Development of mechatronic 1-D strain bioreactor for tissue-engineered oesophagus

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Development of Mechatronic 1-D Strain Bioreactor for Tissue-Engineered Oesophagus

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Abstract

Current treatments for oesophageal cancer are not free of complications. The medical field has been trying to increase the success rate of these treatments, especially for oesophageal substitution, the most common treatment for patients with oesophageal atresia and oesophageal cancer.

This project is part of the Singapore-University of Washington Alliance (SUWA) programme which uses an *in vitro* tissue engineering approach to develop a functional tissue-engineered oesophagus as a substitute for the diseased oesophagus. The project pioneers the bioreactor and mechanical conditioning aspects of the overall SUWA programme and aims to study the effect of mechanical stimulation on oesophageal smooth muscle cells, particularly in cell alignment and proliferation.

The mechatronic bioreactor developed in this project is able to provide a variety of 1-D physiological mechanical stimulation regimes (e.g., amplitude, frequency, stress loading time). Polyurethane membrane with O₂ plasma surface modification (200W 60s) has been evaluated as a suitable scaffold for mechanobiology works. Both components were integrated for mechanical stimulation studies. Mechanical stimulation was applied to the porcine oesophageal smooth muscle cells on polyurethane membrane under different stimulation regimes and the effects on cell alignment and proliferation were analyzed.

Based on cell viability test results, the stimulated visceral smooth muscle cells did not proliferate, indicating that the cells exhibited differentiated phenotype. Cardiovascular cyclic strain at 2.5 – 10% strain for 2 hours/day has influenced the smooth muscle cells to align perpendicular to the direction of applied strain. The 10% strain gave adverse effect to the cells and caused the cells to detach after 4 days of stretching. This alignment phenomenon of porcine oesophageal smooth muscle cells is similar as the literature report for the vascular smooth muscle cells. However in oesophageal cycle at low strain, the cells were parallel to the direction of strain. Further research is required to investigate this phenomenon.

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Chapter 1 – INTRODUCTION

Oesophageal cancer is the 7th most common cancer worldwide [1]. In 2006 there will be about 14,550 new cases of oesophageal cancer and about 13,770 will die of this disease in the USA [2]. Moreover, this cancer is much more common in other countries, approximately 10 to 100 times higher in Iran, northern China, India and southern Africa than in the USA [1, 2]. Since oesophageal cancer is usually not diagnosed at early stages, hence most patients eventually die of this disease. The most common treatment for oesophageal cancer is esophagectomy. However the substitution of diseased esophagus is not satisfactory due to either leaking, or lack of functional peristaltic motion. This project is part of Singapore-University of Washington Alliance (SUWA) programme which uses an *in vitro* tissue engineering approach to treat esophageal cancer. SUWA programme aims to develop functional tissue-engineered esophagus to be implanted in human body to substitute the diseased oesophagus. This project itself focuses on the bioreactor and mechanical conditioning of tissue-engineered oesophagus.

1.1 Background

1.1.1 Oesophagus

The oesophagus is one of the organs of the gastrointestinal (GI) tract (See Figure 1.1). It lies posterior to the trachea, begins at the inferior end of the laryngopharynx

and ends at the superior portion of the stomach. Based on the measurement in 212 individuals, the mean oesophageal length was approximately 23 cm [3]. The outer diameter of the oesophagus is approximately 2 cm at its smallest point [2, 4].

Figure 1.1 Human digestive system. (From CancerbaCUP: Cancer of the gullet information centre, [Online], Available: <http://www.cancerbacup.org.uk/cancertype/gulleetoesophagus>, 2004. [5])

The oesophagus is a complex structure, composed of 4 layers and controlled by nerves arranged as plexuses to perform its functions. The arteries and veins are present within layers in oesophagus to deliver O_2 and nutrition to the cells and remove waste. Figure 1.2 gives a schematic drawing of the oesophagus.

Figure 1.2 Schematic structure of an oesophagus with various components and their functions. (Modified from Tortora, G.J., and Grabowski S.R.: Principles of Anatomy and Physiology, 10th ed. John Wiley & Sons Inc, 2003. [4])

Histology and Physiology

Structurally, the oesophagus consists of 4 layers from deep to superficial, the mucosa, submucosa, muscularis and adventitia [4, 6]. The structure of oesophagus is similar with other gastrointestinal organs, except at the outermost layer. Its adventitia at the outermost layer consists of areolar connective tissue and merges with the connective tissue of surrounding tissue whereas other gastrointestinal organs have serosa, formed by areolar connective tissue with epithelium at the outermost layer.

Figure 1.3 Oesophagus Wall. (From Tortora, G.J., and Grabowski S.R.: Principles of Anatomy and Physiology, 10th ed. John Willey & Sons Inc, 2003 (left) and Burkitt H.G., Young, B., & Heath, J.W.: Wheater's Functional Histology, 3rd ed. Churchill Livingstone, 1999.(right) [4, 7])

The mucosa is a mucous membrane composed of the following [1, 2, 4, 6]:

a. Epithelial layer

The epithelial layer is the innermost layer and contacts the food directly. The upper 2/3 closest to the pharynx, of this layer is nonkeratinized stratified squamous epithelium (See figure 1.4, Labeled E in the figure 1.3) that serves as a protection against abrasion and wear-and-tear from food particles. These cells are able to resist abrasion and heat and heal quickly if damaged. The other 1/3 closest to the stomach is stratified columnar epithelium. It protects and allows mucus- and fluid-secretion into the lumen of the tract.

Figure 1.4 Epithelium of the mucosa layer: (left) Stratified squamous epithelium. (right) stratified columnar epithelium. (From Tortora, G.J., and Grabowski S.R.: Principles of Anatomy and Physiology, 10th ed. John Wiley & Sons Inc, 2003. [4])

b. Lamina propria

The lamina propria is formed by areolar connective tissue (See figure 1.5) that supports the epithelium and binds it to the muscularis mucosae. It contains many blood and lymphatic vessels (Labeled L_y in figure 1.3), mainly mucosa-associated lymphatic tissue (MALT), containing lymphocytes. Near the stomach the groups of oesophageal cardiac glands [6] also secrete mucus to lubricate the bolus and reduce friction.

Figure 1.5 Areolar connective tissue. (From Tortora, G.J., and Grabowski S.R.: Principles of Anatomy and Physiology, 10th ed. John Wiley & Sons Inc, 2003. [4])

- c. A thin layer of smooth muscle fibers called the muscularis mucosae (Labeled MM in the figure 1.3)

The submucosa (Labeled SM in the figure 1.3) consists of areolar connective tissue that binds the mucosa to the muscularis. It has blood, lymphatic vessels and mucus-secreting glands, called the oesophageal glands (Labeled G in the figure 1.3) [4, 8]. It also contains the submucosal plexus (plexus of Meissner), an extensive network of neurons [4]. The submucosal plexus has principal roles in sensing the environment within the lumen, regulating gastrointestinal blood flow and controlling epithelial cell function [9]. It forms part of the Enteric Nervous System (ENS) which is responsible for all digestive processes in the digestive system.

The muscularis layer consists of two layers of muscle tissue in transverse direction: the inner sheet of circular layer (Labeled CM and C in the figure 1.3 and 1.7 respectively) and the outer sheet of longitudinal layer (Labeled LM and L in the figure 1.3 and 1.7 respectively) [4, 5]. In circular layer, the smooth muscle cells are aligned tangentially to the oesophageal lumen whereas the smooth muscle cells are parallel to the oesophageal axis in the longitudinal layer. Both layers contract and relax in a coordinated way to push the food to the stomach. The circular muscles behind the bolus contract to push the bolus downward towards the stomach. Meanwhile the longitudinal muscles ahead of the bolus, contract to shorten this section and widen the oesophagus to receive the bolus (See figure 1.6). These movements have a great physiological advantage by concentrating circular muscle

fibers and also a great mechanical advantage by reducing the level of contractile force required to transport the bolus. Both advantages greatly reduce the circular muscle tone and effort required during oesophageal peristalsis [10].

Figure 1.6 Peristaltic mechanism. (From Tortora, G.J., and Grabowski S.R.: Principles of Anatomy and Physiology, 10th ed. John Willey & Sons Inc, 2003. [4])

In the axial direction, the oesophagus is divided into 3 parts. The upper 1/3 of the muscular layer closest to pharynx consists solely of striated muscle cells that produce voluntary swallowing. At the middle portion, a mixture of striated and smooth muscle cells are found whereas at the lower 1/3 of the muscular layer closest to the stomach, only smooth muscle cells are found.

The smooth muscle cells was controlled by a second plexus of the Enteric Nervous System, myenteric plexus (Auerbach plexus, labeled G in the figure 1.7) which is present between the layers of muscularis [9]. This plexus controls primarily over the peristaltic movement. In addition to the two major plexuses in the enteric nervous

system (ENS), there are minor plexuses within the oesophageal wall [9]. They connect to the central nervous system and contain sensory and motor neurons and interneurons. These minor plexuses allow communication from signal outside digestive system to the digestive system and vice versa. The motor neurons are subdivided into sympathetic and parasympathetic postganglionic neurons.

Figure 1.7 Visceral smooth muscle cells. (From Burkitt H.G., Young, B., and Heath, J.W.: *Wheater's Functional Histology*, 3rd ed. Churchill Livingstone, 1999. [7])

Mechanical Properties

Mechanical properties of the oesophagus from three different groups of researchers were compared. Data from Yamada [11] and Vanags [12] showed similar pattern of mechanical properties of three different section of oesophagus: upper (cervical), middle (thoracic), and lower (abdominal). The ultimate tensile stress of cervical section of the oesophagus is the highest among the other sections in the longitudinal direction whereas the maximum strain of the same section is the highest in transverse direction (See figure 1.8). However, the values of ultimate tensile stress and maximum tensile fracture strain between Yamada [11] and Vanags [12] are different. For example, according to Vanags [12], the ultimate tensile stress and maximum

strain at cervical section in the age group of 19-44 years are 2.19 ± 0.06 MPa and 70 ± 7 % respectively, whereas according to Yamada in the age group of 20-39 years are 0.67 MPa and 133.5% respectively. The work of Egorov [10] supports Vanags [12] works by showing similar values of ultimate tensile stress and maximum strain of the oesophagus in the abdominal section (See table 1.1). The differences in Yamada's [11] and Vanags'[12] research may be due to different physical condition of the human cadaveric oesophagi used and duration of preservation.

Table 1.1 UTS and Max Strain of abdominal section of the oesophagus according to Egorov [10] and Vanags [12]

	Ultimate Tensile Stress	Maximum Tensile Fracture Strain
Egorov <i>et al.</i> [10]	1.289 MPa	57.65%
Vanags <i>et al.</i> [12]	1.42 MPa	60.85%

Different layers in oesophagus have different mechanical properties. According to Yamada, the mucous (both mucosa and submucosa) layer has an ultimate tensile stress, in both longitudinal and transverse directions, about 1.2 times of the whole oesophageal wall. The muscularis and the adventitia comprise about three-fifth of the entire oesophageal wall in the longitudinal direction and about fourth-fifth in the transverse direction. The rupture occurs in all layers at the same time for the longitudinal directions. Nevertheless, the oesophagus fails first in the muscularis layer and the adventitia, then in mucous (both mucosa and submucosa) layers for the transverse direction.

Figure 1.8 Stress-strain curves of the oesophagus in the age group of 20-29 years old. (From Yamada, H.: Strength of Biological Materials. Williams & Wilkins, 1970. [11])

1.1.2 Oesophagus Substitution

In certain cases, surgical removal of oesophagus (esophagectomy) has to be performed due to oesophageal disease. The defective oesophagus is removed and replaced by a plastic tube, or substituted with part of the large intestine (colon), or gastric transposition. Gastric transposition (See figure 1.9) brings the whole stomach to where the oesophagus has been cut and then anastomoses the top of the stomach to the end of the oesophagus. However, these therapies are not free of complications. The oesophageal substitute has poor peristalsis, high incidence of leakage, and precarious blood supply to the transplanted section [1]. A further complication is narrowing of the oesophageal wall. As a result, the patients have to go for another operation to dilate the oesophageal channels. After substitution, the patients may

experience major surgical trauma, along with significant changes in quality of life.

The success rate on oesophageal substitution follow up is only 60-80% [8].

Figure 1.9 Oesophageal Cancer (right) and Gastric transposition (left). (From National Cancer Institute: Esophageal Cancer: Treatment, [Online], Available: <http://www.cancer.gov/cancertopics/pdq/treatment/esophageal/Patient/page4>, 2005. [13])

Two possible causes for the esophagectomy are oesophageal atresia and oesophageal cancer. Oesophageal atresia is a closure of oesophageal channel, which usually occurs in babies shortly after birth [1] whereas oesophageal cancer may arise due to mutations in cells on the inner lining of the lower part of the oesophagus. Acid reflux from the stomach may damage the oesophageal wall and cause these abnormal cells that may narrow the oesophagus and cause difficulty in swallowing.

1.1.3 Tissue-engineered Oesophagus for Replacement of Defective Oesophagus

Tissue engineering is an emerging interdisciplinary field that applies the principles of biology, medicine and engineering to the development of viable substitutes that

replace or support the function of defective or injured body parts [14, 15]. There are two approaches in tissue engineering that can be adopted to create new tissues. One is *in vivo* implantation of cell-containing or cell-free devices (scaffolds) that induce the regeneration of functional human tissue. The surgeon may use the patient's own native tissue (autologous transplantation) from either similar tissue from another site (for example, skin grafts and bone grafts), or a different tissue (for example, substitution of the oesophagus with a section of colon). Other options are to use allogeneic cells and tissues (from one human to another) or xenogeneic transplants (from an animal to a human) [16, 17]. The second approach is *in vitro* construction of bioartificial tissue for later implantation to replace or repair diseased tissues. The cells grow on either natural or synthetic scaffolds and differentiate to form functional tissues for later implantation. Both approaches can be used as new methods for oesophageal substitution. For this project, the author chose to adopt *in vitro* approach for the following reasons.

Replicating a complete *in vivo* environment *in vitro* is impossible due to the complexity of tissue functions. However, the *in vitro* tissue engineering approach attempts to provide culture conditions that mimic the biochemical and biophysical signals that regulate *in vivo* tissue development [18]. *In vitro* tissue engineering approach has the advantage of being able to isolate and control the individual parameters (e.g., cell supportive structure, seeding density, biochemical signals, biophysical forces) to be tested. Therefore, this approach may be used as a tool to study cells' behaviour and morphogenesis in certain environments [19]. As a key

phase of the development of a bioartificial oesophagus, it is important to understand the effect of the environment on the growth of cells in each layer of the oesophagus.

The smooth muscle layer in the oesophagus plays an important role to conduct peristaltic motion. *In vivo*, smooth muscle cells exhibit contractile phenotype which is shown by cell orientation and differentiated, contractile protein expression whereas *in vitro* smooth muscle cells exhibit dedifferentiated, synthetic phenotype which actively proliferates. This phenotype is important to be able to implant functional tissue-engineered oesophagus. In order to retain their phenotype, correct environmental cues have to be given to smooth muscle cells in culture.

There are many factors to consider when growing cells *in vitro*. These include sterilization of all equipment; source of cells; choice of scaffold; nutrient supply; mechanical factors; tissue culture medium and technique; and waste disposal. In living oesophageal tissue, the smooth muscle cells are exposed to mechanical forces. To mimic mechanical forces *in vivo*, mechanical stretching or fluid flow is often applied to cell-scaffold constructs *in vitro*.

The mechanical stimulation has been proved to be able to influence cell proliferation and alignment, causing the cultured smooth muscle cells to exhibit contractile phenotype [20]. However the optimum regimen of mechanical stimulation (e.g., frequency, duration, rate of cyclic strain, etc) remains unknown, as do other biochemical effects.

1.2 Objective

The objective of this project is to study the effect of 1-dimensional tensile cyclic strain on the cultured porcine oesophageal smooth muscle cells' behaviour, focusing particularly on cell alignment and proliferation.

