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Study of Decellularization Methods and Characterization of Porcine Esophagus

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Abstract

In order to reconstruct esophagus due to congenital or acquired esophageal diseases, a number of materials and techniques in tissue engineering have been developed to produce biocompatible and biodegradable scaffolds to support esophageal regeneration. Recently, acellular extracellular matrix (ECM) prepared by cell removal from native tissues (a novel technique called “decellularization”) has shown promising results in regeneration of tissues such as skin, blood vessels and gastrointestinal tracts. Many decellularization methods have been developed but the effects of the methods on the resulting decellularized ECMs have not been fully elucidated. The aim of this project is to study two different decellularization protocols for producing decellularized porcine esophageal ECMs.

In this study, protocols from University of Washington (“UW protocol”) and from Professor Stephen Badylak (“SB protocol”) were employed and compared. UW protocol used Triton X-100, Deoxyribonuclease I and Ribonuclease A (DNase/RNase), and sodium dodecyl sulfate (SDS) for cell removal while SB protocol employed mechanical scraping followed by peracetic acid. Scanning electron micrographs showed that both protocols produced highly porous matrices. Porous network produced by UW protocol was more regular which resembled the native submucosa whereas SB protocol resulted in a rather random and broken structure, probably due to mechanical damage. From the histological results of decellularized

ECMs, both UW and SB protocol could produce ECMs in the absence of any cell nuclei and cell components while collagen and elastin were preserved. Histological and MTS results of cell-seeded on decellularized ECMs suggested that the decellularized ECMs produced by UW protocol promoted cell growth whereas cells were hardly found in the reseeded ECMs fabricated using SB protocol, probably due to damage of the microstructure. In the mechanical test, Elastic moduli of the ECMs derived from UW and SB protocols were 2.1 and 1.6 MPa, respectively while their respective ultimate tensile strengths were 3.8 and 4.7 MPa. Mechanical properties of decellularized ECMs were mostly retained. In conclusion, decellularized porcine esophageal ECMs produced by UW protocol resulted in better preservation of microstructure and supported cell growth, giving them better properties to be a potential candidate as tissue engineering scaffold for esophageal regeneration.

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Chapter 1. Introduction

1.1 Background

Esophagus is a layered tubular structure along the alimentary tract that conveys food bolus from pharynx to stomach through peristaltic movement of muscular layer. In human, esophagus is approximately 23-25 cm long and 2 cm in diameter. Anatomically, it locates posterior to the trachea and its upper one-fifth lies in the neck and the rest fourth-fifth lies in the thorax. The canal begins at the terminal of the laryngopharynx, passes through the mediastinum and diaphragm and ends at the superior portion of the stomach [1, 2].

Figure 1.1.1 shows a cross-section of esophageal wall in which four basic layers can be identified: (1) mucosa; (2) submucosa; (3) muscularis externa and (4) adventitia [3]. Mucosa, or mucous membrane, is the innermost layer of esophageal wall. The typical digestive mucosa can be subdivided into 3 layers: epithelium, lamina propria, and muscularis mucosa. Epithelium is 18 to 20 layers of stratified squamous non-keratinized epithelial cells which line the inner wall of esophagus producing a barrier to resist abrasion, hot and cold temperatures, and chemical attack. The lamina propria is a layer of connective tissue while the muscularis mucosa consists of longitudinal muscle fibres. Submucosa, just external to the mucosa, consists of areolar connective tissue that connects the mucosa to the muscularis externa. It is a moderately dense connective tissue containing blood and lymphatic vessels, lymph

nodules, and nerve fibers. Muscularis externa is responsible for segmentation and peristalsis. Muscle fibers that run circumferentially and longitudinally can be found in this layer. Distribution of smooth and skeletal muscle along the esophagus depends on species. In human esophagus, the first third is skeletal; the second third is transitional with both muscle types; the final third is occupied fully by smooth muscle. Instead of serosa which is more prevalent in other parts of alimentary tract, the outer layer of esophagus is a layer of connective tissue called the adventitia.

Figure 1.1.1: Histological section of esophageal cross-section [4] (M= mucosa; SM= submucosa; MM = muscularis mucosa; IC = inner circular layer of muscularis externa; OL=outer longitudinal layer of muscularis externa and E=stratified squamous epithelium).

In the last decade, about 5,000-10,000 patients were diagnosed each year with congenital or acquired esophageal diseases, and over 30,000 new cases of esophageal carcinoma were discovered throughout the world [5, 6]. Esophageal cancer has been reported as the ninth most common cancer in the world and the low survival rate (10% in 5 years) makes esophageal cancer become one of the deadliest cancers [7-9]. The conventional treatments for esophageal cancer include surgery and chemotherapy including radiation therapy with or without subsequent surgery [10]. For surgery, other gastrointestinal tracts such as stomach [11], jejunum [12] and colon [11] are often used for esophageal reconstruction after removal of diseased portion. However, patients always suffer from post-operative complications due to poor compatibility and functionality of new grafts [12-14]. On the other hand, the procedure of chemotherapy and radiation therapy is highly stressful to patients [13].

Esophageal substitutes made of synthetic non-degradable polymers such as plastics and silicone rubber, have been developed in order to replace or repair damaged or diseased esophagus and has shown its potential use [15-17]. However, problems such as foreign body reaction and stenosis have to be solved before these can be applied. Due to these drawbacks, the application of tissue engineering may provide an alternative solution to produce biocompatible and functional substitutes for esophageal replacement [14].

In tissue engineering, cells and scaffolds are the most essential elements. Cells are the smallest units of tissues and organs. Incorporation of suitable cell types is important for regeneration of specific tissues and organs.

Scaffolds are biomaterials which do not induce toxicity in cells while serve as a template to guide cells to grow and re-organize into functional substitutes. Furthermore, scaffold materials have to be (1) highly porous and interconnected to allow effective cell delivery and nutrient/waste transport, (2) biodegradable or bioresorbable for cells to rebuild their own extracellular matrix (ECM) and (3) appropriate in mechanical properties. The scaffold materials currently in use include synthetic biodegradable polymers such as polyglycolic acid (PGA) and polylactic acid (PLA) [18, 19], natural polymers such as collagen [20] and chitosan [21]. It is hoped that using suitable cell sources and scaffolds with inclusion of essential biological signals, biological and functional tissue can be regenerated. Currently, many tissue and organ types are under study in tissue engineering: skin, cartilage, bone, blood vessels and gastrointestinal tracts [21, 22]. The results are promising and some tissue engineered products such as skin and cartilage have been commercialized [23-25]. The success of tissue engineering may solve the severe shortage of allograft and restore functions of lost or diseased body parts [23, 26]. Due to recent progress in tissue engineering, it is believed that esophageal substitutes created using principles of tissue engineering may provide new hope for esophageal reconstruction.

In esophageal tissue engineering, research using materials such as synthetic biodegradable polymers [27, 28], natural polymers [29-32] and acellular extracellular matrices (ECMs) derived from native tissues as scaffolds [5, 33, 34] have been documented and shown different degree of progress *in vitro* and *in vivo*.

Compared to scaffolds made of synthetic polymers, it was found that acellular

ECM derived from native tissue resulted in higher regenerative capabilities because biological signals conducive to cell adhesion, proliferation and differentiation are retained [35]. Studies also showed that these acellular ECMs are promising in repair and reconstruction of a wide variety of tissues including vascular [36-39], skin [40, 41], tendon [42], bladder [43]. The technique to prepare acellular ECMs is termed decellularization, which is a process of cell removal of native tissues by physical, chemical or enzymatic means in order to acquire a structure devoid of cells. A combination of these methods may be used to achieve effective cell removal[44]. Currently, native tissues such as small intestine submucosa (SIS) [45], urinary bladder submucosa [46] and heart valves [47] have been used to produce decellularized ECMs for tissue regeneration.

Due to its potential use in tissue engineering, decellularized ECM would be an option for scaffold material in esophageal regeneration. However, since ECM composition differs from tissue to tissue, it would be possible to use esophagus as tissue source for decellularization. On the other hand, due to severe shortage of allograft, esophagus from other animals can be considered. Currently, decellularized ECMs in research are mostly from porcine source because human and pig share some common features physically, physiologically and anatomically [48]. Therefore, porcine esophagus could be a possible choice. Furthermore, decellularization process is important because all cell components have to be removed to minimize immunogenicity while essential biological signals have to be retained in the acellular matrix. Therefore, developing a reliable and robust decellularization protocol is

required.

1.2 Objective and Scope

The purpose of this project is to study and evaluate two decellularization protocols proposed by University of Washington (UW protocol) and Professor Stephen Badylak (SB protocol), respectively.

In this two-year study, decellularized porcine esophageal ECMs were prepared using UW protocol and SB protocol. UW protocol relies mainly on enzymatic and chemical treatment while SB protocol is a combination of both mechanical and chemical method. Study the effect of dehydration methods on microstructure of decellularized ECM was carried out. Microstructure, biological aspects, cell-matrix interaction and mechanical properties of resulting decellularized ECMs were studied. The microscopic structure of ECM was viewed and analyzed by scanning electron microscopy (SEM) while the degree of cell removal and ECM components were examined by histology. Cell seeding onto decellularized ECM was also carried out to examine cell-matrix interaction. MTS and histology were employed to study the cell viability and cell distribution within the ECM. Tensile properties of resulting decellularized ECMs were tested using an Instron tensile testing machine. Furthermore, major steps involved in the protocols were studied: For chemical treatment, different treatment times were used while for mechanical scraping in SB protocol, tissues were scraped for different number of times. The outcome was examined by histology and the optimized treatment time and the number of times for

mechanical scraping was determined.

1.3 Thesis Organization

This thesis is organized into five chapters. The background, objective and scope of present research are introduced in Chapter 1. In Chapter 2, a brief literature review from previous research on the esophagus, esophageal cancer and scaffold used in tissue engineering will be presented.

Chapter 3 describes the materials and methods used in this project. These include procedure of decellularization protocols adopted in this project, dehydration methods and examination tools consisting of scanning electron microscopy, histological protocols and mechanical testing. Furthermore, Procedure of cell seeding onto decellularized ECMs is given accompanied with MTS testing for cell viability.

Results and discussion are presented in Chapter 4. The resulting ECMs derived from UW and SB protocols were examined using SEM and histology and the results are discussed. Investigation of dehydration method for sample preparation and steps involved in the protocols are also included. Results in cell seeding and mechanical testing will also be presented in this section.

A summary of the work and a conclusion will be given in chapter 5 while the future work and the prospect of the application of these techniques will be outlined in chapter 6.

Chapter 2. Literature Review

2.1 Esophageal Reconstruction

Esophageal cancer and congenital esophageal anomalies are two common esophageal diseases that require esophageal reconstruction.

Esophageal cancer has 2 main histolytic types: Squamous cell carcinoma developed in squamous epithelium and adenocarcinoma found in glandular tissue [49]. Smoking is widely considered to be the major factor for esophageal cancer; alcohol also contributes to the cause of squamous cell carcinoma while obesity and gastroesophageal reflux are other likely precursors of adenocarcinoma [6, 49, 50]. It has become ninth highest prevalent cancer in the world [5, 6] which patients are mostly found in Asia, Southern and Eastern Africa [50]. Its survival rate is as low as 10% in 5 years [7-9, 50] because noticeable symptoms often develop in the late stage of the cancer which causes most of the treatments futile. American Cancer Society predicts 13,770 patients will die of esophageal cancer among 14,550 newfound cases in 2006, the fact which reflects the severity of cancer developed in esophagus [50].

Surgery is one of the most common options for patients that affected portion is removed and the remaining part of the esophagus is either reconnected with stomach (called stomach “pull-up” or stomach transposition) or grafted with other part of gastrointestinal tract such as colon [51], stomach [11] and jejunum [12]. Although quality of life has been improved for some patients, reflux [13], anastomotic leakage

[12, 14] and stenosis [14] are the common post-operative complications. Other treatments such as chemotherapy and radiation therapy are also being used [10]. However, side effects of these treatments are highly stressful to patients.

Congenital esophageal anomalies are mainly esophageal atresia which affects 1 in 3000 to 5000 births [13]. Patients with esophageal atresia are born to lose connection between proximal and distal esophagus which develop to blind-ending pouches. Several lengthening techniques are used to bridge the gap of the upper and lower esophageal segments. One-fifth of patients were reported with anastomotic leakage after surgery due to several reasons such as ischemia and excessive anastomotic tension [13]. Alternatively, esophageal replacement is required to restore the normal continuity of esophagus in the case. Autografts such as colon [52] and jejunum [53] have been used as substitutes [13]. Anastomotic leakage and stenosis contribute to high mortality and morbidity rate [52, 53].

