Development and characterization of acellular matrices for tissue engineering of the esophagus

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DEVELOPMENT AND CHARACTERIZATION OF ACELLULAR MATRICES FOR TISSUE ENGINEERING OF THE ESOPHAGUS

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Abstract

This project focuses its efforts on the creation of a tissue engineered esophageal replacement for patients afflicted with esophageal cancer. The aim of this project is to develop a decellularization process to produce decellularized matrices for esophageal tissue engineering applications. The research work was categorized into two sections: the development of a decellularization process and the various characterizations of acellular scaffolds treated with the decellularization process developed. Two different decellularization techniques were explored; a chemical process, and a mechanical-chemical process. While both processes utilized peracetic acid as a primary decellularization reagent, the mechanical-chemical process involved mechanical scraping to aid in cell removal. Although both processes were found to be effective in native cell removal, and were non-cytotoxic to host cells, the mechanical-chemical process yielded much better results in terms of extracellular (ECM) protein preservation and cell-matrix interactions. Structural and functional proteins such as collagen type I, IV, laminin and fibronectin were found to be preserved, albeit in lower concentrations, in samples treated with the mechanical-chemical process. Cell-matrix interaction studies using porcine esophageal epithelial cells and fibroblasts showed favourable attachment and growth for these samples, while samples treated with the chemical process were unable to support cell growth and proliferation. Samples treated with the mechanical-chemical process were further characterized by assessing its biomechanical properties, as compared with fresh, untreated samples. While the treated samples exhibited lower strain rate of 61.7%, it displayed higher tensile strength at failure when compared to untreated samples. Based on the preliminary findings, the mechanical-chemical process was selected as the optimized decellularization process for esophageal tissue engineering applications. Future works will focus on identifying native growth factors retained in decellularized scaffolds, and conducting *in-vivo* biocompatibility studies to assess potential foreign body response.

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CHAPTER 1 INTRODUCTION

1.1 Tissue engineering – The answer to organ transplantation

Each year, millions of people suffer end stage organ failure or tissue loss, leading to debilitating national health care costs exceeding US\$400 billion [8]. The problem of organ failure has been addressed with the introduction of organ transplantation into medical practice. Diseased organs are typically treated by performing organ transplantation using living or post mortem donor organs. An estimated 8 million surgical procedures are carried out annually, with organ transplants including kidneys, lungs and heart [8, 9]. Although organ transplantation has yielded many benefits in terms of survival and quality of life for patients, it possesses inherent limitations.

One of the limiting factors is the growing problem of donor shortage. Today, more than 82,000 people are awaiting transplants in the United States, with 17 people dying everyday while on waiting lists for needed organs [2]. Figure 1 illustrates the growing discrepancy between the number of patients on the waitlist and the number of transplants for the past decade.

Between 2005 and June 2006, there were close to 96,000 patients on the waiting list for organ donors, while only 34,000 organ transplants were performed. The statistics for both waiting list and organ transplants during this period are categorized by organ type in Figure 2.

In addition to the donor shortage problem, tissue and organ transplantation are extremely expensive. Not only do patients incur transplantation costs, the surgery itself, and post-operative care, add to the financial burden. As seen in Table 1, the estimated financial cost per transplant as of July 1, 2005 varied between US\$210,000 and US\$890,000. Such exorbitant costs often leave patients and their families unable to cope with the financial strain of organ transplantation, despite its necessity in terms of life saving potential.

Figure 1: Graph showing the difference between waiting list and transplant numbers from the period of 1990 to 2001 [2]

Figure 2: Graph showing the difference between waiting list and transplant numbers from the period of 1990 to 2001 [3]

Because of the shortcomings associated with conventional tissue and organ transplantations, tissue engineering therapies are now hailed as a potential alternative to treat the loss or malfunction of tissues and/or organs without the above mentioned limitations. Tissue engineering is defined as “an interdisciplinary field that applies the principles of engineering and life sciences towards the development of biological substitutes that restore, maintain or improve tissue or organ function” [11]. Strategies for the reconstruction of damaged tissue and organs can be summarized by three fundamental approaches: cell based therapy, bioactive molecule based therapy, and scaffold based therapy [12]. All three concepts work in synergy, with the elements of appropriate cell sources, tissue engineered constructs, and bioreactor technologies coexisting to produce successful applications [13, 14]. In this decade, several products have reached the market. Interpore’s Pro-Osteon coral-derived bone graft material was introduced in 1993 [15] DermaGraft (Advanced Tissue Sciences) [16] and AlloDerm (LifeCell), both tissue engineered constructs comprising human fibroblasts seeded on matrices, have been approved by the U.S. Food and Drug Administration (FDA) [15]. To date, Alloderm has been used successfully in more than 80,000 implants, indicating its success as a tissue engineered product designed to induce rapid remodeling and trigger the regenerative healing process of the body [17].

Table 1: Estimated US average of different transplant costs for each organ in 2005 [4]

ESTIMATED US AVERAGE 2005 FIRST-YEAR BILLED CHARGES PER TRANSPLANT									
Transplant	Services That May Comprise a Negotiated Case Rate				Services Not Usually Part of a Negotiated Case Rate				Total
	Procurement	Hospital	Physician	Subtotal	Evaluation	Follow-Up	Maintenance Therapy Outpatient Immuno-suppressants	Subtotal	
Heart Only	\$74,400	\$240,500	\$34,900	\$349,800	\$20,100	\$81,600	\$27,400	\$129,100	\$478,900
Single Lung Only	41,700	128,600	28,200	198,500	17,200	56,500	27,700	101,400	299,900
Double Lung Only	83,400	236,700	44,700	364,800	27,300	89,700	26,400	143,400	508,200
Liver Only	57,400	155,600	55,800	268,800	21,900	74,800	27,300	124,000	392,800
Kidney Only	50,800	62,900	17,700	131,400	12,300	40,200	26,100	78,600	210,000
Pancreas Only	67,200	98,800	20,500	186,500	12,400	40,800	31,100	84,300	270,800
Intestine Only	74,600	549,400	70,700	694,700	34,300	64,600	20,000	118,900	813,600
Heart-Lung	134,400	323,000	46,500	503,900	21,900	87,200	27,800	136,900	640,800
Kidney-Heart	125,200	296,200	34,900	456,300	20,100	81,600	30,400	132,100	588,400
Kidney-Pancreas	118,000	70,400	20,500	208,900	12,400	40,800	31,000	84,200	293,100
Liver-Intestine	132,000	492,000	70,700	694,700	34,300	74,800	26,400	135,500	830,200
Liver-Kidney	108,200	221,900	55,800	385,900	21,900	74,800	32,800	129,500	515,400
Liver-Pancreas-Intestine	199,200	476,000	70,700	745,900	34,300	74,800	30,600	139,700	885,600
Pancreas-Intestine	141,800	490,600	70,700	703,100	34,300	64,600	30,100	129,000	832,100

1.2 Tissue engineering of the esophagus – A solution to esophageal cancer

There is great potential in the field of tissue engineering to decrease human suffering by providing engineering constructs for functional replacement or repair of diseased tissue/organs. One such example is the application of tissue engineering in the cancer of the esophagus. Esophageal cancer is the eighth most common cancer worldwide, responsible for 462,000 new cases in 2002 [7]. It is also the sixth most common cause of death from cancer with 386,000 deaths [18]. In the United States alone, esophageal cancer incidence totaled 14,520 in 2005, with 13,570 perishing of the disease in the same

year [19]. The UK is also not spared the spread of this cancerous malignancy, with at least 7,500 people diagnosed each year [20]. Yet such statistics are considered low when compared to high risk developing countries such as China and Southern Africa, as seen in Figure 3. In fact, the disturbingly high rates of incidences in these areas have caused epidemiologists to label them as geographical esophageal cancer belts. The 'Asian esophageal cancer belt' comprises Mongolia, China, Kashmir, Iran, Turkmenistan, and Quetta in Pakistan, while the 'African esophageal cancer belt' consists of Eastern and Southern African countries [21].

Cancer of the esophagus has a very poor survival. As indicated earlier, only 16% of the cases in the United States, and 10% in Europe survive at least 5 years. Worldwide, the current 5 year overall survival rate of 14% remains low, even when compared with dismal survival rates (4%) in the 1970s [22]. One reason is the late diagnosis of esophageal cancer at an advanced stage, despite histopathological changes [23]. This is attributed to the ineffective screening tools and guidelines for esophageal cancer [24], and leads to unresectable (incurable) esophageal cancer being present in more than 50% of the patients [25]. Operative approaches in attempting to remove the malignancy, varying from conventional transthoracic esophagectomy to limited excision without thoracotomy, have yielded unsatisfactory results. In one case study, it was shown that approximately 18% of patients suffering from esophageal cancer died from post-operative related conditions after esophagectomy, while 48% of the survivors suffered from a recurrence within 24 months after the operation [26]. In another study, it was reported that an estimated 16% of patients perished from surgery related postoperative complications, while a 6 year overall survival rate was only 39.5% [27]. With such a dismal prognosis and high operative risk, there is an urgent need for an alternative solution to address the rapidly increasing mortality rates of this malignancy.

Figure 3: Graph showing the age-standardized incidence rates for esophageal cancer [7]

1.3 Objective of project

Based on the compelling reasons stated in the above section, this research project aims to alleviate the problems associated with the prognosis of esophageal cancer by creating a functional replacement for the diseased esophagus. A tissue engineering strategy will be employed to develop acellular tissue matrices as bioscaffolds, using the technique of decellularization. There has been tremendous interest in the development of decellularization as a technique to produce bioscaffolds, with many acellular organs and tissues being used successfully in a variety of regenerative medicine and tissue

engineering applications. This class of biologically derived biomaterials has been known for its characteristics such as rapid resorption, support of angiogenesis, lack of immunogenicity, and ability to serve as a template for tissue remodeling. This project seeks to develop a decellularization process to produce acellular matrices for esophageal tissue engineering. The key goals are to (1) establish a decellularization protocol in the efficient removal of all cellular and nuclear matter; (2) minimize adverse effects of the decellularization protocol on the resulting extra cellular matrix, including its structural and functional proteins; (3) determine biomechanical properties; (4) assess the performance of these acellular matrices under cell culture conditions. The animal model of interest in this project is the porcine model, due to the anatomical and physiological similarities of its organs with human organs [28].

CHAPTER 2 LITERATURE REVIEW

2.1 The esophagus

2.1.1 Function of the esophagus

The esophagus is a muscular canal, extending from the pharynx to the stomach, and is bound by upper and lower esophageal sphincters [29]. The average adult esophagus ranges between 23cm and 25 cm, with a diameter of 2 cm [30]. The organization of the tissues reflects their function, which is to transport ingested food from the oropharynx to the stomach. The initiation of swallowing is a voluntary act involving the skeletal musculature of the oropharynx, where a food bolus is subsequently conveyed to the stomach via a strong peristaltic reflex [31]. During rest, the lower esophageal sphincter keeps the lumen closed, thus preventing reflux, and while swallowing, to relax, such that food bolus is able to pass through [32].

Figure 4: Anatomy of the human esophagus (Adapted from the “Gastroenterology and hepatology resource centre, John Hopkins Medical Institution” website) [6]

2.1.2 Anatomy of the esophagus

The esophagus is characterized by four layers, which form part of the esophageal wall. It comprises the mucosa, submucosa, muscularis propria, and serosa, as illustrated in Figure 5. The mucosa consists of a stratified squamous epithelium, lamina propria, and muscularis mucosae [30, 33, 34].

Figure 5: Anatomical illustration of the different layers of the human esophagus (Adapted from “Digestive diseases 2004, NIDDK” website) [5]

The extensibility and motility of the mucosal lining in accommodating peristalsis are reflected in the presence of a nonkeratinized mucosal surface, which lines the whole esophagus. This surface layer of stratified squamous epithelium is composed of multiple layers that show different patterns of differentiation [34]. The basal zone, found in the deepest region of the epithelium, occupies approximately 10-15% of the epithelium (1 – 3 cell layers thickness). Basophilic cells are columnar in nature, with dark nuclei, and are responsible for the renewal of more superficial layers through ‘equivalent’ mitosis [33]. Beyond the basal layers are stratified squamous cells that lie in several layers of which up

to 24 have been counted. These layers, known as the pickle and functional layers, consist of cells that are progressively flattened and demonstrate increasing pyknosis of nuclei as they approach the surface [30, 34]. Located immediately beneath the epithelium, the lamina propria consists of loose areolar connective tissue containing a network of collagenous and elastic fibres. Its function is to support the delicate mucosal epithelium, allowing the epithelium to move freely with respect to the deeper structures, and also provides a form of immune defense [33, 35]. Unlike the epithelium, the lamina propria contains much fewer cells, with scattered lymphocytes and solitary lymph follicles [34]. The muscularis mucosae form the boundary between the mucosa and submucosa. It follows the mucosal longitudinal folds and is composed of smooth muscle fibres [32]. The submucosa is a broad layer of loose to moderately dense irregular connective tissue, which facilitates flexibility of movement for the mucosa during peristalsis. It contains blood vessels, scattered lymphocytes and a delicate nerve network called the Meissner's plexus [30, 31, 33]. The thickest layer of the esophagus comprises the muscularis externa. Striated skeletal muscle is found in the cervical part, and often also in the upper thoracic part of the esophagus. More distally, smooth muscle fibres become more abundant, forming about 50% of the musculature [33, 34]. The smooth muscle fibres are oriented in two directions, forming an inner circular layer, and an outer longitudinal layer. These layers are oriented to assist in the coordinated contractility during peristalsis [31, 35]. The outermost layer of the esophageal wall is the adventitia, which is a connective tissue sheath connecting the esophagus to the surrounding organs [33].

2.2 Cancer of the esophagus

As with all cancers, cancer of the esophagus develops as a result of uncontrolled cell division, leading to tumour formation. With cancer of the esophagus, the malignancy starts from the innermost layer, and grows outwards [36].

2.2.1 Types of esophageal cancer

There are two main types of esophageal cancer: squamous cell carcinoma (SCC) and adenocarcinoma (AC) [37]. Because the entire length of the esophagus is protected by a stratified squamous epithelium, SCC can occur anywhere along the esophagus. AC,

however, develops in glandular tissue, which normally does not cover the entire esophagus. The occurrence of AC is often preceded by columnar epithelial metaplasia and usually arises in the distal end of the esophagus. Worldwide, most esophageal cancers are SCC, with the incidence markedly higher in men than in women. The incidence of esophageal cancer also increases with age, with people over the age of 35 years at greater risk [38]. There has been a sharp increase in Western countries, in relative and absolute numbers of AC of the lower third of the esophagus. In 1975, the ratio of SCC to AC cases in the U.S was 3:1. Since 1999, esophageal AC has been more prevalent, with an overwhelming ratio of 1:5 [23].

2.2.2 Causes of esophageal cancer – Risk factors

Distinct sets of risk factors have been identified for both SCC and AC. The main risk factors for SCC include age, tobacco smoking and alcohol consumption [38]. The incidence of esophageal cancer increases with age, with an estimated 75% of people diagnosed falling between the ages of 55 and 85. It has also been reported that the risk of esophageal cancer is eighteen-fold for a person who drinks more than 13 ounces of alcohol daily for years, and this risk is increased 44 times with the same person smoking 1 to 2 packs of cigarettes [37]. Other risk factors include lye ingestion, achalasia (defect of the lower esophageal sphincter), and frequent drinking of very hot liquids, among others.

In the case of AC, well known risk factors such as long-standing gastroesophageal reflux disease (GERD), Barrett's esophagus (BE), obesity and gender are acknowledged to play a role in its development [38]. Commonly known as 'heartburn', GERD occurs as a result of intermittent lowering of the lower esophageal sphincter pressure, leading to backwash of stomach acid and bile into the esophagus [39]. Continued exposure of the stomach contents to the esophagus causes the normal squamous epithelium to be replaced by abnormal columnar epithelium, due to esophagitis. This, in part, can give rise to the condition of BE [38]. BE is known to precede the development of esophageal AC. The risk of developing AC is 30 to 125 times higher for people who suffer from BE, than

those who don't [5]. Obesity is also an issue, with the risk of dying from this cancer increased by 50% in obese men [37].

