A STUDY ON THE EFFECT OF MECHANICAL STRETCH AND ALIGNED SCAFFOLD ARCHITECTURE ON MORPHOLOGY OF RAT VENTRICULAR CELLS

by

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ABSTRACT

A STUDY ON THE EFFECT OF MECHANICAL STRETCH AND ALIGNED SCAFFOLD ARCHITECTURE ON MORPHOLOGY OF RAT VENTRICULAR CELLS

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Heart disease is one of the major causes of death in developing and industrialized countries. Myocardial infarction leads to remodeling of the left ventricle, which severely affects the pumping efficiency of the heart. In order to provide mechanical support to left ventricle the scaffold material should be elastic and mechanically strong. For the success of cardiac graft in vivo one of the important factor is ability of cardiac myocyte morphology to resemble that in vivo. The goal of this study is to build cardiac graft for myocardial infarction repair. Mechanical stretch is one of the important factors for the development of dynamic tissue like myocardium. We designed micro pattern created scaffold from elastic, biodegradable, biocompatible material Crosslinked urethane dopped polyester (CUPE).To provide mechanical stretch we designed a bioreactor system. The neonatal rat derived cardiac cells were cultured on CUPE films and they were subjected to mechanical stretch in the bioreactor. The results of stretched cells were compared with the cells cultured on polymer films having micropattern on them and films without micropattern which were not subjected to stretch. The results indicated that cells that were subjected to stretch exhibited aligned, rod shaped elongated morphology. Moreover, stretched cells increased in size significantly and demonstrated hypertrophy. The cells on film without micropattern on it was randomly oriented, sparsely distributed and could not show noticeable increase in size.Whereas, the

cells cultured on polymer films with micropattern were comparatively more aligned and elongated. From our findings we concluded that alignment and stretch plays an important role in morphology of the cardiac cells. Also survival and growth of cardiac cells on CUPE films suggests that CUPE is a promising material for cardiac tissue engineering.

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CHAPTER 1

INTRODUCTION

1.1 Statistics of heart disease

Heart disease is the major cause of human mortality in the developing and industrialized countries. According to the annual statistics update published by American heart Association 1,200,000 Americans had silent or recurrent heart attack in 2006. Heart failure basically affects its pumping efficiency. The cause of this dysfunction can be valvular heart disease, hypertension or ischemic heart disease including myocardial infarction [1].

Myocardial infarction leads to the loss functional myocytes. Macrophages, monocytes and neutrophils accumulate into the infarct area causing inflammation. Infarction expands due to the activation of matrix matelloproteases which in turn degrades ECM [2].Collagen ECM becomes thinner and causes the ventricular dilation and increased wall stress in the remaining myocardium. These changes in structure lead to remodeling of the ventricles, which serves as a compensatory mechanism. Increased deposition of collagen fibrous structure resists deformation and rupture [3]. Myocardial infracted tissue looses the regenerative capability and can not meet the loss of cardiac myocyte [4]. This pathological condition causes decline in cardiac output and can not meet the metabolic requirement of the body. Functional inefficiency of the ventricles leads to the end stage of heart failure [1].

Total heart transplantation is the most suitable approach for the end stage heart failure. Unfortunately the number of donor hearts available for transplantation is inadequate compared to the number of patients suffering from heart disease. Despite the current pharmacological and surgical approaches the number of cardiac patients is increasing each year [5]. This has necessitated finding other viable alternative approaches for the treatment of the infracted heart.

1.2 Different approaches to treat MI

Cellular transplantation, LV restraint devices and tissue engineering approaches have emerged as potential alternatives of the total heart transplantation. They are presented in the diagram below.

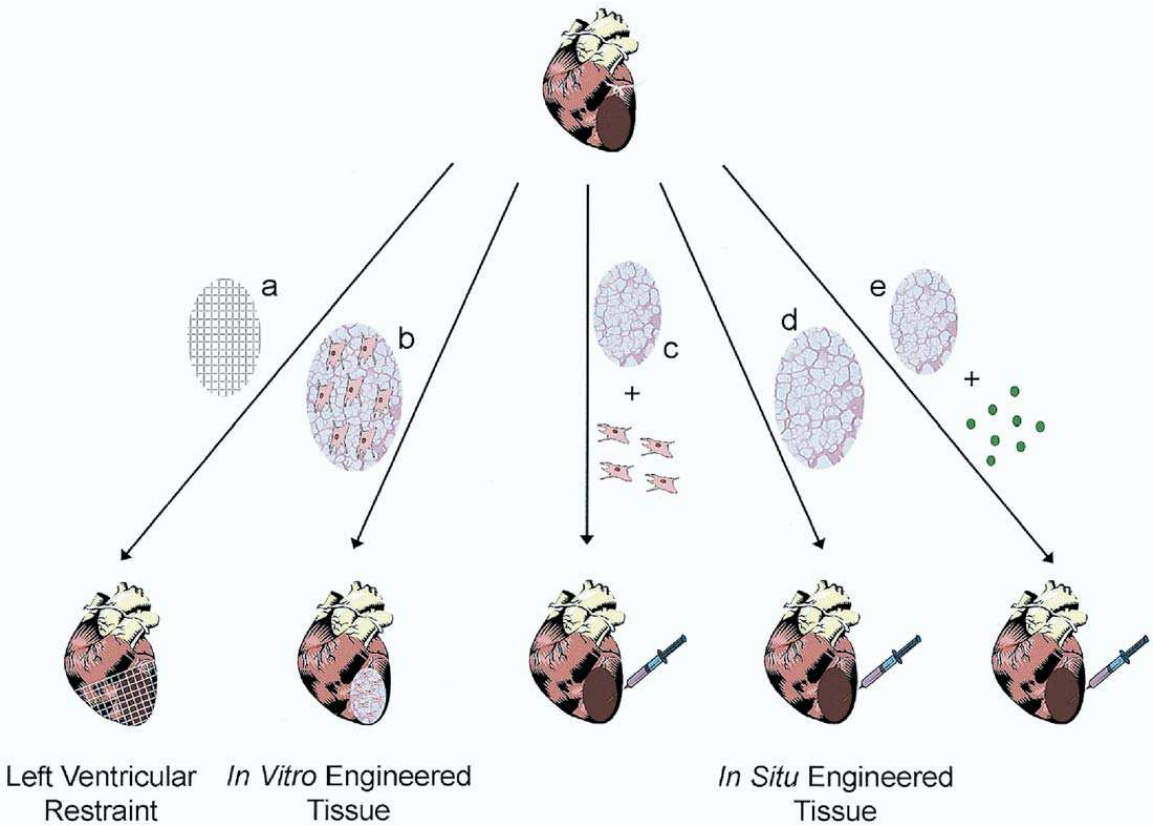


Figure1.1 Different approaches for treatment of myocardial infarction using biomaterials [2].

Depending on the stage of development of the MI three approaches are proposed for treatment

(a) Left ventricular restraint

(b) *In vitro* engineered tissue

(c) *In situ* engineered tissue.

In the early stage of MI cell injection therapy has been successful. While in later stage cardiac patch implantation has been proven to be helpful [6].

1.2.1 LV Restraint

In this approach a biomaterial is wrapped around the wall of infracted myocardium. Kelly *et. al.* used polypropylene mesh. It prevented further deterioration in cardiac function and maintained LV geometry[7] In another approach Acorn Cardiovascular Inc developed LV restrained from knitted polyester mesh which was sutured around both ventricles. The results illustrated decrease in LV end-diastolic volume and myocyte hypertrophy [8]. The shortcoming of this aspect is need of surgery for implantation and worsening of some of the cardiac function [2].

The positive results of several experiments performed with wrapping pre-prepared skeletal muscle to the heart suggested that mechanical constraint by muscle wrap helps in preventing negative remodeling of the infracted heart[9],[10]. Later on several biomaterials were used to restrain the left ventricle, which includes polypropylene[7] and knitted polyester[10].

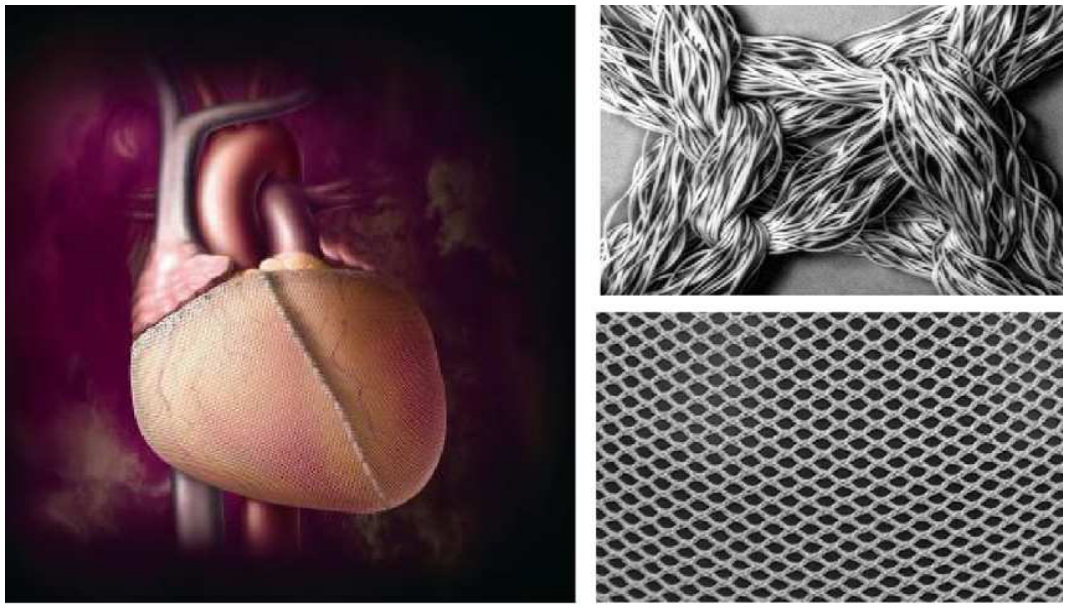


Figure 1.2 Biomaterial approach for ventricular restrain a) Knitted polyester wrapped around left ventricle b) knitted polymer mesh structure [1]

1.2.2 In vitro engineered tissue

An alternative approach to LV restraint and cell injection is developing cardiac tissue *in vitro* which can later be implanted in vivo. It involves implanting the biomaterials with cell seeded onto it onto the infracted zone. Administration of cells alone can yield fruitful results in small scale injuries; while to treat large scale dysfunction in vitro engineered tissue has proved to be viable option [11], owing to its ability to give immediate functionality. Leor *et.al.* has reported improvement in cardiac function by alginate scaffold with fetal cardiomyocyte seeded onto it[12]. This experiment could not shed light on the responsible factors for cardiac function improvement. i.e. biomaterial or cell transplantation, as effect of transplantation of scaffold alone was not examined.

Synthetic polymers offer several advantages over biomaterials. It has defined chemical and mechanical properties, thus it can be prepared without batch to batch variation unlike natural biomaterials. The naturally occurred biomaterials exhibit batch to batch variation depending on its isolation procedure. The properties can be tailored depending on the requirement. The degradation kinetics can be controlled by controlling properties of the polymer [13].

Krupnick et.al employed multiple materials for the cell seeding. Initially the cells were suspended onto collagen and matrigel mixture and later on seeded on poly (L-lactic acid) which was further reinforced with poly (tertrafluroethylene). It was sutured to the infracted wall. The results did not show evidence of aneurismal dilation [14].

Okano *et al*. has used cell sheet engineering approach for implantation of the cardiac patch. It is a matrix free approach and cell sheets are stacked together to form a thick vascularized tissue construct. They used temperature sensitive polymer poly (N-isopropylacrylamide) which is slightly hydrophobic and cell adhesive at 37^{o}C and becomes hydrophilic and cell resistant at 32 ^{o}C. The fetal cardiac myocytes were cultured on PNIPAM treated tissue culture plates. The cell monolayer was detached from the surface by reducing the temperature to 32 ^{o}C. The cell sheet remained intact and was implanted into the animal by putting 3 layers in a single time on top of each other at 2 days interval by performing multiple surgeries. Up to 6 layers maintained 3D pulsatile tissue without causing necrosis. The resulting tissue had

thickness of 1mm [15]. Exclusion of scaffold in this approach eliminates material originated problems; on the contrary it compromises mechanical stability of the graft [13].

Miyahra *et.al*. developed cardiac tissue by cell sheet engineering approach from adipose tissue derived from mesenchymal stem cells. After 4 weeks of the implantation the tissue developed thickness of 600 μm. Though the graft survived and developed thick tissue in vivo the number of cardiomyocytes was very low, limiting the use of mesenchymal stem cells for cardiac repair[16].