Apart from anastomotic leakage and stenosis partly due to operative procedure, current esophageal grafts used for reconstruction are far from ideal. Colonic graft encounters problems such as redundancy over time and slow food transit time while obtaining straight jejunal tube with sufficient blood supply for esophageal reconstruction is difficult [13]. Therefore, these grafts are unable to restore the function of esophagus completely. As knowledge is expanding in engineering, biology and medicine, it would be ideal to generate functional and biological substitutes to replace lost tissues and organs by exploiting useful knowledge from these fields. This concept has been developed into a new and exciting field: Tissue

engineering.

2.2 Tissue Engineering

The emergence of tissue engineering has shown promising results in regeneration of tissues and organs [54-56]. The basic principle involves a biocompatible and biodegradable biomaterial as a temporary template, or “scaffold” which is either pre-seeded with appropriate cell source(s) prior to implantation or directly implanted into patient to produce a construct that structurally and functionally mimic the target tissue or organ. It is hoped that the scaffold material is progressively degraded or resorbed which is timely substituted by the ECM materials secreted by the cells. Cells from autogeneic, allogeneic and even xenogeneic sources are considered and studied. Research in bone-marrow derived cells and stem cells are underway to widen the possible cell sources [57, 58]. Biomaterials that are widely used in tissue engineering include synthetic polymers (e.g. polyglycolic acid and polylactic acid) [18] and natural polymers (e.g. collagen and chitosan). These polymers are processed to create scaffolds with suitable physical, chemical and biological properties to serve as template for tissue and organ regeneration. Recently, naturally-derived xenogeneic extracellular matrices (e.g. decellularized porcine small intestinal submucosa and heart valve) which are derived from native tissue after cell removal have become important candidates for scaffold selection [35].

Commercial tissue engineered skin and cartilage products have been employed clinically [24, 25] and other tissue/ organ types such as bone, liver, blood vessel/

vascular grafts, nerve/ spinal cord, heart/ heart valves and tendon/ ligament are among the top interests currently [22].

However, the current progress of tissue engineering is concluded by one of the pioneer scientists in this field, Robert M. Nerem, as “overpromised and underdelivered” [59]. The current clinical success in tissue engineering is limited to simple tissues such as skin and cartilage [25]. For complex structures such as bone which has great zonal difference in architecture and composition, and blood vessel or gastrointestinal tracts which is a lamination of layers composed of different cell types, are difficult to generate with current technology. Challenges include cell sources that are “off-the-shelf” and immunologically acceptable [59], scaffolds that provides optimal microenvironments to guide tissue regeneration [59, 60] and vascularization [59, 60] and innervation [59] of tissue constructs. Further understanding of developmental biology is necessary to allow us to manipulate gene expression of cells including migration, proliferation and differentiation and design scaffold that provides appropriate physical, chemical and biological signals to guide tissue formation [59, 60].

2.3 Scaffold

Tissue is composed of extracellular matrix (ECM) and cells. ECM is a mixture of glycoproteins (e.g. collagen, fibronectin and laminin), proteoglycans and glycoaminoglycans [61]. ECM has long been recognized to be essential in tissue and organ development [62-65]. Therefore, tissue engineering scaffold aims to provide a

synthetic ECM for seeded or infiltrated cells to reorganize into functional and biological tissue substitute.

By definition, tissue engineering scaffold is a three-dimensional biomaterial that is biocompatible and biodegradable and has an interconnected structure of well-distributed pores with appropriate sizes [66]. Biocompatibility and biodegradability are important because the materials employed should not induce any cytotoxic effect to the seeded or infiltrated cells [67] and no foreign materials should remain by the time when tissue formation is complete. Appropriate porous architecture is also crucial that high surface area and appropriate pore size allows cells to attach and migrate while high porosity with sufficient pore interconnection ensure sufficient nutrient and waste transport [18, 67], continuity of tissue formation and promote vascularization [18, 68]. With more signals related to tissue development discovered, mimicking native ECM architecture in microscale and nanoscale (e.g. microscale undulating surface and nanofibrous structure) has been shown to be important in tissue formation [19, 61]. Furthermore, in the presence of ECM proteins such as collagen, fibronectin and laminin, cell attachment and migration are found to be markedly upregulated [19]. All these exciting and ongoing discoveries serve as solid fundamentals to design better scaffolds in the near future.

To design scaffolds suitable for tissue engineering, material selection and fabrication process are crucial to define scaffold properties. FDA-approved synthetic polymers such as polyglycolic acid (PGA) and polylactic acid (PLA) have been widely used and tested in tissue engineering [18, 19]. Biocompatibility and

biodegradability are main merits of these synthetic polymers. Furthermore, manufacturability to modify its physical shape, mechanical properties, and degradation rate are claimed to be the advantages over natural materials [19, 68]. However, lack of biological signals in these synthetic polymers retards tissue formation [68]. Although PGA and PLA release non-toxic acids during degradation, it results in drastic local reduction in pH which may be harmful to cells if acidic products are not removed efficiently [18]. Furthermore, most of the scaffold fabrication processes involve toxic organic solvent which would induce cytotoxicity even at very low levels [18].

Scaffolds made of natural polymers such as collagen [20] and chitosan [21] have advantages over synthetic polymers that no harmful by-products are released upon degradation or resorption [19]. These materials, for example, collagen has shown to provide arginine-glycine-aspartic (RGD) sequence of amino acids that is important in cell attachment and phenotype modulation [19, 68-70]. However, batch-to-batch variability and relatively lack of strength are disadvantages of natural polymers [18]. Combination of more than one polymer to improve the overall physical and biological properties has been considered another option. For example, poly(lactic-co-glycolic acid) (PLGA), a copolymer of PLA and PGA, can control degradation rate using different polymer ratio [18, 71]. Scaffold made of combination of synthetic and natural polymers may be another material choice to improve biocompatibility, degradation, mechanical strength and repeatability [19, 72].

Some conventional methods for scaffold fabrications include fiber

weaving/knitting, fiber bonding, phase separation, solvent casting/ porogen leaching and electrospinning. In weaving/knitting, PGA fibers are woven/ knitted into the form of porous felts which lack of structure integrity since fibers are woven into a loose network without immobilization at the cross-points. Fiber bonding is the improvement of weaving/knitting process that a second polymer solution is embedded into the woven felt to facilitate bonding of fibers at their cross-points followed by removal of the selective dissolution of second polymer. However, the limited choice of solvent and second polymer restricts the use of this method.

Phase separation between polymer and solvent at low temperature is another method to create porous scaffold. In certain polymer-solvent systems such as PLA-dioxane system, liquid-liquid or liquid-solid separation occurs at low temperature. Porous scaffold is produced after solvent-rich phase is removed by freeze-drying. Solvent casting/ porogen leaching [73] is a process which involves mixing polymer solution with porogen such as salt, sugar and paraffin followed by drying to remove solvent and leaching porogen by second solvent (e.g. water for salt and sugar) that does not dissolve the polymer. Subsequent drying is required for removal of the second solvent. After leaching of the porogen, it leaves a highly porous scaffold. Porogen size is important to control pore size of the resulting scaffold. This method has been reported to produce scaffold no thicker than 3mm since porogen in the deep layers cannot be leached out [18].

Electrospinning involves electrostatic force to create nanofibrous structure. The setup mainly consists of syringe with metallic needle, high-voltage power supply to

generate electric field and a syringe pump to drive the polymer solution to the needle. Power supply is connected to the needle as the fiber connector below the needle is grounded. The fiber is formed when the droplet at the tip of the needle is electrified, elongated and whipped by electrostatic repulsion. Finally, electrospun fibers deposit on the collector below the needle. Average fiber size produced by electrospinning was 100 ± 40 nm which is close to the fiber size of Type I collagen found in native tissue (approximately 50 nm) [19].

An alternative method to produce tissue engineering scaffold is decellularization of xenogeneic tissues which aims to remove all cellular materials while remain all structural (architecture) and biological (e.g. ECM proteins) signals in the matrix devoid of cells. In general, decellularization can be performed by physical, chemical and enzymatic treatment [44]. Sometimes, combination of these treatments is useful in removal of all cellular materials. Main concern of employing xenogeneic tissue-derived scaffold is immunological response which arises from residual cellular matters [47, 62, 74, 75] and chemical residue after chemical treatment [44, 76]. In esophageal tissue engineering, many of the abovementioned materials and methods have been used to create esophageal substitutes which have achieved some promising results.

2.4 Esophageal Tissue Engineering

2.4.1 Use of Synthetic Polymers

Attempts using synthetic non-degradable polymers such as plastic tube (e.g. Celestin tube) [15], silicone rubber tube [16, 17], polyethylene terephthalate (Dacron) [77] and expanded polytetrafluoroethylene (PTFE) [77] reported infection [16], anastomotic leakage [17, 77], stricture [15, 17, 77], fibrosis [16, 77], dislocation [16] and disintegration [15] after implantation for esophageal repair in animal models or human patients. Although some claimed epithelization in short term and one even reported muscular regeneration in dog models after 7 years [17], high complication rates, poor integration with the host and fibrosis due to foreign body reaction, slow tissue regeneration and polymer remnants make these materials unfavorable for esophageal regeneration.

Due to the drawbacks of synthetic non-degradable polymers, scientists move towards new types of synthetic polymers which are more biocompatible and degradable. Beckstead et al. [27] demonstrated stratification of epithelium seeded on poly(L-lactic acid) (PLLA), poly(lactic-co-glycolic) acid (PLGA) and polycaprolactone/ poly(L-lactic acid) (PCL/PLLA) *in vitro*. Zhu et al. [28] cultured esophageal cells (fibroblasts, smooth muscle cells and epithelial cells) on fibronectin- and collagen-grafted poly(L-lactide-co-caprolactone) (PLLC) membrane and concluded both grafted and non-grafted PLLC membrane supported growth of fibroblasts while both smooth muscle cells and epithelial cells showed more notable

growth and attached morphology on protein-grafted surface compared to non-grafted surface. These studies demonstrated the potential use of these synthetic polymers in esophageal tissue engineering and modification with biological signals (e.g. grafting with ECM proteins) can improve the attachment, growth and cell function. However, other physical properties have to be taken into account when using these materials. For example, PLA and PGA are believed to be too stiff for soft tissues [18] which lead to use more pliable co-polymer, PLLC [28].

In parallel with synthetic polymer, researchers are also exploiting the use of natural materials which have good biocompatibility. Collagen, one of the ECM proteins widely found in tissues, has become a good candidate for scaffold material and has been successfully used in skin grafts [24]. However, since collagen-based scaffolds are relatively weak, most of the research efforts related to collagen is focused on hybrid material which is a combination of collagen and synthetic polymers in order to improve mechanical properties and maintain graft integrity during tissue regeneration. Keio and Kyoto University are the main research groups to perform such studies.

2.4.2 Experience in Hybrid Scaffolds

Sato et al. [30] from Keio University first used epithelial-seeded collagen patch transplanted onto the surface of latissimus dorsi muscle of athymic mice to generate esophageal substitutes. Authors believed that pre-seeding of epithelial cells can

prevent stenosis and reinforce the lumen. The histological results showed a layered structure similar to the native esophageal wall at day 16. This demonstrated the regenerative power of collagen. In their next study, a tubular esophageal construct was made of PGA-reinforced collagen scaffold. Purpose of PGA is to maintain the tubular shape [29]. In their later studies, different esophageal cells were incorporated [78, 79] and one of the most notable results was exploiting mesenchymal-epithelial interaction to improve epithelial proliferation and differentiation [79]. However, although histological results suggested their resemblance to native esophagus, no implantation experiment was performed so functionality and integratability of these esophageal constructs are unknown.

