturing to mount samples to tensile testing machine. (Fig. 10) 100% polyester sutures of metric diameter 0.1mm, comparable to a #5/0 surgical suture were passed through the whole thickness of the two edges (1 loop/1.5mm) and looped around two stainless steel rods ($\varnothing=6.5\text{mm}$, $l=30\text{mm}$) that were pre-mounted onto the machine fixture.

Figure 10 Specimens sutured to fixture of Instron 5567.

ECM. (A) Native. (B)

To facilitate the mounting efficiency and modify the suturing method to be more clinically relevant, surgical braided sutures Safil® were used. [PGA, braided, coated, absorbable, #5/0, 1/2c, B | BRAUN, Singapore] The looping ratio was changed from 1 loop/1.5mm to 1 loop/2mm, with references to the commonly used suturing methods in literatures. ^[23,166-168] This provided some insights relating to our ECM applicability in surgical settings. We also custom-designed a fixture to be mounted onto the machine fixture which eases the mounting of specimens as shown in Fig. 11. (Stainless steel D10 X 30mm housing with

Spring Ejector pins Fixture fabrication by Ricko Engineering, Singapore) (*Image and design in Appendix 1*)

Figure 11 Specimen sutured to ejection pin using surgical braided Safil® suture.

3.3.2 Mechanical testing assays

Prior to each test, pre-cycling was performed in order to get reliable and repeatable results on viscoelastic biological materials, allowing for reorientation of macromolecular structures back to their natural alignment.^[127] All specimens were preconditioned with 10 cycles of tensile loading at a rate of 0.05 mm/s to 15% strain, followed by same-rate unloading. Obtaining periodic loops indicates the reorientation of macromolecular structure of the matrix back to its natural alignment. Three sequential mechanical assays were then conducted with 10 min intermissions.^[80,86]

- i. **Cyclic loading:** After pre-cycling, three more straining cycles were continuously performed. Stress-strain data was recorded. Energy dissipation (also known as hysteresis) was calculated based on the area blocked within the stress-strain curves during the last cycle of the dynamic cyclic loading and unloading.
- ii. **Stress Relaxation:** Stretching at 0.5mm/s to 20% strain and then held at the fixed displacement for force-decays over 10 minutes. Relaxation times were estimated

as the mean lifetime taken for the decaying stress level to fall from maximum stressed value at 20% strain to $1/e$ times the value before reaching the equilibrium.

iii. Tensile failure: Stretching at 0.05mm/s until complete cleavage of specimen.

Young's Modulus was calculated as the slope of the linear region of stress-strain curve. Toughness was measured as the total area under the entire stress strain curves during a tensile failure test.

At the beginning of each assay, zero strain point was set at where stress value was balanced at 0.001N (the minimal positive value as shown in the control panel on the equipment). The force-displacement-time data was recorded during all assays; and transformed to stress-strain measurements for data analysis.

3.4 Biaxial mechanical test with CellScale BioTester

Biaxial testing was conducted to characterize the ECM and native tissue's heterogeneous tensile properties. This experiment was performed in collaboration with CellScale [Waterloo, ON, Canada]. We customized a biaxial testing system Biotester 5000 [CellScale]. (Fig. 12) It comprised (from top to bottom) an overhead camera for video tracking, two illumination lamps, BioRake™ sample mounting system with tines, high resolution-controlled extensometers (integrated with the machine control and data collection software); and a fluid chamber at the base. The system's force capacity is 23N with 0.2% accuracy.

Figure 12 CellScale BioTester.

3.4.1 Customizations to CellScale biaxial tester

Our customizations included the following: (*Customization CAD drawings in Appendix 2*)

- i. BioRake™ attachment: longer tines (52mm instead of standard 30mm in length) were used to accommodate the desired specimen size, five downward pointing and four upward-pointing tines for better grip and alignment of thick specimen, tine-to-tine spacing of 2.2mm and 8mm puncture point length. This resembles our specifications in the uniaxial sample mounting. (Fig. 13B)
- ii. BioRake™ base was lengthened and with an additional second magnet for better attachment
- iii. BioRake™ holder was modified to gooseneck shape to support the enlarged BioRake™ base.
- iv. Temperature-controlled fluid chamber: dimensions amended for specimen immersion in phosphate buffered solution at 37°C. (Fig. 13A)

Figure 13 CellScale BioTester customized components and samples in test.

Customized fluid chamber. (A) Customized BioRake. (B) ECM on the tester. (C) Native specimen on the tester. (D)

3.4.2 Sample mounting and testing assays

The BioRake™ composed of nine tungsten tines, five facing down and four facing upwards with 2.2mm tine spacing and 8mm puncture point length. Small Specimens of 25mm (l) x 25mm (w) (n=5 for each sample type) were mounted in their exact orientation of Y-axis (fiber-preferred direction) and X-axis (cross-fiber direction). (Fig. 13C-D) Samples were tested according to the mechanical testing assays as the uniaxial setting detailed in section 3.3.2 except for tensile failure whereby specimens were strain up to 50% nominal biaxial strain due to the equipment constraints. Synchronized video tracking images were collected and force-displacement data were acquired with the integrated software [LabJoy 8.01].

3.5 Development of viscoelastic analytic tool

The aim of developing a new computational analytic tool is to provide a non-bias platform for soft-tissue mechanical characterization. The tool allows the fitting of users' tensile experimental data to a viscoelastic model in order to yield viscoelastic parameters. It enables user to thoroughly evaluate the materials' mechanical properties and allow fair comparison of measurements including the elastic moduli, viscosity moduli and modeled relaxation time.

3.5.1 Methodology and assumptions for analysis

The Zener and other viscoelastic models were implemented to resolve the behavior of native and decellularized pcECM tested under a tensile-relaxation assay comprising two testing modes, namely tensile mode (constant strain-rate) and stress-relaxation mode (constant strain).

Initially, the linear Hookean model ($\sigma = \sigma_0 + (\varepsilon - \varepsilon_0)E$) was fitted on the data registered to produce the Young's modulus (E as the slope) during the tensile mode. Toe border (t_0) was defined as the border separating the toe region from the linear region (LV-region). The elucidated toe border was used later as the starting point from which the Zener and other models were fitted.

The Zener model can be represented as a spring serially connected to a Kelvin-Voigt solid and described according to its typical constitutive differential equation using the viscosity modulus: η , and the elastic moduli: E_1, E_2 .^[137,169,170]

Equation 1

$$\frac{d\sigma}{dt} + \sigma \frac{E_1 + E_2}{\eta} = \frac{d\varepsilon}{dt} E_1 + \varepsilon \frac{E_1 E_2}{\eta}$$

By rearranging the equation, a multivariate linear correlation arises between the stress at time t (σ_t) and three apparently independent variables: $(d\sigma/dt)$, $(d\varepsilon/dt)$ and ε .

Equation 2

$$\sigma_t = \frac{\eta}{E_1 + E_2} \bullet \left(-\frac{d\sigma}{dt} \right)_t + \frac{\eta E_1}{E_1 + E_2} \bullet \left(\frac{d\varepsilon}{dt} \right)_t + \frac{E_1 E_2}{E_1 + E_2} \bullet (\varepsilon_t)$$

Given that $d\sigma$, $d\varepsilon$ and dt could be estimated based on their integral form ($\Delta\sigma$, $\Delta\varepsilon$ and Δt), equation 3 could be stipulated and used as an approximation of the constitutive differential Zener model.

Equation 3

$$d\sigma \approx \Delta\sigma = \sigma_i - \sigma_{i-1}$$

$$d\varepsilon \approx \Delta\varepsilon = \varepsilon_i - \varepsilon_{i-1}$$

$$dt \approx \Delta t = t_i - t_{i-1}$$

$$\Rightarrow \sigma_i = \sigma_0 + \underbrace{\left(\frac{\eta}{E_1 + E_2} \right)}_{\beta_1} \bullet \underbrace{\left(-\frac{\sigma_i - \sigma_{i-1}}{t_i - t_{i-1}} \right)}_{X_1} + \underbrace{\left(\frac{\eta E_1}{E_1 + E_2} \right)}_{\beta_2} \bullet \underbrace{\left(\frac{\varepsilon_i - \varepsilon_{i-1}}{t_i - t_{i-1}} \right)}_{X_2} + \underbrace{\left(\frac{E_1 E_2}{E_1 + E_2} \right)}_{\beta_3} \bullet \underbrace{(\varepsilon_i - \varepsilon_0)}_{X_3}$$

By regressing the stress (σ_t) against X_1 , X_2 and X_3 , the regression coefficients (β_1 , β_2 , and β_3) can be elucidated and used to calculate the elastic moduli: E_1 , E_2 , and the viscosity

modulus: η . As required for multivariate regression, the resulting parameters could only be regarded as estimates of the actual elastic moduli and viscosity.

The non-linear form of the Zener model can be stipulated by integrating Zener constitutive differential equation 1 using boundary conditions that correspond to the stress-relaxation mode from t_1 onwards, with $d\varepsilon/dt=0$. (*Equation development in Appendix 3*) Thus, the integral non-linear model stipulated in Equation 4 can be fitted with the empirical data and optimized to produce E_1 , E_2 and η . While v represents the strain-rate $d\varepsilon/dt$ used during the tensile mode, spanning from t_0 to t_1 .

Equation 4

$$\sigma_t = \begin{cases} t_0 \leq t < t_1 \rightarrow \frac{d\varepsilon}{dt} = v \rightarrow \sigma = \sigma_0 e^{-\frac{t-t_0}{\tau_{rx}}} + \frac{vE_1}{E_1+E_2} \left[(t-t_0)E_2 + \frac{\eta E_1}{E_1+E_2} \left(1 - e^{-\frac{t-t_0}{\tau_{rx}}} \right) \right] \\ t_1 \leq t \rightarrow \frac{d\varepsilon}{dt} = 0 \rightarrow \sigma = \sigma_1 \cdot e^{-\frac{t-t_1}{\tau_{rx}}} + \frac{E_1 E_2 \varepsilon_1}{E_1+E_2} \left(1 - e^{-\frac{t-t_1}{\tau_{rx}}} \right) \end{cases}$$

The equivalent elastic modulus E^* in Zener Model can then be calculated $\left(E^* = \frac{E_1 E_2}{E_1 + E_2} \right)$,

as well as the relaxation time $\left(\tau_{rx} = \frac{\eta}{E_1 + E_2} \right)$.

For comparison, two other models, the Maxwell and Kelvin-Voigt models were also fitted onto the data. The implementation of the Maxwell model is similar to that described for the Zener model (non-linear form of the Zener model equation) but with $E_2=0$. The implementation of the Kelvin-Voigt model is according to equation 5 below.

Equation 5

$$\sigma_t = \begin{cases} t_0 \leq t < t_1 & \rightarrow \frac{d\varepsilon}{dt} = v \rightarrow \sigma = \sigma_0 + \eta v + E v (t - t_0) \\ t_1 \leq t & \rightarrow \frac{d\varepsilon}{dt} = 0 \rightarrow \sigma = \sigma_0 + E v (t_1 - t_0) \end{cases}$$

This failure-enabling implementation of the Zener model was intended to analyze all the tensile-failure data without excluding the failure region. Accordingly, the following three assumptions were made to accommodate data from both the failure and elastic response regions:

- i. The analyzed material is regarded as a bundle of strings, instead of as a uniform monolithic solid. Since all strings belong to the same underlying material, they all have the same elastic moduli and viscosity; however, each string has its own unique failure points. Thus, the superposition of the individually failing strings, throughout the entire bundle, results in a global gradual failure.
- ii. Each string starts its mechanical response as a Zener solid and then fails in a two-stage process. Elasticity loss occurs when the first failure point is reached. This point occurs when the spring denoted by E_1 is fully deformed, causing the Zener solid to lose its major elasticity and start behaving like a *Kelvin-Voigt* solid. Integrity loss occurs when a second point is reached, along the stress-strain continuum, at which the string can no longer hold its structural integrity, causing it to rupture.
- iii. Each failure point (for each string) is denoted as the multiplication product of the strain and stress (stress • strain) at the time of failure. The elasticity-loss measure (defined for each string as $W_E \equiv \text{Stress}_E \bullet \text{Strain}_E$) is normally distributed throughout the bundled material with a mean of $\overline{W_E}$ and a variance of $S_{W_E}^2$. The

integrity-loss measure (defined for each string as $W_I \equiv \text{Stress}_I \bullet \text{Strain}_I$) is normally distributed throughout the bundled material with a mean of $\overline{W_I}$ and a variance of $S_{W_I}^2$.

Hence, equation 6 can be stipulated to describe the stress on a single string, characterized by its own two failure points (W_E and W_I) and the parameters E_1 , E_2 and η shared by all strings.

Equation 6

$$\sigma_{t, W_E, W_I} = \begin{cases} t_0 \leq t < \frac{W_E}{v\sigma_{t_1}} = t_1 & \rightarrow \sigma = \sigma_0 e^{\frac{t-t_0}{\tau_{rx}}} + \frac{vE_1}{E_1 + E_2} \left[(t-t_0)E_2 + \frac{\eta E_1}{E_1 + E_2} \left(1 - e^{-\frac{t-t_0}{\tau_{rx}}} \right) \right] \\ t_1 = \frac{W_E}{v\sigma_{t_1}} \leq t < \frac{W_I}{v\sigma_{t_2}} = t_2 & \rightarrow \sigma = \sigma_0 + \eta v + E_2 v(t - t_1) \\ t_2 = \frac{W_I}{v\sigma_{t_2}} \leq t & \rightarrow \sigma = 0 \end{cases}$$

Finally, when multiplying the above equation by the normal probability density functions

of W_E and W_I (denoted by P_{W_E} and P_{W_I}), and then integrating the product

according to d_{W_E} and d_{W_I} , a new function is generated that describes the overall

stress (σ_t) on a failing bundle of Zener strings.

Equation 7

$$\sigma_t = \int_{W_I=0}^{\infty} \int_{W_E=0}^{\infty} \sigma_{t,W_E,W_I} \cdot \underbrace{\left(\frac{1}{\sqrt{2\pi S_{W_E}^2}} e^{-\frac{(W_E - \overline{W_E})^2}{2S_{W_E}^2}} \right)}_{P_{W_E}} \cdot \underbrace{\left(\frac{1}{\sqrt{2\pi S_{W_I}^2}} e^{-\frac{(W_I - \overline{W_I})^2}{2S_{W_I}^2}} \right)}_{P_{W_I}} \cdot dW_E \cdot dW_I$$

3.5.2 Model simulation using Simulink

Physical networks representing the three mechanical solids (Zener, Maxwell and Kelvin-Voigt) were implemented and simulated using Simulink® R2011a [The MathWorks Inc. MA, USA] supplemented with Simscape® for physical modeling. (Fig. 14) The physical models were simulated using the recommended solver ODE14 extrapolation with a fixed step size.

By connecting basic mechanical elements, the Simscape® environment allows users to create and simulate the time-dependent response of the models-representing physical networks. For instance, springs and dampers are characterized by their respective viscosity and elastic moduli.

Adding reference points and hard-stop elements completes the physical grounding of the network and introduces elastic limits on the springs connected in parallel with hard-stop elements. Specifically, hard-stop elements, included to induce elastic limits, are characterized by strain limits, above which the motion is stopped. Ideal velocity source and ideal force sensors are also included to induce deformation and measure the force exerted by each solid.

The simulation includes the following additional blocks: Step function block creating a time-dependent step-function signal; signal to physical-signal (S→PS) and physical-signal

to signal (PS→S) converters connecting the dimensionless Simulink® environment with the physical Simscape® network; and a solver block determining simulation conditions and output blocks.

Graphical legend for mechanical elements and Simulink® blocks

Mechanical translational elements

spring

damper

hard-stop

reference

Mechanical sources and sensors

ideal translational velocity source

ideal force sensor

Simscape® utilities

Signal to physical signal converter

physical signal to signal converter

Simscape® solver block

Simulink® inout/output ports

step function block

output block

Figure 14 Simulink® models for simulating viscoelastic solids.

3.5.3 Matlab® algorithm

We developed an easy-to-use Matlab® algorithm to fit experimental data and compare it against simulation of other viscoelastic models. Codes were written and executed at Matlab® R2011a [The MathWorks, MA, USA]. This work was done with Dr. Tomer Bronshtein and a paper was published at *Tissue Engineering* ^[124]. (*Scholarly work in Appendix 22*) The function (Zener.m) (*Matlab codes in Appendix 4*) was used to implement a Zener-based analysis for stress–strain data originating from tensile-relaxation or tensile-failure data. For tensile-failure data, only the data delineated between the toe border and the apparent failure border was analyzed, excluding all failure data. The main function used for this analysis was stored in the M-file Zener.m and uses three additional auxiliary functions that were stored in the following M-files: offset.m, get_tv.m and get_young.m. (*Matlab codes in Appendix 5-7*) Data for analysis was stored in the variable data while the average step argument (avg_step) was used to determine the average sampling rate of the raw data. The result of the analysis were graphically displayed and returned as a 5-by-6 matrix. See Examples 1 and 2, below, which demonstrate the analysis of tensile-relaxation and tensile-failure.

Example 1:

```
>> data=tensile_relaxation_data;
>> avg_step=1;
>> Zener(data,avg_step)

ans =
    0.0302    18.9000    28.0020    0.9903    0.0040    0.0000
    0.0494     0.0290     0.1225    0.6850    0.0177     0
    0.0279     0.0312     2.8655    0.9766    0.0050     0
    0.0233         NaN    18.8519    0.8715    0.0102     0
         NaN     0.0185     0.0316    0.6240    0.0182     0
```

The different rows in the above 'ans' matrix refer to the various fitted models. The last three columns in each row refer to the following statistical measures for the fit between

the model and the empirical data: The adjusted correlation coefficient (R^2_{adjusted}); the standard error for the fit (SE); and the significance of the fitted model (as p value), respectively. The first three columns in the first row refer to E , t_0 and t_1 according to the Hook model. The first three columns in the second and third rows refer to E_1 , E_2 and η according to the linear (2nd row) and integral (3rd row) Zener models. The first column in the fourth row and the third column in the fifth row refer to the elastic modulus (E) according to the Maxwell (4th row) and Kelvin-Voigt (5th row) models. The third column in the fourth and fifth rows refer to the viscosity modulus according to the Maxwell (4th row) and Kelvin-Voigt (5th row) models.

Example 2:

```
>> data=tensile_failure_data;
>> avg_step=1;
>> Zener(data,avg_step)

ans =

    0.0091    221.4000    387.8000    0.9891    0.0023    0.0000
    0.0719     0.0107    -0.1211    0.9880    0.0021    0.0000
    0.0084     0.0097   8631.4519    0.9879    0.0030    0.0000
    0.0084         NaN   6023.9505    0.9879    0.0030    0.0000
         NaN     0.0084     0.0000    0.9879    0.0030    0.0000
```

The function (Failing_Zener.m) (*Matlab codes in Appendix 8*) was used to implement a failure-enabling analysis based on the Zener model for data originating from tensile-failure data including the failure region. The main function used for this analysis was stored in the M-file Failing_Zener.m and uses three additional auxiliary functions that were stored in the following M-files: offset.m, get_tv.m and get_young.m. Data for analysis was stored in the variable data. The function required additional three input arguments E_1 , E_2 and η , holding initial estimates for the Zener elastic moduli and

viscosity modulus. These estimates can easily be drawn from our previously demonstrated analysis of tensile-relaxations data or from the literature.

The result of the analysis were graphically displayed and returned as a MAT file (report.mat) containing a 10-by-1 vector. The report vector contained the following data:

- 1) the optimized viscosity modulus (η) and elastic moduli (E_1 , E_2);
- 2) the mean and the variance of the multiplication product resulting from the stress and strain at the elastic failure point;
- 3) the mean and the variance of the multiplication product resulting from the stress and strain at the integrity failure point; and
- 4) a statistical measure of the model fit, error and significance (R^2_{adjusted} , SE and p).

This analysis was executed using a Graphical User Interface (Fig. 15) that allows the user to manually modulate the analysis parameters and receive real-time feedback on how these changes affect the fit between the model and the empirical data.

Figure 15 Screen shot of the graphical user interface.

This screen shot depicts the graphical user interface used in the implementation of the failure-enabling Zener model that enables analysis of the entire tensile-failure data without excluding the failure region. This example is taken from data drawn from a tensile-failure test on a decellularized pcECM sample. The upper plot depicts the modeled and real stress vs. time while the lower plot depicts the real vs. the modeled stress.

The parameters can be modulated by simply moving the sliders between their upper and lower limits, which can also be determined manually. Apart from this manual fine-tuning, the parameters can be optimized automatically to reduce user bias through one of two methods. One method, which was executed upon pushing the SOLVE button, optimized the parameters using the least squares technique by minimizing the sum of squares.

Another method, which was executed with the OPTIMIZE button, optimized the parameters to maximize the R^2_{adjusted} . Both optimization methods can be solved using one

of two solver algorithms: interior point or sequential quadratic programming (SQP). The strings parameter refers to the number of simulated strings that make up the failing solid. With more simulated strings, the analysis was more accurate but also required a much longer execution time. Practically, after a quick analysis using a small number of strings, the number of strings can be increased to smooth the modeled stress curve for presentation purposes. The SDs parameter refers to the number of standard deviations taken from each side of the means representing the elastic and integrity failure points. One hundred strings and three standard deviations should be enough to achieve a fair fit with empirical data. The LAST button was used to retrieve the last values by undoing the action of the SOLVE or OPTIMIZE buttons. The SAVE button created a report file (*report.mat*) on the current path and the LOAD button loaded the parameters from a report file.

3.5.4 Data input and output

The above-mentioned models were fitted to the data collected from mechanical testings of our sample groups (n=5 per group). Data for analysis is given as an N_i-by-3 table with the first column being the sampled time (measured in sec.), and the second and third columns, respectively corresponding to the strain (ϵ_{t_i} , in % of initial length at time 0) and stress (σ_{t_i} , in Pa) at time t_i. For tensile-relaxation data, the algorithm and code produce the viscosity and the elastic moduli (for the various models), optimized through the least squared method, along with the toe region border and statistical measures for the quality of the fitted models ($R^2_{adjusted}$, SE and p value). The dimensions (units) of the resulting measures correspond to the dimensions of the raw data: $\left[\frac{\text{stress unit}}{\text{strain unit}} \right]$ for the

elastic moduli (E , E_1 , and E_2) and $\left[\frac{(\text{stress unit})(\text{time unit})}{\text{strain unit}} \right]$ for the viscosity modulus (η).

For tensile-failure data, analysis according to a failure-enabling implementation of the *Zener* model also produces the coefficient of variance (CV) and means (as $[(\text{Stress unit}) \cdot (\text{Strain unit})]$) of the elastic and integrity failure points.

3.6 Surface and Bulk Biophysical Properties

This section details all the equipment used and experimental/analytical methods employed for Aim 2 to study the surface and bulk biophysical properties of the materials. The orders of appearance of sections follow the chronological sequences of the experiments performed. First the materials' surface properties that govern the interaction of living cells with surfaces were explored. Bulk properties including the hydration content and thermal property were then evaluated. Followed by structure visualization and preliminary study on materials electrical conductivity,

3.6.1 Protein adsorption Study

The adsorption kinetics of a model serum protein onto the ECM was followed to study the protein adsorption capabilities of the pcECM. It enabled us to obtain insights about interaction of living cells with the two surfaces of ECM (top: decellularized pericardium; bottom: decellularized endocardium), in terms of amount of protein adsorbed. Bovine Serum Albumin labeled with fluorescein isothiocyanate (BSA-FITC) was used as a model protein for its well-characterized structure and physiochemical properties ^[146]; and the assay used was modified from those of Ni *et al.* ^[150]; Koh *et al.* ^[145]; and Kim *et al.* ^[151] with modifications to the concentration of solution, sample dimension and range of time points. ECM samples were cut into circular discs for placing into 24-well tissue culture plate. Five

wells were of ECM pericardium layer facing up and five wells of endocardium layer. Five blank wells in the 24-well tissue culture plate were used as control. All wells were exposed to 0.5ml of 50 μ g/ml of BSA-FITC solution [Sigma-Aldrich, St-Louis, MO] prepared in PBS. Plate was agitated on orbital shaker at 125rpm and incubated in dark at 37°C for a range of time points: 1, 3, 5, 15, 30, 60, 90, 120min. Samples were taken out from the suspension and washed with PBS thoroughly such that only the protein firmly adsorbed into the ECM surface was left to be tested. Samples were then placed in new wells and 5mL of Sodium Dodecyl Sulfate (SDS) solution [Sigma-Aldrich, St-Louis, MO] (1% w/v in PBS) were added into individual wells for equilibration of 1 hr in dark at 37°C. The resultant supernatant (BSA-FITC in SDS/PBS) was transferred into new well plate for measurement under Varioskan Flash Spectral Scanning Multimode Reader [Thermo Scientific, MA, USA]. The calibration curve regarding intensity of fluorescence against amount of BSA adsorbed onto matrix was generated by preparing known concentrations of BSA-FITC: 0, 5, 10, 20, 30, and 50 μ g/ml of BSA-FITC in 1 %w/v SDS/PBS. (*Calibration curve in Appendix 12*) Three replicas were taken for each sample in each group. The excitation wavelength for the BSA-FITC concerned was λ =488nm while for detection was λ =530nm.

3.6.2 Fourier transform infrared spectroscopy analysis (FTIR)

Fourier transform infrared (FTIR) has been of expended usage in studying the surface conformation of protein-based biomaterials. [69,145,147,150].

Perkin Elmer® FTIR- Spectrum™ GX [Perkin Elmer Inc., Shelton, USA] was used in attenuated total reflection mode with Golden Gate Single Reflection Diamond ATR [crystal materials: zinc selenide (ZnSe)] at NTU for analyzing surfaces of ECM, native tissues, protein adsorbed ECM surfaces and surface of electrified ECM. Scans were accumulated

in the mid-IR range 600-4000 cm^{-1} with a resolution of 4 cm^{-1} . Data processing was performed with Spectrum™ 5 Software.

During the author's research attachment in the Technion Lab, the below specific equipment available in the Technion Labs were used with which the author prepared the specimens and carried out the data acquisition and analysis. Thermo Scientific- Nicolet 6670 FTIR [Thermo Fisher Scientific Inc., Waltham, MA, USA] was used at smart iTR mode with Diamond plate and concave probe for analyzing surfaces of ECM, native tissues, and reseeded ECM samples. Scans were accumulated in the mid-IR range 600-4000 cm^{-1} with a resolution of 4 cm^{-1} . Data processing was performed with OMNIC Spectra software against a built-in spectrum and peaks library.

3.6.3 Scanning Electron Microscopy (SEM/WETSEM)

Scanning electron microscope was used to visualize the ultra-structures of the specimens. JSM-6360 Scanning electron microscope [JEOL, Tokyo, Japan] was used to visualize ECM, reseeded and native samples. Small specimens of approximate diameter of 5mm were lyophilized overnight and gold-sputtered for 30s at 18mA [JEOL JFC-1600 Auto Fine Coater] and then mounted with double adhesive tape for imaging at 5kV accelerating voltage, 15mm working distance and 68A current. Secondary electrons were detected for SEI images.

During the author's research attachment in the Technion Lab, the below specified equipment available in the Technion Labs were used with which the author prepared the specimens and carried out the data acquisition and analysis. To reveal a better visualization of the reseeded ECM samples, WETSEM technology was implemented using QuantomiX™ Capsules QX 302 [El-Mul Technologies, Ltd., Israel] ^[80,171] (Fig. 16), which

allows high resolution imaging of wet samples under standard scanning electron microscope.

Figure 16 Kits for QuantomiX WETSEM experiment.

From left to right: (1) PTA. (2) Imaging buffer. (3) MP-12 Capsule Plates. (4) QX-302 Capsules.

Samples of about 1mm thick and 3mm in diameter were cut using blades from the endocardium surfaces. They were then washed three times at five min each with double distilled water (DDW). Afterwards, samples were incubated with 1% of phosphotungstic acid (PTA) solution in DDW according to QuantomiX™ sample preparation protocol for QX302. ^[171] PTA is an anionic stain that positively stains charged structures including proteins associated with nuclear DNA and nucleoli as well as mitochondrial matrix.

Upon incubation, samples were washed five times at five min each with DDW. To place the samples inside the specimen dish, the endocardium surface were placed against the bottom membrane of the capsule, which were then sealed with spacer above the back surface of samples. 5µL of imaging buffer was then added to the specimen dish before the closing of the sealing stub.

Σ IGMA Field Emission Scanning Electron Microscopes (FE-SEM) [Zeiss, Germany] at Technion, equipped with the Schottky Thermal Field Emitter and GEMINI column, was used for imaging at EHT 20kV, 5-10mm working distance and 60um aperture. Electron beam can penetrate through the QuantomiX capsule membrane to image the specimen to a depth of up to three microns.

3.6.4 Thermogravimetric Analysis (TGA)

Water content influences the structure, mechanical behaviors and chemical stability of the hydrated scaffolds^[156-159] and thus should be evaluated for the case of our hydrated ECM by temperature-controlled thermo galvanometric analysis (TGA).

Approximately 10 mg of material samples (weighed by CPA225D semi micro balance [Sartorius, Germany] and input for data processing) were loaded to TGA 2950 [TA Instruments, Delaware, USA]. ECM, native tissues, and reseeded ECM samples (n=3) were heated at ramping rate 5°C/min from 25°C-300°C under a controlled nitrogen gas flow of 50 mL/min. Weight change over the heating spans was recorded.

3.6.5 Differential Scanning Calorimetry (DSC)

Thermal property of the protein-based material reflects the compositions and structures of proteins in the materials and their denaturation behavior^[160-164].

In this study, the thermal property of the following groups: ECM, native tissues and reseeded ECM were evaluated by differential scanning calorimetry (DSC). Approximately 5-10 mg of material samples (n>4 for each group) (weighed by CPA225D semi microbalance [Sartorius, Germany] and input for data processing) was sealed in an aluminium hermetic pan [TA Instruments, Delaware, USA]. DSC Q10 V9.9 Build 303 [TA Instruments, Delaware, USA] with DSC Standard Cell FC was used to perform thermal

analysis from 25°C-250°C at an averaged ramping rate of 5°C/min. Universal V4.7A software [TA Instruments, Delaware, USA] was used for data processing. Heat Flow (mW) was recorded through the temperature range against temperature (°C). Transition temperature T_t (°C) was defined by the ‘glass/step transition’ analytic function. Peak melting temperature T_m (°C) was defined by the “signal maximum” analytic function. Heat of fusion (J/g) was defined by the “integrate peak linear” analytic function of the heat capacity during the heating temperature range.