Zimmermann *et.al* developed cardiac graft which was termed as an engineered cardiac tissue (EHT) was developed by seeding the neonatal cardiac cell in collagen type I and matrigel. It was pipetted into custom made circular molds with cell culture medium to obtain desired shape and size of the tissue. The tissue construct was subjected to mechanical stimulus in a custom designed bioreactor for 7 days. EHT remained contractile after implantation in the animal scar tissue for 8 weeks. In a recent study the thickness of EHT is reported to be 450 μm. This graft was shown to improve systolic and diastolic function in rats [13]. Significant decrease in thickness of the graft after transplantation suggests necrosis in the tissue. The limitation of this approach was immunogenicity caused by the collagen gel and limited mechanical strength due to use of collagen. Concentration of collagen above certain level causes decrease in mechanical strength.

Above mentioned experiments yielded cardiac tissue graft of approximately one half millimeters. Cardiac graft of this thickness can not alter the function in diseased myocardium of rat or human which are considerably thicker than this (Thickness of human myocardium is approximately 1 cm [6]).

The major limitations of aforementioned approaches are limitation on thickness of the cardiac graft. Current approaches are able to create graft of thickness approximately 500 to 600 μm due to the constraints placed by oxygen diffusion. Vascularization in the grafts is another hurdle in cardiac tissue engineering. Scarce blood supply in ischemic area exacerbates the survival of graft owing to the huge requirement of oxygen by highly metabolic cardiomyocytes[6].

1.2.3 In situ engineered myocardial tissue

Cellular cardiomyoplasty is one of the strategies of *in situ* tissue engineering. It involves transplantation of viable, functional cells to replace necrotic cardiomyocytes. The cells are injected at the site intravenously, intracoronary or directly injected into the myocardium. This technique has several limitations: The cells are injected via liquid solution, and does not provide temporary matrix for the cells to attach. The cells are administered in the ischemic region of the heart. This area does not have enough blood supply. Also the distribution of the cells is not homogeneous in this region. The cells form cluster between the scar tissue. Consequently the cells can not survive in lack of blood supply and are exposed to heterogeneous milieu [17]. 95 % of the implanted cells are destroyed and leaves debris at the infarction site. This in turn induces inflammatory response which can affect scar remodeling and contractile function of the native tissue [13].

As shown in figure 1 (d), injectable biomaterial is used for cell delivery to the infarct wall. The biomaterial provides substratum for the cell attachment and enhances cell survival. Injectable biomaterial can also be used to provide LV wall support and prevents negative remodeling. This acellular approach is employed to for controlled gene and protein delivery. This technique is minimally invasive and clinically preferable compared to implantation of in vitro engineered cardiac tissue by surgical procedure [2].

Narmoneva *et.al*. had proposed the concept of using injectable gels for myocardial repair. Endothelial cells and cardiomyocytes were co-cultured in self assembling peptide. The presence of endothelial cells leads to the rapid formation of blood vessels. The endothelial cells endorse electrical function of the cardiomyocytes. [18], [19]. Fibrin glue has also been used to sustain cardiac function in infracted heart by maintaining the geometry of the left ventricle [20], [21]. Nevertheless these gels facilitate cells to accumulate and assemble *in situ,* their stiffness is considerably lower than the rat or human myocardium. The Young's modulus of these gels fall in the range of 10 Pa to 20 KPa, [22] while that of rat myocardium is 0.14 MPa and for human myocardium it is 0.2 to 0.5 MPa at the end of diastole [23], [24]. Consequently they can not provide enough mechanical support to the infracted heart.

Chirstman *et.al*. demonstrated the use of fibrin glue as an injectable material and reported neovascularization in the infracted region and decline in infarction expansion [20]. Injection of fibrin glue without skeletal myoblasts has shown to preserve LV ventricular geometry and cardiac function [25].

Contractile cardiac patches have been prepared from natural polymers like collagen and alginate [26] [27] [8] [27] [28] and from synthetic polymers like PGA [29] [12] [30-34].

Several studies have been performed with co culture of cardiomyocytes with endothelial cells [30] [31] [32] [18]and macrophages[30, 33, 34] to induce vascularization.

Thompson *et. al*. [40], Dai *et.al* [41] and Huang *et. al*. [42] has successfully demonstrated use of collagen as injectable biomaterial to heal the infracted heart. Dai *et. al* has reported improved cardiac function by maintaining LV geometry without neovascularization [35]. Kofidis *et. al* [43] and Huang *et. al.* [42] has investigated effects of matrigel. The experiment was done with either the biomaterial alone or the cells with cell culture medium. The results showed improved LV function and increased vasculature. Davis *et .al.* [44] developed an injectable biomaterial from self assembling peptides which forms nanofibers *in situ*. Neonatal cardiomyocytes were also administered alongwith the fibers. The fiber morphology provided favorable environment for cell and vasculature growth. On the contrary, the results demonstrated very little accumulation of endogenous cells to the infracted area. Despite the advantage offered by these injectable materials, they have several limitations which prevent them making ideal therapy for the treatment of MI. Matrigel alone is not ideal for in situ regeneration of cells. The long term effects of alginate, fibrin and collagen are unknown. Self assembling peptide has potential in cell regeneration; however the suitable cell source for myocardial repair has yet to be found [2].In addition to that collagen and alginate are found to be unstable mechanically in vivo. Therefore long term effect of these materials on cardiac function is questionable.

Both in situ and in vitro tissue engineering approach has ability to restore the cardiac function depending on the severity and size of MI affected area. In the early stage of MI cell injection can support the cardiac function. While in the later stage tissue engineered patches are more effective[6].

Pharmacological approaches to treat the heart failure include reduction in workload and protection from humoral toxic substances overexpressed in the heart. This approach is efficient when the

symptoms are not severe [36]. With the marked severity of the symptoms of asynchronous electrical or mechanical function of the heart implantation of pacing devices has become popular[37],[38],[39],[40]. However interventional therapy, surgery or pharmaceutical approaches can not cure the patient during the end stage heart failure .Heart transplantation is one of the options which can be thought of during this stage. Due to the scarce number of the available donors compared to the requirement and constant need of immunospuppressor limits the viability of this strategy. Scientists and surgeons are looking for other alternatives to repair the injured heart muscle.

The contractile function of the infracted myocardium can be improved by implantation of functional cardiac myocytes [41], [42]. In this therapy cell are injected to the infracted area via pericardium, endocardium or coronary arteries. In order to improve the target accuracy the network of cell bandage was prepared *in vitro* and was implanted onto the infracted region [43].

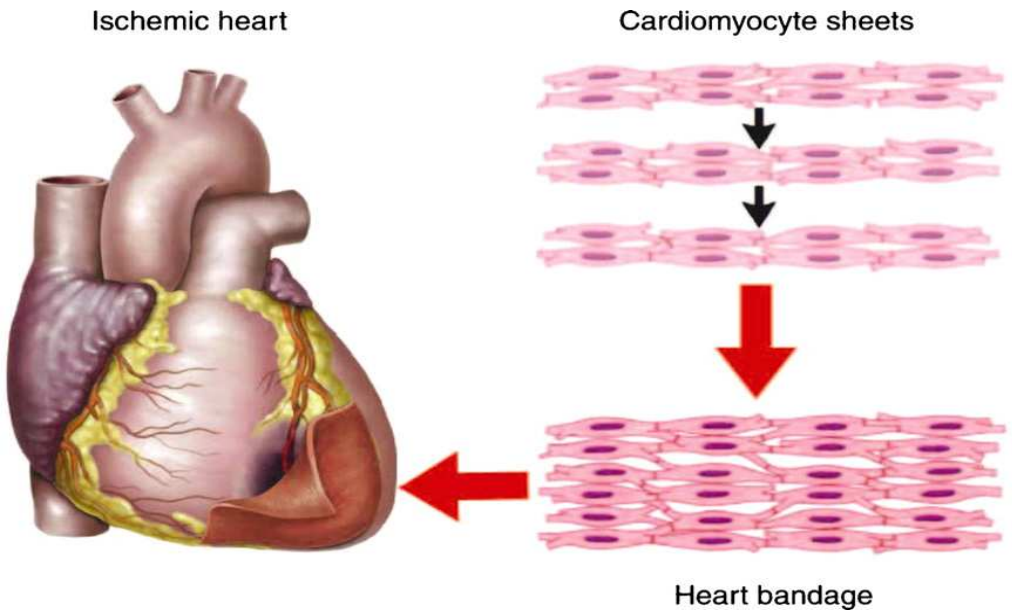


Figure1.3 Schematic of the cell sheet engineering approach. Three layers of the tissue makes cell bandage which is implanted on infracted region [1].

A similar approach involves implantation of man made cell dense heart patch on the infracted zone. This helps in cell delivery and provides mechanical support to the heart wall. It improves cardiac mechanics and reduces cardiac wall stress. [44], [45].

In 3D tissue engineering approaches myocardial tissue is generated in vivo in the presence of natural biology i.e. cells and biomolecules. This approach is based on the fact that regeneration capacity of the living bodies can be maximized in the presence of natural biological environment. 3 D engineered tissues have proven one of the important therapeutic strategy to repair and enhance the function of myocardial infracted heart[46].

Bioreactors are being used in cardiac tissue engineering for providing physiological condition to the artificial tissue construct. For mass transportation of gas and nutrients and for providing regulatory signals like mechanical stretch, electrical signal and hydrodynamic shear Bioreactors play a vital role[6].

Despite the promising results of the seeded constructs two major obstacles remain:

1) Weak mechanical properties compared to the native myocardium being replaced and

2) Inability to maintain thick (i.e., >1mm), viable tissue both *in vitro* and *in vivo* because of the lack of a vascular network within the constructs; the thick tissue design limits the diffusion rate of gases and nutrients to the tissue.

1.3 Objectives of this research project

The goal of this project is to develop a cardiac graft *in vitro*, to provide mechanical support and improve ventricular function of the heart followed by myocardial infarction. To achieve this goal three specific aims were set for this project.

1.3.1 Specific Aims

1) To develop a biomaterial scaffold with micropatterned grooved structure to provide alignment to the cardiomyocytes using an elastic, biodegradable, biocompatible polymer

2) To develop a bioreactor system to apply mechanical cyclic stretch to the cells

3) To examine the effect of mechanical stimulus and on cell growth and Morphology

1.3.2 Impact of this Project

The successful results of this project will have impact in cardiac tissue engineering providing an alternative approach to the total heart transplantation. The prospective outcome of the cardiac graft will open up the possibility to fabricate thick, vascularized tissue for other dynamic organs like heart.

Organization of thesis

Chapter 2 describes the method for making mold by chemical etching technique.

Chapter 3 explains the steps in polymer synthesis. It also illustrates the process of constructing micropatterned polymer film from CUPE prepolymer.

Chapter 4 explains the design and development of the bioreactor. It also illustrates about loading of the polymer scaffold in to the device.

Chapter 5 explains the cell culture protocol on polymer film in the device

Chapter 6 describes the results and discussion of this project.

Chapter 7 briefly mentions about the ongoing and future study with conclusion of the current results.

CHAPTER 2

DEVICE FABRICATION

2.1 Design of the micropattern unit

In order to create micropattern structure on the polymer film, a silicon wafer mold was prepared by chemical etching with TMAH. The mold was prepared on 6 inch silicon wafer. 12 units were created on one mold to get 12 PDMS device at once. Each unit had an area termed punching pad and grooves. The grooved area was 10X10 mm while the punching pad on two sides of the grooves was 7X10 mm. The diagram of this design is illustrated below. For this application the groove size was set to 10 μm; while the separation between grooves was approximately 7 to 8 μm.

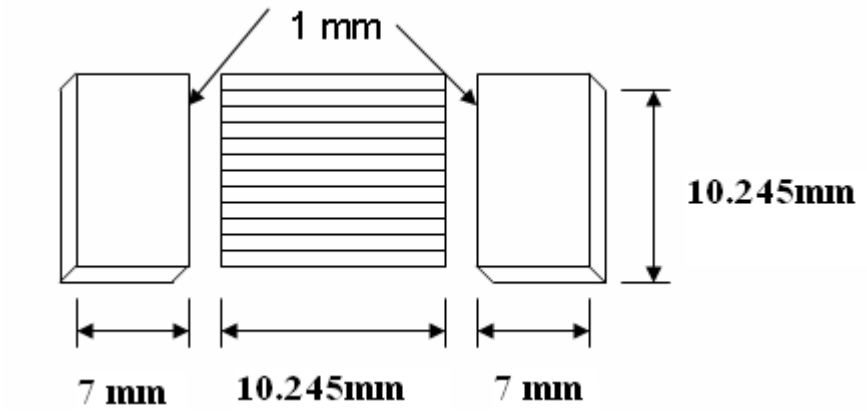


Figure 2.1 Dimension of the unit created on mold

2.1.1 Master mold preparation

Following steps were performed for photolithography and TMAH Etching

1) Cleaning the oxidized wafer

2) Dehydration

3) Spinning

4) Soft Bake

5) Exposure

6) Develop

7) PR on other side of wafer

8) BHF for silicon dioxide

9).Acetone to remove PR

10) TMAH Etching

1) Cleaning the oxidized wafer

The oxidized wafer was cleaned by using piranha clean. 1 H_2SO_4: 1 H_2O_2 in a glass container was added and oxidized wafer was put into it for 10 minutes. After Piranha, wafer was rinsed in running DI water for 5 minutes and blow dried with nitrogen.