1.3 Scope

The scope of this project includes:

1. Understanding the physiological function and metabolic demands of each layer of the oesophagus wall, especially the smooth muscle layer.
2. Designing and developing a mechatronic bioreactor to provide 1-dimensional strain to cells and scaffold.
3. Testing and modification of the mechatronic bioreactor for smooth muscle cell-scaffold construct' analysis.
4. Studying the effect of mechanical stimulation based on the cell orientation and proliferation of the porcine oesophageal smooth muscle cells.

Chapter 2 – LITERATURE REVIEW – OESOPHAGEAL TISSUE ENGINEERING

Several groups of researchers [21-24] have attempted to develop artificial oesophagus using *in vivo* methods. The results have shown some initial success but have not been fully satisfactory due to either stenosis, lack of oesophageal functions, or short survival period.

Miki *et al.* [22] reported that they have been able to develop 20 layers of epithelial stratification which is similar to human oesophageal epithelium in muscle flaps of latissimus dorsi (location: sphenoidal spine) athymic rats. From the same research group, Hayashi *et al.* [23] have reported to develop an artificial oesophagus consisting of human oesophageal epithelial cells as mucosa layer, dermal fibroblasts as submucosa layer, and smooth muscle cells isolated from aortic media as muscularis layer. However both neo-oesophagus have not ever been used in substituting the oesophagus. Their ability to do oesophageal function *in vivo* has not been tested. Another group, Saito *et al.* [21] has attempted to implant an artificial oesophagus, consists of artificial dermis, a latissimus dorsi muscle flap and split-thickness skin in cervical section of oesophagus in Japanese white rabbit. However, the longest survival period was only up to 16 days.

An interesting result has been reported by Grikscheit *et al.* [24]. They seeded mixed oesophageal cells from abdominal section of Lewis rat pups or adults to PGA and

implanted the tissue construct in abdominal section of Lewis rats' oesophagus within 1.5 hours. The mixed oesophageal cells were able to structure themselves to mimic the oesophagus layer. The mucosa layer has developed keratinized stratified squamous epithelial lining, resembling native rat oesophagus. α -actin smooth muscle was seen in the neo-muscularis layer with less developed than the native muscularis layer. The rats survived up to 42 days with success rate of 66%. Of the other 34%, these died or experienced stenosis.

All *in vitro* attempts have used 3 major components: cells that are able to express their differentiated phenotype; biomaterial scaffolds that provide a 3D structural template for cell attachment and tissue growth, and a bioreactor that provides an *in vitro* environment in which cell-scaffold constructs can develop into functional tissues [16, 25, 26]. Besides those major components, *in vitro* tissue engineering also involves biochemical regulation (e.g., growth factors); biophysical regulation (e.g., mechanical loading), and quantitative methods of characterization *in vitro* (molecular, structural, and mechanical properties) and *in vivo* (phenotype stability, survival, vascularization, and mechanical function) [16]. Cells seeded on 3D scaffolds under favorable conditions can actually form constructs with structural and functional features resembling those of native tissue. Several major components in *in vitro* tissue engineering (e.g. cell source, scaffold, bioreactor, cell seeding, etc) will be discussed in detail in the following sections.

2.1 Cell Source and Seeding

Smooth muscle cells that are present in muscularis and partly in the mucosa layer are visceral (single-unit) type of smooth muscle tissue. This type of smooth muscle is also present in the wall of small arteries and veins and of hollow organs such as the other section of the digestive tract, the uterus, gall bladder and the urinary bladder. They are autorhythmic and are activated involuntarily. Since smooth muscle cells are connected to one another by gap junctions, once a neurotransmitter, hormone or autorhythmic action potential stimulates one cell, muscle action potentials spread to neighboring cell, which then contract in unison, as a single unit. They are able to withstand significant stress but only for a short duration [10]. The tension in the smooth muscle cells decreases within a minute and allows the cells to undergo great changes in length while retaining the ability to contract effectively (stress relaxation response).

Ideally, the cells used for tissue engineering should be able to proliferate in a controlled manner and then differentiate *in vitro* [25]. Smooth muscle cells can undergo hypertrophy or proliferate. Even though smooth muscle cells' ability to regenerate is greater than other types of muscle tissue, this ability is still limited compared with other tissue, such as epithelium [18].

The identification of smooth muscle cells from other cell types relies on multiple criteria, including expression of multiple smooth muscle cell selective proteins, the morphologic and functional characteristics of the cell, and, at least *in vivo*, the

anatomical location of the cell. At this time no single marker alone can serve as definitive marker for the identification of smooth muscle cells [27].

2.1.1 Morphology of Smooth Muscle Cells

Figure 2.1 Anatomy of smooth muscle cell. (From Tortora, G.J., and Grabowski S.R.: Principles of Anatomy and Physiology, 10th ed. John Wiley & Sons Inc, 2003. [4])

Smooth muscle cells have a unique morphology that can be differentiated from other cell types. In a relaxed condition, the smooth muscle cells are between 30-200 μm long which is thicker in the middle (3-8 μm) and taper at each end [4] (See figure 2.1). They usually vary to some extent with the tissue of origin, the age, species, and length of time that the cells have been in culture [28].

In early days of primary culture, smooth muscle cells can still retain their contractile phenotype which can be differentiated easily from fibroblasts [28] (See figure 2.2A where m is smooth muscle cell and f is fibroblast). The smooth muscle cells from newborn guinea pig vas deferens that have been grown in culture for less than 7 days

has a ribbon or spindle shape whose size are is approximately $100\text{-}200\text{ }\mu\text{m} \times 10\text{-}15\text{ }\mu\text{m}$ with an oval or sausage-shaped nucleus containing two or more nucleoli. The cytoplasm is phase dense and homogenous and contains few visible inclusions. From the same smooth muscle tissue, fibroblasts are varied in size of $50\text{-}200\text{ }\mu\text{m} \times 20\text{-}50\text{ }\mu\text{m}$ and shape. They are usually flat with a large clear nucleus containing one to five dark, prominent nucleoli. Their cytoplasm contains granular in the region of the nucleus and is clear at the periphery.

However after 7 days in culture, the morphology of smooth muscle cells from newborn guinea pig vas deferens changed. The smooth muscle cells have modulated to synthetic phenotype which are broader and have less dense phase than contractile smooth muscle cells and have a larger nucleus and often more nucleoli (See figure 2.2B). This morphology is similar with fibroblasts. A major distinguishing feature between both is a defined basal lamina in smooth muscle cell which cannot be found in fibroblasts [28].

Another distinguishing characteristic is the concentric growth pattern in fibroblasts and hill-valley growth pattern in confluent smooth muscle cell culture, forming as many as 10-15 cell layers on the hill pattern and 1-3 cell layer on the valley pattern [28, 29]. Figure 2.3C shows the hill-valley growth pattern from monkey aorta in 4th culture whereas Figure 2.3B shows the concentric growth pattern in dermal fibroblasts from monkey skin in 4th culture. The arrow in figure 2.3A shows the nodule in smooth muscle cell >10th subculture from rabbit aorta. The characteristic of hill and valley growth pattern is still observed.

Figure 2.2 Morphology of smooth muscle cells. (From Chamley-Campbell, J., Campbell, G., Ross, R.: The smooth muscle cell in culture, *Physiological Review* 59 (1): 1-51. [28])

Figure 2.3 Hill-valley growth pattern in smooth muscle cell culture and concentric growth pattern in fibroblast culture. (A. From Fallier-Becker, P., Rupp, J., Fingerle, J., Betz, E.: Smooth muscle cells from rabbit aorta. In "Cell culture technique in heart and vessel research" (H.M. Piper, ed.), pp. 271-289. Springer-Verlag, Germany. [29, 30]. B and C. From Chamley-Campbell, J., Campbell, G., Ross, R.: The smooth muscle cell in culture, *Physiological Review* 59 (1): 1-51. [28])

The smooth muscle cells from adult human aorta shows cellular polymorphism after 7 days in culture [30]. Four major cellular types can be distinguished from smooth muscle cell culture (See figure 2.4).

1. Polygonal cell, with endoplasm is localized in the central part of the cells and evenly surrounded by lamellar ectoplasm
2. Stellate cell, with endoplasm division into long radial processes and center localization of nucleus. After spreading, stellate cells take on the appearance of polygonal cells.
3. Asymmetric cell, with irregularly shaped endoplasm.
4. Elongated cell with elongated endoplasm which occupies a larger part of the cell.

Figure 2.4 Cellular Polymorphism of smooth muscle cells *in vitro*. (Smirnov, V.N. and Orekhov, A.N. 1995. Smooth muscle cells from adult human aorta. In Piper, H.M. (ed), *Cell culture technique in heart and vessel research*, pp. 271-289. German, Springer-Verlag. [30])

2.1.2 Phenotype of Smooth Muscle Cells

The muscularis of the oesophagus consists of smooth muscle cells which are aligned in two directions, circumferential and longitudinal. They exhibit a contractile, differentiated phenotype and reside in a mechanically dynamic environment [20, 31]. They are highly specialized cells with unique contractile properties and electrical activities. Contractile proteins such as α -actin, smooth muscle myosin light chain, smooth muscle α -tropomyosin are present and important for their differentiated function [27]. In addition, differentiated smooth muscle cells also express a number of proteins that are part of the cytoskeleton and/or are purported to be involved in the regulation of contraction, such as calponin, smooth muscle 22 α , h-caldesmon, γ -vinculin, and desmin [27]. These proteins can be used as differentiation markers for smooth muscle cells.

The smooth muscle cells may shift from a contractile, differentiated phenotype to a less differentiated, synthetic phenotype which is characterized by high DNA synthesis for cell proliferation, no thick filaments, large amount rough Endoplasmic Reticulum (ER) and ribosomes, and minimal myosin expression. In human, the cells have to change to a proliferative and secretory active state (synthetic phenotype) before they are able to synthesize DNA and divide [28, 32]. This phenomenon makes the concept of differentiation in smooth muscle cells complex. In culture, their conversion from contractile phenotype to synthetic phenotype occurs during early passage [30, 32] and they are able to retain its differentiated phenotype depending on conditions of culture. This characteristic of smooth muscle cells is

distinctive from other cell types due to their dependency on environmental cues even in mature organism rather than being relatively fixed and inherent to the cell itself [27].

2.1.3 Cell seeding

In tissue engineering *in vitro* approach, the cells are seeded on the scaffolds to produce cell-scaffold construct for *in vivo* implantation. This cell seeding procedure is essential for the successful tissue construct *in vitro*, especially to maintain cell-cell communication [15, 19]. The ideal results of cell seeding are a high cell density, and spatially uniform distribution of attached cells in 3D scaffolds [25, 33].

There is a certain minimal cell seeding density to maintain structural and functional construct integrity [19]. Too low cell seeding density results in loss of the construct's mechanical integrity and biochemical composition [19]. It has been shown that cultured chondrocytes in tissue engineered cartilage proliferate better and produce more extracellular matrix with higher seeding densities [34], it may be expected that this is true for visceral smooth muscle cells. Minimum of cell seeding density is important for smooth muscle cells differentiation. Confluent smooth muscle cells are able to regain their contractility when the cell seeding density is above $10^4/\text{ml}$ on culture plate [28].

The cell seeding in a dynamic environment is proved to be able to increase cell seeding density [16, 19]. Carrier *et al.* [19] have demonstrated that cell seeding is more efficient in spinner flask than in static environment or rotating vessels.

2.2 Scaffold and Surface Modification

Scaffolds play an essential role in tissue engineering both *in vitro* and *in vivo*. Cell attachment, migration, proliferation and 3D spatial arrangement are dependent on the composition and structure of the scaffolds [16, 35, 36]. Moreover, human oesophagus smooth muscle cells are anchorage-dependent, like other mammalian cells. They do not survive or proliferate without adhering to a substrate. In living tissues, extracellular matrix (ECM) functions as the substrate or biological scaffold. The extracellular matrix consists of several types of collagen (mostly collagen types I, III, IV), fibronectin, laminin, glycosaminoglycans and different types of growth factors and inhibitors [26, 37]. They influence the behaviour and phenotype of the cells which reside in extracellular matrix. *In vitro*, the scaffolds serve as an extracellular matrix for the cells. They guide new tissue growth by their structural and surface properties such as pore size, hydrophilicity, types of materials, etc.

Biomaterials for scaffold purposes can be simply divided into 2 types: naturally derived materials such as chitosan, collagen, acellular tissue matrices and synthetic polymers such as PGA (Poly Glycolic acid), PLLA (Poly-L-lactic acid), PLGA (Poly(lactic-co-glycolic) acid), and PUs (Polyurethanes). Sometimes both naturally derived and synthetic materials may be mixed, resulting in semisynthetic materials.

Nowadays, naturally derived materials are widely used as scaffold in tissue engineering since they facilitate cell attachment and maintenance of differentiation function[36]. Beckstead, B.L. *et al.* observed that cells seeded on natural scaffolds show superior cell organization and phenotype compared to synthetic scaffolds [38].

An ideal biomaterial for use as a scaffold for direct or later implantation should be biocompatible, promote cellular interaction, tissue development, and differentiation to the desired phenotype and degrade in the body [16]. However for physiological models of tissue in vitro, the scaffolds do not need to be biodegradable or to possess mechanical compliance with human tissue [39]. In some application, the scaffolds are exposed to mechanical stimulation. To withstand mechanical stimulation, the scaffold must be elastic and capable of withstanding cyclic mechanical strain without cracking or significant permanent deformation for time periods ranging from days to months [20, 31].

Several other physical characteristics of scaffolds, such as a pore size, porosity and an absence of an impermeable skin layer on external surface are also important factors to affect tissue formation via nutritional diffusion and cell migration [40]. Past researches reported that 90% of porosity is suitable for migration of most cell types including smooth muscle cells whereas the pore size 150-200 micron is the optimal characters for the adhesion and proliferation of SMCs [40, 41].

The following sections explain several biomaterials used in tissue engineering field.

2.2.1 Chitosan

Chitosan is a natural polymer that is derived from chitin, found in crustacean shells. It is known to be biocompatible [36, 42-45], enzymatically biodegradable [43, 45, 46], have mechanical benefit [42, 44] and adhesive properties [44], able to accelerate wound healing [43, 46, 47] and easily fabricated into bulk porous scaffolds, beads or films [45]. Properties of porous chitosan polymer such as microstructure, crystallinity and mechanical strength can also be varied by altering chitosan concentration, freezing rate and the molecular weight and percent deacetylation of the starting material [45]. Previous researches showed that chitosan can be used as bone graft substitute with hydroxyapatite [42, 46] and in scarless wound healing skin [43, 46, 47].

Although many advantages Chitoson as a biopolymer have been reported, several groups of researchers have shown that chitosan does not support cell proliferation *in vitro*. Human epidermoid carcinoma cells showed signs of rounding-off leading to death after 7 days in culture [36]. Another 10% extra Fetal Calf Serum (FCS) had to be added so that the cells gained their morphology and further proliferation. Rat dermal fibroblasts growth and proliferation in culture was also inhibited in the presence of chitin and its derivative [47]. It may happen due to chitosan bound and inactivated some growth factors present in FCS. This statement was supported by the founding that cell proliferation in high concentration of chitosan was not inhibited in cultures without FCS. Another experiment using collagen-chitosan gels showed that the cell growth was greatly inhibited in collagen-chitosan gel substrates

compared to pure collagen substrates [44]. In addition to that, the cell growth inhibition was increased as the concentration of the chitosan increased. This condition applied until the proportion of chitosan reached 75%. Even though chitosan has been shown to inhibit cell proliferation, it does not affect cell viability *in vitro* [44].

Zhu Xiao *et al.* reported that argon plasma has been used to increase cell attachment and growth on chitosan [43]. It effects chitosan surface chemistry and does not alter its bulk properties. Since argon is an inert gas, its plasma only modified and reorganized the chemical groups that were already present on the surface.