2.2.3 Current treatments for esophageal cancer

The selection of a treatment plan for esophageal cancer is only possible after the cancer has been diagnosed and staged. Staging is a process of determining the spread of the cancer using imaging techniques. It ranges from stage 0 (the earliest stage of esophageal cancer where the cancer cells are limited to the epithelium), to stage IV (spreading of esophageal cancer to more distant lymph nodes and distant sites). With the early stages of cancer, treatment options that are available include surgery, chemotherapy, and radiation therapy. In the case of metastatic or unresectable esophageal cancer, palliative therapy is the only option offered to relieve pain since the disease is incurable at this late stage [37].

As with mid stage cancers (pT1 and pT2), esophagectomy is the most common operative approach for macroscopically curative resections [26]. This procedure requires the removal of the diseased part of the esophagus, along with a small amount of the proximal stomach, as seen in Figure 6(a). For reconstruction of the gastrointestinal continuity after esophagectomy (Figure 6b), the entire stomach, or a gastric tube construction from the greater gastric curvature is transposed through the chest and anastomosed with the esophageal remnant [40]. However, the prognosis for esophageal cancer after radial resection is worse than that for other gastrointestinal malignancies. The disease recurs in approximately half the patients after curative resections [41]. To reduce the high incidence of recurrence in cervical and superior mediastinal lymph nodes, extended lymphadenectomy is routinely performed as an alternative [42]. Yet one of the major disadvantages of such procedures is its high rate of morbidity and mortality, with rates in the range of 1-12% for mortality, and 50%-60% for morbidity rates [43, 44].

2.2.4 Tissue engineered alternatives

Much effort has gone into the development of an artificial esophagus, to circumvent post-operative complications in surgical procedures, and to improve the quality of life in patients after esophageal cancer surgery [45-51]. Early attempts with materials such as woven Teflon and Marlex mesh resulted in failure due to anastomotic leakage, stenosis, and dislocation of the artificial construct [49-51]. Later trials by Takimoto *et al.* and Miki *et al.* using bilayered scaffolds of collagen and biodegradable polyesters or silicones [45, 47, 48] have yielded more promising results, with the regeneration of a stratified squamous epithelium, esophageal glands and muscle tissue in the submucosa similar to native esophagus up to 12 months. However, problems of stenosis and contraction of the regenerated esophageal tissue occurred with the removal of the silicone stent. The use of small intestinal submucosa (SIS) and urinary bladder (UBM) acellular matrices as scaffolds for esophageal reconstruction by Badylak *et al.*, have also shown potential, but for graft stricture complications in complete tubes [52]. While a variety of methods and materials have been explored in developing artificial esophageal constructs, many of them are still far from clinical applications.

2.3 Design and selection of tissue engineered scaffolds

The tissue engineering paradigm in creating tissue engineered constructs is to isolate cells from a patient, grow them on three dimensional biomimetic scaffolds under controlled culture conditions, and direct new tissue formation into the scaffold *in vivo* which can be degraded over time [53]. Hence, the selection of biomaterial scaffolds is an important role in most tissue engineering strategies. The scaffold serves as a mechanical support for cell growth, progenitor cells undergoing differentiation into specific cell types, and inductive growth factors that can modulate cellular activities [54]. Listed below are several criteria which should be included in the design of tissue engineered scaffolds for successful applications.

2.3.1 Biomimetic microenvironment

A scaffold that is to be used successfully in its desired application ought to provide an appropriate regulation of cell behaviour, in terms of cell adhesion, proliferation, differentiation, and migration, in order to promote the development of functional new tissue. In designing scaffolds, one approach is to mimic the functions of extracellular molecules naturally found in tissues. Therefore, a thorough understanding is required of the features of the native substrata (extracellular matrix) for overlying cellular structures. Researchers have discovered that features such as topography and the composition of the native extracellular matrix (ECM) play an important role in cellular behaviour. The extracellular matrix represents a collection of structural (collagens, elastin) and functional proteins (proteoglycans, glycoproteins), which provide an ideal environment in the regulation of cell behaviour [12]. Its topography spans a hierarchical number of structures ranging from micro scale down to nanoscale (e.g. the 66nm repeat banding of collagen fibres) [55], with each playing a critical role in directing cell function. Much work has been reported on fabricating scaffolds that replicate the *in vivo* geometry and dimensional size scale, and it has been proven that such design in scaffolds aid in the maintenance of an *in vivo* like cell phenotype [55-57].

2.3.2 Biocompatibility

The concept of biocompatibility revolves around the interaction between a biomaterial and its environment. It can be defined as the ability of a material to perform with an appropriate host response in a specific application [58, 59]. Biocompatibility comprises several aspects, namely, cytotoxicity, immunological response, local tissue responses (healing process), blood compatibility, carcinogenic, teratogenic, and mutagenic responses. Cytotoxicity is a phenomenon which takes place when a biomaterial or its degraded products exhibit a toxic effect, causing death at the cellular level. *In vitro* tests for cytotoxicity are explained in detail by Hanson *et al* [60]. The local tissue response involving the stages of inflammation, wound healing and foreign body response are considered part of the host response to injury. Upon implantation of a biomaterial, the initial inflammatory response is initiated, leading to the infiltration of neutrophils, monocytes and fibroblasts. Monocytes transform to macrophages, and attempt to phagocytose what the body views as a foreign body. A foreign body response is triggered when the biomaterial is unable to be broken down by the macrophages, leading to a fibrous encapsulation by the action of fibroblasts depositing collagen around the biomaterial. Immunological response is defined as the response of a host body defending itself against invasion by foreign antigens, often found on biomaterials [59]. This leads the immune system to kill cells via the complement and classical pathways. It is noted that while cytotoxicity is considered to be an *in vitro* evaluation, both local tissue responses and immunological responses can only be ascertained in an *in vivo* situation.

2.3.3 Biodegradation and bioresorption

Biodegradation and bioresorption are important criteria which a biomaterial should possess, in order to avoid inflammation while supporting the reconstruction of a completely normal tissue. With the permanent presence of a biomaterial *in vivo*, the risks of inflammatory and foreign body responses are more likely to occur. Care must be taken to ensure that the rate of degradation and concentration of degradation products are at a

tolerable level such that they will not trigger inflammation or toxicity, and can be removed from the body via metabolic pathways [61].

2.3.4 Mechanical properties

A biomaterial should possess appropriate mechanical properties to regenerate tissues with predefined shapes and sizes. One of the main roles of biomaterial scaffolds is to serve as a platform in bringing desired cell types into contact in an appropriate three-dimensional environment [62]. Hence, biomaterials must provide temporary mechanical support sufficient to withstand *in vivo* forces exerted by surrounding tissue and to maintain a potential three dimensional space for tissue development. This mechanical support of the biomaterials should be sustained until the engineered tissue has sufficient mechanical integrity to support itself, and can be achieved by an appropriate selection of mechanical and degradative properties of the biomaterials. Depending on the applications of these scaffolds (e.g. soft and hard tissue engineering), scaffold processing techniques can also play a role in determining on the mechanical properties of a particular scaffold [63].

2.4 **Types of scaffold materials**

Exogenous ECMs for tissue engineering can be fabricated from two classes of biomaterials, namely (1) naturally occurring biomaterials and (2) synthetic biomaterials. The selection for a biomaterial for use as scaffolding plays a key role in the development of a tissue engineered product or application. Not only must the biomaterial possess biocompatibility, it must also elicit a desirable cellular response, by having the ability to control and manipulate cellular interactions [64].

2.4.1 Naturally occurring scaffolds

The main naturally derived biomaterials in scaffolds are of varying chemical natures. They comprise polypeptides, polysaccharides, polyesters and inorganic materials. Some examples include biomaterials such as collagen, alginate, and acellular tissue matrices [61, 65, 66].

Collagen is the most abundant and ubiquitous structural protein in the human body, and is found in high concentrations in tendon, skin, bone and fascia [67]. Because of its integrin

binding domains (e.g. RGD sequences), it is able to assist in the growth, differentiation, replication and metabolic activity of most anchorage dependent cell types in culture. Toolan *et al* reported that chondrocytes retained their phenotype and cellular activity when cultured on collagen [68, 69], while Silver *et al* proved that different cell types grown on collagen matrices displayed the identical morphology and metabolism of cells *in vivo* [70, 71]. Much work has also been reported on collagen based artificial ECM for dermal replacements and skin graft substitutes [16, 72, 73], indicating its success as a substrate for new tissue growth.

Alginate, a polysaccharide isolated from sea weed, has been used in several applications ranging from injectable cell delivery vehicles and cell immobilization matrices [74, 75]. It is relatively biocompatible and has been approved by FDA for human use in wound dressing [66].

Acellular tissue matrices are collagen rich matrices that are fabricated by the removal of cellular components from tissues, a process termed as decellularization. Upon implantation, they are replaced and remodeled by ECM proteins synthesized by transplanted cells. More will be elaborated upon in Sections 2.5 and 2.6.

2.4.2 Synthetic scaffolds

The design and synthesis of synthetic, biodegradable polymers for scaffold development is currently an important research challenge in the field of tissue engineering. The most commonly investigated classes of such polymers include the poly (α -hydroxy acids), poly (glycolic) acid, poly (lactic) acid and their copolymers [64]. These polymers are widely used in tissue engineering, and have gained FDA approval for human use in a variety of applications e.g. sutures [62, 64, 66, 76]. Although the degradation products of PGA, PLA, and PLGA are slightly toxic to cells due to their low pH, they are nevertheless eliminated from the body via the respiratory route [62, 77]. The degradation rate of these polymers can also be tailored from weeks to years by alteration of their crystallinity, molecular weight and copolymer ratio of lactic acid to glycolic acids. Because these polymers are thermoplastic, they can be easily formed into porous, three dimensional shapes through an astonishing array of fabrication techniques such as gas foaming, fibre extrusion and bonding, phase separation, emulsion freeze drying, three dimensional

printing, and porogen leaching [78-81]. These techniques play a critical role in achieving topographical, spatial, chemical and immunological control over cells, and thus create more functional tissue engineering constructs that are capable of supporting cell and tissue growth, with the ability to match the properties of organs they are to replace [62].

2.4.3 Natural vs. synthetic scaffolds

It is widely acknowledged that biological materials have some remarkable advantages over synthetic materials. Not only do they possess biological recognition, they are superior in terms of physiological activities such as selective cell adhesion [65, 66]. They also possess mechanical properties similar to natural tissues, and are biodegradable in nature. However, certain issues such as the risk of viral infection, antigenicity, and batch to batch variations have to be contended with still. While the greatest limitation of synthetic materials lies in its lack of cell-recognition signals, it offers several advantages that have led to widespread use. Synthetic materials can be manufactured reproducibly on a large scale, and properties such as mechanical strength, macrostructure, and degradation time can be readily controlled and manipulated [77, 82].

2.5 **The extracellular matrix**

The ECM is a structural material secreted by cells populating a given organ/tissue, and lies immediately beneath the epithelium, while surrounding the connective tissue cells [83]. The composition and structure of the ECM are influenced by factors determining the phenotype of resident cells, such as mechanical forces and biochemical expression. The ECM, in turn, serves as a medium between cells [84]. As such, the ECM is considered to be in dynamic equilibrium with its cells, affecting their attachment, migration, proliferation and three-dimensional spatial arrangement [12]. A description of the different structural and functional proteins comprising the ECM is discussed below.

2.5.1 Composition of ECM

The ECM is a complex mixture of structural and functional proteins, glycosaminoglycans (GAGs) and small molecules arranged in a unique, three-dimensional architecture [85]. Some of the more prominent proteins are reviewed below.

2.5.1.1 Collagens

Collagen is the most abundant protein in both human and mammalian tissue. More than 20 distinct types of collagen have been identified, each with a unique biological function [85]. Based on their structure and supramolecular organization, they can be grouped into fibril-forming collagens, fibril-associated collagens, network-forming collagens, transmembrane collagens, and basement membrane collagens [86]. The most widespread family of collagen is represented by the fibril-forming collagens (Types I, II, III, V and XI). Type 1 collagen is the most widely studied structural protein present in tissues, and is the major collagen of tendons, skin, ligaments and many interstitial connective tissues. Its main function is to provide strength for tissues subjected to multi-axial mechanical loading [84]. In bone, it defines considerable biomechanical properties involving load bearing, tensional strength, and torsional stiffness [86]. Collagen type III is a homotrimer of three $\alpha 1$ (III)-chains and is widely distributed in tissues comprising collagen type I. Commonly found in organs such as lung, liver, spleen and vessels, it is an important component of reticular fibres in such interstitial tissue. This molecule also contributes to mixed fibrils with collagen type I, and is abundant in elastic tissues. An example would be the submucosa of tissues where flexibility and compliance are required for appropriate function, e.g. urinary bladder [84]. Another form of collagen, type VI, is known as a microfibrillar collagen. Upon secretion into the ECM, collagen type VI tetramers aggregate into filaments and form an independent microfibrillar network in virtually all connective tissues. It functions as a connecting unit between GAGs and larger structural proteins such as collagen type I, thus providing a gel like consistency to the ECM [83]. In summary, collagen is associated with other structural and functional proteins to impart distinctive tissue properties, therefore serving the body in a wide variety of functions.

2.5.1.2 Fibronectin

This protein exists as a multi-functional glycoprotein in the ECM [87]. It is a dimeric molecule of 250 000 MW subunits, and is found in both soluble and tissue isoforms [88]. Fibronectin is known to play an important role in cell adhesion, migration and

differentiation. Its primary function is to attach cells to various ECMs, via specific high-affinity binding sites for cell surface receptors. These binding domains contain Arg-Gly-Asp (RGD) subunits, a tripeptide that is important in cell adhesion. The protein molecule also contains regions that bind collagen, heparan sulfate, and fibrin [83]. Fibronectin is abundant in the ECM of submucosal tissues, basement membranes, and interstitial tissues [7]. Because of its cell friendly properties, it is commonly used as a coating for synthetic scaffold materials to promote host biocompatibility [89, 90].

2.5.1.3 Glycosaminoglycans

Embedded within the ECM are various mixtures of GAGs, depending on the tissue location of the ECM in the host, and its microenvironment. These molecules exist as linear polysaccharide chains, and are covalently bonded to core proteins to form proteoglycans [83]. GAGs participate in events which regulate cell adhesion, migration and proliferation, and also play a role in the mechanical development of matrix assembly. They bind growth factors and cytokines, promote water retention, and contribute to the gel properties of the ECM [85]. Some of the major classes of GAGs found in the ECM include hyaluronic acid (HA), chondroitin sulfate, heparin, and heparan sulfate [91]. Its desirable wound healing properties has made hyaluronic acid one of the most extensively studied GAG molecules for use as scaffolds in tissue engineering applications [92-94].

2.5.1.4 Integrins

Cells require a variety of cell-surface adhesion receptors to interact with the ECM, the most established of which is the integrin superfamily. Integrins are transmembrane glycoproteins possessing the same basic $\alpha\beta$ heterodimeric structure [95]. They recognize specific peptide sequences in their ligands, such as the RGD sequence in ECM proteins including collagen, fibronectin and laminin. Integrin-ligand interactions trigger a spectrum of signal transduction pathways with wide ranging effects on cell survival, proliferation and gene transcription [95]. Not only do they serve as cell adhesion molecules, they also function as signaling molecules, in allowing cells to respond to local changes in the composition of ECM ligands [83].