2) Dehydration

The wafer was baked using hot plate for 10 minutes at 195 C for dehydration. After that it was allowed to cool down for 1-2 minutes.

3) Spinning

PR S1813 was put on the wafer surface and was spin at 4000 rpm for 40 seconds. Spinning was started at 100rpm/sec at start till 500 rpm. It took 5 sec. Then the speed was increased to 4000rmp at rate of 1000 rpm/sec for 40 seconds.

4) Soft Bake

For soft bake, wafer was heat up your for 60 sec at 115C using hot plate

5) Exposure

Using G Line OIA Aligner, wafer was exposed for 9 seconds.

6) Develop

After Exposure, wafer was put into developer for 40-45 seconds to improve the quality of results.

7) BHF for silicon dioxide

BHF etch rate for Si_2O is 100 nm per minutes. Depending on it wafer was allowed in BHF solution to remove Si_2O.

8) Acetone to remove PR

Wafer was put in beaker filled with enough acetone to immerse for 10 min. It removed the entire PR that was applied to the wafer. It was dipped in methanol for 10 minutes after acetone dip and then washed with running DI water and blow dried with nitrogen.

9) TMAH Etching

For each 4 ml of 25% TMAH 1 ml of DI water was added to make 5 ml of 20% TMAH solution. 3000 ml solution was made by adding 2400 ml of 25% TMAH in 600 ml of DI water. Then it was compensated for evaporation by adding 50 ml of DI water. The solution was heated till 90 C using hot plate, Probe, Thermometer and Al Foil to cover the beaker. It took around one hour to reach 90^{o}C, after that wafer was put into TMAH solution for 150 minutes using Teflon wafer carrier. Then etched wafer was put into DI water and was dried.

The diagram of this process flow is shown below.

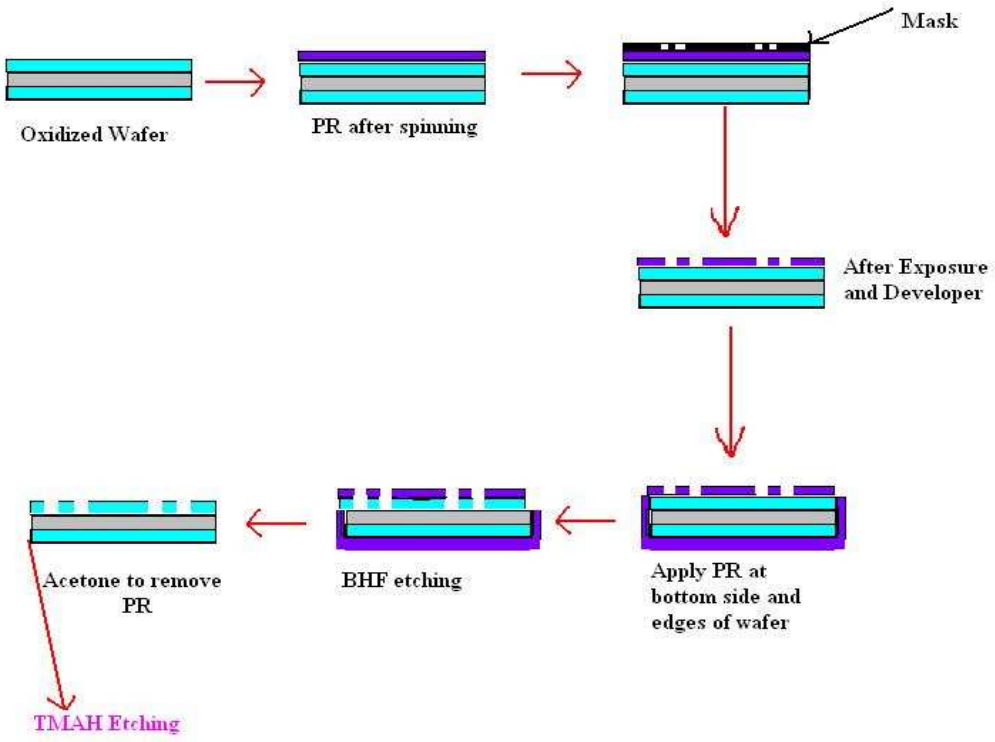


Figure 2.2 Process flow diagram of TMAH etching of wafer

2.1.2 Casting PDMS mold

Sylgard 184 kit was used to prepare Polydimethylsiloane (PDMS) molds. Prepolymer and curing agents were taken in ration of 10:1 w/w. The mixture was stirred carefully until prepolymer and curing agent was mixed properly. The mixture was put in desiccators to remove the bubbles. The mold was wrapped with aluminum foil to hold the PDMS on top of it. Aluminum wrapped mold was put on the hot plate. Desiccated PDMS was poured onto the mold to cover the entire surface. The PDMS was cured at 90^{o} C for 10 min. The PDMS was allowed to cool for few minutes and then was peeled off form the master mold using a scalpel. The master mold can be used to make more PDMS devices by following the same steps as mentioned above.

2.2 Characterization of grooves on master mold

The micropattern on the master mold was characterized by confocal microscopy and profilometer. Confocal microscopy gives 3 D view of the grooves as shown in figure 2.3.The grooves are v-shaped. Figure 2.4 is the profilometer of the micropattern structure. The width of the grooves is 15.9 µm and depth is 7.51 µm.

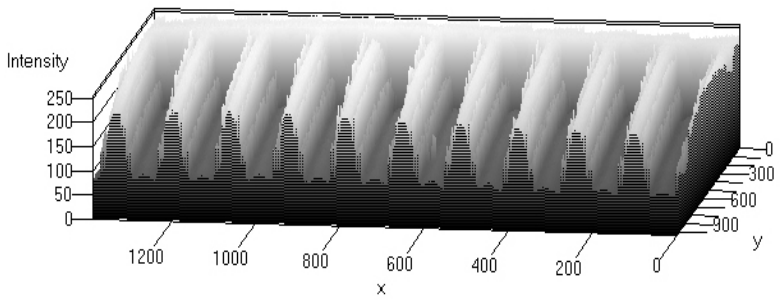


Figure 2.3 Confocal microscopy image of grooves

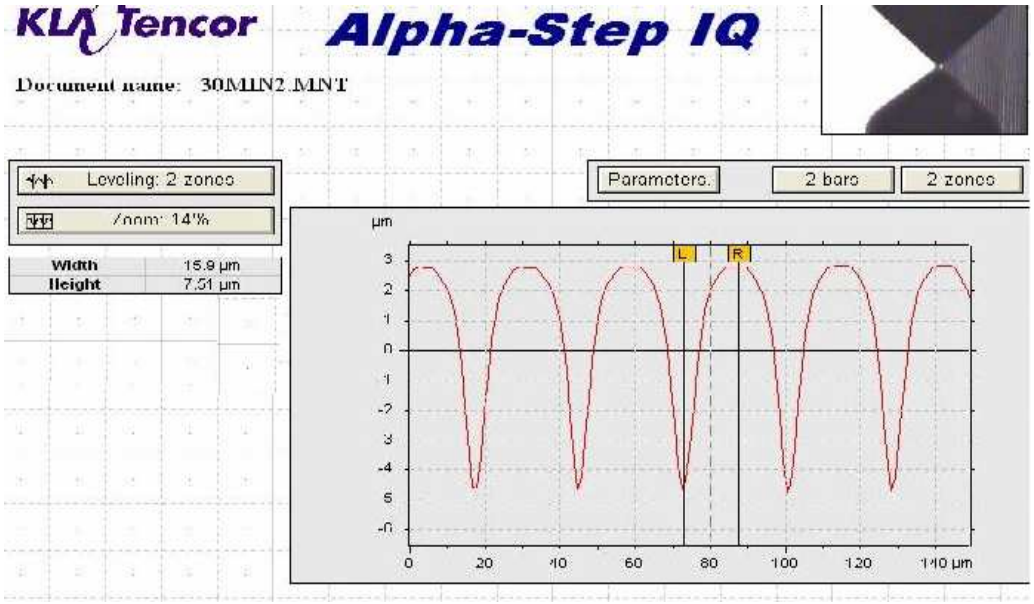


Figure 2.4 Profilometer of the micropattern structure

CHAPTER 3

PREPARATION OF MICROPATTERNED POLYMER THIN FILM

3.1 Requirement of the scaffold for cardiac graft

The ideal scaffold material for tissue engineering application depends on the ability of the material to mimic the natural ECM and support cell growth. The material should have controllable degradation mechanism and high degree of immune tolerance *in vivo* [47]. To improve the functionality of the diseased myocardium the substrate should mechanically, electrically and functionally integrate with the native myocardium. The ideal material should have the properties similar to native myocardium. The substrate should be contractile, mechanically strong, vascularized, autologus and electrophysiologically stable [48]. High mechanical strength can not be obtained from conventionally used polyesters polymers like PGA, PLA, PCL and their copolymers being thermoplastic. Increase in the crosslinking density makes them brittle rather than improving mechanical properties. They are successfully used for nerve guides and blood vessels in vitro. Due to their mechanically weak behavior they can not be used for in vivo applications[49]. Polyurethanes are strong and elastic but undergoes permanent deformation when subjected to cyclical mechanical load environment [49]. Porous and fibrous scaffolds are inherently stiff and can not be used as a scaffold where contractility is the basic requirement. Also, these scaffolds do not degrade completely [60],[5] and offer poor cell alignment and morphology [50]. They undergo plastic deformation when subjected to continuous long term mechanical stress. The tailor made synthetic polymers which are soft and elastic can provide mechanical support and cues for the cell growth before they can develop their own physiological ECM [13].

To mimic the mechanically dynamic function of the myocardium the scaffold material should be soft, elastic and mechanically stable. The scaffold should be able for mechanotransduction between the cells and the surrounding environment. [5]. In addition to mimic the function of the native tissue the scaffold material should be strong enough to handle during surgical procedure in *in vivo* study.

3.2 Selection criteria for CUPE

To overcome aforementioned limitations of conventionally used polymers Dey *et al* developed Crosslinked urethane-doped polyester (CUPE). This polymer has combined properties of elasticity and biocompatibility coming from polyester and high mechanical strength coming from polyurethane. Increased crosslinking density provides increase elasticity to the polymer. The polymer consists of all ester bond crosslinking making the polymer biodegradable. Doping of Urethane compound increases hydrogen bonding and improves mechanical strength of CUPE. Being synthetic polymer its chemical properties and mechanical properties can be tailored depending on the application. Concentration of urethane bonds, polymerization condition and diol used for polymer are responsible factors for mechanical properties and degradation rate of CUPE. By changing these parameters the properties of CUPE can be changed. Material chemistry plays an important role in cell growth. Cell friendly material allows cell attachment and differentiation [11].The tensile strength of CUPE is 41.07±6.85 MP and elongation at break is 222.66±27.84%.

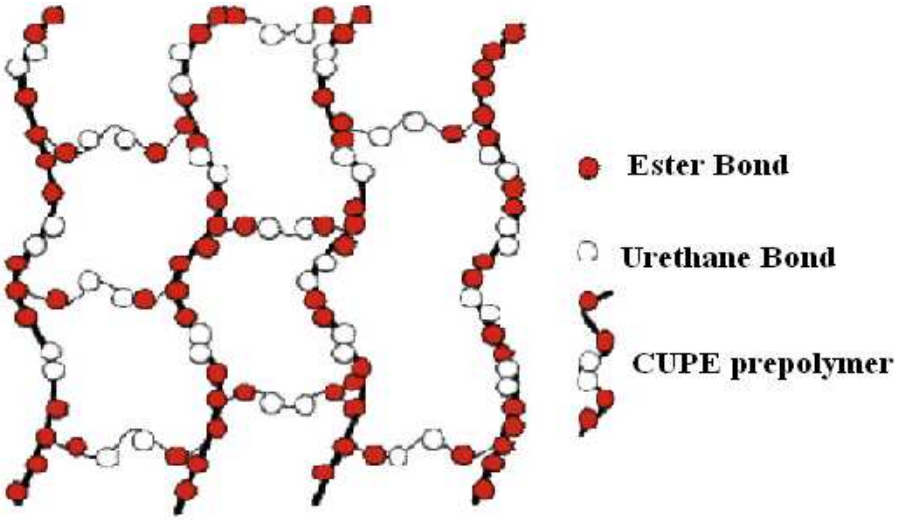


Figure 3.1Crosslinked structure of CUPE network [59]

3.3 Synthesis of polymer

Monomers citric acid and 1, 8 octane diol were bulk polymerized at 160^{O}C with monomer feeding ration of 1:1.1. After melting of the polymer the temperature was lowered to 140^{O}C. POC prepolymer was obtained after polymer mixture was stirred for 1 hour. To purify the prepolymer the polymer solution was precipitated dropwise in DI water and lyophilized. 1, 4 dioxane was used as a solvent for purified POC to prepare 2.5% of the polymer solution. The polymer system was kept at 55^{O} C to react with 1,6 hexamethyl diisocynate in the presence of catalyst stannous octoate. After 6 hours the FTIR was performed on sample of polymer using a Nicolet 6700 FTIR spectrometer (Thermo Fisher Scientific). The FTIR curve is shown below. The process was stopped after confirming the absence of isocyanate peak at 2267cm^{-1} in FTIR curve.