2.2.2 Acellular Tissue Matrix

Acellular tissue matrix or decellurized matrix is an extracellular matrix harvested from living tissue, normally taken from porcine small intestinal submucosa (SIS) or human skin (e.g.: AlloDerm®). An advantage of using SIS *in vivo* is that it rapidly degrades and can be completely excreted from body if it is used as substrate *in vivo* [48, 49]. The use of hydrated SIS as scaffold *in vitro* also results in better cell adherence. It is reported that human dermal microvascular endothelial cells adhere more to hydrated SIS *in vitro* than to plastic dishes coated with one of the several known components of the SIS extracellular matrix: collagen Type I, collagen type IV, fibronectin and laminin [37].

Multiple groups have reported using both AlloDerm[®] and SIS in an in situ xenograft model. Chen and co-workers are succeeded in performing recovery of a patch of defective wall in dog oesophagus after several weeks using SIS [49]. Isch and colleagues have also performed oesophagus regeneration in the mucosa layer of cervical oesophagus in a dog with AlloDerm[®] (As cited by Chen, M.K. [49]) after two months. Despite the successful studies reported, the use of acellular matrix alone has not been successful for small diameter tubular structures, especially those that require peristaltic function. This is due to inability to maintain a tabularized form and the lack of peristaltic capability during remodeling [35].

2.2.3 Polyurethanes

Polyurethanes (PUs) are block copolymers which consist of alternating soft blocks and hard blocks [50]. Segmented PUs can be represented by three basic components in the following general form: $P - (D(CD)_n-P)_n$, where P is the polyol (the soft blocks), D is the diisocyanate, and C is the chain extender. The combination of the chain extender (C) and the diisocyanate (D) is referred to as the hard segment.

PUs have been widely used in biomedical applications due to its outstanding mechanical properties and biocompatibility. They combine flexibility with high strength, wear resistance and a degree of hardness. They can be manufactured in wide range of forms such as sheets, foam, tubes or rods.

PUs are known to have good blood compatibility [51-53] due to their resistance to cell adhesion and proliferation. Various methods such as plasma treatment have been introduced to modify PUs' surface chemistry to change their surface polarity, wettability and adhesiveness. PUs are known to be hydrophilic and their degree of hydrophilicity depends on their chemical composition [52]. Plasma treatment was reported to be able to remove contamination [54] and alter the hydrophilicity of PUs' surface, significantly effecting (positively and adversely) the interaction with the cells, specifically to Vero cells [53].

Wettability is a good initial characterization of PUs surface. Figure 2.5 demonstrates a difference in wettability. Figure 2.5A shows how a water droplet might appear on a hydrophobic surface ($\theta > 90^\circ$) whereas figure 2.5B shows how a water droplet might appear on a hydrophilic surface ($\theta < 90^\circ$). Wettability can be quantified using contact angle measurement. For an ideal, homogeneous solid surface, contact angles measure the force balance between the attraction of molecules within the droplet to each other versus the attraction or repulsion of those droplet molecules experience towards the surface molecules in microscopic level [55, 56]. This balance is described by the Young-Dupre equation:

$$\cos \theta = \frac{(\gamma_s - \gamma_{SL})}{\gamma_{LV}} \quad (1)$$

where θ is the contact angle on the surface, γ_s is the surface energy at solid-vapor interface, γ_{SL} is the surface energy at solid-liquid interface, and γ_{LV} is the surface energy at liquid-vapor interface. Five techniques are available to be employed to measure contact angle: static or sessile drop method (Figure 2.6A), Wilhemly plate method (Figure 2.6B), captive air bubble method (Figure 2.6C), capillary rise method

(Figure 2.6D), and the tilted-drop measurement (Figure 2.6E). Out of 5 techniques, static or sessile drop method is the most commonly used technique.

Figure 2.5 The difference in wettability. (From Stein, Jeanette: Contact angles, [Online], Available: <http://www.uweb.engr.washington.edu/research/tutorials/contact.html>, 2004 [56])

Figure 2.6 Techniques used for contact angle measurement (From Stein, Jeanette: Contact angles, [Online], Available: <http://www.uweb.engr.washington.edu/research/tutorials/contact.html>, 2004 [56])

2.2.4 Poly(L-lactide-co- ϵ -caprolactone)

Poly(L-lactide-co- ϵ -caprolactone) (PLCL) scaffolds are highly elastic and able to maintain a complete recovery under cyclic loading in culture media. They consist of ϵ -caprolactone (PCL) as soft matrix and L-lactide (LLA) as hard domains [40]. Their mechanical properties were comparable to those of human cartilage, skin and coronary artery [40] and depend on the ratio between the basic materials. PLCL can also be fabricated by electrospinning to produce nanometer-sized fibers that resemble the native ECM. It was reported that the cells attach to and organize well around the fibers with diameter smaller than the diameter of the cells [41].

PLCL scaffolds are biocompatible and biodegradable. *In vivo* studies showed that there was an increase in formation of collagen and number of smooth muscle cells seeded on tubular PLCL scaffolds in rats [40]. The implanted scaffolds showed a slow degradation in 15 weeks where caprolactone units degraded faster than lactide did. *In vitro* experiments showed favorable interactions between smooth muscle cells and endothelial cells on PLCL scaffold for up to 7 days [41].

2.3 Bioreactor

A bioreactor is designed to be a controllable system to promote cell growth, maturation, and tissue differentiation *in vitro* [16, 17, 57]. It supplies nutrients through culture media, removes waste products away from the cell-scaffold

construct, monitors oxygen concentration and pH, and sometimes provides mechanical stimulation to the cell-scaffold construct.

Nutrients and oxygen must be delivered to the cells, and metabolic wastes removed, to prevent cell necrosis [26, 58]. It occurs between a porous scaffold and the surrounding medium through diffusion [26]. In static environments (See figure 2.7B), the external diffusion between the medium and the outer layer of the cell-scaffold construct occurs slowly; hence it may not be able to meet the metabolic requirements of the cells for longer period [59].

Bioreactors such as spinner flasks (See figure 2.7A) and rotating wall vessels (See figure 2.7C) are designed to provide a more dynamic environment for the cell-scaffold construct. Spinner flasks create an isotropic turbulent flow that is generated by the magnetic stirrer whereas rotating wall vessels produce laminar rotational flow [59]. The rotating wall vessel is designed to stimulate microgravity environments [59]. However, even though these bioreactors can mitigate the external diffusion issues, the internal diffusion limitations within the porous network of the scaffold still remain [60]. A flow perfusion bioreactor offers the solution for this issue [59-61]. It is designed to direct the fluid flow to the cell-scaffold construct (See figure 2.8). Besides overcoming the internal diffusion limitations, fluid flow also produces fluid shear stress to the cell-scaffold construct resulting in cell proliferation [60].

Figure 2.7 Bioreactors. (From Sikavitsas, V.I., Bancroft, G.N., and Mikos, A.G.: Formation of three-dimensional cell/polymer constructs for bone tissue engineering in a spinner flask and a rotating wall vessel bioreactor. *Journal of Biomedical Materials Research* 62(1), 136-148, 2002. [59])

Figure 2.8 Flow perfusion through porous scaffold. (From Bancroft, G.N., Sikavitsas, V.I., and Mikos, A.G.: Design of a flow perfusion bioreactor system for bone tissue-engineering applications. *Tissue Engineering* 9(3), 549-554, 2003. [60])

Several groups of researchers have focused on designing a flow perfusion system whose flow resembles the mammalian blood pressure waveform. Histology, functional characteristics and strength of the tissue-engineered constructs may be improved by exposure to pulsatile flow waveform as would be encountered due to blood circulation during embryonic development [62, 63]. Mechanical ventilators [61-63], peristaltic/pulsatile pumps [57, 61, 64, 65] and semi-compliant tubing [61]

have been used to produce laminar flow condition similar to mammalian physiological conditions.

Besides nutrients, oxygen is also essential for cell metabolic activity. The requirement for oxygen varies largely, depending on cell type [15]. Based on a simple mathematical model, the critical distance between the gas phase (air) and a confluent monolayer of cells in an unstirred aqueous layer, at which the oxygen concentration at the cell surface becomes limiting is 950 μm for a confluent monolayer of hepatocytes [15]. Other researchers have reported that to meet the oxygen requirements of a monolayer of hepatocytes, the bioreactor has to ensure the critical distance is shorter than approximately 100-150 μm [64]. However since hepatocytes are highly metabolically active cells, these critical distance may not be applied to smooth muscle cells.

Spatial cell distribution also plays a role in cell differentiation. Bursac *et al.* (2003) demonstrated that cardiomyocytes in 3D constructs maintained their phenotype better than if cultured as confluent monolayers [66]. Therefore, most of the bioreactors are designed to accommodate 3D tissue constructs such as tubular construct.

Since mechanical stimuli induce cell differentiation, several bioreactors are designed to produce mechanical stimuli either through fluid flow or cyclic strain. Pulsatile fluid flow through a tubular construct and stretching in axial direction can provide circumferential and axial strain respectively. Some bioreactors are computer controlled to allow total control over the frequency, amplitude, and duration of the

strain [62, 67]. Some have combination from above bioreactor's functions to meet the requirement of the experiment. For example, the bioreactor in figure 2.9 has been designed as a 3-dimensional rotating bioreactor with continuous fluid flow perfusion.

Figure 2.9 Three dimensional rotating bioreactor with fluid flow perfusion. a. Disassembled bioreactor, b. Connected bioreactor with tubular construct. (From Gabouev, A.I., Schultheiss, D., Mertsching, H., Köppe, M., Schlote, N., Wefer, J., Jonas, U.: *In vitro* construction of urinary bladder wall using porcine primary cells reseeded on acellularized bladder matrix and small intestinal submucosa. The International Journal of Artificial Organs, 26 (10), 935-942, 2003 [68])

2.4 Mechanical Stimulation on Cells

Cells within the body always interact with other cells to build a functional organ. During these interactions, the cells experience 2 main physical forces: body forces (gravity, inertia) and surface forces. These surface forces can result from applied forces such as a muscle contraction, fluid motion, and adhesion of cells to the extracellular matrix.

2.4.1 Mechanical Forces on Oesophageal Smooth Muscle Layer *in vivo*

Smooth muscle cells in internal organ (e.g., oesophagus) or blood vessels are always exposed to mechanical stresses due to hydrodynamic stresses induced by pulsatile flow (See figure 2.10) and contraction (e.g., peristaltic motion) (See figure 2.11). They are aligned in one direction to form efficient functional configuration [69]. These stresses seem to be the most influential factor for their orientation *in vivo* [69]. In blood vessels, vascular smooth muscle cells are aligned parallel to the direction of strain [69, 70].

Figure 2.10 Arterial blood pressure waveform. (From Karamanoglu, Mustafa: A system for analysis of arterial blood pressure waveform in humans. Computer and Biomedical Research 30: 244-255. 1997 [71])

Figure 2.11 Oesophageal peristaltic pressure recording of a 5 ml barium swallow. (From Castell, D.O.: The Esophagus, 2nd ed. Little, Brown and Company, 1995. [3])

2.4.2 Mechanical Forces on Cells *in vitro*

Cell interaction with their environment plays a vital role for cellular behaviour such as cell adhesion, migration, orientation, proliferation, differentiation, and metabolism [72]. In order to study the cell behaviour, *in vitro* experiments have tried to replicate as close as possible with the condition *in vivo*. The physical force mentioned in previous section can be applied on *in vitro* experiment to give some mechanical effects to the cultured cells in at least two ways: by inducing shear stress to the contract surface through fluid flow and applying direct mechanical stimulation on the cell-scaffold construct [17, 18].

Some researchers have reported that mechanical stimulation has positive effects for the cell growth and development of many cell types. Cell growth rate [73-76], mechanical properties of the tissue [73, 76] and collagen synthesis [74, 75] have been increased due to the application of strain. Some cells have responded by enhancing matrix elaboration [73]. However several studies have shown that excessive cyclic stretch will give adverse effect to the cells [18, 77]. Human aortic smooth muscle cell-scaffold construct was demonstrated deteriorate when the mechanical stimulation (10% radial cyclic strain) was extended up to 8 days [77].

The effect of mechanical stimulation varies between different species and cell types, and particular dependent on cell type [74, 75, 77, 78]. Human aortic smooth muscle cells are highly sensitive to cyclic strain and exhibits the largest increase in collagen and matrix protease production compared to rat aortic smooth muscle cells and human dermal fibroblasts [77]. Human and porcine coronary smooth muscle cells exhibit a highly differentiated phenotype, but less response to mechanical stimuli in cell proliferation, collagen synthesis, and LDL (Low Density Lipoprotein) degradation than noncoronary smooth muscle cells [78]. Hence even though smooth muscle cells in the body have the same structure, their response to mechanical stimulation is cell-type dependent.

Smooth muscle's response to mechanical stimulation is also dependent on type of substrate. Cyclic strain on canine coronary smooth muscle cells grown on elastin reduced cell proliferation while it had no effect on cell grown on collagen [70]. Another result also supports the statement. Bovine aortic smooth muscle cells on 2D

polyurethane membrane aligned perpendicular in the direction of strain where they aligned parallel with the direction of strain when they were grown on 3D collagen gel [72].

2.4.2.1 The effect of mechanical stimuli on cell proliferation

Mechanical stimuli applied on visceral smooth muscle cells were reported to increase cell proliferation. Cultured rat myometrial (uterus) smooth muscle cells on flexible culture plate in single static mechanical stretch (25% elongation) showed a rapid and transient increase in c-fos expression, gene that can influence the rate of transcription after 30 min of stretch [79]. Another group of researchers assessed the cell growth from DNA synthesis measured by the aphidicolin sensitive specific activity of DNA. DNA synthesis of guinea pig bladder smooth muscle cells was increased by tension applied in Kreb solution [80]. Furthermore, the simultaneous exposure of ovine bladder smooth muscle cells on fibronectin silastic membrane to mechanical strain (25% elongation at 1 Hz) and sustained hydrostatic pressure (40 cm H₂O) results in increases of Heparin Binding Epidermal Growth Factor (HB-EGF) mRNA levels which play a role in cell proliferation and collagen type III synthesis [81].

Similar result is also reported on vascular smooth muscle cells, which are more commonly used in research. Mechanical stimuli increased cell proliferation [31, 77, 78, 82-86]. Some were expressed through DNA synthesis [85], the amount of rough endoplasmic reticulum, or expression of specific growth factor (Platelet-Derived Growth Factor A and B) and their receptors [84, 85].

As mentioned earlier, the effect of mechanical stimuli is cell-type dependent. Even though most of previous researches reported increases in cell proliferation, porcine aortic smooth muscle cells subjected to cyclic deformation show decrease in cell proliferation [70]. Pulmonary arterial smooth muscle cells show no effect of mechanical stimuli on cell proliferation [70].

2.4.2.2 The effect of mechanical stimuli on cell alignment

Mechanical stimulation on smooth muscle cells induces cellular alignment. Three factors of mechanical stimulation regime: amplitude, stress loading time and frequency are responsible for the control of cellular orientation. Higher values of those factors resulted in more profound increases in the average orientation angles [69].

Most researchers have reported that vascular smooth muscle cells align perpendicular to the strain applied (shown by arrow in figure 2.12 and 2.13) [69, 70, 72, 86]. On the other hand, vascular smooth muscle cells grown on 3D collagen lattice appeared to align parallel with strain direction [87]. This occurred because components of extracellular matrix (e.g., collagen fibers) tend to align in parallel with strain direction which influenced smooth muscle cells to orientate along with the extracellular matrix [72]. Buck *et al.* [88] explained the orientation as a cellular avoidance reaction to stretching in the direction of their long axis.