3.6.6 Electrical Property

An electrical characterization was performed to obtain preliminary insight of the effect of cell presence on the conductivity of the pcECM as the last objectives of Aim 3 as stated in Scope of Project section 2.3. Inherent resistance to direct current of the ECM specimens was measured with the PowerPac™ Basic Power Supply [Bio-Rad, Hercules, CA, USA] in our custom-made chamber. (*Prototype and raw design in Appendix 10*) (Fig. 17) Voltage of 3-30 V was applied across the 2cm length of the specimen, with the surface area of approximately 0.7cm² (width of specimen: 1cm and thickness: 0.7cm). Resistance (R in Ω) referred to the slope of the voltage (in V) vs. current (in A) curve. Resistivity was calculated by multiplying resistance with the area of application divided by length of sample ($\rho = RA/l$ in Ωcm). Conductivity equals to the reciprocal of resistivity ($1/\rho$ in $\Omega^{-1}\text{cm}^{-1}$).

Figure 17 Electrical characterization set-up.

Custom-made Electric chamber with PowerPac™ Basic Power Supply (A) ECM sample gripped in the holders. (B)

3.7 Cell culture

The focus of this thesis is to evaluate our ECM constructs upon being recellularized by human cardiomyocyte-relevant cells. Aiming to obtain clinically meaningful cell numbers, the potential of human mesenchymal stem cells to expand on the construct was studied.

In this project, Poietics™ human bone marrow-derived mesenchymal stem cells (hBM-MSCs) [Lonza, Basel, Switzerland] were used as cardiomyocytes-model cells.

Culture media for MSCs is made up of Alpha modified minimum essential medium eagle (α MEM) [Sigma-Aldrich, St-Louis, USA], 10% heat inactivated fetal bovine serum (FBS) [Gibco, Life-Technologies, CA, USA], supplemented with 5 ng/ml fibroblast growth factor human recombinant (bFGF) [Millipore, MA, USA], and 1% antibiotic-antimycotic (ABAM) [Sigma-Aldrich, St-Louis, USA].

1M of frozen cells in vial as purchased were thawed and cultured on TC plate (15cm diameter) and grown in 25mL culture media with 0.1% bFGF in a humidified 37°C, 5% CO₂ incubator. Culture flasks with filtered cap were in use when contamination was in concern, and 40-60 mL culture media with 0.1% bFGF was used. On the following day, old medium with non-adherent cells were removed and cell plates were replenished with fresh media. Cells were allowed to grow for 3-4 more days to about 75% confluences (~3-4M on each TC plate).

Confluent cells on TC plate were washed with sterile phosphate buffered saline (PBS) [Gibco, Life-Technologies, CA, USA] followed by trypsination with Trypsin-EDTA Solution [Sigma-Aldrich, St-Louis, USA], 15-20mL per TC plate. Cell suspension was then centrifuged at

200G for 5 min to obtain the cell pellets. The cell pellets were re-suspended in culture media to a concentration of 2M cells/mL. They were then placed on new TC plates for subculture for an additional week prior to subsequent experiments.

Medium was replenished every two days. Passage 10 was not exceeded during all seeding-related experiments. Cell mortality before seeding was examined with Trypan Blue staining [Sigma-Aldrich, St-Louis, USA].

3.8 Static Construct Culture

In this thesis, static construct culture refers to the cultivation of reseeded ECM construct in tissue culture flasks of cell culture media, without the use of a dynamic bioreactor. The impact of cells on the ECM towards a native-myocardium-mimetic construct was studied in terms of cell viability and proliferation, as well the material properties concerned in Aim 1 and 2.

3.8.1 ECM disinfection

Thick ECM specimens were disinfected by washing with 70% filtered Ethanol on laboratory rocker for one day, with new 70% filtered Ethanol replenished every 3 hours; followed by one day of PBS with 2% ABAM washing, replenished every 3 hours. Ethanol has been used as part of the decellularization and disinfection protocols for our preparation of acellular ECM samples in the lab ^[80,86]. It was chosen as for sterilization the ECM before seeding owing to its ease of preparation and application; as well as its sufficient effectiveness in killing microbes or fungal spores at 70%. Other sterilization methods (such as ethylene oxide, gamma radiation, electron beam radiation) that have been reported in decreasing the mechanical properties of biological scaffold materials ^[21] were hence avoided. One

day before seeding, the sterile specimens were placed into our custom-made seeding chamber (Polyethylene box 86 X 86 X 40mm with polycarbonate) (*Seeding chamber images in Appendix 9*) where ECMs were incubated with respective culture media overnight. The seeding chamber was designed to be of low-cell binding surfaces with compartment dimensions fitting our large (25x70x10mm) and small (20x30x10mm) specimens, therefore confining the surfaces to achieve maximum cell seeding. The PE and PC chosen are also thermally stable in high temperature, which allowed them to be autoclaved. ^[150,172] Two hours before seeding, media was removed and scaffolds were left to partially dry in a Biological Safety Cabinet.

3.8.2 Seeding methods evaluation

The seeding method experiment aims to compare the different commonly used seeding methods on biomaterials to compare their respective efficacy for the case of our ECM. ^[11,77,141,142,173] Sterile ECM scaffolds were cut into the size of Ø16.5mm to be fit in 24 well plates. MSCs were re-suspended in culture medium at ~1M cells /mL then being reseeded by the following four methods:

- 1) Even pipettation over the surface;
- 2) Needle injection with a B|BRAUN Ø 0.5x25mm 25G needle;
- 3) Centrifugation at 45G for 2min;
- 4) Vacuum induced cell seeding in which cells were delivered to the ECM bulk using a vacuum driving force generated by a standard for 2 min.

For pipette surface seeding, 10 shots of 100µL cell suspension were pipetted slowly and evenly over the endocardium surface of the ECM in a 24-well plate. Needle injection seeding was performed using a 25-gauge x1" hypodermic needle [Sterican®, B. Braun,

Melsungen, Germany] with 3 mL BD Luer-Lok™ disposable syringe [Becton, Dickinson and Company, Singapore]. Ten shots of 100µL cell suspension were injected into the approximate mid-layer bulk of the ECM. Centrifuge seeding was done by placing the ECM at the tip of 15mL centrifuge tube and submersing with 1mL of cell suspension. The 15ml tube was subsequently centrifuged at 45G in the Thermo Scientific IEC CL30 Centrifuge with S41 Swing out motor (16.1cm radius) [Thermo Scientific., MA, USA] for 2min at room temperature, with re-suspension of tube-bound cells after each centrifuge cycle for three times. For vacuum pump seeding, ECM was placed on the filtering paper of the Nalgene® PES filter unit [Thermo Fisher Scientific, NY, USA] subjected to 2min of 2bar vacuum suction with 1 mL cell suspension pipetted on top over the time span.

After all respective seeding methods, reseeded ECM were returned to a 24-well plate and incubated for attachment for 90 min in a 37°C, 5% CO₂ incubator followed by fully submerging in 1 mL fresh culture media per well for further culturing.

3.8.3 Static construct culture upon pipette surface seeding

Confluent cells on TC plate were washed with PBS followed by trypsination. Cell suspension was centrifuged at 200G for 5 min, and then the cell pellets obtained were re-suspended in culture medium to a concentration of 2M cells/mL. In the seeding chamber, cell suspension was pipetted slowly and evenly over the endocardium surface of the ECM at a density of 1M/cm². Seeded scaffolds were incubated in the seeding chamber for 90 min (optimized time to allow cell attachment). After 90min, the seeded ECM was placed into a T75 culture flask with filter cap. It was fully submerged in 25mL culture media for further culturing. Media was changed every two days and cell viability was monitored over specific time ranges as detailed in the results sections.

3.8.4 Cell viability and proliferation

Cells' adherence and density upon seeding were evaluated using AlamarBlue® assay [Life-Technologies, CA, USA] according to manufacturer's instructions ^[174] over specific time ranges as detailed in the results sections. Briefly, old medium was removed and the seeded scaffolds were washed in PBS then transferred to a new flask such that only cells adhered to the ECM were counted. Culture medium with 10% v/v AlamarBlue® was added to each flask and incubated for four hours. 100 µL of incubated solution were transferred to well with four replicates in a 96-well plate for fluorescent reading [Thermo Scientific Varioskan Flash Spectral Scanning Multimode Reader]. Quantitative examination was done against a calibration curve of known cell quantities performed in 6-well plates. *(Calibration curve in Appendix 13)* Afterwards, scaffolds were washed gently and replenished with fresh media for further culture.

Cell visualization was performed upon day 1 of seeding to confirm cell existence. Cells were pre-labeled with Dil (1, 1-diotadecyl-3, 3, 3, 3-tetramethylindocarbocyanine perchlorate [Life-Technologies, CA, USA]) in order to stain plasma membrane. One day prior to seeding, 75% confluent cells were washed with PBS. Working solution of Dil stain was prepared by dilution in FBS free-culture medium at 1:200 ratios. FBS was avoided as the serum protein can reduce the effective dye concentration for quality labeling. 25 mL of dye solution was added to each TC plate and incubated for 30 min. Afterwards, cells were washed with FBS-free medium three times, and then incubated overnight in medium to get rid of burst-released dye. On the next day, stained cells were harvested by trypsinization, and then seeded onto the ECM by surface pipettation. Five hours prior to imaging, samples with pre-stained cells were rinsed with PBS three times. They were then immersed in 25 mL of 4% paraformaldehyde (PFA) [Sigma-Aldrich, St-Louis, USA] at 4°C

fridge for fixing for 4 hours. 30 min prior to imaging, samples were washed thoroughly with PBS three times for the ease of handling since PFA is toxic. Just before the imaging, samples were transferred to a Petri dish filled with PBS. Stained ECMs were observed using TCS SP5 confocal-multiphoton (MP) microscope. [Leica, Wetzlar, Germany]

3.9 Dynamic Construct Culture

In this thesis, dynamic construct culture refers to the cultivation of reseeded ECM construct with the use of dynamic perfusion bioreactors. The impact of cells on the ECM towards a native-myocardium-mimetic construct was studied in terms of cell viability and proliferation, as well the material properties concerned in Aim 1 and 2.

3.9.1 Bioreactor systems

Dynamic culturing systems were set up for the purpose of facilitating the supply of oxygen and nutrients across the reseeded ECM constructs through perfusion. (Fig. 18) A perfusion type of bioreactor was chosen as it has been shown to greatly improve the efficiency of cell viability as well as nutrient and oxygen transport compared to other bioreactor systems. [3]

Figure 18 Bioreactor system.

Schematic of the flow of the bioreactor system and the actual set up demonstration in accordance to the schematic.

The bioreactor system consisted of:

- 1) A reservoir containing medium with required growth factors and metabolites,
- 2) An oxygenator connected to the incubator providing the necessary oxygen content (95%); temperature (37°C) and humidity (95%),
- 3) A peristaltic pump with tubing allowing pulsate fluid flow of 12.7ml/min (optimized based on the initial construct to whole-heats' mass ratio), and
- 4) A bioreactor unit that house the ECM constructs.

Two similar bioreactor systems have been used in this study which were set up respectively in NTU (during initial studies and optimizations) and Technion (during the author's research attachment in the Technion Lab for actual data acquisition and analysis). Both systems were employed under the same medium flow system with the only difference being the housing bioreactor unit, as showed in Fig. 19 and 20 respectively. Both bioreactor units consisted of transparent covers to allow close monitoring of the system. Catheter connection inlet and outlet port were used to connect the tubing to the two perfusion catheters at the two ends of the construct. Bath chambers were where the cell-reseeded pcECM construct sat. Air conduits were placed for allowing pressure balance with filtered air. Medium outlet ports (either on the bioreactor base for the NTU unit, or on the cylindrical wall for the Synthecon™ unit) were placed for the outflow of medium to allow efficient medium changing.

Figure 19 Custom-made bioreactor unit used at NTU.

Schematics of the NTU bioreactor unit (A). Disinfected ECM aseptically connected to the perfusion bath inside the bioreactor unit (B).

Particularly, the Synthecon™ unit, composed of autoclavable cylindrical wall with the bath chamber enclosed, could help reduce the risks of contamination. The unit bath chamber was modified to allow the cell-reseeded pcECM to sit flat inside in the middle of the bath. This ensured the sample was fully immersed in the medium and was being well fed with the culture media through the holes on the bath chamber walls.

Figure 20 Synthecon™ bioreactor unit used at Technion.

Cell seeded ECM on custom-made construct chamber in the Synthecon™ bioreactor unit (A-B) Construct being well fed with the perfused culture media through the holes (C)

3.9.2 System disinfection

Autoclavable parts of the bioreactor system were autoclaved; while non- autoclavable parts were soaked in ethanol overnight before the day of assembly for disinfection. After the complete assembly, the system was cleaned before construct placement with the sequential procedure of: one flush of ethanol, two cycles of ethanol perfusion; one flush of PBS, and two cycles of PBS perfusion. 100-200mL of the respective solution was used for each flush or cycle.

3.9.3 Pre-seeding ECM handling

The perfusion capability of the ECM was tested prior to the commencement of the long-going dynamic-culture experiment. A small amount of red-colored medium was slowly perfused into the coronary artery and the flow of medium along the artery towards the vessels and capillary branches until the other end of the construct had to be observed for the construct to be qualified and color-tagged for future use. (Fig. 21)

Figure 21 Perfusion capability check of potential dynamic-culture construct.

Medium was injected to the artery (A). Smooth medium flow to the end of the construct indicates a qualified sample (B). Sample being tagged for subsequent experiment (C).

Selected ECM specimens were disinfected by washing the ECM with 40mL 70% filtered ethanol in flasks on laboratory rocker for 3 days followed by 2 days of 40mL PBS with 2% ABAM washing. Fresh solution of ethanol and PBS was replenished every day. One day before seeding, ECMs were aseptically placed inside the construct chamber of the bioreactor unit with the two end-catheters connected the perfusion channel. The unseeded ECM together with entire bioreactor system were perfused with culture media overnight for contamination check before the commencement of the culturing experiment. Two hours before seeding, the medium inside the system was drained and the glass cover of the bioreactor unit was taken off carefully. Constructs staying in the bioreactor unit were left to partially dry in a biological safety cabinet.

3.9.4 Seeding and culturing

Confluent human bone marrow-derived mesenchymal stem cells (MSC) on tissue culture plates were washed with PBS followed by trypsination. Cell suspension was centrifuged at 200G for 5 min, and then the cell pellets obtained were re-suspended in culture medium to a concentration of 2M cells/mL. Cell suspension was pipetted slowly and evenly over the endocardium surface of the ECM in the bioreactor unit at a density of 1M/cm². (Fig. 22A) The bioreactor unit hosting the reseeded scaffold was then transferred to an incubator and incubated for 90 min (optimized time to allow cell attachment in our lab). (Fig. 22B) After 90 min, the seeded ECM was subjected to dynamic culture conditions using pulsate flow of oxygenated culture media. (Fig. 22C) Fresh media was replenished every two days.

Figure 22 ECM construct in bioreactor unit.

Seeding (A). Incubation (B). Culturing (C).

3.9.5 Cell viability and proliferation

To monitor cell proliferation within the closed environment of bioreactor, AlamarBlue® assay was performed differently from the static culture condition over specific time ranges as detailed in the results sections. 10% AlamarBlue® in 100 mL medium was prepared and being contained in reservoir. It was then perfused from the reservoir through the perfusion tubing and catheters into the construct inside the bioreactor unit placed inside the incubator. 2 mL of reacted solution was sampled upon incubation for four hours. Same as static culture samples, 100 μ L of incubated solution were transferred to well with four replicates in a 96-well plate for fluorescent reading. Quantitative examination was done against a calibration curve of known cell quantities. (*Calibration curve in Appendix 13*)

3.10 Biaxial mechanical test –in collaboration with BGU

After obtaining a dynamically cultured construct, biaxial testing was again conducted to reveal their heterogenous tensile properties in comparison to their native tissue, acellular ECM and statically cultured counterparts (n=3 for each group). This experiment was performed in collaboration with Prof. Jacob Bortman's Heart Modeling team at Ben

Gurion University (BGU). The biaxial tester (as described in Section 3.10.2) was custom-made by Prof. Jacob Bortman's Heart Modeling lab at BGU. The author prepared the reseeded samples and brought over to the BGU lab where she carried out the whole process of setting up the tester, calibration, testing and data analysis, with the training and assistance kindly provided by the BGU team. For the freshly-harvested native porcine heart tissues biaxial testing, the author and the BGU team together moved the tester from BGU to Lahav C.R.O. preclinical services to perform the testing. The author sincerely thanks the assistance and hospitality the team had rendered to make this experiment possible.

3.10.1 Specimen

ECM and reseeded ECM samples (n=3 per group) were prepared at the tissue culture lab at Technion and transported to BGU. Acellular ECMs were kept in PBS in flask. Reseeded constructs were kept in filtered capped flasks with culture media, and were incubated overnight before the day of testing in the tissue culture facilities generously provided by Prof. Smadar Cohen's lab at BGU. Each construct was cut into square samples sizes ranging from 3cm x 3cm to 5cm x 5cm. (Fig. 23)

Figure 23 Reseeded ECM for biaxial tensile testing.

Constructs in media in flask (A). Reseeded ECM cut into square dimension for biaxial testing (B-C).

Native tissue experiments were performed at Lahav C.R.O. – Comprehensive pre-clinical Services, where freshly harvested adult porcine hearts were obtained right after pigs' execution by electric shock. Hearts were cut into specific specimen sizes (n=3). (Fig. 24)

Figure 24 Native tissues for biaxial tensile testing.

Lahav C.R.O. – Comprehensive pre-clinical Services (A). Freshly harvested adult porcine heart (B). Native left ventricle wall (C).

3.10.2 Custom-made biaxial tester and sample mounting

The heart tissue biaxial testing system at BGU comprised the following components:

- i. Four uniaxial motors with a 25mm motion range, 80N force capacity and 5mm/sec maximum velocity.
- ii. Four load cells connected between the sample clamps and motors with load capacity of 20N and accuracy $\pm 15\%$.
- iii. Four samples clamps, each with a metal clamp base and a metal fixture, samples were gripped in between with sharp needles punching through the thickness.
- iv. An overhead camera [Basler] acA2500-14gm, resolution of 1944x2592, pixel size of $2.2 \mu\text{m} \times 2.2 \mu\text{m}$, 14 frames per second, 12 bit.

All system components were wire-connected to the controlling and testing interface in the computer. (Fig. 25)

Figure 25 Biaxial tester system set up at BGU

As demonstrated in Fig. 26, square samples were placed on top of the metal clamp base on the sample mounting stage. Four metal fixtures were then placed on the four edges of the specimen aligned with their respective metal clamp base location. Sharp needles (10mm puncture point length, 5mm tine-to-tine spacing) were then used to punch through the thickness of the specimen to fix the edges to the clamp.

Figure 26 Reseeded ECM specimen mounting for biaxial tensile testing.

The square biaxial specimen (A). Sample mounting stage (B). Clamped specimen (C).

The clamped specimens were then removed from the sample mounting stage; and connected to the four corresponding load cell heads. Attention has to be paid to ensure the cross-fiber and fiber-preferred directions were mounted onto X (across load cell 1-2) and Y direction (across load cells 3-4) of the equipment accordingly.

Samples were immersed in a water bath of 1L electrolyte of Plasma-Lyte A Injection pH 7.4 (Multiple Electrolytes Injection, Type 1, USP) [Baxter, USA] with 20mL of Esracain 2% (20mg/mL Lidocaine HCl) [Rafa Laboratories Ltd., Jerusalem, Israel], 15mL of Sodium Bicarbonate 8.4% and 40mL Potassium Chloride 14.9%. Plasma-Lyte is a sterile, non-pyrogenic isotonic solution that closely mimics human plasma for intravenous administration^[175-177]; where Esracain is commonly used in open heart surgeries settings as an intravenous injection for cardiac arrhythmia by inducing cardiac standstill .^[178,179]

Oxygen was diffused into the bath for fresh harvested native tissue and cell-reseeded samples by means of a silicone tube with holes punched and place at the bottom of the bath chamber. Immediately before the commencement of test, cosmetic mica powders were sprinkled onto the surface of the samples as markers for image processing purposes.

(Fig. 27)

Figure 27 Clamped biaxial samples in electrolyte bath.

Oxygen tubes placed under samples at the bottom of bath chamber (A) Powder markers sprinkled over the sample surface. (B)

3.10.3 User interface and testing protocols

Figure 28 User interface of the BGU biaxial tester.

Fig. 28 displayed the user interface of the BGU biaxial tester for data acquisition. First, calibrations of four load cells were done with different masses of weights to verify their accuracy and sensitivity. The coefficients of slopes of the calibration curve (voltage against mass) were transformed and then input as the load cell constants at the yellow box so the load experienced by each load cell as measured as voltage during experiment could be automatically computed for the calculation of corresponding stresses in X and Y direction.

(Calibration curve in Appendix 14)

Within the orange box was the real time measurement of the amplitude of loading in X-direction (across load cells 1-2) and Y-direction (across load cells 3-4). Real-time imaging of the deformation of the samples from our camera above the system was shown in the blue square. Experiment name (EXP NAME), file saving path, speed of stretch and maximum elongation were input in the red box. The three purple boxes controlled the forward and backward movements of the load cells and recorded the current position of each load cells. Thus the size of the sample could be defined as the width equals to the distance across load cells 1-2 and the length equals to the distance across load cells 3-4. The green box showed the nine options of stretching speed ratio between the X and Y directions. The ratio 0/10 corresponds to 0mm/s (i.e. 0% elongation) in the X-direction and 0.1 mm/s elongation in the Y direction, which can be related to a uniaxial testing but with the four edges clamped. The ratio 15/15 corresponds to an equibiaxially stretching of 0.15 mm/s elongation for both directions.

Prior to the biaxial data acquisition, samples were equibiaxially preconditioned with 7 cycles of stretching to 10% elongation at 0.1 mm/s.

3.10.4 Data processing

Loads were measured by the four load cells and computed into corresponding stress measurements. For strain measurement, apart from the straightforward displacement information as given by the X-Y motor, the real deformation and strain of the specimen were also monitored by the video tracking from the overhead camera and were extensively processed and calculated by image processing. Forty-six pictures were captured throughout the experiment time span of each elongation ratio of each sample.

(Example of Images captured during a biaxial test in Appendix 21)

A Matlab function “takemarkerJpg.m” (*Matlab codes in Appendix 15*) was used to divide each of the 46 images into 900 grids of markers. (Fig. 29)

Figure 29 Image divided into 900 grids.

Out of the 900 grids, those 90 grids located in the central region of specimen with a distance from the clamping edge were chosen to be defined as markers. (Fig. 30)

Figure 30 Selected grids were denoted as markers for motion tracking.

Subsequently, motion tracking was conducted by a Matlab function “drawMarkerMovement140305.m” (*Matlab codes in Appendix 16*) which visualizes the movement pathway of markers during the specimen deformation. Nine of the markers as indicated in Fig. 31 as red circles were picked, one at the center four at the corners and four at the mid-points of edges. Markers were manually picked based on the criteria of a clear movement pathway with minimum noise data points and being well distant (at least one marker in between) from each other across the central region of specimen. Ultimately, four quadrilateral “Elements” were formed by connecting the markers.

Figure 31 Motion tracking of markers.

Finally, the analysis was performed by a Matlab function “MotherOfAllExp.m” (*Matlab codes in Appendix 17*) to integrate the results of the execution of measurements of

1) Cauchy Stress measured by calibrated load cells, by the function “CauchyStresses140116.m” (*Matlab codes in Appendix 18*);

2) Motor Strains based on the motor-measured deformation, by the function “MotorsStrains140126.m” (*Matlab codes in Appendix 19*);

3) Image-processed strain as the real deformation of “Elements 1-4” representing four sections of specimen moving towards the four corners directions during the biaxial stretching,

by the function “ImageProssStrains140702.m” (*Matlab codes in Appendix 20*)

Stress-strain curves were then presented for both X and Y directions (Fig. 32), where stress equals to the Cauchy Stress (S_{xx} , S_{yy}); and elongation strain (E_{xx} , E_{yy}) based on the Motor Steps, as well as the image-processed strains of the Elements 1-4.

Figure 32 Stress-strain curves output from image processing in biaxial testing.

3.11 Statistical analysis

Statistical methods or algorithms used in this thesis work were available in the Microsoft Office Excel statistical tools and functions.

Student's T-test was used for evaluate statistical significances in the differences of means of two data sets, which assume to be originated from same population before and after a treatment (distributions with equal population means). In this thesis work, the treatment refers to the decellularization of ECM and hence, for instance acellular ECM and native tissues. Alpha value of 0.05 was used. Results are expressed as the mean $\pm$ standard deviation of $n\geq 3$ and a two-tailed p -value of $p<0.05$ was considered significant.

Student's t-Test Assuming Unequal Variances was used for two data sets with different data points came from distributions with unequal variances. Alpha value of 0.05 was used. Results are expressed as the mean $\pm$ standard deviation of $n\geq 3$ and a two-tailed p -value of $p<0.05$ was considered significant.

One-way (Single factor) ANOVA (analysis of variance) was used to determine the significance of effect of a single factor in more than two groups of data sets. In this thesis work, the single factor refers to presences of cells. Alpha value of 0.05 was used. p values are expressed to determine the significances, which correspond to the relative ratio of variance between groups and the variance within group. $p<0.05$ was considered significant.

For regression analysis, all R^2 are adjusted ($R^2_{adjusted}$) to correct for sample size and model complexity bias. A correlation of $R^2_{adjusted}\geq 0.9$ was required for valid analysis.

Chapters 4-9. Results and Discussion

These six chapters present deliberate the results and discussions obtained in achieving our goal to experimentally show improved biophysical functionality of reseeded ECM-based cardio-mimetic constructs through optimized culturing methods; and to rationalize the results with computational modeling and correlation among the biophysical properties of the biomaterials.

Chapter 4 first presents the results and discussion of the recellularization strategies to obtain the reseeded ECM constructs through optimized culturing methods.

Chapters 5 to 9 then present the biophysics, with the emphasis on biomechanics, of the reseeded ECM construct, in comparison to acellular ECM and the native porcine myocardium.

Chapter 5 covers the uniaxial mechanical testing. Chapter 6 details the analysis and evaluation with the developed viscoelastic computational modeling tool. Chapter 7 covers biaxial mechanical testing and summarizes the mechanical testing results obtained across chapter 5-7. Finally, chapter 8 and 9 presents the entire collections of surface and bulk biophysical characterizations respectively.

Chapter 4. Recellularization strategies of the acellular pcECM

We hypothesize that with optimized culture conditions, our reseeded porcine cardiac extracellular matrix constructs could be a suitable cardio-mimetic construct for myocardial tissue engineering.

This chapter presents the results and discussion of the recellularization strategies used for obtaining the reseeded ECM constructs. The optimized seeding and culturing methods were performed as in sections 3.6.1, 3.7-3.9. Protein adsorption capabilities of the pcECM surfaces was first evaluated to facilitate the decision of which surface the cells should be seeded on for a better cell-substrate interaction outcome. Four commonly used cell-seeding methods were then employed to repopulate ECM with human bone-marrow derived mesenchymal stem cells (MSCs) in order to select the most efficient one for subsequent recellularization experiments. Finally, three different culturing regimes in static and dynamic conditions were employed. They are compared in term of efficacy of culturing methods and cell proliferation.

4.1 Protein adsorption study on ECM

The protein adsorption capabilities of the pcECM surfaces was evaluated by adsorbing Bovine Serum Albumin labeled with fluorescein isothiocyanate (BSA-FITC) onto the epicardium and endocardium surfaces of the pcECM over different incubation spans, followed by fluorescent measurement to quantify the protein adsorbed against the calibration curve. Comparison was made against the surface of the same area of 24-well tissue culture plate. Fig. 33 presents the BSA adsorption kinetics over 120min on the three surfaces.

Figure 33 Protein adsorption kinetics of ECM.

Bovine serum albumin (BSA) was adsorbed over 120min on the pericardium and endocardium surfaces of ECM, compared to tissue culture plate. Surface area of each sample disc= 2.14cm². Data were collected in triplicates of five independent samples (n=5) in each of the three groups of surfaces.

The result showed a consistently higher BSA adsorption on the two surfaces of ECM than tissue culture plates (TC plate) for all time points, indicating a higher protein-substrate interaction between the BSA and ECM surfaces than that of BSA with TC plate.

TC plate demonstrated a typical adsorption kinetic profile as most synthetic materials. The adsorption was rapid in the initial stage and eventually reached a plateau.^[145,180] For our ECM, BSA continuously adsorbed onto the surfaces, with the endocardium bottom layer having significantly higher adsorption. At 120min, BSA adsorbed on ECM-pericardium, endocardium and TC plate were 2.52 ± 0.24 , 3.16 ± 0.6 , and 0.78 ± 0.12 μg respectively.

Adsorption on pericardium and endocardium were significantly higher than TC plate ($p < 0.03$) while no significance difference was found between pericardium and endocardium ($p = 0.11$). Nonetheless, it showed that our ECM is highly surface-active in terms of protein adsorption, which may indicate favorable cell adhesion of it over TC plate.^[144,150,151] For the subsequent recellularization experiments, we chosen the endocardium surface of the ECM as it showed a slightly higher protein-substrate interaction outcome.

4.2 Comparing seeding methods

MSCs was recellularized onto our ECM construct using four seeding methods commonly used in the literatures.^[11,77,141,142,173] They include static pipetting, needle injection, vacuum pump and centrifuge seeding, as detailed in Materials and Methods section 3.8.2. The initial cell seeding efficiency was determined using AlamarBlue® measurement five hours after seeding. As presented in Fig 34, static pipetting, needle injection and vacuum pump seeding methods all yielded respectable efficiencies of over 40% of initial seeding cell numbers, except for centrifuge seeding which provided cell attachment of only 25% of the initial cell seeding numbers. The 40% percentage (post 5-hr) of hBM-MSCs attachment was lower than what we previously obtained for rat MSCs on our ECM scaffold (about 80% post-90min)^[86] but higher than what we previously obtained for HUVEC on

our ECM scaffold (about 30% post-90min) ^[87], which indicates the different seeding performance of different types of cells onto our ECM scaffolds.