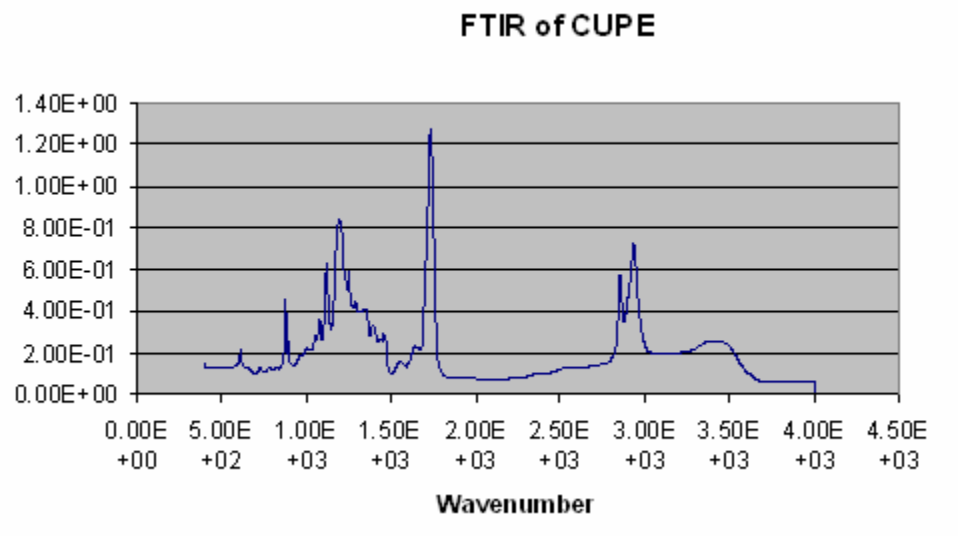


Figure 3.2 FTIR curve for CUPE

3.4 Thin films of CUPE polymer

Thin uniform polymer films were prepared by solvent casting of CUPE prepolymer. The PDMS molds were cleaned with 70% ethanol to remove debris. A box shaped structure was prepared with nonsticky aluminium foil to hold the desired volume of the polymer solution on PDMS mold. The picture of this mold is shown below. The nonsticky side of the foil faces the side of the polymer. Nonsticky nature of

the foil helps in easy peeling of the polymer film. The aluminum foil was wrapped firmly with the PDMS device so as to prevent any leakage or slippage of the polymer solution underneath the PDMS device.

Figure 3.3 Solvent casting of CUPE on micropatterned PDMS

3 ml of polymer solution was added onto each PDMS device. The device were kept overnight at room temperature to allow the solvents to evaporate from the polymer solution. On the other day it was transferred to the oven at 80 ^{O}C for postpolymerization. After 12 hrs the polymer films were peeled off from the PDMS device with the help of forceps. The hydrophobicity of the PDMS device helps in detachment. The micro patterned structure transferred on the polymer film was confirmed by observing the samples under microscope.

The polymer films were kept back in the oven at 80 ^{O}C for additional 36 hours for post polymerization to allow further crosslinking. Incubation at 80^{0} C allows the polymer to crosslink which improves the mechanical strength of the polymer film. The –COOH group in the polymer react with each other at 80^{0} C. This lowers the acidicity of the polymer and brings it near to the physiologic pH level. This reduces the release of acidic product from the polymer in cell culture medium which is essential for cell survival.

3.4.1 Transfer of pattern onto the polymer film

The micropattern on the polymer film was confirmed by light microscopy. As shown in the figure below the width of the groove on the polymer film was 15.12 µm, while the width of the grooves on silicon wafer was approximately 13 to 15 µm. The microscopic image of the polymer film confirmed that the pattern on the silicon wafer master mold was transferred on the polymer film.

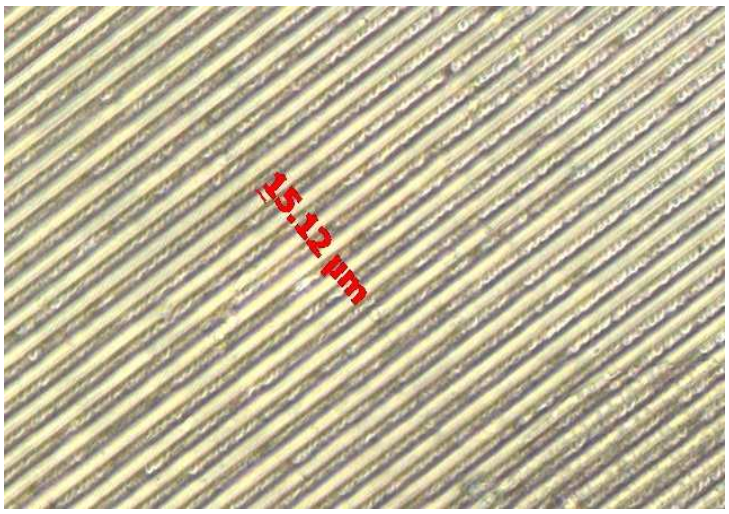


Figure 3.4 Microscopic view of the micropatterned polymer film

CHAPTER 4

DESIGN AND FABRICATION OF BIOREACTOR

4.1 Bioreactor design

Bioreactors play an important role in the development of the dynamic tissues such as cardiac tissue. Mechanical and chemical cues to the cells during in vitro culture support functional development of the tissue [6].

To apply uniaxial mechanic stretch to cardiac graft during cell culture *in vitro* a bioreactor system was designed and fabricated.

The custom designed bioreactor is made up of control unit and stretching unit. Stretching unit consists of T-75 flask, moving bar and fixed bar. The control unit consists of a linear stepper motor and driving circuit. The moving bar is connected to the stepper motor.

In the proposed design the controller runs the stretching unit at a constant frequency of 1.5 Hz. The unit is designed to run for 10 days. To offer flexibility, the circuit consists of an ‘off’ switch which allows the stepper motor to stop manually. The controller provides displacement of the moving bar by 2 mm. Our construct is approximately 1.9 cm in the length in the direction of stretching. So the unit applies approximately 10% stretch on the construct. Depending on the slight variation in length of the film, and the loading condition in the bioreactor the stress applied on the sample varies from approximately 10 to15 % .This bioreactor assembly can be used to simulate the heart beat, resulting in improving contractile nature and mechanical property of the bioengineered cardiac tissue in vitro.

4.2 Fabrication of Bioreactor

4.2.1 Material

The custom designed bioreactor assembly is made up of the nontoxic material. The list of the material is given in the table.

Table 4.1 Materials used in fabrication of Bioreactor

Bioreactor	Material Used
Moving bar	Nylon 6-6
Fixed bar	Nylon 6-6
Connecting Rods	Delrin
Sample holding bar	Delrin
U shaped channel(To provide path for the movement of the bar)	Polycarbonate
Base and Top cover	Delrin
T-75 Flask	Polyethylene
Case of stepper motor	Stainless Steel
Screws, Nuts, Bolts	Stainless Steel

The Bioreactor assembly is made up of cell culture chamber and the driving mechanism. The cell culture chamber comprises T-75 flask and two bars made up of delrin to hold the samples. The sample holding bars are connected to fixed bar and the moving bar with 3 L shaped delrin rods on each side. These rods have one end on the sample holding bar passing through the holes made in sample holding bar. The other ends are passing from the holes made in T-75 flask and fixed with screws to the moving bar and fixed bar. This connection was made with screws to allow flexibility of redesigning the system. Two bars are placed in the opposite sides of the T-75 flask. One of them can be fixed with the system after loading the sample at appropriate distance depending on the sample size. The other bar is

connected with the driving mechanism and it applies uniaxial phasic stretch on the sample. Both ends of these bars are placed in a U shaped polycarbonate channel to allow freedom of movement. These channels are placed on the acrylic tube to adjust the height level of the channels with the holes of the T-75 flask.

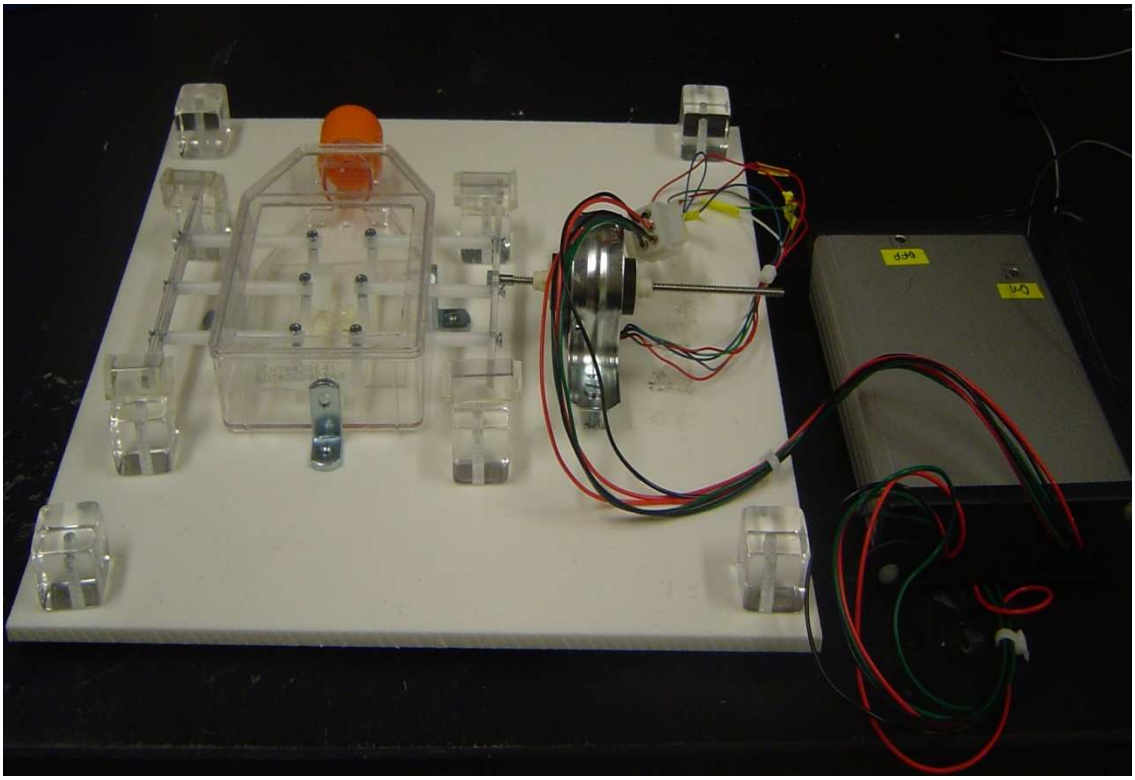


Figure 4.1 The top view of the bioreactor device with control circuit

4.2.2 Control Circuit

The driving mechanism comprises a linear stepper motor (model # 35DBM10B1U-L from Portescap, Danhar Motion Company) and a control circuit. The cell culture chamber and the stepper motor are mounted on the delrin base. The motor is enclosed with delrin sheets to prevent any possible contamination. The cell culture chamber and the stepper motor is further enclosed with a delrin enclosure for ease of sterilization. The whole assembly was put in the incubator.

The movement of the moving bar in each bioreactor is controlled by two separate linear stepper motors (model # 35DBM10B1U-L from Portescap).Movement of both the stepper motors are controlled by

a single control unit. The stepper motor is connected to the moving bar with its shaft. The movement of the motor is controlled by a microcontroller (16F877A). The microcontroller is programmed to run the motor for 5 days at 1.5 Hz. The stepper motor moves to and fro in a defined path range of 1 mm. This displacement range results in 10 to 15 % of uniaxial stretch on the loaded samples. The sample holding bar is kept equidistant throughout its length to ensure equal application of the tensile stretch on all three samples. The sample dimensions are 26X10 mm.

Fixing the samples with the sample holding bar occupies 3 mm length of the sample from both sides. It results in total of 6 mm length on the sample holding bar which in turn leaves 20 mm long sample subjected to 10% of uniaxial stretch.

