Development of tissue-inspired scaffolds : integrating biology with materials science

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**DEVELOPMENT OF TISSUE-INSPIRED SCAFFOLDS:
INTEGRATING BIOLOGY WITH MATERIALS
SCIENCE**

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ABSTRACT

The lack of a definitive strategy for soft tissue regeneration highlights the need to evaluate new approaches for soft tissue replacement that is of clinical relevance. As tissue engineering presents a promising strategy for soft tissue regeneration, the focus of this thesis is mainly on the development of tissue-inspired scaffolds for adipose tissue engineering applications. In order to achieve this objective, studies were carried out to evaluate the efficacy of two novel materials, namely adipose tissue-derived extracellular matrix (ECM) material and bullfrog skin-derived collagen.

Taking inspiration from the native tissue itself, decellularized tissue-derived ECM is regarded as a promising material for tissue engineering applications. However, it has been shown that different decellularization methods play an influential role over the property of the final decellularized ECM material. Two novel decellularization methods were established during the course of this thesis for the isolation of ECM material from adipose tissue without the need of any harsh chemicals. The ECM material isolated using these two methods led to the preservation of more biological components including growth factors, matricellular proteins and structural proteins, as compared to previously established decellularization methods.

The extracted ECM material was subsequently used as a bioactive material to enrich other biomaterials for tissue engineering applications for the first time. It

was found that the ECM material was an effective enrichment due to the well preservation of various valuable components within ECM material. This ECM enrichment improved the cell-material interaction for both synthetic (i.e. Polycaprolactone) and natural (i.e. Ovalbumin) materials. In addition, the ECM coating was observed to have minimal pro-inflammatory response and improved *in vitro* wound healing, endothelialization, and adipogenesis.

In parallel, *in vitro* and *in vivo* studies were also carried out to demonstrate how bullfrog skin-derived collagen could be a promising alternative source of collagen for the fabrication of tunable acid-soluble collagen (AC) scaffolds. The material properties and cell proliferation capabilities of the AC scaffolds could be further modulated using different concentrations of crosslinker to suit various tissue engineering applications. In addition, a decrease in both *in vitro* and *in vivo* degradation rate was observed with increasing crosslinker concentration.

Overall, by combining engineering tools and techniques with biological assays and animal studies, adipose tissue-derived ECM material and bullfrog skin-derived collagen were shown to be promising materials for soft tissue engineering applications. The novel concept of using ECM material to improve cell-material interactions demonstrates the potential exploitation of adipose tissue as useful bioactive coating material. Overall, an ECM-containing hybrid scaffold is an acellular scaffold with cellular benefits and is therefore a great stepping-stone towards the development of engineered adipose tissue replacement for clinical applications.

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LIST OF ABBREVIATIONS

2D	Two dimensional
3D	Three dimensional
AB	AlamarBlue TM
AC	Acid-soluble collagen
ASCs	Adipose tissue-derived mesenchymal stem cells
ATRP	Atom transfer radical polymerization
BAT	Brown adipose tissue
BDE	1,4-butanediol diglycidyl ether
bFGF	Basic fibroblast growth factor
BIBB	2-bromoisobutryl bromide
BSE	Bovine spongiform encephalopathy
DI	Distilled
DSC	Differential Scanning Calorimetry
ECM	Extracellular matrix
EDAC	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide
FBS	Fetal Bovine Serum
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
FMD	Foot and mouth disease
FTIR	Fourier transform infrared
GA	Glutaraldehyde
GAG	Glycosaminoglycans
H&E	Hematoxylin and Eosin
HUVECs	Human umbilical vein endothelial cells
LpECM	Lipoaspirate-derived ECM
MAAS	Methacrylate sodium
M _i	Initial mass
M _f	Final mass
NHS	N-hydroxysuccinimide
OVA	Ovalbumin
PBS	Phosphate buffered saline solution
PCL	Polycaprolactone
PGA	Polyglycolide
PET	Poly(ethylene) terephthalate
PLA	Poly lactide
PLLA	poly-L-lactic acid
PLGA	Poly(lactic-co-glycolic acid)
PTFE	Polytetrafluoroethylene
R _a	Root-mean-square roughness
SC-CO ₂	Supercritical Carbon Dioxide
SD	Standard deviation