2.5.1.5 Growth Factors

Growth factors are bioactive molecules which reside in the ECM, contributing to a wide array of biologic signals. Though present in extremely small amounts, they act as potent modulators of cell behaviour. Growth factors tend to exist in multiple isoforms, each with its specific biologic activity [84]. Examples of growth factors found in the ECM include vascular endothelial cell growth factor (VEGF), fibroblast growth factor (FGF), epithelial cell growth factor (EGF), and transforming growth factor beta (TGF-beta), among others [85].

2.5.2 The basement membrane

The basement membrane is a unique ECM that underlies a variety of epithelial cells. Its architecture is very much different from the usual fibrillar network of connective tissue matrices. The connective tissue ECM comprises a porous network allowing cell movement while providing structural support. In comparison, the basement membrane is a somewhat dense, sheet-like structure at the interface between epithelial and other tissues. Its unique organization contributes to its functions as a support, a sieve and a barrier, keeping cells and proteins on one side [88]. Not only does it modulate key cellular processes of the overlying epithelium, it also serves to focus growth factors and their receptors during early epithelial morphogenesis [96].

2.5.2.1 Composition of basement membrane

The basement membrane is composed of a complex mixture of constituents including collagen type IV, laminin, entactin/nidogen, and sulfated proteoglycans. In that, the basement membrane is no different from other matrices, with cell-adhesive glycoproteins, collagens for stability, and carbohydrate rich proteoglycans [97]. Two of the major components in the basement membrane are discussed below.

2.5.2.1.1 Collagen type IV

This collagen is present within the basement membrane of most vascular structures, and also within tissues possessing an epithelial cell component [98]. It is arguably the most important structural constituent, integrating laminins, nidogens, and other components into the visible two-dimensional stable supramolecular aggregate. The ligand affinity of collagen type IV for endothelial cells has led it to be widely used as a biocompatible coating for biomaterial-blood interfaces in medical devices [85].

2.5.2.1.2 Laminin

Laminin is a major component of the basement membrane, and consists of three disulphide-linked chains forming a characteristic cross shape [85]. Up to 12 distinct laminin isoforms have been isolated [11]. While its primary role is to anchor cells to the basement membrane (adhesion to collagen type IV via nidogen links), it has a variety of effects on adjacent cells. Laminin 1 induces differentiation in epithelial cells, while laminin 5 is involved in cell adhesion and migration [11]. Degradation of laminin *in vivo* could lead to liberation of growth factor activity that stimulates growth in regions of tissue damage, thus triggering tissue remodeling.

2.6 Decellularization

It has been highlighted that naturally derived scaffolds possess many latent advantages compared to synthetic scaffolds when used in tissue engineering applications (Section 2.4.3). In particular, naturally occurring matrices derived from ECM (e.g. small intestinal submucosa) have gained much recognition as scaffolds which elicit an integrative type of tissue response, unlike the typical fibrous encapsulation caused by synthetic biomaterials [16]. Such desirable responses have been contributed to the complex mixture of structural and function proteins comprising the native ECM (Section 2.5), hence making the ECM an ideal biologic scaffold for tissue engineering applications. Decellularization is a technique that has been developed by many research groups to isolate the ECM from various tissues for applications such as tissue graft constructs and cell culture substrates [99-106]. This technique, as defined by Atala *et al.*, is the removal of parenchyma tissue, leaving behind the three-dimensional interstitial structure of connective tissue [107]. The primary goal of decellularization is to efficiently remove all cellular and nuclear material,

while minimizing any adverse effects on the composition, biological activity, and mechanical integrity of the resulting ECM. In the following sections, the varying mechanisms of decellularization developed by different research groups will be explored, along with the advantages and disadvantages of such a technique employed, while also considering the applications of these acellular matrices in the tissue engineering field.

2.6.1 Methods of decellularization

The most commonly utilized methods of decellularization involve a combination of different treatments. These methods serve to disrupt the cellular membrane by cell lysis and release cytoplasmic and nucleic contents to aid in the development of an acellular ECM. The ECM must be adequately disrupted during the decellularization process to allow for exposure of cells to reagents of choice, while providing a path for cellular matter to be removed from the tissue. Depending on the type and source of ECM, different decellularization methods tend to yield varying degrees of success. It is known that any processing step designed to facilitate cell removal will alter the native three-dimensional architecture of the ECM. Therefore, a decellularization process must be chosen with the intention to develop acellular tissues, yet retain native biological and mechanical properties. There are three broad categories of decellularization methods, namely (1) physical, (2) chemical, and (3) enzymatic processes. Listed below is a brief description of each category.

2.6.1.1 Physical methods

There are several methods of decellularization used by different researchers, which have been classified as physical methods. These include snap freezing, sonication and mechanical agitation [108]. Snap freezing has been utilized extensively in the decellularization of tendinous and ligamentous tissue, as reported by Roberts *et al.* [101, 109]. In theory, intracellular crystals are formed, which causes cell lysis, when tissue is rapidly frozen. However, the rate of temperature gradient must be controlled to avoid causing damage to the resulting ECM as a result of the formation of the intracellular crystals. Also, while freezing is successful in disrupting the cellular membrane, other processes must be incorporated to facilitate the removal of cellular and nucleic content.

Mechanical agitation [102, 104, 110] and sonication [100] are usually used in tandem with chemical treatments to assist in cell lysis and the subsequent removal of cellular debris. Mechanical agitation takes many forms, including magnetic stir plates, orbital shakers, and low profile stirrers. It is noted that the processing parameters of speed, volume of reagent, and duration of mechanical agitation must be optimized depending on the tissue being decellularized.

2.6.1.2 Chemical methods

2.6.1.2.1 Acid treatments

Acid treatments are generally used to remove nuclei acids and solubilize cell cytoplasm. Examples of acid solutions used include acetic acid, peracetic acid (PAA) and hydrochloric acid. This thesis will concentrate on PAA and its uses in decellularization, as it is the primary decellularization reagent used in this research work.

Peracetic acid or peroxyacetic acid is the peroxide of acetic acid. It is available in the form of a quaternary equilibrium mixture containing acetic acid ($\text{CH}_3\text{CO}_2\text{H}$), hydrogen peroxide (H_2O_2), water (H_2O) and PAA ($\text{CH}_3\text{CO}_3\text{H}$) as shown in the following equation [111]:

PAA is a clear, colourless liquid with no foaming capability. It is soluble in water in all proportions and in polar organic solvents, but its solubility in aromatic solvents is limited. PAA solution is produced from the reaction of acetic acid or acetic anhydride with hydrogen peroxide (HP) in the presence of sulfuric acid, which acts as a catalyst. Also, a stabilizer or a sequestering agent is utilized during the production of PAA. The reaction is allowed to continue for up to 10 days to yield maximum amounts of PAA as its product. PAA is considerably less stable than HP. A 40% PAA solution loses 1% to 2% of its active ingredients per month, while a 30% to 90% HP solution loses less than 1% per year. Dilute PAA solutions are even more unstable, with a 1% solution losing half its strength through hydrolysis in 6 days [112]. However, commercially available PAA

solutions (10% - 15%) used in the industry are a lot more stable compared to higher and lower end strength solutions.

PAA is a well-known and widely used disinfectant in many industries. The disinfection efficiency of PAA towards microorganisms can be ranked as following on a general basis: Bacteria > viruses > bacterial spores > protozoan cysts [113]. Its disinfectant activity is based on its rapid penetration into microorganisms, and the release of active oxygen. It is likely that sensitive sulfhydryl and sulfur bonds in proteins, enzymes, and other metabolites are oxidized and that double bonds are reacted [112]. It is also suggested that PAA disrupts the chemiosmotic function of the lipoprotein cytoplasmic membrane and transport through dislocation or rupture of cell walls [114]. Therefore, it is equally effective against outer membrane lipoproteins, facilitating its action against Gram-negative cells. Additionally, intracellular PAA may also oxidize essential enzymes, thus causing vital biochemical pathways, active transport across membranes, and intracellular solute levels to become impaired [115].

Due to its effectiveness against a wide spectrum of bacteria and viruses, PAA is utilized in many industrial and environmental applications. PAA has been found to be an efficient bactericidal, virucidal and fungicidal agent in wastewater disinfection. Baldry and French found PAA to be an effective disinfectant for secondary effluent and stated that the ease of implementing PAA treatment without expensive equipment, the broad spectrum activity even in the presence of organic matter, and the lack of environmentally damaging by-products make PAA a favourable agent for wastewater treatment [116]. PAA has also been used for environmental applications such as a disinfectant for ion exchangers, cooling towers, combined sewer overflows and membrane hollow fibres [112]. It has been shown to be effective for pathogen reduction in biosolids, reduction in solid odours, and food sludge debulking [112]. Another application of PAA is in the medical and pharmaceutical industry. It has been listed in the Centres of Disease Control of the United States as a chemical sterilant and high-level disinfectant. Large antimicrobial spectrum, short exposure time, and nontoxic decomposition products make PAA an ideal disinfectant for medical equipment [112].

Because of the advantages that PAA possesses, its use as a decellularization reagent was investigated in the course of this research project. PAA is known to be an extremely

strong oxidizing agent that disrupts cell wall permeability by oxidizing sulf-hydrl (-SH) and sulfur (S-S) bonds [117], which aids in decellularization. Hydrogen peroxide present in PAA is another strong oxidizing agent that generates reactive hydroxyl radicals. These radicals attack membrane lipids, DNA, and essential cell components [118]. More importantly, PAA is known to inactivate catalase, an enzyme known to detoxify free hydroxyl radicals [112]. Metal ions present within cells help to catalyze hydroxyl radical formation, thus increasing the efficacy of PAA in decellularization. PAA has also been demonstrated to act on the bases of the DNA molecule, rendering it effective in decellularization.

Even though an extensive body of data exists to support the effectiveness of PAA in disinfection and sterilization processes, most of the research is focused on the use of PAA to disinfect hospital equipment and reusable, medical instruments. Little information exists in support of the use of PAA as a sterilant for naturally occurring bioscaffolds, and even less information is available regarding its use as a decellularization agent. Thus far, PAA has been found to be efficient in removing cellular matter from thin ECM structures and disinfecting them. Decellularized scaffolds treated with PAA at concentrations approximately 0.1-0.15% (w/v) were found to retain many important components of native ECM proteins and GAGs such as laminin, fibronectin, hyaluronic acid and heparin [91, 119, 120]. PAA was also able to preserve the structure and function of many growth factors present in native ECM, including basic fibroblast growth factor [121].

2.6.1.2.2 Non-ionic detergents

This class of detergents is used in a wide variety of decellularization processes, because of their relatively mild effect on tissue structure. The mechanism of non-ionic detergents lies in its ability to disrupt lipid-lipid and lipid-protein interactions, while leaving protein-protein interactions intact. This leads to the hypothesis that protein structures retain their functional conformation despite being exposed to non-ionic detergent treatments [122]. One of the most extensively studied non-ionic detergents utilized in decellularization processes is polyethylene-glycol-octylphenyl-ether, more commonly known by its trade name of Triton X-100. Various research groups have explored Triton X-100 treatments in decellularization, ranging from exposure durations of hours to days [99, 103, 123-125].

However, the efficacy of the treatments varied depending on the type of tissue being decellularized. In the decellularization of heart valves, Grauss *et al.* reported complete cell removal within 24 hours of Triton X-100 treatment, yet cellular remains were still observed in adjacent myocardium and aortic walls. The treatment also led to a complete loss of GAGs and basement membrane proteins in the resulting ECM [105, 125]. Conversely, there was no reported loss of GAGs when anterior cruciate ligaments were treated with Triton X-100 even after 4 days, despite incomplete decellularization [104]. It has been suggested that a combination of Triton X-100 along with other decellularization processes would work better in attaining complete cell removal, thus optimizing a given decellularization protocol.

2.6.1.2.3 Ionic detergents

It has been reported that while ionic detergents are effective in solubilizing both cytoplasmic and nuclear cellular membranes, it is known to denature proteins by interfering with protein-protein interactions [122]. Commonly used ionic detergents include sodium dodecyl sulfate (SDS) and sodium deoxycholate [102, 124, 126-128]. Unlike Triton X-100, SDS treatments are harsher, resulting in a disruption of the native tissue architecture and loss of collagen integrity. However, it does ensure a complete removal of nuclear remnants, and cytoplasmic proteins, hence its continued use by several researchers. Treatments with sodium deoxycholate were found to have similar results compared with SDS treatments; however its effect on native tissue architecture was more damaging.

2.6.1.2.4 Hypotonic and hypertonic treatments

Hypotonic and hypertonic solutions rely on the mechanism of osmotic shock to trigger cell lysis. Some of these solutions include deionized water or low ionic strength solution [83, 102, 104, 124]. As reported by Dahl *et al.*, 11 hours of hypotonic and hypertonic solutions was adequate to cause cell lysis, but it was incapable of removing cellular remnants from the tissue [124]. Again, it is recommended that this particular form of treatment be coupled with other decellularization processes to optimize a particular decellularization protocol.

2.6.1.2.5 Chelating agents

Chelating agents form ring-shaped molecular complexes, which binds and isolates central metal ions. Studies have proven that divalent cations are crucial in cell attachment to ECM proteins such as collagen and fibronectin [129, 130]. By binding to the cations that are present at the cell adhesions to the ECM, these agents are able to assist in cellular removal.

2.6.1.3 Enzymatic methods

This form of decellularization functions by separating cellular components from the ECM. Enzymes commonly used include proteases, calcium chelating agents, and nucleases [131, 132]. Proteases, such as trypsin, are highly specific enzymes that cleave the peptide bonds between arginine and lysine residues. Nucleases such as endonucleases catalyze the hydrolysis of terminal bonds of deoxyribonucleotide or ribonucleotide, resulting in degradation of DNA and RNA [133]. The use of enzymes in decellularization experiments has been studied, and its effects are varied. In decellularization of porcine pulmonary valves, cellular removal was complete using a combination of 0.05% trypsin and 0.02% EDTA for 24 hours [134]. However, a treatment of 0.5% trypsin and 0.05% EDTA for 17 hours left cells non-viable, but still present in the resulting ECM [135]. It has also been discovered that the use of enzymes presents certain adverse effects on the resulting ECM after treatment. Prolonged treatment with trypsin/EDTA led to undesirable changes in the ECM structure of porcine pulmonary valves, along with a substantial reduction in ECM protein content such as laminin and fibronectin. Elastin and GAGs content also suffered a decrease, and as much as 50% reduction in tensile strength of the ECM was observed [134, 135]. Due to the inherent damage caused by enzymatic treatments to the ECM, it is best to limit the exposure of such treatments in decellularization.

2.6.2 Advantages and limitations of decellularization

2.6.2.1 Advantages

With decellularization, the resulting ECM that emerges is one that possesses the same three-dimensional interstitial structure, and has the same shape and size as the native organ. In the case of synthetic scaffolds, they often have to be shaped into the desired configuration of the organ, to ensure that the reconstructed organ may function properly *in vivo*, with the correct interstitial structure and microenvironment. Therefore, the choice of appropriate material and processing technique is critical. Also, the shaping process may produce undesirable effects on mechanical strength, or scaffolds with irregular three-dimensional geometries [107]. As described in Section 2.5, the composition of the ECM is a complex mixture of structural and functional proteins, glycosaminoglycans, glycoproteins and other molecules arranged in a tissue specific histoarchitecture. The diversity of collagen types, the association with non-collagenous molecules, would make it a difficult challenge in trying to reconstruct such a composite scaffold *in vitro* with synthetic materials. However, with an optimal decellularization process, such difficulties are negligible with the rich source of structural and functional proteins preserved in their relative concentrations and orientation. Another advantage of utilizing decellularization to prepare naturally occurring ECM as scaffolds or cell substrates is the presence of all growth factors in the same relative amounts and three-dimensional architecture. It is hypothesized that the ECM, during the course of degradation, acts as a controlled release vehicle for such growth factors, when used in tissue engineering applications [136].