The Control circuit is made up of 16F877A PIC microcontroller and ULQ2003A Darlington array. The microcontroller is programmed to run the stepper motor for 10 days at 1.5 Hz with travel range of 1 mm. The circuit is equipped with manual on off switch to stop the motor before its preprogrammed running duration. The signal from the microcontroller is given to Darlington array ULQ2003A. The darlington array amplifies the current and sends the signal to the stepper motor.

The diagram below represents the connection between stepper motor, PIC controller and Darlington array.

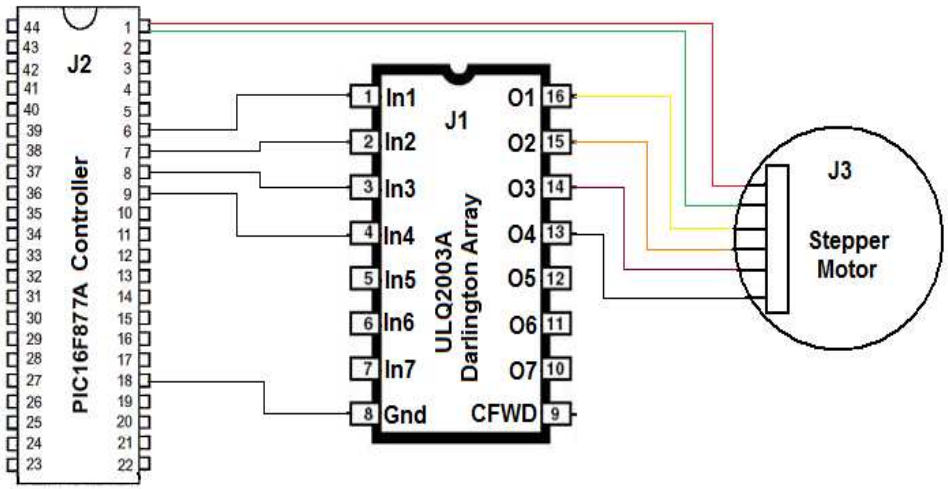


Figure 4.2 Connection diagram for stepper motor and PIC controller

The circuit assembly was fixed in an enclosure for ease of sterilization and to make the unit portable.

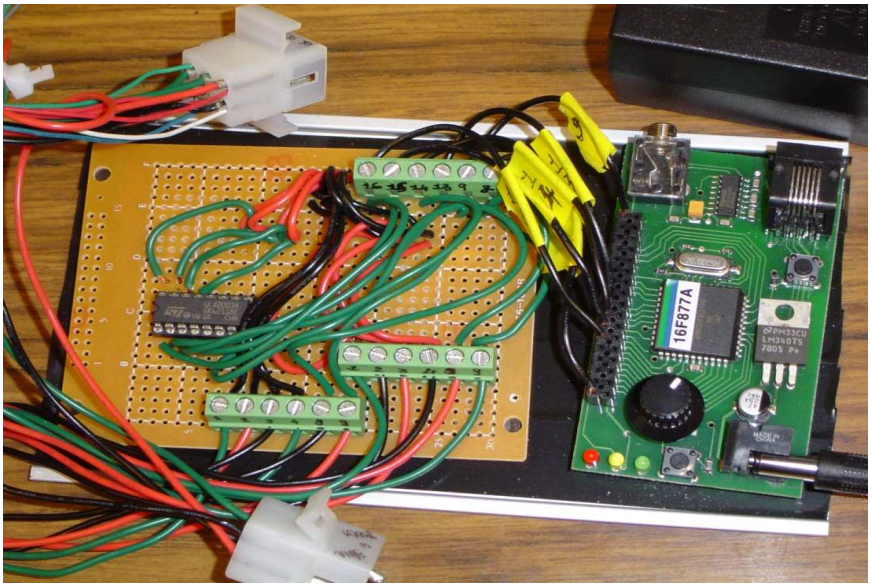


Figure 4.3 Control circuit for the Bioreactor which contains Darlington Array ULQ2003A, PIC microcontroller 16F877A unit with manual on off switch, Connectors for stepper motors and Power Adapter

4.3 Stretching parameters selection

Three micro patterned polymer films were fixed on the sample holding bar of the moving bar and fixed bar with UV light curable medical grade glue in each of the custom designed bioreactor. 3 day old rat derived cardiomyocytes were cultured on CUPE polymer film for 5 days. All the samples were subjected to 10% stretch of the original length of the substrate for 5 days at a frequency of 1.5 Hz. The entire assembly was held in incubator at 37^0 C with 5% CO_2 concentration.

4.4 Loading the film into bioreactor

The dimension of the sample holder is allowed to incorporate 4samples together in each bioreactor. A total of eight samples were loaded in a single batch. The samples were attached with the holding bar using UV light curable medical grade glue.

4.5 Sterilization of the device

The whole device was sterilized 70% ethanol. It was kept under UV light overnight for sterilization. The cells were cultured according to the protocols described in the chapter 5.The device was transferred into the incubator.

CHAPTER 5

CELL CULTURE ON POLYMER FILM

5.1 Collecting cardiomyocytes from P3 animal

The cardiomyocytes were derived from 3 days old neonatal Sprague Dawley rats using the protocols approved by the animal care facility protocols at the University of Texas at Arlington. The left ventricles of the 3 day old neonatal rats were minced. The minced ventricular tissue was put into ice cold trypsin prepared in L -15 medium with the concentration of 50µg/ml for overnight at 4^{o}C to dissociate the cells. On the other day the mixture of tissue and trypsin was transferred to the 2 ml of 10% FBS in DMEM / F-12 to stop trypsinization. This mixture was centrifuged at 1200 rpm for 5 min. The supernatant was removed. Collagenase (1500 U, Worthington) was prepared in L- 15 medium with concentration 240 U/ mg. 5 ml of collagaenase was added in cell pellet mixed well. The mixture was incubated at 37^{o}C for 45 minutes. The cells were triturated_with fire polished glass pipette. The cell strainer was put onto the centrifuge tube and soaked with L- 15 medium. The cell suspension was filtered through the cell strainer to remove the cell chunks. The mixture was centrifuged at 1200 rpm for 5 min. The supernatant was removed and 10 ml of 10% FBS in DMEM/F 12 was added in the cell pellet. The cells were seeded in the t 75 flask and incubated at 37 ^{o}C for 45 min. To prevent fibroblast population in the cell culture the supernatant was removed after 45 min which is rich in cardiomyocyte population. The cardiomyocytes were reseeded in T 75 flask. The cell culture medium was changed on every other day during the cell culture in T 75 flask.

The experiment was done with two types of control groups and experimental group. The positive control group is the samples with thin polymer film having grooved structure on it. The negative control group is the sample without grooves on the polymer film. The number of the samples for each group was kept 3. For the control group the samples were fixed to the 35 mm tissue culture plate with medical grade UV curable glue. (Photo of the samples fixed to the tissue culture plate with UV glue). 3 samples of the experimental group were also fixed to the holding bar of the bioreactor with the medical grade UV curable

glue. The samples were sterilized under UV light overnight. On the other day the samples were kept in PBS to get rid off all the debris and acidic products released from the polymer. The samples were supplied with fresh PBS on the other day. After 48 hours in PBS, the samples were coated with collagen type I.

5.2 Coating with Collagen type I

To enhance the cell attachment to the surface the substrate were treated with extracellular matrix coating. The substrates were coated with collagen type I (285 U/ml) with the concentration of 50µg /ml in 0.02 N acetic acid. The samples were incubated at 37 ^{o}C for 2 hours to allow polymerization of the collagen. To remove unbound collagen the samples were washed twice with sterilized DI water. The cell culture medium was put in water bath at 37 ^{o}C. After 20 min the samples were submerged in prewarmed cell culture media and incubated for 24 hours.

The release of the acidic product form the CUPE thin films turned the color of the media from pink to yellow. The samples were supplied with fresh media and incubated for 24 hours to get rid of all the acidic products and to prevent cell from all the possible source of contamination.

The CUPE polymer swells in the presence of PBS and cell culture media. After 4 days in wet condition the samples were swollen. To maintain the stretch level of 10% of the resting length of the sample, the fixed bar was adjusted to a new position according to the increase in resting length of the experimental samples. The cells were cultured on the control groups and experimental group as described below.

5.3 Cell culture

The cells were seeded on the T 75 flask for one week during which they reached confluency. The cells were passaged from the T 75 flask to the samples by following protocols.

Previously aliquated Trypsin EDTA was put in water bath to bring its temperature to 37^{o}C. The cell culture media was taken out of the flask and was given trypsin EDTA. To activate the trypsin EDTA the flask was put into the incubator for 5 min. The cell suspension was taken out into the centrifuge tube and was mixed with 1 ml of SBTI (soybean trypsin inhibitor) to neutralize the effect of trypsin. The cell suspension was centrifuged at 1200 rpm for 5 min. The supernatant was discarded and the cell pellet was

given 10 ml of 10% FBS. The cells were dissociated physically using pipette tips. Equal volume (100 µl) of cell suspension was given to all the samples. Initially the amount of cell culture medium was kept below the height of the samples in bioreactor to give enough time for the cells to adhere to the surface and the samples were put in the incubator (37^{o}C, 5% CO_2). After 3 hours the t-75 flask in the bioreactor was given enough media so that the samples can submerge into it. The cells were allowed to settle and become confluent on the polymer film surface in the bioreactor for 24 hours after seeding. The device was started running on the other day with 10% stress, 1.5 Hz frequency for 5 days. The media was changed in the control samples every other day during this period. The T 75 flask in the bioreactor was given enough media (80 ml), so that the samples can survive in the media.

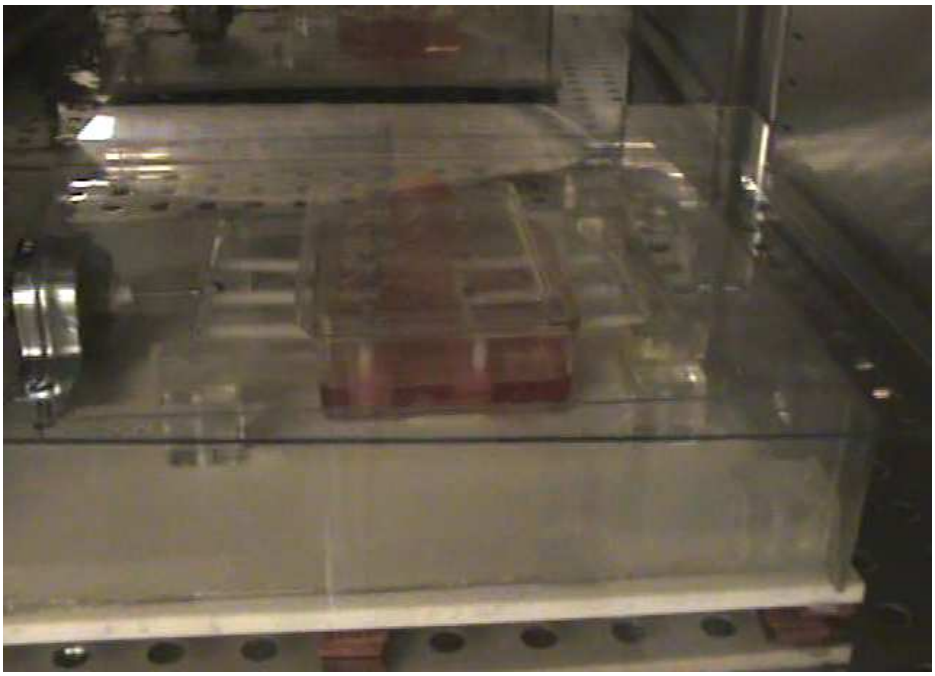


Figure 5.1 Top view of the bioreactor inside the incubator. The device was covered with acrylic sheet.