SDS	Sodium doecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Scanning electron microscope
TCP	Tissue culture plastic
TCP-ECM	ECM coated TCP
TEA	Triethylamine
TGA	Thermo-gravimetric Analyzer
T _n	Denaturation temperature
TSE	Transmissible spongiform encephalopathy
VEGF	Vascular endothelial growth factor
WAT	White adipose tissue
WCA	Static water contact angles

1. INTRODUCTION

1.1 Research Background

There is currently no definitive repair or regenerative strategy for extensive soft tissue defects caused by congenital abnormalities, trauma (e.g. significant burns), tumour resection (e.g. carcinoma removal and mastectomy), degenerative disorders or aging [1-3]. The most common treatment for such defects is the use of autologous fat tissue, since excess adipose tissue (fat) can be found readily throughout the whole body and obtained via liposuction for transplantation to a target location [2,4,5]. However, results from autologous fat grafts have been poor and largely unpredictable as transplants are typically resorbed (graft volume loss of 40-60%) and eventually replaced by oil cysts and fibrous tissues [2,6]. In addition, the current practice of *in vitro* cell expansion followed by subsequent cell infiltration into a scaffold prior to implantation has not been successful for large defects. At the same time, wound management is also a challenge during soft tissue regeneration. Mismanagement of large defects, repeated trauma or underlying congenital conditions can all result in incomplete healing and formation of chronic wounds, which are typically non-responsive to most treatments [7]. Therefore, constructive healing strategies are necessary in the early stages of wound management to prevent wounds from progressing into a chronic state. Clinically, autologous flaps are currently the gold standard as a result of the

presence of growth factors, cytokines, and intact vasculature, but donor site morbidity restricts its availability and overall usefulness [5,8]. Tissue engineering strategies are also employed in cosmetic procedures. Instead of repairing or regeneration of tissue, cosmetic procedures are used mainly for altering or enhancing the body, in other word, to change the appearance of the body [9]. Generally, the strategies employed can be classified into two categories, one being cosmetic surgical procedures such as breast augmentation, liposuction and nose reshaping; the other being minimally-invasive procedures, for example injection of botulinum toxin, soft tissue fillers and laser hair removal etc. [10]. Similar to the reconstructive procedures, the main concern of the cosmetic procedures is that of the long-term outcome. As such, investigation into new approaches for engineering soft tissue is required.

Tissue engineering presents an alternative solution for soft tissue regeneration [11]. The integration of material science together with new biological knowledge suggests the possibility of using tissue engineering strategies to solve the clinical problem [12]. Currently, the most commonly used strategy involves the incorporation of biofactors into scaffolds fabricated from resorbable materials to create bioengineered tissues [5,6,13-15]. These scaffolds constructed from synthetic, natural, or hybrid materials serve as structural supports for cell proliferation, and to facilitate tissue regeneration and host integration [15,16]. This approach has become progressively significant in the field of tissue

engineering [17,18]. Nevertheless, these non-autologous materials also face limitations such as long-term resorption, suboptimal mechanical properties, unpredictability, contraction of fibrous capsule, and adverse immune responses [8].

Therefore, as the clinical demand for soft tissue repair in reconstructive surgeries and cosmetic procedures increases, there is a greater need for further in-depth research on soft tissue engineering. It is envisioned that soft tissue engineering using tissue-engineered adipose substitutes would facilitate the regeneration of new tissues and has a great potential to becoming an alternative solution to current clinical problems [5]. Since this is an emergent field, the exploration of novel and functional biomaterials will be critical in order to identify suitable materials that will improve cell-material interaction and tissue regeneration.

1.2 Motivation, Hypothesis and Novelty

As tissue engineering is currently the most promising strategy for soft tissue regeneration, many studies have incorporated the use of cell-seeded scaffolds. However, as this is a developing field, further exploration of this tissue engineering strategy is essential to optimise and ultimately achieve the clinical goal of adipose tissue replacement. The main challenges such as vascularization and long-term sustainability are still the present obstacles for tissue engineered

substitutes that are used in clinical trials for soft tissue repair. The choice of the biomaterial is critical in developing tissue engineered substitutes as the properties of the biomaterial can regulate cellular functions and affect cell-material interactions. Motivated by the unmet clinical needs and the challenges of tissue engineering, studies were carried out to evaluate the efficacy of various materials more suitable for soft tissue engineering applications as well as to investigate new approaches for tissue regeneration.