Figure 2.12 Smooth muscle cells aligned perpendicular to the strain applied. (From Smith, P.G., Garcia R., and Kogerman L.: Mechanical strain increases protein tyrosine phosphorylation in airway smooth muscle cells. *Experimental Cell Research* 239, 353-360, 1998. [89])

Figure 2.13 Comparison of smooth muscle cells under cyclic strain (left) and static culture (right). (From Smith, P.G., Garcia, R., and Kogerman, L.: Strain reorganizes focal adhesions and cytoskeleton in cultured airway smooth muscle cells. *Experimental Cell Research* 232, 127-136, 1997. [89])

2.4.2.3 The effect of mechanical stimuli on cell differentiation, mechanical properties, and other cell's functions

Mechanical stimulation also induces contractile phenotype of smooth muscle cells that would make the tissue contraction feasible [31, 82, 89]. The contractile phenotypic smooth muscle cells were more spindle shaped and elongated along the

long axis of the cell [89]. Kanda *et al.* discussed that when cellular proliferative activity is mechanically suppressed due to confluency and mechanical confinement, their phenotypic modulation from the synthetic to contractile state occurs regardless on 2-D or 3-D environment [72]. However if the vascular smooth muscle cells has undergone more than 5th subculture, they remains in permanent synthetic phenotype.

Mechanical integrity of the engineered tissue is also enhanced by increment in collagen synthesis [20, 31, 77, 78], matrix production [83], formation of stress fibres [78, 82] and focal adhesion and spreading [78, 82] due to cyclic strain. Increase in the expression of specific genes (c-fos [90]) and phenotype specific proteins (tyrosine phosphorylation [82], LDL (Low Density Lipoprotein) degradation [78]) and reduction in the expression of specific gene (c-jun, fra-1) [90] were also reported as the result of applied strain.

Chapter 3 – METHODOLOGY

This chapter explains the procedures or protocols that have been used in this project. They are summarized in figure 3.1. The first three sections describe the preparation of the basic components that were necessary for this project: how to cultivate and grow cells in vitro, construct scaffolds, and set up a bioreactor system. The cells were then seeded on scaffold to evaluate the cell behaviour on scaffold. The selected scaffold was then integrated with cells in a bioreactor system. Finally mechanical stimulation was applied to cell-scaffold construct for analysis.

Figure 3.1 Outline Procedures.

3.1 Design of Bioreactor and Control System

A bioreactor with mechanical stimulation is needed in this project to give a mechanical environment cues to the cells. As there has been little research into the effect of mechanical stimulation on oesophageal smooth muscle cell-scaffold

constructs, a one dimensional mechatronic bioreactor was modified from an existing bioreactor and manufactured. Few reasons that a bioreactor was self-designed even though there are a number of commercial bioreactors in the market are they tend to be high in cost and may not fulfill the specifications of this project. Flexcell International Corporation provides wide range of choices of bioreactors with two dimensional strain which is not suitable with the specification of the bioreactor required in this project. Just recently (2004) B-bridge International Incorporation started to commercialize one dimensional strain bioreactor.

3.1.1 Bioreactor Requirements

Material and Equipment

The bioreactor should be made of biocompatible, preferably non metallic material. The material must be able to be sterilized by steam autoclaving (121°C, 2.2 atm) or immersion in 70% ethanol. A transparent material will be an advantage so that any contamination inside the bioreactor can be seen.

Operating Environment

The bioreactor shall be designed to operate at 37°C temperature, 5%CO₂ incubator.

Bioreactor Performance

The bioreactor should have mechanical stimulation by direct stretching (approximately 10% elongation). The direct stretching mechanism should produce one dimensional strain cyclically with consistent movements. The user should be able to prescribe the strain regimen through a computer control interface.

3.1.2 Conceptual Design

Several control systems can be used to achieve 1-D strain. Servo motors or stepper motors were considered as actuators for the system. Due to complexity of pneumatic systems and the usage of air/liquid in the system, it is not favorable for the overall system. Servomotors can provide automatic control of position or speed in response to a control signal. Stepper motors respond with a fixed rotation (step) for each pulse and can produce a very precise angular position by controlling the number of pulses delivered to the motor. Both motors have to be attached to ball screw to convert the rotation into linear motion. Since the movement of the bioreactor is fixed for certain number of cycles and for simplicity, the stepper motor was chosen. The final schematic diagram of the mechanical stimulation system of the bioreactor is presented in figure 3.2.

Figure 3.2 Schematic diagram of mechanical stimulation system of the bioreactor.

3.1.3 Bioreactor Components

Table 3.1 lists the equipments chosen to meet the requirement listed in section 3.1.1. Two sets of mechanical stimulators were machined to accommodate the experiments. The existing mechanical stimulator was still used for static control while 2 sets of modified version were used to induce mechanical stimulation to cell-scaffold construct. Motor drivers 1 and 2 have similar specification to drive the linear actuator.

Table 3.1 Equipments for bioreactor.

No	Equipment	Manufacturer	Model	Remarks
1	<div>Motion Controller</div> <div> </div>	Galil Motion Control, Inc	DMC-1417 (PCI Card)	Run in Microsoft Windows XP
2	<div>Motor Driver 1</div> <div> </div>	General Controls Electronics, Inc	Dragon Driver TM Bipolar Chopper	
3	<div>Motor Driver 2</div> <div> </div>	Servo Systems Co.	IB463	

4	DC Power Supply		RS Components	ISO-Tech IPS 1810H	
5	Linear Actuator		HSI motor	HSI Size 14 hybrid linear actuator	Resolution: 0.003175 inches/step
6	Mechanical Stimulator 1 (existing design)		NTU (K.S. Chian)		Self specified
7	Mechanical Stimulator 2 (modified version-a)		NTU (A.C. Ritchie)		Self specified
8	Mechanical Stimulator 3 (modified version-b)		NTU (A.C. Ritchie, S. Wijaya)		Self specified
9	Cell Culture Multiwell Plate		Grenier Bio-one International Asahi Techno Glass Corporation (Iwaki Glass Co., Ltd)		No of wells: 6, 12, 24, 96

Figure 3.3 Mechanical Stimulator. A. Existing design, B. Modified version-a, C. Modified version-b, incorporates an aluminium moving plate to increase stiffness.

Figure 3.4 Basic principle of stretching mechanism (side view).

The mechanical stimulator is made of two stacked plates (See figure 3.4). The lower plate is fixed and the upper plate is moved by the linear actuator. It has clamping mechanism to grip the scaffold. The initial separation between the two clamps can be changed according to the requirement, but for these tests it was set at 1.5 cm. The location of the clamps has already been arranged to hold 6 scaffolds in a 6 well culture plate, one for each well.

3.1.4 Calibration

Before usage, the bioreactor needs to be calibrated. Its reliability and accuracy were examined based on two parameters: strain and frequency which were varied to

optimize the regime of mechanical stimulation. Program Test (See Appendix A1) was written in DMC Smart Terminal, a software to communicate with DMC PCI card, to examine the bioreactor on its reliability and accuracy. For calibration purposes, the strains produced were compared with the input values, ranging from 0.2 – 2 mm with 0.2 mm interval to obtain the relationship between the input value and output strain. A timer and digital vernier were used to capture the duration and output displacement, respectively. The frequency was calculated by the formula $f=1/T$ where f is frequency and T is time period. The time period for 10.5 cycles was recorded, thus the period of 1 cycle can be obtained by dividing the time measured with 10.5.

Figure 3.5 Calibration schematic diagram.

To confirm the consistency of the bioreactor, the bioreactor was run for 15 hours inside the humidified environment of the incubator (37°C, 5%CO₂). Program Mech (See Appendix A2) was written to produce uniaxial cyclic strain with user control over the number of cycles, the frequency and amplitude through speed control and elongation input, respectively. 10% cyclic strain was produced with the speed of 900 counts/s and acceleration/deceleration of 9216 counts/s² for 54000 cycles (approximately 15 hours). The variation throughout the period was calculated. All statistical analysis is expressed as mean ± standard deviation.

3.2 Cell Isolation, Growth and Characterization

For this research, porcine cells are used instead of human cells due to higher availability and similar differentiated metabolic function [64] and size. Porcine oesophagi were obtained from a local abattoir. The cells were then isolated from the oesophagus, grown, and sub cultured in the culture medium and kept in the 5%CO₂ incubator Sanyo MCO – 17AIC (SciMed (Asia) Pte. Ltd, Singapore).

The following composition of culture medium was used:

1. 89% DMEM (Dulbecco's Modified Eagle's Medium)/High Modified (JRH Biosciences, Inc., Victoria, Australia and Research Instruments, Singapore) mixed with 4500 mg/l glucose, 4mM L-glutamine, without Sodium Pyruvate
2. 10% Fetal Bovine Serum (FBS), origin South America (BioClot GmbH, Germany, Lot No. 3249)

3. 1% Antibiotics Antimycotic Solution (AAS) (Sigma-Aldrich Pte Ltd, Singapore, Cat No. K2393) Stabilised with 100 units penicillin, 1 µg streptomycin and 25E-2 µg amphotericin B per ml
4. 0.2% Kanamycin Solution (Sigma-Aldrich Pte Ltd, Singapore, Cat No. K0254)

3.2.1 Cell Isolation

The following procedure was used to clean porcine oesophagus:

1. Porcine oesophagus was collected from a local abattoir. It was kept in a container with an ice pack and processed within two hours from collection.
2. Porcine oesophagus was cut perpendicular to the axis at upper part of abdominal section of the oesophagus (blue colour in figure 3.6) into a small piece (length = ± 1.5 cm).
3. The piece of tissue was then immersed in 1%vol antibiotic PBS (Phosphate Buffer Saline) solution inside 50 ml centrifuge tube before continuing to the next process.

Figure 3.6 Sections in oesophagus.

Smooth muscle cells were then isolated from the muscularis layer as follows:

1. Digestion Solution #1 (5 ml culture medium + 3.2 mg collagenase type I from clostridiopeptidase (Sigma-Aldrich Pte Ltd, Singapore) and digestion Solution #2 (5 ml culture medium + 3.2 mg collagenase + 1.2 mg/353 μ l elastase type III from porcine pancrease (Sigma-Aldrich Pte Ltd, Singapore) were prepared and filtered using 0.2 μ m filter.
2. All following procedures were done in Biological Safety Cabinet Class II (Gelman Sciences, Inc, Michigan, USA). The oesophagus tube section was cut longitudinally to form a sheet. The epithelial layer and connective tissue of the oesophagus wall was then removed. The remaining tissue was minced and put in the digestion solution #1 and incubated at 37°C for 40 min.
3. 5 ml culture medium was added into the tube.
4. The solution was centrifuged at 1000 rpm for 1 minute.
5. The supernatant was discarded.
6. The tissue was put in 6 cm culture dish and minced with scalpel. It was then put in the digestion solution #2 and incubated at 37°C for 1 hour.
7. The cells were passed together with the solution through cell strainer (100 μ m Nylon membrane).
8. They were centrifuged at 1000 rpm for 5 minutes.
9. The supernatant was discarded and 1 ml culture medium was added.
10. Ten 6 cm diameter culture dishes were prepared with 4 ml culture medium each in it.
11. 100 μ l cell solution was put into the 6 cm culture dish. This primary cell culture was defined as passage 0.

12. The medium was changed every 3 to 4 days. Any bacterial contamination will change the medium colour from pink to yellow

3.2.2 Cell Line

After 3 weeks, the primary cell cultures grew confluent inside the dishes. They were ready to be used or multiplied in a new container. The process of transferring the cells from one culture vessel to another due to the cells reaching confluence is termed passage/subculture. The cells were subcultured as follows:

1. The medium in 6 cm diameter culture dish was aspirated using a glass pipette.
2. PBS was added and aspirated to clean the previous medium.
3. 2 ml 0.25% trypsin – EDTA Solution, Sigma-Aldrich Pte Ltd, Singapore (Cat No. K2373) was added and the solution was incubated at 37°C for 3 minute.
4. The cells were detached from the dish base and transferred into 50 ml centrifuge tube.
5. Culture medium was added with ratio trypsin : culture medium = 1:1.
6. The mixed solution was centrifuged at 1000 rpm for 5 minute.
7. The supernatant was discarded.
8. 1 ml culture medium was added and the cells and culture medium are ready to be transferred.
9. Cells were counted using a haemocytometer (Paul Marienfeld GmbH & Co. KG, Germany). 18 µl trypan blue (Sigma-Aldrich Pte Ltd, Singapore) and 2 µl cell solution were mixed in small tube and centrifuged. 10 µl of the

mixture was put in the haemocytometer and cell counting was done under inverted microscope Zeiss Axiovert 25 CRL (Carl Zeiss Pte. Ltd., Singapore) or Olympus CKX41 (Olympus Imaging Singapore Pte. Ltd., Singapore).

3.2.3 Cell Seeding Density

Cell seeding density on mechanical stimulation studies was calculated based on the area of cloning ring (0.785 cm^2 with $r = 0.5 \text{ cm}$). For example: The number of cells per well is 5×10^4 cells, the cell seeding density is $5 \times 10^4 \text{ cells} \div 0.785 \text{ cm}^2 = 6.37 \times 10^4 \text{ cells/cm}^2$. Three different cell seeding densities were tested on PU membrane: $1.27 \times 10^4 \text{ cells/cm}^2$, $6.37 \times 10^4 \text{ cells/cm}^2$, and $1.27 \times 10^5 \text{ cells/cm}^2$.

3.2.4 Cell Characterization

The cells were characterized from their morphology under light microscope and stained with monoclonal mouse anti-human smooth muscle actin (Dako, SPD Scientific Pte Ltd, Singapore), anti myosin; and anti desmin (Chemicon International, Inc., Research Biolabs Pte Ltd, Singapore). Table 3.2 lists the composition (for 2 sets) of each solution used for staining.

Table 3.2 The composition of the solution.

Solution	Total	Goat Serum	BSA	PBS
PBS/0.1%BSA	50 ml		50 mg	
4% blocking medium	3 ml	120 µl	7.5 mg	3 ml
2% solution for Antibody (2%serum)	6 ml	120 µl	15 mg	6 ml
		2% serum	Antibody	
Primary antibody (anti- smooth muscle α-actin, anti myosin, anti desmin)	0.5 ml	495 µl	5 µl	
Mouse IgG1control	0.5 ml	499 µl	1 µl	
Secondary antibody (mouse immunoglobulins/biotin)	1 ml	990 µl	10 µl	
Streptavidin, Texas-red conjugate	1 ml	980 µl	20 µl	
Dapi	1 ml	900 µl	100 µl	

The procedure of immunohistochemical staining is explained as follows:

1. The cells (3×10^4) were grown on two cover slips in two 3 cm diameter culture dishes for three days in the culture medium.
2. The medium was aspirated and the glass slip was washed using PBS and shifted to new 3 cm diameter culture dishes.
3. 100% cold MeOH/Glutaric dialdehyde (Sigma-Aldrich Pte Ltd, Singapore) was added to the culture dishes. Then the dishes were kept in freezer (-20°C) for 20 minutes. After that the solution was aspirated.
4. Then cover slips were washed $3 \times$ with PBS for 15 minutes each.
5. 3 ml 4% blocking medium was divided into 2 and poured into each dish. They were kept overnight.
6. The medium was discarded.

7. The cover slips were shifted to other places while the dishes were washed with PBS.
8. The primary antibody was given to one of the cover slips while IgG1 Negative control (Dako, SPD Scientific Pte Ltd, Singapore) was given to the other one. Both were kept overnight.
9. Then cover slips were washed 3× with PBS/0.1%BSA solution for 15 minutes each.
10. Secondary antibody biotin (Dako, SPD Scientific Pte Ltd, Singapore) was given to the cover slips for 2 hours and then they are washed 3× with PBS/0.1%BSA (Sigma-Aldrich Pte. Ltd., Singapore) solution for 15 minutes each.
11. Third antibody (streptavidin-Texas-red) (OncongeneScience, SPD Scientific Pte Ltd, Singapore) was given and kept for 2 hours in dark environment. The following processes will all be kept in the dark condition. After that they were washed 3× with PBS solution for 15 minutes each.
12. Dapi solution was given for 1 minute and they were rinsed with PBS 3×.
13. The cover slips were then moved to glass slides on Dako® Fluorescent mounting medium (SPD Scientific Pte Ltd, Singapore, Cat No. S3023) to preserve the colour.
14. The glass slides were examined using fluorescence microscope Zeiss Axioskop 2 Mot Plus (Carl Zeiss Pte. Ltd., Singapore).