Thevenot *et. al.* also studied the effect of four seeding methods on cell attachment with their mouse fibroblasts seeded PLGA salt-leached scaffolds.^[142] Our results resembled what they obtained that surface and injection seeding were superior to centrifuge seeding in terms of initial cell attachment number. They have also observed that the attached viable cells dropped from 75% of seeding numbers (post 3hr) to 62% (post 6hr), which may explains the decrease of attached cells we observed for post-90min to post-5hr.

Figure 34 Initial cell attachments of the four seeding methods.

* $p < 0.05$. (n=3)

The 40% attachment of the seeding cells was obtained 5 hours after the initial seeding with the optimized attachment time of 90min.

Fig. 35 displayed the cell growth measurement by AlamarBlue® assay carried out on day 1, 3, 7 and 10 from the day of reseeding. The result showed that for all seeding methods,

MSCs proliferated in the ECM to about three-fold of initial seeding numbers over the ten days of culture, reconfirming the highly cell supportive surface of the ECM. [18,53,86]

Figure 35 Cell proliferation upon the four seeding methods.

Surface area of each sample disc= 2.14cm². (n=4 sample per group)

By comparing Fig. 34 and 35, we observed a further reduction of attached cells from 40% (post 5hr) to 15% (post 1 day) but a gradual increase from day 1 onwards until day 10. Although needle injection and vacuum pump seeding showed significantly higher initial loading in Fig. 34, alternative seeding methods have no significant impact on MSCs proliferation within our ECM after 10 days of culture as shown in Fig. 35. The gradual proliferation resembled what we previously obtained for MSC and HUVEC on our biochemically modified ECM scaffold [87]. Hence, given that the different seeding methods do not offer significant advantage over the simplest form of surface pipetting seeding, and that as suggest from Thevenot *et. al.* the viable cells (post 3hr and 6hr) of surface seeding is among the highest of other seeding methods [142], we applied surface pipetting seeding for its ease of execution of the subsequent recellularization experiments.

4.3 Observation on reseeded samples

After one day of seeding, reseeded ECM with cells DiI pre-stained were fixed and viewed under confocal microscope to confirm cell existence. Pink dots of cells aggregates were visible on the reseeded ECM surface (Fig. 36A) and different sizes and forms of cells clusters were observed at regions of interest that visualize the presence of cells on mainly the seeding surface during the initial culture. (Fig. 36B-D)

Figure 36 DiI Staining of cells and confocal microscopy images.

Pink staining of cell aggregates on ECM. (A) Magnifications of ROI visualizing the presence of cells on the seeding surface. Green-squared ROI. (B) Magnification of red-squared ROI. (C) Magnification of orange-squared ROI. (D)

From two days upon recellularization with MSCs, we observed that reseeded ECM construct shrank with an intermediate texture between non-seeded ECM and native heart tissue from handling. (Fig. 37) This may demonstrate that the cells resided in our ECM and started changing their extracellular environment.

Figure 37 Comparison of the three samples appearances.

"Loose and fluffy" texture of acellular ECM (A). "Firm" texture of native (B). Reseeded-ECM of "intermediate" texture (C).

AlamarBlue® assay determined viable cell numbers against a calibration curve of known concentrations of cells. As viable cells reduced the resazurin (blue colour) to resorufin (pink) within the AlamarBlue® solution, the color change in the solution after incubation could visually indicate cell growth within the construct, while the pink color patches found on the reseeded ECM could facilitate the locating of the cell clusters. Fig. 38 displayed an example of the progressively changing colours of AlamarBlue® solution within a static-reseeded ECM over a culture span of two weeks. The shrinkage of the construct was also obvious in these four images.

Figure 38 Color changes of the AlamarBlue® in reseeded ECM construct.

For dynamic-reseeded ECM, the major colour changes of AlamarBlue® solution from blue to pink were evident along the artery in which oxygenated culture media was perfused. (Fig. 39A) This visually showed that cells were attracted to the area where nutrients and oxygen were freshly supplied. The pink color patches found on the reseeded ECM differentiate the locations of the growing cell clusters from the less-densely cell populated portions of the ECM. (Fig. 39B) The dynamic-reseeded ECM at the end of the culture spans was found to become a more shrunk construct than the static-reseeded ECM, with a texture close to native heart tissues from handling. (Fig. 39C)

Figure 39 Images of dynamic-reseeded ECM construct.

(A) Dynamic-reseeded ECM taken out from the bioreactor after 4-hr of AlamarBlue® solution perfusion showed evident pink colour changes along the artery areas. (B) Pink patches on the AlamarBlue®-incubated reseeded construct facilitated the location of cell clusters. (C) A shrunk dynamic-reseeded ECM with texture similar to native myocardium at the end of the culture span.

4.4 Cell viability and proliferation

Two similar perfusion bioreactor units under the same medium flow system were employed in NTU and Technion respectively for the study of the dynamic-cultivation of our reseeded ECM constructs. (as detailed in section 3.9.1) The custom-developed bioreactor unit was used in NTU during initial studies and optimizations, whilst the Synthecon™ bioreactor unit was used in Technion during the author's research attachment in the Technion Lab for the actual data acquisition. Following initial studies

and optimizations at NTU, the author focused on the analysis of the results obtained from the Synthecon™ bioreactor system which were used as dynamic reseeded group data presented within most of this thesis.

Three construct culture regimes were employed and compared. For static-alone culture (Stat), after ninety min of attachment, reseeded ECM was kept in flask for culture statically up to seventeen days. For Dynamic-alone culture (Dym), after ninety min of attachment, reseeded ECM was immediately moved to bioreactor bath chamber and undergone perfusion culture dynamically up to fourteen days. The third regime combined the static and dynamic culture (Stat-Dym). After ninety min of attachment, reseeded ECM was kept in flask for culture statically up to ten days, and then was moved to the bioreactor bath chamber and undergone perfusion culture dynamically for another ten days.

Seeding efficiency was measured at day 1 upon the reseeded. Fig. 40 revealed the comparison of ratio in percentage of the cell numbers after one day of culture to their initial seeding numbers between the 'Stat' and 'Dym' cultures.

Figure 40 Seeding efficiency measured at day 1 on stat and Dym culture.

* $p < 0.05$ ($n=6$ for static; $n=3$ for dynamic)

Attachment efficiencies measured after day 1 were $13.8 \pm 3.8\%$ of the initial seeding numbers for 'Stat' culture; and $7.42 \pm 1.8\%$ for 'Dym' culture. 'Stat' gave a significant higher cell attachment numbers than 'Dym' ($p=0.01$).

This result suggested that it might be important to allow time for the cells to settle and reside on the ECM upon seeding, before subjecting them to a dynamic culture environment in which the medium perfusion may possibly wash away the cells before they attached onto the ECM.

Cell growths on the three groups of samples at different time points were followed over their culture span. The cell numbers normalized to the cell numbers measured day 1 post-seeding are presented in Fig. 41.

Figure 41 Cell proliferation comparison of the three culture regimes.

Stat: static, Dym: dynamic and static-dynamic combined culture.

For 'Dym' samples, minimal cell growth was observed, with the cell quantities at day 14 only measuring $948 \pm 3.46k$ of cells. Together with the cell attachment results shown in Fig. 40, it might suggest that the deficiency of the initial attachment of cells may hinder the subsequent cell growths. 'Stat' samples showed a steady growth over 2 weeks, with the cell quantities at day 17 measuring $2.96 \pm 1.22M$ of cells. For 'Stat-Dym' samples, a better cell growth can be clearly observed in comparison to their 'Stat' and 'Dym' counterparts. Maximum cell quantity was measured at day 20 of $7.6 \pm 1.9M$ of cells. One of specimen showed almost a 6.5 fold of the cell quantities measured at the day 1 post-seeding. This provided a clue that the cells could grow better if the dynamic culture was performed some times after the static culture, which might allow time for the MSC to settle and reside well on the ECM before undergoing perfusion.

Chapter 5. Uniaxial mechanical testing

Ventricular myocardium experiences non-stop dynamic cyclic loading over a human life span. Thus, any heart tissue substitute requires a stiff and strong material to withstand the tension and pressure, at the same time it needs flexibility to accommodate contracting motions during blood pumping. As discussed in the Introduction section 1.5.1, tensile testing seems to be a more relevant and applicable method on characterizing the macro-mechanical properties of our soft and thick biological materials. Yet in order to securely mount the wet and thick specimens onto the commercial tensile tester, a sample mounting method had to be developed.

In our uniaxial tensile experiments, we sutured the two edges of the specimens onto two ejection pins, which were then fitted onto the equipment standard fixtures. The efficacy of the method, the effect of different suture materials and specimen dimensions are first discussed in section 5.1.

Section 5.2 details the results and discussions of the experimental data of our materials' mechanics under three mechanical testing assays. The similarities and discrepancies between the properties of acellular ECM and native myocardium are first highlighted. Then the results of our reseeded ECM-based cardio-mimetic construct with human bone-marrow derived mesenchymal stem cells (MSC) are presented as the evidence of the recovery of biomechanical properties towards that of native myocardium.

5.1. Evaluation of sample mounting method

When testing wet and thick specimens, most commercially available grips fail to impose sufficient gripping force to prevent slippage. In contrast, some of them introduced over-intense local stress at the gripping region that cause premature deformation of the specimen even before the actual test. In this study, the method of using sutures to stitch the two ends of specimen onto mounting fixtures was tried out as inspired by surgical procedures of how wounds are closed or how implants are connected to host tissues^[9,20,23,166-168].

From our handling, this method could not only avoid the introduction of local compressing pressure at gripping regions, but could also maintain the thickness of specimen (which is the most important figure of interest in this study) by the stitching loops at the edges of specimen around the fixture rods. The samples were securely mounted onto the tensile machine and all the three tensile testing assays were performed smoothly without specimen slippage or misalignment or premature fracture.

The additional advantage of this suturing method is that we could obtain some clinically-relevant insights as in how our ECM construct could be transplanted to connect with host myocardial tissues by suturing with reference to the literature ^[9,20,23,166-168]. Given that there might be possible tearing of tissues at the stitching sites at higher tension levels, the results yielded might also suggest the suture holding strength or the tear resistance of the materials.

The custom-designed fixtures composed of spring ejector pins have eased the mounting of specimens by a simple action that “insert” the samples to the equipment grips. It also greatly facilitated continuous samples testing as a second samples can be sutured onto another pair of pins while the first sample is in test.

5.1.1 Effect of suture materials used on Young's Moduli

Two kinds of suture materials were used to stitch the two edges of the specimen onto our spring ejection pin fixtures, namely, 100% polyester sutures and surgical braided PGA sutures.

Fig. 42 displays the comparison of the Young's Moduli of ECM and native specimens mounted on fixture by the two different kinds of sutures. No significant difference of the same sample between the two suture materials was measured. ECM was found significantly stiffer than native samples ($p \leq 0.02$) for both suture materials.

Figure 42 Young's Moduli of specimens mounted by two kinds of sutures.

ECM and native tissue specimens were mounted onto the tensile tester fixture by two different kinds of sutures: 100% polyester sutures and surgical braided PGA sutures. * $p < 0.05$ ($n=3$)

5.1.2 Effect of specimen dimensions used on Young's Moduli

Young's modulus, measured by fitting the linear region of the stress-strain curve under a uniaxial tensile failure test, should be an intrinsic material property of the specimen by definition, regardless of the specimen dimension and sample gripping methods. [122,181,182]

Therefore, we compared the two specimen dimensions used as stated in Method section 3.1. Large ECM specimens refer to 30mm x 70mm and small ECM specimens 30mm x 30mm. The full thickness of samples including the intact epicardium and endocardium was maintain.

Observation on the deformation and fracture behaviors between the specimens revealed no obvious differences. No significant difference in Young's modulus of the ECM samples was measured (Fig. 43). Standard deviation among sample replicates was lower for small specimens than larger specimens, suggesting a higher uniformity within the smaller specimens, which actually favor experimental controls and comparison.

Figure 43 Young's moduli of specimens in two dimensions.

ECM specimens were into two different dimensions and tested. Large ECM: 30mm x 70mm and small ECM: 30mm x 30mm. (n=5)

5.2 Uniaxial tensile testing results of the three sample groups

Myocardial tissue engineered construct with inadequate modulus has shown to fail in reducing infarcted myocardial wall stress and attenuating LV dilation; whilst inadequate

flexibility can result in inefficiency to perform myocardial contraction and expansion. [3,16,183] Any mismatch in elasticity can hinder the proper integration with host tissue and the conduction of mechanical stimulating cues from the host myocardium.

In addition, substrates with similar elasticity as native tissue have been shown to direct MSCs growth and expression of precursor proteins for typical cells present in the tissue, give rise to a more functional biomedical device. [148,184] Hence, the mechanical properties of our three sample groups, namely, acellular ECM, reseeded ECM construct and native tissues were tested in their full thickness using the three sequential mechanical assays consisting of cyclic loading, stress relaxation, and tensile failure assays.

5.2.1 Observations on specimens deformations and failure behaviors

Fig. 44 displayed a close examination of the deformation and fracture behaviors of the samples.

Figure 44 Representative images of samples in uniaxial tensile test.

It could be observed that ECM exhibits a cone-like fracture surface as a more 'ductile' material compared to native tissues with a 'brittle' flat fracture surface, occurring near to the stitching region. The reseeded ECM gives an intermediate deformation and fracture profile between the acellular ECM and the native tissues, indicating the recovery of structural properties due to the presence of cells.

5.2.2 Cyclic loading

A cardio-mimetic scaffold should withstand the physiological working conditions of the heart with repeated cyclic loading of 15%-17% longitudinal strain.^[3,11,34,35] To address the scaffold mechanical compatibility of our constructs with the cyclic working loads in the human heart, dynamic cyclic loading up to 15% strain were performed for three cycles on our samples upon preconditioning.

Figure 45 Representative stress-strain curves of cyclic loading.

Due to viscoelasticity of soft biological materials, hysteresis is often observed between the loading and unloading curves, where not all the energy expended during loading can

be recovered upon force removal. ^[120,169] In our study, all materials displayed a resilient loop stress- strain profile, (Fig. 45) reflecting that energy can be stored within the biological tissue for dissipation. This may suggest the possible recovery of original shape of the tissues following the physiological-like pumping action.

The similar stress-strain profile of the ECM and native tissues qualitatively indicated the similar capacity in cyclic working loads of the materials, and an apparent recovery of cyclic loading behavior was found with the reseeded samples, which closely resembling the native tissues compared to acellular ECM.

Energy dissipation was measured by area blocked within the stress-strain curves during the last cycle of the dynamic cyclic loading and unloading, and presented in Fig. 46. ECM dissipation was $6.78 \pm 1.93 \text{ kJ/m}^3$ while that of native myocardium was $7.77 \pm 1.85 \text{ kJ/m}^3$, with no significant difference between. The measurement of reseeded ECM was $7.22 \pm 1.27 \text{ kJ/m}^3$, remaining no significantly different.

Figure 46 Comparison of energy dissipations of the three samples.
(n=5)

5.2.3 Stress relaxation

Samples were stretched at 0.5mm/s to 20% of strain; and then held at the fixed displacement for over 10 minutes to reveal their stress relaxation behavior.

Under stress-relaxation tests, all materials exhibited viscoelastic behaviors as demonstrated in Fig. 47 where samples held at a specific constant strain exhibited a force-decay over time. Reseeded ECM was found to be more native resembling with lower maximum and equilibrium stress levels.

Figure 47 Representative stress-time curves of stress relaxation.

According to the graphic presentations, ECM seemed to be of a relatively greater force decaying and shorter relaxation time compared to native tissue. Yet, in order to comprehensively evaluate their viscoelastic properties and accurately determine the viscoelastic parameters, we proposed an analytic tool for implementing a single-relaxation-time model as detailed in section 4.2.

5.2.4 Tensile failure

The last assay in the sequential tensile testing involved the stretching of samples until their fractures. Fig. 48 displayed the representative stress-strain curves of our three sample groups under the tensile failure test.

The tensile-failure stress-strain profiles of all the samples were found to be comparable to some previously reported biological soft tissues, revealing a toe region prior to the linear tensile region, followed by the linear elastic region, the ultimate stress and the eventual failing.^[36,123]

Figure 48 Representative stress-strain curves of tensile failure.

Toe region corresponds to the non-linear high deformation-low force behavior as observed during the initial 10% strain.^[36,123] It could be attributed to the crimped collagen fibers that are heterogeneously distributed throughout the tissue before the loading.^[185-188] As displacement increase, they are recruited and the straightening of the crimped fibers occurs. Upon the un-crimping of fibers, the stress level reaches the linear tensile

region. The slope of the linear region of the stress-strain curve can then be measured as Young's Modulus. It generally reveals strength of the material against deformation (i.e. modulus), and it is mainly contributed by collagen fibers that bear loads biological materials. [36,123,185]

ECM has a steeper slope at the linear elastic region, a higher ultimate strength and a lower deformation strain when compared to native tissues. It was encouraging to observe that the strain-to-failure stress-strain profiles of the MSCs-reseeded construct has shifted to be more native-resembling, indicating the recovery of deformation profile from the decellularized ECM to reseeded ECM towards native heart tissues; with a trend of less steep slope in linear elastic region, decreasing ultimate stress, and increasing ultimate strain. Fig. 49 presents the calculated Young's moduli of the three samples.

Figure 49 Comparison of Young's Moduli of the three samples.

* $p < 0.05$ ($n=5$)

A significant higher Young's modulus of ECM ($1.38 \pm 0.76 \text{ MPa}$) was measured than native tissues ($0.25 \pm 0.11 \text{ MPa}$) ($p=0.02$). This result is consistent with the findings of existing

literature, which reveal a stiffer decellularized extracellular matrix than native tissues. For instance, Wang *et al.*^[119] tested the tensile failure performances of their 2mm thick cardiac patch with decellularized porcine myocardial scaffold on uniaxial machine [Mach-1, Biosyntech] after 10 cycles of preconditioned loading at a ramp speed of 0.1 mm/s. They also utilize a biaxial mechanical testing system to load the specimens up to a tension of 30 N/m^[189]. They found that both thin slices of native myocardium and acellular scaffold showed anisotropic behavior where the fiber-preferred direction was stiffer than cross fiber-preferred direction. They also observed that a stiffer stress–strain behavior upon the removal of the muscle cells from native myocardium. Ott *et al.*^[98] stretched their decellularized rat left ventricle using a biaxial mechanical testing machine [Instron] equibiaxially to 40% strain. Samples were elongated in increments of 4% strain and then being allowed to relax for 60 s at each strain value. They obtained similar results on the moduli increase after cell removal, as well as and heterogeneous behaviors.

Numerous literatures have discussed the correlation between the composition of the biological tissues and protein-based biomaterials and their mechanical properties [37,78,116,119,123,190,191]. We therefore suggest that the stiffer mechanical properties of the ECM is probably due to a higher proportion of collagenous proteins in the ECM after cell removal, resulting in a denser and more compact scaffold composed mainly of collagen; while more elastin stored in the intact vasculature in the native tissues contribute to a high strain before ultimate stress reached.

Young's modulus of reseeded ECM (0.69 ± 0.1 MPa) was substantially reduced from acellular ECM ($p > 0.05$) although it is still significantly higher than native tissues. This reflects that the presence of cells recovered the mechanical properties of the decellularized cardiac tissue, probably owing to the cells secreting their ECM proteins or

adhering onto collagen fibers to give flexibility and reduce the modulus. Hence, potential synthesis of new collagen by the reseeded cells should be investigated by chemical assays.

Toughness of the samples were measured by the area blocked under the entire stress-strain curve in the tensile failure test and presented in Fig. 50.

Figure 50 Comparison of toughness of the three sample groups.

(n=5)

The toughness of ECM, native and reseeded was measured as $2.19 \pm 0.22 \text{ MJ/m}^3$, $1.75 \pm 0.33 \text{ MJ/m}^3$, and $2.28 \pm 0.35 \text{ MJ/m}^3$ respectively. Despite the seemingly difference in ultimate stresses and ultimate strains, the toughness of the three groups materials remain not significantly different from each other. This possibly indicates that the overall collagenous structural framework was preserved upon decellularization.

5.3 Summary of uniaxial tensile testing results

Table 1 Summary of uniaxial tensile measurements of the three sample groups.

	ECM	Reseeded ECM	Native
Energy dissipation (kJ/m ³)	6.78	7.22	7.77
Standard deviation (S.D.)	1.93	1.27	1.85
Young's modulus (MPa)	1.38	0.69	0.25
Standard deviation (S.D.)	0.76	0.1	0.11
Toughness (MJ/m ³)	2.19	2.28	1.75
Standard deviation (S.D.)	0.22	0.35	0.33
Sample size (n)	5	5	5

As summarized in Table 1 of the biomechanics studied under uniaxial tensile testing, our ECM construct was shown to closely mimic the stress-strain profile as that of native tissues, with the reseeded ECM displaying improved resemblance. Energy dissipation and toughness were found to be of no significant difference between the three groups, while Young modulus of the ECM was significant higher than native tissues. Reseeded ECM has shown a substantial, although not significant, reduction of the modulus from the acellular ECM, with a deformation and fracture profiles closer to that of the native tissues. This suggests the potential reversibility of the biomechanics upon recellularization of our ECM-based cardio mimetic construct.

Chapter 6. Viscoelastic analytic tool

The use of decellularized tissues or other complex biomaterials as scaffolds for tissue engineering inevitably requires a thorough investigation of their viscoelastic and failure properties. However, such investigation is often limited by the lack of easy-to-use analytic tools to analyze the ample data resulting from standard mechanical tests.

The biomedical potential of our novel decellularized pcECM motivated us to develop such analytic tools in order to efficiently analyze its viscoelastic properties. These tools could also be applicable to other soft tissues. We developed an algorithm that fits the experimental data to the Zener viscoelastic model executed by an easy-to-use MATLAB® code. The development of the algorithm has been presented in Method section 3.5 and the MATLAB® programming codes are presented in Appendix 4-8.

This chapter presents and discusses the results of the viscoelastic and failure analysis performed by the tool. Section 6.1 first discusses the superiority of our chosen viscoelastic Zener model compared to the other two existing models as mentioned in Introduction section 1.5.4. Section 6.2 evaluates the delineation of toe border performed by the tool. Section 6.3 presents the analysis results on acellular ECM, reseeded ECM construct and native tissues with elaborated discussions. Afterwards, section 4 discusses the overall performance evaluation and prospects of the analytic tool developed. Finally, section 6.5 discusses the overall comparison of viscoelastic properties of the three sample groups and the significance of the findings.

6.1 Comparison to existing models

To begin with, we first compared the Zener model with two other viscoelastic models, namely the Maxwell and the Kelvin-Voigt, to verify the quality of fitting for the complete stress relaxation data; as well as the elastic Hook model to ensure the linear region fitting was valid. A screen shot of the graphical outputs on a typical ECM sample is presented in Fig. 51.

Figure 51 Models fitting on stress relaxation data of a typical ECM sample.

The three upper plots, displayed from left to right, represent 1) the strain-rate profile for the stress-relaxation experiment over the experiment time span of 10min; 2) the fit between the Hook model and data drawn from the tensile mode only; and 3) the fit between the data and the linear approximation of the Zener model. The three lower plots, displayed from left to right, represent the fit between the data and the 4) Zener, 5) Maxwell and 6) Kelvin-Voigt models, respectively. E1, E2 represent the initial estimates for the Zener elastic moduli and Eta represents the initial estimates for the Zener viscosity modulus. $R^2_{adjusted}$, SE and p represent the statistical measures of the model fit, error and significance respectively.

The fit between the linear region of our data and linear Hook model showed an $R^2=0.99$, which confirm that the linear region fitting was valid. The fit between our data and the linear approximation of our Zener model showed an $R^2=0.69$ while $R^2=0.976$ was achieved for the fully developed Zener model. The fit against the Maxwell and Kelvin–Voigt models are of $R^2=0.87$ and 0.62 respectively. It was clearly shown that out of the four viscoelastic models (linear Zener, Zener, Maxwell, and Kelvin–Voigt), which can all mathematically accommodate both tensile and stress-relaxation modes, only the Zener model was found to correspond to the mechanical response data collected from both testing modes.

When comparing the several models' fitting capabilities on the ECM and native tissue (Fig. 52), a close correlation of $R^2>0.94$ between the Zener model and experimental data was achieved and found much superior to two other common viscoelastic models. The quality of fit for the data drawn from the linear region was highly comparable to that of the Hookean ideal spring model.

Figure 52 Comparison of model fit of the four elastic and viscoelastic models.

(n=5)

6.2 Delineation of toe border

Biological materials usually displayed a non-viscoelastic toe region, right before the linear tensile region in a stress–strain curve. ^[36,123] For a better fit of the viscoelastic model to experimental data, the toe region has to be delineated such that the borders between the toe region and the linear region can be clearly defined for the calculation of the Young's modulus.

To do so, we have first fitted the data to the linear Hook model to produce the Young's modulus E by calculating the slope. For stress-relaxation data, after fitting the linear model, the rest data was fitted to the Zener model within the interval of t_0 , which was optimized after the toe region, until the point of transition between the tensile linear and the stress-relaxation modes (t_1). For tensile-failure analysis, t_0 was defined at the end of the toe region and t_1 would be designated as the beginning of the viscoelastic region. Hence, the t_0 is now better determined to serve as a more accurate starting point for other analyses concerning the toe border. It also allows a more fair comparison among different materials by using this non-biased computational method.

We compare the strain values of the toe regions between the tensile-relaxation tests (at strain rate of 0.5 mm/s) and the tensile-failure tests (at strain rate of 0.05 mm/s) on the ECM and native tissue samples. (Fig. 53)

Figure 53 Comparison of strain values of toe regions.

ECM and native tissue samples were tested at strain rate of 0.5 mm/s for the tensile-relaxation tests and at strain rate of 0.05 mm/s for the tensile-failure tests. (n=5)

As discussed previously in section 5.2.4, toe region corresponds to the non-linear high deformation-low force behavior as observed during the initial 10% strain ^[36,123], which might be due to that collagen fibers are heterogeneously distributed throughout the tissue and are crimped before the loading. Within the toe region, as displacement increases, they are recruited and the straightening of the crimped fibers occurs. Hence, with no significant difference found in all groups, it may suggest that the crimping and un-crimping behaviors of the collagen fibers under low degree of loading were preserved upon decellularization.

6.3 Viscoelastic analysis results of the three sample groups

As discussed in Introduction section 1.5, most existing analytical methods relied on graphical evaluation or estimations of a single mechanical testing mode for the relaxation time. This could hinder possible comparison between materials due to the lack of common ground. As relaxation time is a parameter that critically reflects the viscoelasticity of

materials, it should be carefully defined. Here with our Zener model analytic tool, it is now possible to mathematically calculate the relaxation time. The model is capable to resolve the elastic moduli and viscosity modulus, and failure profiles of samples tested under tensile-relaxation and tensile-failure tests, and hence allowing theoretical calculation of equivalent elastic modulus, relaxation time, and determining the load limits governing the failure of materials. This section presents the analysis results on acellular ECM, reseeded ECM construct and native tissues, along with elaborated discussions. The viscoelastic behavior and failure profiles observed for both the ECM and native tissue were found to be highly correlated with our Zener model. ($R^2 > 0.94$) (Data not shown) The experimental data derived from our reseeded constructs remain highly correlated with $R^2 > 0.93$, which further validated our model analysis capability on a range of biological samples.

6.3.1 Zener elastic moduli and viscosity moduli

Our Zener model-implementing algorithm resolved the Zener elastic moduli (E_1 and E_2) and viscosity moduli (η). Fig. 54 displays the measurements on acellular ECM, reseeded ECM construct and native tissues.

Figure 54 Zener modeled viscoelastic measurements of the three sample groups.

Our Zener model measured the modeled elastic moduli (E_1 and E_2) (A) and viscosity moduli (η) (B) of acellular ECM, reseeded ECM construct and native tissues. * $p < 0.05$, ** $p < 0.01$ ($n=5$)

The measurements between acellular ECM and native tissues revealed that E_1 significantly increases following decellularization, where E_2 significantly decreases for the decellularized ECM. (Fig. 54A) The equivalent elastic modulus E^* , as measured by the ratio of the product of $(E_1 \cdot E_2)$ to the sum of $(E_1 + E_2)$ and supposed to reflect the overall modeled elasticity of the material, was calculated to be about 4-fold larger for the acellular ECM than the native tissue. This result is consistent with our finding and discussion in chapter 5, implying an increased modulus due to decellularization.

No significant difference in the modeled viscosity modulus (η) was found between acellular ECM and native tissues (Fig. 54B), implying that the viscosity was preserved following decellularization. The results of the reseeded ECM showed a promising reduction in the elastic modulus E_1 and increase in E_2 , while remaining not significantly different in the modeled viscosity modulus (η) when compared to acellular ECM. This restoration of viscoelastic properties should be attributed to the presence of cells that give flexibility to the construct towards resembling the native properties.

To validate the finding that ECM is stiffer than native tissues, we performed a comparison on the elastic moduli calculated according to the various implemented models; namely, the elastic modulus E for the Hook model (i.e., Young's modulus), the Maxwell model and the Kelvin-Voigt model and E_1 for the Zener model. (Fig. 55)

Figure 55 Elastic moduli measured by the four elastic and viscoelastic models.

Elastic moduli were calculated according to the various implemented models; namely, the elastic modulus E for the Hook model (i.e., Young's modulus), the Maxwell model and the Kelvin-Voigt model and E_1 for the Zener model. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ($n=5$)

Under all models, elastic moduli of the acellular ECM were significantly higher than native tissues. The average of all elastic moduli of acellular ECM under all models was significantly higher than native tissues with $p < 0.001$. This reaffirm our conclusion that acellular ECM is stiffer than native tissue, which could be attributed to a denser and more compact ECM mainly composed of collagen fibers, and could also be attributed to the loss of cellular components

6.3.2 Zener modeled relaxation time

With the use our developed Zener model analytic tool, we were able to obtain a mathematically determined, non-biased value of relaxation times, instead of relying on graphical estimations. (Fig. 56) The Zener modeled relaxation times measures the ratio between the modeled viscosity modulus (η) and the sum of the modelled elastic moduli ($E_1 + E_2$).