In general, it is essential for the tissue-engineered materials to have properties that closely mimic that of the natural cell microenvironment. Natural biomaterial attracts our attention as it generally has better biological properties. Hence, the first main biomaterial studied in this thesis is that of decellularized adipose tissue. The decellularized adipose tissue ECM material contains different biological components including structural proteins, functional proteins, growth factors and proteoglycans [19-21]. It has been used as a scaffolding material as a whole for its ability to support growth and adipogenic differentiation of adipose tissue-derived mesenchymal stem cells (ASCs) [20,21]. Various methods typically include a combination of physical, chemical and enzymatic treatments were developed to extract the ECM material from adipose tissue [19-22]. However, it is difficult to preserve the native adipose tissue architecture due to the large proportion of lipid within the adipose tissue. Moreover, there was study which showed that different decellularization methods affect both the ECM components and architecture [19].

Therefore, the hypothesis of the studies in this thesis involving ECM material is that the decellularized adipose tissue could be applied as a bioactive coating material rather than a scaffolding material to support different types of cells for its various biological components, thus demonstrating a broader range of potential applications. In addition, a physical method that eliminates the use of any harsh chemicals (acid, alkaline, enzyme, detergent) could be used to decellularize the adipose tissue successfully within a shorter processing time and preserve more biological components in the adipose tissue ECM. In addition, Hence, two new novel methods, which do not require the usage of any harsh chemicals, were developed for isolating extracellular matrix (ECM) material from adipose tissues that are currently treated as clinical waste. These methods not only cut down the processing time, but also preserve more biological components than previously established methods. Matricellular proteins were found for the first time in the adipose tissue-derived ECM materials isolated using our novel physical methods, whilst there was no matricellular proteins found using previously established method. For the first time, the application of the entire ECM containing different types of biological components as a biological coating to improve cell-material interactions was demonstrated by culturing three different cell types including immortalized human keratinocyte (HaCaT cells), adipose tissue-derived mesenchymal stem cells (ASCs) and human umbilical vein endothelial cells (HUVECs). The ECM-containing hybrid scaffolds could be a new option as well as a new direction of utilizing the adipose tissue-derived ECM material for tissue

engineering applications.

The second biomaterial investigated is collagen, which is a commonly used natural material in soft tissue engineering due to its low cytotoxicity and immunogenicity [23,24]. Currently, most commercially available collagen-based products are limited to those sources such as human, cows, pigs and sheep [24]. Risks of disease transmission are problems commonly associated with the acellular collagen scaffolds, which are derived from mammalian sources [23-26]. Several studies showed that bullfrog skin is a rich source of collagen and does not have these diseases [27-29]. Ongoing studies have also shown that bullfrog skin-derived collagen has good antioxidant properties [29]. However, it has never been fully characterized and used as biomaterial for tissue engineering applications. Hence, we hypothesize that the bullfrog skin is a viable alternative to current sources of collagen as a scaffolding material for biomedical applications. In this thesis, collagen from bullfrog skin was successfully extracted and fabricated into scaffolds for the first time. Both *in vitro* and *in vivo* degradation profiles of collagen scaffolds could be modulated by the amount of chemical crosslinker. For the first time, the application of bullfrog skin-derived collagen as a scaffolding material for soft tissue engineering was demonstrated and it was shown to be biocompatible in both *in vitro* and *in vivo* studies. Overall, bullfrog skin-derived collagen was found to be a promising, cheap and safe alternative source of non-mammalian collagen that does not pose any risk of disease transfer.

1.3 Objectives and Scope

The main objectives of this project are summarized as follows:

1. To develop new methods for decellularizing the ECM material from adipose tissue (fat graft and lipoaspirate) and to characterize the isolated ECM;
2. To investigate the potential of using the entire ECM as a bioactive coating material to improve the biocompatibility and cell-material interactions;
3. To design and fabricate novel ECM-containing hybrid scaffolds;
4. To evaluate the *in vitro* and *in vivo* biocompatibility of the ECM material;
5. To isolate the collagen from bullfrog skin, followed by construction and characterization of the bullfrog skin collagen scaffold;
6. To measure the *in vitro* and *in vivo* stability of bullfrog skin-derived collagen scaffold;
7. To investigate the cell-material interactions of both ECM material and bullfrog skin collagen *in vitro* and *in vivo*.