3.3 Scaffold Properties and Preparation

Smooth muscle cells are anchorage dependent, therefore they have to be attached on substrate in order to grow and proliferate. This substrate can be an extracellular matrix, natural or synthetic biomaterial. The following section explains protocols to produce several scaffold and consideration in selecting the suitable scaffold.

3.3.1 Scaffold Requirement

Many scaffolds are available for tissue engineering use. However to obtain a suitable scaffold for the mechanical stimulation experiment, a thorough evaluation was made to meet the project requirement. The criteria for selection were as follows:

1. The scaffold should be biocompatible and promote cell attachment and growth.
2. The scaffold should be elastic and resilient; having at least 10% elongation without any permanent deformation when the cyclic strain is applied.
3. Porous scaffold is an advantage with pore size less than 20 μm to avoid cell loss to the culture medium.
4. The scaffold should be able to be produced repeatedly with consistent properties.
5. The scaffold should be chemically stable and must not degrade or release reacted monomers, solvents, or plasticizers into the culture medium.
6. The scaffold should allow surface modification for protein immobilization or surface treatment.
7. Ease of handling in integrating with bioreactor

3.3.2 Scaffold Preparation

Several scaffolds were tested and observed to meet the requirement. The following sections show the protocols to construct those scaffolds.

3.3.2.1 Chitosan and its modification

4wt% chitosan gel is prepared as follows:

1. Solution #1: 12.5 g chitosan (Dalian Xindie Chitin Company Limited, Dalian, China) was mixed with 200 ml DI (deionized) water and stirred for 1 hour.
2. Solution #2: 6 g acetic acid was added to 94 ml DI water.
3. Solution #2 was added slowly in small portions to solution #1.
4. The mixed solution was stirred overnight to obtain chitosan gel.

Air-dried chitosan was prepared by casting and drying the chitosan gel in biological safety cabinet for 1 day whereas freeze dried chitosan is prepared by casting and freezing the chitosan gel in -80 °C overnight. Then the freeze-dried chitosan was put in room temperature to defreeze. To neutralize the acetic acid, the chitosan scaffold was immersed in 5wt% NaOH solution for 2 hours. It is then washed in deionized water until the neutral pH was obtained. The chitosan scaffold was stored in 70% ethanol until it was used.

To enhance cell attachment, the 4wt% chitosan gel is mixed with 40wt% collagen (South Korea origin, MW <1000). Several ratios were done such as 1:1, 7:3 and 6:4. The ratio is calculated based on the dry weight of the chitosan and collagen.

Chitosan collagen Genipin was prepared initially by cross linking between chitosan and genipin for 4 hours. The percentage of the solution was assumed to remain unchanged, 4wt%. Then it was mixed with collagen with different ratio of dry weight of the chitosan genipin and collagen.

3.3.2.2 Poly(L-lactide-co- ϵ -caprolactone)

Poly(L-lactide-co- ϵ -caprolactone) copolymer (PLCL) was synthesized from PLA (LASIA H100J, Tg 58°C, mp 165°C) and PCL (Sigma-Aldrich Pte. Ltd., Singapore, MW 80,000, mp 60°C) with the ratio of L-lactic Acid (PLA): caprolactone (PCL) equal to 84:16. The copolymer was then dissolved in 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) at a concentration of 0.04 g/ml and then was placed in a 30 ml plastic syringe fitted to a 26G (gauge size) needle. A pneumatic pump was used to maintain the pressure inside the plastic syringe at 0.2 psi. Electrospinning was carried out at a fixed voltage of 30 kV across two oppositely charged electrodes placed 100 mm apart. The fibrous scaffolds were dried under vacuum at room temperature.

3.3.2.3 Polyurethane Membrane

12% PU (w/w) was made by dissolving pallet ChronoFlex® AL-80A (CardioTech International, Inc., Massachusetts, USA) into Tetrahydrofuran (THF) (Sigma-Aldrich Pte. Ltd., Singapore) as a solvent. It was cast and air-dried for 2 days. The cast PU membrane was then put inside the vacuum oven to remove the THF completely.

A commercial PU, Tegaderm™ was used in comparison with PU cast in the laboratory. Tegaderm™ is normally used for wound dressing. It is impermeable and coated with acrylic adhesive [91].

3.3.2.4 Surface Treatment with Argon and Oxygen Plasma

The scaffolds were placed in the plasma reaction chamber. Micro-RIE Series 800-II (Technics) was used for oxygen plasma surface treatment while March Instruments (March Plasma System, Inc., California, USA) for argon plasma. The chamber was flushed with nitrogen gas and then the gas inside was pumped out. Oxygen was introduced with the constant flow rate of 20 ml/min while argon at 350 ml/min. Plasma was created by the application of power 200 W for different time duration at a radio frequency of 13.56 MHz. Then the gas flow was stopped and the pressure was increased to the atmospheric pressure by adding nitrogen gas.

3.3.3 Material Testing

All materials underwent the following evaluations to test their suitability for the project: toxicity test, mechanical testing, morphology examination, wettability, and cell culture.

3.3.3.1 Toxicity Test

Toxicity test using direct contact method [92] was done to evaluate the cytotoxic potential of materials for biomedical applications. This method can be used directly to evaluate materials or as a reference against or supporting other cytotoxicity test methods. This method requires positive and negative controls to undergo the same procedure as the specimen. The material was cut to obtain flat surface, having an area of 100-250 mm² to be placed in direct contact with confluent monolayer cells. Both material and cells were kept in culture medium DMEM in 37°C, 5%CO₂ incubator for 24 hours. Then qualitative examination was carried out. The material was deemed to show toxicity if microscopic examination reveals the following: malformation, degeneration, sloughing, or lysis of cells extending beyond the perimeter of the specimen of material or moderate to severe reduction of cell layer density. The observation was graded according to this scale:

- 0 indicates no detectable zone around or under the specimen
- 1 indicates a few malformed or lysed cells under the specimen
- 2 indicates a zone of lysis under the specimen and less than 0.5 cm beyond the specimen

- 3 indicates a zone of lysis 0.5 to 1 cm beyond the specimen
- 4 indicates a zone of lysis greater than 1 cm beyond the specimen

3.3.3.2 Mechanical Testing

A computer controlled mechanical testing machine, Instron 5566 (Instron Corporation, Massachusetts, USA) was used to measure the mechanical properties. The sample was cut in dog bone shape according to the dimensions shown in figure 3.7 and attached to the holders (gauge length: 27 mm) of the machine. A constant extension rate of 25 mm/min was applied and the computer continuously recorded the tensile load and deformation in unit length of the specimen.

The data recorded was converted to a stress strain curve. The ultimate tensile strength was obtained from the equation $\sigma = F/A$, where σ is the tensile strength (MPa), F is the maximum load applied (N) before rupture and A is the initial area (m^2) of the specimen. The strain plotted is the engineering strain or deformation per unit length given as $(l - l_0)/l_0$ where l is the extended unit length and l_0 is the initial length of the specimen.

Figure 3.7 Tensile test specimen geometry with dimensions in mm.

3.3.3.3 Morphology

Morphology of the scaffold was analyzed using visual examination for macroscopic analysis, examination under Scanning Electron Microscope (SEM) JSM-5600LV (Jeol, Ltd., Tokyo, Japan), and/or optical microscope (upright/inverted microscope) for microscopic analysis. The sample was dried in the vacuum oven and sputter coated with gold film immediately before SEM examination.

3.3.3.4 Wettability

Wettability was measured by the contact angle between a droplet on the polymer surface. Static or sessile drop method was employed. 10 μ L deionized water was dropped onto PUs surface and the droplet was shape captured by a high magnification digital camera (Navitar Incorporation and Sanyo). The contact angle was analyzed using First Ten Ångstrom (FTÅ) software.

3.3.3.5 Cell Culture

The scaffolds were sterilized in 70% alcohol and put inside the culture media for 24 hours to check whether any reaction had occurred based on the media colour. The cells were obtained from the protocol outlined in section 3.2.2. They were seeded on the scaffold within a glass cloning ring with cell seeding density of 6.37×10^4 cells/cm² on sterile 24 well or 12 well culture plates. They were put inside the 37°C incubator at 5%CO₂. The culture media was changed every 2 to 3 days.

The following observations were performed: visual examination for transparent scaffolds and/or SEM examination and/or cell viability/proliferation.

To prepare cell-scaffold constructs for SEM, they were fixed using 2.5% Glutaraldehyde for 30 minutes and dehydrated in graded ethanol solution (70%, 80%, 90%, and absolute alcohol) for 15 minutes each. After that the specimens were dried in vacuum oven and sputter coated with gold immediately.

The CellTiter96[®] AQueous One Solution Cell Proliferation Assay, Promega Corporation, Singapore was used to study the number of viable cells in proliferation. The MTS tetrazolium compound inside the assay is bio-reduced by cells into a coloured formazan product that is soluble in tissue culture medium. The conversion is assumed to be caused by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells [93]. The assay was put in the culture media with 1 to 5 concentrations. This mixed solution replaced the old culture media. They were kept in the incubator for 3-4 hours and after that the formazan in every 100µL mixed media from each sample were measured for their light absorbance in ELx800™ Absorbance Microplate Reader (BioTek Instruments, Inc., Vermont, USA) at 490 nm.

3.4 Mechanical Stimulation

In vivo oesophagus is always present in a dynamic environment due to peristaltic motion and arterial blood pressure. Mimicking *in vivo* oesophageal mechanical forces, oesophageal smooth muscle cells *in vitro* were stimulated with two regimes in 1 dimensional strain. One regime mimics the arterial blood pressure (Cardiovascular cycle), whereas another regime mimics peristaltic motion (Oesophageal cycle).

Porcine oesophageal smooth muscle cells, between 3rd – 5th passage were seeded on PU membrane with cell seeding density 6.37×10^4 cells/cm². The cells remained in static culture for 2-3 days for cell attachment before mechanical stimulation commenced.

3.4.1 Cardiovascular Cycle

The cardiovascular cycle is based on the arterial blood pressure waveform. Its frequency is similar to the heart beat, set at 1 Hz (60 cycles per minute). As can be seen in figure 3.8, one cycle of cardiovascular cycle was broken down into 5 different motions which were commanded by the computer program. Table 3.3 shows the displacement path and duration of each motion.

Figure 3.8 Cardiovascular Cycle. A. Arterial blood pressure waveform (From Karamanoglu, Mustafa: A system for analysis of arterial blood pressure waveform in humans. Computer and Biomedical Research 30: 244-255. 1997 [71]), B. The displacement in cardiovascular cycle.

Table 3.3 Displacement path and duration of cardiovascular cycle.

Part of Cycle	Displacement	Duration
C1	$0.933 \times \text{total displacement}$	0.124 s
C2	$0.067 \times \text{total displacement}$	0.124 s
C3	$- 0.5 \times \text{total displacement}$	0.144 s
C4	$- 0.5 \times \text{total displacement}$	0.505 s
C5	0	0.103 s

An initial experiment using 10% strain was carried out with the simplified cardiovascular cycle shown in figure 3.8 where the construct went the same path and timing to reach the displacement and return. Subsequent experiments followed the cardiovascular cycle shown in figure 3.8 at a reduced strain magnitude of 2.5% strain.

3.4.2 Oesophageal Cycle

Oesophageal cycle is based on the pressure waveform at oesophageal wall during eating [3]. Since the porcine smooth muscle cells used in the experiment were extracted from the lowest 1/3 of the oesophagus closest to the stomach, the pressure waveform marked with grey square in figure 2.11 (page 38) was chosen to be the model of oesophageal cycle (Figure 3.9). Amplitude of 2.5% strain is chosen to compare with the construct under cardiovascular cycle. The displacement path and duration of the oesophageal cycle is provided in table 3.4

Figure 3.9 Oesophageal Cycle. A. Peristaltic pressure (Modified from From Castell, D.O.: The Esophagus, 2nd ed. Little, Brown and Company, 1995). [3], B. The displacement in oesophageal cycle

Table 3.4 Displacement path and duration of Oesophageal cycle.

Part of Cycle	Displacement	Duration
E1	Total displacement	1.28 s
E2	0 (hold)	0.32 s
E3	- Total displacement	1.6 s
E4	0 (hold)	10.59 s

Several programs were written and applied to provide oesophageal and cardiovascular cyclic strain to cell-scaffold constructs for 2 days (See Table 3.5). Then observation on cell orientation and test on cell viability were made.

Table 3.5 List of programs.

No	Program Name	Type of Cycle	Frequency	Displacement (mm)	No. of Cycles
1	Mech (Appendix A2)	Modified Cardiovascular Cycle	0.833 Hz	1.5 (10%)	7200
2	MechEso (Appendix A3)	Oesophageal Cycle	0.0725 Hz	0.375 (2.5%)	420
3	MechCar (Appendix A4)	Cardiovascular Cycle	1.08 Hz	0.375 (2.5%)	7200 or 21600

3.5 Assessment on Cell Function

Cell-scaffold constructs under mechanical stimulation were assessed in comparison with the constructs in static culture, particularly with regard to cell proliferation and cell alignment.

1. Cell proliferation

Cell proliferation was studied using The CellTiter 96® AQueous One Solution Cell Proliferation Assay® whose protocol has been explained in the section of 3.3.3.5.

2. Cell alignment

Cell alignment can be seen under light inverted microscope due to transparency of the scaffold. To get better contrast, the constructs were also studied under SEM.

Chapter 4 – RESULTS AND DISCUSSION

4.1 Bioreactor

The bioreactor as shown in figure 4.1 is user-controlled through DMC-1417, a programmable single-axis motion controller. The user can program the motion controller using DMC Smart Terminal software in computer. Then controller DMC-1417 will send a signal received from the user to the stepper motor driver to run the linear actuator. The linear actuator then moves the attached mechanical stimulator.

Figure 4.1 Mechanical stimulation system.

4.1.1 Materials and Components of Mechanical Stimulator

Materials of the new mechanical stimulator that come into contact with cells and culture media are chosen to prevent leading of cytotoxic material into the cell culture area. The existing design came with metal screws which were found to give adverse effects on cell growth, probably due to the release of metal ions to the cells.

In addition, the existing design had only a solenoid and a spring to pull and release the moving plate. It gave abrupt motion which resulted in sudden loading to the cells. The new design of bioreactor both modified version-a and version-b incorporates the motion controller and linear actuator which are able to control the movement precisely, and the movement is smooth. No metal components come into contact with the cells or culture media. The only difference between both versions is the aluminium moving plate to increase the stiffness. The summary of the improvement in performance of the new bioreactor design is listed in table 4.1.

Table 4.1 Summary of the improvement in performance of the new bioreactor design

	Existing design	Modified version - a	Modified version - b
Material in contact with cell-culture	Metal	Nylon and PolyCarbonate	Nylon and PolyCarbonate
Control System	Solenoid and a spring	Motion controller	Motion controller
Moving plate		PolyCarbonate	Aluminium

All bioreactors are able to be sterilized using 70% ethanol. In the humidified incubator, they were kept inside the semi-transparent containers so that the activity occurring inside can be seen.

4.1.2 Bioreactor System Performance

Program Mech has been written to produce 1 dimensional cyclic strain to the scaffold gripped. The program also allows several variables to be controlled such as:

- Waveform and frequency

In figure 4.2, holding time I (H1) is the period of time when the cell-scaffold construct is exposed to maximum strain and holding time II (H2) is the relaxation period. Forward time (T1) is the duration to reach maximum strain and T2 is backward time (T2) is the duration to reach the starting position. Both of them can be controlled indirectly through the speed, acceleration and deceleration commanded in the program. By controlling T1, H1, T2, and H2, the frequency of the cyclic strain can be controlled.

- Maximum Displacement (Percentage Elongation)
- Number of Cycles

Figure 4.2 Variables controlled in mechanical stretching.