Figure 56 Zener modelled relaxation times of the three sample groups.

(n=5)

Despite the variations between the Zener elastic moduli of the native and decellularized pcECM, their Zener modelled relaxation times were found to be of no significant difference. Reseeded ECM construct also remained no significantly different relaxation time as compared to ECM and native tissues. These new crucial results of similar modelled relaxation times of the three sample groups demonstrate their similar viscoelastic biomechanics.

6.3.3 Zener modelled elastic and integrity limit

The failure points, designating the loss of elasticity and integrity in a tensile failure experiment, were assumed to be normally distributed and measured as the multiplication products of the stress•strain at the time of the each failure. The determinations of them were detailed in Method section 3.5. Fig. 57 presents the Zener modelled elasticity and integrity limits of acellular ECM, reseeded ECM construct and native tissues.

Figure 57 Zener modelled elasticity and integrity limits of the three sample groups.

** $p < 0.01$ ($n=5$)

The elastic limit was significantly larger for the acellular ECM compared with native tissue while the integrity limit of it was significantly lower than native tissue. Reseeded ECM was found to significantly increase the integrity load limits from acellular ECM towards native tissues, indicating that the stress-strain behavior approaching specimen failure of the reseeded ECM was closed to that of native tissue.

An increase in the elastic limit was however found. The elastic limit was calculated as the multiplication products of the stress•strain at the time of elastic failure (i.e. maximum stress at linear elastic region multiplied by the strain value at that point). As the corresponding stress and strain values at the elasticity failure point of the reseeded ECM were lying in between those of native tissues and ECM, they could have made up to a higher measurement of the multiplication products of the stress•strain that is higher than both the ECM and native tissues values.

6.4 Performance evaluation and prospect of the analytic tool developed

As we have extensively discussed, a single average Young's modulus value is not sufficient to characterize or compare different biomaterials, especially in applications where the matching of the viscoelastic mechanical characteristics is crucial to the eventual success of the implanted scaffold or device. Truncating the stress–strain data or excluding response regions to fit simpler models may be biased by the user data interpretation. This could substantially influence the analysis and produce inaccurate and unreliable measures jeopardizing the evaluation of the mechanical compatibility and applicability of biomedical constructs.

In this chapter, we have demonstrated an efficient and easy-to-use analytic tool for further mechanical characterization of biomaterials, providing more accurate and substantial information regarding the viscoelastic behavior and failure profiles of biomaterials. The determinations of the Zener modelled relaxation times as well as the elasticity and integrity limits obtained in this study are novel. This modelled information could be more indicative on the viscoelastic and failure behaviors viscoelastic properties of materials than graphically estimation or calculation from simple linear fittings. In particular, this additional information resulting from our analytic tool can now be used to evaluate the mechanical compatibility and applicability of other constructs as scaffolds for tissue engineering.

Further research is required to revalidate this analysis on a variety of other materials; however, we remain confident that this algorithm can be used as a common platform for soft-tissue analysis, providing comparable and user independent measures to characterize the viscoelastic response and failure profiles.

6.5 Summary of viscoelastic analysis results

The striking mechanical similarities in viscoelasticity between the acellular ECM, reseeded ECM and native tissues were thoroughly characterized; and demonstrated here for the first time. The results confirm our expectations as outlined in the chapter 5. The collagen-rich protein-based scaffold, and not the cells themselves, was supposed to govern the overall mechanical behavior of the biomaterials.

The comparison between the acellular ECM and the native myocardium tissue does shed a new light on the mechanical contribution from the presence of cells. The native tissue with high population of cells was shown to possess lower elastic limit, while increasing its integrity limit probably due to the additional flexibility and deformability provided by the cells. These changes generally can be attributed to the increase in the mechanical predominant collagen content per weight of the ECM compared to the native tissue.

Recellularization of human-relevant cells was shown to facilitate the recovery of the modulus and integrity limit while maintain the similarity in viscosity and relaxation time. This similarities in viscoelasticity, along with previously reported results in chapter 5 which shown similar elastic hysteresis and toughness between the samples, indicate the potential mechanical applicability of the recellularized pcECM for cardiac tissue engineering upon modulus reduction with improved recellularization. Further investigation of their biomechanics should be evaluating the anisotropic behavior of the samples, which are going to be covered in the coming chapter 7.

Chapter 7. Biaxial mechanical testing

In chapters 5 and 6, uniaxial tensile testing and viscoelastic evaluation of the acellular ECM, reseeded ECM and native tissues were discussed. The encouraging findings revealed similarities of the three sample groups in elastic hysteresis, toughness and viscoelasticity. They indicate a high potential of mechanical applicability of the recellularized pcECM for cardiac tissue engineering, upon modulus reduction with improved recellularization.

Yet, uniaxial testing alone is not sufficient to thoroughly evaluate the complex biological tissues. Hence, we conducted biaxial tensile testing to obtain insight of how the materials behave under a physiological-like biaxial stretching condition. Therefore, two sets biaxial mechanical testing were performed as detailed in Method sections 3.5 and 3.10 to evaluate acellular ECM, native tissues and reseeded ECM. Both the methodology and the results obtained are discussed in this chapter. The similarities and discrepancies between the properties of acellular ECM and native myocardium are first highlighted, followed by showing improved biomechanical functionality of the reseeded ECM-based cardio-mimetic construct.

Particularly in section 7.2, both statically and dynamically cultured reseeded ECM constructs (cultivated over 2 weeks) were extensively studied under a custom-made biaxial tensile tester developed by Prof. Jacob Bortman's team in Israel Ben Gurion University (BGU). Acellular ECM samples were re-tested in this set up; while native tissues were obtained from Israel Lahav C.R.O. preclinical services and re-tested in this set up as well.

7.1 Biaxial tensile testing on ECM and native samples by CellScale Biotester

Biological tissues are loaded in all directions in physiological conditions, and most possess anisotropic nonlinear behavior. ^[112,116-120] In order to perform biaxial tensile testing with an equipment that could accommodate our thick and soft tissues, we customized a CellScale BioTester 5000 with BioRake™. The BioRake™ composed of nine tungsten tines, five facing down and four facing upwards with securely grip the four edges of the square specimens without introducing forced compression to it.

Testing protocol was in accordance to the uniaxial setting detailed in section 3.3.2 including the three assays of cyclic loading; stress relaxation and tensile stretch conducted continuously, except that for tensile failure whereby specimens were strain up to 50% nominal biaxial strain due to the equipment constraints. Specimens were stretched in both Y (fiber-preferred) and X (cross-fiber) directions equibiaxially at the same rate.

7.1.1 Heterogeneity of the two samples

Video tracking of deformation images were reviewed and analyzed using a LabJoy software developed at CellScale in association to the Biaxial Biotester. (Fig. 58) The principle of following the deformation is by focusing the camera lens to track the sprinkled mica powder on the specimen surfaces.

Figure 58 Representative video tracking images in biaxial tensile testing.

ECM specimen at (A) Initial state (B) Stretched to 50% strain. (C) ECM specimen being focused on the sprinkled graphite powder for deformation tracking. (D) Central region of the specimen was defined as region of interest to be analyzed. (E) Central region was divided into 9 grids for analysis. (F) Deformed grids observed during stretching. (G) Displacement motion of each grid was shown by the yellow mark. (H) Deformation of a native sample.

Figures 58A-B depict the inhomogeneous deformation of specimen from their initial state (Fig. 58A) to 50% strain (Fig. 58B) as evident by the different locations of tines. Figures 58C-H displayed the central region of interest of the specimen, which was divided into 9 grids to follow their deformation movement. Displacement motion of each deformed grid

was shown by the yellow marks in Fig. 58G-H displaying the non-uniformity in deformation on both ECM and native samples. These visual evidence is in accordance to the general fact that heart tissues are composed of many different components that orient heterogeneously within the tissues, [3,11,26,28,77] including both collagen and elastin fibers.

7.1.2 Anisotropy of the two samples

Different loading curve profiles were shown for the fiber-preferred direction (Y) and cross fiber direction (X) in both ECM and native tissue in all the three assays, with the fiber-preferred directions (Y) requiring higher force to deform than the cross fiber directions (X). This highlights the anisotropy of the samples.

Figure 59 Representative stress-strain graphs of biaxial cyclic loading.

Along with the uniaxial results presented in section 5.2, both of the materials in both of their stretched directions showed resilient behavior with hysteresis during cyclic loading (Fig. 59) and force decaying during stress relaxation due to their viscoelasticity (Fig. 60). In accord with the uniaxial results, higher stress levels were measured for the ECM than for the native tissue in all the testing assays.

Figure 60 Representative stress-time graphs of biaxial stress relaxation.

However, highly fluctuating loading curve profiles at low stress levels were obtained when compared to the uniaxial testing, indicating the interferences of loading forces between the two fiber directions when being stretched equibiaxially.

7.1.3 Comparison of biaxial moduli

Under biaxial tensile stretching, both ECM and native tissues displayed stress-strain profiles (Fig. 61A) that echoing existing literature of some biaxial tensile testing results of biological tissues. [29,78,119,192] Fig. 61B demonstrated that Young's moduli in the Y-direction are significantly larger than X-direction by a factor ranging from 1.2- 3.0 for both materials ($p < 0.009$ for ECM and $p < 0.002$ for native tissue). In accord with the uniaxial results, ECM was stiffer than native tissues under uniaxial loading ($p = 0.1$). The Young's moduli of ECM in X and Y-axis were 0.9 ± 0.55 MPa and 1.71 ± 0.67 MPa; whereas those of native tissues were 0.75 ± 0.23 MPa and 0.96 ± 0.28 respectively ($n = 5$). Interestingly, when comparing the Young's modulus of ECM-X against Native-X, the significant difference observed was only of $p = 0.06$, indicating a resemblance of the modulus in the cross-fiber direction of the ECM to the native tissues.

Comparison of anisotropy ratio of ECM and native tissues measured as the ratio of Y modulus against X modulus of the same specimen are shown in Fig. 61C. ECM is significantly more anisotropic than native tissues, suggesting that the fibers are more aligned in the same direction along the fiber-preferred (Y) direction. The difference between the anisotropy ratios of ECM and native tissues may be attributed to the unidirection of the perfusion of decellularizing detergents in washing of vasculatures and fibers during that might somehow align the fibers along the perfusion axis (i.e. Y-axis). Yet the difference was not substantial ($p=0.0491$).

Figure 61 Biaxial tensile test results of ECM and native samples.

Representative stress-strain curves of biaxial tensile stretch. (A) Comparison of Young's Moduli in the X and Y direction of ECM and native tissues. (B) Comparison of anisotropy of ECM and native tissues. (C) * $p<0.05$ ($n=5$)

To summarize, our biaxial results suggested that ECM highly retains the biaxial tensile properties, which reflects the collagenous fibers constructing the major structural framework. Similar heterogeneity as shown by the video tracking images; whereas similar anisotropic behaviors as shown in the Young's moduli comparison. We hypothesized that recellularization will lead our ECM to a further native resembling construct. Two major recellularization strategies were undertaken, respectively: 1) using static tissue culture techniques through standard tissue culture protocol and 2) dynamic cultivation using a custom developed bioreactor system that mimics the physiological conditions *in vitro*. Their biaxial results after cell-reseeded constructs were cultured over 2 weeks are presented in section 7.2, which were obtained in collaboration with Prof. Jacob Bortman's team at Israel Ben Gurion University (BGU) and Lahav C.R.O. preclinical services.

7.1.4 Limitations of the sample mounting method

Due to the standardized design of the commercial set-up of CellScale BioTester, the method of suturing used for uniaxial testing could not be applied to this equipment. In order to employ comparable specimen size and mounting method as in the uniaxial setting, we used five downward facing and four upward-facing thin tungsten tines to grip the samples. Metallic tines appeared to introduce higher local stress at the puncture region during our handling when compared to the suturing threads. However, as we were only stretching the specimen to 50% strain for analysis, minimal premature tearing or breakages were observed among our sample populations and the thickness of the samples could be maintained between the long puncture point lengths of the tines.

Yet a drawback of this gripping method could be the difficulties to maintain the flatness of the specimen when puncturing the five downward-facing tines onto the specimen after it had been already affixed to the four upward-facing tines. This might explain the

difference in measurements when compared to uniaxial tensile testing, particular for native tissues. Developments of fixtures should be developed that allow the specimen to be flattened and aligned at its four edges. Puncturing using thin needles across the thickness could be a more effective way to mount.

7.2 Biaxial test of reseeded constructs at BGU

Although it had already been shown that our ECM possesses similar mechanical properties native tissues under uniaxial and biaxial loading, it was hypothesized that reseeded (both statically and dynamically) should further enhance the native-mimicking biomechanics of the construct by reducing the stress values and approaching the native tissue stress-strain profile. Hence, biaxial testing was performed In Israel Ben Gurion University (BGU) and Lahav C.R.O. preclinical services with Prof. Jacob Bortman's team after cell-reseeded constructs were cultured over two weeks. The biaxial tester (as described in Section 3.10.2) was custom-made by Prof. Jacob Bortman's Heart Modeling lab at BGU. The author prepared the reseeded samples and brought over to the BGU lab where she carried out the whole process of setting up the tester, calibration, testing and data analysis, with the training and assistance kindly provided by the BGU team. For the freshly-harvested native porcine heart tissues biaxial testing, the author and the BGU team together moved the tester from BGU to Lahav C.R.O. preclinical services to perform the testing.

7.2.1 Evaluation of sample mounting method

Due to the design of metal base and fixtures, the method of suturing used for uniaxial testing could hardly be applied to this equipment. The gap available on the metal fixture

was too small for an effective stitching operation of the suturing, resulting in non-uniform thread distribution along the edges as can be observed in Fig. 62.

Figure 62 An attempt of using sutures to mount specimen onto biaxial tester.

Therefore, in order to use specimen size and mounting method as much comparable as in the uniaxial setting, sharp needles were used to punch through the thickness of the specimen in order to fix the edges to the clamp as shown in Fig. 63 and as detailed in section 3.10.2.

Figure 63 Native tissue specimen under biaxial tensile test at BGU.

Similar to the mounting method with CellScale biaxial tester, metallic tines appears to introduce higher local stress at the puncture region during our handling when comparing to the suturing threads. Yet here we were only stretching the specimen to 15% strain and

no premature tearing or breakages were observed. We also overcame the problem with the gripping tiles as discussed in section 7.1.4 that the needle punching method used hereby can be efficiently perform with the flatness of the specimens maintained .

7.2.2 Biaxial tensile testing results of the four sample groups

Samples were equibiaxially stretched at 0.15 mm/s elongation up to 15% strain after preconditioning. Video tracking images, user interface and data processing methodologies were explained in details in section 3.10 and all the Matlab programming codes are presented in Appendix 15-20. Fig. 64 displays the representative stress-strain curves of our four sample groups including the reseeded ECMs.

Figure 64 Representative biaxial stress strain curves of the four sample groups.

E: ECM, R: Statically reseeded ECM, D: Dynamically reseeded ECM, N: Native tissues, Y: fibers direction, X: cross-fiber direction.

A clear descending trend was observed from ECM (Ey, Ex) to reseeded ECMs in static condition (Ry, Rx) to dynamic reseeded ECMs (Dy, Dx) towards native samples (Ny, Nx). This encouragingly indicate that the biaxial mechanical properties of our ECM construct in

a physiological-relevant condition could be reversed towards native tissue properties upon successful recellularization.

For all four groups, Young's moduli in the Y-direction are significantly larger than X-direction by a factor ranging from 1.5-2.1 (Fig. 65), highlighting the anisotropy of the materials.

Figure 65 Comparison of biaxial Young's Moduli of our four sample groups.

Y: fiber-preferred direction. X: cross fiber direction.

A clear descending trend was observed from ECM to Reseeded ECMs (static and dynamic with no significant difference) towards native samples. The X and Y moduli of the ECM are significant larger than those of the two reseeded groups (where no significant difference was measured between static and dynamic), indicating a substantial improvement of the material' biomechanics with the presence of cells in a biaxial setting. Yet, native samples are still of significant lower X and Y moduli than those of the other three groups. A summary of the Young's moduli measurements of the four sample groups in their two respective directions is presented in table 2.

Table 2 Summary of biaxial tensile testing measurements of the four sample groups.

	Acellular ECM	Statically- reseeded ECM	Dynamically- reseeded ECM	Native tissues
X-Modulus	0.81	0.57	0.41	0.11
Standard deviation	0.19	0.14	0.14	0.06
Y-Modulus	1.27	0.87	0.83	0.2
Standard deviation	0.14	0.09	0.02	0.03
Anisotropy ratio	2.1	2.05	1.75	1.58
Standard deviation	0.23	0.66	0.32	0.27
Sample size (n)	4	3	3	3

Anisotropy was measured as the ratio of Y modulus against X modulus of the same specimen. (Fig. 66) No significant difference was measured among the four groups, indicating that the antistrophic nature of the ECM was almost unaffected by decellularization or recellularization. The native and dynamic cultured samples revealed a slightly lower ratio. These may indicate that with the presence of cellular component, the materials behave more like a mesh instead of fibers aligned along the fiber-prefer (Y) direction.

One-way (Single factor) ANOVA analysis of the anisotropy ratios was conducted to determine the significance of effect of a single factor (assumed to be the presences of cells) in the four groups of data sets. The *p*-value of 0.4 indicates that the presences of cells have no significant impact on anisotropy ratios in the four groups of samples.

Figure 66 Comparison of anisotropy ratios of the four sample groups.

To conclude, promising biaxial results of the recellularized ECM constructs were obtained in collaborative experiments with Prof. Jacob Bortman's team at Israel Ben Gurion University (BGU) and Lahav C.R.O. preclinical services. Our ECM construct was shown to closely mimic the stress-strain profile as that of native tissues, with the reseeded ECM displaying improved resemblance.

ECM Young's Moduli were still significantly higher than native tissues. Nonetheless, when recellularized, it has shown a significant reduction of the modulus from the acellular ECM. With the similar anisotropies measured among the four sample groups, it may suggest that the ECM construct highly retains the biaxial tensile properties. This might reflect the preservation of the collagenous fibers constructing the major structural framework of the ECM upon decellularization.

7.3 Summary of the entire mechanical results covered by chapter 5-7

For any engineered constructs that are intended to be implanted *in-vivo*, one needs to evaluate their mechanical properties as they could crucially determine their ultimate integration and regenerative success. [3,11,43,99,100] Hence in chapter 5-7, we studied the biomechanics of our recellularized ECM constructs, in comparison to their acellular counterpart and native porcine myocardium. Their uniaxial tensile properties, viscoelastic properties and biaxial tensile properties were revealed experimentally and computationally. All results from the three tests revealed similar deformation and stress-strain profiles between ECM and native tissues except the different modulus.

Energy dissipation, viscoelasticity, toughness and anisotropy were similar among the sample groups. These striking mechanical similarities between the acellular ECM, reseeded ECM and native tissues were thoroughly characterized; and demonstrated here for the first time, shedding a new light on the mechanical contribution from the presence of cells.

Reseeded ECM has shown significant reduction of the modulus from the acellular ECM, with a deformation profiles shifting closer to that of the native tissues, suggesting the potential reversibility of the biomechanics upon recellularization of our ECM-based construct. These results supported our hypothesized that recellularization will lead our ECM to a further native resembling cardio mimetic construct for myocardial tissue engineering and cardiac regenerative therapy.

To reveal a complete material profile of our novel pcECM other than biomechanics, as well as to contribute to the literature in the characterization of native myocardial tissues, several critical surface and bulk biophysical properties are studied, which are going to be covered in chapter 8-9.

Chapter 8. Surface biophysical characterizations

Surface characteristics of substrates have shown to affect and regulate cell attachment and even determine cell fate. This chapter covers the surface biophysical characterizations on the ECM, native tissues and reseeded ECM, thus addressing Aim 2 and 3 in the Scope of Project.

Section 8.1 presents the results on surface functional groups analysis by Fourier Transform Infrared spectroscopy (FTIR) performed as detailed in Methods section 3.6.2. Section 8.2 presents the ultra-structure visualization of the materials by Scanning Electron Microscopy (SEM) performed as detailed in Methods section 3.6.3. Finally, section 8.3 summarizes the results obtained in the whole chapter on the surface biophysical characterization.

In the discussion of each section, similarities and discrepancies between ECM and native samples were first identified, along with explanations and supporting evidences. Then the results of the representative reseeded samples are discussed. Highlights of improved biophysical functionality of the reseeded ECM-based cardio-mimetic construct are emphasized to evaluate the reversibility of biophysical properties upon recellularization towards native like properties. Correlations among the surface characterizations on the biomaterials were deduced and discussed.

8.1 Surface analysis by Fourier Transform Infrared spectroscopy (FTIR)

Fourier transform infrared spectroscopy (FTIR) has been of expended usage in studying the surface conformation of protein-based biomaterials. ^[69,145,147,150]. Surfaces of ECM and native tissues as well as the protein-adsorbed ECM surfaces were evaluated by Perkin Elmer® FTIR- Spectrum™ GX in attenuated total reflection mode; whereas the comparison of surface conformations of ECM, native tissues and reseeded ECM samples were performed with Thermo Scientific- Nicolet 6670 FTIR in smart iTR mode. In both cases, scans were accumulated in the mid-IR range 600-4000 cm⁻¹ with a resolution of 4 cm⁻¹.

Table 3 below summarizes the peaks obtained in our experiments, along with their corresponding functional groups. The identification of peaks have been either observed and discussed in the list of literatures^[69,145,193]; or have been published in existing libraries available online. Libraries referred here include 1) BioProcess International Technical article^[153]; 2) IMB Jena Image Library of Biological Macromolecules^[194]; 3) Department of Chemistry, University of Maine, Orono^[195]; 4) Department of Chemistry & Biochemistry, University of California Los Angeles^[196]; and 5) Department of Chemistry, California State University, Stanislaus^[197]. Peaks of interests are further highlighted during discussions.

Table 3 FTIR peaks observed in this work and their corresponding functional groups

Peak (cm ⁻¹)	Functional group	Peak (cm ⁻¹)	Functional group
3500-3000 band	Amide A about 3500 and amide B about 3100 Mainly due to N-H stretching (95%)	2029	C-H alkane stretching C≡C alkyne Stretching
3280- 3225 inside amide A and B Band	Amide A and B hydrogen bond	1977	C-H fingerprint region C≡C alkyne Stretching
3090 inside amide A and B Band	Resonance of vibration between the first overtone of amide II and N-H stretching.	1740	Secondary amines N-H stretch
2920	C-H alkane stretching	1700-1600 band	Amide I and Amide II peptide carbonyl groups (NH- CO-(CH ₂) ₂ -COO-) C=O stretching (70-85%) C-N stretching (10-20%).
2850	N-H or O-H stretching of aliphatic group	1580-1510	Amide II N-H stretching (40-60%) C-N stretching (18-40%) C-C stretching (10%) Aromatic C=C Bending C=O stretch Stretching

2360	N=C=N of diimides	1460-1440	Secondary amines C-H bending
2510	O-H Stretching	1180-1140	Secondary amines/ Amide II C-N/ C-H/ C-O Stretching
2260-2100	C≡C alkyne Stretching	1080-1040	Primary amines C-H Stretching

8.1.1 Comparison of ECM and native tissue surfaces

Comparison of the FTIR spectra of ECM and native tissues were first performed in order to identify the similarities and discrepancies between the two groups. Fig. 67 presents the FTIR Spectra obtained by Perkin Elmer® FTIR- Spectrum™ GX.

For all ECM and native tissue surfaces, characteristic absorption bands of proteins were detected. The major band at 3500-3000 cm^{-1} corresponds to the N-H stretching vibrations of amide A and B [147]. And the major band at 1660-1600 cm^{-1} corresponds to the C=O stretching vibrations with contribution of C-N stretching of Amide I and II. [145]

The peak at 2360 cm^{-1} , which corresponds to N=C=N stretching of diimides [147], was patently found on both ECM and native endocardium surfaces compared to the other layers. (Fig. 67A-B) This may explain the difference between the amounts of BSA-FITC adsorbed on ECM pericardium and endocardium as observed and discussed in section 4.1. We suspect that the diimides bond might possibly act as an active surface functional group to mediate protein adsorption. Further investigation of the reaction between diimides to BSA might be conducted to verify the idea but this is beyond the scope of this work.

Absorbance of the peak at 1550 cm^{-1} , which mainly corresponds to the N-H bond of amide II [145], was particularly high on native tissue endocardium and myocardium surfaces when compared to the ECM surfaces. (Fig. 67B-C) This might suggest that a more intact cellular layer could give rise to a higher amount of amide II.

Three extra stretches were only found on ECM surfaces but not native tissues. (Fig. 67C) 2920 cm^{-1} correspond to C-H stretching of alkane groups; 2855 cm^{-1} corresponds to N-H or O-H stretching of aliphatic group; 1740 cm^{-1} corresponds to N-H stretching of secondary amines. [69,145,193] This might suggest that the process of decellularization may have

fragmented some of the collagen fibers or broke some bonds between polypeptide molecules chains, which could possibly give rise to the additional peaks.

Figure 67 FTIR Spectra of ECM and native tissues.

Perkin Elmer® FTIR- Spectrum™ GX obtained FTIR Spectra on ECM surfaces (A), and native surfaces (B). Comparison of the spectra ECM and native endocardium surfaces. (C)

8.1.2 Comparison of ECM and BSA-adsorbed ECM surface

Upon protein adsorption study as discussed in section 4.1, FTIR was performed to verify the existence of adsorbed BSA. Fig. 68 presents the FTIR spectra obtained in comparison to non-adsorbed ECM endocardium surface.

Figure 68 FTIR Spectra for the confirmation of BSA adsorption on ECM endocardium.

The reduction of 3500-3000 cm⁻¹ corresponds to the N-H stretching vibrations of amide A and B ^[147] were observed. The heightened stretches as highlighted in brown at 2920 cm⁻¹ (correspond to C-H stretching of alkane groups); 2855 cm⁻¹ (corresponds to N-H or O-H stretching of aliphatic group); 1740 cm⁻¹ (corresponds to N-H stretching of secondary amines)^[69,145,193] were observed. And the extra peaks at 1455 cm⁻¹ (corresponds to C-H bending of secondary amines) and 1170 cm⁻¹ (corresponds to C-N/ C-H/ C-O stretching of secondary amines or amide II) ^[194-197] were observed. All these observations may reflect the conformation of the bovine serum albumin, and hence this FTIR spectrum might be possibly used to serve as an indicator to confirm BSA adsorbed onto substrate surface.

8.1.3 Comparison of ECM, native tissue and reseeded ECM surfaces

To identify any change in the surface functional groups after cell reseeding, FTIR was performed on reseeded scaffold at Technion using Thermo Scientific Nicolet 6670 FTIR with data processing performed with OMNIC Spectra software. It was expected that reseeding would improve the native-resembling surface conformation as compared to decellularized ECM. For instance a reduction of the extra peaks of 2920, 2855 and 1740 cm^{-1} which were evidently found on decellularized ECM surfaces; as well as an increase intensity of presence of 3500-3000 cm^{-1} , 1660-1600 cm^{-1} , and 1550 cm^{-1} which were previously found particularly higher on native endocardium and myocardium surfaces.

Fig. 69 presents the representative FTIR spectra of reseeded ECM in comparison to the surface conformations of ECM, native tissues with the above-mentioned specific peaks highlighted. The acellular ECM and native FTIR spectra were comparable to what we had previously obtained by Perkin Elmer® FTIR- Spectrum™ GX. For the reseeded ECM, the results went along with our expectation that reseeding improved the native-resembling surface conformation as compared to decellularized ECM. Intensities of peaks at 2920 cm^{-1} (correspond to C-H stretching of alkane groups); 2855 cm^{-1} (corresponds to N-H or O-H stretching of aliphatic group); 1740 cm^{-1} (corresponds to N-H stretching of secondary amines) [69,145,193] were reduced by about 0.05 absorbance unit. Intensities of peaks at 3500-3000 cm^{-1} (corresponds to the N-H stretching vibrations of amide A and B [147]), 1660-1600 cm^{-1} (corresponds to the C=O stretching vibrations with contribution of C-N stretching of Amide I and II. [145]), and 1550 cm^{-1} (corresponds to the N-H bond of amide II [145]), were increased by about 0.05 unit. This affirms the reversibility of the surface conformational properties of the ECM towards native like properties upon recellularization, and encouragingly supported our hypothesis.

Figure 69 Representative FTIR spectra of the three sample groups.

8.1.4 Extended evaluation with OMNIC Spectra built-in library

To make the best use out of the OMNIC Spectra Software capabilities and obtain more information of our FTIR spectra, we further evaluated our results against the existing OMNIC Spectra built-in library.

Figure 70 OMNIC Spectra software function of Peaks identification.

Top: ECM sample. Bottom: highlighted peaks match corresponded to aromatic amino acid groups.

One of the functions in the software allow peak identifications and spectra search performed against the OMNIC Spectra built-in library. For example, Fig. 70 shows that on a reseeded ECM spectrum, peaks at 2920, 2855 and around 1500 cm^{-1} were highlighted and were indicated to be corresponded to aromatic amino acid groups with 55% of match. Up to date, there was no record of heart tissues spectrum in the entire OMNIC Spectra library. We were only able to find that our ECM samples have a 73.70% of spectra match to human skin and 65.46% of spectra match to whole human blood. (Fig. 71) We anticipate

that our spectra, especially that of the native heart tissues could be incorporated into such library. This could provide a reference for biomaterial surface conformation characterization to fellow researchers who are also working with heart tissues or myocardial tissue engineering. We also suggest that FTIR could be employed to evaluate the surface conformational properties of the recellularized or engineered tissue, such that their reversibility towards native like properties could be verified in similar fashion as what we have performed.

Figure 71 OMNIC Spectra software function of Spectrum search.

Top: ECM sample. Bottom green: human skin spectrum. Bottom red: whole human blood spectrum

8.2 Ultra-structure visualization by Scanning Electron Microscopy (SEM)

After identifying the reversibility in surface conformational properties of the recellularized construct towards native like properties by FTIR, scanning electron microscopy was used to visualize the ultra-structure of its surface, in comparison to the features of ECM and native heart tissues.