A decade of study records of using a bilayered construct made of collagen and silicone rubber as substitutes for esophageal repair in dogs were conducted by research group in Kyoto University. Their studies indicated that collagen improves esophageal regeneration while silicone rubber imparts rigidity, prevents infection, leakage and dislocation at the anastomotic site upon regeneration. However, early studies showed that removal of silicone stent during premature regeneration of tissue beneath epithelium resulted in stenosis [80]. The further investigation was made to obtain the optimal stenting time of 4 weeks [31] to prevent stenosis which was parallel to the recent study by another research group using polyurethane as stent [81]. This bilayered construct was successfully repaired cervical esophageal segment up to 10 cm long [82] with fully regenerated layered structure consisting of epithelium, submucosa, glands and smooth muscle layers in both circular and longitudinal

directions [83]. However, the later studies in repairing intrathoracic esophageal segments were unsuccessful due to the lack of regeneration of skeletal muscle [84-86].

The above studies demonstrated good regenerative power of scaffolds made of collagen. Based on this fact, it appears that decellularized ECM would also be a potential scaffold option because it consists of all natural ECM proteins that may be important in tissue regeneration.

2.4.3 Decellularized ECM in Esophageal Tissue Engineering

To prepare ECM from native tissues, all cell components within tissue have to be removed completely while natural ECM proteins are retained. This process is termed decellularization. In esophageal tissue engineering, commercial decellularized skin product, Alloderm has shown to promote higher degree of stratification of esophageal epithelium *in vitro* compared to synthetic polymer [27].

Professor Stephen Badylak, one of the pioneer researchers in decellularization reported esophageal repair in dog models using acellular ECM derived from decellularization of porcine small intestinal submucosa (SIS) [5] and urinary bladder submucosa (UBS) [5, 33]. It was found that decellularized ECM was remodeled progressively and skeletal muscle cells were identifiable by day 50 which was absent in hybrid scaffolds; however, the regeneration was relatively slow that partial epithelial coverage was only observed by day 35 compared to about 28 days of full

epithelization in hybrid scaffolds [5]. All decellularized ECMs were patent for all patch repairs but the segmental repairs with decellularized ECMs alone resulted in stricture [5, 33]. On the other hand, implanting decellularized ECM alongside the muscle tissue remained patent without stricture for segmental repairs and tissue was found to be remodeled from a non-compliant tube to a structure that is histologically and biomechanically similar to native esophagus. Decellularized ECM prepared from elastic porcine aorta was also used in patch repair which showed complete tissue regeneration with layered structure and innervation [87]. These studies demonstrated native ECM poses a great hope for esophageal repair and regeneration.

2.5 Decellularization

ECM has long been recognized to be essential in tissue and organ development [62-65]. The composition and ultrastructure of the ECM are determined by factors that affect cell phenotype including mechanical forces, biochemical milieu, oxygen requirements, pH and the inherent gene expression patterns. In turn, ECM influences cell phenotype [88]. Therefore, ECM can vary from tissue to tissue and undergo continuous assembly and remodeling. The rationale behind decellularization is that with an acellular structure formed by native ECM proteins retained in its original spatial organization after cell removal, it is believed that cellular processes such as cell migration, proliferation and differentiation are initiated in a programmed manner leading to tissue regeneration.

Tissue sources for decellularization include porcine skin [40, 72], porcine small

intestinal submucosa (SIS) [5, 36-43, 45, 89-98], porcine urinary bladder submucosa (UBS) [46] and porcine heart valve (HV) [47, 76, 99-104]. SIS has shown good progress in regeneration of different tissue types *in vivo* such as tendon [42, 105], bladder [43, 46, 91, 98, 106, 107], Achilles tendon [93], vascular[36-39, 74, 89, 92, 96, 97, 107-111], meniscus [94], skin [40, 41], larynx [95] and esophagus [5, 33]. SIS also demonstrated good compatibility with different cell types *in vitro* [[41, 89, 92]. UBS has been the scaffold candidate for esophagus [33] and urinary bladder [46] while commercial products of decellularized porcine heart valve, SynerGraft, have been used as substitutes for heart valves with mixed results. One of the tragic results of using SynerGraft was reported in Europe when three child patients implanted with SynerGraft died of early failure of SynerGraft due to immunological-induced inflammation and calcium depositions [99]. Although many studies showed that decellularization is able to reduce immune response which is induced by cellular materials from syngeneic and allogeneic tissue [100, 101, 112], a small amount of remaining cell remnants and antigens within the ECM may be enough to induce adverse immune reaction. Nevertheless, success to overcome immunological issue would render xenogeneic tissue “off-to-shelf” availability which would solve the current severe allograft shortage.

Decellularization is a process involving removal of cell components while retaining biological signals by physical, chemical or enzymatic means [44]. In most of the current decellularization protocols, a combination of these methods may be applied to achieve complete decellularization. Physical methods include deep

freezing, mechanical scraping and agitation; chemical method involves treating sample with alkaline, acid, detergents, hypertonic and hypotonic solutions and enzymatic method relates to the use of enzymes. An extensive review of current method has been performed by Gilbert et al. [44].

A complete decellularization protocol may involve four steps [44]:

1. Lyse cells: Cell lysis involves breakage of cell membrane in order to release cytoplasmic materials. It can be performed by mechanical agitation, sonication and osmotic shock with hypo- or hyper- tonic solution such as deionized water (hypotonic) and solution at high salt concentration (hypertonic). Freezing in rapid manner (snap freezing) also proves effective to break cell membranes by ice crystal formation. However, ice formation could be damaging to ECM microstructure.
2. Untie cellular components from the ECM: Cells are bound tightly to ECM through integrin-ECM interaction. Trypsin and EDTA which are widely used in cell dissociation for cell culture effectively disrupt cell-ECM interaction. Trypsin is a pancreatic serine protease which cleaves specifically amide and ester bonds of lysine (Lys) and arginine (Arg) which are important in cell adhesion. EDTA is a chelating agent of divalent metallic ions such as Mg^{2+} and Ca^{2+} which are employed in cell adhesion. Prolonged trypsin treatment is known to remove important ECM proteins [44]. Non-ionic detergent, Triton X-100 may also be useful to disrupt lipid-lipid and protein-lipid interaction while retain the protein-protein interaction. Therefore, cell-cell and cell-matrix interaction are damaged.

Furthermore, Triton X-100 also causes permeabilization of cell membrane which can lead to further cell lysis [113].

3. Solubilize cytoplasmic and nuclear cellular components: After all cellular materials are released and untied, they are solubilized by chemicals such as acid, alkaline and sodium dodecyl sulfate (SDS) [113-116]. Pre-treatment with endonucleases and exonucleases to further digest DNA and RNA can be useful [104, 113, 114, 117].
4. Remove cell debris: Solubilized cellular materials and cell debris remaining in the ECM are ready to remove by soaking in buffered saline with constant shaking or agitation to allow diffusion. Post-treatment also includes removal of remaining chemicals and ECM sterilization.

2.6 Decellularization of Esophagus

Considering that the structural components can be varied from tissue to tissue [88], decellularized ECM derived from esophagus itself would be the best candidate to reduce the difference in chemical composition between decellularized ECM and host tissue. There is limited research on decellularization of esophagus. One recent study compared protocols using deoxycholic acid and Triton X-100, respectively to decellularize rat esophagus [34]. Treatment with deoxycholic acid showed better-preserved microstructure and lower DNA content in decellularized ECM compared to Triton X-100 (Figure 2.6.1). Authors believed that strong detergent Triton X-100 chemically damaged the ECM structure. *In vitro* study showed that esophageal

epithelial cells are stratified into 3 to 4 layers on decellularized esophagus in 7 days (Figure 2.6.2).

Although decellularization methods are able to remove cellular components, they may also concurrently remove essential ECM components from the ECM. Collagen and elastin are main ECM components found in esophagus. Collagen distributes widely in animal tissues and amino acid sequence of collagen is important in cell adhesion and phenotype modulation [69, 88, 118]. Moreover, it contributes to tensile and compliant properties of many soft tissues [20, 119]. On the other hand, elastin renders elastic properties [120, 121].

Figure 2.6.1: (A) Native esophagus; (B) Decellularized esophagus using deoxycholic acid; (C) Decellularized esophagus using Triton-X 100 [34].

Figure 2.6.2: (A) Native esophagus; (B) Epithelial-reseeded decellularized esophagus [34].

Rat esophagus can be used as an animal model in research but it would not be a good candidate as esophageal substitute for human due to the difference in size. It is found that pig and human share some common features physically, physiologically and anatomically [48] and amino acid sequence is highly conserved between these two species [122]. Therefore, using porcine esophagus as study object would be more suitable than rats.

In this project, two different decellularization protocols were used to decellularize porcine esophageal ECM. The first one proposed by University of Washington is based on the sequential cell removal procedure outlined by Gilbert et al. [44] and this protocol is a combination of chemical and enzymatic treatments. The second one proposed by Professor Stephen Badylak is more dependent of physical means to remove cell components. Two protocols were compared in terms of microstructure, ECM proteins and cytocompatibility. Furthermore, optimal time for each protocol would be determined to provide effective decellularization within the shortest period.

Chapter 3. Methodology

3.1 Decellularization of Porcine Esophagus

3.1.1 Collection and Pre-treatment of Porcine Esophageal Samples

All fresh porcine esophagi were harvested at local abattoir operated by the Agri-Food & Veterinary Authority (AVA) of Singapore. Esophagi were removed from healthy, 5½ month old pigs weighing about 75 kg. All dissection tools and glassware were sterilized by autoclaving or soaking in 70% ethanol before use. At the abattoir, porcine esophagi were cut into 4-cm segments. Segments were then rinsed with deionized water (about 10 minutes) followed by 0.9% saline solution. The esophageal samples were then transported back in cold sterile saline. Back in the laboratory, esophageal segments were immersed in phosphate buffer solution (PBS) if the blood was not cleaned off completely. Segments were stored in PBS at 4°C for use in decellularization.

3.1.2 Decellularization

The rationale behind decellularization is to remove all cellular components from the original tissue while preserving the microstructure of ECM, structural proteins and biomechanical properties. Two decellularization protocols from University of Washington (UW protocol) and Prof. Stephan Badylak (SB protocol) were employed

to decellularize esophageal tissues.

3.1.2.1 UW Protocol

Materials: Tris-EDTA buffer concentrate (Fluka), antibiotic antimycotic solution (AAS, Sigma A5955), pepstatin (Sigma), leupeptin (Sigma), ethylenediaminetetraacetic acid solution (EDTA, Fluka), sodium dodecyl sulfate (SDS, Fluka), phenylmethylsulfonyl fluoride (PMSF), sodium dihydrogenphosphate (Na_2HPO_4 , Fluka), potassium orthophosphate (KH_2PO_4 , Fluka), Deboxynuclease I (DNase I, Sigma) and Ribonuclease A (RNase, Sigma), Hank's balanced salt solution (HBSS, Sigma H9269), phosphate buffered saline (PBS) and Triton X-100 (Sigma).

Procedure: First, the antibiotic solution (20 μl PMSF, 100 μl AAS, 10 μl Pepstatin, 10 μl Leupeptin/10 ml stock solution) and Serensen's Phosphate Buffer (3.9 g Na_2HPO_4 , 1.15g KH_2PO_4 / 500 ml) should be made.

Muscularis externa was removed from the tissue with a scalpel and a scissor. The remaining mucosa-submucosal layer was treated in chemicals in the following sequence: (1) 100 ml hypotonic Tris-EDTA buffer (pH = 8.0) containing protease inhibitor PMSF (0.35 mg/1) for 24 hours with constant stirring; (2) 100 ml of 1 % Triton X-100 combined with 1.4 ml antibiotic solution and 5mM EDTA for 48-96 hours with constant stirring, solution was changed every 24 hours; (3) 100 ml Serensen's phosphate buffer solution consisting of RNase (0.125 mg/ml)/DNase (0.2

mg/ml) and 1.4 ml antibiotic solution for 2-6 hours; (4) 100 ml 0.5 % SDS added 1ml AAS for 24-72h with constant stirring and solution was changed every 24 hours; (5) 100 ml HBSS at 4°C for 48 hours. Except step (3), all steps were carried out at 4°C while 37°C was required for step 3.

3.1.2.2 SB protocol

Materials: PBS, distilled water, acetic acid (Merck), Hydrogen peroxide (H₂O₂, Merck), sodium hydroxide (NaOH, Merck) and phenolphthalein (Sigma).