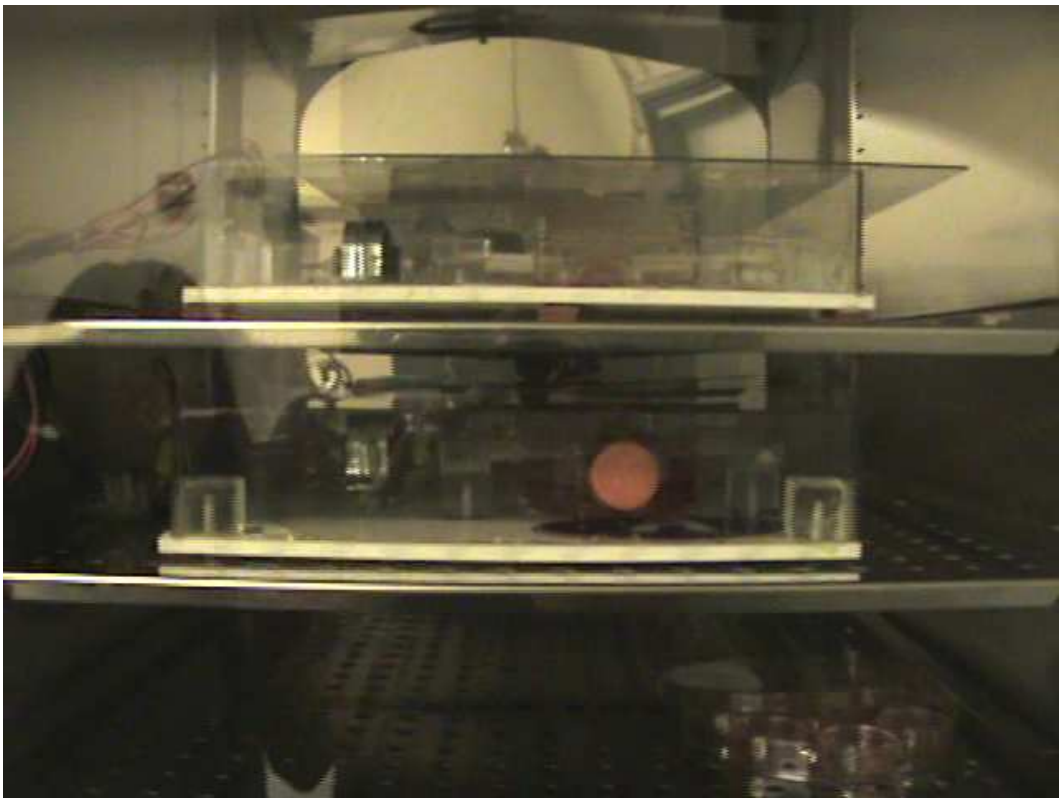


Figure 5.2 Two bioreactors in incubator

After 5 days the device was stopped. Three samples in the bioreactor were carefully removed with the scalpel from holding bar. The samples were placed in the custom made holder in 35 mm tissue culture plate to avoid curling after removing from bioreactor. The control samples and the experimental samples were fixed with 4% paraformaldehyde. After 2 hours all the samples were washed with 1X PBS. The samples were kept at 4 ^{o}C in 1X PBS.

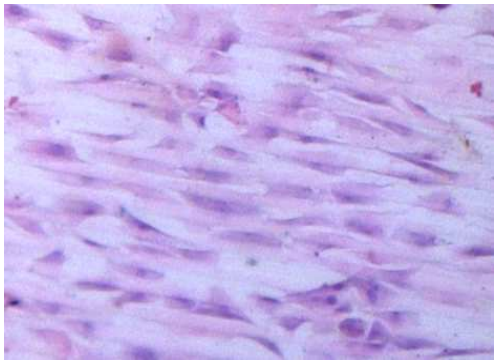
The bioreactor flask was cleaned and washed with DI water and was prepared for the next experiment.

CHAPTER 6

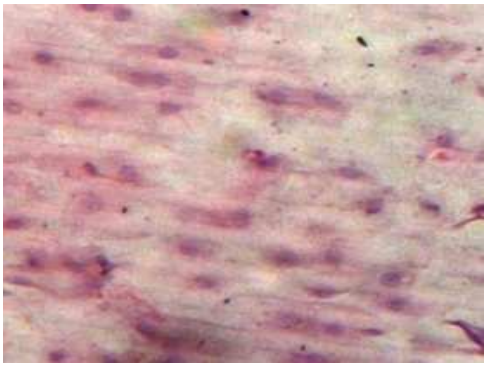
RESULTS AND DICUSSION

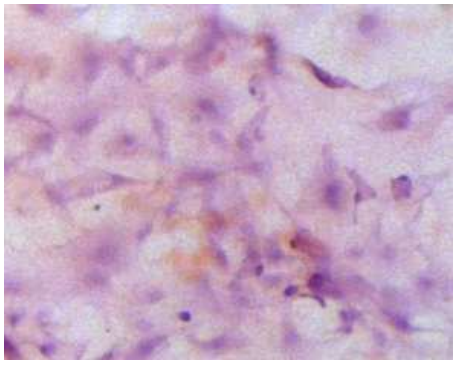
H & E staining of the samples illustrate obvious difference in morphology of the cells between control samples and treatment samples. The pictures were taken with Zeiss Axiovert 430 light microscope with 10X magnification.

6.1 Elongation



(a)





(b) (c)

Figure 6.1 Elongation in cardiomyocytes (a) sample subjected to mechanical stress (b) Cardiomyocytes cultured on grooved CUPE polymer film (c) Cardiomyocytes cultured on CUPE films without grooves.

Figure 6.1 (a) shows the response of the cardiomyocytes subjected to stretch for 5 days. The cells exhibit elongated, rod shaped structure similar to that found *in vivo.* Unstretched cells culture on

micropatterned CUPE films as shown in figure6.1. (b) and cells cultured on CUPE films without micropattern on it as shown in figure6.1. (c) have increased in size, but do not display elongation after culture period. The degree of elongation in stretched and unstretched samples were quantified and the distribution of elongation is presented in form of graph as shown in fig below.

6.1.1 Quantification

The quantification was done with Image J software. Grid was applied in an image and from one image 20 cells were picked up for quantification. A total of 400 cells were quantified for each condition. Two axes passing through the nucleus of the cells were measured. The longest axis passing through the nucleus was called major axis, while the shortest axis passing through the nucleus was called minor axis. The measurement of the ratio of these axes gives idea about relative elongation in three different experimental conditions. The graph of elongation ration vs. %of cells was plotted as shown below.

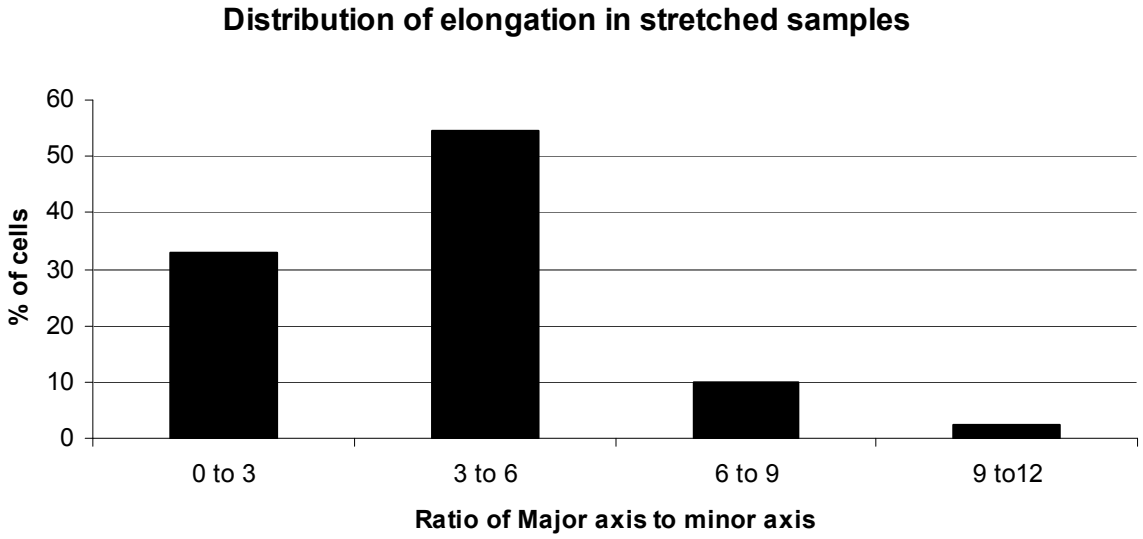


Figure 6.2 Distribution of elongation in cells in samples exposed to stretch

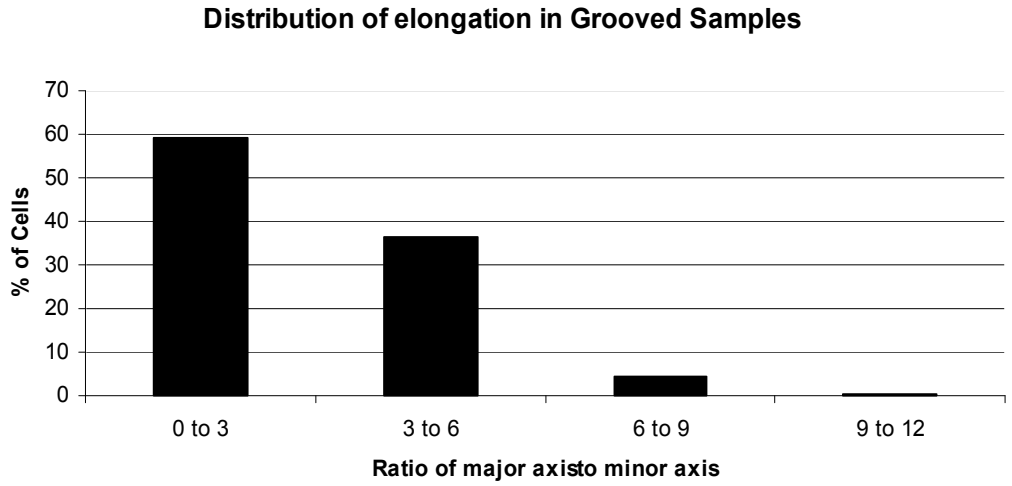


Figure 6.3 Distribution of elongation in cells on CUPE film with grooves

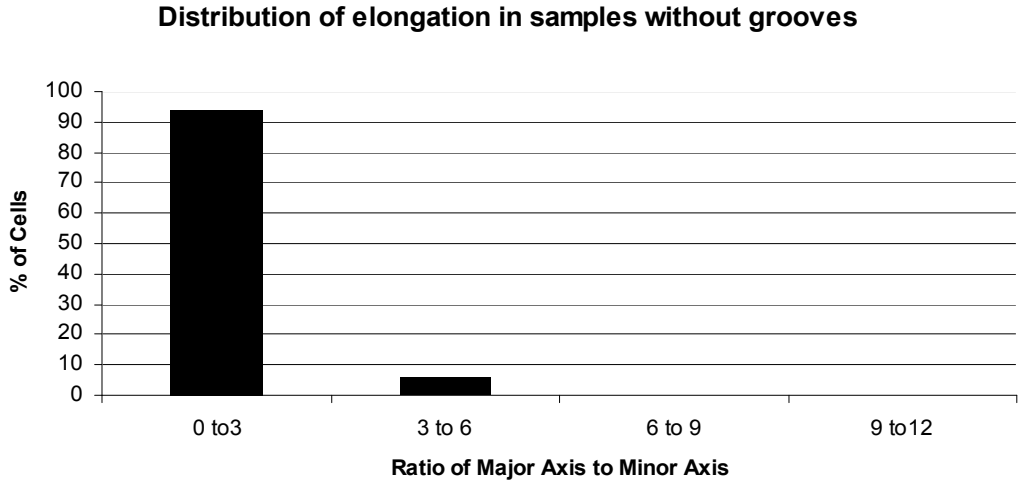
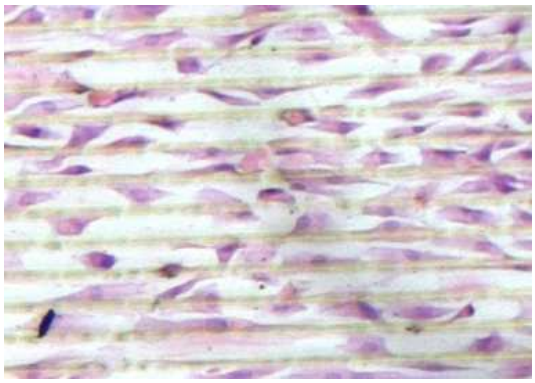


Figure 6.4 Distribution of elongation in cells on CUPE film without grooves

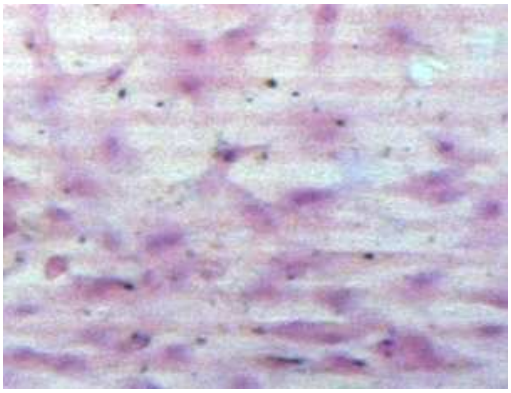
As shown in the figure 6.2 almost 55% of the cells had elongation ratio from 3 to 6 in stretched samples. Approximately 10% of the cells had ratio from 6 to 9, while 3% of the cells had ratio from 9 to 12. This indicates that approximately 68% of the cells have ratio 3 or above.

In samples without stretch and with grooves as shown in figure 6.3, approximately 60% of the cells have ratio below 3. 35 % of the cells have ratio from 3 to 6, 4% has ratio from 6 to 9 while only 1% of the cells have ratio greater than 9. As shown in figure6.4 in unstretched samples without grooves most of the cells i.e. approximately 92% have ratio below 3. Only 5% of the cells lie in the range of 3 to 6; while no cells had elongation ration greater than 6.