2.6.2.2 Limitations

One of the major concerns of decellularized matrices is the issue of xenotransplantation and its subsequent effect of immunogenic rejection. It is known that the major barrier to xenotransplantation is the presence of natural antibodies to the terminal galactose alpha 1,3 galactose (alpha-gal) epitope. This epitope is expressed on the cellular membrane of all mammals except humans and old world primates [137, 138]. Although ECM derived from porcine tissues contains small amounts of the gal epitope, it appears that the quantity and distribution, or the subtype of immunoglobulin response to the epitope is such that complement activation does not occur. A study conducted by Badylak *et al.* showed that gal^{-/-} mice with naturally occurring anti-gal antibodies failed to show any

significant effect upon graft remodeling when implanted with porcine derived ECM [139].

2.6.3 Tissue engineering applications of decellularization

Xenogeneic-sourced acellular ECM has been used as scaffolds in the replacement and reconstruction of many tissue types. This section discusses the use of ECM in a variety of tissue engineering applications, and forms the rationale for applying such a technique in the field of esophageal tissue engineering.

2.6.3.1 Reconstitution of skin

Acellular ECMs are excellent substitutes in the area of skin engineering. Examples of tissue engineered skin include acellular human dermis (Alloderm®) and porcine SIS (OaSIS™). Studies have shown that these acellular scaffolds positively affect the healing of chronic ulcers and traumatic skin wounds. Not only do they provide bulk as dermal substitutes, they also serve as ideal substrates for analogous keratinocyte growth [106, 140]. Another category of tissue engineered skin substitutes consists of composite cellular scaffolds with neonatal fibroblasts and keratinocytes, such as Dermagraft™ and ApliGraft®. These have also proven to be excellent scaffolds, though the distinction between acellular scaffolds, and acellular scaffolds with cellular components, has yet to be defined.

2.6.3.2 Arterial graft engineering

The use of acellular matrices, especially porcine SIS, has been investigated broadly in the area of vascular grafts. It has been reported that these xenogeneic grafts displayed exceptional long-term patency and extensive remodeling of blood vessel wall structures [105]. In a study involving the replacement of infrarenal aorta with SIS scaffolds in dog models [105], it was shown that a confluent endothelial cell growth was detected on the surface within 28-42 days. An intimal layer consisting of organized layers of collagen, together with an external layer of contractile spindle shaped cells, stained positive for smooth muscle actin, was detected. These results proved the success of SIS ECM in promoting organized and differentiated cell types instead of fibrous encapsulation.

Promising results were also seen with these scaffolds in venous graft reconstruction [141] and venous valve replacements [142].

2.6.3.3 Lower urinary tract engineering

One of the technologies for lower urinary tract regeneration lies in the development of acellular collagen-based tissue grafts (xenogeneic or autologous) derived from stomach, bladder and small intestine. Pioneering work done by Tanagho [143-145] and Atala [99, 146, 147] in animal models of urethral, uterine, bladder wall and vaginal replacement have led to the successful use of these tissue grafts in human patients. These studies have reported that the implanted scaffolds degraded rapidly, leaving behind organized and functional smooth muscle and site appropriate urothelium [143]. Sutherland *et al.* have also reported good bladder regeneration in rat bladders using allografts of acellular rat stomach and bladder [148]. The results demonstrated full thickness bladder regeneration, and suggested that cell to cell signaling facilitated the induction and development of bladder smooth muscle and regeneration. SIS has also shown great promise as an acellular tissue graft for the reconstruction of the urinary tract. Kropp has described the use of SIS in bladder augmentation of both rats and dogs, and within three months, the implanted tissue graft was indistinguishable from the native bladder, with the remodeled tissue displaying typical contractile activity [149].

2.6.3.4 Orthopedic applications

ECM scaffolds have been successfully utilized in the field of orthopaedic engineering, especially soft tissues such as musculotendinous and ligamentous tissues. Some of these commercial ECM derived products that are available in the market for such applications include RESTORE™, Cuff Patch™, Graft Jacket™ (the orthopaedic version of Alloderm), and TissueMend™ [150]. Implantation of these scaffolds has shown the remodeled tissue to possess bundles of musculoskeletal tissue instead of the usual scar tissue formation at the site of injury.

2.7 Histological and histochemical methods

Histology is the study of minute structures of cells, tissues and organs in relation to their function. Histological sections are usually prepared for study with the aid of a light microscope. Tissue preparation includes fixing samples in a fixative to prevent autolysis, embedding the sample in an embedding medium for sectioning, and mounting thin sections on adhesive-coated glass slides. The slides are then viewed under a light microscope. Depending on the component of interest in the tissue section, different staining procedures are used to produce an insoluble coloured compound that labels the particular component visible under the light microscope. Described in the following sections are three commonly used histological methods used to stain different components in animal tissue.

2.7.1 Hematoxylin and Eosin (H & E) staining

H & E staining is one of the most common nuclear and simple counterstaining procedures used to study microscopic anatomy. The nuclei of cells are stained a dark colour, and a lighter, contrasting colour is imparted to the cytoplasm and ECM. The hematoxylin dye consists of haematein (the principle oxidation product of hematoxylin), and Al^{3+} ions [151]. The cationic dye-metal complex present in the dye solution binds to the DNA in tissue sections, as the Al^{3+} ions have high affinity for DNA, thus staining it a dark blue. Eosin Y is then used as a counterstain, colouring the other tissue components a pale pink. It is an anionic dye that binds principally to ionized cationic groups of protein molecules such as the ϵ -amino group of lysine and guanidino group of arginine [151]. Since nearly all proteins contain these two amino acids, eosin is often bound to almost all structures in the tissue.

2.7.2 Trichrome staining

Trichrome staining involves staining techniques in which two or more anionic dyes are used together with a heteropolyacid such as phosphomolybdic acid (PMA) or phosphotungstic acid (PTA) solution. These acids are water and alcohol soluble crystalline compounds, and are usually applied to the sections sequentially, between treatments with different dyes. Tissue sections stained with trichrome dyes result in cell

nuclei being coloured black, collagen blue, and cytoplasm and muscle a dark red. The mechanism for trichrome staining depends on the attraction between cationic dyes and the free negatively charged groups of collagen-bound ions of heteropolyacid [152]. The dyes used to stain collagen in trichrome techniques are all amphoteric, and are bound by ionic forces to PMA or PTA, which is itself attached electrovalently to the tissue section. Since the cytoplasmic stains used in the trichrome dyes are anionic, they will not attach to the free negatively charged sites of the bound heteropolyacid molecules. There are different trichrome techniques that are currently practiced, namely Masson's trichrome, Mallory's trichrome and Heidenlain's AZAN. These techniques are done in stages, which allow some control over the intensity of colour in cytoplasm and collagen.

2.7.3 Immunohistochemical staining

Immunohistochemical (IHC) staining is based on the affinity between antigens and antibodies. When a molecule of antigen is in contact with a molecule of its antibody, the two combine to form an antigen-antibody complex in a reaction similar to the 'lock and key' mechanism demonstrated by an enzyme and its substrate. The complex is held together by non-covalent bonds such as hydrophobic interaction, hydrogen bonding and ionic attraction. When an antigen which forms part of the tissue section is mixed with a solution containing appropriate antibodies, it forms an insoluble antigen-antibody complex which usually precipitates. The complex can then be labeled by conjugating them with fluorochromes or with histochemically demonstrable enzymes. The different techniques of IHC staining are based on the use of labeled antibodies. Such techniques include direct fluorescent antibody methods, enzyme-labeled antibody methods, and avidin-biotin methods. The use of a particular method depends on the type of tissue section being evaluated, the need to enhance sensitivity of the labeling, and the ability to suppress attachment of reagents to tissue by mechanisms other than specific antigen-antibody affinity [153].

CHAPTER 3 METHODOLOGY

This section describes the methodologies used in my research work on the tissue engineering of the esophagus. Section 3.1 describes the anatomical and structural characterization of a porcine esophagus using histological methods and electron microscopy. Section 3.2 provides the basic experimental set up and the different protocols developed to optimize the decellularization process for porcine esophageal tissue. Section 3.3 describes the studies on cell interactions with the decellularized scaffolds. Finally, section 3.4 elaborates on the methods used to characterize the decellularized scaffolds.

Figure 7: Summary of methodologies

3.1 Characterization of Native Porcine Esophagus

3.1.1 Harvesting of porcine esophageal tissue

Porcine esophagi were harvested from adult pigs at a local abattoir. The length of the esophagus was dissected between the cervical and the lower esophageal sphincter portions, and the mid-segment of 5cm was resected. The excised esophageal tissue was then stored in sterile phosphate buffered saline (PBS) supplemented with an antibiotics cocktail of penicillin, kanamycin and streptomycin (1:100). This antibiotics cocktail was subsequently used to supplement all storage solutions for treated samples. The physiologic saline solution was kept chilled at 4°C as the samples were transported back to the laboratory.

3.1.2 Characterization of native porcine esophageal tissue

Histological and scanning electron microscopy imaging were used to characterize the native porcine esophagus. The procedure for sample processing and histochemical staining would be described in Section 3.3.2. The slides were then viewed under light microscope (Olympus CKX41) at magnifications between 40X and 400X, and the images were captured using Olympus optical DP controller. To ascertain the general topography and histoarchitecture of the samples, scanning electron microscopy (SEM) was used. Both adluminal and abluminal surfaces were observed under SEM at different magnifications. The procedures for sample preparation for SEM would be described in Section 3.3.1.

3.1.3 Characterization of native basement membrane (BM)

Porcine esophagus was harvested and the muscularis externa layer removed to leave behind the endomucosa, as described in Section 3.1.1. A piece of endomucosa measuring 3 mm by 3mm was excised, and incubated in 50 units/ml of dispase overnight at 4°C to remove the native epithelium and expose the basement membrane. Both adluminal and abluminal surfaces of the treated tissue were viewed under SEM and light microscope.

3.2 Decellularization set up and experiments

This section covers the different decellularization processes that were developed and evaluated. The following flow chart below summarizes the different steps involved in the decellularization processes.

Figure 8: Flowchart of decellularization processes

3.2.1 Development of chemical- based (PAA) decellularization protocol

3.2.1.1 Material preparation

In the laboratory, the abattoir-harvested section of the esophagus was thoroughly rinsed with sterile deionized water for 5 minutes at room temperature. The tissue was then split longitudinally to form a sheet (approximately 1.5cm by 5 cm). The muscularis externa

and serosal layers were mechanically removed, leaving behind the endomucosa. The endomucosa was stored in sterile deionized water for 30 minutes at 4°C prior to decellularization. The decellularization reagent, peracetic acid (PAA), was prepared by mixing appropriate amounts of hydrogen peroxide (30%), acetic acid (glacial), and deionized water. The pHs of the different PAA solutions were also measured using a pH/conductivity meter (Fisher Scientific AR20). Listed below are the different formulations of the decellularization reagent that were used in the various decellularization experiments, and their respective pHs.

Table 2: Different PAA formulations and their pHs

PAA %	Components in peracetic acid solution (by vol %)			pH of PAA
	Glacial acetic acid	Hydrogen Peroxide	Deionized water	
1	2	1	97	4
5	10	5	85	3
10	20	10	70	3
15	30	15	55	2
20	40	20	40	2
25	50	25	25	2

The effects of treatment time and concentration reagent were then studied as described below.

3.2.1.2 Effect of treatment time and reagent concentration

The objective of this experiment is to study the effect of treatment time and reagent concentration on the porcine esophageal tissue, and the extent of decellularization. Different concentrations of the decellularization reagent were prepared, ranging from 10% to 25% PAA (vol/vol). 50 ml of each reagent was then dispensed into 100 mm glass petri dishes containing the endomucosal sample. The samples were immersed in this reagent for pre-determined periods ranging from 2 to 24 hours. At the pre-determined treatment time, the endomucosa was removed from the decellularization reagent and placed in an orbital shaker containing sterile PBS solution. After 15 minutes of agitation at 150 rpm, the PBS solution was changed to remove residual traces of PAA. This

process was repeated until the pH of the sample reached 7. After each change, the pH of the sample was determined using a universal indicator strip. This neutralization process was subsequently used in all decellularization processes highlighted in the thesis. The neutralized sample was then stored in sterile PBS supplemented with the antibiotic cocktail and kept in a fridge at 4°C, after which it was characterized using H & E staining, and SEM.

3.2.2 Development of chemical-mechanical decellularization protocol

3.2.2.1 Investigation of optimal scraping tool

This study aims to evaluate and compare the efficacy of using a toothbrush, and a gauze-wrapped glass slide in the chemical-mechanical decellularization process. Both the toothbrush and gauze-wrapped slide were sterilized in 70% ethanol before use. Native endomucosa was extracted and placed in a shallow rectangular petri dish containing 30ml of 5% PAA solution. Both surfaces (adluminal and abluminal) of the sample were scraped with either the toothbrush or the edge of a gauze-wrapped glass slide for 100 and 200 times. After scraping, the sample was neutralized as previously described. The treated samples were then examined for the extent of decellularization using SEM and H & E staining.

3.2.2.2 Effect of mechanical scraping and reagent concentration

The objective of this experiment is to determine the effect of mechanical scraping and varying reagent concentrations on the decellularization of porcine esophageal tissue. Different concentrations of the decellularization reagent were prepared, ranging from 1% to 10% PAA (vol/vol). Native endomucosa was placed in a shallow rectangular petri dish containing 30 ml of the decellularization reagent. The sample was then scraped for 200, 250 and 300 times respectively with a sterilized gauze-wrapped glass slide. After the treatment, it was neutralized and characterized as described above.

3.3 Methods of characterization for decellularized samples

3.3.1 Scanning electron microscopy (SEM) –sample preparation & imaging

For SEM, the samples were transversely cut into 5 mm sections and fixed in 2.5% glutaraldehyde for a minimum of 12 hours. The treated samples were then rinsed in PBS, dehydrated in a graded ethanol series from 50% to 100%, and finally dehydrated in a critical point dryer (BAL-TEC CBD 030). Dried samples were subsequently mounted on aluminum mounting stubs and sputtered with gold palladium alloy (JEOL JFC-1600 Auto-fine Coater). Imaging was conducted on a JEOL JSM-5600LV.

3.3.2 Histological examination

3.3.2.1 Sample processing

Treated samples, measuring 10 mm by 10 mm, were fixed in 10% neutral buffered formalin for 8 hours, and subsequently dehydrated with an increasing series of alcohol concentrations. Xylene was used to remove alcohol in the samples, prior to paraffin embedding. The paraffin block containing the sample was mounted on a microtome (Leica RM2125), and serial sections of 7 μ m were obtained. The sections were mounted on adhesive coated glass slides for better tissue adhesion, and left to dry on a flat plate at 40°C for an hour. The complete tissue processing protocol can be found in Appendix A.

3.3.2.2 Hematoxylin and Eosin (H & E staining)

To determine acellularity, paraffin sections of formalin-fixed samples were stained with H & E dye. Sections were first deparaffinized in xylene and a decreasing series of alcohol concentrations, before rehydrating in deionized water. They were then stained with hematoxylin and eosin dyes for 10 minutes and 1 minute respectively. After staining, the sections were rinsed in increasing grades of alcohol concentrations and xylene before being sealed with glass cover slips for protection and long-term storage. Sealed sections were analyzed using routine bright field (BF) light microscopy, and images captured. Cell nuclei are stained deep purplish-black, while cytoplasmic material are stained light pink, when viewed under a light microscope.

3.3.2.3 Masson's Trichrome staining

Masson's trichrome dye was used to stain for collagen type I to evaluate the effect of decellularization on the sample. Sections were paraffinized as described above, and rehydrated in deionized water. They were then stained with Weigert's Iron hematoxylin solution, Biebrich Scarlet-Acid Fuchsin solution, phosphomolybdic-phosphotungstic acid (PMA-PTA) solution and Aniline Blue solution for 10, 15, 14 and 10 minutes respectively. Stained sections were then rinsed briefly in deionized water and differentiated in 1% acetic acid solution for 1 minute. Post-staining rinsing and cover-slipping for the sections were carried out as described above. Under BF light microscopy, cell nuclei stained deep purplish-black, collagen a deep blue, while muscles and cytoplasm are stained red.