The aim of this thesis is to create acellular scaffolds with cellular benefits for adipose tissue regeneration. Therefore, the thesis is mainly focused on the adipose tissue, and other types of soft tissue are not within the scope of study. In addition, as the biomaterial and scaffold is the key focus of the study, the cellular aspects of tissue engineering such as the cell source, cell line and the cell signalling pathways, are not evaluated in the present thesis. Overall, this thesis explores in detail two materials, adipose tissue-derived ECM and bullfrog skin-derived

collagen. In the first part of the thesis, in order to explore proper decellularization methods to extract adipose tissue-derived ECM material as bioactive coating material for soft tissue engineering applications, a comparative study was carried out to explore the effect of different methods on the properties of isolated ECM material. The ECM material isolated using the newly established physical method was characterized and compared with conventional chemical and enzymatic methods in terms of removal of cells, lipids, and DNA content, preservation of different types of biological components, processing time and cost. The subsequent studies focused on the use of the entire ECM as a bioactive coating material (surface and bulk) instead of a scaffolding material directly. Hence, different ways of coating the entire ECM material were explored i.e. direct coating, chemical modification and development of ECM-containing hybrid material. Various techniques were used to confirm the successful coating of ECM material such as contact angle analysis, Fourier transform infrared (FTIR) spectroscopy and immunostaining. Four different types of cells including human monocytic leukemia cells (THP-1 cells), HaCaT cells, ASCs and HUVECs were cultured on the ECM coating to test the immune response and the effect of ECM coating to the different types of cells. The material properties of different types of ECM-containing hybrid scaffolds were subsequently characterized and correlated to their ability to affect ASCs attachment and proliferation. In addition, an alternative source of collagen was isolated from bullfrog skin, which does not pose any risk in transferring diseases. In the second part of the thesis, bullfrog

collagen scaffolds were fabricated using different concentrations of crosslinkers to tune the properties of scaffolds including mechanical property, swelling ability and both *in vitro* and *in vivo* degradation profiles. Furthermore, the capability of bullfrog collagen scaffolds for supporting ASCs attachment and proliferation were also analysed and correlated to the different concentrations of crosslinker.

1.4 Thesis Organization

The whole thesis is divided into 8 chapters:

Chapter 1 introduces the background of this research and the motivation of using tissue-inspired strategy to address the unmet medical needs of soft tissue repair and regeneration. The novelty, objective and scope are also included in this chapter.

Chapter 2 presents an overall literature review of topics related to the studies carried out including the structure and function of soft tissue, soft tissue engineering, biomaterials, scaffold design and biofunctionalization.

Chapter 3 lists the necessary materials and the source of adipose tissues used in this study. Detailed research methodology and experimental procedures are also described in this part to show the extraction process, fabrication method, characterization techniques, as well as *in vitro* cellular studies and *in vivo* animal

studies.

Chapter 4 shows the results of comparative studies on different decellularization methods and discusses the advantages of the newly developed methods over conventional methods.

Chapter 5 discusses the feasibility of applying the entire ECM material as a bioactive coating material and the effectiveness of this coating material for improving cell-material interactions of different cell types, all of which are involved in the soft tissue regeneration processes.

Chapter 6 illustrates how the entire ECM material could be used to create novel ECM-containing hybrid scaffolds and shows the advantages of this material over conventional materials used currently.

Chapter 7 provides the results of collagen isolation from bullfrog skin and the detailed characterization of the collagen properties. This chapter investigates in detail the material and biological properties of tunable bullfrog skin-derived collagen scaffolds.

Chapter 8 contains the overall conclusion of the thesis and also includes future recommendations for this work.

2. LITERATURE REVIEW

2.1 Soft Tissue Replacement

2.1.1 Overview

Currently, soft tissue replacement is still facing clinical challenges due to the lack of a definitive strategy [4,5]. At the same time, there is a growing need for soft tissue replacement due to cancer, trauma including burns, aging and congenital abnormalities, in both reconstructive and cosmetic surgery [1,2]. According to a recent report from the American Society of Plastic Surgery, 5.7 million reconstructive procedures were performed in USA in 2013, of which 4.4 million were due to tumor resection procedures [10]. In addition, there were about 15.1 million cosmetic procedures which involved the use of different kinds of synthetic or natural material fillers [10]. In most cases, the need for surgical intervention is due to the loss of adipose tissue, which has a limited capacity to regenerate.