Procedure: Muscularis externa was pre-removed, as described in the UW protocol. The remnant cells and superficial layer of tunica mucosa were removed by scraping with toothbrush for 50-150 times in a water-filled disk. The scraped sample was then placed in 150-ml peracetic acid solution (0.2 % w/w) at 4°C for 48-96 hours with constant stirring and solution was changed every 24 hours. Here, low concentration of peracetic acid solution (0.2 % w/w) used in SB protocol [89] was prepared by mixing acetic acid with H₂O₂ solution. Briefly, 2-ml glacial acetic acid was blended with 3-ml 31 % H₂O₂ solution. Then, mixture was diluted to 1000 ml by adding distilled water. Titration with NaOH (0.01 M) with phenolphthalein as indicator was used to measure the concentration of peracetic acid solution. Decellularized sample was rinsed extensively in PBS (pH = 7.0) at 4°C for 24 hours with constant stirring at 200 rpm.

3.2 Sample Storage

Decellularized ECMs were soaked in PBS for 2 hours before storage. It was then replaced by fresh PBS and the samples were stored at 4°C.

3.3 Scanning Electron Microscopy (SEM)

The microstructure and morphology of untreated esophagus and decellularized samples were viewed under scanning electron microscopy. Before observation under SEM, samples were dehydrated and then gold sputtered. Since decellularized ECMs are delicate, three dehydration methods, namely, critical point drying (CPD), freeze-drying (FD) and air-drying (AD) were investigated to determine the optimal drying approach which resulted in least damage to the sample microstructure.

3.3.1 Sample Dehydration for SEM Analysis

Before dehydration, the samples should be fixed first. The fresh or decellularized esophagus was washed in PBS for about 2 hours. The samples were cut into 1-cm² pieces, which were then fixed chemically with glutaraldehyde-osmium tetroxide fixative for 40 minutes. Briefly, the fixative was prepared from stock solutions made up with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4). First, these two solutions were cooled to 0°C in an ice bath. After cooled, one part of glutaraldehyde solution was mixed with 2 parts of osmium tetroxide solution. The fixed samples were stored in PBS at

4°C overnight.

3.3.1.1 Critical Point Drying (CPD)

Before critical point drying (CPD), samples were dehydrated in 70%, 80% and 90% ethanol sequentially. Ethanol acts as intermediate fluid, which was later replaced by CO₂ as medium or transitional fluid in critical point drying. In critical point dryer (BAL-TEC, CPD030), the sample was flushed with liquid CO₂ for 6 to 7 times to replace ethanol followed by drying sample near critical point of CO₂ (31°C and 73.8 bar).

3.3.1.2 Freeze Drying (FD)

Decellularized ECMs were pre-frozen at -20°C for at least 2 hours before freeze-drying. Freeze dryer (Martin Christ, Alpha 1-2) was operated at -53°C and 0.3 mbar in order to induce water sublimation and samples were freeze-dried for 24-48 hours.

3.3.1.3 Air Drying (AD)

Decellularized ECMs were air-dried in a biohazard hood (27°C, 1 atm). During air-drying, samples became whiter and thinner. The process was terminated when the color and weight of decellularized ECM stopped to change. About 7-9 days were required.

3.3.2 Gold Sputtering

All dehydrated samples were gold sputtered (Jeol, JFC-1600) for 120 seconds at pressure between 8 and 9 Pa and coating current of 20 mA before SEM examination.

3.3.3 Scanning Electron Microscopy (SEM)

Microstructures of untreated and decellularized esophageal samples were observed under SEM (10 kV) at different magnifications. For untreated samples, cross sections of muscularis externa and esophageal wall were investigated while cross-sections, mucosal and submucosal surfaces were examined for decellularized samples.

3.4 Histological Analysis

Histological analysis was used to qualitatively determine the degree of cell removal and remaining ECM components in resulting decellularized ECMs. In general, sample preparation for histological study involves 4 major steps: (1) fixation, (2) dehydration, clearing and embedding, (3) section and (4) staining.

3.4.1 Fixation, Dehydration, Clearing and Embedding

Sample was fixed with 10% formalin solution (Merck) for 8 hours followed by

sequential dehydration in 70 %, 80 % and 90 % ethanol for 45 minutes in each step. Finally, it was immersed three times (45 minutes each) in 99 % ethanol. Xylene (Merck) was used as clearing agents. The dehydrated sample was immersed in xylene 3 times (45 minutes each). To facilitate sectioning, sample was infiltrated with a liquid embedding medium, and then frozen to form a solid block. Embedding sample in paraffin was carried out twice for 1 hour each time. All above processes were carried out in tissue processor (Leica, TP 1020).

3.4.2 Re-embedding and Sectioning

To facilitate sectioning process, re-embedding sample in paraffin from wax dispenser (Leica, EG 1160) was carried. The re-embedded sample was sectioned using rotary microtome (Leica, RM 2155) to obtain 7 μm -thick sections. The thin section was then immersed in distilled water (500 ml) with tissue adhesive solution (5 ml) at 42°C in water bath (Leica, HI 1210) in order to ensure that sections are wrinkle-free. Finally, section-attached slide was placed on the 40°C flattening plate (Leica, HI 1220) for dehydration.

3.4.3 De-Paraffinizing Process

The sample attached on glass slide was de-paraffinized before staining. In this de-paraffinizing process, slide was first be immersed in xylene three times for 3 minutes

each to remove paraffin followed by dipping in 100 %, 95 % and 80 % ethanol for 10 times in each grade and re-hydration in distilled water for 30 to 60 seconds.

3.4.4 Staining

Dyes were used to stain the cell and tissue components for light microscopy of the cells and extracellular materials.

3.4.4.1 Hematoxylin & Eosin (H&E)

The H&E method [123] is used to stain the cytoplasm and nuclei in order to determine the extent of decellularization.

Materials: 70%, 95% and 100% Ethanol, Xylene (Merck), Harris haematoxylin (Sigma), Eosin (Sigma), Acid-alcohol (140 ml of 100% ethanol, 1 ml of concentrated hydrochloric acid), Scott's blue solution (Sigma).

Procedure: Slides containing the samples were stained in the following sequence:

(1) Stain in Harris haematoxylin for 6 minutes → (2) rinse in deionised water for 2-3 min. → (3) wash away excess dye → (4) 4 dips in acid-alcohol → (5) staining in Scott's blue solution for 5 minutes → (6) washing under running tap water → (7) 3 dips in eosin solution → (8) rinse for about 30 seconds → (9) dehydration in 70% and 95% ethanol for 30 seconds each and in two changes of 100% ethanol for 1 minute each → (10) clear in two changes of xylene for 10 minutes each → (11) mounting

coverslips on the sample using a resinous mounting medium. All the rinses were carried out in running tap water. Under inverted light microscope (Olympus DP70, CKX41), nuclei were stained dark blue while collagen and the cytoplasm of most cells appeared red to pink after H&E staining.

3.4.4.2 Masson's Trichrome method

In order to detect collagen fibers and distinguish collagen from cytoplasm and from cell nuclei, Masson's Trichrome method [124] was used in this experiment.

Material: Wiegert's iron-haematoxylin (Sigma), Bouin's fixative solution (Sigma), Beibrich's Scarlet-acid fuchsin (Sigma), phosphomolybdic-phosphotungstic acid solution (PMA-PTA, Sigma), Aniline blue solution (Sigma), 100% ethanol, Xylene (Merck), 1% Acetic acid solution (Merck).

Procedure: Slides containing the samples were stained in the following sequence: (1) Stain in Bouin's fixative solution at 55-60°C for 45 minutes → (2) rinse for 30 seconds → (3) immerse in Wiegert's iron-haematoxylin for 10 minutes → (4) rinse for about 30 seconds → (5) soak in Beibrich's scarlet-acid fuchsin for 5 minutes → (6) rinse → (7) immerse in PMA-PTA solutions for 12 minutes → (8) transfer to aniline blue solution and stain for 5 minutes → (9) rinse briefly → (10) differentiate in 1% acetic acid solution for 1 minute → (11) rinse → (12) dehydrate in three changes of 100% ethanol → (13) clear in xylene → (14) mount coverslip on the sample using a resinous mounting medium. All rinses were conducted in distilled water. Under

inverted light microscope (Olympus DP70, CKX41), Cell nuclei appeared in dark blue, cytoplasm and muscle were stained red and collagen showed blue after Mason's Trichrome staining.

3.4.4.3 Orcein Method

In order to demonstrate the presence of elastin in the decellularized ECM, orcein method [125] was used in the experiment. Orcein is used for staining elastic fibers and laminae.

Material: Orcein solution (1 g of orcein from Sigma, 100 ml of 70% alcohol), Toluidine Blue (1g of Toluidine Blue O from Sigma, 200 ml of water, 2ml of glacial acetic acid), Xylene (Merck), 95% and 100% ethanol.

Procedure: Slides containing the samples were stained in the following sequence: (1) stain in pre-warmed (37°C) orcein solution for 45 minutes → (2) rinse in 70 % ethanol → dip in acid-alcohol for 2-3 seconds → rinse under running tap water for at least 30 seconds → counterstain in Toluidine Blue at pH 4 → dehydrate in 95 % ethanol and two changes of 100 % ethanol for 15 minutes each → rinsed in two changes of xylene for 15 minutes each → mount coverslip on the sample using a resinous mounting medium. Under inverted light microscope (Olympus DP70, CKX41), elastic fibers and laminae were stained brown and other tissue components appeared blue and purple.

3.5 Cell Viability of Decellularized Porcine Esophagus

After decellularization, in order to test the cytotoxicity and cell-ECM interaction of the scaffold, it was necessary to perform cell seeding on the decellularized scaffolds. In this experiment, smooth muscle cells isolated from porcine esophagus were cultured and seeded on the decellularized ECMs and the effect would be analyzed using histological test.

3.5.1 Primary Culture of Porcine Esophageal Smooth Muscle Cells

Material: PBS, 1% antibiotic antimycotic solution (AAS, Sigma A5955), Dulbecco modified eagle medium (DMEM, HyClone SH30022), collagenase type I (Sigma C0130), fetal bovine serum (FBS, Sigma),

Procedure: The digestion solution for cell disaggregation should be prepared first. It contained 5ml DMEM, 0.625 ml FBS, 6.4 mg collagenase type I and 1 % AAS. The solution should be filtrated through 0.22 μ m filter to remove microorganisms. The cell culture medium composed of 79 % (v/v) DMEM, 20 % (v/v) FBS and 1 % (v/v) AAS was also prepared. Under sterile conditions, the esophageal muscle tissue taken from muscularis externa was placed in 50-ml centrifuge tube containing 10-ml chilled PBS with 1 % AAS for cleaning first. Then, the esophageal muscle tissue was cut into 1cm length segments. The segment was put into the digestion solution and incubated in 37 °C water bath with stirring for 3-4 hours. 5ml cell culture medium was then added to stop the digestion process. The mixture was centrifuged at 1000 rpm for 5 minutes.

The supernatant was then aspirated and discarded. The sediment was resuspended with cell culture medium and filtered through a cell strainer to remove large pieces of tissue debris. The suspension was centrifuged again at 1000 rpm for 5 minutes. After that, cells were counted using a hemocytometer and seeded at about 2.5×10^5 cells per ml of culture medium. 5-ml cell culture medium was added to each flask and stored in 37 °C, 5 % CO₂ humidified incubator (Sanyo). After 72 hours for cells to attach, the medium was then changed. The cell culture medium would be changed twice a week since then.

3.5.2 Cell Seeding

After a 14-day primary culture of porcine esophageal smooth muscle cells (SMCs), confluence was reached and subculture was carried out. Decellularized ECMs derived from both UW and SB protocols were seeded with SMCs and cell-seeded ECMs were cultured for 21 days. Staining was employed to examine the harvested ECMs.

Material: 70 % ethanol, PBS, cell culture medium (89 % DMEM, 10 % FBS, 1 % AAS, 0.2 % Kanamycin from Sigma), 0.25 % trypsin/ EDTA (Sigma T4049).