8.2.1 SEM of ECM, reseeded and native samples

JSM-6360 Scanning electron microscope was used to visualize ECM, reseeded and native samples by detection of secondary electrons at 5kV accelerating voltage. (Fig. 72)

Acellular ECM (Fig. 72A) was found to be a dense and compact mesh of weaved fibrous structures. This finding echoes our mechanical testing that the possible reason of the stiffer acellular ECM could be caused by this dense and compact mesh mainly composed of collagen fibers. Nonetheless, this interconnected porous network might also provide an advantage of our ECM scaffold over less-porous scaffold materials in terms of facilitating the cell loading and infiltration processes.

SEM image of the native tissue (Fig. 72C) revealed a dense intact cell layer, having many cellular features, which are probably the presence of endothelial cells that line the endocardium layers. Reseeded ECM samples (Fig. 72B) encouragingly revealed layers of cellular features forming tissues growing onto the fibers.

Figure 72 SEM images of the three sample groups.

Scanning Electron Microscopy images were collected by JSM-6360: (A) Acellular ECM. (B) Reseeded ECM. (Representative among a combined collection of statically and dynamically reseeded samples) (C) Native tissue. Scale Bar: 10 μm.

8.2.2 WETSEM images of reseeded samples

To obtain a better visualization of the reseeded ECM samples in their wet conditions, Zeiss SIGMA Field Emission Scanning Electron Microscopes obtained WETSEM images of reseeded samples utilizing the QuantomiX technology. They were produced by the detection of either secondary electrons (SE2) or angle selective backscattered electrons (AsB). PTA was used to stain charged cellular structures components such as nuclear DNA and nucleoli. It also intensely stain mitochondrial matrix, which shows brighter contrast than the surrounding fibers and buffers.

Figure 73 WETSEM representative images of reseeded ECM.

Representative images were chosen among a combined collection of statically and dynamically reseeded samples. (A) Specimen touching the membrane of the QuantomiX capsule. Scale bar: 600 µm. (B) Dense layer of aligned and orientated cell tissue. Scale bar: 100 µm. (C) Stretched cells grown along fibers observed at the cross section of sample. Scale bar: 25 µm. (D) Thin microfibers possibly secreted by cells. Scale bar: 10 µm.

Fig 73A showed the areas of specimen touching the membrane of the QuantomiX capsule where Fig. 73B revealed a dense layer of aligned and orientated cell tissue formation on the hydrated surface of the reseeded construct. Closer examinations at magnification of 3000X observed at the cross section of sample revealed that stretched cells grown along fibers. (Fig. 73C) At magnification of 6000X, we were able to observe features surrounding cells in contrast of the imaging buffer. The thin fibrous features seen in Fig. 73D might possibly reveal the newly secreted extracellular matrix and microfibers, which are of charged structures around the cell membrane.

8.3 Summary of surface characterizations

Summarizing our findings in chapter 8 on surface characterizations of samples, we have shown that recellularized ECM possesses reversibility of surface biophysical properties towards native-like properties. The discrepancies in surface functional groups between ECM and native samples (as analyzed by Fourier transform infrared (FTIR) spectroscopy) were bridged upon recellularization. Ultra-structure visualization of the reseeded ECM-based cardio-mimetic construct by Scanning Electron Microscopy (SEM) revealed formation of cellular tissues on the fibrous ECM scaffold, bridging towards the intact cell layer found on native tissues. The two surface characterizations correlate with each other and both suggested that the presence of cells could alternate the surface properties of the constructs. These results also support the findings that we obtained in the biomechanical characterizations study. The higher modulus of the acellular ECM could be caused by its dense and compact mesh of collagen fibers. Upon recellularization, the modulus was reduced that probably due to the presence of cells, which provide higher flexibility and deformability to the reseeded constructs.

Chapter 9. Bulk biophysical characterizations

This chapter covers the bulk biophysical characterizations on the ECM, native tissues and reseeded ECM to address Aim 2 and 3 in the Scope of Project.

Section 9.1 presents the evaluation of hydration content by Thermogravimetric Analysis (TGA) performed as detailed in Methods section 3.6.4. Section 9.2 presents the results on thermal properties by Differential Scanning Calorimetry (DSC) performed as detailed in Methods section 3.6.5. Section 9.3 presents the electrical characterization performed to obtain preliminary insight of the effect of cell presence on the conductivity of the pcECM. Finally, section 9.4 summarizes the results obtained in the whole chapter on the bulk biophysical characterization.

In the discussion of each section, similarities and discrepancies between ECM and native samples were first identified, along with explanations and supporting evidences. Then the results of the representative reseeded samples collected are discussed. Highlights of improved biophysical functionality of the reseeded ECM-based cardio-mimetic construct are emphasized to evaluate the reversibility of biophysical properties upon recellularization towards native like properties.

9.1 Evaluation of hydration content by Thermogravimetric Analysis (TGA)

The level of hydration has substantial influence in hydrated biomaterials' thermal stability and biomechanical properties.^[155,158,159,198] Hence, thermogravimetric analysis was done to evaluate the hydration content of the ECM, native tissues and reseeded ECM constructs with absolute temperature-controlled TA Instruments TGA 2950. Samples of each group were weighed and their changes in weight over the heating span were recorded and presented in Fig. 74.

Figure 74 Overlay of representative TGA traces of the four sample groups.

TA Instruments TGA 2950 measured weight changes (%) against temperature (°C). From top to bottom: native tissue (red dash), dynamically reseeded ECM (green line), statically reseeded ECM (purple line) and acellular ECM (blue dot).

It was observed that all samples are composed of a high content of volatiles of over 70% of their weights, indicating the hydration levels might indeed have substantial influences in the biomaterials' thermal stabilities and biomechanical properties.

The chart graph Fig. 75 presents the comparison of hydration content of ECM, statically reseeded ECM, dynamically reseeded ECM, and native tissue. ECM was having the highest hydration content, which is probably attributed to its porous structure that encourages liquid infiltration as we have shown by SEM in section 8.2.

Figure 75 Comparison of hydration content of the four sample groups.

* $P < 0.05$ (n=3)

When comparing the dry weight of contents left behind upon vaporizing of volatiles in the samples, we observed a clear trend of increasing contents from acellular ECM ($6.93 \pm 0.66\%$) to statically reseeded ECM ($12.02 \pm 0.77\%$) to dynamically reseeded ECM ($16.87 \pm 5.09\%$) and towards native tissue ($18.76 \pm 0.99\%$). ECM was having a significantly lower dry weight of contents than the two groups of reseeded sample as well as the native tissues, demonstrating that there was some loss of solid composition upon decellularization, and with recellularization, a higher solid content was recovered.

The major compositions of the ECM would mostly be dense packed collagen I and III fibers and extracellular matrix components; while that of native tissues would be a collection of proteins and extracellular matrix components as well as blood and different cell types. Their compositions of ECM and native myocardium have been presented in the Introduction sections 1.3.4 and 1.2.2 respectively.

One-way (Single factor) ANOVA analysis of the TGA hydration content results was conducted to determine the significance of effect of a single factor (assumed to be the presences of cells) in the four groups of data sets. The p -value of 0.002702 indicates that the presences of cells significantly influence the hydration levels and hence the dry weight solid contents in the four groups of samples.

To summarize, the TGA results of reseeded ECM samples went along with our expectation that a higher solid content could be achieved upon recellularization, owing to the additional presence of cells and ECM proteins compared to the decellularized ECM. These provide extra evidence to support of our hypothesis in additional to the FTIR and SEM results discussed in chapter 8.

9.2 Thermal analysis by Differential Scanning Calorimetry (DSC)

Thermal properties of the protein-based materials can reflect the compositions and structures of proteins in the materials and their denaturation behavior^[160-164]. Hence, in this study, acellular ECM, statically reseeded ECM, dynamically reseeded ECM, and native samples were thermally evaluated by differential scanning calorimetry (DSC). TA Instruments DSC Q10 was used to perform thermal analysis from 25°C-250°C and sections

9.2.1 to 9.2.3 detail the results and discussions on the observation of thermograms, transition temperature, melting temperatures and the heat of fusion.

9.2.1 Observation on DSC thermograms

The representative thermograms of the four sample groups are displayed in Fig. 76. All the four sample groups showed thermal stability up to about 70°C. Upon which, baseline shifts were observed, relating to the physical process transition behaviors of the materials that reflect the molecular or chain mobility^[154]. After the transition, endothermic events were followed.

Figure 76 Representative DSC thermograms of the four sample groups.

DSC thermograms of heat flow (mW) (Exothermic Up) against temperature (°C) were obtained on our four sample groups: acellular ECM, (A) statically reseeded ECM, (B) dynamically reseeded ECM, (C) and native tissue. (D)

Complex endothermic peaks were observed for most native tissues at higher temperatures ranging from 100 °C - 200 °C. (Fig. 76D) The complex peaks might reveal the multiple degradation processes of the multi-components^[154,199-201], which could be related to the various collagen types, extracellular matrix components, different cell types and other biological components that have higher molecular weights. These could be further verified by chemical assays, and the water masking factor should be taken in account for the peaks at around boiling temperature, and hence dehydrated sample or a second run of the DSC heating can be further done. Truong *et al.* reported the DSC trace of their crosslinked resilin-mimetic protein which displayed similar complex thermal profile.^[154]

In contrast, a narrow and sharp single dominant endothermic peak ranging from 90 °C - 120 °C was observed for acellular ECM samples (Fig. 76A). This may reflect that the major composition of the ECM could be of minimal types of components. With reference to our published data on proteomics, the dominant proteins of the ECM would be collagen I (about 54% of coverage).^[86] Li *et al.*^[155]; Fathima *et al.*^[202]; Leikina *et al.*^[203]; Lee *et al.*^[204]; and McClain *et al.*^[205] all reported single-peak DSC traces of their protein-based materials.

Reseeded ECM samples showed a shift of the thermogram profiles towards native-like profile of endothermic peaks at higher temperature range. (Fig. 76B-C) Recovery of some of the complex peaks was also observed. They probably correspond to the multiple degradation processes of the newly existed cellular components as well as the possible new secretion of extracellular matrix proteins by the cells.

9.2.2 Transition and peak melting temperatures

The two major thermal events were recorded and compared in Fig. 77, namely the transition temperature T_t (°C) and the peak melting temperature T_m (°C). A summary of the DSC measurements of the four sample groups is presented in table 4.

Figure 77 Comparison of T_t and T_m of the four sample groups.

TA Instruments DSC Q10 measured the averaged first detectable transition and peak melting temperatures of acellular ECM, statically reseeded ECM, dynamically reseeded ECM, and native tissue. * $P < 0.05$ ($n > 4$)

T_t was defined as the onset defined by the extrapolation of the rising edge of the endothermic peak to intercept the baseline.^[154,204] In some literature, transition was also known as the first detectable step transition within the DSC thermogram. Such step transition have been observed in the reported DSC traces by Macocinschi *et al.* and Lee *et al.*^[164,204] Fig. 77 revealed that T_t of acellular ECM was significantly lower than that of the native tissues. This suggests that less specific heat capacity was required for moving the peptide chains inside the acellular ECM.^[158,164,200], which might due to possible reduction

in the intermolecular forces and the degree of cross-linking of its amorphous component (i.e. elastin ^[206]) caused by the decellularization process.

Since the mobility of the chains to flow is related to the ductility of the materials, this finding may also explain the long plastic deformation (also known as necking or yielding) before fracture observed on the ECM during the mechanical testing. The examination of the mechanical deformation and fracture behaviors revealed that ECM was behaving more ductile with cone-like fracture surface. In contrast, the native tissues exhibited a brittle-like flat fracture surface after voids were formed within the stretched tissues and fiber-by-fiber breakage occurred.

In addition, molecular dynamics of protein molecules are also dependent on the state of water ^[154,156-159,200] and thus hydration level has substantial influence in thermal stability and mechanical properties of materials ^[155,158,159,198]. Correlating to the TGA results previously stated in section 9.1, the higher level of hydration in ECM could lead to more swollen collagen fibers and lower denaturation temperature, ^[156] resulting in more ductile material behaviors of collagenous tissues.

T_m was defined as the highest point of the dominant endothermic peak. As suggested by literature ^[155,164,202], T_m is related to the triple-helix-to-random-coils disintegration of crystalline collagens. Our results show that T_m of acellular ECM as well as the two reseeded ECM constructs were significantly lower than the native tissues. This may suggest some extent of possible destruction to the collagen triple helical structure during the decellularization process ^[204,207], including the intrahelical and the intermolecular cross-linking of collagen fibers. A wide range of melting temperatures has been reported in literatures. For instance Li *et al.* ^[155] reported a T_m of 40°C of their calf-skin collagen solution. Fathima *et al.* ^[202] reported a T_m of 62°C of their rat-tail tendon collagen. Lee *et*

al. ^[204] reported a T_m of 65°C of their bovine pericardium. Skrzyński *et al.* ^[207] reported a T_m of 94.5°C of their intervertebral disc. And Truong *et al.* ^[154] reported a peak T_m of 287°C of their crosslinked resilin-mimetic proteins. To the best of our knowledge, this is the first calorimetric characterization performed on a porcine myocardium as well as its decellularized and recellularized forms.

Reseeded ECM samples have revealed certain recovery of the native tissues DSC trace profile with complex peaks at higher temperature range as shown in Fig. 76, suggesting that the presence of cells could give rise to structural changes in the collagen-based materials. Yet their respective T_t and T_m are still significant lower than those of native tissues. We anticipate that by further improving the recellularization to obtain higher cell number as well as the ingrowth of the cells to the bulk of the constructs, a larger extent of recovery could be achieved.

9.2.3 Heats of fusions

Heat of fusion is calculated as the area under the endothermic peak. It is known to be the enthalpy change during the denaturation or de-polymerization of collagen fibers ^[158,199,200,204] associated with the breakage of hydrogen bonds between adjacent polypeptide chains of collagen molecules. ^[155,164,207]

Figure 78 Comparison of heats of fusions at melting of the four sample groups.

* $P < 0.05$ ($n > 4$)

As shown in Fig. 78, heat of fusion of acellular ECM was significantly higher than the native tissues. This result may indicate the higher extent of hydrogen bonds between the triple helixes and around the collagen molecules within the highly hydrated acellular ECM construct. Encouragingly, reseeded samples have shown to recover the native-resembling heat of fusion with no significance difference found between the two reseeded sample groups and native tissues. Table 4 summarizes the entire collection of DSC results of the acellular ECM, statically reseeded ECM, dynamically reseeded ECM, and native tissue.

Table 4 Summary of DSC results of the four samples groups.

	Acellular ECM	Statically- reseeded ECM	Dynamically- reseeded ECM	Native tissues
First detectable transition temperature T_t (°C)	77.31	81.04	89.66	108.56
Standard deviation (S.D.)	3.61	15.86	14.60	17.79
Peak melting temperature T_m (°C)	107.62	112.76	113.31	135.43
Standard deviation (S.D.)	12.06	16.03	7.63	14.92
Heat of fusion at melting ΔH_f (Jg ⁻¹)	1650.33	1221.57	1236.45	1330.83
Standard deviation (S.D.)	129.43	562.70	294.02	235.05
DSC Sample size (n)	6	7	4	6

Comparing to the mechanical findings obtained in chapter 5-7, it seems contradictory that native myocardium, having a higher T_m that reflect a higher degree of intra or inter molecular crosslinking of collagens, possessed a lower modulus. It should be noted that T_m corresponds mainly to the disintegration of the collagen triple helixes into random coils. Yet the modulus could be affected also by the hydrogen bonds ^[155,164,207], ratio of collagen to elastin or denseness of the collagen fibers ^[98,119,124,208], as well as the presence of cells ^[119,123], which are often of lower modulus than collagen itself. The complex structure of the native tissues, could be part of the reason for the change in mechanical behaviours

and modulus observed, where remodeling of collagens might have occurred. Reseeded ECM has shown significant reduction of the modulus from the acellular ECM, with a deformation profiles shifting closer to that of the native tissues, suggesting the potential reversibility of the biomechanics upon recellularization of our ECM-based construct.

To conclude, the DSC results revealed that decellularization process might induce changes in the molecular structure of collagen that alters their denaturation behaviors. The DSC results of reseeded ECM samples have shown complex exothermic peaks at higher temperature range with decreased heats of fusions. This indicates that the recovery of thermal properties of the ECM constructs towards native tissue could be achieved upon recellularization, providing bonus evidence to support our hypothesis in addition to the previously obtained findings from FTIR, SEM and TGA.

9.3 Preliminary study of effect of cells on conductivity of pcECM

As discussed in section 1.2.4, electrical waves are propagated rapidly across the myocardium along membranes of adjacent CMs by gap junctions. Physiological voltage across myocardium has been reported in the range of 3-8V with the resistivity reported to be in the range of 200-600 Ωcm depending on the temperature & applied frequency setting in experiments. Moreover, as discussed in section 1.3.3, applying electrical pacing signals has been demonstrated to induce synchronous contractions and encourage differentiation and proliferation of stem cells into cardiomyocytes-like cells. Therefore, we were curious to study the inherent electrical conductivity of the pcECM; as well as the effect of cell presence on the conductivity of the material after repopulating the ECM construct with MSCs.

9.3.1 Electrical properties of ECM

The electrical resistance to direct current was measured in our custom-made electric chamber connected to a Bio-Rad PowerPac supply. Fig. 79 displays the images of the experiment, the current-voltage measurement and FTIR of the electrified sample surface.

Figure 79 Electrical characterization experiment of ECM.

Steam was evolved from ECM beyond 20V of voltage application. (A) Black particles were evolved around 23V of voltage application. (B) Current-Voltage measurement. (C) FTIR of the electrified ECM compared to ECM. (D)

During the electrical application of ECM, it was observed that steam started to evolve from the ECM when 20V or higher voltages were applied. (Fig. 79A) Upon reaching 23-25V application, the currents went up continuously tending to infinity without further voltage increase and black particles were found to appear on the sample surface. (Fig. 79B-C)

The stable resistance (R) within the range of 10V-20V was calculated as $616.01 \pm 141.08 \Omega$ (n=7); while resistivity, as calculated by resistance multiplying area of application divided by length of sample ($\rho = RA/l$), was $421.44 \pm 104.60 \Omega \text{cm}$ and hence the averaged conductivity ($1/\rho$) was $2.37 \times 10^{-3} \Omega^{-1} \text{cm}^{-1}$. This result is comparable to the literature that resistivity value for randomly-oriented human heart muscle in the range of 200-600 Ωcm depending on the temperature & applied frequency setting in experiments. [38-42] Moreover, to the best of our knowledge, it is the first attempt of electrical conductivity evaluation of a decellularized biological tissue.

To evaluate the possible conformational chemistry of the black particles evolved, FTIR analysis was performed on the electrified samples and evaluated with FTIR table 3 presented in section 8.1. (Fig. 79D) The peak at 2510 cm^{-1} corresponds to O-H stretching vibration. The peaks at 2159, 2029 and 1977 cm^{-1} all fall in the ranges corresponding to the of $\text{C}\equiv\text{C}$ triple bonds alkynes stretching, which was probably caused of the burnt collagen resulting in charcoal-like composition.

9.3.2 Electrical properties of reseeded ECM

Conductivity of constructs was expected to be improved with the presence of cells. The electrical properties of reseeded ECM was studied and images of the experiment are displayed in Fig. 80.

Figure 80 Reseeded ECM on voltage application.

Medium migration towards cathode (A & C) foaming and shrinking (B & D)

It was observed that above 4V, the pink culture media slowly migrated from the anode (red wire, positive) towards the cathode (black wire, negative end). (Fig. 80A-B) White foam started to evolve from the cathode end when 15V or higher voltages were applied. (Fig. 80B) Eventually a dark burnt-mark line was observed in the middle of the samples. (Fig. 80C) Upon 20V of voltage application, the currents went up continuously tending to infinity without increase of voltage, and the shrinkage of specimen was observed. (Fig. 80D)

For the reseeded construct, the stable resistance (R) within the range of 4V-8V was calculated as $359.77 \pm 40.96 \Omega$; while resistivity, as calculated by resistance multiplying area

of application divided by length of sample ($\rho=RA/l$), was $239.85\pm27.31\ \Omega\text{cm}$ and hence the averaged conductivity ($1/\rho$) was $4.17\times10^{-3}\ \Omega^{-1}\text{cm}^{-1}$. Again, this result is comparable to the literature that resistivity value for randomly-oriented human heart muscle in the range of 200-600 Ωcm depending on the temperature & applied frequency setting in experiments.

[38,40-42]

It was clearly shown that with the incorporation of cells, the resistivity of the construct was significantly reduced from $616.01\pm141.08\Omega$ to $359.77\pm40.96\Omega$ ($p=0.0023$). The conductivity of the construct was improved from $2.37\times10^{-3}\ \Omega^{-1}\text{cm}^{-1}$ to $4.17\times10^{-3}\ \Omega^{-1}\text{cm}^{-1}$, proving that the presence of cells facilitate electrical propagation. Fig. 81 displays the representative voltage-current curves of ECM and reseeded samples ($n=3$) within their respective stable resistance ranges.

The dielectric properties of tissue and cells have been discussed in literature^[42,209-211] but this is out of the scope of this study as here we aimed to obtain preliminary impression of the inherent conductivity of construct and its recellularized form. Yet, we suspect that the migration of cell-medium to one end of the electrode (as observed in Fig. 80) might be due to the reason that the dielectric nature of the reseeded material polarized under an applied electric field and that cells could be induced to migrate under a small D.C. electric fields.

Figure 81 Voltage-Current measurement of acellular ECM and reseeded ECM.

It was also interesting to realize that the stable resistance range of the reseeded ECM was 4V-8V which is comparable to the physiological voltage across human myocardium reported to be in the range of 3-8V.

To conclude, we have shown that our ECM-based constructs possess resistivity comparable to the literature value for heart muscle in the range of 200-600 Ωcm and the recellularized ECM with human stem cells revealed a more conductive construct. To the best of our knowledge, it is the first attempt of electrical conductivity evaluation and comparison between decellularized biological tissues and their recellularized counterparts. We have shown that with the presence of cells, conductivities of constructs were significantly improved. This preliminary study of the effect of cell presence on the conductivity of the pcECM might serve as a basis for any potential electrical stimulated reseeded construct studies in the future work.

9.4 Summary of bulk biophysical characterizations

Summarizing our findings in chapter 9 on bulk characterizations of recellularized ECM cardio-mimetic construct, we have shown that it possesses certain reversibility of the bulk biophysical properties towards native-like properties. The discrepancies in solid weight content between ECM and native samples, as analyzed by thermogravimetric analysis (TGA), were bridged upon recellularization, most likely due to the presences of additional cells and ECM proteins.

Differential scanning calorimetry (DSC) results revealed that some extent of molecular structures of proteins might be altered during the decellularization. Reseeded ECM constructs showed recovery towards native-like thermal properties with the detection of complex exothermic peaks, higher temperature range and decreased heats of fusion.

In the preliminary study of the inherent electrical resistivity of the ECM pre and post-recellularization, it was clearly demonstrated that the presence of cells enhanced the conductivity of the constructs with the resistivity of the construct significantly reduced, proving that the presence of cells facilitate electrical propagation.

All the bulk characterizations suggested that the presence of cells could substantially alternate the bulk properties of the constructs towards native tissue, providing extra evidences to support our hypothesis in addition to the finding we obtained in the biomechanical and surface characterizations.

Chapter 10. Conclusion and future prospects

In this last chapter of the thesis, the important conclusions drawn from experimental findings obtained throughout chapter 4-9 are captured in section 10.1. The significance and novelty of this project is substantiated in section 10.2. Section 10.3 covers numerous recommendation of potential future works, which can further address the gaps and limitations lied in this current work.

10.1 Highlights of findings

Our leading hypothesis is that acellular porcine cardiac extracellular matrix scaffold (pcECM), when reseeded with human bone-marrow-derived mesenchymal stem cells (MSC) and undergone optimized culture conditions, can possess biophysics comparable to native myocardium. To evaluate this hypothesis, we pursue three aims to study the biomechanics, surface biophysics and bulk biophysics of recellularized ECM construct, in comparison to the acellular ECM and native porcine myocardium tissues.

We optimize the recellularization methods of MSCs in terms of seeding surface, seeding methods and construct cultivation regimes. The results show that cells adhere and proliferate on our ECM construct, particularly when dynamically cultivated using our designed perfusion bioreactor system. There are significant changes to the appearances and cell cluster sizes due to the present of cells and the cell-secreting extracellular matrix.

We characterize the mechanical properties of the samples experimentally and computationally. Uniaxial and biaxial tensile testing were conducted, and an analytic tool was developed for modeling experimental results and evaluating the viscoelasticity of the materials. All results show similar deformation and stress-strain profiles between the ECM and native tissues with no significant difference in energy dissipation, viscoelasticity, toughness and anisotropy. We find that ECM has a higher Young's modulus than native tissue, but reseeded ECM shows significant reduction shifting closer to that of the native tissues. This suggests that recellularization of our ECM-based construct can reversibly change the mechanical properties of our ECM. The results also support our hypothesis that recellularized ECM constructs can possess biomechanics comparable to native myocardial tissue.

Fourier transform infrared spectroscopy analysis (FTIR) reveals the surface conformation of the samples. We find that reseeded ECM demonstrates improved native-resembling surface conformation when compared to decellularized ECM. The intensities of certain FTIR peaks that were evident in native tissues increase, whereas those that were evident in acellular ECM decrease. Scanning electron microscopy (SEM) visualize the ultra-structure of the samples, with the use of WETSEM™ technology. We observe cellular features growing onto the fibrous structure of the ECM scaffold, and dense layer of aligned cells forming on portions of the endocardium surface of the constructs. The results affirm the reversibility of surface properties of the ECM towards native like properties upon recellularization.

Thermo galvanometric analysis (TGA) evaluates the hydration contents of the samples. Results of reseeded ECM demonstrates a higher solid content with the additional presence of cells and ECM proteins when compared to the decellularized ECM. Differential scanning calorimetry (DSC) study the thermal properties of the samples. The reseeded ECM shows similar heat of fusion as native tissues, with a small extent of recovery in the transition temperature, peak melting temperature, and the complexity of exothermic peak profile. This indicates that recellularization can facilitate the recovery of thermal properties of the ECM constructs towards native tissue. Our preliminary electrical characterization results provide bonus evidence to our hypothesis that recellularization can enhance the inherent electrical conductivity of the ECM constructs.

To conclude, we have accomplished our goal to experimentally show improved biophysical functionality of reseeded ECM constructs through optimized culturing methods. Hence, it can potentially be a suitable cardio-mimetic construct for myocardial tissue engineering.

10.2 Significance and Novelty

The novelty of this project is greatly consolidated with our full-thickness decellularized porcine cardiac extracellular matrix (pcECM), which is a novel biomaterial on its own, with close resemblance to the native myocardium. We establish three areas of knowledge surrounding the pcECM for a better understanding of its material profile:

- 1) Its mechanical properties;
- 2) Its surface and bulk biophysical properties;
- 3) Effect of different recellularization methods on construct properties.

Another significance of this project is the establishment of knowledge on the native myocardium's material properties. To the best of our knowledge, it is the first time an extensive material characterization of the left ventricular myocardium is reported, despite its importance for any scaffold intending to serve as cardiac tissue construct. The thorough correlations among the characterizations of the biological tissues, including mechanical, surface conformational, hydration content and thermal properties, are as well elaborated and deduced hereby for the first time in the literature, to the best of our knowledge.

Contributing to the general biomaterial field, we establish a mechanical characterization regime that can be applicable to any soft and thick tissue, including sample mounting, uniaxial and biaxial testing instrumentations, testing protocols, and analytic modeling. They are validated with our experimental results on ECM constructs and native tissues. Such characterization is previously not standardized due to the lack of a reliable sample mounting method, a productive continuous testing protocol, an easy-to use analytic tool, and a common ground for comparison. In particular, we develop a new Matlab-operated analytic tool which allows the fitting of experimental data to a viscoelastic model with high

correlation. It facilitates the yielding of non-biased mechanical measurements, which can benefit all tissue engineers when biological tissues or scaffolds are concerned.

We experimentally demonstrate that our pcECM itself possesses a close biophysical functionality as native myocardium with a few discrepancies; and by recellularizing it with the culturing methods optimized in this thesis, the resemblance is improved. We study and compare two seeding surfaces, four seeding methods, two bioreactor units and three construct cultivation regimes to yield a well-recellularized ECM-based cardio-mimetic construct. Uniaxial and biaxial tensile testing together with the developed viscoelastic analytic tool show their similar biomechanics except modulus. Fourier transform infrared spectroscopy analysis and scanning electron microscopy display the reversible surface properties. Differential scanning calorimetry and thermogravimetric analysis reveal their recovery of thermal properties. In addition, an electrical experiment shows that the presence of cells improved conductivity.

Overall, this thesis proves a high potential of our recellularized porcine cardiac extracellular matrix scaffold, with properties matching closely with native myocardium, to become a suitable cardio-mimetic construct for myocardial tissue engineering. Its well-characterized material profile as established in this thesis also makes the pcECM a high-fidelity model for any relevant cardiac therapeutic drugs or molecular studies.

10.3 Proposals of future works

This thesis work is completed in a 4 years PhD candidature timeframe and still leaves behind certain knowledge gaps that open rooms for future investigations. The following sections are proposals for future work that could be the next stages beyond this project.

10.3.1 Validation of the developed mechanical characterization regime

One novelty of this project lies in the establishment of a mechanical characterization regime that is applicable to soft and thick tissues. It includes the sample mounting method, continuous testing protocol, and the viscoelastic analytic tool. To test the applicability of the methodology on another mechanical tester, a preliminary experiment has been performed on an Instron 5943 with pneumatic action grips. (Fig. 82A)

The direct mounting of samples onto the pneumatic action grips had failed to impose sufficient gripping force and failed to prevent the specimens from slippage (Fig. 82B-C). All the three testing assays were basically failed to perform as the specimens slipped during the pre-cycling process. Fig. 82D showed the stress-strain curve of a forced trial of tensile failure test on a small ECM sample mounted by pneumatic action grips that gave an abnormal profile.

Figure 82 Failed tensile test with pneumatic action grips on Instron 5943.