2.1.2 Current clinical approaches

The clinical approaches currently used to repair and reconstruct soft tissues include the use of autologous tissues, allogenic biomaterials, xenogenic natural materials and synthetic materials [30]. The choice of treatment for soft tissue defects is related to several factors, including the severity of defect, cost of surgery, and condition of patients. Briefly, for large volume defects, especially after large tumor removal procedures, the first choice of treatment is the transplantation of an

autologous flap, where a vascularized whole tissue is transferred to the defect site [31,32]. However, the graft or flap transfer technique has its own limitations. As a major surgical procedure, it is very costly and time consuming. In addition, it often results in scarring and donor site morbidity thus leaving a secondary defect [5]. The transplanted tissue graft or flap may also suffer from volume loss in the long term [6,30]. Therefore, other materials and less invasive approaches are used to repair the soft tissue defects, especially in the situation of small volume defects. At present, lipoaspirate material is mainly used as a filler material due to its availability and lack of immune rejection issues [33-35]. However, it also suffers from long term resorption due to the lack of vascularization, as well as the overall replacement by oil cysts and fibrous tissues [30,36]. Apart from this, allogenic and xenogenic natural materials have also been investigated as an implant and filler material for their good biological properties. However, the main problem is that these materials can be easily absorbed *in vivo*, which causes unnecessary volume loss and requires multiple treatments [37]. Synthetic materials are also used in the treatment of soft tissue defects for their tunable properties. However, due to the intrinsic poor biological properties, it is difficult for such materials to integrate with the host tissue [38]. As a result, these synthetic materials have poor long term outcomes with undesirable fibrous contraction, volume loss and shift in position over time [8].

2.1.3 Adipose tissue engineering

Currently, clinical approaches for soft tissue regeneration are still mainly focused on volume restoration, rather than tissue regeneration and functional restoration [39]. Therefore, researchers have been trying to resolve this issue by using the adipose tissue engineering approach, which not only resolves the issue of volume loss, but also results in regeneration and repair of the original functions of the tissue [40,41]. In general, a typical tissue engineering strategy involves the incorporation of cells into scaffolds to create a three dimensional (3D) microenvironment that provides the appropriate biological factors for supporting cell growth and tissue regeneration [6,15,42-44] (Figure 1).

Figure 1. The three key components of tissue engineering, cells, scaffolds and signals.

There are various strategies for soft tissue engineering including cell-based therapy, injectable cell-carrier based treatment and scaffold-based tissue engineering [45]. Many studies have concluded that the use of cell-seeded scaffolds is the most promising strategy for soft tissue regeneration [46-50]. The most commonly used cell source in adipose tissue engineering is adipose tissue-derived stem cells (ASCs) [51-53]. ASCs can be easily isolated and obtained from adipose tissue using well established protocols [54,55]. In addition, it can be differentiated into mature adipocytes under desired conditions and has minimal *in vivo* immune response [51-55]. The 3D scaffolding material is another important part of this strategy, where it can be made into various forms and shapes such as injectable carriers, tubes, plugs and spongy forms according to the requirements of the specific application [56]. The basic steps involved in this scaffold-based strategy are shown in Figure 2. Briefly, ASCs isolated from human lipoaspirate are cultured and expanded in an *in vitro* environment. Subsequently, ASCs are seeded onto the 3D scaffold with appropriate medium and growth factors to allow cells attach, grow and proliferate over a certain time. Lastly, the ASC-seeded scaffold is implanted to the defect site, where ASCs eventually differentiate into mature adipocytes *in vivo*. Meanwhile, the scaffold is gradually degraded and remodeled as the new adipose tissue is being regenerated. Some key requirements that are essential for tissue engineering substitutes to support soft tissue regeneration are listed in Table 1. For example, biocompatibility, which refers to the ability of a material to elicit an appropriate response to host tissue in a

specific application [57], is always an important requirement for any tissue engineered implants. Also, long-term viability and functionality, which is associated with adequate vascularization, is crucial for soft tissue regeneration.