Procedure: The decellularized ECM was sterilized in 70 % ethanol for 6-12 hours. After that, the scaffold was immersed in sterile PBS for 2 days to wash away ethanol. After rinsed by sterile distilled water, each type of decellularized ECM was cut into 12 1-cm² segments. 6 samples of each type of ECM were kept in a 24-well plate and

incubated in complete medium without cells for 21 days. These were served as backgrounds and 6 wells in the same plate were incubated with medium only to serve as blanks. The rest of 6 samples of each type of ECM were kept in another 24-well plate before seeding. Confluent porcine esophageal SMCs were trypsinized from 25-cm² T-flask with 1-ml 0.25% Trypsin-EDTA solution. After dissociated cells were counted by hemocytometer, about 2.5×10^5 cells per ml of culture medium were seeded on mucosa surface of each decellularized scaffold. Furthermore, another 6 wells without scaffolds in the same plate were seeded with same amount of cells which were served as reference. 1-ml cell culture medium was added in each well. Cell-seeded ECMs were cultured for 21 days. During this period, cell culture medium was changed twice a week. The results were analyzed by H&E and Mason's Trichrome method mentioned above. The MTS assay would also be used to analyze the cell viability.

3.5.3 MTS Assay

MTS assay was to determine the number of viable cells in culture. CellTiter 96[®] AQueous One Solution reagent (Promega) was used in this experiment. Metabolism in viable cells produces "reducing equivalents" that could reduce the tetrazolium product, MTS, into an aqueous, soluble formazan product. The amount of the colored formazan product was proportional to the number of viable cells in culture.

On day 21, the scaffolds and medium were transferred to a new plate in order to

test the cells that grew on the ECMs only. The old plate was checked under light microscope for any cells growing on the well substratum.

100 μ l of MTS solution was added to each well containing 500 μ l medium followed by incubation in 37°C, 5% CO₂ incubator for three and a half hours. The scaffolds were removed and then the product solution from each well was transferred to a 96-well microplate. The absorbance was measured by microplate reader (Tecan) at wavelength of 490nm.

3.6 Mechanical Testing

3.6.1 Apparatus

Tensile property of porcine esophagi and decellularized ECMs were tested by tensile testing machine (Instron tester, Instron 5566).

3.6.2 Specimen Preparation

Since there was no standard for tensile test of soft tissues or biological materials, the standard specimen dimensions in accordance with the American Standard Test Method (ASTM) for tensile properties of plastics D638-03 were used. About 32 samples including 18 untreated esophagi, 7 decellularized ECMs produced by UW and 7 by SB protocol were tested. All samples were cut into the standard shapes (dumbbell) shown in Figure 3.6.1 and the standard dimensions are given in Table 3.1.

Figure 3.6.1: Specimen model used in tensile test.

Table 3.1: Specimen dimensions

Dimensions (see figure)	Type (mm)	Tolerances (mm)
W-Width of narrow section	3.18	± 0.5
L-Length of narrow section	9.53	± 0.5
WO-Width overall	9.53	± 3.18
LO-Length overall	63.5	no max
G-Gauge length	7.62	± 0.25
D-Distance between grips	25.4	± 5
R-Radius of fillet	12.7	± 1
T-Thickness	Measured by dial gauge	

3.6.3 Tensile Test

All the tensile tests were conducted at room temperature 22°C under open air condition. First, the thicknesses of all specimens were measured with dial gauge. The test speed was set at 0.5mm/second. One specimen was then mounted on the testing machine through 2 sets of grips. After mounting, the tensile test was started and the specimen was pulled uniaxially until rupture. Load and extension during testing were recorded by machine-connected PC. By using two relations: stress = load/cross-sectional area and strain = extension/original length, stress-strain curves of the specimen can be created accordingly. The modulus, stress and strain were calculated from the load and extension data.

Chapter 4. Results and Discussion

4.1 General Aspects of Decellularized Esophagus

In general, decellularized ECM was a whitish, translucent and fibrous matrix with epithelial ridges obviously being removed. The UW protocol-derived ECM appeared more intact compared to that from SB protocol which was apparently a looser and more swelling structure. Compared to undecellularized tissue which has 0.45 ± 0.12 mm in thickness (exclusive of epithelium), the thickness of decellularized ECMs produced using UW and SB protocols were 0.53 ± 0.04 mm and 0.72 ± 0.02 mm, respectively. The increase of thickness can be attributed to swelling of tissue after removal of cellular materials which has been observed in many studies. After cells and non-polar lipid materials were removed from the matrix, the structure becomes more hydrophilic and water would fill up the voids resulting in matrix swelling and subsequent increase in thickness.

Courtman *et al.* [104] observed a 50% swelling of decellularized heart valve using a protocol similar to UW protocol when SDS was included in the final step. Combined with their previous study on decellularization of pericardial tissue [117], they concluded that SDS would increase swelling due to disruption of hydrogen bond in triple helix of collagen and enhancement of hydrophilicity of elastin that could increase the susceptibility of elastase attack. The exclusion of SDS in the final step resulted in lower degree of swelling (25%) in pericardial tissue. In this study, it was

found that UW and SB protocol increased the ECM thickness by 20% and 60%, respectively. Apparently, UW protocol produced decellularized ECM with lower degree of swelling even though SDS wash in the final step was included. However, the thickness was increased by 60% using SB protocol. This is probably due to higher water invasion into a more broken ECM structure caused by mechanical scraping and the hydrolytic effect of peracetic acid.

A comparison of these two protocols (Table 4.1) reveals UW protocol relies mainly on chemical treatment with three important cell component removing agents whereas SB protocol is a relatively simpler process requiring only 1 chemical (peracetic acid, pH=3.2) as the agent to solubilize the cytoplasmic materials, disrupt nucleic acid [44] and also sterilize the ECM. In terms of process time, UW and SB protocol took 11 to 14 days and 3-6 days, respectively. It appeared that the longer chemical effect of UW protocol may contribute to resulting thinner ECM.

Table 4.1: UW and SB protocols

	UW Protocol	SB Protocol
Mechanical treatment	<ul style="list-style-type: none"> • Pre-removal of muscularis externa 	<ul style="list-style-type: none"> • Pre-removal of muscularis externa • Mechanical scraping of mucosal surface
Chemicals	<ul style="list-style-type: none"> • Tris-EDTA buffer (24 hours) • 1% Triton X-100 (2-6 days) • DNase /RNase (6-18 hrs) • SDS (2-4 days) 	<ul style="list-style-type: none"> • Peracetic acid
Total chemical treatment time	• 11-14 days	• 3-6 days

In general, it is preferable to employ a decellularization technique that minimizes the damage to or alteration of the proteinaceous matrix. In UW protocol, four chemicals were used and they play different role in the process: A hypotonic solution, Tris-EDTA buffer was used to provide osmotic shock to induce cell lysis which is to release cytoplasmic components from the cell interior. It is efficient for cell lysis, but does not effectively remove the cellular remnants [65, 102, 104, 108, 116, 117, 126]. Triton X-100 is the most widely studied nonionic detergent for cell extraction by destroying lipid-lipid and lipid-protein interactions that exist in cell-cell and cell-ECM adhesion. Exposure of tissue to Triton X-100 for periods ranging from several hours to 14 days [98, 103, 105, 106, 116, 126, 127]. SDS is an ionic detergent which removes nuclear remnants and cytoplasmic proteins but tends to disrupt native tissue structure, remove glycosaminoglycans and damage collagen [76, 109, 115, 116, 127-129]. DNase and RNase are enzymes that degrade nucleic components of cells [113]. Although UW protocol was desirable to remove cells without significant damage to ECM, degradation and hydrolysis which affected by these chemicals would inevitably impair the structure and components of ECM [113].

On the other hand, SB protocol employed scraping as its major decellularization procedure. This procedure removes cells directly from the tissue while it would damage the structure of ECM significantly. Furthermore, scraping introduced overextension and breakage of surface fibers. Therefore, many broken fibers were hung loosely on the ECM surfaces during this procedure making the resulting ECMs appear thicker than UW ones. In terms of process time, UW protocol took twice

longer than SB protocol because each step requires different treatment times to give effective results. However, longer treatment time in UW protocol implies higher cost in scale-up production. Furthermore, DNase used in the process is delicate which also increases the production difficulty. Results and discussion in the following sections will elaborate on the effect of treatment on different aspects of resulting decellularized ECMs.

4.2 ECM Microstructure: SEM Study

The microstructures and morphologies of fresh esophagus and decellularized esophageal ECM were investigated through SEM micrographs. Furthermore, optimal dehydration method was determined for SEM sample preparation in order to obtain the high-quality SEM images for decellularized ECM.

4.2.1 Microstructure of Untreated Esophagus

A cross-section of esophagus in Figure 4.2.1 showed that esophagus is primarily composed of two separable layers: muscularis externa and mucosa-submucosal layer. A dense muscular layer was displayed in Figure 4.2.2. Cross-section of mucosa-submucosal layer in Figure 4.2.3 and submucosal surface in Figure 4.2.5 clearly showed a relatively loose porous structure in submucosa formed by cross-linked collagen fibers which can be easily distinguished from muscular and mucosal layers. Pores were elongated and distributed evenly throughout the layer. Mucosal surface was non-porous, as shown in Figure 4.2.4. Since submucosa is porous before decellularization, it was expected that ECM would be even more porous after cell components were removed.

The microstructures of decellularized ECMs using 2 different protocols are presented in the following sections. Before that, optimal dehydration method for SEM imaging of decellularized ECM was chosen among 3 methods and the results were shown in the next section.

Figure 4.2.1: SEM micrograph (35 $\times$) of the cross-section of untreated esophageal wall (mu-sub: mucosa-submucosal layer; me: muscularis externa).

Figure 4.2.2: SEM micrograph (400 $\times$) of the cross-section of untreated esophageal muscularis externa.

Figure 4.2.3: SEM micrograph (400 $\times$) of the cross-section of mucosa-submucosal layer after muscular removal from the untreated esophageal wall (sub = submucosa; mu = mucosa).

Figure 4.2.4: SEM micrograph (400 $\times$) of non-porous mucosal surface of untreated esophagus.

Figure 4.2.5: SEM micrograph (400×) of the submucosal surface of untreated esophagus after muscular removal.

4.2.1 Effect of Dehydration Methods on SEM Preparation

Before the microstructure of decellularized porcine esophagus ECM was studied, an investigation on sample dehydration methods was carried out. Three methods were considered: (a) critical point drying (CPD); (b) freeze drying (FD) and (c) air-drying (AD). Their respective SEM micrographs were shown in Figure 4.2.6 to Figure 4.2.14.

Of the 3 methods, FD was demonstrated to be the most suitable dehydration. It can be easily explained through the cross-section views (Figure 4.2.6, Figure 4.2.9 and Figure 4.2.12) that only freeze-dried decellularized ECM retained the porous

network which resembled the microstructure observed before decellularization (see Figure 4.2.3) whereas other dehydration methods ended up a compact, closed structure due to collapse of porous structure during dehydration. Furthermore, both mucosal and submucosal surfaces of FD sample were better-preserved with pores which appeared to be the sites cells were situated before decellularization. Apparently, these surface morphologies could not be retained using CPD or AD.

Although CPD has been claimed to be useful to minimize damage on biological cells and tissues, the results showed that the procedure of CPD would damage the microstructure of decellularized samples which are seemingly more fragile. According to Spector [130], samples drying at critical point (31°C and 73.8 mbar) of CO₂ would suffer from shrinkage resulting in collapsed microstructure observed in sample dehydrated by CPD. Moisture content and the tissue itself on the other hand weighed down the microstructure during AD and shrinkage over long drying was also expected to contribute to the compact microstructure and non-porous surfaces. Spector [130] also pointed that shrinkage can be minimized in FD. This was probably because the microstructure was “frozen” (fixed) at temperature as low as -53°C while the solid water (ice) was being vaporized (sublimation) and removed directly at vacuum pressure (0.3 mbar). Due to the merit of FD, it was used as dehydration method for the following SEM examinations.

Figure 4.2.6: SEM micrograph (200 $\times$) of the cross-section of decellularized ECM by CPD.

Figure 4.2.7: SEM micrograph (200 $\times$) of the mucosal surface of decellularized ECM by CPD.

Figure 4.2.8 SEM micrograph (200 $\times$) of the submucosal surface of decellularized ECM by CPD.

Figure 4.2.9: SEM micrograph (200 $\times$) of the cross-section of decellularized ECM by FD.

Figure 4.2.10: SEM micrograph (200 $\times$) of the mucosal surface of decellularized ECM by FD.

Figure 4.2.11: SEM micrograph (200 $\times$) of the submucosal surface of decellularized ECM by FD.

Figure 4.2.12: SEM micrograph (550 $\times$) of the cross-section of decellularized ECM by AD.

Figure 4.2.13: SEM micrograph (200 $\times$) of the mucosal surface of decellularized ECM by AD.