Instron 5943 tensile tester. (A) ECM samples mounted by the Instron pneumatic action grips. Front view (B) and side view. (C) Stress-strain curve showing an abnormal profile probably due to the slippage of samples. (D)

In contrast, we sutured a small ECM specimen around two long stainless steel rods and fit the rods onto pneumatic grips. (Fig. 83A) The pneumatic grips securely held the two long stainless steel rods while the sutures around the rods securely held the ECM specimen throughout the test. (Fig. 83E-J) We successfully performed all assays including pre-cycling, cyclic-loading and stress relaxation and tensile failure. (Fig. 83B-D) with a modulus measured as 1.18MPa that is highly comparable to those obtained from Instron 5567.

Figure 83 Preliminary tensile test with sutures on Instron 5943.

ECM specimen was sutured around two long stainless steel rods to be mounted onto pneumatic grips. (A) Representative curves of the successfully performed uniaxial testing assays on Instron 5943. Pre-cycling and cyclic loading. (A) Stress relaxation. (B) Tensile failure. (C) Deformation of sutured small ECM specimens tested. Front view: (E-G) Side view: (H-J)

We also suggest studying the effect of cell proliferation over the culture span to the mechanical properties, in order to attempt to develop the correlation between cell numbers and the extent of changes to the mechanics.

10.3.2 Co-culture and Multiple-culture of cells

Human myocardium is composed of a large mix of varieties of cells as discussed in Introduction section 1.2.2. There are various non-cardiomyocyte cell types present in a healthy myocardium for the proper functioning of the heart tissues. Co-existence of different kinds of cardiac-relevant cells could be beneficial for the growth of stem cells and the ultimate construct functionality^[12,28,212,213].

Hence, apart from human bone-marrow derived mesenchymal stem cells (MSC) which could act as cardiomyocyte model cells, other cell types might also be reseeded onto the scaffold for possible improved functionality of the MTE construct. For instance, endothelial cells mainly lie in the vasculature in the heart tissues and they facilitate the transportation of nutrition and oxygen; as well as secreting essential growth factors. ^[3,11,26] Hence, experiments of coculture and even multiple-cell types on our ECM construct could be performed to give further insights on the cell-interacting capability of our ECM, as well as how different types of cell could individually or jointly influence the biophysical properties of their extracellular matrix.

Preliminary uniaxial mechanical testing results of coculturing MSCs and HUVECs on our thin ECM constructs have been obtained as shown in Fig. 84.

It can be clearly seen that the stress-strain profiles of the cells-reseeded construct has shifted to be more native-resembling, indicating the recovery of deformation profile from

the decellularized ECM to cells-reseeded ECM towards native heart tissues; with a trend of decreasing modulus, decreasing ultimate stress, and increasing ultimate strain. There are also apparent recovery of mechanical behavior in the cyclic loading and stress relaxation testing assays with the recellularized samples closely resembling the native tissues compared to decellularized ECM.

Figure 84 Preliminary results of mechanical testing of MSC/HUVEC cocultured ECM.

Representative graphs of the three uniaxial tensile test assays: cyclic loading (A), stress relaxation (B) and tensile failure (C) on our six groups of samples : 1) Acellular ECM (pcECM), 2) native porcine myocardium (Native), 3) MSC monocultured ECM (ECM-MSC), 4) HUVEC monocultured ECM (ECM-HUVEC), 5) MSC-HUVEC simultaneous cocultured (Co-sim), and 6) HUVEC-MSC sequential cocultured (Co-seq). The orders of labeling in the figure legends were presented according to the trend of the maximum stress point of each curve from highest to lowest for the ease of following. (n=5)

HUVEC monoculture was found having little impact on modulating the mechanical properties of the reseeded ECM, which we found reasonable since HUVEC are mainly lining along the blood vessel within the myocardium and has relatively smaller contribution to the flexibility and deformation of the bulk of the tissues. During cyclic loading and stress relaxation, MSC monoculture showed the closest resembling towards the native tissue; while during tensile failure, co-sequential HUVEC-MSC coculture resemble the best in terms of decreasing ultimate stress, and increasing ultimate strain. These findings should be further investigated as future works.

10.3.3 Dynamic cultivation with electromechanical stimulations

We believe that further improving the cell ingrowth and proliferation on the ECM constructs will eventually lead us to our goal for improved functionality of the reseeded ECM-based constructs with more native-resembling properties, and even differentiation into cardiomyocytes-like phenotype and contractile functions.

As discussed in Introduction section 1.5.3, many sophisticated designs of bioreactors with the introduction of electromechanical stimulation have been of increasing interest over recent years.^[26,89-91] A bioreactor system is also under ongoing development in our lab at Technion to achieve the goal of obtaining an adequately myocardium-resembling construct with better cell ingrowth and even cardiac-cell type differentiation and maturation. (Fig. 85)

Figure 85 Schematic of our custom-developed bioreactor with stimulation means.

To mimic closely the heart pumping motions, we are introducing a multi-axis mechanical stimulation by mean of an inflatable latex balloon placed underneath the construct inside the culturing chamber. Inflation is mediated by computer-controlled pumping of fluids or pressurized air towards the bottom of construct. Electrical stimulation is applied to the construct by two electrode plates placed at two of the walls of the construct chamber. This bioreactor system is expected to provide a highly native mimicking environment for cells ingrowths and even differentiation.

10.3.4 Cardio-mimetic construct evaluation upon stimulated culture

The recellularized samples being cultivated under the above-mentioned improved bioreactor should be characterized as described in this thesis for comparison and evaluating the extent of reversibility of biophysics towards native-like properties. Cell viability, proliferation, morphology as well as distribution should be studied against the results in this thesis. Potential synthesis of new collagen by the reseeded cells should be investigated by chemical assays as discussed in section 5.2.4. In addition, immuno-staining could also be performed to check for markers regarding the possible differentiation of BM-MSCs into CM-like cells. Such as cardiomyogenic-cell markers: α -sarcomeric actinin; GATA-4; β -MyHC; cardiac troponin 1 and Connexin-43, as well as ECM-remodeling-related

genes: collagen I, III; fibronectin and MPM 2. Any successful differentiation of hBM-MSCs into CM-like cells may give rise to contracting tissue constructs. Upon which, contractile force measurement such as excitation threshold (E.T.) and maximum capture rate should be measured and compared against physiological values and published literature. ^[93,214]

10.3.5 Advanced stem cell sources for recellularization of construct

As discussed in Introduction section 1.3.1, the use of embryonic stem cells (ESCs)^[61,62] and inducible pluripotent stem cells (iPSCs)^[63] have been getting attention in the field of MTE. Their pluripotency possess high potential for possible differentiation into cardiomyocytes (CM) relevant cells. To obtain any possible FDA approval for clinical application, both cell sources are still undergoing extensive investigation. With the hope that such approval could be reached in future, the technologies developed, characterized and resented in this thesis would no doubt be also applicable to these cell types.

10.3.6 Comparison with other potential MTE-relevant products

In this thesis, we have experimentally shown the closely matched biophysical properties of our ECM comparable to native myocardium. We have also shown that improvement in native-resembling properties could be achieved by optimized recellularization, suggesting that the ECM could be a highly suitable cardio-mimetic construct for myocardial tissue engineering. Hence, we are interested in comparing our ECM-based cardio-mimetic construct with other potential MTE-relevant products in terms of mechanical, surface and bulk biophysical properties as well as cellular behaviors *in vitro* to iterate the importance of a close material properties match in enabling better cell functions.

10.3.7 Animal trials

A milestone test before human clinical trials would be the implantation of our construct in large human-equivalent animal models such as pigs. Transplantation surgery to augment or replace the infarcted myocardium should be performed to study the *in vivo* performance of our ECM-based cardio-mimetic construct including integration, host response functionality and biodegradation. Upon positive animal trials results, the potential of the construct to be a suitable cardio-mimetic construct for myocardial tissue engineering would be reaffirmed. The last stage of the development of this cardiac regenerative medicine therapy would be the pre-clinical human trials to step forward in putting the ECM-based cardio-mimetic construct as an off-the-shelf biomedical product for diseased myocardium replacement.

Appendices

1. Specimen mounting fixture design

Specimen mounting fixture of Stainless steel $\varnothing 10 \times 30\text{mm}$ housing comes with spring ejector pins was designed by the author and fabricated by Ricko Engineering, Singapore

2. Biaxial Biotester Customization CAD drawings

BioRake Bases: The standard BioRake base was lengthened to accommodate the desired specimen size. For a better affix of the base, a second magnet was added. The pictures below show a standard base and a new custom BioRake base.

Rake Holders: stainless steel rake holders are modified to fit larger BioRake bases.

Fluid Chamber: fluid chamber is shaped to accommodate specimens and more fluid.

3. Development steps of Zener integral Equations

$$\frac{d\sigma}{dt} + \sigma \frac{E_1 + E_2}{\eta} = \frac{d\varepsilon}{dt} E_1 + \varepsilon \frac{E_1 E_2}{\eta}$$

$$\frac{d\sigma}{dt} + \sigma \frac{E_1 + E_2}{\eta} = \varepsilon \frac{E_1 E_2}{\eta}$$

$$a = \frac{E_1 + E_2}{\eta}, b = \varepsilon \frac{E_1 E_2}{\eta}$$

$$\frac{d\sigma}{dt} + a\sigma = b \Rightarrow \frac{d\sigma}{dt} = -a\sigma + bX = \sigma - \frac{b}{a} \Rightarrow \sigma = X + \frac{b}{a}$$

$$\frac{d\left(X + \frac{b}{a}\right)}{dt} = \frac{dX}{dt} = -aX \Rightarrow \frac{dX}{X} = -a \cdot dt$$

$$\int_{X_1}^{X_2} \frac{dX}{X} = -a \int_{t_1}^{t_2} dt \Rightarrow \ln\left(\frac{X_2}{X_1}\right) = -a\Delta t$$

$$\Rightarrow \frac{X_2}{X_1} = e^{-a\Delta t} \Rightarrow \frac{\sigma_2 - \frac{b}{a}}{\sigma_1 - \frac{b}{a}} = e^{-a\Delta t}$$

$$\sigma_2 = \frac{b}{a} + \left(\sigma_1 - \frac{b}{a}\right)e^{-a\Delta t}$$

$$\tau_{rx} = \frac{\eta}{E_1 + E_2} = 1/a$$

$$\Rightarrow \sigma_2 = \frac{E_1 E_2 \varepsilon}{E_1 + E_2} + \sigma_1 \cdot e^{-\Delta t / \tau_{rx}} - \frac{E_1 E_2 \varepsilon}{E_1 + E_2} \cdot e^{-\Delta t / \tau_{rx}}$$

$$\sigma_2 = \sigma_1 \cdot e^{-\Delta t / \tau_{rx}} + \frac{E_1 E_2 \varepsilon}{E_1 + E_2} \left(1 - e^{-\Delta t / \tau_{rx}}\right)$$

4. Matlab programming code: Zener.m

```
%% Main function
function [out] = Zener(full_data,avg_step)
% The main function (Zener.m) is used to implement a Zener based
analysis
% for stress-strain data originating from tensile-relaxation or
% tensile-failure data. For tensile-failure data, only the data
% delineated between the toe border and the apparent failure border
will
% be analyzed, excluding all failure data. The input data for analysis
% (full_data) is given as an N-by-3 matrix of stress-strain data; with
% the first column being the sampling time, the second being the sampled
% strain and the third being the sampled stress. See more detailed
% description of the input/output arguments in the methods part on the
% main article. The average step argument is used to determine the
% average sampling rate of the raw data. For example, although data is
% sampled in variable steps that reflect on the stress sensitivity, an
% average step of 0.2 sec means that the overall number of samples
taken
% is five times the test duration (in seconds). When the average step
% equals zero, all data points will be sampled.
% Data is offset according to the first data point of each column to
% correct for instrument variations and mis-calibrations that may
results
% in negative first data points.
full_data=offset(full_data);
% The Zener viscoelastic moduli are evaluated using the linear form of
% the constitutive model. These evaluated moduli will be later used as
% initial values for optimization utilizing the non-linear Zener model.
Lout=linearzener(full_data,avg_step);
% The toe region border (as the end of the toe region - t0) and the
end
% of the 'elastic' region (t1) are calculated. For tensile-relaxation
% data, t1 corresponds to the time of transition from tensile mode
% (constant strain-rate) to stress-relaxation mode (constant strain).
For
% tensile-failure data, t1 corresponds to the apparent failure border.
[E,SE_E,t0,t1,R2adj,p]=get_young(full_data,false);
Hout=[E,t0,t1,R2adj,SE_E,p];
% Data is truncated, offset and sampled. The data reflecting the toe
% region is excluded from all analysis. For tensile-failure data, the
% data above t1 is also excluded.
[data,tv,ttype,t0,~,f0]=correct_data(full_data,t0,t1,avg_step);
% Negative moduli values, which may arise from the linear Zener model
are
% corrected before inputted as initial estimations for optimization
% utilizing the non-linear Zener model.
K1=abs(Lout(3));
K2=abs(Lout(2));
D=abs(Lout(1));
% Moduli are optimized according to the Zener model.
subplot(2,3,4);
Zout=solve_model(K1,K2,D,data,tv,ttype,'Zener',full_data,t0,f0);
% Moduli are optimized according to the Maxwell model.
subplot(2,3,5);
Mout=solve_model(K1,K2,D,data,tv,ttype,'Maxwell',full_data,t0,f0);
% Moduli are optimized according to the Kelvin-Voigt model.
subplot(2,3,6);
KVout=solve_model(K1,K2,D,data,tv,ttype,'Kelvin-
Voigt',full_data,t0,f0);
% Function output arguments are defined.
```

```

out=[Hout;Lout;Zout;Mout;KVout];
end
%% Linear modeling
function [out] = linearzener (full_data,avg_step)
% Given the same input arguments as the main function, and utilizing
the
% linear form of the Zener model, this function will produce initial
% estimations for the viscoelastic moduli along with statistical
measures
% for the quality of the fit.
% Sub-plotting for the hook model output to be produced by the function
% get_young executed next.
subplot(2,3,2);
% The toe region border (as the end of the toe region - t0) and the
end
% of the 'elastic' region (t1) are calculated. For tensile-relaxation
% data, t1 corresponds to the time of transition from tensile mode
% (constant strain-rate) to stress-relaxation mode (constant strain).
For
% tensile-failure data, t1 corresponds to the apparent failure border.
[~,~,t0,t1]=get_young(full_data,true);
% Data is truncated, offset and sampled. The data reflecting the toe
% region is excluded from all analysis. For tensile-failure data, the
% data above t1 is also excluded.
[data,tv,~,t0,~,f0]=correct_data(full_data,t0,t1,avg_step);
% Data is parsed to three vectors: t, x & f.
t=data(:,1);
x=data(:,2);
f=data(:,3);
% Plotting extension rate vs. time.
subplot(2,3,1);
plot_tv([tv(1,1);tv(2:end,1)+t0],tv(:,2));
% Defining the independent variables for regression.
v=interp1(tv(:,1),tv(:,2),t);
dfdt=(f(2:end)-f(1:end-1))./(t(2:end)-t(1:end-1));
dfdt=[dfdt(1);dfdt];
% Regression of the linear Zener equation.
[B,~,resid]=mvregress([dfdt,v,x],f);
% Calculating the statistical measures for the fit between the sampled
% data and the linear form of the Zener model.
SSE=sum(resid.^2);
F=B(1)*dfdt+B(2)*v+B(3)*x;
R_squire=corr(F,f)^2;
N=size(t,1);
dFm=3;
dFt=N-1;
dFe=dFt-dFm;
R_squire_adj=1-(1-R_squire)*(dFt)/(dFe);
SSM=sum((F-mean(f)).^2);
SE=sqrt(SSE/dFe);
p=1-fcdf((SSM/3)/(SSE/dFe),dFm,dFe);
% Calculating the Zener moduli K1, K2 and D corresponding to E1, E2
and
% Eta.
K1=-B(2)/B(1);
K2=B(3)*K1/(K1-B(3));
D=-B(1)*(K1+K2);
% Plotting the linear Zener model.
subplot(2,3,3);
plot_linearzener(full_data,t+t0,F+f0,SE,R_squire_adj,p,K1,K2,D);
% Function output arguments are defined.
out=[K1,K2,D,R_squire_adj,SE,p];
end
%% Non-linear modeling

```



```

function [out] = solve_model
(K1,K2,D,data_,tv_,ttype_,mtype_,full_data,t0,f0)
% This function is used to resolve empirical tensile-relaxation and
% tensile-stress data according to three viscoelastic models: Zener,
% Maxwell and Kelvin-Voigt. Note that tensile-failure data inputted to
% this function should be truncated to exclude the apparent failure
% region. The function receives the following input arguments:
estimates
% of Zener viscoelastic moduli (K1, K2 and D); the truncated data for
% analysis (data_); the strain-rate vs. time look-up-table (tv_); the
% mechanical test type (ttype_) being tensile-relaxation or
% tensile-failure; the type of the model used to resolve the data
% (mtype_); the complete raw data (full_data); and the time (t0) and
% stress (f0) at the toe border. This function will then produce an
% output including the optimized viscoelastic moduli and statistical
% measures (R2adj, SE and p) for the fit between the empirical data
and
% the model used.
% Declaration of global variables (data, tv, ttype and mtype) to be
later
% used in sub-functions for moduli optimization.
global data; data=data_;
global tv; tv=tv_;
global ttype; ttype=ttype_;
global mtype; mtype=mtype_;
% Determining the initial values (KDi) and lower boundaries (KDl) for
the
% moduli optimization. In case data is modeled not according to the
Zener
% model, a single initial elastic modulus is calculated based on K1
and
% K2.
if strcmp(mtype,'Zener')
KDl=[0,0,0];
KDi=[K1,K2,D];
else
KDl=[0,0];
KDi=[K1*K2/(K1+K2),D];
end
% Determining the upper boundary for the optimized moduli (KDh).
KDh=10^5*KDi;
% Optimization of the viscoelastic moduli (KD) between their upper
(KDh)
% and lower (KDl) boundaries starting from their initial values (KDi)
% using the optimization function fmincon with an interior-point
% algorithm. The optimization uses the target function KDtoSSE which
% receives a vector of the three moduli and returns the sum of squares
% (SSE) between the empirical data (stored in the global variable:
data)
% and the model characterized by the moduli and the model type (stored
in
% the global variable: mtype).
options = optimset;
options = optimset(options,'Display', 'off');
options = optimset(options,'Algorithm', 'interior-point');
KD=fmincon(@KDtoSSE,KDi,[],[],[],[],KDl,KDh,[],options);
% The vector output of the optimization process (KD) is parsed into
% single moduli according to the implemented model.
if strcmp(mtype,'Zener')
K1=KD(1);
K2=KD(2);
D=KD(3);
elseif strcmp(mtype,'Maxwell')
K1=KD(1);

```

```

K2=0;
D=KD(2);
else
K1=[];
K2=KD(1);
D=KD(2);
end
% A graphical output is produced depicting the full data (full_data)
% overlaid with the modeled stress resulting from the applied model
using
% the optimized moduli. This function will also produce statistical
% measures (R2adj, SE and p) for the fit between the empirical data
and
% the model used.
[R2adj,SE,p]=plot_model_stats(K1,K2,D,full_data,t0,f0);
% Moduli output is corrected according to the model used.
if strcmp(mtype,'Kelvin-Voigt')
K1=NaN;
K2=KD(1);
D=KD(2);
elseif strcmp(mtype,'Maxwell')
K1=KD(1);
K2=NaN;
D=KD(2);
end
% Function output arguments are defined.
out=[K1,K2,D,R2adj,SE,p];
end
function [SSE] = KDtoSSE (KD)
% This function receives a vector of the three moduli (KD) and returns
% the sum of squares (SSE) between the empirical data (stored in the
% global variable: data) and the model characterized by the moduli and
% the model type (stored in the global variable: mtype).
% Declaration of global variables that were previously defined by the
% parent function solve_model.
global data;
global tv;
global ttype;
global mtype;
% Parsing the moduli vector (KD) to the respective moduli (K1, K2 and
D)
% according to the selected model. In the simplest case of a Kelvin-
Voigt
% model, SSE is linearly calculated and the function returns.
if strcmp(mtype,'Zener')
K1=KD(1);
K2=KD(2);
D=KD(3);
elseif strcmp(mtype,'Maxwell')
K1=KD(1);
K2=0;
D=KD(2);
else
K=KD(1);
D=KD(2);
v=interp1(tv(:,1),tv(:,2),data(:,1));
dt=[0;data(2:end,1)-data(1:end-1,1)];
x=cumsum(v.*dt);
SSE=sum((K*x+D*v)-data(:,3)).^2);
figure(1);
title([mtype,' SE=',num2str(sqrt(SSE/((size(data,1)-1)-2)))]);
return;
end

```

```

% Numeric analysis is applied to determine the sum of squares (SSE)
under
% non-linear viscoelastic models (i.e., Zener and Maxwell). For
% tensile-relaxation data, the numeric evaluation will include two
% intervals: one spanning from t0 to t1, in which the strain-rate is
% constant; and a second, spanning from t1 and until the last data
point,
% in which the strain is constant. The stress values in the first time
% interval are calculated by the function: zener_const_v (for constant
% strain-rate). The stress values in the second time interval are
% calculated by the function: zener_const_x (for constant strain). For
% tensile-failure data, all data between t0 and the failure border (t1)
% are evaluated using the function: zener_const_v, data outside those
% borders (t0 up to t1) is excluded from tensile-failure analysis.
F1=0;
SSE=0;
if strcmp(ttype,'relax')
t1=tv(3,1);
tli=find(t1<=data(:,1),1);
x1=data(tli,2);
for i=2:tli
t=data(i,1)-data(i-1,1);
v=interp1(tv(:,1),tv(:,2),data(i,1));
F2=zener_const_v(t,v,K1,K2,D,F1);
SSE=SSE+(F2-data(i,3))^2;
F1=F2;
end
for i=tli+1:size(data,1)
t=data(i,1)-data(i-1,1);
F2=zener_const_x(t,x1,K1,K2,D,F1);
SSE=SSE+(F2-data(i,3))^2;
F1=F2;
end
else
for i=2:size(data,1)
t=data(i,1)-data(i-1,1);
v=interp1(tv(:,1),tv(:,2),data(i,1));
F2=zener_const_v(t,v,K1,K2,D,F1);
SSE=SSE+(F2-data(i,3))^2;
F1=F2;
end
end
% The standard error (SE) is calculated and displayed, for each
% iteration, to track optimization progress.
figure(1);
title([mtype, ' SE=', num2str(sqrt(SSE/((size(data,1)-1)-2)))]);
end
function [F2] = zener_const_v (t,v,K1,K2,D,F1)
% This function produces the stress (F2) at time t (after F1 was
% measured) under the Zener or Maxwell models for constant strain-rate.
% The function receives the following input arguments: the elapsed time
% (t) between measuring F1 and determining F2; the constant strain-
rate
% (v); the viscoelastic moduli (K1, K2 and D); and the initial stress
at
% time zero (F1).
% Calculating relaxation time (T).
T=D/(K1+K2);
% Calculating F2 - the stress at time t for constant strain-rate.
F2=F1*exp(-t/T)+((1-exp(-t/T))*(D*K1/(K1+K2))+t*K2)*(v*K1/(K1+K2));
end
function [F2] = zener_const_x (t,x,K1,K2,D,F1)
% This function produces the stress (F2) at time t (after F1 was
% measured) under the Zener or Maxwell model for constant strain. The

```

```

% function receives the following input arguments: the elapsed time (t)
% between measuring F1 and determining F2; the constant strain (x);
the
% viscoelastic moduli (K1, K2 and D); and the initial stress at time
zero
% (F1).
% Calculating the relaxation time (T).
T=D/(K1+K2);
% Calculating F2 - the stress at time t for constant strain.
F2=F1*exp(-t/T)+(K1*K2*x/(K1+K2))*(1-exp(-t/T));
end
%% Graphical output functions
function [] = plot_tv (tv)
% This function produces a graphical output for the look-up-table tv
% depicting the strain-rate vs. time along the test-run.
plot(tv(:,1),tv(:,2));
tmin=tv(1,1);
tmax=tv(end,1);
vmin=min(tv(:,2));
if vmin<0
vmin=1.1*vmin;
else
vmin=0.9*vmin;
end
vmax=1.1*max(tv(:,2));
axis([tmin tmax vmin vmax]);
xlabel('Time');
ylabel('Strain rate');
title(['Test time (excl. failure): ',num2str(tmax)]);
end
function [] = plot_linearzener (data,t,F,SE,R_squre_adj,p,K1,K2,D)
% This function receives raw data (data) and two vectors (t and F)
% depicting the stress (F) vs. time (t) profile according to the linear
% Zener model. Along with additional input arguments, such as the
% statistical measures for the fit (i.e., R_squre_adj and p) and the
% resulting viscoelastic moduli (i.e., K1,K2 and D) this function will
% produce a graphical output summarizing the fit between the sampled
data
% and the linear form of the Zener model.
plot(data(:,1),data(:,3),'.',t,F,'.');
tmin=data(1,1);
tmax=data(end,1);
Fmin=min(min(data(:,3)),min(F));
Fmax=max(max(data(:,3)),max(F));
axis([tmin tmax 0.9*Fmin 1.1*Fmax]);
xlabel('Time');
ylabel('Stress');
legend('Real','Model');
title(['Linear Zener: SE=',num2str(SE,1),' R',char(178),...
'adj.=',num2str(R_squre_adj,4),' p=',num2str(p,4)]);
line1=['E1=',num2str(K1,2)];
line2=['E2=',num2str(K2,2)];
line3=['Eta=',num2str(D,2)];
text((tmin+tmax)/2,+1*(Fmax-Fmin)/10+(Fmin+Fmax)/2,line1);
text((tmin+tmax)/2,(Fmin+Fmax)/2,line2);
text((tmin+tmax)/2,-1*(Fmax-Fmin)/10+(Fmin+Fmax)/2,line3);
end
function [R2adj,SE,p] = plot_model_stats (K1,K2,D,full_data,t0,f0)
% This function produces a graphical output presenting the full data
% (full_data) overlaid with the modeled stress resulting from the
applied
% model when using a given set of moduli. The function will also produce
% statistical measures (R2adj, SE and p) for the quality of the fit
% between the empirical truncated data (stored in the global variable:

```

```

% data) and the applied model. The function will require the following
% input arguments: the viscoelastic moduli (K1, K2 and D); the complete
% data (full_data) and the toe border time (t0) and stress (f0).
% Declaration of global variables.
global data;
global tv;
global ttype;
global mtype;
% Determining data size (n) for later statistical calculations.
n=size(data,1);
% Calculating the statistical degrees of freedom for the model (dFm),
% total (dFt) and error (dFe).
dFm=2;
dFt=n-1;
dFe=dFt-dFm;
% Numeric analysis is applied to determine the stress response under
the
% applied viscoelastic model. In the simplest case of a Kelvin-Voigt
% model, the modeled stress (Fmodel) is linearly calculated. When other
% models are applied (i.e., Zener or Maxwell), the stress response is
% numerically evaluated using non-linear functions for either constant
% strain-rate (zener_const_v) or constant strain (zener_const_x). For
% tensile-relaxation data, the numeric evaluation of the modeled stress
% will include two intervals: one spanning from t0 to t1, in which the
% strain-rate is constant; and a second interval, spanning from t1
until
% the last data point, in which the strain is constant. For
% tensile-failure data, all data between t0 and the failure border (t1)
% are evaluated using constant strain-rate.
if strcmp(mtype,'Kelvin-Voigt')
K=K2;
v=interp1(tv(:,1),tv(:,2),data(:,1));
dt=[0;data(2:end,1)-data(1:end-1,1)];
x=cumsum(v.*dt);
Fmodel=K*x+D*v;
elseif strcmp(ttype,'relax')
v=tv(1,2);
t1=tv(3,1);
tli=find(t1<=data(:,1),1);
x1=data(tli,2);
F1=zener_const_v(data(1:tli,1),v,K1,K2,D,0);
F2=zener_const_x(data(tli+1:end,1),x1,K1,K2,D,F1(end));
Fmodel=[F1;F2];
else
Fmodel=zeros(n,1);
for i=2:n
t=data(i,1)-data(i-1,1);
v=interp1(tv(:,1),tv(:,2),data(i,1));
Fmodel(i)=zener_const_v(t,v,K1,K2,D,Fmodel(i-1));
end
end
% Based on previously defined degrees of freedom (dFm, dFe and dFt),
% statistical measures (R2adj, SE and p) are calculated for the quality
% of the fit between the empirical truncated data (stored in the global
% variable: data) and the modeled stress (Fmodel).
R2=corr(Fmodel,data(:,3))^2;
R2adj=1-(1-R2)*(dFt)/(dFe);
R2adj(R2adj<0)=0;
SSE=sum((Fmodel-data(:,3)).^2);
SSM=sum((Fmodel-mean(data(:,3))).^2);
SE=sqrt(SSE/dFe);
p=1-fcdf((SSM/dFm)/(SSE/dFe),dFm,dFe);
% Graphical output is produced to present the real vs. modeled stress.
plot(full_data(:,1),full_data(:,3),'.',data(:,1)+t0,Fmodel+f0,'.');