3.3.2.4 Immunohistochemical staining (IHC)

Immunohistochemical staining was performed using a streptavidin-biotin peroxidase method. 7 μ m tissue sections that were fixed in formalin and paraffin-embedded were deparaffinized and treated with peroxidase block for 20 minutes to quench endogenous peroxidase activity. Following a rinse in PBS, the sections were incubated using a blocking serum for 30 minutes to block non-specific binding of antibodies. Tissue sections to be tested for extracellular proteins such as fibronectin, collagen type IV, and laminin, were then incubated in mouse anti-human fibronectin (1:50), mouse anti-human collagen type IV (1:100), and mouse anti-laminin (1:50) respectively for 2 hours. This was followed by another incubation with the secondary antibody, biotinylated mouse anti-goat immunoglobins, for 30 minutes. These sections were then incubated in streptavidin solution for 10 minutes, before visualizing with diaminobenzadine (DAB) substrate. The reaction was monitored under a microscope, and the slides rinsed in PBS to stop the development of the DAB substrate when the desired darkness of labeling was achieved. This protocol was carried out at room temperature.

3.3.3 Microbial assay

Microbial assays were conducted to determine the sterility of acellular samples. Agar broth was prepared using bactoagar, bactopectone, yeast and sodium chloride, according

to the manufacturer's instructions. The liquid broth was sterilized by autoclaving, and when cooled to 60°C, dispensed into 60 mm polystyrene petri dishes. Decellularized samples 30mm by 10 mm were then minced into 1 mm² fragments in 3 ml of low serum culture media and left to incubate for 24 hours at 37°C, 5% CO₂. After the incubation period, the supernatant was collected and streaked onto the cooled agar plates. Streaking was done with the use of a sterile pipette tip to spread consecutive lines of supernatant across the top of the agar plate. After streaking, the agar plates were placed in an incubator at approximately 30°C to allow for colonies, if any, to form. After an overnight incubation, the plates were removed from the incubator and examined visually for signs of bacterial colonies. Untreated porcine esophageal tissue was used as a positive control, while sterile deionized water acted as the negative control. The procedure was carried out in a laminar hood to ensure sterility.

3.3.4 Cytotoxicity assay

Cytotoxicity was conducted to determine the basic, *in-vitro* biocompatibility for the treated scaffolds. Testing was based on ISO 10993-5 standards. Samples measuring 9 cm² were cut to size and sterilized by immersion in 70% ethanol for 8 hours on an orbital shaker. The samples were then rinsed 5 times in 40 ml of sterile distilled water, for approximately 15 minutes each, to remove residual ethanol. They were then placed into 6-well plates containing 3 ml of full tissue culture media in each well. Tissue culture media consisted of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics-antimycotics cocktail. Samples were incubated in the full tissue culture media for 24 hours in 37°C. Meanwhile, L929 mouse fibroblasts were plated in 24-well plates at a cell seeding density of 5x10⁴ cells/ml to achieve 70% confluency. After the 24-hour incubation, the media containing the samples was extracted and inoculated to the 24-well plate containing 3T3 mouse fibroblasts. Incubation periods were fixed at 24 and 48 hours respectively. After the pre-determined incubation period, the plate containing inoculates were viewed under a microscope to analyze the morphology of the fibroblasts. Latex rubber measuring 9 cm² was used as a positive control, while tissue culture polystyrene plates were used as a negative control.

3.3.5 Tensile Testing

Mechanical testing was performed on untreated porcine esophageal tissue and decellularized scaffolds with reference to ASTM D638-03 [154]. Dumbbell shaped specimens (Type V) were die cut from both untreated and decellularized samples. Before testing, the thickness of the sample in its unloaded state was measured using a dial gauge. For tensile testing, the ends of the sample were mounted on cardboard paper strips to ensure ease of handling and gripping. The sample was placed into the grips of an Instron mechanical tester (Instron 5566). Care was taken to ensure that the sample was vertical. 1 kN load cell was used, and the strain rate was set at 1mm/min. The sample was tested to failure. It was also doused with PBS every 5 minutes to prevent dehydration. Stress-strain curves for each sample were generated, and properties such as ultimate tensile strength, maximum strain and elastic modulus were determined. Data were given as mean of 8 samples; differences between the means of the untreated and decellularized samples were tested for statistical significance using Student's t-test, with $p < 0.05$ considered to be significant.

3.4 **Cell-matrix interactions**

3.4.1 Culture of porcine esophageal fibroblasts (PEFs)

Primary cultures of porcine esophageal fibroblasts were obtained from Dr. P.S. Mhaisalkar. Once the cells reached confluence, they were plated in 75 cm² tissue culture flasks and maintained in a humidified incubator at 37°C and 5% CO₂. Cells were fed with DMEM supplemented with 10% FBS, and an antibiotics cocktail of penicillin, streptomycin, amphotericin B, and kanamycin (1:100). This medium cocktail was termed collectively as full culture media. The media was replaced every three days, and cells were passaged when they reached 95% confluency, and the passage number was recorded.

3.4.2 Culture of porcine esophageal epithelial cells (PEECs)

The protocol for maintaining PEECs and PEFs were the same. For PEECs, growth factors such as epithelial growth factor (EGF), insulin, cholera toxin, apotransferrin and

hydrocortisone were added to the full culture medium. Primary cultures of PEECs were also obtained from Dr. P.S. Mhaisalkar. The cells were plated in 75 cm² tissue culture flask and cultures were maintained at 37°C in 5% CO₂ atmosphere in full culture medium. The medium was replaced every 3 days.

3.4.3 In-vitro seeding of PEFs

Treated samples previously prepared were cut into 1 cm circular discs measuring 1.88cm² with a custom made die cutter. The samples were rinsed in sterile PBS before being placed in 24-well plates, and subsequently incubated with full culture media for 4 hours. PEFs in their fourth passage were trypsinized, and the cells were seeded at 1x10⁵ cells/cm² seeding density, on the adluminal surface of the sample. The cell culture was maintained under culture conditions of 37°C and 5% CO₂ for 3 days. At the pre-determined period, the recellularized samples were sacrificed for SEM and histological examination.

3.4.4 In-vitro seeding of PEECs

The protocol for cell seeding on decellularized scaffolds was the same as that described above for PEFs. PEECs in their fourth passage were trypsinized, and seeded at the same seeding density on the adluminal surface of the sample. The cell culture was maintained under culture conditions of 37°C and 5% CO₂ for 14 days, after which it was sacrificed for SEM and histological examination.

CHAPTER 4 RESULTS AND DISCUSSION

4.1 Characterization of native porcine esophagus

Currently, not much is known about the histological anatomy of porcine esophagus. Though it is widely recognized that human organs are anatomically similar to pigs [28], these similarities may not be mirrored in the microstructure of organs, specifically the esophagus. This study aims to understand the microstructure of porcine esophageal ECM, and to determine its suitability as a decellularized scaffold. Another objective in this study is to identify the location and characterize the basement membrane (BM) of native porcine esophagus.

4.1.1 Topographical examination of a porcine esophagus

Under SEM, the adluminal surface (Figure 9a) of the native porcine esophagus was observed to be a continuous sheet. This represented the epithelium surface comprising a monolayer of squamous porcine epithelial cells. In contrast, the abluminal surface (Figure 9b) appeared as a widely dispersed fibrillar network of collagen, with large and coarse fibres.

Figure 9(a): SEM micrograph of the adluminal surface of porcine esophagus

Figure 9(b): SEM micrograph of the abluminal surface of porcine esophagus

4.1.2 Histological examination of a porcine esophagus

The characteristic layers of the porcine esophageal wall were stained with H & E, as seen in Figure 10(a) to (f). Intact cell nuclei were stained purplish black, while cytoplasmic contents and structures were stained light pink. Figure 10(a) showed the complete esophageal wall, consisting of the endomucosa (EM), a collective term for the mucosa and submucosa, and the muscularis externa (ME). It can be seen from Figure 10(b) that the muscularis externa consisted of smooth and skeletal muscle bundles, while displaying a scarcity of connective tissue. Due to the scarcity of collagenous tissue that was available for decellularization, the ME was usually stripped away. Therefore, only the endomucosa was decellularized to provide the acellular ECM.

Figure 10(a): H & E staining of the native esophagus at 100X. (i) Endomucosa, (ii) Muscularis externa

Figure 10(b): H & E staining of the muscularis externa at 100X. (iii) Circular muscle, (ii) Longitudinal muscle

Figure 10(c): H & E staining of (i) non-keratinizing stratified squamous epithelium, (ii) lamina propria at 200X

Figure 10(d): H & E staining of the native epithelium showing the basal to superficial layers of cells in (iii) the basal zone, (iv) superficial zone at 400X

The non-keratinized stratified squamous epithelium consisted of 8-12 layers of cells. The underlying support of the epithelium is the lamina propria (LP), as seen in Figure 10(c). Positioned along this basal zone were the basal cells, recognized by their cuboidal shape (Figure 10d), as opposed to the flattened squamous cells in the superficial layers (uppermost layer).

Figure 10(e): H & E staining of the lamina propria and (i) muscularis mucosa at 400X

Figure 10(f): H & E staining showing the native submucosa at 400X

Although both the lamina propria and submucosa (SBM) are composed of connective tissue, their structures are different. The lamina propria (Figure 10e) is relatively more cellular, while in the submucosa (Figure 10f), blood vessels and submucosal glands can be seen within its more fibrous structure [31-33]. The boundary between the lamina propria and submucosa is formed by the muscularis mucosa. From Figure 10(e), it was observed that the muscularis mucosa comprises smooth muscle fibres.

4.1.3 Native basement membrane

The basement membrane is typically positioned between parenchymal cells and connective tissue [88]. In the porcine esophageal tissue, the basement membrane (Figure 11a) was located at the interface between the dense sheet of epithelium and the collagen network of the lamina propria. Under histological examination (Figure 11b), the basement membrane was represented by the underlying stroma of the innermost layer of basal cells, along the fingerlike projections of the epithelium. In order to study the

topography of this unique matrix, the basement membrane was exposed by removal of the epithelium (as described in Section 3.1.3). SEM examination revealed the basement membrane to be made up of a closely-knit network-forming collagen. As seen in Figure 11(c), the pores were in the nanoscale range, which corroborated with the findings of other types of BMs [88, 97, 119, 155].

Figure 11(a): SEM micrograph showing cross section of epithelium-LP interface at 3000X. (i) Epithelium, (ii) LP

Figure 11(b): H and E staining showing the epithelium-lamina propria region of native tissue. (i) Location of BM

Figure 11(c): Topography of the porcine esophageal epithelial basement membrane

It had been suggested that BMs act as a molecular sieve, and serve as channels for small molecules [88]. Although the nanoscale topography of BMs is important in cellular

processes [96, 97], the presence of the BM proteins also serves to influence critical cellular functions [88]. In developing a suitable decellularization process for porcine esophageal tissue, it is therefore important that the structure of the BM was retained.

4.2 Development of chemical-based (PAA) decellularization protocol

In our preliminary studies, we had found PAA to be highly effective in removing cells from porcine esophageal tissue. Peracetic acid is widely utilized in many decellularization processes as a disinfectant [156-158]. However, its use as a decellularization reagent on porcine esophageal tissue has not been evaluated as yet, to our knowledge. Upon completing the experimental matrix described in Section 3.2.1, the results were summarized in the table below.

Table 3: The effect of different treatments on the epithelium and interstitial matrix of treated samples

	Effect on epithelium				Effect on interstitial matrix			
	10%	15%	20%	25%	10%	15%	20%	25%
2 hrs	No effect	++	+++	+++	No effect	No effect	No effect	---
4 hrs	+	+++	+++	+++	-	--	---	---
6 hrs	++	+++	+++	+++	--	---	---	---
12 hrs	+++	+++	+++	+++	---	---	---	---

Legend:			
+	Minimal delamination	-	Minimal degradation
++	Partial delamination	--	Partial degradation
+++	Full delamination	---	Full degradation

The decellularization treatments were evaluated by examining their effect on two different regions of the esophageal tissue - the epithelium, and the rest of the tissue, termed collectively as the interstitial matrix. The effect on epithelium was measured by the delamination of the epithelium from the adluminal surface, while the effect on the

interstitial matrix was measured in terms of tissue degradation. The degradation of the matrix was defined as the extent of damage to the collagenous tissue structure after different decellularization treatments, under histological observation. There were three classifications of degradation: minimal, partial and full degradation. Minimal degradation was seen in treated samples that had a mostly intact tissue structure resembling the native histoarchitecture of untreated porcine esophageal tissue. Samples which retained gross structural features, but contained pronounced openings in the collagenous matrix, were deemed as partially degraded, while full degradation was used to describe samples that showed a complete disintegration of the collagenous matrix. Sections 4.2.1 and 4.2.2 describe in detail the effect of different treatment parameters on these esophageal layers.

4.2.1 Effect of treatment time

Figures 12(a) to (f) show the effect of treatment time on porcine esophageal tissue. The samples had been treated with 10% PAA ranging from 2 to 24 hours. The effect of the different treatments on the epithelium of the samples was assessed. Both the 2 and 4-hour treatments with 10% solution had negligible effect on the samples. Large areas of native epithelium remained attached to the adluminal surface after the treatments. This was reflected as a smooth, continuous lining under SEM (Figures 12a and c), while histological examination (Figures 12b and d) showed the epithelium to be firmly attached to the underlying lamina propria.

Figure 12(a): SEM micrograph showing the adluminal surface of sample after 2hr, 10% treatment

Figure 12(b): H & E staining of the sample after 2hr, 10% treatment at 200X.

(i) Intact epithelium attached to LP

Figure 12(c): SEM micrograph showing the adluminal surface of sample after 4hr, 10% treatment

Figure 12(d): H & E staining of the sample after 4hr, 10% treatment at 200X. (i) Intact epithelium, (ii) Pronounced openings in collagen

However, when the samples were treated for 6 hours, the epithelium was successfully delaminated in some areas. Figure 12(f) shows that the exposed areas consisted of a meshwork of collagen. Subsequent H & E staining confirmed that these areas were part of the lamina propria, when compared to H & E stained sections of native esophageal tissue. It was observed, though, that the collagen bundles were fragmented, with no continuity in the dense meshwork.

Figure 12(e): SEM micrograph showing the adluminal surface of sample after 6hr, 10% treatment. (i) Intact collagen bundles, (ii) Fragmented collagen

Figure 12(f): H & E staining of the sample after 6hr, 10% treatment at 200X. (i) Pronounced openings, (ii) Portions of delaminated epithelium

Figure 12(g): SEM micrograph showing the adluminal surface of sample after 12hr, 10% treatment. (i) Intact collagen bundles, (ii) Fragmented collagen

Figure 12(h): H & E staining of the sample after 12hr, 10% treatment at 200X. (i) Pronounced openings, (ii) Portions of delaminated epithelium

The effect of the increasing treatment time on the abluminal surface of the samples was more pronounced. The 2-hour treated sample (Figure 12b) showed that the structure remained intact, resembling native esophageal tissue (Figure 10e). However, the 4 and 6-hour treated samples showed that the submucosa had become fragmented. Unlike the compact collagen mesh observed in lamina propria of native esophageal tissue, pronounced openings were seen in the treated samples. Additionally, part of endomucosae was beginning to detach from the rest of the tissue, especially along the strips of muscularis mucosae. This indicated the harsh effect of increasing treatment time on the samples. Samples treated for 12 hours (Figures 12g and h) resulted in a complete loss of structural integrity.