Accuracy of the Elongation

The system accuracy has been tested by analyzing the input value of displacement in the program and output displacement. Table 4.2 shows the percentage error of the output displacement at ten different speeds and accelerations (See Appendix B1). The displacements were limited to 0.2 - 2 mm. Since the cells cannot experience high tensile strain, a maximum value of $\pm 10\%$ was set. For all experiments, the specific acceleration and deceleration (multiples of 1024 counts/s^2) were chosen so that the linear actuator would reach a constant velocity in less than 0.1 s. Using statistical analysis, the average of percentage error is $1.15 \pm 1.8\%$. The biggest possible value of the error is 0.059 mm. Since the error is within acceptable limits, the performance of the bioreactor from elongation aspect is considered accurate.

Table 4.2 Percentage Error of output displacement.

Input Displacement (mm)	Speed (counts/second)									
	100	200	300	400	500	600	700	800	900	1000
0.2	0	5	0	5	10	5	5	5	10	5
0.4	2.5	2.5	2.5	0	0	2.5	0	2.5	2.5	0
0.6	0	0	1.67	0	0	1.67	0	1.67	0	0
0.8	1.25	1.25	0	1.25	0	1.25	1.25	0	2.5	2.5
1	1	0	0	0	1	0	1	2	1	1
1.2	0.83	0	1.67	0	1.67	0.83	0.83	0.83	0.83	0
1.4	0	0	1.43	0	0.71	1.43	0.71	0	1.43	0.71
1.6	0	1.25	1.25	1.25	1.25	0.63	0	0.63	0.63	0
1.8	0.56	0.56	0.56	0.56	1.11	0	1.11	0	0	0
2	0	0.5	0	0	0	0.5	0	0	0.5	0.5

Accuracy of the Frequency

The accuracy of the frequency is tested using the time period. The experiment results showed that the amount of time to reach maximum strain was different approximately 84.67 ± 8.5 milliseconds with the ones in calculated results (See Appendix B2). Starting torque, static and kinetic friction, acceleration and deceleration might cause the motor take longer time to reach maximum displacement and come back to original position. As a stopwatch was used to measure the time taken for 10.5 cycles, there may be a small error due to the measurement method. Table 4.3 shows the time required to complete one cycle from different speed and distance with no holding time.

Table 4.3 Time (second) required in completing one cycle.

Distance (mm)	Speed v (counts/s) and Acceleration/Deceleration a (counts/s ²)									
	v = 100	200	300	400	500	600	700	800	900	1000
	a = 1024	2048	3072	4096	5120	6144	7168	8192	9216	10240
0.2	1.14	0.67	0.51	0.43	0.37	0.35	0.33	0.31	0.29	0.28
0.4	2.14	1.15	0.83	0.68	0.58	0.52	0.47	0.44	0.42	0.39
0.6	3.16	1.67	1.16	0.94	0.78	0.69	0.59	0.54	0.52	0.48
0.8	4.17	2.17	1.51	1.17	0.98	0.87	0.73	0.68	0.62	0.59
1	5.16	2.66	1.84	1.43	1.17	1.04	0.89	0.79	0.75	0.68
1.2	6.20	3.18	2.18	1.68	1.39	1.19	1.03	0.95	0.84	0.78
1.4	7.19	3.68	2.52	1.94	1.58	1.34	1.18	1.07	0.98	0.88
1.6	8.21	4.19	2.85	2.18	1.80	1.52	1.33	1.19	1.09	0.98
1.8	9.20	4.69	3.18	2.44	1.99	1.68	1.48	1.31	1.18	1.08
2	10.20	5.19	3.52	2.68	2.19	1.87	1.61	1.44	1.29	1.17

Frequency of heart beat in physiological condition is ≈ 1 Hz, which means the heart takes 1 second to fulfill one cycle. Theoretically, taking 10% elongation (1.5 mm) and no holding time, the speed of approximately 800 counts/s is chosen to produce 1 Hz cyclic strain. However due to the time error of ≈ 169.34 ms/cycle, the speed of 900 counts/s with acceleration and deceleration of 9216 counts/s² is chosen instead.

Figure 4.3 shows the time-displacement graph (See Appendix B3) at that speed, acceleration and deceleration. The gradient of 3.553 is the velocity in mm per second. It equals to 895.24 counts/s (See Appendix B4) that results in error of 0.53% from the input speed. The value of -0.3144 mm (approximately 79 counts or 88.5 milliseconds) is the error due to mostly starting torque, static friction, acceleration and deceleration error.

Figure 4.3 Time-displacement graph at $v=900$ counts/s, $a=9216$ counts/s².

Reliability of the Bioreactor

The bioreactor was run inside the humidified incubator (37°C and 5%CO₂). The speed and acceleration used were 900 counts/s and 9216 counts/s² respectively. The y axis in figure 4.4 shows the position in the encoder (Resolution: 0.003175 inches/step). Throughout 54,000 cycles (approximately 15 hours), the cyclic displacement positions remained constant (Variation = ±3.68 counts). Figure 4.4 shows only the positions of the last 40 cycles. The minus sign gives the direction of the rotation. It is therefore concluded that the linear actuator has a good reliability inside the humidified incubator.

Figure 4.4 Reliability test results.

The evaluation of the bioreactor includes material and its performance. It is biocompatible and able to fulfill the requirements in section 4.1.1. The bioreactor can provide 1 dimensional cyclic strain with accurate position and time delay of ≈ 169.34 ms/cycle. It can function well in humidified incubator (37°C and $5\%\text{CO}_2$) for 15 hours.

4.2 Smooth Muscle Cells

Cells in muscularis layer were extracted using the protocol mentioned in section 4.2.1. They were grown in culture dish/flask, subcultured, and frozen in liquid nitrogen for future experiment. Before they are used for experiment, the cells are characterized to confirm smooth muscle cell-type.

4.2.1 Cell Characterization

Morphology

Figure 4.5 shows the hill-valley growth pattern which characterizes smooth muscle cells in confluent culture. They exhibit no cell alignment and might have lost their contractile phenotype since they have been in static culture for approximately two months (subculture 3).

Figure 4.5 Hill-valley growth pattern of smooth muscle cells. Arrows indicate hills.

Contractile and cytoskeleton protein expression

Figure 4.6 Immunohistochemical staining. A. anti α -actin, B. anti myosin, C. anti desmin.

The cells were stained with antibody α -actin and myosin to reveal their contractile proteins and desmin as one of the cytoskeleton proteins that regulates contractile protein. Figure 4.6 shows these protein expressions. Figures in right column show their respective negative controls. Nuclei were stained in blue with Dapi stain.

4.2.2 Cell Seeding Density

The cell seeding density is important for cell-scaffold evaluation. For cell viability/proliferation analysis, the cell seeding density cannot be too much due to the limitation of cell proliferation assay reagents. The reagent reacts with area of cells in contact that is not effective on multilayer cells. Experiment on bioreactor needs glass cloning ring to secure the cells on scaffold during cell seeding. Within the area of glass cloning ring, 0.785 cm^2 , high cell seeding density tends to result in multilayer cells.

Figure 4.7A shows no confluence inside the cloning ring, figure 4.7B shows confluent cells inside the cloning ring with little piled-up cells, and figure 4.7C shows confluent cells inside the cloning ring with multi layers of cells surrounding the cloning ring. From the cell activity graph (See figure 4.8), similar light absorbance is recorded for seeding densities of $6.37 \times 10^4/\text{cm}^2$ and $1.27 \times 10^5/\text{cm}^2$, as the cells formed a multilayer due to the high density of cells at $1.27 \times 10^5/\text{cm}^2$. Therefore a cell seeding density of $1.27 \times 10^5/\text{cm}^2$ was used in all future tests to avoid multi-layer of cells on the scaffold.

Figure 4.8 Cell Activity at day 3 with different cell seeding density.

4.3 Evaluation on Cell-Scaffold Construct

Several scaffolds made of polyurethanes, chitosan, etc shown in figure 4.9 were investigated for their suitability for mechanical strain bioreactor. They are elastic and known to be biocompatible from literature. For future discussion, surface of the scaffold cast on the mould, facing to the mould was named the bottom side b and surface facing to the air was named the top side a (See figure 4.10).

Figure 4.9 Scaffolds. A. PU on mould (150 × 150 × 2 mm), B. Air-dried Chitosan on mould (120 × 120 × 2 mm), C. Freeze-dried Chitosan, D. PLCL, and E. Tegaderm™.

Figure 4.10 Two sides of scaffold on mould.

4.3.1 Air Dried Chitosan

Cast air-dried chitosan is yellowish transparent with thickness of less than 0.5 mm. It is resilient to withstand cyclic strain for 15 hours in alcohol solution in room temperature without permanent deformation. Figure 4.11A, B, C show the morphology of the scaffold on both sides. No pores are found in the scaffold, so the cells can only grow on the side of the scaffold where the cells are seeded.

Smooth muscle cells were seeded on each side of the scaffold. The result showed cells only grew on side B (See figure 4.10D); however the cell attachment was found to be very low. Therefore, it was concluded that air dried chitosan does not support cell attachment. Furthermore, cell proliferation assay graph (See figure 4.12) supports this result by showing no viable cell present in the construct.

Figure 4.11 Air-dried chitosan. A. Side a view $\times 100$, B. Side a view $\times 300$, C. Side b view $\times 100$, D. Cells on side B $\times 100$ at day 3.

Figure 4.12 Cell viability on air-dried chitosan.

4.3.2 Freeze Dried Chitosan Collagen

Freeze-dried chitosan was cast to allow cells in-growth by having pores. The maximum thickness is 2 mm due to limitation of the mould. The morphology of freeze-dried chitosan was shown in figure 4.14. It has irregular pore sizes, ranged up to 200 μm . The pores cut deep into the scaffold but not through the scaffold due to the thick layer at the bottom side B (see figure 4.14D).

Due to poor attachment on air dried chitosan scaffold, modification with collagen was applied to freeze-dried chitosan. The chitosan was mixed with collagen under different dry weight concentration. Three different weight concentrations of chitosan collagen: 1:1, 6:4, 7:3 were examined. No difference in morphology was observed among the three concentrations. As with air-dried chitosan, the three different composition chitosan collagens are also elastic, as can be seen in figure 4.13. Chitosan collagen 7:3 can withstand strain up to 200%. The collagen present in the substrate weakens the mechanical integrity of the scaffold. The higher concentration of collagen in the scaffold results in weaker scaffold. Only Chitosan collagen 7:3 had sufficient strength to be tested in the tensile testing machine. The chitosan collagen 1:1 and 6:4 scaffolds were very fragile.

Porcine smooth muscle cells with cell seeding density of $1.27 \times 10^4/\text{cm}^2$ were seeded on the top side a. Cell attachment on three different weight concentrations of the scaffolds were compared. The results are shown in figure 4.15-4.17. Arrows on the figures show the cell attachment. On continuous observation from day 2 to day 5

showed that the cells do not proliferate. This may be due to the presence of chitosan, a derivative of chitin. Mori *et al.* [47] have found also that chitin and its derivative products show no acceleratory effect on the proliferation of cultured fibroblasts.

Figure 4.13 Stress-strain curve of chitosan collagen 7:3.

Figure 4.14 Morphology of freeze-dried chitosan collagen. A. Top Side A, B. Cross Section, C. Bottom Side B, D. Cross section $\times 50$.

Figure 4.15 Cell attachment on chitosan collagen 1:1 at day 3. Arrows indicate cells.

Figure 4.16 Cell attachment on Chitosan Collagen 6:4. Arrows indicate cells.

Figure 4.17 Cell attachment on chitosan collagen 7:3. Arrows indicate cells

4.3.3 Freeze Dried Chitosan Collagen Genipin

Freeze-dried chitosan collagen genipin has similar morphology to freeze-dried chitosan collagen (See figure 4.18A, B, C). The bottom side B has more openings that link to the pores inside the scaffold. The pores are interconnected through the scaffold. Its colour is opaque bluish due to presence of genipin. It binds free amino groups in chitosan which may hinder cell attachment.

The initial plan of improving the cell attachment by introducing genipin in the substrate was not successful. The smooth muscle cells, seeded with cell seeding density of 1.27×10^5 cells/cm², attached on the scaffold for initial three days as can be seen in figure 4.18D. The calculation of living cells in waste for 15 days confirmed that some living cells detach from the scaffold after day 3 (See figure 4.20). This results in difficulty in finding cell attachment after 5 days under SEM examination. Moreover, the morphology between the scaffold and top cells is very similar that cause them to be difficult to differentiate. Figure 4.19 shows the result of SEM examination for cell attachment on continuous observation days with right column showed the cell attachment at 10° from the surface.

Figure 4.18 Chitosan collagen Genipin 114. A. Top side A, B. Bottom side B, C. Cross Section, D. Cells attaching at day 3. Arrows indicate cells.

Figure 4.19 Cell Attachment on chitosan collagen genipin. Arrows indicate cells. A. Day 3, B. Day 5, C. Day 7, D. Day 12.

Figure 4.20 Cells detached from the scaffold

4.3.4 Poly(L-lactide-co-ε-caprolactone)

Electrospun Poly(L-lactide-co-ε-caprolactone) (PLCL) provides nanofibers with diameter of the fiber up to approximately 1 μm, as can be seen in figure 4.21. Its appearance is white opaque. The thickness of the electrospun scaffold can be

tailored to meet the requirement of the application. This electrospun material has very limited pores between the fibers.

The fibers in electrospun PLCL are randomly aligned which affects the cell orientation during cell attachment. Figure 4.23D illustrates that the cell seeding on random orientation fiber results in no cell alignment. The actin fibers in smooth muscle cells also show random orientation (See figure 4.25).

Smooth muscle cells were seeded with cell seeding density 6.37×10^4 cells/cm². In culture dish, this cell seeding density will grow confluent inside the cloning ring at day 2. However the cells do not distribute well on PLCL scaffold (See figure 4.22). They piled up to form multilayer cells with several cells extending their cytoplasm to anchor on to the scaffold (See figure 4.23A and 4.23C). Continuous observation of cell attachment (See figure 4.22) showed that the piled up layer of cells was washed away with time. Cell proliferation assays support this observation by showing a decrease in light absorbance at wavelength 490 nm (See figure 4.24). Another set of experiment was done with different cell seeding density of 1.27×10^4 cells/cm². However the cell proliferation assay result showed similar decrement.

Figure 4.21 Morphology of PLCL. A. Top side $\times 100$, B. Top side $\times 1000$, C. Top Side $\times 4000$, D. Bottom side $\times 1000$

Figure 4.22 Cells on PLCL. A. Day 3, B. Day 5, C. Day 7, D. Day 10, E. Day 14.

Figure 4.23 Cell attachment on PLCL. A. Day 3 $\times 300$, B. Day 3 $\times 1000$, C. Day 3 $\times 1400$, D. Day 5 $\times 300$.

Figure 4.24 Cell Activity on SMC- PLCL construct.

Figure 4.25 Actin Fibers on PLCL construct (left). Negative Control (right).

4.3.5 Tegaderm

TegadermTM is a thin, semitransparent polyurethane membrane with acrylic adhesive. Since it is commercially made for wound dressing, its chemical properties are maintained to be constant. It swells in contact with aqueous solutions. Its thickness is less than 10 μm which makes it fragile and difficult to handle (e.g., fixing into bioreactor). Under SEM examination, both sides were similar in appearance and morphology (See figure 4.26A and 4.26B).

Smooth muscle cells were seeded with cell seeding density of 3.18×10^4 cells/cm². The cells attached on the tegadermTM surface very poorly, as can be seen in figure 4.26C and 4.27D. However, toxicity test has showed that tegadermTM was graded to scale 0, which means it is not cytotoxic to living cells. In order to improve cell attachment and growth on tegadermTM, two types of treatments were applied to tegadermTM: chemical treatment and plasma surface treatment. Chemical treatment consists of hexane, acetone and alcohol. Group I tegadermTM was immersed in

Acetone, Hexane (Merck & Co., Inc. NJ, USA) for 4 hours, continued in Hexane (Merck & Co., Inc. NJ, USA) for 12 hours, then absolute Alcohol for 1 hour. Group 2 was immersed in Hexane for 12 hours and absolute Alcohol for 1 hour. Group 3 was immersed in absolute Alcohol for 17 hours. All groups experienced O₂ plasma treatment at 200W for 20s. The cells growing at day 7 was shown in figure 4.27A, 4.27B and 4.27C. Figures 4.27D, 4.27E, and 4.27F show the cell attachment on O₂ plasma treated PU without any chemical treatment. From this discussion, only O₂ plasma treatment is able to enhance cell attachment and growth.