```

```

xlabel('Time');
ylabel('Stress');
legend('Real','Model');
title([mtype,': SE=',num2str(SE,1),' R',char(178),...
'adj.',num2str(R2adj,4),' p=',num2str(p,4)]);
tmin=full_data(1,1);
tmax=full_data(end,1);
Fmin=min(min(full_data(:,3)),min(Fmodel+f0));
Fmax=max(max(full_data(:,3)),max(Fmodel+f0));
axis([tmin tmax (1-0.1*sign(Fmin))*Fmin (1+0.1*sign(Fmax))*Fmax]);
% Displaying the previously calculated statistical measures.
switch mtype
case 'Zener'
line1=['E1=',num2str(K1,2)];
line2=['E2=',num2str(K2,2)];
line3=['Eta=',num2str(D,2)];
case 'Maxwell'
line1=['E=',num2str(K1,2)];
line2=['Eta=',num2str(D,2)];
line3='';
otherwise
line1=['E=',num2str(K2,2)];
line2=['Eta=',num2str(D,2)];
line3='';
end
text((tmin+tmax)/2,+1*(Fmax-Fmin)/10+(Fmin+Fmax)/2,line1);
text((tmin+tmax)/2,(Fmin+Fmax)/2,line2);
text((tmin+tmax)/2,-1*(Fmax-Fmin)/10+(Fmin+Fmax)/2,line3);
end
%% Auxiliary functions
function [data,tv,ttype,t0,x0,f0] = correct_data
(full_data,t0,t1,avg_step)
% This function receives the raw data (data) and the time borders
% delineating the analysis (t0 and t1) as well as the average sampling
% step (avg_step) and corrects the data to fit the suggested analysis.
% The test type (ttype) is determined using the function get_tv.
[~,ttype]=get_tv(full_data);
% The data is truncated to exclude the toe region (for data resulting
% from all tests) and the failure region for tensile-failure data only.
% After cropping, data is offset again.
if strcmp(ttype,'relax')
data=full_data((full_data(:,1)>=t0),:);
f0=data(1,3);
x0=data(1,2);
data=offset(data);
else
data=full_data((full_data(:,1)>=t0)&(full_data(:,1)<=t1),:);
f0=data(1,3);
x0=data(1,2);
data=offset(data);
end
% Extension rate look-up-table (tv), indicating the strain-rate vs.
time,
% is determined for the truncated offset data.
[tv,~]=get_tv(data);
% For tensile-failure data, the last time point for analysis (in the
% look-up-table - tv) is corrected to correspond with the apparent
% failure time (t1).
if strcmp(ttype,'fail')
tv(end,1)=t1;
end
% Data is truncated again to fit the look-up-table.
data=data(data(:,1)<=max(tv(:,1)),:);

```

```

% Analyzed data is sampled according to the sampling index calculated
by
% the function get_sampling based on the truncated data and the average
% step.
data=data(get_sampling(data,avg_step),:);
end
function [I] = get_sampling (data,avg_step)
% This function will produce the sampling index (I) used to sample raw
% data for analysis (based on the stress sensitivity). The function
% receives two input arguments: truncated or full data (data); and the
% average step (avg_step) which is used to determine the average
sampling
% rate of the data. For example, an average step of 0.2 sec means that
% the overall number of samples taken is five time the test duration
(in
% seconds). Sampling will not only decrease data size and by that
% increase execution speed, but will also improve the fidelity of the
% analysis. In particular, for tensile-relaxation tests, large changes
in
% the stress are commonly recorded in a relatively short time during
the
% tensile mode and the beginning of the stress-relaxation mode. However,
% stress variations are usually quite low during the rest of the much
% longer stress-relaxation mode. As such, if all data points are taken
% (i.e., no sampling) or if data is sampled using a fixed step size,
the
% resulting models may be biased towards the longer time-intervals that
% do not necessarily reflect on the stress variability.
% When the average step equals zero, the sampling index (I) will be
all
% true and the function will return.
if avg_step==0
I=true(size(data,1),1);
return;
end
% The number of samples (Ns) that will be drawn from the data is
% calculated based on the overall test time and avg_step.
Ns=round((max(data(:,1))-min(data(:,1)))/avg_step);
% Stress differential column (df) is derived from the data.
df=data(2:end,3)-data(1:end-1,3);
% Time differential column (dt) is derived from the data.
dt=data(2:end,1)-data(1:end-1,1);
% The sampling index (I) is calculated.
L=cumsum(sqrt(df.^2+dt.^2).*abs(df./dt));
L=round(Ns*L/L(end));
I=L(2:end)>L(1:end-1);
I=[true;true;I];
I(end)=true;
end

```

5. Matlab programming code: offset.m

```
function [data] = offset (data)
% Due to instrument variations and differences in calibration, some
% stress-strain data start at offset positions exhibiting negative
strain
% and/or stress values at time zero and therefore need to be adjusted
and
% offset before analyzed. This function receives an N-by-3 matrix and
% returns the same matrix where each column is offset according to the
% first value.
N=size(data);
data=data-repmat([data(1,1),data(1,2),data(1,3)],N(1),1);
end
```

6. Matlab programming code: get_tv.m

```
function [tv,test_type] = get_tv (data)
% This function receives an N-by-3 matrix containing stress-strain data
% (data) and returns a look-up-table (tv) describing the strain-rate
vs.
% time; and the type of mechanical test used to derive this data
% (test_type), being tensile-relaxation or tensile-failure.
% Data is offset according to the first data point of each column.
data=offset(data);
% The time column from the data matrix is transformed to a time vector
% (t) smoothened by first order moving average.
t=(data(2:end,1)+data(1:end-1,1))/2;
% The extension rate (v) is calculated as dx/dt.
dx=(data(2:end,2)-data(1:end-1,2));
v=dx./(data(2:end,1)-data(1:end-1,1));
% Tensile-failure test is being concluded if the strain is
monotonically
% increasing throughout the entire test. In that case, only one
% strain-rate value should exist throughout the test (v_on), which is
% calculated as the average strain-rate (the mean of v). The border of
% the failure region (t_on) is determined as the point in which the
% stress equals the maximum reached through the monotonically
increasing
% part of the stress curve. Having the strain-rate and failure time
and
% given the test type, the function then returns.
if all(dx>0)
v_on=mean(v);
n=size(data);
f=(data(2:end,3)+data(1:end-1,3))/2;
df=smooth((data(2:end,3)-data(1:end-1,3)),n(1)/9);
toff_corrected=((t>max(t)/10)&(df<0));
if all(toff_corrected==0)
t_on=max(t);
else
t_on=t(f==max(f(t<=min(t(toff_corrected)))));
end
tv=[0,v_on;t_on,v_on];
test_type='fail';
return;
end
% Determining the data interval (ton, as a logical indexing vector)
```



```

% during which the strain-rate is larger than the average measured
% throughout the entire experiments. This interval corresponds to the
% data collected during the tensile mode under tensile-relaxation tests.
ton=v>mean(v);
% Determining the data interval (toff, as a logical indexing vector)
% during which the strain-rate is smaller than the average measured
% throughout the entire experiments. This interval corresponds to the
% data collected during the stress-relaxation mode under
% tensile-relaxations tests.
toff=v<mean(v);
% Determining the strain rate throughout the stress-relaxation mode.
% Although this value can be assumed as zero (from the test conditions)
% this calculation can be used to assess the fidelity of the raw data
and
% the ability of this function to correctly evaluate the strain rate
% profile of the raw data.
v_off=mean(v(toff));
% The duration of the stress-relaxation mode (t_off) is determined as
the
% time during the test in which the lowest strain-rate (v_off) is
% maintained.
t_off=max(t(toff))-max(t(ton));
toff_corrected=(v>=v_off)&(v<mean(v));
if round(min(t(toff_corrected)))==0
i=find(round(t(toff_corrected))~=0,1);
il=find(toff_corrected,i);
il=il(i);
toff_corrected=[false(il-1,1);toff_corrected(il:end)];
end
% The duration of the tensile mode (t_on) is determined as the time
% during the test in which the highest strain-rate (v_on) is maintained.
f=(data(2:end,3)+data(1:end-1,3))/2;
t_on=t(f==max(f(t<=min(t(toff_corrected)))));
v_off=0;
v_on=mean(v((ton)&(t>=0.1*t_on)&(t<=0.9*t_on)));
% Function output arguments are defined.
tv=[0,v_on;t_on-(t_on+t_off)/1e6,v_on;t_on,v_off;t_on+t_off,v_off];
test_type='relax';
end

```

7. Matlab programming code: get_young.m

```
function [E,SE_E,t0,t1,R2adj,p] = get_young (data,plot_res)
% Hookean model for data drawn from tensile-relaxation and
% tensile-failure tests. The function receives two input arguments:
the
% full data (including the toe and failure regions) as an N-by-3
% stress-strain matrix (data); and a Boolean (plot_res), that if true,
% will order the function to produce a graphical output as well. The
% and statistical measure (R2adj and p) for the fit between the data
% delineated by t0 and t1 and the Hook model. Practically, the lower
% border of the elastic region (t0) can be also regarded as the toe
% border.
% Data is offset according to the first data point of each column.
data=offset(data);
% Determining the strain-rate look-up-table (tv), indicating the
% strain-rate vs. time, and the test type (testtype)
[tv,testtype]=get_tv(data);
% Data is truncated to exclude the failure non elastic region.
data=data(data(:,1)<=tv(2,1),:);
% Global variables x and f are being declared and defined as a column
% vectors to store the strain and stress data.
global x; x=data(:,2);
global f; f=data(:,3);
% The lower limits of the toe border strain and the apparent failure
% strain are defined.
x0l=[0,x(find(f>=0.5*max(f),1))];
% The higher limits of the toe border strain and the apparent failure
% strain are defined.
x0h=[x0l(2),x(end)];
% The initial values of the toe border strain and the apparent failure
% strain are defined (x0i) as the average between their upper and lower
% limits.
x0i=(x0l+x0h)/2;
% modulus analysis, is corrected for tensile-relaxation data to reflect
% upon the strain reached at the maximal stress; occurring upon
% transition from the tensile mode to the stress-relaxation mode.
if strcmp(testtype,'relax')
i=find(f>=max(f),1);
x0l(2)=x(i-1);
x0i(2)=x(i);
x0h(2)=2*x0i(2)-x0l(2);
end
% Optimization of the strain borders (x0(1) and x0(2)) between their
% upper (x0h) and lower (x0l) limits starting from their initial values
% (x0i) using the optimization function fmincon with an interior-point
% algorithm. The optimization uses the target function opt2linear which
% receives a vector of the two borders (as strain) and returns the
% standard error (SE) of the Hookean model fitted on the empirical data
% (stored in the global variables: x and f). In this case, optimizing
by
% minimizing the standard error yielded better correlation (as adjusted
% R-square) between the Hook model and the empirical data than when
% minimizing the sum of squares (SSE).
warning('off','stats:regress:NoConst');
options = optimset;
options = optimset(options,'Display', 'off');
options = optimset(options,'Algorithm', 'interior-point');
x0=fmincon(@opt2linear,x0i,[],[],[],[],x0l,x0h,[],options);
% The time borders delineating the elastic region (t0 and t1) are
% determined according to the optimized strain borders (x0(1) and
x0(2)).
t0=min(data(x>=x0(1),1));
```

```

t1=min(data(x>=x0(2),1));
% The stress in the toe region is regressed vs. the strain.
x1=x(x<=x0(1));
f1=f(x<=x0(1));
[B1,~,~,~,~]=regress(f1,x1);
% The stress in the elastic region is regressed vs. the strain.
x2=x((x>x0(1))&(x<=x0(2)))-x0(1);
f2=f((x>x0(1))&(x<=x0(2)))-B1(1)*x0(1);
[B2,~,~,~,STATS2]=regress(f2,[x2 ones(size(x2))]);
% Statistical measures (SE_E, R2adj and p), for the fit of the Hook
% model, are calculated based on the results of the previous regression
% of the stress (vs. strain) in the elastic region.
E=B2(1);
SE_E=sqrt(STATS2(4));
n=size(x2,1);
R2adj=1-(1-STATS2(1))*(n-1)/(n-2);
p=STATS2(3);
% If the Boolean plot_res is true then plot_get_young will be executed
to
% produce a graphical output as well.
if plot_res
plot_get_young(x,f,x0,B1,B2,E,SE_E,t0,R2adj,p);
end
end
function [SE] = opt2linear (x0)
% This function calculates the standard error (SE) of a two-linear
model
% fitted on tensile data stored in the global variables x and f. The
% tensile data should include the toe region but not the apparent
failure
% region (for tensile-failure data) nor the stress-relaxation region
(for
% tensile-relaxation data). The function receives an input vector (x0)
% indicating the border strain values delineating the elastic region
on
% which the Hook model will be fitted.
% Declaration of global variables x and f - previously defined by the
% parent function. X and f respectively store the strain and stress
% values of the data to be fitted with the two-linear model.
global x;
global f;
% The first linear model will be fitted onto the toe region between
the
% first data point and the data point in which the strain reaches the
% lower border of the elastic region (or the toe border) defined by
x0_1.
% The second linear model (Hook) will be fitted onto the elastic region
% that spans between the lower (x0_1) and upper (x0_2) borders of the
% elastic region.
x0_1=x0(1);
x0_2=x0(2);
% Two vectors corresponding to the strain (x1) and stress (f1) in the
toe
% region data are derived from the full data (stored in x and f) by
% excluding all data above the toe border.
x1=x(x<=x0_1);
f1=f(x<=x0_1);
% The stress in the toe region is regressed vs. the strain and the sum
of
% squares for the toe region (SSE1) is calculated accordingly.
n1=size(x1,1);
[B1,~,~,~,STATS1]=regress(f1,x1);
SSE1=STATS1(4)*(n1-2);
% Two vectors corresponding to the strain (x2) and stress (f2) data in

```

```

% the elastic region are derived from the full data (stored in x and
f)
% by excluding all data below the toe border.
x2=x((x>x0_1)&(x<=x0_2))-x0_1;
f2=f((x>x0_1)&(x<=x0_2))-B1(1)*x0_1;
n2=size(x2,1);
% The stress in the elastic region is regressed vs. the strain and the
% sum of squares for the elastic region (SSE2) is calculated accordingly.
[~,~,~,~,STATS2]=regress(f2,x2);
SSE2=STATS2(4)*(n2-2);
% The total sum of squares (SSE) is calculated as the sum of SSE1 and
SSE2.
SSE=SSE1+SSE2;
% The standard error (SE) for the fit of the two-linear model with the
% empirical tensile data spanning the toe and the elastic region is
% calculated based on SSE and the statistical degrees of freedom.
SE=sqrt(SSE/(n1+n2-3));
end
function [] = plot_get_young (x,f,x0,B1,B2,E,SE_E,t0,R2adj,p)
% This function produces a graphical output presenting the analyzed
% statistical measure for the fit of the Hook model. This function will
% require the following input arguments: the stress-strain data (x and
% f); a vector containing the strain values delineating the elastic
% region (i.e., the elastic region borders - x0); two vectors holding the
the
% regression coefficients arising from the regression of stress vs.
% strain in the toe region (B1) and in the elastic region (B2); the
% point (t0) in which the toe border is reached; and statistical
measures
% for the fit of the Hookean model to the tensile data (R2adj and p).
plot(x,f,'.',[x0(1),x0(2)],...
[x0(1)*B1(1),(x0(2)-x0(1))*B2(1)+x0(1)*B1(1)],'-','LineWidth',2);
axis([x(1) x(end) 0.9*min(f) 1.1*max(f)]);
title(['Hook: (from: ',num2str(t0),'s) R',char(178),...
'adj=',num2str(R2adj,4),' p=',num2str(p,4)]);
line1=['E=',num2str(E,1),char(177),num2str(SE_E,1)];
text((x(1)+x(end))/3,(min(f)+max(f))/2,line1);
legend('Real data','Elastic region');
xlabel('Strain');
ylabel('Stress');
end

```

8. Matlab programming code: Failing_Zener.m

```

function [] = Failing_Zener (data,K1,K2,D)
% This is the main function used to analyze and resolve tensile-failure
% data according to the tensile-failure implementation of the Zener
model.
% The input data for analysis (full_data) is given as an N-by-3 matrix
of
% stress-strain data; with the first column being the sampling time,
the
% second being the sampled strain and the third being the sampled
stress.
% Apart from the raw data, the function receives three more input
arguments
% (K1, K2 and D) that store the initial estimates for the viscoelastic

```

```

% moduli according to the Zener model. These moduli will be later
optimized
% and reported along with mechanical and statistical information
% characterizing the failure profile of the analyzed material.
% Declaration of global variables to be used during the execution of
the
% GUI (fail.fig) that is used to implement this analysis.
global vars;
global init_flag;
global GUI_data;
global Nstrings;
global NSD;
global solver;
global solve_or_opt;
% Initial definition of the number of strings (Nstrings) used to
simulate
% failure on a solid made up from a bundle of such strings.
Nstrings=100;
% Initial definition of the number of standard deviation (NSD) taken
from
% each side of the means describing the average failure in a bundle of
% strings.
NSD=3;
% Initial definition of the solver algorithm to be used in fmincon.
solver='interior-point';
% Initial definition of the optimization method implemented to fit the
% empirical tensile-failure data on the model (solve_or_opt). If the
option
% 'solve' is selected, the optimization will minimize the sum of squares
% (SSE) between the real and modeled stress. If 'opt' is selected,
% optimization will be done to maximize the resulting adjusted R-square
% value.
solve_or_opt='solve';
% Data is offset according to the first data point of each column to
% correct for instrument variations and mis-calibrations that may
result in
% negative first data points.
data=offset(data);
% Truncating all data at the end of the experiment with stress values
lower
% than 1 percent of the maximal stress reached during the test. This
is
% used to exclude near-zero stress values commonly apparent in the end
of
% tensile-failure data arising from arbitrary stress fluctuations
within
% the measurements. If not excluded, this truncated data, will bias
the
% model towards the near-zero stresses achieved after the catastrophic
% failure is reached.
data=data(1:find(data(:,3)>=0.01*max(data(:,3)),1,'last'),:);
% Data is offset again
data=offset(data);
% Global flag init_flag is set as true to indicate that the following
% execution of the function solve_bundle_fail will be its initial
% execution, called from the main function and not from the GUI.
init_flag=true; %#ok
% The primary function in this analysis (solve_bundle_fail) is executed
for
% the first time. Apart from the input argument shared with the main
% function (data, K1, K2, and D), the function solve_bundle_fail also
gets
% four empty input arguments and an additional Boolean argument. The
four

```

```

% empty arguments correspond with the means and variance (as CV) of
the
% elastic and integrity failure points. Since these failure measures
are
% unknown yet, and need optimization, they are empty. The Boolean
argument
% (set as true) indicates that the function should optimize the moduli
and
% determine the failure measures and not only produce a graphical
output.
% The output argument of this function (out) is a vector containing
the
% following information: The three optimized moduli; the mean and CV
for
% the elasticity failure point; the mean and CV for the integrity loss
% point; and three statistical measures for the fit between the
empirical
% data and the model (R2adj, SE, and p).
out=solve_bundle_fail(data,K1,K2,D,[],[],[],[],true);
% Global flag init_flag is set as false to indicate that the next
execution
% of the function solve_bundle_fail will be called from the GUI.
init_flag=false;
% The moduli and failure measures resulting from the previous execution
of
% solve_bundle_fail are stored into the global variable vars that will
be
% used by the GUI.
vars=out(1:7);
% Data for the GUI (GUI_data) is stored into a global variable.
GUI_data=data;
% The GUI (fail) is executed.
fail;
end

```

9. Seeding chamber

Polyethylene (PE) box 86 X 86 X 40mm come with Polycarbonate (PC) Cover, compartment sizes of 3cmx7cm; 3cmx3cm was designed by our lab and fabricated by Ricko Engineering, Singapore.

10. Electrical chamber

Polycarbonate (PC) electrical chamber was designed by our lab and fabricated by Ricko Engineering, Singapore.

11. Porcine whole heart dissection

Cut the left ventricle from lateral side surface along the interventricular septum down to apex. The whole left ventricular wall upon complete dissection is a think curved volume.

Make a minor cut at the tip to flatten out the ventricle as highlighted by the yellow circle. The total dimension of the slab is approximately 70-90mm (w) x 70-80mm (l) x 10-15mm (t). The yellow rectangle highlights the lipid white edge. The corresponding y-axis and x-axis should be carefully noted and labeled accordingly during the biaxial test. Samples are cut within the left ventricle area, preferably the central region.

12. Calibration curve of BSA adsorption.

13. Calibration curve of AlamarBlue® MSC counting.

14. Calibration curve of load cell of the BGU biaxial tester

15. Matlab programming code: takemarkerJpg.m

```
%% This Matlab code written by Avihai Spizzichino, 2014
%%M.Sc Student|Department of Mechanical Engineering
%%Ben-Gurion University of the Negev%%

function []=takemarkerJpg(Path,Start,End)
%(filename,row,col)
for f=Start:End
    path=[Path,num2str(f),'\\'];
    filename='pig0001.jpg';
    fullname=[path filename];
    row=30;
    col=row;
    rr=exist(fullname,'file');
    if rr~=0
        I =imread(fullname);
        N=size(I);
        Irow=N(1);
        Icol=N(2);
        NPC=floor(Icol/col); %Num of pixels in one col
        NPR=floor(Irow/row); %Num of pixels in one row

        for i=0:row-1
            for j=0:col-1
                A=I (NPR*i+1:NPR*(i+1),NPC*j+1:NPC*(j+1));
                marker=[path,'marker',num2str(i*col+j+1),'.jpg'];
                imwrite(A,marker,'jpg');
            end
        end

        imshow(I)
        hold on
        y=linspace(1,Irow,2);
        for i=0:row
            x=linspace(NPC*i+1,NPC*(i+1),2);
            plot(x,y,'LineWidth',1,'LineStyle','-','Color',[1 1 1]);
        end
    end
end
```

```

end
x=linspace(1,Icol,2);
for i=0:col
    y=linspace(NPR*i+1,NPR*i+1,2);
    plot(x,y,'LineWidth',1,'LineStyle','-','Color',[1 1 1]);
end
%# grid domains
xg = 1:NPC:NPC*col;
yg = 1:NPR:NPR*row;
%# label coordinates
[xlbl, ylbl] = meshgrid(xg+NPC/2, yg+NPR/2);
%# create cell arrays of number labels
lbl = strtrim(cellstr(num2str((1:numel(xlbl))')));

hgsave([path 'grid0']);

for i=1:2:row
    for j=1:2:col
        counter=(i-1)*row+j;
        text(xlbl(i,j),                                ylbl(i,j),
lbl(counter),'color','w',...

'HorizontalAlignment','center','VerticalAlignment','middle');
        counter2=(i)*row+j+1;
        text(xlbl(i+1,j+1),                                ylbl(i+1,j+1),
lbl(counter2),'color','w',...

'HorizontalAlignment','center','VerticalAlignment','middle');
    end
end

hgsave([path 'grid1']);
%hgexport(gcf,[path 'grid']);
print(gcf, '-djpeg', [path 'grid1']);
close all

open([path 'grid0.fig']);

for i=1:2:row
    for j=1:2:col
        %counter=(i-1)*row+j;
        text(xlbl(i,j),                                ylbl(i,j),[num2str(i)
num2str(j)],'color','w',...

'HorizontalAlignment','center','VerticalAlignment','middle');
        %counter2=(i)*row+j+1;
        text(xlbl(i+1,j+1), ylbl(i+1,j+1), [num2str(i+1)
num2str(j+1)],'color','w',...

'HorizontalAlignment','center','VerticalAlignment','middle');
    end

end

hgsave([path 'grid2']);
%hgexport(gcf,[path 'grid']);
print(gcf, '-djpeg', [path 'grid2']);

close all
delete([path 'grid0.fig']);
end
end

```

16. Matlab programming code: drawMarkerMovement140305.m

```
%% This Matlab code written by Avihai Spizzichino, 2014
%%M.Sc Student|Department of Mechanical Engineering
%%Ben-Gurion University of the Negev%%

function []=drawMarkerMovement140305( ElongPath,PIGPath)
cd(PIGPath);
load('PIG.mat');
End=size(DATA.ElongationRatio,2);
for ExpNum=1:End % Number of Ratios
    fprintf('\n \n ***Now draw Exp Num %d***\n',ExpNum);
    NewPath=[ElongPath num2str(ExpNum)];
    cd(NewPath);
    open('grid1.fig');
    M=size(DATA.ElongationRatio(1,1).Location,2);
    l=size(DATA.ElongationRatio(1,ExpNum).Location{1,1}.X,2);
    x=rand(1,1);
    y=rand(1,1);
    for i=1:M % Number Of merker with location, i=Marker Num
        x=DATA.ElongationRatio(1,ExpNum).Location{1,i}.X;
        y=DATA.ElongationRatio(1,ExpNum).Location{1,i}.Y;
        if min(x)==0

plot(x,y,'MarkerSize',2,'Marker','+','LineStyle','none',...
        'Color',[0 0 0]);
        else
plot(x,y,'MarkerSize',2,'Marker','+','LineStyle','none',...
        'Color',[1 0 0]);
        end
    end

    name=[DATA.ElongationRatio(1,ExpNum).Label{1,1}];
    expdata=['I need to insert experiment data: Elongation ratio, size,
Oparetor, Date, Male\Fe..' name];
    text(50,50,expdata,'color','k',...
        'HorizontalAlignment','left','VerticalAlignment','middle');

    hgsave([PIGPath '\\' num2str(ExpNum) ' ' name '_Move']);
    print(gcf, '-djpeg', [PIGPath '\\' num2str(ExpNum) ' ' name
'_Move']);
end
close all;
end
```

17. Matlab programming code: MotherofAllExp.m

```
%% This Matlab code written by Avihai Spizzichino
%%M.Sc Student|Department of Mechanical Engineering
%%Ben-Gurion University of the Negev%%

function []=MotherOfAllExp...
    (Path2PIG)%,lengthxx12mm,lengthyy34mm,thick,PxPermm)

Start=1;
End=6;

for ExpNum=Start:End
    fprintf(['MotherOfAllExp, ExpNum' num2str(ExpNum) '\n']);

    a=xlsread([Path2PIG '\EngP.xlsx'],ExpNum);
    lengthxx12mm=a(3,2); lengthyy34mm=a(3,6);
    thick=a(3,9); PxPermm=a(5,3);

    ImageProssStrains140702(ExpNum,Path2PIG);
    MotorsStrains140126(ExpNum,Path2PIG,PxPermm)

    CauchyStresses140116(ExpNum,Path2PIG,lengthxx12mm,lengthyy34mm,thick)
    SecondPiolaK140126(ExpNum,Path2PIG);
    SecondPiolaK_Kzero140702(ExpNum,Path2PIG)

    SE_ExperimentGraph140127(ExpNum,Path2PIG);
    SE_ExperimentGraph_Kzero140702(ExpNum,Path2PIG)
    Cauchy_E_ExperimentGraph140127(ExpNum,Path2PIG);

end
end
```

18. Matlab programming code: CauchyStresses140116.m

```
%% This Matlab code written by Avihai Spizzichino, 2014
%%M.Sc Student|Department of Mechanical Engineering
%%Ben-Gurion University of the Negev%%

function
[]=CauchyStresses140116(ExpNum,Path2PIG,lengthxx12mm,lengthyy34mm,thi
ck)
fprintf('\n Cauchy Stresses \n');
load([Path2PIG '\PIG.mat']);

Load12_1=DATA.ElongationRatio(1,ExpNum).Load.Load1;
Load12_2=DATA.ElongationRatio(1,ExpNum).Load.Load2;
Load34_3=DATA.ElongationRatio(1,ExpNum).Load.Load3;
Load34_4 =DATA.ElongationRatio(1,ExpNum).Load.Load4;
Motor1step=DATA.ElongationRatio(1,ExpNum).MotorStep.Motor1step;
Motor2step=DATA.ElongationRatio(1,ExpNum).MotorStep.Motor2step;
Motor3step=DATA.ElongationRatio(1,ExpNum).MotorStep.Motor3step;
Motor4step=DATA.ElongationRatio(1,ExpNum).MotorStep.Motor4step;

A=[Motor1step Motor2step Motor3step Motor4step];
M=size(A,1);
Load12=(Load12_1(1:M,:)+Load12_2(1:M,:))/2;
Load34=(Load34_3(1:M,:)+Load34_4(1:M,:))/2;
```

```

Load12=Load12./1000*9.80665;          %[gr2Kg*Gravity of Earth] =>
[Kg*m/sec^2]
Load34=Load34./1000*9.80665;          %[gr2Kg*Gravity of Earth] =>
[Kg*m/sec^2]

for l=1:M
    UVmotor1(l,1:2)=[-(A(l,1)-A(l,1)),0];    %the displacement of eng 1
on x[mm]- left=(-)
    UVmotor2(l,1:2)=[A(l,2)-A(l,2),0];      %the displacement of eng 2
on x[mm]- right=(+)
    UVmotor3(l,1:2)=[0,(A(l,3)-A(l,3))];    %the displacement of eng 3
on y[mm]- up=(+)
    UVmotor4(l,1:2)=[0,-(A(l,4)-A(l,4))];  %the displacement of eng 4
on y[mm]- down=(-)
end

V0=lengthxx12mm*lengthyy34mm*thick;
Newlength34=lengthyy34mm+abs(UVmotor4(:,2))+abs(UVmotor3(:,2));
Newlength12=lengthxx12mm+abs(UVmotor2(:,1))+abs(UVmotor1(:,1));
NewThick=V0./(Newlength34.*Newlength12);
Axx=NewThick.*Newlength34;
Ayy=NewThick.*Newlength12;
Axx=Axx./10^6; %mm^2 -> m^2
Ayy=Ayy./10^6; %mm^2 -> m^2
Sigmaxx=Load12./Axx;
Sigmayy=Load34./Ayy;

load([Path2PIG '\Analysis.mat']);
title={'SigmaXX' 'SigmaYY'};
Analysis.ElongationRatio(1,ExpNum).CauchyStress.title=title;
XL=[Sigmaxx Sigmayy];
Analysis.ElongationRatio(1,ExpNum).CauchyStress.data=XL;

save([Path2PIG '\Analysis'], 'Analysis');

close all;

```

19. Matlab programming code: MotorsStrains140126.m

```

%% This Matlab code written by Avihai Spizzichino, 2014
%%M.Sc Student|Department of Mechanical Engineering
%%Ben-Gurion University of the Negev%%

function []=MotorsStrains140126(ExpNum,Path2PIG,PxPermm)

fprintf('\n Motors - Strains \n');
% read data text file and calculate C-Sigma and 2nd P-K S from load,
and epsilon
%from motor steps

load([Path2PIG '\PIG.mat']);

Motor1step=DATA.ElongationRatio(1,ExpNum).MotorStep.Motor1step;
Motor2step=DATA.ElongationRatio(1,ExpNum).MotorStep.Motor2step;
Motor3step=DATA.ElongationRatio(1,ExpNum).MotorStep.Motor3step;
Motor4step=DATA.ElongationRatio(1,ExpNum).MotorStep.Motor4step;

A=[Motor1step Motor2step Motor3step Motor4step];
M=size(A,1);