4.2.2 Effect of reagent concentration

The effect of reagent concentrations on treated (2-hour) samples can be seen in Figures 13(a) to (i). Unlike the 10% PAA treatment, samples treated with the 15% PAA treatment were more successful, as shown in Figures 13(c) and (d). The exposed surface consisted of collagen bundles which made up the lamina propria. However, the extent of epithelium removal was still limited. Full epithelium removal only occurred at 20%, and uniform tracks of collagen bundles were seen across the entire adluminal surface, as shown in Figure 13(e). SEM examination at 5000X (Figure 13g) revealed that the collagen bundles

comprised thick tendrils of collagen entwined to form a dense mesh. These collagenous fibres were found to be less than 1 μm in diameter. Increasing the reagent concentration to 25%, however, had a detrimental effect on the adluminal surface. Figure 13(h) shows a disrupted network of broken collagen fibres, with no visible alignment of collagen bundles previously observed in the 20% PAA treated samples.

Figure 13(a): SEM micrograph showing the adluminal surface of sample after 10%, 2 hr treatment

Figure 13(b): H & E staining of the sample after 10%, 2 hr treatment at 200X.
(i) Intact epithelium attached to LP

Figure 13(c): SEM micrograph showing the adluminal surface of sample after 15%, 2-hour treatment. (i) Collagen bundles
(ii) Native epithelium

Figure 13(d): H & E staining of the sample after 15%, 2 hr treatment at 200X.
(i) Partially exposed adluminal surface

Figure 13(e): SEM micrograph showing the adluminal surface of sample after 20%, 2 hour treatment

Figure 13(f): H & E staining of the sample after 20%, 2 hr treatment at 200X.
(i) Fully exposed adluminal surface

Figure 13(g): SEM micrograph showing the adluminal surface of sample after 20%, 2 hour treatment (i) Exposed collagen fibrils

Figure 13(h): SEM micrograph showing the adluminal surface of sample after 25%, 2 hour treatment

Figure 13(i): H & E staining of the sample after 25%, 2 hr treatment at 200X.
(i) Disintegration of collagen matrix

In summary, different treatment schemes had varying effects on the underlying tissue structure of the porcine esophagus. Both 10% and 15% PAA treatments (Figures 13d and f) resulted in partial decellularization but did not cause any visible damage to the lamina propria and submucosa. The gross histological features were also preserved. However, it was found that complete decellularization occurred only with samples treated in 20% PAA solution. No visible cell nuclei were seen in the endomucosa after H & E staining (Figure 13f). In addition, no visible damage was observed in the lamina propria and submucosa. Samples treated with 25% PAA solution (Figure 13i) were swollen and distended, with unrecognizable features due to extensive disintegration in the collagenous matrix.

4.2.3 Optimization of chemical-based decellularization protocol

Peracetic acid was selected as a decellularization reagent in decellularizing porcine esophageal tissue because of its ability to disrupt cell wall ability. The efficacy of the decellularization process was determined by 2 parameters: treatment time and reagent concentration.

Samples treated with increasing treatment times of up to 6 hours still showed significant epithelium attached. This was attributed to the nature of the esophageal lining. As discussed in Section 2.1.2, the mucosal epithelium of porcine esophageal tissue comprises 8-20 layers of cells. These cells are tightly bound to each other via desmosome junctions [30, 33, 35], thus providing a barrier and protective layer against injury. Due to the tightly bound epithelium, decellularization of the adluminal surface was diffusion dependent. The diffusion of PAA solution into the lamina propria was limited because of the number of epithelial cell layers, thus making complete decellularization difficult to achieve. On the other hand, the esophageal submucosa has a much looser and more porous collagen network. This made it easier for PAA to diffuse into the sample, and aided the decellularization process. Hence, for a given period of treatment, the abluminal surface was decellularized faster than the adluminal surface. Therefore, by the time the adluminal surface was fully decellularized, the abluminal surface would have degraded.

The effect of reagent concentration can be explained by the presence of reactive hydroxyl radicals in the PAA formulation, as described in Section 2.6.1.2.1. With increasing reagent concentration, more hydroxyl radicals were available to accelerate the decellularization process. This resulted in progressive cell removal with increasing concentration in a given period of time.

Based on the above results, a 2 hour- 20% PAA treatment was selected because the treated samples were decellularized without visible adverse effects on the matrix. These samples were then evaluated based on the effectiveness of the decellularization treatments, and the results will be discussed in Section 4.4.

4.3 Development of chemical-mechanical decellularization protocol

It had been shown that PAA treatment alone could not fully decellularize a sample without damaging the interstitial ECM. In this study, a combination of PAA treatment and mechanical scraping was evaluated. Mechanical scraping has been used successfully by several researchers to delaminate layers of tissues from organs [12, 84, 108]. This is especially effective in thin tissues such as small intestinal submucosa and urinary bladder [52, 85, 89, 140]. This method had been reported to cause minimal disruption to the three-dimensional architecture of the ECM. The parameters of interest in this research study were (1) type of scraping tool, (b) reagent concentration, and, (c) number of scraping cycles.

4.3.1 Selection of scraping tool

Two scraping tools, a toothbrush and a gauze-wrapped slide, were used to scrape the surfaces of porcine esophageal tissue in 5% PAA solution. A concentration of 5% was arbitrarily selected to avoid the harsh effects of higher PAA concentrations on the samples.

The treated samples were assessed to determine which tool was more effective in cell extraction, while minimizing damage to the ECM.

Table 4: The effect of different scraping tools on the delamination of native epithelium and collagen fibril alignment on the abluminal surface

	Delamination of epithelium		Collagen fibril alignment	
	100X	200X	100X	200X
Brush	No effect	+	No	No
Gauze wrapped slide	+	++	Yes	Yes

Legend:
+ Minimal delamination
++ Partial delamination
+++ Full delamination

4.3.1.1 PAA treatment with 100 scraping times

Figures 14(a) to (f) show the effect of scraping with the toothbrush, and gauze-wrapped slide, on the samples after scraping 100 times in 5% PAA. The scraping treatment with the gauze-wrapped slide was successful in removing the upper layers of native stratified epithelium as seen in Figure 14(a).

Figure 14(a): H & E staining of the sample after scraping with the gauze for 100 times. (i) Fewer layers of epithelium

Figure 14(b): H & E staining of the sample after scraping with the brush for 100 times. (i) Fully intact epithelium

Figure 14(c): SEM micrograph of the adluminal surface after scraping with the gauze for 100 times

Figure 14(d): SEM micrograph of the adluminal surface after scraping with the brush for 100 times

Figure 14(e): SEM micrograph of the abluminal surface after scraping with the gauze for 100 times. (i) Alignment of collagen fibres after scraping

Figure 14(f): SEM micrograph of the abluminal surface after scraping with the brush for 100 times

This result corroborated with SEM examination (Figure 14c), where collagen fibrils were seen on the adluminal surface of the sample in certain areas. However, the same treatment was less successful for samples scraped with the toothbrush. Both H & E (Figure 14b) and SEM (Figure 14d) micrographs show an intact epithelium still attached to the adluminal surface of the sample. Samples scraped with a gauze-wrapped slide displayed a coarse network of thick collagen fibres under SEM, and possessed a general collagen fibre alignment (Figure 14e). While the brush-scraped sample displayed the

same collagen structure, its fibril arrangement was random and haphazard (Figure 14f). This was attributed to the scraping tool used. During the gauze-scraping process, the force exerted was equally distributed across the length of the gauze in contact with the sample. This caused the collagen fibre bundles to align in the scraping direction. In the case of the brush, its soft bristles tended to buckle under the force exerted during scraping. Therefore, fibres were not forced to align, but rather were pulled in a haphazard manner with the collagen fibrils being tangled between the bristle gaps (approximately 3mm in width).

4.3.1.2 PAA treatment with 200 scraping cycles

Figures 15(a) to (f) show the effect of different scraping tools on the samples after increasing the number of scraping cycles to 200 times. As previously discussed, a larger portion of native epithelium was removed in gauze-scraped samples (Figures 15a and c). However, significant amounts of epithelium were still present on brushed-scraped samples, as seen in Figures 15(b) and (d). The effect of the scraping treatments on the abluminal surfaces of the brush and gauze-scraped samples were similar to that previously discussed above.

Figure 15(a): H & E staining of the sample after scraping with the gauze for 200 times. (i) Portions of delaminated epithelium

Figure 15(b): H & E staining of the sample after scraping with the brush for 200 times. (i) Fully intact epithelium

Figure 15(c): SEM micrograph of adluminal surface after scraping with gauze for 200 times

Figure 15(d): SEM micrograph of adluminal surface after scraping with brush for 200 times.
(i) Native epithelium

Figure 15(e): SEM micrograph of abluminal surface after scraping with gauze for 200 times

Figure 15(f): SEM micrograph of abluminal surface after scraping with brush for 200 times.

The gauze-wrapped slide was selected as the scraping tool as it was more effective in removing the native epithelium and cellular matter than the brush under same scraping conditions.

4.3.2 Effect of reagent concentration

Figures 16 and 17 show the H & E micrographs of samples treated with different reagent concentrations, and scraped for 200 times using gauze-wrapped slides. The effect of this treatment on the epithelium-lamina propria and muscularis mucosa-submucosa region were then studied.

4.3.2.1 Epithelium-lamina propria region

The epithelium-lamina propria regions of the treated samples are shown in Figures 16(a) to (d), in order of increasing concentration. Samples treated in 1% PAA solution and scraped 200 times were unaffected, as shown by the intact epithelium. As seen in Figure 16(b), only the upper stratified squamous layers of epithelial cells were removed. A significant number of cells, especially those forming the basal layers, still adhered to the underlying lamina propria. Increasing the PAA concentration to 5% helped to remove more layers of epithelial cells. Figure 16(c) also shows that the basal layer of cells had started to delaminate from the lamina propria. With the 10% PAA treatment (Figure 16d), the majority of the epithelium was successfully removed, exposing the denuded lamina propria surface. However, native cells were still observed in the lamina propria.

Figure 16(a): H & E staining of the untreated sample. (i) Full native epithelium

Figure 16(b): H & E staining of the sample scraped in 1% PAA. (i) Fully intact epithelium

Figure 16(c): H & E staining of the sample scraped in 5% PAA.
(i) Delaminating native epithelium

Figure 16(d): H & E staining of the sample scraped in 10% PAA.
(i) Exposed lamina propria. (ii) Native cells in LP

4.3.2.2 Muscularis mucosa (MM)-submucosa region

Figures 17(a) to (d) show the effect of increasing reagent concentrations on the muscularis mucosal-submucosal regions of treated samples. When compared with the untreated control, the 1% PAA-treated sample showed less native cells. This was seen in Figure 17(b), where much fewer dark-stained cell nuclei were observed. This trend was also shown in 5% and 10% treated samples, where progressively fewer cells were stained within the interstitial matrix. However, the increasing concentration had an undesirable effect on the collagenous structure. The presence of small openings in an otherwise compact collagenous bundle structure was first observed in 5% PAA –treated samples (Figure 17c). In the 10% PAA-treated samples (Figure 17d), the effect was more pronounced. Widespread disintegration of the collagenous tissue was observed, with fragmentation observed in the lamina propria and submucosa.

Figure 17(a): H & E staining of the untreated sample (MM-submucosal layers).

Figure 17(b): H & E staining of the sample scraped in 1% PAA.
(i) Native cells present in strips of MM

Figure 17(c): H & E staining of the sample scraped in 5% PAA.
(i) Openings in collagenous matrix

Figure 17(d): H & E staining of the sample scraped in 10% PAA.
(i) Degradation of collagenous matrix

4.3.3 Effect of mechanical scraping

Figures 18 and 19 show the H & E stained samples that were treated in 1% PAA solution and scraped for different number of times. The two areas of interest to be examined were the epithelium-lamina propria and the muscularis mucosa-submucosa regions.

4.3.3.1 Epithelium-lamina propria region

As previously discussed, samples were not affected when treated in 1% PAA and scraped for 200 times (Figure 18b). Samples treated in 1% PAA and scraped for 250 times were more successful, as shown in Figure 18(c), where the epithelium was fully removed. However, some shrunken cell nuclei were still detected in the lamina propria region. By increasing the number of scraping to 300 times, full decellularization was achieved. From Figure 18(d), it could be seen that the sample was relatively acellular, with no intact cell nuclei observed throughout the lamina propria.

Figure 18(a): H & E staining of the untreated sample (epithelium-lamina propria layers)

Figure 18(b): H & E staining of the sample after scraping for 200 times

Figure 18(c): H & E staining of the sample after scraping for 250 times
(i) Native cells present in MM

Figure 18(d): H & E staining of the sample after scraping for 300 times

4.3.3.2 Muscularis mucosa-submucosa region

Figures 19(a) to (d) show the muscularis mucosal-submucosal regions of H & E stained samples after different scraping treatments. Samples treated in 1% PAA solution and scraped for 200 times were not fully decellularized, as shown in Figure 19(b). The muscularis mucosae and submucosa retained substantial numbers of dark stained cell nuclei. Samples scraped for 250 times had fewer native cells in the submucosa and muscularis mucosae located near the adluminal surface, as shown in Figure 19(c). Only a treatment of 300 scraping times was successful in full decellularization, with no visible cell nuclei observed in the entire region (Figure 19d).

Figure 19(a): H & E staining of the untreated sample (MM-submucosal layers)

Figure 19(b): H & E staining of the sample after scraping 200 times (i) Native cells present in strips of MM and submucosa

Figure 19(c): H & E staining of the sample after scraping for 250 times (i) Native cells present in strips of MM

Figure 19(d): H & E staining of the sample after scraping for 300 times

4.3.4 Optimization of chemical-mechanical decellularization protocol

Mechanical scraping has been successfully used in decellularizing thin tissue structures. For thicker tissues and organs, a combination of mechanical and chemical decellularization processes was required. The effects of reagent concentration and scraping times were summarized in Figure 20.

Figure 20: Pictorial representation of experimental matrix and the process parameters for optimized decellularization.

Three reagent concentrations were evaluated, namely 1%, 5% and 10%. The final concentration was chosen based on its ability to decellularize whilst minimizing any damage to the ECM. Although combining scraping with increasing PAA concentrations helped to expedite the removal of the epithelium, degradation of the ECM occurred. However, samples treated with a fixed PAA concentration and varying scraping times showed similar epithelium removal without collagen degradation. This was due to the scraping action, which physically removed epithelial cells, making it easier for the PAA solution to diffuse into the matrix. Hence the lowest PAA concentration (1%) treated sample combined with 300 scraping times was able to achieve the same level of

decellularization as treatments using higher PAA concentrations, whilst avoiding any damage to the interstitial matrix. Samples were prepared using this selected process, and assessed for its effectiveness in Section 4.4.

4.4 Comparison & selection of developed decellularization processes

Two different decellularization processes were established, namely the 2 hour-20% PAA, and the 1% PAA-300 scraping times treatments. To evaluate the effectiveness of these processes, several criteria were used. These criteria included (a) acellularity, (b) preservation of structural and functional ECM proteins, (c) retention of basement membrane proteins, (d) potential cytotoxicity, and, (e) cell-seeding studies.

Acellularity is one of the most important factors in determining successful decellularization. Due to its potential use as a xenogeneic material in various tissue engineering applications, the presence of native cells would be a case of immunological concern. H and E staining would be used to evaluate the extent of decellularization in the treated samples.

Another criterion of importance is the retention of major ECM and BM proteins. One of the advantages that naturally occurring materials have over synthetic materials is the array of native ECM and BM proteins that are inherent in the biomaterials. These native proteins, in turn, influence the processes of cell attachment, migration and proliferation. By conducting immunohistochemical staining, we hope to select a decellularization process that is able to retain these proteins, and therefore contribute to superior cell-matrix interactions.

A common concern with decellularized samples is the presence of soluble reagents and toxins after treatment which may lead to adverse effects in *in-vitro* and *in-vivo* experiments. A cytotoxicity assay using NIH/3T3 mouse fibroblasts was conducted to evaluate if the rinsing steps in the decellularization process was sufficient to remove any residual traces of the decellularization reagent.