Figure 2. The scaffold-based approach typically used in tissue engineering applications.

Table 1. Key requirements for soft tissue substitutes [58-60]

<i>Biocompatibility</i>
Minimal immune responses
Degradation profile compatible with tissue regeneration
<i>Bioactivity</i>
Regeneration of functional soft tissue
Incorporation of vascularization
<i>Sustainability</i>
Long-term viability and functionality
Minimal donor-site morbidity

Many approaches that use cell-seeded 3D scaffolds as cellular supports to mimic the natural microenvironment have been tested with the aim of improving the tissue regenerative response [61-63]. However, limited success has been achieved in regenerating new tissues due to challenges in adipose tissue engineering techniques. One of the main challenges is vascularization or the ingrowth of blood vessels, which is necessary for the engineered adipose tissue substitutes to survive and integrate *in vivo* [59,64,65]. For this reason, large engineered adipose tissue substitutes do not perform well due to the lack of vascularization after implantation [5,15]. Diffusion only functions within a distance of 150-200 μm , and it takes days and even up to weeks for the growth of new blood vessels into the implants [64-68]. Therefore, most large volume implants are often observed to fail in the *in vivo* environment. Hence, it is crucial to explore new materials or scaffolding approaches to improve vascularization properties, in order to successfully regenerate soft tissue.

2.2 Physiology of Adipose Tissue

2.2.1 Overview

In order to successfully repair adipose tissue defects and regenerate the new tissue, it is very important to understand the physiology of the native adipose tissue. Adipose tissue, which is also known as fat, can be found abundantly in humans and it represents about 10-29% of the total body weight of adult humans with

normal weight [6,69,70]. It is a multifunctional and highly specialized connective tissue, distributed throughout the whole human body [2,8]. There are two main categories of adipose tissue, namely brown adipose tissue (BAT) and white adipose tissue (WAT) [5,71]. These two types of adipose tissues have significant differences in terms of their location, colour, structure and function [2].

2.2.2 Brown adipose tissue (BAT)

BAT is most prominent during the neonatal period, where it is crucial for the baby to survive temperature changes at birth [5,72]. BAT is responsible for the heat generation via non-shivering thermogenesis to maintain the temperature as it is able to break down intracellular triglycerides [5,72,73]. The highly vascularized tissue contains numerous, large mitochondria that gives BAT its unique brown color [72-75]. Morphologically, the brown adipocyte has a rounded nucleus at the center with many small intracellular lipid droplets surrounding it [75,76]. In newborns, BAT is the main adipose tissue that distributes throughout the whole body, and can be found even within the WAT [5,72]. However, the BAT content in adults is negligible as it is gradually replaced by WAT when the body ages [77,78].

2.2.3 White adipose tissue (WAT)

In the normal adult human body, WAT is distributed throughout the body and is yellowish/white color in appearance [70,79]. It can be categorized according to

the location: subcutaneous fat where WAT forms a layer between the skin and muscle, intra-abdominal and visceral fat where WAT mainly surrounds the internal organs such as stomach, heart and kidney, and other sites where WAT can even be found in bone marrow and muscle [70,79]. WAT can even facilitate the regulation of energy for the whole body through lipid storage and consumption [6,80,81]. It not only serves as energy storage, but also functions as heat insulation for maintaining the body temperature and mechanical protection for the organs [80,81]. Apart from these functions, WAT is also regarded as an endocrine secretory organ that is involved in a wide range of functions beyond simply fat and energy storage. WAT is a source of multiple paracrine, autocrine, and endocrine factors e.g. steroid hormones, fatty acids, and prostaglandins [6]. From the cellular point of view, WAT is mainly composed of mature adipocytes that contain a single large droplet of lipid in the vacuole [75,78]. Almost 90% of cell volume is filled with this intracellular lipid, thus causing the overall color of WAT to be yellowish white in color [76,78]. As compared to brown adipocytes, white adipocytes contain fewer and smaller mitochondria, and are less densely vascularized [6].

For this thesis, the focus will be on WAT regeneration and replacement as it is the predominant form of adipose tissue in adult human beings [5,6,8,77,82,83].