```

```

UVmotor1=rand(M,2);
UVmotor2=rand(M,2);
UVmotor3=rand(M,2);
UVmotor4=rand(M,2);
XYmotor1=rand(M,2);
XYmotor2=rand(M,2);
XYmotor3=rand(M,2);
XYmotor4=rand(M,2);

rr=exist([Path2PIG '\EngP.xlsx'],'file');
if rr~=0
    EngP=xlsread([Path2PIG '\EngP.xlsx'],ExpNum);
    if ExpNum==EngP(1,1) && size(EngP,2)>=7
        x1=EngP(1,2); % x left
        x2=EngP(1,3); % x right
        y1=EngP(1,4); % y left & right
        x3=EngP(1,5); % x up & down
        y4=EngP(1,6); % y up
        y3=EngP(1,7); % y down
    end
else
    x1=input('Please insert X position(Px) of Left eng :');
    x2=input('Please insert X position(Px) of Right eng :');
    y1=input('Please insert Y position(Px) of Right & Left eng :');

    x3=input('Please insert X position(Px) of Up & Bottom eng :');
    y4=input('Please insert Y position(Px) of Upper eng :');
    y3=input('Please insert Y position(Px) of Bottom eng :');
end

for l=1:M
    UVmotor1(l,1:2)=[-(A(1,1)-A(1,1)),0];
    UVmotor2(l,1:2)=[A(1,2)-A(1,2),0];
    UVmotor3(l,1:2)=[0,(A(1,3)-A(1,3))];
    UVmotor4(l,1:2)=[0,-(A(1,4)-A(1,4))];
    XYmotor1(l,1:2)=[x1 y1]+UVmotor1(l,1:2).*PxPermm;
    XYmotor2(l,1:2)=[x2 y1]+UVmotor2(l,1:2).*PxPermm;
    XYmotor3(l,1:2)=[x3 y3]+UVmotor3(l,1:2).*PxPermm;
    XYmotor4(l,1:2)=[x3 y4]+UVmotor4(l,1:2).*PxPermm;
end
UVmotor1Px=UVmotor1.*PxPermm;
UVmotor2Px=UVmotor2.*PxPermm;
UVmotor3Px=UVmotor3.*PxPermm;
UVmotor4Px=UVmotor4.*PxPermm;

syms f1(xi,eta)
f1(xi,eta)=0.25*(1-xi)*(1+eta);

syms f2(xi,eta)
f2(xi,eta)=0.25*(1+xi)*(1+eta);

syms f3(xi,eta)
f3(xi,eta)=0.25*(1-xi)*(1-eta);

syms f4(xi,eta)
f4(xi,eta)=0.25*(1+xi)*(1-eta);

ExxMotor1=rand(M,1);
EyyMotor1=rand(M,1);
ExyMotor1=rand(M,1);
EzzMotor1=rand(M,1);
ExzMotor1=zeros(M,1);
EyzMotor1=zeros(M,1);

```



```

lambdaM11=rand(M,1);
lambdaM22=rand(M,1);
lambdaM33=rand(M,1);
kM11=rand(M,1);
kM22=rand(M,1);

% This function defined only by the first row
X=f1*XYmotor1(1,1)+f2*XYmotor4(1,1)+f3*XYmotor3(1,1)+f4*XYmotor2(1,1);
% X(xi,eta)=x11*f1+x21*f2+x31*f3+x41*f4
dXxi=diff(X,'xi');
dXeta=diff(X,'eta');

Y=f1*XYmotor1(1,2)+f2*XYmotor4(1,2)+f3*XYmotor3(1,2)+f4*XYmotor2(1,2);
% Y(xi,eta)=y11*f1+y21*f2+y31*f3+y41*f4
dYxi=diff(Y,'xi');
dYeta=diff(Y,'eta');

J=diff(X,'xi')*diff(Y,'eta')-diff(X,'eta')*diff(Y,'xi'); %Jacobian

%Calculate U and V for every step at time.
for l=1:M

U=f1*UVmotor1Px(l,1)+f2*UVmotor4Px(l,1)+f3*UVmotor3Px(l,1)+f4*UVmotor
2Px(l,1);

V=f1*UVmotor1Px(l,2)+f2*UVmotor4Px(l,2)+f3*UVmotor3Px(l,2)+f4*UVmotor
2Px(l,2);

%Humpry - Cardiovascular Solid Mechanics, eq 5.14
dUdX=1/J*(dYeta*diff(U,'xi')-dYxi*diff(U,'eta'));
dUdY=1/J*(-dXeta*diff(U,'xi')+dXxi*diff(U,'eta'));

dVdX=1/J*(dYeta*diff(V,'xi')-dYxi*diff(V,'eta'));
dVdY=1/J*(-dXeta*diff(V,'xi')+dXxi*diff(V,'eta'));

lambda1=1+dUdX;
lambda2=1+dVdY;
k1=dUdY;
k2=dVdX;
lambda3=lambda1*lambda2-k1*k2;

lambdaM11(1,1)=1+dUdX(0,0);
lambdaM22(1,1)=1+dVdY(0,0);
kM11(1,1)=dUdY(0,0);
kM22(1,1)=dVdX(0,0);
lambdaM33(1,1)=lambdaM11(1,1)*lambdaM22(1,1)-kM11(1,1)*kM22(1,1);

ExxMotor=0.5*(lambda1^2+k2^2-1);
ExyMotor=0.5*(lambda1*k1+lambda2*k2);
EyyMotor=0.5*(lambda2^2+k1^2-1);
EzzMotor=0.5*(lambda3^2-1);

ExxMotor1(l)=ExxMotor(0,0);
ExyMotor1(l)=ExyMotor(0,0);
EyyMotor1(l)=EyyMotor(0,0);
EzzMotor1(l)=EzzMotor(0,0);
end

load([Path2PIG '\Analysis.mat']);

for i=1:4
title={'Xmotor' num2str(i)} ['Ymotor' num2str(i)]...
['UmotorPx' num2str(i)] ['VmotorPx' num2str(i)]};

```

```

Analysis.ElongationRatio(1,ExpNum).Motor.MotorDisplacement.Motor(1,i)
.title=title;
switch i
case 1
A=[XYmotor1 UVmotor1Px];
case 2
A=[XYmotor2 UVmotor2Px];
case 3
A=[XYmotor3 UVmotor3Px];
case 4
A=[XYmotor4 UVmotor4Px];
end

Analysis.ElongationRatio(1,ExpNum).Motor.MotorDisplacement.Motor(1,i)
.data=A;
end

title={'MotorLambda1(0,0)' 'MotorLambda2(0,0)' 'MotorKappa1(0,0)'
'MotorKappa2(0,0)' 'MotorLambda3(0,0)'};
Analysis.ElongationRatio(1,ExpNum).Motor.DeformationGrad.title=title;
F=[lambdaM11 lambdaM22 kM11 kM22 lambdaM33];
Analysis.ElongationRatio(1,ExpNum).Motor.DeformationGrad.data=F;

title={'e11(xx)' 'e12(xy)' 'e13(xz)' 'e22(yy)' 'e23(yz)' 'e33(zz)'};
Analysis.ElongationRatio(1,ExpNum).Motor.GreenStrainInTheCenter.title
=title;
E=[ExxMotor1 ExyMotor1 ExzMotor1 EyyMotor1 EyzMotor1 EzzMotor1];
Analysis.ElongationRatio(1,ExpNum).Motor.GreenStrainInTheCenter.data=
E;

save([Path2PIG '\Analysis'], 'Analysis');

close all;

% xlswrite('Motor Displacement data.xls',title);
% A=[XYmotor1 UVmotor1Px XYmotor2 UVmotor2Px XYmotor3 UVmotor3Px
XYmotor4 UVmotor4Px];
% xlswrite('Motor Displacement data.xls',A,1,'A2');
% fprintf('Saved file: "Motor Displacement data.xls" \n');
%
% title={'MotorLambda1(0,0)' 'MotorLambda2(0,0)' 'MotorKappa1(0,0)'
'MotorKappa2(0,0)' 'MotorLambda3(0,0)'};
% xlswrite('MotorF.xls',title,1);
% F=[lambdaM11 lambdaM22 kM11 kM22 lambdaM33];
% xlswrite('MotorF.xls',F,1,'A2');
% fprintf('Saved file: "Motor F.xls" \n');

% title={'e11(xx)' 'e12(xy)' 'e13(xz)' 'e22(yy)' 'e23(yz)' 'e33(zz)'};
% xlswrite('Motor_Green Strain in the center.xls',title,1);
% E=[ExxMotor1 ExyMotor1 ExzMotor1 EyyMotor1 EyzMotor1 EzzMotor1];
% xlswrite('Motor_Green Strain in the center.xls',E,1,'A2');
% fprintf('Saved file: "Motor_Green Strain in the center.xls" \n');

```

20. Matlab programming code: ImageProssStrains140702.m

```
%% This Matlab code written by Avihai Spizzichino, 2014
%%M.Sc Student|Department of Mechanical Engineering
%%Ben-Gurion University of the Negev%%

function []=ImageProssStrains140702(ExpNum,Path2PIG)

fprintf('\n Image processing - Strains \n');
NumberOfPointsToCheck=4;    % we assume homogenius deformation and we
check 4 deffrent points,  each base on 4 markers.

% MarkerNumbers=rand(4,4);
% for i=1:4
%for j=1:4
%    MarkerNumbers(i,j)=input(['Insert marker ' num2str(i) ' possition
(number): ']);
%end
% end

rr=exist([Path2PIG '\Book1.xlsx'],'file');
if rr~=0
    Markers=xlsread([Path2PIG '\Book1.xlsx'],ExpNum);
else
    Markers=rand(NumberOfPointsToCheck,4);
    for ii=1:NumberOfPointsToCheck
        for jj=1:4
            Markers(ii,jj)=...
                input(['Please insert Marker Num for'...
                    'element ' num2str(ii) 'corner ' num2str(jj) ': ']);
        end
    end
end

%[405 408 525 528 ;405 408 525 528;405 408 525 528;405 408 525 528];

rr=exist([Path2PIG '\Analysis.mat'],'file');
if rr~=0
    load([Path2PIG '\Analysis.mat']);
end

load([Path2PIG '\PIG.mat']);
Analysis.ElongationRatio(1,ExpNum).Label=...
    DATA.ElongationRatio(1,ExpNum).Label;

Numofmark=size(DATA.ElongationRatio(1,ExpNum).Location,2);

for find1=1:NumberOfPointsToCheck
    for find2=1:4
        for lock=1:Numofmark

a=DATA.ElongationRatio(1,ExpNum).Location{1,lock}.MarkerLabel;
            if a==Markers(find1,find2)
                NewMarkers(find1,find2)=lock;
            end
        end
        if NewMarkers(find1,find2)==0;
            error (['Marker Number ' num2str(Markers(find1,find2)) '
did not found']);
        end
    end
end
```

```

for ElementNum=1:NumberOfPointsToCheck

    for Pina=1:4

        b(:,1)=DATA.ElongationRatio(1,ExpNum).Location{1,NewMarkers(ElementNum,Pina)}.X;

        b(:,2)=DATA.ElongationRatio(1,ExpNum).Location{1,NewMarkers(ElementNum,Pina)}.Y;
        N=size(b,1); % Num of rows
        M=2; % Num of Col
        for l=1:N-1 % calculate delta per row - u(x,y)
            & v(x,y).
                b(l,M+1)=b(l+1,1)-b(l,1);
                b(l,M+2)=b(l+1,2)-b(l,2);
            end

            if Pina==1
                A=b;
            else
                K=min(size(A,1),size(b,1));
                A(1:K,(Pina-1)*4+1:4*Pina)=b(1:K,:); % A: x1 y1 u1 v1 x2
                y2 u2 v2...
            end

            Analysis.ElongationRatio(1,ExpNum).ImagePross.ElementNum{1,ElementNum}
            }.PinaNum(Pina).MarkerLabel=Markers(ElementNum,Pina);

            Analysis.ElongationRatio(1,ExpNum).ImagePross.ElementNum{1,ElementNum}
            }.PinaNum(Pina).X=b(:,1);

            Analysis.ElongationRatio(1,ExpNum).ImagePross.ElementNum{1,ElementNum}
            }.PinaNum(Pina).Y=b(:,2);

            Analysis.ElongationRatio(1,ExpNum).ImagePross.ElementNum{1,ElementNum}
            }.PinaNum(Pina).U=b(:,3);

            Analysis.ElongationRatio(1,ExpNum).ImagePross.ElementNum{1,ElementNum}
            }.PinaNum(Pina).V=b(:,4);
        end

        N=size(A,1);

        syms f1(xi,eta)
        f1(xi,eta)=0.25*(1-xi)*(1+eta); %????????? ???? ?????????? ??????

        syms f2(xi,eta)
        f2(xi,eta)=0.25*(1+xi)*(1+eta);

        syms f3(xi,eta)
        f3(xi,eta)=0.25*(1-xi)*(1-eta);

        syms f4(xi,eta)
        f4(xi,eta)=0.25*(1+xi)*(1-eta);

        Exx1=rand(N,1);
        Eyy1=rand(N,1);
        Exy1=rand(N,1);
        Ezz1=rand(N,1);
        Exz1=zeros(N,1);
        Eyz1=zeros(N,1);

```

```

lambda11=rand(N,1);
lambda22=rand(N,1);
lambda33=rand(N,1);
k11=rand(N,1);
k22=rand(N,1);
k11Kzero=zeros(N,1);
k22Kzero=zeros(N,1);
lambda33Kzero=rand(N,1);
Exx1Kzero=rand(N,1);
Eyy1Kzero=rand(N,1);
Exy1Kzero=rand(N,1);
Ezz1Kzero=rand(N,1);
Exz1Kzero=zeros(N,1);
Eyz1Kzero=zeros(N,1);

% This function defined only by the first row
X=f1*A(1,1)+f2*A(1,5)+f3*A(1,9)+f4*A(1,13); %
X(xi,eta)=x11*f1+x21*f2+x31*f3+x41*f4
dXxi=diff(X,'xi');
dXeta=diff(X,'eta');

Y=f1*A(1,2)+f2*A(1,6)+f3*A(1,10)+f4*A(1,14); %
Y(xi,eta)=y11*f1+y21*f2+y31*f3+y41*f4
dYxi=diff(Y,'xi');
dYeta=diff(Y,'eta');

J=diff(X,'xi')*diff(Y,'eta')-diff(X,'eta')*diff(Y,'xi');
%Jacobian

%Calculate U and V for every step at time.
for l=1:N

    U=f1*A(1,3)+f2*A(1,7)+f3*A(1,11)+f4*A(1,15); %
    U(xi,eta)=u1*f1+u2*f2+u3*f3+u4*f4
    V=f1*A(1,4)+f2*A(1,8)+f3*A(1,12)+f4*A(1,16); %
    V(xi,eta)=v1*f1+v2*f2+v3*f3+v4*f4

    %Humphry - Cardiovascular Solid Mechanics, eq 5.14
    dUdX=1/J*(dYeta*diff(U,'xi')-dYxi*diff(U,'eta'));
    dUdY=1/J*(-dXeta*diff(U,'xi')+dXxi*diff(U,'eta'));

    dVdX=1/J*(dYeta*diff(V,'xi')-dYxi*diff(V,'eta'));
    dVdY=1/J*(-dXeta*diff(V,'xi')+dXxi*diff(V,'eta'));

    %symbol functions
    %lambda1=1+dUdX;
    %lambda2=1+dVdY;
    %k1=dUdY;
    %k2=dVdX;
    %lambda3=1/(lambda1*lambda2-k1*k2);
    %lambda3Kzero=1/(lambda1*lambda2);

    %Exx=0.5*(lambda1^2+k2^2-1);
    %Exy=0.5*(lambda1*k1+lambda2*k2);
    %Eyy=0.5*(lambda2^2+k1^2-1);
    %Ezz=0.5*(lambda3^2-1);

    %ExxKzero=0.5*(lambda1^2-1);
    %ExyKzero=0;
    %EyyKzero=0.5*(lambda2^2-1);
    %EzzKzero=0.5*(lambda3Kzero^2-1);

    %Vectors of lambda and kappa

```

```

        lambda11(1,1)=1+dUdX(0,0);
        lambda22(1,1)=1+dVdY(0,0);
        k11(1,1)=dUdY(0,0);
        k22(1,1)=dVdX(0,0);
        lambda33(1,1)=1/(lambda11(1,1)*lambda22(1,1)-
k11(1,1)*k22(1,1));

        Exx1(1,1)=0.5*(lambda11(1,1)^2+k22(1,1)^2-1);
        Exy1(1,1)=0.5*(lambda11(1,1)*k11(1,1)+lambda22(1,1)*k22(1,1));
        Eyy1(1,1)=0.5*(lambda22(1,1)^2+k11(1,1)^2-1);
        Ezz1(1,1)=0.5*(lambda33(1,1)^2-1);

        lambda33Kzero(1,1)=1/(lambda11(1,1)*lambda22(1,1));
        Exx1Kzero(1,1)=0.5*(lambda11(1,1)^2-1);
        Exy1Kzero(1,1)=0;
        Eyy1Kzero(1,1)=0.5*(lambda22(1,1)^2-1);
        Ezz1Kzero(1,1)=0.5*(lambda33(1,1)^2-1);

    end

    %%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
    fprintf(['Element No.' num2str(ElementNum) ' done \n']);

    title={'lambda1(0,0)'      'lambda2(0,0)'      'k1(0,0)'      'k2(0,0)'
'lambda3(0,0)'};

    Analysis.ElongationRatio(1,ExpNum).ImagePross.ElementNum{1,ElementNum
}.DeformationGradient.title=title;
    F=[lambda11 lambda22 k11 k22 lambda33];

    Analysis.ElongationRatio(1,ExpNum).ImagePross.ElementNum{1,ElementNum
}.DeformationGradient.data=F;

    title={'lambda1(0,0)'      'lambda2(0,0)'      'k1(0,0)'      'k2(0,0)'
'lambda3Kzero(0,0)'};

    Analysis.ElongationRatio(1,ExpNum).ImagePross.ElementNum{1,ElementNum
}.DeformationGradientKzero.title=title;
    F=[lambda11 lambda22 k11Kzero k22Kzero lambda33Kzero];

    Analysis.ElongationRatio(1,ExpNum).ImagePross.ElementNum{1,ElementNum
}.DeformationGradientKzero.data=F;

    title={'E11(xx)'      'E12(xy)'      'E13(xz)'      'E22(yy)'      'E23(yz)'
'E33(zz)'};

    Analysis.ElongationRatio(1,ExpNum).ImagePross.ElementNum{1,ElementNum
}.GreenStrainInTheCenter.title=title;
    E=[Exx1(:,1) Exy1(:,1) Exz1(:,1) Eyy1(:,1) Eyz1(:,1) Ezz1(:,1)];

    Analysis.ElongationRatio(1,ExpNum).ImagePross.ElementNum{1,ElementNum
}.GreenStrainInTheCenter.data=E;

    title={'E11(xx)'      'E12(xy)'      'E13(xz)'      'E22(yy)'      'E23(yz)'
'E33(zz)'};

    Analysis.ElongationRatio(1,ExpNum).ImagePross.ElementNum{1,ElementNum
}.GreenStrainInTheCenterKzero.title=title;
    E=[Exx1Kzero(:,1) Exy1Kzero(:,1) Exz1Kzero(:,1) Eyy1Kzero(:,1)
Eyz1Kzero(:,1) Ezz1Kzero(:,1)];

```

```

Analysis.ElongationRatio(1,ExpNum).ImagePross.ElementNum{1,ElementNum
}.GreenStrainInTheCenterKzero.data=E;

end

save([Path2PIG '\Analysis'], 'Analysis');

close all;
% title={'X1' 'Y1' 'U1' 'V1' 'X2' 'Y2' 'U2' 'V2' 'X3' 'Y3' 'U3'
'V3' 'X4' 'Y4' 'U4' 'V4'};
% xlswrite(['Displacement data_' ElementNo '.xls'],title);
% xlswrite(['Displacement data_' ElementNo '.xls'],A,1,'A2');
% fprintf(['Saved file: "Displacement data_' ElementNo '.xls" \n']);

% title={'Exx(-1,1)' 'Exx(0,1)' 'Exx(1,1)' 'Exx(-1,0)' 'Exx(0,0)'
'Exx(1,0)' 'Exx(-1,-1)' 'Exx(0,-1)' 'Exx(1,-1)'};
% xlswrite(['Green Strain_' ElementNo '.xls'],title);
% xlswrite(['Green Strain_' ElementNo '.xls'],Exx1,1,'A2');
%
% title={'Eyy(-1,1)' 'Eyy(0,1)' 'Eyy(1,1)' 'Eyy(-1,0)' 'Eyy(0,0)'
'Eyy(1,0)' 'Eyy(-1,-1)' 'Eyy(0,-1)' 'Eyy(1,-1)'};
% xlswrite(['Green Strain_' ElementNo '.xls'],title,2);
% xlswrite(['Green Strain_' ElementNo '.xls'],Eyy1,2,'A2');

% title={'Exy(-1,1)' 'Exy(0,1)' 'Exy(1,1)' 'Exy(-1,0)' 'Exy(0,0)'
'Exy(1,0)' 'Exy(-1,-1)' 'Exy(0,-1)' 'Exy(1,-1)'};
% xlswrite(['Green Strain_' ElementNo '.xls'],title,3);
% xlswrite(['Green Strain_' ElementNo '.xls'],Exy1,3,'A2');
%
% title={'Ezz(-1,1)' 'Ezz(0,1)' 'Ezz(1,1)' 'Ezz(-1,0)' 'Ezz(0,0)'
'Ezz(1,0)' 'Ezz(-1,-1)' 'Ezz(0,-1)' 'Ezz(1,-1)'};
% xlswrite(['Green Strain_' ElementNo '.xls'],title,4);
% xlswrite(['Green Strain_' ElementNo '.xls'],Ezz1,4,'A2');
% fprintf(['Saved file: "Green Strain_' ElementNo '.xls" \n']);
%
% title={'lambda1(0,0)' 'lambda2(0,0)' 'k1(0,0)' 'k2(0,0)'
'lambda3(0,0)'};
% xlswrite(['F_' ElementNo '.xls'],title,1);
% F=[lambda11 lambda22 k11 k22 lambda33];
% xlswrite(['F_' ElementNo '.xls'],F,1,'A2');
% fprintf(['Saved file: "F_' ElementNo '.xls" \n']);
%
% title={'e11(xx)' 'e12(xy)' 'e13(xz)' 'e22(yy)' 'e23(yz)'
'e33(zz)'};
% xlswrite(['Green Strain in the center_' ElementNo '.xls'],title,1);
% E=[Exx1(:,5) Exy1(:,5) Exz1(:,1) Eyy1(:,5) Eyz1(:,1) Ezz1(:,5)];
% xlswrite(['Green Strain in the center_' ElementNo
'.xls'],E,1,'A2');
% fprintf(['Saved file: "Green Strain in the center_' ElementNo
'.xls" \n\n']);

```

21. Example of Images captured during a biaxial test.

22. Scholarly works

1. Gigi Chi Ting Au-Yeung, Tomer Bronshtein, Freddy Yin Chiang Boey, Subbu S. Venkatraman, Marcelle Machluf. **2nd runner-up Poster Presenter Award** at 11th Joint Symposium on Materials Science and Engineering for the 21st Century, 19–22 June 2011. Singapore. *Decellularized Porcine Cardiac Extracellular Matrix for Cardiac Tissue Engineering*.
2. Gigi Chi Ting Au-Yeung, Tomer Bronshtein, Freddy Yin Chiang Boey, Subbu S. Venkatraman, Marcelle Machluf. **Poster presentation** at 5th MRS-S Conference on

- Advanced Materials, 20-22 March 2012. Singapore. *Biomechanics of porcine cardiac extracellular matrix construct for myocardial tissue engineering.*
3. Gigi Chi Ting Au-Yeung, Tomer Bronshtein, Freddy Yin Chiang Boey, Subbu S. Venkatraman, Marcelle Machluf. **Oral presentation-Technical program** at Biomedical Engineering Society 6th Scientific Meeting (BES6SM), 19 May 2012. Singapore. *Biomechanics of porcine cardiac extracellular matrix construct for myocardial tissue engineering.*
 4. Gigi Chi Ting Au-Yeung, Tomer Bronshtein, Udi Sarig, Freddy Yin Chiang Boey, Subbu S. Venkatraman, Marcelle Machluf. **Poster presentation** at 9th World Biomaterials Congress 2012 (WBC), 1-5 June 2012. Chengdu, China. *A new Viscoelasticity Analysis Tool for Soft Biomaterial Scaffolds Verified by Experimental Results of Decellularized and Native Porcine Cardiac Tissues.*
 5. Udi Sarig, Gigi Chi Ting Au-Yeung, Yao Wang, Tomer Bronshtein, Nitsan Dahan, Freddy Yin Chiang Boey, Subbu S. Venkatraman, Marcelle Machluf. Journal Paper at **Tissue Engineering Part A 18 (19-20): p 2125-37, 2012.** *Thick acellular heart extracellular matrix with inherent vasculature: Engineering of myocardial replacement tissue.* ^[86]
 6. Gigi Chi Ting Au-Yeung, Tomer Bronshtein, Udi Sarig, Freddy Yin Chiang Boey, Subbu S. Venkatraman, Marcelle Machluf. **Poster presentation** at UK-Singapore Materials Workshop, 6-7 December 2012. Singapore. *Decellularized Porcine Cardiac Extracellular Matrix for Cardiac Tissue Engineering.*
 7. *Tomer Bronshtein, *Gigi Chi Ting Au-Yeung, Udi Sarig, Evelyne Bao-Vi Nguyen, Priyadarshini S Mhaisalkar, Freddy Yin Chiang Boey, Subbu S Venkatraman, Marcelle Machluf. (*equal contribution) Journal Paper at **Tissue engineering. Part C, Methods 19 (8): p 620-30, 2013.** *A Mathematical Model for Analyzing the*

Elasticity, Viscosity, and Failure of Soft Tissue: Comparison of Native and Decellularized Porcine Cardiac Extracellular Matrix for Tissue Engineering. ^[124]

8. Gigi Chi Ting Au-Yeung, Tomer Bronshtein, Udi Sarig, Freddy Yin Chiang Boey, Subbu S. Venkatraman, Marcelle Machluf. **Gold Award Oral presentation- Technical program** at Biomedical Engineering Society 7th Scientific Meeting (BES7SM), 19 May 2013. Singapore. *Recellularization and Bioreactor System for myocardial tissue engineering.*
9. Gigi Chi Ting Au-Yeung, Udi Sarig, Tomer Bronshtein, Freddy Yin Chiang Boey, Subbu S. Venkatraman, Marcelle Machluf. **Oral presentation** at 5th International Conference on Mechanics of Biomaterials and Tissues 2013 (ICMoBT5), December 2013. Sitges, Spain. *Experimental and computational study on biomechanics of recellularised porcine cardiac extracellular matrix scaffold for myocardial tissue engineering*
10. Gigi Chi Ting Au-Yeung, Marcelle Machluf. **Winner of Student Competition (3rd place)** at Israel Society of Medical and Biological Engineering (ISMBE) Biomedical Engineering Annual Conference 2014, February 2014. Haifa, Israel. *Effect of decellularization and stem cells-recellularization on materials properties of porcine myocardial extracellular matrix for heart tissue engineering.*
11. Two manuscripts were under preparation at the time of this thesis submission.
(One of which as first author)

23. Curriculum Vitae of Author

Chi Ting AU-YEUNG (Gigi) was born on 21 May 1988 in Hong Kong Special Administrative Region (HKSAR) of China. Her primary and secondary school educations were completed at Sacred Heart Canossian School in 2000 and Ying W Girls' School 2007 respectively.

In 2010, Gigi obtained her Bachelor's degree (B. Eng) with First class honor and Dean's honor in Product Analysis with Engineering Design, Department of Mechanical Engineering, The Hong Kong Polytechnic University (PolyU). During her 3 years of tertiary education, she had received prestigious awards including Sir Edward Youde Memorial (SEYM) Scholarship 2008/09, Professional Validation Council of Hong Kong Industries Scholarship 2009, and HKSAR Government Scholarship 2009/10 for recognizing the most outstanding engineering undergraduates in Hong Kong. She had spent one semester in Dublin Institute of Technology, Ireland during Jan-June 2009 as Student Exchange Programme. She also had her first encounter of scientific research on biomaterials under supervision of Prof. Kin-tak Lau (Alan) on projects related to bio-composites, natural fiber-reinforced materials and bone mechanical properties by surface indentation.

After finishing her B.Eng examinations in August 2010, Gigi joined the School of Materials Science and Engineering (MSE) at Nanyang Technological University (NTU) Singapore, with the recommendation of Prof. Yin Chiang Freddy BOEY (Chair of MSE then and Provost of NTU 2014). She embarked her Ph.D. research on this thesis titled "Biophysics of Recellularized Porcine Cardiac Extracellular Matrix (ECM) for Myocardial Tissue Engineering (MTE) " under the co-supervision of Prof. Subbu S. VENKATRAMAN at NTU and Prof. Marcelle MACHLUF at Technion Israel Institute of Technology. From Jan-Mar 2014, she did a research attachment in Prof. Marcelle Machluf's Lab for cancer drug delivery and cell based technologies at Technion where she performed some of the crucial

experiments included in this thesis. The thesis has proved her hypothesis that reseeded acellular ECM scaffolds possess close biophysical properties matching the native myocardium to be a native-mimetic myocardial tissue engineered implant as a cardiac regenerative therapy for post-myocardial infarction patients.

During her four years of PhD candidature, Gigi published two journal papers and two were under preparation or review at the time of thesis submission. She attended three overseas conferences and five local conferences; and had received awards including Poster Presenter Award at 11th Joint Symposium on Materials Science and Engineering for the 21st Century, Gold Award-oral presenter at Biomedical Engineering Society 7th Scientific Meeting; and Winner of ISMBE Student Competition at Israel Society of Medical and Biological Engineering (ISMBE) Biomedical Engineering Annual Conference 2014.

Her research expertise and interests include mechanical engineering; material characterization; mechanical testing; uniaxial and biaxial tensile testing. Biomedical engineering; biomaterials; biomedical devices; biomechanics. Differential Scanning Calorimetry; Thermogravimetric Analysis; Fourier transform infrared spectroscopy; Scanning Electron Microscopy; WETSEM; QuantomiX technology. Cell culture; cell viability assay; protein adsorption; confocal microscopy; histology. Extracellular matrix; myocardium; myocardial Infarction; heart modeling; bioreactor. nanotechnology; instrumentation development; rapid prototyping; product design; industrial design and project management.

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