Figure 4.26 SMCs and Tegaderm™ under SEM examination. A. Tegaderm™ ×35, B. Tegaderm™ ×600, C and D. Cells growing on Tegaderm™ at day 3.

Figure 4.27 Cell attachment on different treated plasma. A. Group 3 tegaderm™, B. Group 2 Tegaderm™, C. Group 1 tegaderm™, D and F. O₂ plasma treatment only at day 5, E. O₂ plasma treatment only at day 10.

4.3.6 Polyurethane Membrane

Polyurethane (PU) membrane is transparent, strong, biocompatible and very elastic. Toxicity test result graded PU in scale 0, which indicates no detachable zone around and under the specimen. Therefore it is not cytotoxic to cells. Under light microscope, it consists of arrays of small crystals which appear in darker line in figure 4.28D. These lines are surrounded by scattered light lines that act as the amorphous components of polyurethane membranes. Under SEM examination, both top and bottom sides were looked the same. During tensile test (n=5), the membrane was pulled up to 475% before failure. Its ultimate tensile strength is 30.92 ± 5.5 MPa with modulus of elasticity is 2.29 ± 0.31 MPa (see figure 4.29).

Figure 4.28 Morphology of PU membranes. A. SEM $\times 35$, B. SEM $\times 500$, C. Light microscope $\times 40$, D. Light microscope $\times 400$.

Figure 4.29 Stress-strain behaviour of PU membrane. Note: The strain is $(l - l_0)/l_0$.

Smooth muscle cells were seeded on PU membrane with cell seeding density of 3.18×10^4 cells/cm². The scaffold itself was sterilized using 70% alcohol. Immersion in Alcohol made the transparent membrane into opaque, which might indicate changes to the surface properties. Hence, it was sterilized by spraying 70% alcohol. After 14 days in culture, the cells could not reach confluence as can be seen in figure 4.30A and 4.30B. They tend to pile up to form multilayer cells. These multilayer cells cannot digest cell proliferation assays effectively. To improve the cell distribution and proliferation, PU membrane was treated with either oxygen or argon Plasma. On plasma treated PU with power of 200W and exposure time of 60s, smooth muscle cells were seeded. The cell proliferation and distribution were improved after 14 days in culture (see figure 4.30C, D). The cells grew in multilayer after they reached confluence. In macro scale as can be seen in figure 4.30E, Ar plasma treated PU supports cell proliferation better than O₂ plasma treated PU, indicated greater area of cell attachment. Figures 4.31 show the smooth muscle cells growing on O₂ plasma (200W 20s) PU membrane until day 14.

Figure 4.30 Cells growing on PU membrane. A and B. Untreated membrane, C Ar 200W 60s, D O₂ 200W 60s, E Comparison between Ar and O₂ plasma treatment.

Figure 4.31 SEM examination of SMCs growing on oxygen plasma treated PU membrane (200W 20s). A. Day 2, B. Day 5, C. Day 7, D. Day 9, E. Day 12, F. Day 14.

Figure 4.32 Contact angle.

Contact angle measurement was conducted to examine plasma treated PU characteristic which may affect the cell proliferation. The untreated PU membrane has contact angle varied from 77° to 88° . The plasma treated PU membranes have reduced contact angles as shown in table 4.4. They are more hydrophilic compared with the untreated PU membrane. The result also shows that O_2 plasma treated PU can be more hydrophilic than Ar plasma treated PU. This occurred because O_2 plasma treatment has introduced oxygenated functional groups which are able to enhance cell attachment up to certain degree. Due to the inert nature of argon, its plasma treatment only introduced free functional groups. Some bind with functional groups in the air whereas some remain free.

At the same power of 200W and exposure time of 60s, argon and oxygen plasma treatment have close values of contact angle. However the cell proliferates at different rates. This result shows that there are other factors affecting the cell proliferation besides contact angle. Due to limitation of the project scope, this matter will not be discussed further.

Regardless of any values of contact angles, in macro scale argon plasma treatment supports cell proliferation by having greater area of cell attachment than with O₂ plasma treatment in figure 4.33. The exception occurred with O₂ plasma treatment at 200W for 300s exposure time. It has the biggest area of cell attachment, approximately 1.5 cm diameter. After reaching the maximum area of attachment at 200W 300s, the area of cell attachment with O₂ plasma treatment decreased. Different cell behaviour occurred with argon plasma treatment. The smooth muscle cells proliferated along with the increase in plasma exposure time, reached maximum in 300s exposure time and maintained the same proliferation rate in the increase of plasma exposure time. Three hundred seconds of exposure time may provide the maximum free functional groups which can bind to any components in the air. Major cell apoptosis occurred with 200W 150s argon plasma treatment for unknown reasons.

In micro scale, argon plasma treatment gave consistent cell growth pattern in different exposure time. The cell growth pattern and morphology are shown in figure 4.34A and 4.34B. O₂ plasma treatment gave more complex of cell behaviour. Increasing the exposure time up to 300s increase the cell proliferation however results in uneven cell distribution (See figure 4.34E, 4.34F, and 4.34G). Therefore, O₂ plasma with exposure time above 60s is not considered for future experiment even though 300s exposure time gave the biggest area of cell attachment.

Table 4.4 Contact angles on treated PU membrane.

Power - Time	Ar Plasma	O ₂ Plasma
100W 30s	41.45 ± 2.38°	44.06 ± 1.70°
100W 60s	38.14 ± 1.41°	39.63 ± 2.45°
200W 30s	38.34 ± 2.15°	37.47 ± 2.15°
200W 60s	39.89 ± 2.06°	37.18 ± 1.83°
200W 150s	37.10 ± 2.44°	29.05 ± 1.92°
200W 300s	36.81 ± 1.51°	15.06 ± 1.20°
200W 600s	29.66 ± 0.82°	9.10 ± 0.28°

Figure 4.33 Area of cell attachment under argon and oxygen plasma treatment.

Figure 4.34 Cells growing on PU under light microscope. A. Ar plasma at 200W 600s ×40, B. Ar plasma at 200W 600s ×400, C. O₂ plasma at 200W 30s ×40, D. O₂ plasma at 200W 60s ×40, E. O₂ plasma at 200W 300s ×40, F. O₂ plasma at 200W 600s ×40, G. O₂ plasma at 200W 600s ×400.

Consideration of using argon or oxygen plasma treatment to give better cell proliferation and attachment has been discussed. Argon plasma treatment has consistent cell growth pattern with bigger area of cell attachment, higher cell proliferation. However the control of components binding to the free functional

groups on PU membrane is difficult to monitor. Thorough research needs to be done before future usage. O₂ plasma treated PU has higher cell proliferation than untreated PU with providing oxygenated functional groups on the membrane. However 60s is the maximum exposure time before the cell distribution becomes uneven. Hence oxygen plasma treatment at 200W 60s is chosen for mechanical stimulation research.

Out of 6 scaffolds that have been tested (See table 4.5 for the summary), polyurethane membrane with O₂ plasma treatment was selected. It was chosen due to its ability to support cell growth up to 14 days, the longest among other scaffolds. It promotes cell proliferation and gives better cell distribution. It is elastic and easy to handle.

Table 4.5 Summary of evaluation on scaffolds

	Air-dried Chitosan	Freeze-dried chitosan (collagen/genipin blended)	PLCL	Tegaderm	PU
Morphology	Yellowish transparent, no pores	Irregular pore size	Limited pores between fibers (dia 1µm), white opaque	Thin, semitransparent membrane	Transparent membrane
Elasticity	Strong and elastic, can withstand 10% cyclic strain for 15 hours	Strain up to 200%	Elastic	Very elastic	Strong and very elastic, strain up to 475%
Toxicity	Not tested	Not tested	Non-toxic	Non toxic	Non toxic
Cell attachment	Poor	Ok	The cells piled up to multilayer cells	After plasma surface treatment, the attachment was very good	After plasma surface treatment, the attachment was very good
Cell proliferation	Not seen, the cells died after three days	Not seen, the cells died after seven days	Not seen, the cells detached after 14 days	Very good	Very good
Cell activity	None	Not tested	Decreasing	Not tested	Stable
Others				Difficult to handle due to its thickness	Chosen for mechanical stimulation experiment

4.4 Mechanical Stimulation

One dimensional stretching with different regimes was applied to smooth muscle cell – polyurethane membrane construct to study the effect of mechanical stimulation on cell-scaffold construct. Smooth muscle cells were seeded on polyurethane membrane with cell seeding density 6.37×10^4 cells/cm². The cells were remained in static culture for 2-3 days. Subsequently, three different factors in mechanical stimulation regime: amplitude, stress loading time and frequency of mechanical stimulation were varied and applied to cell-scaffold construct.

4.4.1 The effect of mechanical stimulation on cell morphology, orientation and proliferation

Mechanical strain under any types of cycle on visceral smooth muscle cells results in cell alignment, as can be seen under light microscope in figure 4.35A, 4.35B and under SEM examination in figure 4.36A, 4.36B. This alignment is important to achieve a functional layer smooth muscle cells, similar with their alignments *in vivo*. In general, smooth muscle cells in static culture show random alignment, however they may display local alignment. (See figure 4.35E). During this research, a small constructs under mechanical stimulation showed random alignment. This may be attributed to poor clamping, failing to grip the scaffold firmly. So, instead of stretching the scaffold, the clamps caused the loose scaffold to flex up and down.

Compared to cells in static culture (See figure 4.35C and 4.35D), smooth muscle cells under mechanical stimulation have denser cytoplasm, smaller nucleus, and narrower cytoplasm. This morphology is close to the differentiated, contractile phenotype of cells *in vivo*. However further research is needed to confirm whether they are in differentiated, contractile phenotype.

Cell proliferation assays (See figure 4.37) shows that no cell proliferation on stimulated cells ($\leq 2.5\%$ strain). There are two possibilities, either the cells were dying or the cells were not in synthetic phenotype which usually actively proliferates. However looking at the morphology of the cells, they did not show any signs of rounding off due to cell apoptosis. The stimulated cells were most probably in differentiated, contractile phenotype. The cell proliferation assays on static control cells were consistent. This is probably due to their reaching confluence. The number of dying cells was equal to the rate of cell proliferation.

Figure 4.35 The effect of mechanical stimulation on cell-scaffold construct on day 2 of stretching (Light Microscope). A, B. Stimulated SMCs, C, D, E. Static SMCs.

Figure 4.36 The effect of mechanical stimulation on cell-scaffold construct on day 2 of stretching (SEM). A, B. Stimulated SMCs, C, D. Static SMCs.

Figure 4.37 Cell proliferation assay of static and stimulated SMCs (Eso Cycle – 0.0725Hz, 2.5% strain for 2 hrs; Cardio Cycle – 1.08 Hz, <2.5% strain for 2 hrs).

The following sections explain the effects of three different factors in mechanical stimulation regimen on cell alignment in detail.

4.4.2 The effect of amplitude in mechanical stimulation regime on SMC-PU construct

Cardiovascular cycle with two different amplitude of strain: 10% and 2.5% were applied to the cell-scaffold construct for 2 hours. The cells were aligned perpendicular to the direction of strain, as can be seen in figure 4.38A and 4.38B. This finding supports the works of Kanda [69, 72, 87], Kakisis [70], and Kim [86]. Their results have shown that vascular smooth muscle cells align perpendicular to the direction of strain. At 10% strain, some cells were lost after 4 days of stretching (See figure 4.39). It may be due to the higher strain giving adverse effects and causing the cells lysis. PU membranes may also play a part in cell detachment as there are no pores to maintain the cells in place if they experience fluid shear.

In some experiments, for 2.5% nominal strain, the actual strain was slightly lower due to manufacturing discrepancies which is caused by the small amount of deformation of the plates and clamps holding the scaffolds. The unexpected result is shown in figure 4.38C. The cells were aligned parallel to the direction of strain. According to Kanda [72], the cells aligned parallel to the direction of strain due to the influence of collagen matrix. The extracellular matrix normally follows the direction of applied strain which later guides the cell orientation. However in this

experiment, the cells were grown on non-collagen based scaffold for period of 4 days. Compared to the works of Kanda [72], they applied mechanical strain to vascular smooth muscle cells for 4 weeks which give permissible times for the cells to synthesize collagen. In this experiment, it is unlikely that the visceral smooth muscle cells have developed collagen matrix to guide them aligned in parallel to the applied strain at day 4. A possible explanation is due to small strains guiding the cells to align in their long axis to the direction of strain. Most published research reports the effect of strain of 5% and above on cell alignment. Kanda's publication mentioned the discussion from Buck *et al.* that the cell orientation is due to cellular avoidance reaction to stretching in the direction of their long axis. It is possible that at above 5% strain, the cells attempt to reduce the strain effect by positioning themselves perpendicular to the direction of strain. However in small amplitude of strain, the cells follow the direction of strain. Another reason may be simply because visceral and vascular smooth muscle cells behave differently at small strain. Further research is required.

Figure 4.38 SMCs aligned under different amplitude of mechanical stimulation regime after two days of stretching. A. 10%, B. 2.5%, C. <2.5%, D. Static culture. Right figures in higher magnification $\times 400$. Arrows indicate the direction of stretching.

Figure 4.39 Cell loss after 4 days of stretching.

4.4.3 The effect of stress loading time in mechanical stimulation regime on SMC-PU construct

Cardiovascular cycle with 2.5% amplitude of strain was applied with two different stress loading time: 2 hours and 6 hours per day. After two days of stretching, both groups show the cells were aligned perpendicular to the strain direction (See figure 4.40). These pictures were taken 24 hours after stretching, i.e. on the 3rd day. These results explain that two hours per day of stretching for two days is enough to align the smooth muscle cells. However the optimum stress loading time requires further research.

The influence of two different stress loading time on cell morphology was observed. Even though the cells at 6 hours of mechanical stimulation per day are aligned, they were looked over-stretched in irregular shape. Most publications who have done above 6 hours of stress loading time on smooth muscle cells did not report any overstretched cells. However the effect of mechanical stimulation depends on cell-

type. Six hours of stress loading time may affect adversely on porcine oesophageal smooth muscle cells:

Figure 4.40 SMCs under two different stress loading time after 2 days of stretching. A, B. for 6 hours, C, D. for 2 hours. Solid line indicates the cell alignment. Arrow indicates the direction of stretching.

4.4.4 The effect of frequency in mechanical stimulation regime on SMC-PU construct

Two different cycles: oesophageal cycle and cardiovascular cycle were applied at 2.5% strain to cell-scaffold construct for approximately two hours per day. The input command has been calculated so that the frequency of cardiovascular cycle was 1

Hz. The output frequency of cardiovascular cycle was measured to be 1.08 Hz. The input command for oesophageal cycle has been calculated to be 0.0725 Hz. Its output frequency was measured, equal to 0.074 Hz. Hence the effect of two different frequencies: 1.08 Hz and 0.074 Hz in mechanical stimulation regime were analyzed.

Both groups of smooth muscle cells show alignment after two days of stretching (See figure 4.41). However, at 1.08 Hz frequency, the cells were aligned perpendicular to the direction of strain whereas at 0.074 Hz frequency, the cells were aligned parallel to the direction of strain. Few publications have ever discussed the effect of different frequencies on cell-scaffold constructs. It is suggested that the frequencies at low amplitude of strain (2.5%) play a major contribution in affecting the cell behaviour to minimize the effect of cyclic strain. At higher strain, the amplitude may affect the cells greater than the frequency. At high frequency, the cells experience more stresses due to over-reactive of cell metabolism (e.g. lactic acid build up). Hence, they position themselves perpendicular to applied strain to avoid more stresses in the cells.