2.3 Biomaterials

2.3.1 Overview

As shown in Figure 1, the three main components that involved in tissue engineering strategy are scaffolds, cells, and biological factors. In order to successfully develop tissue engineered substitutes, it is very important to identify suitable biomaterials that can act as the structural supports (i.e. scaffolds) for the cells. A large number of biomaterials including both synthetic and natural polymers have been explored as soft tissue engineered substitutes [8,77,84,85]. The advantages and disadvantages (biocompatibility, mechanical property, bioactivity and chemical stability) of these materials for the soft tissue applications have been reported [15,86-88]. However, it should be pointed out that at present, none of them can solely fulfil all the requirements necessary for the clinical needs of soft tissue regeneration.

2.3.2 Synthetic biomaterials

Synthetic biomaterials have been widely explored in soft tissue applications due to their batch-to-batch consistency, and tunable material properties including degradation rates, molecular weight, density, mechanical properties and chemical stabilities [8]. Numerous studies have been carried out for soft tissue regeneration using the biodegradable polymers such as polyglycolide (PGA), polylactide (PLA), polytetrafluoroethylene (PTFE), poly-L-lactic acid (PLLA), polycaprolactone (PCL), poly(lactic-*co*-glycolic acid) (PLGA) and polyethylene terephthalate (PET)

[5,84,89-108]. Despite the tunability of synthetic materials, the main drawback is the lack of naturally-derived molecules, which causes poor cell-material interactions and integration with the host tissue [109].

2.3.3 Natural biomaterials

On the other hand, natural biomaterials, which are isolated from native tissues or some other natural systems, have also attracted great interest for soft tissue applications [87,88,105]. Due to the similarity in the composition and structure of these natural biomaterials to the ECM in nature, they generally have better biocompatibility, cell-material interactions, and good integration with the microenvironment at the host tissue site [109]. Various natural polymers, including collagen, gelatin, alginate, fibrin, chitosan, hyaluronic acid and silk have been used for soft tissue engineering applications [109,110].

Among the natural materials, collagen is one of the most commonly used structural proteins in soft tissue engineering research such as adipose tissue engineering and wound healing studies [5,24,58,111]. There are 29 distinct collagen types that have been discovered and characterized, and all these types display a typical triple helix structure [24,112]. Among them, collagen types I, II, and III are known to form collagen fibres, and these are also the few types which are most commonly used in the production of collagen-based biomaterials [24]. Collagen type I is currently the gold standard and most commonly used collagen

type in the field of tissue engineering [23,24,112,113]. It can be found in bone, skin, tendons, ligaments, and cornea, representing over 90% of total collagen in the body [24]. The most significant advantage of collagen over other materials is its low cytotoxicity and immunogenicity, in addition to its good biocompatibility for soft tissue regeneration [23,114-116]. Hence, collagen has been made into different scaffold forms including gel, sheet, film and sponge, and has been proven to support cells in both *in vitro* and *in vivo* environments [24,111,116,117]. Moreover, collagen type I can be extracted from various sources. The common sources for tissue engineering applications include human skin, bovine skin and tendons, and porcine skin [24,88,118]. Some examples of commercially available collagen products from different sources for soft tissue repair are shown in Table 2.

All these collagen sources have their own advantages and disadvantages. When the issue of biocompatibility and immunogenicity is considered, human source is still the best among all the other sources of collagen. However, the main issue is that of a donor shortage as well as the ethical issue of using human samples [24,119]. When collagen extracted from mammalian sources (i.e. bovine and porcine) are used, the main concerns are the safety issues, viral diseases and inadequacy of standardized sterilization techniques to completely eliminate the possibility of infective agent transmission [24,120]. For example, concerns have been raised due to the risks of transmission of bovine spongiform encephalopathy

(BSE), foot and mouth disease (FMD) and transmissible spongiform encephalopathy (TSE) from these mammalian sources [23-29]. Therefore, an alternative source of collagen for tissue engineering application is highly desirable. Many studies have indicated that the bullfrog skin is a rich source of collagen type I, without the threat of BSE, TSE or FMD [27-29]. Moreover, Huang *et al.* [29] showed that bullfrog skin-derived collagen has good antioxidant properties. However, the use of bullfrog skin-derived collagen as a biomaterial for tissue engineering applications has never been explored. As such, the use of bullfrog skin-derived collagen as a scaffolding material is demonstrated in Chapter 7.