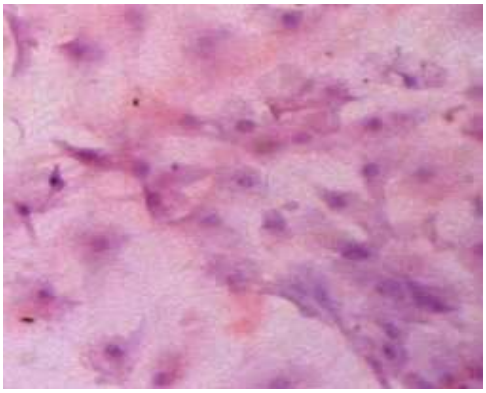
6.2 Alignment



(a)



(b)



(c)

Figure 6.5 H & E staining of the samples reveals alignment of cells in different conditions (a) sample subjected to mechanical stress (b) Cardiomyocytes cultured on grooved CUPE polymer film (c) Cardiomyocytes cultured on CUPE films without grooves.

H & E staining of the samples reveals alignment in the samples. In the stretched samples as shown in figure 6.5(a) cells are arranged parallel to the grooves and most of them are confined in the grooves and shows array type structure. In unstretched samples without grooves few cells are arranged between consecutive grooves making disarray. While the sample without grooves shows random arrangement of the cells.

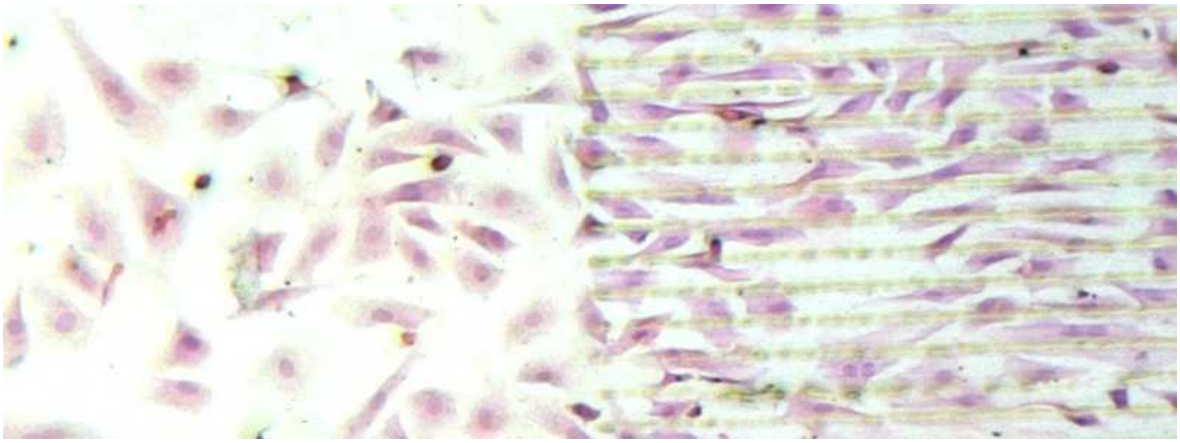


Figure 6.6 Interface between the grooves and flat surface on CUPE films in stretched samples

As shown in figure 6.6, there is remarkable difference in alignment in the grooved region and flat region on the same CUPE film which was exposed to stretch. Most of the cells in area on the right are confined in grooves and gives aligned structure while those cells on the left half of the film which are attached to the area which does not have grooves are randomly oriented.

6.2.1 Quantification

Using Image J orientation of the cells was quantified. As explained earlier for this quantification also 400 cells were selected for each condition. The angle of the major axis of the cells with respect to a reference line was measured. The graph of angle vs. % of cells for each condition was plotted as shown below.

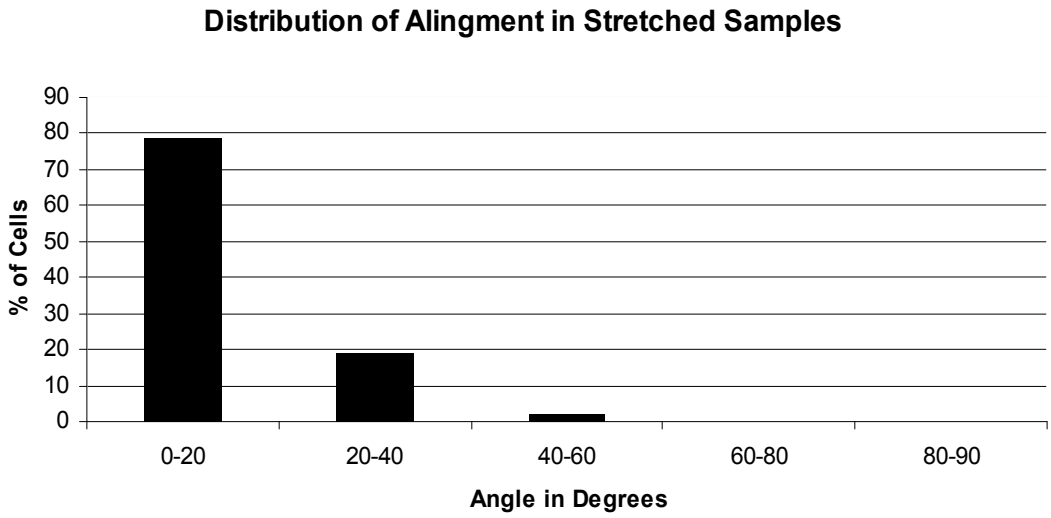


Figure 6.7 Distribution of alignment in cells in samples exposed to stretch

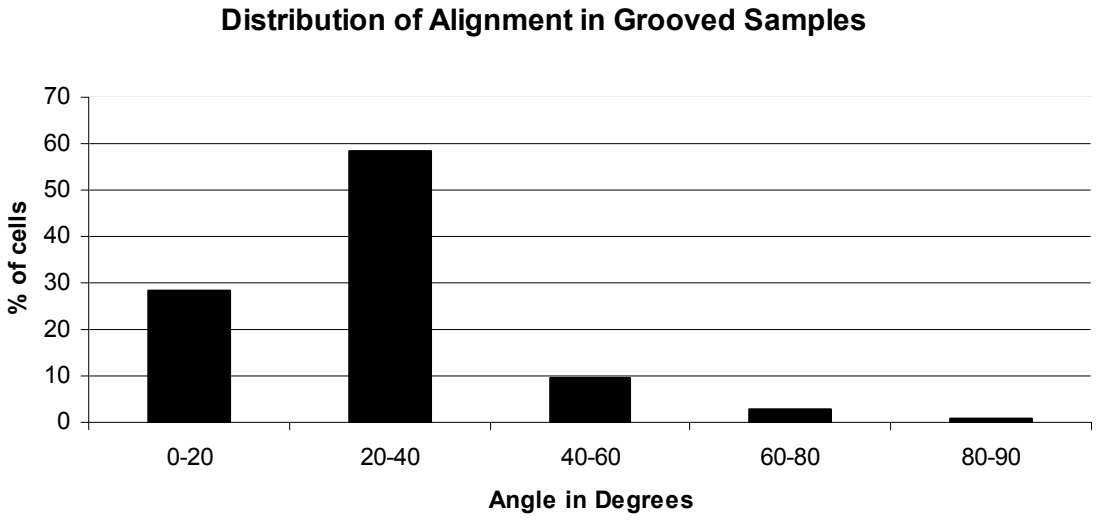


Figure 6.8 Distribution of alignment in cells on CUPE film with grooves

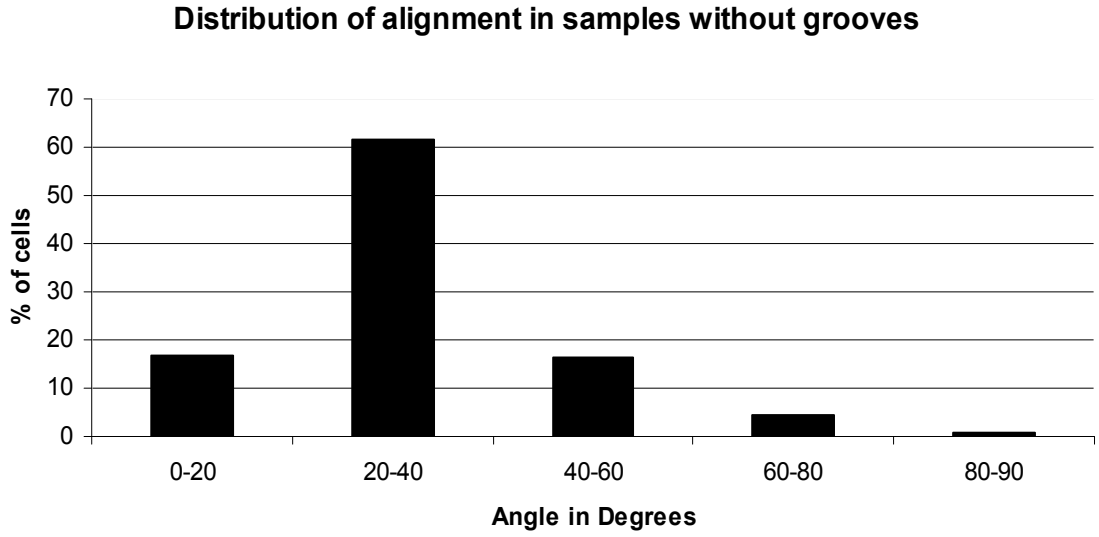
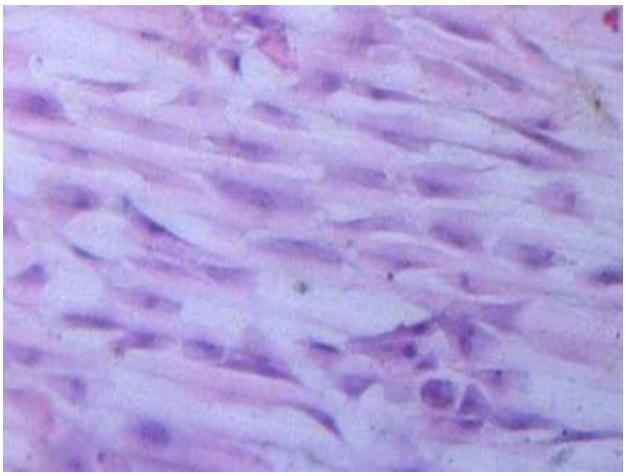


Figure 6.9 Distribution of alignment in cells on CUPE film without grooves

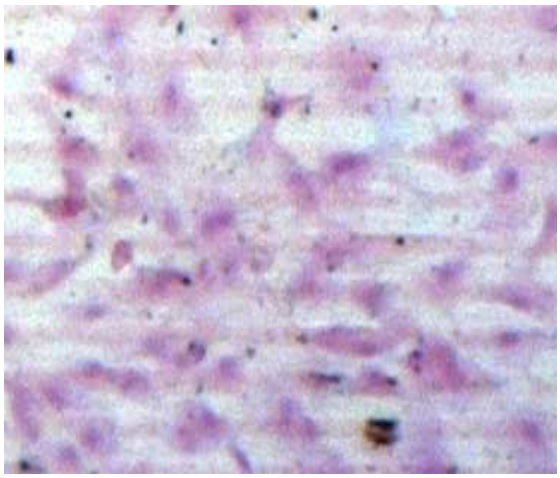
The cells lying in this range were defined as aligned, while the cells lying in the range greater than 20^{o} are defined as randomly oriented. As shown in figure 6.7 almost 80% of the cells are in the range from 0 to 20^{o} . In samples with grooves a total of 29% cells were aligned and 71% of the cells were randomly oriented, while in samples without grooves only 18% of the total cells were aligned and remaining 82 % were randomly oriented.

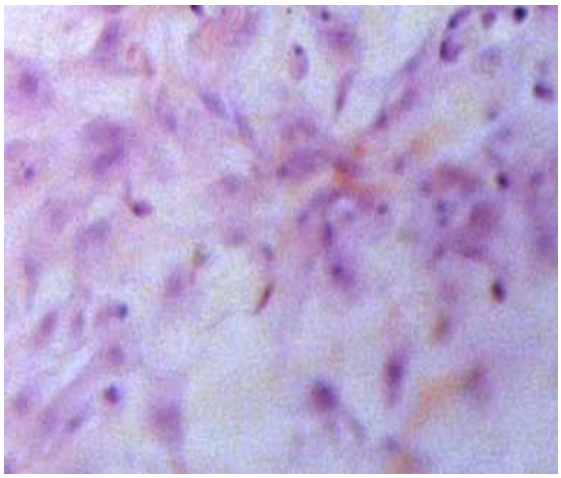
6.3 Hypertrophy

H & E stained samples were used to analyze increase in cell size which is a hallmark of maturation, after culture period in all three different conditions.