Figure 4.41 SMCs under different frequency after 2 days of stretching. Arrows indicate the strain direction. A, B. Under oesophageal cycle ($f = 0.074$ Hz), C, D. Under cardiovascular cycle ($f = 1.08$ Hz). Note: Nuclei are more distinct in B although in both B and D, the SMCs exhibit their characteristic spindle shape.

The effect of amplitude and frequency on cell-scaffold construct was summarized in table 4.6.

Table 4.6 Summary of the effect of mechanical stimulation

Strain/Frequency	<2.5%	2.5%	10%
Oesophageal cycle ($f < 1$ Hz)	not tested	Parallel	Not tested
Cardiovascular cycle ($f \geq 1$ Hz)	Parallel	perpendicular	Perpendicular

Chapter 5 – CONCLUSIONS AND FUTURE WORKS

The objective behind this project was to study the effect of mechanical stimulation on the cultured smooth muscle cells, particularly on cell alignment and proliferation. Therefore, a bioreactor with mechanical stimulation, porcine oesophageal smooth muscle cell line and suitable scaffold were established. Then, they were integrated to study on mechanical stimulation effects, on the smooth muscle cells.

From the results of this project, the following conclusions are drawn:

1. The bioreactor can repeatably stimulate cells with a variety of 1-D physiological regimes (e.g., oesophageal cycle, cardiovascular cycle) and strain magnitudes up to 25% with high accuracy in displacement position.
2. A suitable scaffold with biocompatibility, elasticity, chemical stability and surface modification allowance is required for this project. Polyurethane membrane with 200W 60s O₂ plasma surface modification was selected after evaluation among the six scaffold.
3. Amplitude of strain and frequency affect the cell alignment. Cyclic strain with high amplitude (above 2.5%) and high frequency (above 1 Hz) produced perpendicular alignment whereas cyclic strain at low amplitude (less than 2.5%) and low frequency (less than 1 Hz) produced parallel alignment. More testing is required to confirm this parallel alignment.
4. Duration of 2 hours per day for two days of stretching is able to influence the cell alignment. However, the optimum stress loading time requires further research.

5. All stimulated smooth muscle cells did not proliferate, indicating possible differentiated phenotype. However, further research on contractile or cytoskeleton protein expression is required to describe the phenotype of stimulated smooth muscle cells.
6. Mechanical strain in one dimension can directly influence the alignment of smooth muscle cells. It can therefore be used in the development of functional sheets of smooth muscle cells for oesophageal tissue engineering.

One dimensional mechanical stimulation has influenced cell alignment and proliferation. However more experiments are needed to study the effect of mechanical stimulation at small strain and lower frequency. Moreover, in order to know the effect of mechanical stimulation to cell's functions, further analysis is needed such as contractile protein and other differentiated protein expression. Study of the effect of mechanical stimulation on oesophageal cells growing on a 3D porous scaffold constructs will be beneficial to pursue the mechanical stimulation study of tubular constructs.

In the current experiment, the smooth muscle cells form one sheet of multilayer cells and stimulated in one dimensional direction. Compared to the oesophageal cells *in vivo*, they form tubular. The cells *in vivo* experience mechanical stimulation in all possible directions, mainly due to cardiovascular pressure and peristaltic motion. The following steps are recommended to achieve functional tissue-engineered oesophagus *in vivo*.

1. To study the effect of mechanical stimulation to other cell types such as fibroblasts, epithelium,
2. To establish tubular construct consists of co-culture between smooth muscle cells and other cell types, if possible using acellular oesophageal matrix as scaffold and tubular flow through bioreactor with consistent nutrition, and
3. To obtain the optimum mechanical conditioning of tubular construct that is able to stimulate differentiated phenotype prior to implantation

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GLOSSARY

Adventitia: The outer coat of the wall of various organs, consists of loose connective tissue, without *serosa*

Allogeneic: of foreign cells/tissue used for human to human transplantation

Aphidicolin: antiviral antibiotic that inhibit the growth of eukaryotic cells

Areolar: a form of fibrous connective tissue in which the fibers are loosely arranged

Confluent: occupying the whole surface area

Deep: situated far from the surface

Differentiated: mature cells which have distinctive characteristics and perform specific function

Esophagectomy: surgical removal of oesophagus

Hydrophilic: easily interact with water.

Hydrophobic: repels water

Hypertrophy: increase in the size of an organ or part due to enlargement of its cell rather than to cell multiplication

Inferior: below another structure

Keratinized epithelium: an epithelium in which keratin layer is formed

Lamellar: arranged in thin plates or scale

Latissimus dorsi muscle flap: muscle tissue attached to sphenoidal spine

Lymphocytes: white cells of the blood that are mainly responsible to the production of antibody

Morphogenesis: the development of form and structure of the organs and the whole body

Mucosa: mucous membrane

Mucus: The free slime of the mucous membranes, secreted by the glands

Muscularis: the muscular layer that covers a hollow organ

Muscularis mucosae: muscular layer of *mucosa*

Phenotype: characteristic displayed by tissue/organism under a particular set of environmental factors, regardless of the actual genotype

Plexus: network

Polymorphism: the regular and simultaneous occurrence in a single origin.

Proliferation: reproduction or multiplication of cells

Serosa: one form of connective tissue which is present in the internal cavity of the body

Serum: blood plasma with no clotting factors

Submucosa: a tissue layer beneath a mucous membrane

Superficial: situated near the surface

Superior: above another structure

Stratified: arranged in the form of layers or strata

Visceral: of the organs in the great cavities of the body, especially applied to the organs in the abdomen

Xenogeneic: of foreign/tissue used for animal-human transplantation

APPENDICES A

APPENDICES B

APPENDICES A

Appendix A1 – Program Test

#TEST	\\Name of the program
MT 2	
KS1	
IN"Enter Displacement (mm): ",L1	\\User input
IN"Enter Speed (counts/s): ",S	
IN"Enter total time (s) ",TA	
C=0	
L2=X*L1/100	\\Calculation
L3=L2/25.4	
L4=L3*6400	
S1=S/251.97	
A=(S/0.1)+250	
MG "Displacement (counts) =",L4{F4.2}	\\Output message
MG "Displacement (mm) =",L2{F4.2}	
MG "Speed (cts/s) =",S{F4.2}	
MG "Speed (mm/s) =",S1{F4.2}	
#LOOP	
DE 0	\\Initial position
DP 0	
PA L4	\\Forward Movement
SP S	
AC A	
DC A	
BG	
AM	
MG "Cycle =",C {F7.0}	
MG "Position I =",_TP{F5.1}	
PA 0	\\Home
SP S	
AC A	
DC A	
BG	
AM	
MG "Position II =",_TP{F3.1}	
C=C+1	
JP#E,C>=10	\\Repeat
JP#LOOP	
#E	
PA L4	\\Maximum Displacement
SP S	
AC A	
DC A	
BG	
EN	

Appendix A2 – Program Mech

```

#MECH                                \\Name of the program
MT 2
KS1
IN"Enter scaffold length (mm): ",L1  \\User input
IN"Enter elongation (%): ",X
IN"Enter cycle time (ms): ",T
IN"Enter hold time I (ms): ",H1
IN"Enter hold time II (ms): ",H2
IN"Enter total time (s) ",TA
C=0
L2=X*L1/100                          \\Calculation
L3=L2/25.4
L4=L3*6400
S1=900
S2=900
S3=S1/251.97
S4=S2/251.97
A1=(S1/0.1)+250
A2=(S2/0.1)+250
MG "Elongation (counts) =",L4{F4.2}  \\Output message
MG "Elongation (mm) =",L2{F4.2}
MG "Fwd Speed (cts/s) =",S1{F4.2}
MG "Fwd Speed (mm/s) =",S3{F4.2}
MG "Backwd Speed (cts/s) =",S2{F4.2}
MG "Backwd Speed (mm/s) =",S4{F4.2}
MG "Fwd Acc (cts/s2) =", A1{F4.2}
MG "Backwd Acc (cts/s2) =",A2 {F4.2}
#LOOP
DE 0                                \\Initial position
DP 0
PA L4                               \\Forward Movement
SP S1
AC A1
DC A1
BG
AM
MG "Cycle =",C {F7.0}
MG "Position I =",_TP{F5.1}
WT H1                               \\Waiting Period
PA 0                                \\Home
SP S2
AC A2
DC A2
BG
AM
MG "Position II =",_TP{F3.1}
WT H2                               \\Waiting Period
C=C+1
JP#LOOP,C<=(1000*TA/T)              \\Repeat
EN

```

Appendix A3 – Program MechEso

#MECHESO	\\Name of the program
MT2	
KS1	
IN"Enter scaffold length (mm): ",L1	\\User Input
IN"Enter elongation (%): ",X	
IN"Enter cycle time (ms): ",T	
IN"Enter hold time I (ms): ",H1	
IN"Enter hold time II (ms): ",H2	
IN"Enter total time (s): ",TA	
C=0	
L2=X*L1/100	\\Calculation
L3=L2/25.4	
L4=-1*L3*6400	
S1=147	
S2=118	
A1=2048	
A2=2048	
MG"Elongation (counts) =", L4{F4.2}	
MG"Elongation (mm) =", L2{F4.2}	
#LOOP	
DE 0	\\Initial Position
DP 0	
PA L4	\\Forward Movement
SP S1	
AC A1	
DC A1	
BG	
AM	
MG"Cycle =",C{F7.0}	\\Output Message
MG"Position I =", _TP{F5.1}	
WT H1	\\Waiting Period
PA 0	\\Home
SP S2	
AC A2	
DC A2	
BG	
AM	
MG"Position II =", _TP{F3.1}	
WT H2	\\Waiting Period
C=C+1	
JP#LOOP,C<=(TA*1000/T)	\\Repeat
EN	

Appendix A4 – Program MechCar

```

#MECHCAR                                \\Name of the program
MT2
KS1
IN"Enter scaffold length (mm): ",L1      \\User Input
IN"Enter elongation (%): ",X
IN"Enter cycle time (ms): ",T
IN"Enter hold time I (ms): ",H1
IN"Enter hold time II (ms): ",H2
IN"Enter total time (s): ",TA
C=0
L2=X*L1/100                             \\Calculation
L3=L2/25.4
L4=L3*6400
L5=0.9333*L4
L6=0.5*L4
S1=949
S2=70
S3=434
S4=167
A1=9216
A2=1024
A3=6144
A4=2048
MG"Elongation (counts) =", L4{F4.2}
MG"Elongation (mm) =", L2{F4.2}
#LOOP
DE 0                                     \\Initial Position
DP 0
PA L5                                   \\Forward Movement
SP S1
AC A1
DC A1
BG
AM
PA L4
SP S2
AC A2
DC A2
BG
AM
MG"Cycle =",C{F7.0}                    \\Output Message
MG"Position I =", _TP{F5.1}
WT H1                                  \\Waiting Period
PA L6                                  \\Backward Movement
SP S3
AC A3
DC A3
BG
AM
PA 0                                    \\Home
SP S4
AC A4
DC A4
BG

```


Appendix A

```
AM
MG"Position II =", _TP{F3.1}
WT H2                                \\Waiting Period
C=C+1
JP#LOOP, C<= (TA*1000/T)            \\Repeat
EN
```

APPENDICES B

Appendix B1 – Accuracy Displacement Test

Experiment Sheet
Accuracy Displacement Test

Input Value (mm)	Real Displacement (mm)									
	100 cts/s	200 cts/s	300 cts/s	400 cts/s	500 cts/s	600 cts/s	700 cts/s	800 cts/s	900 cts/s	1000 cts/s
0.2	0.2	0.19	0.2	0.19	0.22	0.19	0.19	0.19	0.22	0.19
0.4	0.39	0.39	0.41	0.4	0.4	0.39	0.4	0.39	0.41	0.4
0.6	0.6	0.6	0.59	0.6	0.6	0.59	0.6	0.59	0.6	0.6
0.8	0.79	0.79	0.8	0.79	0.8	0.79	0.81	0.8	0.82	0.78
1	0.99	1	1	1	0.99	1	0.99	0.98	1.01	0.99
1.2	1.19	1.2	1.22	1.2	1.22	1.21	1.21	1.21	1.19	1.2
1.4	1.4	1.4	1.38	1.4	1.41	1.42	1.41	1.4	1.42	1.39
1.6	1.6	1.58	1.58	1.58	1.62	1.61	1.6	1.61	1.59	1.6
1.8	1.79	1.81	1.79	1.79	1.82	1.8	1.82	1.8	1.8	1.8
2	2	2.01	2	2	2	1.99	2	2	1.99	2.01

Appendix B2 – Time Error

$$\text{Theoretical time} = \frac{\text{Input Displacement (counts)}}{\text{Input Speed (counts / s)}}$$

$$\text{Time Error} = \text{Real Duration} - \text{Theoretical Time}$$

Time Error
To Reach the Displacement

Input value of Displacement	Time Error (second)									
	100 cts/s	200 cts/s	300 cts/s	400 cts/s	500 cts/s	600 cts/s	700 cts/s	800 cts/s	900 cts/s	1000 cts/s
0.2	0.0651	0.0818	0.0877	0.0883	0.0859	0.0917	0.0942	0.0937	0.0892	0.0915
0.4	0.0612	0.0689	0.0807	0.0899	0.0860	0.0920	0.0927	0.0964	0.0971	0.0930
0.6	0.0696	0.0770	0.0765	0.0897	0.0900	0.0933	0.0812	0.0829	0.0934	0.0893
0.8	0.0690	0.0759	0.0824	0.0808	0.0878	0.0969	0.0792	0.0904	0.0884	0.0932
1	0.0627	0.0721	0.0782	0.0853	0.0794	0.1005	0.0862	0.0812	0.0957	0.0866
1.2	0.0749	0.0777	0.0816	0.0860	0.0881	0.0923	0.0819	0.0949	0.0855	0.0881
1.4	0.0682	0.0743	0.0841	0.0872	0.0854	0.0840	0.0856	0.0957	0.0961	0.0891
1.6	0.0714	0.0795	0.0824	0.0840	0.0913	0.0862	0.0893	0.0913	0.0978	0.0864
1.8	0.0665	0.0794	0.0791	0.0852	0.0891	0.0855	0.0907	0.0878	0.0861	0.0888
2	0.0625	0.0741	0.0807	0.0821	0.0883	0.0953	0.0863	0.0906	0.0858	0.0808

$$\text{Average of Time Error} = 84.672 \pm 8.506 \text{ ms}$$

Appendix B3 – Time-Displacement Graph

Line Equation from Time-Displacement Graph

Graph	Line Equation	Gradient or Speed		Error
		mm/s	count/s	%
100 count/s	$y = 0.3971 x - 0.0322$	0.3971	100.06	0.06
200 count/s	$y = 0.7994 x - 0.0716$	0.7994	201.42	0.71
300 count/s	$y = 1.1838 x - 0.093$	1.1838	298.28	0.57
400 count/s	$y = 1.5926 x - 0.1452$	1.5926	401.29	0.32
500 count/s	$y = 1.9854 x - 0.1656$	1.9854	500.26	0.05
600 count/s	$y = 2.416 x - 0.2388$	2.416	608.76	1.46
700 count/s	$y = 2.8046 x - 0.2507$	2.8046	706.67	0.95
800 count/s	$y = 3.2064 x - 0.304$	3.2064	807.91	0.99
900 count/s	$y = 3.554 x - 0.3144$	3.554	895.24	0.53
1000 count/s	$y = 4.0676 x - 0.3921$	4.0676	1024.91	2.49
Mean Error				0.81 ± 0.73

Appendix B4 – Calculation of Conversion count - mm

Lead Screw = 0.0003125 inches/step
 = 0.0625 inches/revolution
 = 1.5875 mm/revolution

Encoder Resolution = 400 counts/revolution
 = 6400 counts/inch
 = 251.9685 counts/mm