Figure 4.2.14: SEM micrograph (200×) of the submucosal surface of decellularized ECM by AD.

4.2.2 Microstructure of Decellularized ECM

After freeze-drying, decellularized ECMs were gold-sputtered and observed under SEM. SEM micrographs for decellularized ECMs derived from UW and SB protocols were shown in Figure 4.2.15 and Figure 4.2.20, respectively. Cross-section view of UW protocol-derived ECM (Figure 4.2.15) showed a porous network which appeared more open than the untreated esophagus (Figure 4.2.3) and the microstructure was well-preserved. On the other hand, a randomly porous structure was observed in ECM produced by SB protocol (see Figure 4.2.18), especially for the area near the mucosal surface where the microstructure was somewhat random compared with Figure 4.2.15. This might be probably due to aggressive mechanical brushing involved in SB protocol.

For UW protocol, both mucosal (Figure 4.2.16) and submucosal surface (Figure 4.2.17) of ECM were porous but the former was less porous. In contrast, for SB protocol-derived ECM, mucosal surface (Figure 4.2.19) was virtually non-porous and submucosal surface (Figure 4.2.20) was randomly porous. Obviously, the microstructure of mucosal surface was damaged by mechanical treatment. The surface with broken fibers generated during scraping was shrunk after dehydration. This would damage the surface integrity resulting in impermeable surface. Without mechanical scraping involved in UW protocol, surface morphologies were well-preserved because there was no direct mechanical contact on the ECM surface during decellularization.

SEM micrographs have shown that UW protocol produced highly porous and organized structure. Furthermore, the microstructure was well-preserved which resembled the untreated ECM whereas SB protocol would damage the structure of ECM and could hardly retain the native morphologies of mucosal and submucosal surfaces. Studies have demonstrated that 3D presentation of RGD-peptide sequence is far more important than the mere presence of the sequence. Badylak *et al.* [89] showed that endothelial cells attached in a higher extent on hydrated form of decellularized SIS than air-dried form. Obviously, it is due to the disruption of 3D microstructure of ECM which subsequently caused the undesirable rearrangement of all biomolecular signals on the ECM. It strongly implies the importance of microstructure conservation to tissue regeneration.

Next, degree of decellularization was evaluated by histology and functions of

critical steps involved in decellularization protocol were studied and the process time was optimized.

Figure 4.2.15: SEM micrograph (500 $\times$) of the cross-section of decellularized ECM by UW protocol.

Figure 4.2.16: SEM micrograph (500 $\times$) of the mucosal surface of decellularized ECM by UW protocol.

Figure 4.2.17: SEM micrograph (500 $\times$) of the submucosal surface of decellularized ECM by UW protocol.

Figure 4.2.18: SEM micrograph (200 $\times$) of the cross-section of decellularized ECM by SB protocol.

Figure 4.2.19: SEM micrograph (400 $\times$) of the mucosal surface of decellularized ECM by SB protocol.

Figure 4.2.20: SEM micrograph (400 $\times$) of the submucosal surface of decellularized ECM by SB protocol.

4.3 Histology

The objective of decellularization is to remove all cellular materials such as DNA and RNA while important natural ECM materials such as collagen and elastin are retained. To examine the efficacy of a decellularization protocol, degree of cell removal, presence of ECM materials have to be evaluated and histology allows us to localize cells and other ECM components.

4.3.1 Degree of Decellularization: H&E Staining

Haematoxylin and eosin (H&E) staining allows to localize nuclei and cytoplasm of cells embedded in the tissue. After decellularization, it is hoped that all the cell materials are removed and H&E staining of decellularized ECM should show absence of cell materials.

The H&E stained sections of untreated porcine esophagus were shown in Figure 4.3.1 and Figure 4.3.2. Nuclei and cytoplasm were stained purple and pink, respectively. Epithelium and the underlying layers (including basement membrane, lamina propria, muscularis mucosae and submucosa) were easily distinguished in 10× magnification (Figure 4.3.1), epithelial cells were identifiable but stained nuclei could only be spotted in 40× magnification (Figure 4.3.2). The H&E staining of untreated sample serves as a reference to evaluate the degree of cell removal of porcine esophagus after decellularization.

Figure 4.3.1: Histological section (H&E, 10×) untreated esophagus after removal of muscularis externa. Stratified epithelium (ep) lines the esophageal wall and beneath epithelium is a dense structure with some pockets of porous networks scattered near the bottom side.

Figure 4.3.2: Histological section (H&E, 40×) untreated esophagus after removal of muscularis externa (ep = epithelium). At this magnification, cell nuclei (purple dots) of epithelial cells are conspicuous and higher number of undifferentiated epithelial cells can be seen on the basement membrane (dark purple wavy contour).

4.3.1.1 UW protocol

UW protocol includes five major steps which have their particular functions. Among all steps, 3 of them which are cell extraction/rinse by Triton-X 100, nuclear digestion by DNase/RNase and nucleic acid/cell extraction by SDS are the most crucial in UW protocol. Therefore, studies on process time and function for each of these important steps were carried out individually and H&E sections were used to examine the degree of decellularization.

Figure 4.3.3: Histological section (H&E, 40 $\times$) of decellularized porcine esophagus (by UW protocol) after 2-day cell extraction/rinse. Epithelium (ep) appeared to be intact after treatment compared to untreated sample.

Figure 4.3.4: Histological section (H&E, 40×) of decellularized porcine esophagus (by UW protocol) after 4-day cell extraction/rinse. Delamination of epithelium (ep) occurred and most of cell nuclei in the upper epithelium were extracted but cells were still found near the cell membrane (dark stained wavy contour).

Figure 4.3.5: Histological section (H&E, 40×) of decellularized porcine esophagus (by UW protocol) after 6-day cell extraction/rinse. Delamination of upper epithelium (ep) and separation of basement membrane occurred but there was no obvious difference in number of nuclei-containing layers compared to 4-day treatment.

The decellularization progress after 2-, 4- and 6-day cell extraction/rinse was shown from Figure 4.3.3 to Figure 4.3.5. In this step, 1% Triton X-100 was used. As a non-ionic surfactant (detergent), Triton X-100 was useful for removal of cells and has been used widely [125]. Triton X-100 contains agents that can disrupt and/or degrade cellular constituents such as cell membranes and proteins. Early studies showed that enzyme activity from mammalian muscle and nerve tissue could be stopped in 0.5% or 1% Triton X-100 [131, 132] due to cell lysis and destruction of cellular components.

The results showed that there was no notable change between untreated esophagus (see Figure 4.3.2) and ECM after 2-day treatment (Figure 4.3.3). On day 4 (Figure 4.3.4), although the image was not in focus in some area, it demonstrated epithelium was delaminated about 1/3 of original thickness. However, delamination did not proceed further after day 4 (Figure 4.3.4) as the epithelium of decellularized ECM on day 6 (Figure 4.3.5) showed no obvious difference compared to that on day 4. Although Ozeki *et al.* [34] showed a more degrading structure after 3-day Triton X-100 treatment, it appeared that since porcine esophagus is apparently thicker than rat esophagus, longer Triton X-100 treatment in porcine esophagus would cause less damaging effect on the ECM structure. Non-ionic detergents have been widely used in decellularization process due to its mild damaging effect on ECM than ionic detergent [104, 105, 114, 115, 117, 127, 128]. It disrupts lipid-lipid and lipid-protein interactions, while leaves protein-protein interaction intact [128, 133]. As a non-ionic detergent, Triton X-100 has achieved mixed results in decellularization. Triton X-100

alone has shown not to be able to completely remove cellular materials [103, 105, 127, 128]. Higher degree of decellularization with Triton X-100 has to combine with other steps such as enzymatic digestion and further wash steps [104, 111, 117]. In this study, it showed that Triton X-100 alone is insufficient to achieve complete cell removal of esophageal tissue, even treatment for up to six days. On the other hand, if treatment time was prolonged, the structure of ECM was found to be slightly disrupted. The use of Triton X-100 was to loosen the tight “bond” between cell and cell and also between also cell and matrix. This step was enough to “loosen up” the cells from the tightly bound structure but still far from effective to remove cellular materials such as cell nuclei which were still heavily stained in the treated sample.

From this analysis, since there is no obvious difference between results on day 4 and 6, 4 days would be effective for this step. After 4-day Triton X-100 treatment, the samples were treated with DNase/RNase solution to remove nucleic materials.

Figure 4.3.6: Histological section (H&E, 10×) of decellularized porcine esophagus (by UW protocol) after 6-hr nuclear digestion. Epithelium was fully removed but cells within the underlying layer were not obvious at this magnification.

Figure 4.3.7: Histological section (H&E, 40×) of decellularized porcine esophagus (by UW protocol) after 6-hr nuclear digestion. At this magnification, a small number of cell nuclei (purple dots) scattered.

Figure 4.3.8: Histological section (H&E, 40×) of decellularized porcine esophagus (by UW protocol) after 12-hr nuclear digestion. Cell nuclei are hardly found after treatment.

Figure 4.3.9: Histological sections (H&E, 40×) of decellularized porcine esophagus (by UW protocol) after 18-hr nuclear digestion.

The treatment solution contains Deoxyribonuclease I (DNase I) and Ribonuclease A (RNase A) which are ubiquitous enzymes that catalyze the degradation of nucleic acids primarily through the hydrolysis of phosphodiester bonds of DNA and RNA, which are the most important components that are composed of a cell nucleus. As the widely used enzymes [113] for degrading nucleic components, DNase/RNase were applied for digesting nuclei in this experiment and it was expected that most of nuclei could be removed.

Freyman [134] showed that DNase treatment less than an hour could reduce double-stranded DNA (dsDNA) content by 4 times compared to non-treated bone marrow while collagen content in DNase-treated sample remained comparable to native tissue. It showed the effectiveness of DNA on clearing cellular DNA without damaging native collagen. In this study, DNase performed effectively on DNA removal. Figure 4.3.6 presented histological section of decellularized porcine esophagus after 6-hour nuclear digestion at low magnification (10×). Obviously, this step resulted in further delamination of epithelium that most of the cell nuclei were digested and hardly found in the mucosa. On the other hand, only a small number of purple-stained nuclei could be seen within the submucosa at high magnification (40×, Figure 4.3.7).

In order to further remove cell nuclei in submucosa, this process was prolonged to 12 and 18 hours, respectively. After 12-hour nuclear digestion, almost no cell nuclei could be spotted in the submucosa (Figure 4.3.8). Extended nuclear digestion for 18-hour (Figure 4.3.9) gave the similar results compared to 12-hour digestion.

Therefore, nuclear digestion for 12 hours is optimal based on this result. This result was also parallel to the findings of Courtman *et al.* on pericardial tissue and heart valve that DNase/RNase treatment after cell-cell and cell-matrix disruption by Triton X-100 was important and effective in DNA and RNA removal.

According to Chen *et al.* [115] on decellularization of skin, it is important to remove the epidermal layer to obtain a cell-free underlying dermal matrix. Similarity can probably apply in this study here. Epithelium and epidermis are both stratified layers with cells tightly bound to each other. If these cell layers are not removed, cells underneath in dermis (in this case which is submucosa) are far from the surface due to these tightly bound layers which could pose a diffusion resistance to all chemicals and enzymes reaching these cells. Furthermore, epithelium and glands in submucosa in normal esophagus have shown to be positive for HLA-ABC and HLA-DR antigens (on the surface of these cells) which indicate high possibility of inducing immune reactions upon implantation if they are not completely removed [75]. Therefore, complete removal of epithelium is required. DNase/ RNase treatment resulted in an epithelium-free matrix with some cells scattering in the underlying layer.

To clear cell remnants in underlying layer, nucleic acid/cell extraction by SDS was carried out. This step was important for decellularization because it was used as final clearance of all cell components.

Figure 4.3.10: Histological section (H&E, 10×) of decellularized porcine esophagus (by UW protocol) after 2-day nucleic acid/cell extraction. The overall structure was still intact as untreated sample and the pockets of porous networks were not destructed after treatment.

Figure 4.3.11: Histological section (H&E, 40×) of decellularized porcine esophagus (by UW protocol) after 2-day nucleic acid/cell extraction. Cell nuclei were not found in the layer giving an acellular structure.

Figure 4.3.12: Histological section (H&E, 40×) of decellularized porcine esophagus (by UW protocol) after 4-day nucleic acid/cell extraction.