4.4.1 Acellularity

Acellularity was determined by observing the number of visible hematoxylin-stained cell nuclei present in the treated samples. As seen in Figures 21(a) and (b), both samples were substantially acellular, due to the lack of intact cell nuclei. However, since visual observation is not a definitive method, the issue of quantifying the extent of cell removal will be addressed in future work.

Figure 21(a): H & E staining of the sample after 2 hour, 20%PAA treatment at 200X

Figure 21(a): H & E staining of the sample after 1%PAA, 300 scraping times treatment at 200X

4.4.2 Characterization for ECM proteins

The native ECM contains a complex mixture of structural and functional proteins which influence critical cell biological activities [12, 84]. The treated samples were evaluated to assess if crucial ECM proteins were preserved after the decellularization processes. Two ECM proteins of interest were selected, collagen type I and fibronectin. Collagen type I is a vital structural protein that provides strength for tissues subjected to loading, and fibronectin contributes to the cell adhesion properties of the ECM [120, 136].

4.4.2.1 Characterization for collagen type I

The samples were stained with Masson's trichrome stain to identify the presence and distribution of collagen type I in the tissue matrices. Masson's trichrome dye stains collagen blue, muscles and cytoplasm red, and nuclei black. All samples underwent identical staining sequences and conditions to ensure that the eventual staining intensity

was not affected by different staining times. Figures 22(a) and (b) show that the distribution of collagen type I varied across the different layers of native (untreated) tissue. The staining for collagen was intense in the lamina propria region, as seen in Figure 22(a). This represented the high collagen content within this region. In comparison, the submucosa consisted of coarse and thick fibrils that are less entwined. Hence, the collagen staining (Figure 22b) was much lighter, reflecting the open structure and lower collagen content present.

Figure 22(a): Trichrome staining of the untreated sample (epithelial-lamina propria layers) at 200X. (i) Epithelium

Figure 22(b): Trichrome staining of the untreated sample (muscularis mucosal-submucosal layers) at 200X. (i) Strips of MM

Figure 22(c): Trichrome staining of the sample (lamina propria) after 1% PAA-300 scraping times treatment (i) Darker staining in LP

Figure 22(d): Trichrome staining of the sample (submucosa) after 1%PAA-300 scraping times treatment

Figure 22(e): Trichrome staining of the sample (lamina propria) after 2 hour-20% PAA treatment

Figure 22(f): Trichrome staining of the sample (submucosa) after 2 hour-20% PAA treatment

Figures 22(c) to (f) show the samples processed with the 1%PAA-300 scraping times, and 2 hour-20% PAA treatments respectively. While both samples stained positive for collagen type I, more protein was preserved in the former treatment. The distribution of collagen type I in the sample treated with 1% PAA and scraped 300 times was comparable to the untreated control. This could be seen in Figures 22(c) and (d), with darker staining highlighting the dense collagen content in the lamina propria, and fainter staining in the sparse collagen network within the submucosa. This implied that the 1% PAA-300 scraping times treatment had not substantially damaged the collagen quantity. However, the 2 hour-20% treated sample showed degradation in collagen quantity. From Figures 22(e) and (f), very faint staining was observed. The harsh treatment of high PAA concentration on the collagen fibres was a suggested reason for the lighter staining.

At present, the effects of PAA on collagen type I inherent in porcine esophageal tissue have not been studied. Hodde *et al.* had demonstrated that porcine SIS treated with 0.1% - 0.15% PAA were able to retain native ECM proteins after sterilization [156], but nothing was discussed about the effect of different PAA concentrations and exposures on collagen type I. However, collagen oxidation has been studied using different oxidative systems including metal ion/H₂O₂, γ -radiolysis, and metal ion/ascorbate systems [159, 160]. It is reported that collagen proteins subjected to oxidation can result in fragmentation, increased susceptibility to proteolysis, and amino acid modification [160]. In characterizing the oxidation-induced changes in collagen protein thermal stability by

thermodynamic methods, Komsa-Penkova *et al.* showed that increasing oxidation of collagen using metal ion/H₂O₂ systems resulted in discrete reduction of collagen thermal stability and a corresponding increase in collagen destruction. They had also suggested that the discrete stability reduction appeared to be associated with oxidation of the side chain amino groups, and not with the accumulation of collagen fragments [161]. It had been shown that affinity for PMA-PTA during Trichrome staining was depressed if amino groups present in proteins were removed. Due to PAA's strong oxidizing ability, the native collagen present in the samples underwent oxidation, and likely caused amino acid modification. A sample treated with 20% PAA was likely to incur more oxidative damage, and subsequent amino acid modification, than a sample treated with 1% PAA. This would have affected the PMA-PTA affinity for the staining of collagen protein, hence resulting in lighter staining for the 2 hour-20% PAA treated sample, as opposed to the 1% PAA-300 scraping times treated sample.

Another factor that affected the dye binding ability was the presence of hydrogen bonds. When these bonds were disrupted due to the pH change from its physiological environment of pH 7.2 to pH 2, the dye was unable to bind to PMA-PTA attached to tissue [162]. It was hypothesized that the high PAA concentration in the 2 hour-20% PAA treatment had also altered the protein conformation due to the pH change, thus diminishing the binding ability of the dye, for reasons stated above. It must be emphasized that the qualitative results from Masson's Trichrome staining only identified the presence and distribution of collagen type I in treated samples, and were not able to quantify the amounts of collagen type I left after the treatments.

4.4.2.2 Characterization for fibronectin (Fbn)

Figures 23(a) to (d) show untreated and treated samples that were immunohistologically stained for the functional protein, fibronectin. Figure 23(a) shows the presence of positively stained fibronectin in an untreated sample. Fibronectin was found in all the layers of the esophageal wall, being particularly dense surrounding the underlying lamina propria and muscularis mucosae regions. In the sample treated with 1% PAA and scraped 300 times (Figure 23c), fibronectin staining was localized at the lamina propria. Lighter staining was observed in the muscularis mucosae. This implied that fibronectin was not

fully preserved. For the sample treated in 20% PAA for 2 hours (Figure 23d), very faint fibronectin staining was observed in certain areas of the lamina propria. This indicated that fibronectin was damaged after the treatment.

As discussed in the earlier section, SIS scaffolds sterilized with 0.1% - 0.15% PAA were found to retain fibronectin and GAGs [156]. However, Vissers *et al.* showed that fibronectin was susceptible to oxidative damage when exposed to hydroxyl radicals generated in different systems [163]. By measuring the oxidation of susceptible amino acids and the changes in the molecular weight of fibronectin due to cross-linking or fragmentation, they were able to determine the effects of hydroxyl radical and H_2O_2 exposure. It was found that that exposure to H_2O_2 alone had no effect on fibronectin. However, when fibronectin was exposed to hydroxyl radicals, the damage incurred ranged from the loss of amino acids, such as tryptophan, to fibronectin fragmentation.

The above explanation would account for the damage sustained after the 2 hour-20% PAA treatment, as reflected by the faint staining. Compared to the 1% PAA-300 times scraping treatment, a sample treated with 20% PAA would generate a lot more reactive hydroxyl radicals, thus increasing the oxidative damage to fibronectin.

Figure 23(a): Untreated sample stained with anti-Fbn Ab (positive control) viewed at 200X (i) Regions of Fbn

Figure 23(b): Untreated sample stained with anti-Fbn Ab (negative control) viewed at 200X

Figure 23(c): Treated sample (1% PAA-300 time scraping) stained with anti-Fbn Ab viewed at 200X
(i) Regions of darker Fbn Ab staining

Figure 23(d): Treated sample (2 hour-20% PAA) stained with anti-Fbn Ab viewed at 200X. (i) Region of faint Fbn staining

4.4.3 Characterization for BM proteins

The native BM is a sheet-like extracellular matrix separating epithelial tissues and mesenchymal cells from connective tissues [88]. One criterion in assessing the established decellularization processes was the characterization of BM proteins after treatments. Two BM proteins of interest were selected, collagen type IV and laminin.

4.4.3.1 Characterization for collagen type IV (cIV)

In native porcine esophageal tissue (Figure 24a), collagen type IV was found primarily in the basement membrane. This was defined as the region tracing the fingerlike projections of the lamina propria under the basal layer of the epithelium. IHC staining showed the presence of collagen type IV localized at the sample surface after treatment with 1% PAA and scraping for 300 times (Figure 24c). However, the lighter staining could indicate a lesser quantity of collagen type IV after the treatment. In comparison, the sample treated with 20% PAA for 2 hours (Figure 24d) showed minimal staining for the protein, suggesting an almost complete loss of collagen type IV.

Figure 24(a): Untreated sample stained with anti-cIV (positive control) viewed at 200X
(i) Regions of cIV Ab staining

Figure 24(b): Untreated sample stained with anti-cIV (negative control) viewed at 200X

Figure 24(c): Treated sample (1% PAA-300 scraping times) stained with anti-cIV Ab viewed at 200X
(i) Diffuse cIV staining in LP

Figure 24(d): Treated sample (2 hour-20% PAA) stained with anti-cIV Ab viewed at 200X

4.4.3.2 Characterization for laminin

As expected, the highest concentration of laminin was found at the epithelial basal cell layer-BM interface in the untreated sample (Figure 25a). Figure 25(b) shows that the 1% PAA- 300 scraping times treated sample stained positive for laminin in the lamina propria. Diffusely staining laminin was also present in the remnants of tissue vasculature. No visible laminin staining was seen in the sample that underwent the 2 hour- 20% PAA treatment, as seen in Figure 25(d).

Figure 25(a): Untreated sample stained with anti-laminin Ab (positive control) viewed at 200X
(i) Laminin Ab staining in LP

Figure 25(b): Untreated sample stained with anti-laminin Ab (negative control) viewed at 200X

Figure 25(c): Treated sample (1% PAA-300 scraping times) stained with anti-laminin Ab viewed at 200X
(i) Diffuse laminin Ab staining

Figure 25(d): Treated sample (2 hour-20% PAA) stained with anti-laminin Ab viewed at 200X

IHC staining showed that collagen types I and IV, fibronectin, and laminin were preserved in samples treated in 1% PAA and scraped 300 times, albeit in lower concentrations. Brown *et al.* and Hoddes *et al.* had reported the presence of laminin and fibronectin in porcine SIS and UBM after exposure to 0.1% PAA treatment [119, 120]. Badylak *et al.* had demonstrated that PAA has no adverse effect on substrates for *in vitro* cell culture, with growth factors and glycosaminoglycans being preserved [91, 121]. Although the 1% PAA-300 scraping times treatment caused a slight loss in protein concentration, this effect was minimal if the structure and function of the ECM proteins

were conserved, and can be used successfully in tissue engineering applications. The 2 hour-20% PAA treatment caused a complete loss in the ECM proteins, especially in laminin and collagen type IV. This was likely due to the high concentration of PAA used. It is well known that pH variations affect protein folding and conformation [164]. The low pH (pH=2) of 20% PAA solution may have altered the ionization states of amino acid side chains, thereby changing protein charge distributions and hydrogen bonding requirements.

4.4.4 Cytotoxicity assay

An *in-vitro* cytotoxicity assay with NIH/3T3 mouse fibroblasts was conducted for treated samples in accordance with ISO 10993-5 standards. This assay is a qualitative indicator based on the morphological examination of cell damage when in direct contact with the eluted samples. Figures 26 (a) to (f) show the photomicrographs of the assay conducted.

Figure 26(a): Positive control at 24 hours

Figure 26(b): Negative control at 24 hours

Figure 26(c): NIH/3T3 fibroblasts after incubation with treated sample (1% PAA-300 scraping times) eluate

Figure 26(d): NIH/3T3 fibroblasts after incubation with treated sample (2 hour-20% PAA) eluate

As expected, the positive control eluate was toxic to the cells. Within 24 hours (Figure 26a), there was massive lysis observed in the incubated cells. Cells had rounded up from the surface and were floating in the media. Cellular debris was observed in the media as well. Cells incubated in both samples' eluate (Figure 26c and d) adhered just as readily as the cells in the negative control (Figure 26b). Rapid growth was observed during the exposure period of 24 hours, with the cell proliferation comparable to that of the negative control, under visual observation. No significant morphological changes were observed in the cells that were in contact with the sample eluate. It could be concluded that samples treated with both decellularization treatments were non-toxic to NIH/3T3 mouse fibroblasts, at present eluate concentration.

4.4.5 *In-vitro* cell-seeding study with PEFs

Figures 27(a) to (e) show the SEM and H & E micrographs of decellularized and recellularized samples treated with 20% PAA for 2 hours. The samples were seeded with porcine esophageal fibroblasts (PEFs) in a three-day culture. From Figure 27(b), it can be seen that the cellular response was unfavourable. The seeded PEFs were sparsely scattered along the adluminal surface of the matrix. Upon closer examination (Figure 27c), the host cells exhibited a rounded morphology, indicative of their inability to adhere

and spread on the matrix surface. H and E stained sections (Figure 27e) also revealed the absence of host cells, implying host cells failed to attach to the sample.

Figure 27(a): SEM micrograph showing the adluminal surface of treated sample (2 hour-20% PAA)

Figure 27(b): SEM micrograph showing PEFs on the adluminal surface of treated sample (2 hour-20% PAA)

Figure 27(c): SEM micrograph showing rounded morphology of seeded PEFs on treated sample (2 hour-20% PAA)

Figure 27(d): H & E staining of the treated sample (2hour-20% PAA) at 200X

Figure 27(e): H & E staining showing the absence of PEFs on treated sample (2hour-20% PAA) at 200X

Figures 28(a) and (e) show the results of treated samples (1% PAA-300 scraping times) seeded with PEFs for 3 days. When comparing Figures 28(a) and (b), it was noted that the meshwork of fine collagen fibres could no longer be seen on the adluminal surface of the sample. Instead, a smooth dense sheet was observed, and lamellopodia formation in cells could be seen in some areas (Figure 28c). The lamellopodia helped cells to anchor themselves to the underlying collagen fibrils, thereby exhibiting a flat morphology. The confluent monolayer of host fibroblasts was also observed in treated samples stained with H & E. While only eosinophilic staining was observed in the structure of the decellularized sample (Figure 28c), hematoxylin dye stained for the presence of cells lining the lamina propria (Figure 28e).

Figure 28(c): SEM micrograph showing PEFs on the adluminal surface of treated sample (1% PAA-300 scraping times)

Figure 28(d): H & E staining of the treated sample (1% PAA-300 scraping times) at 200X

Figure 28(e): H & E staining of the PEF-recellularized sample at 200X.
(i) Monolayer of seeded PEFs

By comparing the cell-seeding studies for both treatments, it was clear that successful recellularization depended on factors other than acellularity. In Sections 4.1.2 and 4.1.3, it was shown that the samples treated in 1% PAA and scraped 300 times retained proteins such as fibronectin and laminin better than samples treated in 20% PAA for 2 hours. Evidently, the presence of these proteins in the chemical-mechanically treated sample had contributed to its ability to support cell growth and proliferation.

4.4.6 Final decellularization process

In the course of our research study, two decellularization treatments were developed, with PAA as a primary decellularization reagent. The chemical-based decellularization treatment involved treating samples in 20% PAA solution for 2 hours to remove native cells in porcine esophageal tissue. While H & E stained samples were deemed acellular after the treatment, it was unable to preserve structural and function proteins. It was highly possible that its inability to support cell growth and proliferation was due to the resulting damage and loss of these proteins. By comparison, samples treated in 1% PAA and scraped 300 times were able to achieve the same level of decellularization. This treatment was also more effective in preserving collagens type I and IV, laminin and fibronectin, and the presence of these proteins likely contributed to the favourable cell-matrix interactions seen. Due to its effectiveness, this process was selected as the final decellularization process in treating porcine esophageal tissue.