(a)





(b) (c)

Figure 6.10 H & E staining of the samples showing hypertrophy of cells in different conditions (a) sample subjected to mechanical stress (b) Cardiomyocytes cultured on grooved CUPE polymer film (c) Cardiomyocytes cultured on CUPE films without grooves.

As shown in figure 6.10 (a) the increase in the size of the cells after culture period is maximum in stretched samples. While in unstretched samples the increase in cell size was comparatively lower as shown in figure 6.10(b) and 6.10(c). There was not noticeable difference in cell size in samples with grooves and samples without grooves.

Comparative increase in cell size for three different conditions was quantified with Image J. 400 cells for each condition were selected for quantification as explained with the measurement of above two

parameters. The periphery of each cell was traced and measured. The data was plotted in the form of graph for each experimental condition as shown in figure 20.

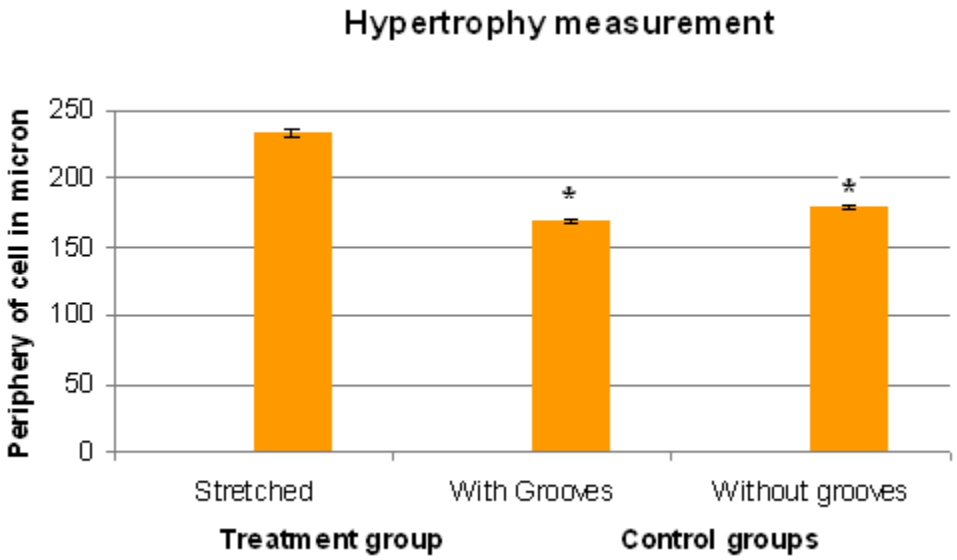


Figure 6.11 Measurement of hypertrophy in cells for different experimental condition (* P<0.05).

As shown in graph the average size of the cells in stretched samples was 230 µm in stretched samples. For sample with grooves it is 168 µm and for samples without grooves it is 178 µm. Pair wise T-test was done for treatment group with each of the control group. For both the tests the value of P is less than 0.001. This indicates that there is significant difference in cell size stretched samples and samples with grooves. Also a significant P value for T-test for the comparison between stretched and samples without grooves implies a considerable difference in cell size between these two groups.

6.4 Discussion

Cardiomyocytes were cultured on CUPE polymer films. CUPE is biocompatible, elastic and biodegradable material. Studies are done with NIH 3T3 fibroblast to prove the compatibility of the material *in vivo*[59]. Scaffold chemistry has major effect on the cell attachment and growth. The results illustrate that CUPE supports cardiomyocyte cell culture. Survival, growth and in vivo like cell morphology suggest that the chemistry of the CUPE supports cell growth.

Improved cell morphology in different experimental condition suggests that stretch induces morphological changes in the cardiomyocytes. CUPE is elastic, biocompatible material. Ideal scaffold for cardiac tissue engineering should be able for the mechaotransduction. i.e. the film should be able to transfer forces from the environment to the cells[59].. This is critical features for the survival of the graft in vivo; the reason being in order to pace with the natural environment in vivo the graft has to be able to transfer the stimuli to the cells. In this experiment the remarkable difference in the results obtained from stretched and unstretched samples suggests that mechanical stress applied to the CUPE films has been transferred to the cells attached to them. Thus CUPE is appropriate material for cardiac tissue engineering application.

Size of the scaffold can be controlled. Properties of the material can be controlled by controlling several parameters during synthesis. CUPE being elastic, mechanically strong material [59] will be able to provide mechanical support to the LV ventricle and maintain the geometry alongwith providing functional cardiac cells at the infarction site; when implanted *in vivo* .

To facilitate the proper pumping function of the heart it is essential for a heart patch to provide passive diastolic constraint to infracted region[7].Poor mechanical supporting ability of collagen gel scaffold is one of the shortcomings of the engineered cardiac graft designed by Zimmermann *et al.* Type I collagen which is used as a scaffold material in this approach has young's modulus of 20 to 80 Pa for 1-3 mg/ml. [61]. This is considerably lower than that of rat myocardium which is 0.14 MPa at the end of diastole [1]. One of the other shortcoming with the use of collagen type I as a scaffold material is that as collagen concentration increases, mechanical strength of the construct decreases. Experiments done by Okano *et al.* used cell sheet engineering approach. It does not require matrix from any biomaterial, hence the mechanical support provided by this graft to infracted site is limited. In this experiment the CUPE has tensile strength of 41.07±6.85 MP, which is very high, compared to the native myocardium. Thus, CUPE can be expected to provide enough mechanical strength *in vivo*.

Successful outcome of the experiment shows that the custom made bioreactor system is compatible with CUPE scaffolds and cell survival. The bioreactor system provides manual control to change the stimulation protocols by changing the controlling parameters. This flexibility will allow studying

the effect of various parameters on the response of the cardiomyocyte growth. This bioreactor system can be utilized in future to stretch all types of tissues which require external haemodynamic force for the growth. The stretching parameters can be adjusted by changing parameters in the control circuit and can be made to be feasible depending on the requirement of individual tissue.

The results indicate that micropattern created scaffold and stretch has significant influence on the cell growth and morphology. The cells which are exposed to stretch show morphology which is similar to the adult rat ventricular cell morphology. These cells are elongated and rod shaped. Stretched cells remain confined to grooves and make parallel array like structure, which is characteristic of myocardium. They exhibit remarkable increase in size i.e. hypertrophy compared to the unstretched cells.

Native cardiac tissue has aligned cardiac muscle fibers containing myofilaments organized parallel to the major axis of the elongated cells. Spatial constraints laid by grooves makes them to remain confined in the grooves and gives rise to aligned, parallel, elongated structure. Cardiomyocyte exhibit attachment dependent phenotype. Attachment plays an important role in growth and survival of cardiomyocytes.[62].Cell adhesion gives proper alignment to the cardiomyocytes[63].Cardiomyocytes are found to respond to the geometry of their environment and changes phenotype accordingly[63]. To guide the cell growth *in vitro* for myocardial tissue, aligned geometry of the scaffold is desirable Coating of collagen type I on the grooves structure helps the cells to attach on the surface. This highly oriented architecture is crucial for the electromechanical coupling of the cells [64].Studies have shown that poor organization of cardiomyocytes leads to electrical asynchrony. [1]. The velocity of action potential is greater and similar to that of *in vivo* in oriented cardiomyocytes compared to disorganized structure of the cells [64]. This is owing to the fact that consecutive cardiomyocytes in cardiac tissues are connected at their end through intercalated disks [64]. Aligned morphology of cardiomyocytes helps them arrange in a fashion which can allow them to have end-to end contact. The collagen treatment on CUPE film before cell seeding helps in cell attachment by providing directional cues on the grooves architecture. High number of cells and aligned pattern of cells in stretched samples as shown in fig. has high probability to form cell-cell junction through intercalated disks and turning into functional tissue *in vivo.* Cells in control

samples, as shown in fig. are disorganized and have limited chance of making end to end connection. Therefore, their possibility of turning into electrically synchronous, functional tissue is very low.

The dimension of the grooves also has influence in giving rise to aligned cell structure. The micropattern on CUPE film was designed according to the size of the cardiomyocytes. The cardiomyocytes are 100 to 150 μm in length and 10 to 20 μm in width [65].

This dimension and helps the cardiomyocytes to confine in the grooves grow with major axis keeping almost parallel to the groove direction. From fig 6.7, 6.8, 6.9 graph shows that almost 80% of cells were aligned in stretched samples, 29% in samples with grooves and 18% in samples without grooves. High number of cells in grooved samples compared to samples without grooves indicates that grooves guide in cell alignment. Fig 6.7 shows that in stretched samples only the cells attached on grooves exhibit aligned structure, while the cells outside the grooves had random orientation. Highest number of aligned cells in stretched samples, and fig 6.6 implies that both stretch and grooves are crucial factor to give aligned structure.

The results presented above shows there is considerably more number of cells in stretched samples which undergo this phenotypic change and exhibit hypertrophy compared to control samples which are not exposed to cyclic stretch. This implies that stress cause hypertrophy in the cells. Several studies have shown that forces applied on cells cause hypertrophic and hyperplastic changes. During the cell cycle of cardiomyocyte they undergo from hyperplastic to hypertrophic phenotype change [66]. During hypertrophic phase of the cycle the cells change their shape from round shape to oval shape. The cell functionality can mimic to that in vivo if during the culture they are provided the same factor as in *in vivo* condition[11]. Mechanical stimulus is thought to provide in vivo like context for the growth of cardiomyocyte in vitro.

Mechanical stress has been considered the primary cause of cardiac hypertrophy [67],[68].Cardiac hypertrophy is a normal physiological response of the heart to meet the increased demands of pumping action by cardiomyocytes. [59].Several experimental findings support this hypothesis. For instance, isolated heart expressed upregulation of cardiac load stimulated protein synthesis. The results of this experiment imply that mechanical stress plays an important role in

developing hypertrophy in cardiomyocytes in vitro also. Here this mechanical stimulation causes hypertrophy in the cells which turns their morphology from neonatal rat ventricular cells to the adult myocardium like morphology.

The results suggest that mechanical stress causes morphological change in cells. The rod shaped, elongated cells arrange in aligned fashion and undergo maturation by increasing cell size. This morphology of the cells is similar to that found *in vivo* in adult rat ventricular cells.

CHAPTER 7

FUTURE STUDY AND CONCLUSION

7.1 Future Study

The survival of the cardiac graft *in vivo* will be studied in animal model. To build a thick, vascularized tissue in the animal, 10 bioreactor devices will be fabricated. More number of bioreactors allows availability of large number of cardiac grafts via batch production. A single device allows loading of 4 CUPE films in a single experiment. With 10 such bioreactor we expect to have 40 cardiac grafts from one experiment ideally. Large number of cardiac graft will allow implantation of multiple grafts layer by layer in animal model. A bunch of the grafts will be transplanted in a single surgery onto the gastrocnemius muscle of the animal model. At a certain time interval another bunch of the grafts will be implanted in the animal. The time duration between two consecutive surgeries will allow implanted graft to have vascularization and connection with host tissue. To prevent necrosis and provide sufficient nutrients to the cells vascularization is a key requirement. During the implantation of the graft holes will be created in the graft to make it porous and allow blood vessels to form through the multiple layers. This approach has advantage to maintain mechanical strength of the material in the bioreactor system. Pores will be created in the scaffold after application of mechanical stress; this will increase the chances of the survival of the graft in bioreactor during application of stress.

The survival of the graft in the animal will be studied for 15 days period. After 15 days the tissue at the implantation site will be sliced and stained with markers for blood vessels.

7.2 Conclusion

The overall aim of this project was to develop thick, vascularized cardiac graft to repair myocardial infarction. The specific aims were to develop a biodegradable, elastic micropatterned scaffold, to design a bioreactor system to apply mechanical stimulus and to observe the morphological changes in the cardiomyocytes due to effect of this mechanical stress. The successful results of this project demonstrate that CUPE is elastic and biocompatible. Cardiomyocytes can survive and grow on the CUPE

films. The mechanical strength of CUPE is high enough for its application in cardiac tissue engineering. The CUPE films can survive in the bioreactor system during application of 10 to 15% of stress. The custom designed bioreactor system is compatible with CUPE substrate and cardiomyocytes. The feasibility of setting the stretching protocols depending on the requirement will allow this system to use for the study of effect of different stretching protocols in cell morphology.

Micropatterned structure on the polymer film along with applied stretch makes the cells to arrange in aligned manner. The stretch causes morphological change in the cell shape and size. After the application of stretch the cell become elongated and undergoes hypertrophy. Stretched samples exhibit cell morphology which is similar to that found *in vivo* for adult cardiac cells. Following these results, there is high possibility of to build a thick, vascularized graft *in vivo* which can survive and improve cardiac function.

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