SDS is anionic detergent that has been applied in many decellularization protocols [105, 113-115, 117, 128, 129]. As a useful tool for the separation of protein, it was expected that SDS could extract cells and nucleic acid completely. The histological images proved that this ionic detergent was effective in solubilization of cellular materials.

Figure 4.3.10 and Figure 4.3.11 presented the histological sections at 2 different magnifications after 2-day treatment. Obviously, it was shown that almost all cell components were removed after 2-day treatment. Nevertheless, since this is the final step in cell removal, it is reasonable to prolong the treatment for another 2 days to ensure complete removal of cell components (see Figure 4.3.12). SDS is an amphipathic molecule and its hydrophobic region binds to protein that is believed to increase the negative charge of the protein [135]. Therefore, it improves the solubility

of proteins and it would be highly effective to remove cellular proteins from tissue. Compared to other detergents, SDS was reported to remove nuclear remnants and cytoplasmic proteins, for example, vimentin more completely [116]. Chen *et al.* [115] also showed a beneficial effect of extended SDS wash on complete cell removal in dermal layer of skin.

In general, the UW protocol could produce decellularized ECM successfully. Histological images had demonstrated that each chemical used in the protocol perform their functions in extracting cells and nucleic acid resulting in acellular ECM. Ozeki *et al.* [34] used one-step protocol consisting of a concoction of Triton X-100, EDTA and DNase/RNase and ended up in higher DNA content. The reason of higher DNA content is obvious due to misuse of EDTA in the cocktail which is a chelating agent of divalent ions (Mg^{2+} and Ca^{2+}) in order to reduce DNase activity. The presence of EDTA would definitely retard the effect of DNase. In UW protocol, although both chemicals were involved, they were not concurrently used so the activity of DNase involved in the final step was not affected. However, this protocol has disadvantages. It consists of a combination of chemicals. Any residue of chemicals might induce adverse effect on tissue regeneration [76]. Furthermore, this protocol is comparatively time-consuming and the cost would increase for scale-up production.

4.3.1.2 SB Protocol

SB protocol is relatively simple which involves mechanical scraping of both mucosal and submucosal surfaces followed by cell lysis using 0.2% peracetic acid. Figure 4.3.13 to Figure 4.3.15 showed the effect of increasing number of times of mechanical scraping (50, 100 and 150 times each for both surfaces). In general, Cell layers were effectively peeled off by mechanical scraping and increasing the number of times of scraping definitely resulted in higher degree of decellularization. However, scraping could damage the delicate ECM structure that some broken ECM fragment hung on the surface and the degree of damage appeared to be increased when higher number of times of mechanical scraping was employed.

Nevertheless, from these histological sections, mechanical scraping for 100 times were shown to be more effective in cell removal than that for 50 times but relatively less damaging to ECM than that for 150 times. 150-time scraping appeared to eliminate most of the fibers on the surface. Therefore, 100-time mechanical scraping was considered to be optimal for this step.

Figure 4.3.13: Histological section (H&E, 40×) of decellularized porcine esophagus (by SB protocol) after 50-time mechanical scraping. Delamination of epithelium (ep) from the basement membrane (bm) occurred.

Figure 4.3.14: Histological section (H&E, 10×) of decellularized porcine esophagus after 100-time, mechanical scraping. (by SB protocol). Epithelium was fully removed while further thinning of underlying layers and rupture of pockets of porous networks in submucosa occurred. A small amount of cells (nuclei in black) scattered.

Figure 4.3.15: Histological section (H&E, 10×) of decellularized porcine esophagus (by SB protocol) after 150-time mechanical scraping. Porous network disappeared leaving a dense structure with a small amount of cells (nuclei in black) scattered.

Figure 4.3.16: Histological section (H&E, 40×) of decellularized porcine esophagus (by SB protocol) after 2-day peracetic acid treatment. Almost no cell nuclei were found.

Figure 4.3.17: Histological section (H&E, 40 $\times$) of decellularized porcine esophagus (by SB protocol) after 4-day peracetic acid treatment.

After cell removal by scraping, the sample was then immersed in 0.2% peracetic acid solution. Figure 4.3.16 and Figure 4.3.17 displayed the H&E-stained sections after lysis by peracetic acid for 2 and 4 days, respectively. Peracetic acid used in this protocol has been widely used as disinfectant in livestock industry due to its oxidizing effect on the cell wall of a wide spectrum of microorganisms [136]. This strong oxidant made of acetic acid and peroxide might also be able to stimulate the cell separation and hydrolyzation of proteins.

In patent filed by Padmini *et al.* [137], strong oxidizing agents like peracetic acid can produce free radicals such as hydroxyl radicals ($\text{OH}\bullet$) and reactive oxygen species ($\text{O}_2\bullet$) which cause reaction with lipid membrane and DNA destruction. This can be shown in the histological images from this study that cellular remnants were removed thoroughly after 4-day treatment. Based on DNA quantification of

decellularized SIS, Padmini *et al.* [137] also found the synergistic effect of hydrogen peroxide and peracetic acid to achieve higher DNA removal.

From this result, SB protocol is also able to effectively remove cell components giving an acellular ECM. However, although mechanical scraping can remove cells effectively in a short period, ECM structure was destroyed according to SEM results which might be important in cell re-seeding and tissue regeneration [62]. On the other hand, UW protocol not only can remove cell effectively, but also preserve the ECM integrity with a relatively ordered porous network that resembled the native esophageal submucosa.

4.3.2 Presence of collagen: Masson's Trichrome

Figure 4.3.18 showed the histological micrograph of the untreated esophagus stained by Masson's Trichrome method for collagen. Nuclei appeared dark blue, the cytoplasm and muscle were stained in red color and collagen showed in blue color. Figure 4.3.19 and Figure 4.3.20 showed the stained sections of samples prepared by decellularization with UW and SB protocol, respectively. Taking the untreated esophagus (Figure 4.3.18) as reference, both decellularized ECMs were shown to be free of cells leaving a network abundant in collagen.

Figure 4.3.18 : Histological section (Masson's Trichrome, 40×) of untreated esophagus. Collagen (blue) is present in the underlying layer of epithelium (ep).

Figure 4.3.19: Histological section (Masson's Trichrome, 40×) of decellularized porcine esophagus by UW protocol.

Figure 4.3.20: Histological section (Masson's Trichrome, 40×) of decellularized porcine esophagus by SB protocol.

Collagen is the most abundant protein in animals and provides the frame work for all multiple-cellular organisms [118]. It is the chief structure protein that makes up connective tissues. Furthermore, peptide sequences RGD and DGEA that exist in collagen are believed to improve cell adhesion [69]. Therefore, preservation of collagen in decellularized ECM is beneficial in cell re-seeding and tissue regeneration. On the other hand, of the many components in the ECM, collagen is the primary determinant of tensile properties [20] and resists multi-dimensional stresses in highly compliant tissues such as blood vessels and gastrointestinal tract [119]. The presence of collagen structure is important to render proper mechanical properties in decellularized ECM.

Chemical and enzymatic treatments were known to destroy ECM collagen in some extent during cell removal [44, 129]. UW protocol employed 2 detergents (Triton X-100 and SDS) and two enzymes (DNase and RNase). Triton X-100 is

nonanionic detergent which has shown least damage on ECM collagen in most of the studies [104, 117, 128, 129]. On the other hand, DNase and RNase are weak enzymes which only target on DNA and RNA from lysed cells [104, 117] and they have been shown to retain collagen content in the ECM [134]. However, there are mixed results on reservation of ECM proteins when SDS was used. Courtman *et al.* [104, 117] observed increased swelling and decreased thermal stability in the pericardium and heart valve treated with SDS, suggesting destabilization of the collagen matrix. Hudson *et al.* [128, 129] also found the destructive nature of anionic detergent on nerve ECM structure. On the other hand, Chen *et al.* [115] showed collagen crosslinks were unaffected in dermal layer after SDS treatment. The use of SDS on decellularization protocol is probably tissue-dependent.

The results of the present study have shown that collagen present in both decellularized ECMs. However, compared to original esophagus, collagen stained less intense in the decellularized ECMs. There were even some open areas within acellular matrix. This might be due to SDS loosening collagen bundles in UW protocol due to disruption of hydrogen bond in collagen triple-helix conformation [117] and mechanical damage of collagen fibrous structure in SB protocol.

4.3.3 Presence of Elastin: Orcein Method

Using Orcein method, elastin and laminae were stained brown. Figure 4.3.21 and Figure 4.3.22 showed that both protocols preserved elastin well. Esophagus is a highly elastic organ and elastic fibers formed by elastin contribute the elasticity of the tissue. As such, it plays an important role in the functional properties of tissues and organs that require flexibility and elasticity [138]. Elastin fibers act in the physiological range and provide the necessary resilience to recover from tissue deformations. It is resilient and displays mechanical properties similar to rubber that allow it to linearly store energy with little loss [120, 121] and it can extend up to 50-70% of original length under physiological loads [110]. Therefore, preservation of elastin is important in decellularized ECM since it contributes to the elastic property of native tissue.

Figure 4.3.21: Histological section (Orcein, 400×) of decellularized porcine esophagus by UW protocol

Figure 4.3.22: Histological section (Orcein, 400x) of decellularized porcine esophagus by SB protocol.

Histological images stained by Masson's Trichrome and orcein method revealed decellularized ECMs produced using UW and SB protocol were acellular as suggested in H&E results. Furthermore, essential ECM components, collagen and elastin, were well-preserved throughout the ECM. In terms of acellularity and preservation of ECM components, both protocols are comparable. However, it appeared that UW protocol preserved microstructure better than SB protocol suggested in SEM findings. The results in cell seeding onto the decellularized ECM might allow us to understand the importance of microstructure on cell-matrix interaction.

4.4 Cell Viability of Decellularized ECM

4.4.1 MTS Assay

After cell culture of PESMC-seeded decellularized ECMs for 21 days, MTS assay was used to analyze the cell viability. The data of absorbance and absolute absorbance of MTS assay was shown in Table 4.2 and Table 4.3 with Figure 4.4.1 showing the configuration of sample allocation in 96-well plate.

There was no significant difference in absorbance between unseeded ECMs produced by UW and SB protocols, which were 0.528 and 0.526, respectively and close to blank control (medium only) which had a mean of 0.538. In the cell-seeded wells (without scaffolds), the mean absorbance was as high as 1.196. The absorbance was relatively lower for seeded decellularized ECMs but it was higher than unseeded ECMs and blank control implying the presence of viable cells. The similar conclusion can be drawn from Table 4.3 where absolute absorbance for both ECMs were positive.

Table 4.2: Absorbance of MTS assay, Mean and SD

	1	2	3	4	5	6	Mean	SD
A	0.812	0.805	0.785	0.775	0.829	0.790	0.799	0.018
B	0.560	0.554	0.542	0.549	0.533	0.562	0.550	0.010
C	1.201	1.192	1.180	1.204	1.211	1.186	1.196	0.011
D	0.531	0.532	0.519	0.524	0.529	0.535	0.528	0.005
E	0.519	0.524	0.533	0.521	0.518	0.537	0.526	0.007
F	0.543	0.532	0.540	0.531	0.539	0.548	0.538	0.006

Table 4.3: Absolute absorbance (minus background reading), Mean and SD

	1	2	3	4	5	6	Mean	SD
A-D	0.282	0.273	0.266	0.251	0.300	0.255	0.271	0.017
B-E	0.040	0.030	0.009	0.028	0.015	0.025	0.025	0.010
C-F	0.660	0.660	0.640	0.673	0.672	0.638	0.657	0.014

Figure 4.4.1 The 96-well plate designed for MTS test

By comparison of cell-seeded ECMs produced by 2 protocols, the mean absolute absorbance was much higher for ECMs produced by UW protocol than those by SB protocol. This result suggested there were more viable cells detected on ECMs derived by UW protocol. However, MTS results of seeded scaffold were significantly lower than controls. This result might not be conclusive because MTS test depends on the reaction between viable cells and dye solution. Since decellularized ECM is three-dimensional, the reaction might be diffusion-limited. Secondly, cells seeded on the ECM surface might migrate into the ECM. Therefore, not all cells within the ECM were exposed to dye solution during the test resulting in lower absorbance. Finally,

The seeded surface area of decellularized ECM (1 cm^2) was only half of that of the well surface (2 cm^2) so more cells seeded on the well exposed to the MTS solution, resulting in higher reading. If these values were normalized by area (absorbance/area), the mean absorbance of UW-derived samples was higher than monolayer of cells, suggesting non-cytotoxicity of materials and good cell-matrix interaction. However, one has to be careful of the residue of chemicals left in the decellularized ECM which could exhibit cytotoxicity to cells [76]. A thorough wash protocol after treatment has to be carried out before it is put into use. In order to further understand cell-matrix interaction, histology was carried out in order to visualize the cells within the ECM

4.4.2 Histology

Figure 4.4.2 and Figure 4.4.3 showed the histological images (H&E) of cultured smooth muscle cells on the decellularized ECMs produced by UW protocol and SB protocol, respectively. The nuclei displayed blue; collagen and the cytoplasm of most cells showed the color of red to pink. These two images showed clearly that UW protocol by far resulted in better cell distribution that cells could be observed across the ECM whereas cells could hardly be seen on the decellularized ECM produced using SB protocol.