4.5 Further characterization for decellularized scaffolds using optimized process

4.5.1 Cell studies with porcine esophageal epithelial cells (PEECs)

Figures 29(a) to (d) show the results of a 14-day culture of PEECs on decellularized scaffolds treated with the chemical-mechanical process. Both SEM and histological examination revealed a monolayer of PEECs on the adluminal surface of the decellularized scaffold (Figures 29(b) and (d)). The phenotype of the cultured cells was similar to native esophageal epithelial cells, displaying the cobblestone morphology typical of epithelial cells [165]. Upon closer examination with light microscopy (Figure 29d), one was able to observe the attached cells exhibiting a flattened morphology along the luminal edge of the scaffold. This observation was important as cell adhesion strength correlates with good cell spreading, higher DNA synthesis and metabolism [166]. Two reasons were suggested to explain the favourable response of the host cells on acellular matrices, namely the (1) topography of the basement membrane surface, and (2) presence of preserved ECM proteins. It is widely known that nanoscale substratum topography modulate cell behaviours independent of ligand-receptor mediated pathways [55, 56, 96]. It is also recognized that many ECM components facilitate attachment, spreading, and formation of focal adhesions presenting multiple sites for cell interactions with many different types of cell surface receptors, and by promoting clustering of integrins [83, 95, 119]. These reasons could have contributed to the cell growth patterns observed.

Figure 29(a): SEM micrograph showing the adluminal surface of decellularized scaffold

Figure 29(b): SEM micrograph showing PEECs on the adluminal surface decellularized scaffold

Figure 29(c): H & E staining of the decellularized scaffold at 200X

Figure 29(d): H & E staining of the recellularized scaffold at 200X. (i) PEECs monolayer on adluminal surface

The ability of decellularized porcine esophageal matrices in supporting PEEC growth and proliferation is encouraging. Researchers have shown that the presence of autologous stratified epithelium is vital to the success of esophageal regeneration. In addition to providing barrier function, an autologous stratified epithelium stimulates muscle regeneration [165]. Therefore, it is an important component in the tissue engineering of an implanted esophagus. The differentiation and stratification of PEECs on decellularized porcine esophageal matrices will be addressed in future work.

4.5.2 Mechanical properties

Decellularization processes involve the removal of native cells, potentially creating voids and defects within the collagen matrix that may lead to changes in the mechanical properties of the ECM structure. The purpose of this study was to compare the changes in the mechanical properties of native tissue and samples treated with the final decellularization process. The study was conducted by subjecting samples to a uniaxial tensile load in the longitudinal direction, and testing them to failure.

Stress-strain plot for native esophageal tissue reflected the typical relationship for biological tissue, with an initial non-linear toe region, followed by a linear region, and culminating in failure.

Table 5: Mechanical properties of native and decellularized tissue

	Modulus (MPa)		Tensile stress at break (MPa)	Elastic stress at break
	Toe region	Linear region		
Untreated tissue	0.012 ± 0.4	1.60 ± 0.65	2.20 ± 0.52	5.06 ± 1.23
Decellularized tissue	0.015 ± 0.7	4.93 ± 1.47	4.98 ± 1.33	3.11 ± 0.52

Figure 30: Typical stress-strain curves for native & decellularized esophagus

The mechanical properties for both native and decellularized tissue were summarized in the above table. The mean ultimate tensile strength (n=8) for native esophageal tissue was 2.2 ± 0.52 MPa. This was comparable to human esophagi in the age group of 19-44 ($\sigma = 2.19 \pm 0.06$ MPa) [167]. The plot for the treated scaffolds (n=8) showed a higher incline, with a much shorter initial toe region. In treated scaffolds, the ultimate tensile strength (UTS) was significantly higher than that of native esophageal tissue, with a value of 4.98

± 1.33 MPa. However, the treatment had left the scaffold less extensible, displaying 61.7% of the original elastic strain. The modulus of untreated tissue was found to be 1.60 ± 0.65 MPa, while that of treated tissue was 4.93 ± 1.47 MPa. A student's two-tailed t test was conducted and p was found to be less than 0.05, indicating that the two treatments were significantly different.

The mechanical properties of soft connective tissues have been extensively studied. Its behaviour depends primarily on the response of the tissue structure and its constituents. The composition of soft collagenous tissues comprises mainly collagen fibres, elastin fibres, and amorphous ground substance of mucopolysaccharides [168]. The stress-strain relationship is common among soft tissues, displaying a non-linear curve with the tissue becoming stiffer with strain. This can be explained by the contribution of collagen crimping in collagenous tissues. Lanir reported that under morphological observation, the collagen fibres were arranged in a three dimensional but primarily planar wave array [169]. When a uniaxial tensile load is applied, the collagen fibres uncrimp and reorient while elastic fibres, among other components, bear the load. As strain is increased further, the stress-strain curve undergoes a nonlinear transition associated with uncrimped collagen becoming the primary load-bearing element [170]. Being a soft tissue, the untreated esophageal tissue displayed a similar curve as described above (Figure 30). At low strain levels, the behaviour is dominated by the elastin, and at high strain levels, by collagen.

The stress-strain curve for the sample treated with the final decellularization process was markedly different from that of the untreated tissue, with a decreased extensibility but higher UTS values. There were two postulated explanations which could account for the decreased elasticity and strain at break. Due to the mechanical scraping of the sample in the longitudinal direction, the natural collagen crimping was lost and the wavy pattern of collagen fibres was straightened out. Therefore, the loading was transferred directly to the collagen fibrils in the tissue, which had relatively little elasticity. This resulted in a lower value for the strain at break, as seen in the strain reduction. This phenomenon was also observed by Bolland *et al.* in their work with decellularized porcine bladder matrices

[171]. Their results revealed that decellularized bladder matrices showed an increase in the collagen phase slope, and decreased failure strain values, which they attributed to an alteration in the ultrastructural properties of the collagen fibres.

Another theory involved the preservation of elastin, which imparts elasticity to biological tissues [172]. If elastic fibres had been affected by the decellularization treatment, the subsequent loss of elastin would have led to less elastic structures. However, this can only be verified by characterization of the elastin component in treated scaffolds, which will be addressed in future experiments.

It was also noted that the treated samples displayed higher UTS values than the untreated samples. This higher UTS value was associated with the exposure of the collagenous matrix to PAA solution. Due to the reactivity of oxygen-free radicals generated by PAA, the collagen fibres underwent oxidation and formed cross-links such as dityrosine and disulfide bridges, causing the sample to become stronger. Gilbert *et al.* and Freytes *et al.* had reported similar findings with porcine urinary bladder matrices subjected to sterilization treatments using 0.1% PAA for 2 hours [173, 174].

Mechanical testing of decellularized porcine esophageal samples revealed that they exhibited a lower extensibility, but showed greater stress values at break. However, it must be emphasized that the collagen fibres in the esophagus are not homogenous, and are anisotropic in nature [167, 171]. Uniaxial testing alone would not be able to fully describe the properties of the esophagus, and more characterization will be conducted in the future to understand the structure-function relationship of the porcine esophagus.

CHAPTER 5 CONCLUSION

Cancer of the esophagus is one of the few malignancies showing a sharply increasing incidence in developed countries. Patients diagnosed with esophageal cancer have a poor prognosis, often due to late diagnosis. Tissue engineering offers a potential alternative in the form of a tissue-engineered esophagus. The overall aim of this project is to develop an acellular ECM- based scaffold for tissue engineering of the esophagus.

In achieving this goal, decellularization was proposed to produce acellular scaffolds towards the aim of esophageal reconstruction. Ideally, these scaffolds should also possess preserved ECM structure and components, similar biomechanical properties as native tissue, and the ability to support cell growth and proliferation. Many cell extraction processes developed for a variety of organs and tissues were found to be lacking. This was due to lengthy and time consuming protocols, and the use of chemicals and enzymes that were damaging to ECM proteins.

Two decellularization processes were developed and assessed in fulfilling the above criteria. Both processes involved the use of peracetic acid as a combined decellularization reagent and a sterilizing agent. Parameters such as reagent concentration, treatment time and scraping cycles were optimized to achieve acellularity. One process involved treating samples in 20% PAA solution for 2 hours, while the other involved scraping samples for 300 times in 1% PAA solution. The developed decellularization processes were then assessed on the above-mentioned criteria. Samples treated with 1% PAA solution and scraped 300 times were found to be more superior. Histological studies demonstrated that the resulting ECM after decellularization is a cell-free matrix that retained the primary structure of native esophagus. Light microscopy confirmed the retention of main structural and functional proteins, namely collagen type I and fibronectin, and basement membrane proteins collagen type IV and laminin. While samples treated with both decellularization processes were found to be non-toxic at the selected concentrations, only samples treated in 1% PAA solution and scraped 300 times were able to support PEF growth and proliferation.

To further characterize the selected decellularization process, the mechanical properties of the treated samples were evaluated. Treated samples displayed a higher tensile strength at failure, while exhibiting 61.7% of strain found in native tissue. PEECs cultured on the treated samples showed favourable cell attachment and growth by days 3 and 14, with host cells exhibiting the correct morphology.

Future work includes identifying the growth factors in these acellular matrices, inducing the formation of a stratified squamous epithelium and conducting *in vivo* biocompatibility studies.

CHAPTER 6 FUTURE WORK

A decellularization process for porcine esophageal tissue was developed after comparing two different treatments. Aspects of the study involved selecting and optimizing the process based on factors such as acellularity, retention of major ECM proteins, and potential cytotoxicity. The future work can be divided into two areas: (a) characterization and (b) applications for decellularized scaffolds

6.1 Characterization of decellularized porcine esophageal matrices

6.1.1 Quantitative DNA analysis

Acellularity is one of the most important criteria in assessing the efficacy of a decellularization process. The presence of residual native cells may provoke increased innate and cellular immune responses in a host, thus reducing the possibility of long term remodeling of tissue engineered constructs. In this research project, acellularity was determined by visual observation of visible hematoxylin-stained cell nuclei in treated samples. To further evaluate the extent of decellularization, a quantitative analysis of native DNA content is proposed. A PicoGreen DNA quantification kit will be used with a microplate fluorometer to compare double-stranded DNA concentrations in treated samples against fresh controls

6.1.2 Quantitative collagen type I analysis

Collagen type I is one of the most abundant proteins in tissues, providing the strength required to accommodate mechanical loading. In our biochemical analysis of decellularized samples, Masson's Trichrome staining was used to identify collagen present. However, this analysis was not able to quantify the amount of collagen preserved after the treatments. In this proposed study, we aim to investigate the efficacy of our decellularization process in preserving this structural protein. This experiment involves quantifying the hydroxyproline content present in the decellularized samples. Another aspect of this study is to quantify the amount of denatured collagen by subjecting the samples to α -chymotrypsin digestion and measuring the hydroxyproline content.

6.1.3 Identification of growth factors present in decellularized scaffolds

Although much research has been conducted on the role of the ECM in mediating cell processes such as cell migration and proliferation, few studies have attempted to study the effect of the ECM on tissue repair. Developmental studies have demonstrated that growth factors present in the ECM can regulate the synthesis and deposition of ECM components, and in turn, these synthesized ECM components regulate the availability and activity in growth factors [84, 85]. The proposed study aims to identify the growth factors that are conserved in the matrices after decellularization, in order to better understand the role that the ECM scaffolds play in remodeling and wound healing processes.

6.2 Applications for decellularized porcine esophageal matrices

6.2.1 Regeneration of autologous stratified epithelium in acellular matrices

Initial attempts at patch or circumferential esophageal repair involved using a variety of materials ranging from PGA, silicone/collagen hybrids, and porcine aorta *in vivo* [45, 47, 48, 51, 52]. These studies demonstrated that coverage of the graft by adjacent epithelium was extremely slow, requiring two to three weeks for complete re-epithelialization, and resulted in complications. However, when autologous cells were seeded on constructs and subsequently implanted, re-epithelialization occurred within two weeks and was accompanied by enhanced regeneration of the mesenchymal tissue. We have shown that our decellularized constructs were able to support PEEC growth and proliferation. However, no stratification of PEECs was observed on the construct after a 14-day study. This proposed experiment aims to induce the presence of a fully stratified epithelium in creating a construct for esophageal tissue engineering. It has been shown that varying Ca^{++} levels in cell culture has an effect on differentiation and proliferation of cells [165]. By optimizing Ca^{++} levels in cell culture conditions, we will be able to re-epithelize acellular scaffolds for both *in-vitro* and *in-vivo* applications.

6.2.2 In-vivo biocompatibility studies

This study aims to study the foreign body response of the decellularized scaffolds in an *in-vivo* environment by conducting subcutaneous studies in small animals. While *in-vitro* biocompatibility studies are useful in assessing scaffold cytotoxicity, *in vivo* studies can determine the scaffolds's ability to be healed within the host. The biocompatibility studies will assess the inflammatory response by determining potential fibrous encapsulation thickness, neutrophil invasion, macrophage density, and microvascularization. Such data will verify the scaffold's esophageal regeneration potential and ability to avoid complications.

CHAPTER 7 REFERENCES

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CHAPTER 8 APPENDICES

8.1 **Appendix A – Formulation of PAA**

	Components in peracetic acid solution (by vol %)		
PAA %	Glacial acetic acid	Hydrogen Peroxide	Deionized water
10	20	10	70
15	30	15	55
20	40	20	40
25	50	25	25

8.2 **Appendix B – Histochemical protocols**

The following histochemical techniques were developed by NTU-SUWA researchers in the University of Washington, and these protocols are specific in the use of porcine esophageal tissue.

8.2.1 Protocol for tissue processing

- 1) Place samples in 10% formalin for 8 hours
- 2) Dehydrate samples using graded series of ethanol as described, with each step taking 45 minutes:
 - 70 vol % ethanol
 - 95 vol % ethanol
 - 100 vol % ethanol
 - 100 vol % ethanol
 - 100 vol % ethanol
- 3) Clear samples in 3 changes of xylene at 45 minutes each.
- 4) Immerse samples in separate paraffin baths at 55 °C for an hour each.
- 5) Samples are now ready for embedding and sectioning.

8.2.2 Protocol for Hematoxylin & Eosin staining (H&E)

- 1) Place deparaffinized slide in filtered hematoxylin for 12 – 16 minutes, followed by a rinse in tap water to remove excess stain.
- 2) Dip slide in acid alcohol (4 dips), followed by a rinse in tap water.
- 3) Place slide in Scott's Blue solution for 5 minutes. Check the intensity of the stain under microscopic examination.
- 4) Rinse slide in tap water to remove excess stain.
- 5) Place slide in working Eosin stain (2 dips).
- 6) Dehydrate slide in graded series of ethanol and xylene as described in Section 8.1.1.
- 7) Cover slip the slide using Permount, and allow slide to dry overnight in a fume hood.

8.2.3 Protocol for Masson's Trichrom staining

- 1) Place deparaffinized slide in Bouin's fixative for 45 minutes at 55-60°C, followed by a rinse in tap water to remove excess stain.
- 2) Place slide in Weigert's iron hematoxylin stain for 10 minutes, followed by a rinse in distilled water.
- 3) Place slide in Biebruch's scarlet-acid fushsin stain for 5 minutes, followed by a rinse in distilled water to remove excess stain.
- 4) Place slides in freshly made phosphomolybdic-phosphotungstic acid solution for 14 minutes. Allow excess stain to drain off the slide before placing the slide in Aniline blue for 5 minutes. Rinse in distilled water to remove excess stain.
- 5) Place slide in fresh 1% acetic acid for 1 minute.
- 6) Dehydrate slide in ethanol and xylene as described in Section 8.1.1.
- 7) Coverslip slide with Permount and allow slide to dry overnight in a fume hood.

8.3 Appendix C – Mechanical studies

8.3.1 Tensile stress-strain curves for untreated porcine esophageal tissue

Stress-Strain Curve of Untreated Porcine Esophagus

8.3.2 Tensile stress-strain curves for treated tissue