Because the color contrast could not be clearly made under low magnification with H&E staining method, Masson's Trichrome method was also used to analyze the cell distribution.

Figure 4.4.4 and Figure 4.4.5 showed the histological sections (Masson's Trichrome) of cell-seeded ECMs produced by UW protocol. The nuclei were stained dark blue, the cytoplasm and muscle displayed red and collagen appeared blue. With images at 100 $\times$ and 400 $\times$ magnifications, it could be clearly observed that cell layers were formed on the surface.

Figure 4.4.2: Histological section (H&E, 40 $\times$) of SMCs-seeded decellularized porcine esophagus produced by UW protocol after 21-day culture. Cells were found to be layering up on the decellularized ECM.

Figure 4.4.3: Histological section (H&E, 40×) of SMCs-seeded decellularized porcine esophagus produced by SB protocol after 21-day culture. Almost no cells were found within the ECM.

Figure 4.4.4: Histological section (Masson's Trichrome, 10×) of SMCs-seeded decellularized porcine esophagus produced by UW protocol after 21-day culture. Cell layers obviously grew and infiltrated into the collagen-abundant ECM.

Figure 4.4.5: Histological section (Masson's Trichrome, 40×) of SMCs-seeded decellularized porcine esophagus produced by UW protocol after 21-day culture. Cell nuclei were identifiable within the cell-seeded ECM and the thickness of cell layer exceeded 100 μm .

Figure 4.4.6: Histological section (Masson's Trichrome, 10×) of SMCs-seeded decellularized porcine esophagus produced by SB protocol after 21-day culture. Cells were hardly found within the collagen-abundant ECM.

Figure 4.4.7: Histological section (Masson's Trichrome, 40 $\times$) of SMCs-seeded decellularized porcine esophagus produced by SB protocol after 21-day culture. At this magnification, only a few cell nuclei were spotted on the ECM surface.

Figure 4.4.6 and Figure 4.4.7 presented the staining results (Masson's Trichrome) for cell-seeded ECM produced by SB protocol. Obviously, cells could hardly be seen across the ECM. Therefore, from the H&E and Masson's Trichrome staining images, UW protocol demonstrated to produce better decellularized ECM to support cell growth compared to SB protocol. While the scaffold derived from SB procedure could hardly support the cell seeding.

This result allows us to understand the importance of preservation of ECM of tissue. ECM is crucial in regulating cell functions via cell-matrix interactions, cytoskeletal organization, and integrin-mediated signaling [70, 139]. The main components of ECM are glycoproteins and in most animals, the most abundant glycoprotein in the ECM is collagen. Collagen is one of the important adhesion proteins that enhance cell attachment and proliferation through specific interactions between domains, such as RGD in collagen molecules and integrins in cell

membranes [70]. ECM also contains many other components: proteins such as fibrin and elastin, minerals such as hydroxylapatite, or fluids such as blood plasma or serum with secreted free flowing antigens [41, 140].

Given this diversity, ECM serves a number of functions, such as providing cells with anchorage sites and support and regulating intercellular communication. Therefore, if the organization of spatial structure proteins and other important components in the ECM were damaged after decellularization, it might result in poor cell-matrix interaction which was suggested in the cell seeding results on decellularized ECMs produced by SB protocol. On the other hand, ECM from UW protocol preserved not only the structural components, collagen and elastin, but also retain the microstructure integrity. Promotion of cell growth on this type of ECM demonstrated the importance of structural integrity of the decellularized ECM which is parallel to the findings of Badylak *et al.* that 3D presentation of peptides was far more important than the mere presence of all the biological cues [89].

On the strategies of esophageal regeneration *in vitro*, Ozeki *et al.* [34] suggested pre-epithelization prior to implantation would be beneficial to prevent scar formation and improve wound healing and *in vitro* seeding of smooth muscle cells was important to resume muscular function of esophagus and support the tube integrity. In this study, cell layers of smooth muscle observed in UW protocol-derived ECM was likely the precursor of smooth muscle. Appropriate mechanical or other biological cues are probably important for the maturation of smooth muscle.

4.5 Mechanical Test

All the esophageal samples showed non-linear stress-strain behavior. Figure 4.5.1 and 4.5.2 show the load-extension curves and stress-strain relationships of mucosa-submucosal layer, muscular layer and decellularized ECM. Values of load and extension were first recorded by the PC connected to Instron machine which were then computed as stress and strain by using two relations: $\text{stress} = \text{load}/\text{cross-sectional area}$ and $\text{strain} = \text{extension}/\text{original length}$. Stress-strain curves of the specimen can be generated accordingly assuming invariable cross-sectional area. Therefore, the stress in Figure 4.5.2 refers to engineering stress.

Figure 4.5.1: Load-extension curves of mucosa-submucosal layer, muscle layer (muscularis externa) and decellularized ECM using UW protocol. All curves were obtained at crosshead speed of 0.5mm/s.

Figure 4.5.2: Stress-strain curves of mucosa-submucosal layer, muscle layer (muscularis externa) and decellularized ECM using UW protocol. All curves were obtained at crosshead speed of 0.5mm/s.

The ultimate tensile stresses (UTS) of mucosa-submucosal layer, muscle layer and decellularized ECM were 7, 2.1 and 3.9 MPa, respectively. Therefore, decellularized ECM inherits part of mechanical strength from the native tissue which was stronger than muscular layer. However, UTS of mucosa-submucosa was almost reduced by half after decellularization. On the other hand, their respective Elastic moduli were 7.8, 2.1 and 5.3 MPa, respectively. It implied that decellularized ECM is less stiff than the native tissue. This difference might be due to ECM structural change during decellularization. Mucosa-submucosal layer is mainly composed of collagen, the predominant compositional and structural component found in ECMs of connective tissue [20] which contribute to tensile property of the tissue. After

decellularization, structural components might be degraded or damaged in some extent resulting in weaker tensile property and becoming less stiff.

Figure 4.5.3: Stress-strain curves for decellularized porcine esophagi derived from UW and SB protocol. All curves were obtained at the extension rate of 0.5 mm/s.

Figure 4.5.2 showed 2 stress-strain curves of decellularized ECMs prepared by UW and SB protocol, respectively. The respective UTS were 3.8 and 4.7 MPa while the respective Elastic moduli were 2.1 and 1.6 MPa. Obviously, although the reduction in mechanical strength was significant in decellularized ECM from SB protocol but was still slightly stronger than those produced by UW protocol. On the other hand, decellularized ECM from SB protocol was less stiff than those from UW protocol, implying the effect of fiber breakage during mechanical scraping. The significantly higher reduction in mechanical strength for ECM derived from UW protocol was probably because of the chemical degradation of structural materials.

Longer treatment time might also cause higher degree of degradation.

In previous studies, loss of mechanical strength in a certain extent has been observed for most of the decellularization protocols used [44, 104, 105, 117] which was due to loss of cells and soluble ECM components, and removal or re-arrangement of structural ECM components that are mainly responsible for the ECM mechanical properties. Even most of the ECM structure was preserved after decellularization, cells were not able to be removed completely which would be highly immunogenic once implanted and graft failure will pursue quickly. Therefore, decellularization is a trade-off process between cell removal (to reduce or eliminate immune reaction) and preservation of native ECM (to retain biological cues and mechanical properties). Loss of ECM components and mechanical properties is almost inevitable to achieve complete cell removal.

Although both protocols would induce weakening of mechanical properties of native tissue, mechanical testing results showed that the UTS and elastic modulus of decellularized ECMs were still on the same order as the native tissue. Therefore, most of the mechanical properties were preserved after decellularization using both protocols. In the future mechanical testing on decellularized ECM, other mechanical aspects such as viscoelasticity have to be conducted under different loading frequency. Test on stress relaxation is also a valuable tool to evaluate the viscoelastic properties of decellularized ECM [104, 117].

Chapter 5. Conclusion

Decellularized tissues have shown potential use as tissue engineering scaffolds for regeneration of many different tissue types. In this study, two decellularization protocols were employed to produce decellularized esophageal ECMs. UW protocol relies on treatment of three chemicals (Triton X-100, DNase/RNase and SDS) while SB protocol combines mechanical scraping and treatment in peracetic acid. The microstructure, histology, cell-matrix interaction and mechanical properties of decellularized ECMs were studied.

Both protocols produced whitish, translucent membrane. Microstructure of decellularized ECMs from UW protocol showed organized, porous surfaces and cross-section. The porous network resembled the structure prior to decellularization. On the other hand, SB protocol resulted in nonporous surfaces and randomly porous interior, probably due to mechanical damage by scraping. Histology results showed both protocol were able to achieve complete decellularization and retention of essential structural components, collagen and elastin. From this study, it was found 4-day cell extraction by Triton X-100, 12-hour nuclear digestion by DNase/RNase followed by 4-day nuclei acid/ cell extraction by SDS proved effective in complete decellularization for UW protocol while acellularity can be achieved by 100-time mechanical scraping followed by 4-day peracetic acid treatment in SB protocol. Porcine esophageal smooth muscle cells were cultured on decellularized ECMs for 3

weeks. Both MTS and histology suggested that decellularized ECMs prepared by UW protocol supported cell growth. From the histological sections, cell layers were clearly found across the ECM while cells were hardly spotted within the ECM produced by SB protocol. It implied that the microstructure plays an important role in tissue regeneration resulting in better cell growth in well-preserved decellularized ECMs. Finally, mechanical test revealed that the mechanical strength and stiffness of decellularized ECMs were diminished after decellularization. The weakening of mechanical properties might be due to chemical degradation in UW protocol and fiber breakage during mechanical scraping in SB protocol. Nevertheless, Elastic moduli and UTS were still in the same order as the native tissue, so most of the mechanical properties were preserved after decellularization.

Decellularized esophageal tissues derived from UW protocol was shown to support cell growth, preserve collagen and elastin and retain most of the mechanical properties. Therefore, UW protocol has a higher potential to produce better tissue scaffold for esophageal regeneration compared to SB protocol.

Chapter 6. Future Work

In the future work, studies will focus on building up a novel decellularization method to remove all cells and cell debris while preserving the microstructure and biological and biomechanical function of ECM.

The former work has shown that decellularization experiments which followed UW and SB protocols were successful in acquiring decellularized porcine esophageal ECMs. From this study, many areas deserve further study.

Cell-matrix interaction is a major modulator of cellular phenotype. It might improve quality of tissue construct. Future studies should include quantification of matrix properties such as porosity, fiber diameter, hydrophobicity and protein content and correlating them with cell signaling such as protein secretion and mRNA expression.

Mechanical aspects require more attentions since graft-host tissue mismatch has been one of major factors of implant failure. In this study, uniaxial tensile test was carried out on ECM sheets. It is known that esophagus is a tubular structure and the implants will experience *in vivo* mechanical forces from all different directions. Therefore, an implantable graft should not only fulfill mechanical demand uniaxially, but also multidimensionally. One of the important mechanical parameters for tubular structure is burst pressure and it would be important to test this parameter for the tubular graft in the future. Furthermore, the study has to be carried out on how the

treatments involved in the decellularization protocol can affect the resulting mechanical properties. This study will allow us to design a protocol which can minimize the loss of mechanical properties while decellularization.

For future clinical applications in humans, decellularized tissues should be subjected to biocompatibility and biodegradability examination *in vivo* by animal testing. Graft-host response can be characterized by acute inflammation, chronic inflammation, granulation tissue formation, foreign body reaction and foreign body giant cell formation.

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