Other than isolating collagen itself, other researchers have also explored the use of the decellularized tissues for tissue engineering applications [88,118,121-127]. The decellularization process removes most antigenic parts including cells and DNA components. Hence, the resulting decellularized tissue exhibits itself as the most promising solution for soft tissue regeneration [128]. Table 2 shows some examples of clinical products that are made from decellularized tissue for soft tissue applications. Further details of the decellularization of adipose tissue is found later in section 2.5.

Table 2. Examples of commercial products comprised of either collagen or decellularized tissues [24,88,118]

Products	Source
Acellular Dermal Matrix	
AlloDerm®	Human
AlloMax™ Surgical Graft	Human
DermaMatrix	Human
GraftJacket®	Human
Karoderm™	Human
SureDerm™	Human
Dermalogen™	Human
Collagen scaffold	
Fibrogen®	Human
OASIS™ Wound Matrix	Porcine
SurgiAid®	Bovine
E-Z Derm®	Porcine
CollaWound	Bovine
Zyderm® and Zyplast ®	Bovine

2.4 Scaffold

2.4.1 Overview

Scaffolds act as structural supports for the cells to attach, grow, migrate, proliferate and differentiate in a three-dimensional (3D) environment into specific tissue [38,60,129]. Currently, it is widely accepted that 3D scaffolds are preferred to two dimensional (2D) structures as they are more capable of mimicking the actual structural complexity *in vivo* [60]. Ideally, scaffolds should mimic the native cellular microenvironment in order to support tissue regeneration. Therefore, it is necessary to study the ECM material before developing it into a scaffold for adipose tissue engineering applications. In this thesis, the characterization of

decellularized ECM material is discussed in detail in Chapter 3.

2.4.2 Nature's own scaffold - the ECM

In general, the ECM, which is often regarded as nature's own scaffold, is a complex combination of different biological components including structural proteins, functional proteins, growth factors, and proteoglycans [128]. The ECM provides cells with the necessary signals for important cellular processes to take place [130-132]. The 3D ECM network is tissue-specific in terms of the composition and the architecture, and functions not only as a structural support, but also influences cell fate, including cell attachment, growth, proliferation and differentiation, and remodels the injured tissue [128,130-133]. This cell-enriched ECM eventually contributes to the tissue architecture and function as it is a dynamic 3D network, and undergoes constant remodeling [134]. Cell-ECM interactions occur at the specific receptors (e.g. integrin) along the ECM, which is not only one way, but reciprocal [135-137]. Indeed, cells are also constantly changing the properties of ECM via creating, secreting, breaking down and rearranging the ECM components [136,137]. On the other hand, any change of the properties of ECM can also influence the cell behavior [136,137]. Overall, it is likely that the tissue specific 3D microstructure, surface chemistry, and biological composition of the ECM, contributes as a whole to the function of the ECM itself. Therefore, many decellularization protocols have been used on a wide range of human tissues including the dermis, liver, bladder, heart valves, arteries, skeletal

muscle, placenta, and more recently, the adipose tissue. These ECM have been used in the fabrication of biological scaffolds for tissue engineering applications.

2.4.3 Key requirement of scaffold

Taking inspiration from the native ECM, an ideal scaffold for soft tissue engineering should be three-dimensional, with high porosity and an interconnected network that enables nutrient transportation [60,114,138]. Different forms of 3D scaffolds have been produced using various biomaterials and fabrication techniques. Regardless of the application, some key requirements have to be considered when designing a scaffold (Table 3). Firstly, a scaffold has to be biocompatible and not harmful to the host tissue [38,58]. It must allow cells to adhere, migrate, proliferate and function normally on the surface and eventually integrate with the host tissue. Also, a scaffold must be non-toxic and have negligible immune response *in vivo* [58]. A scaffold should also have controllable biodegradation and resorption rates that match the tissue *in vivo*. Particularly for adipose tissue engineering, sufficient space is required for cells to differentiate, since there is an increase in cell volume as the ASCs develop into lipid-filled mature adipocytes [139,140]. Furthermore, the by-product from degradation should also be non-toxic and non-immunogenic [58]. In terms of scaffold architecture, it has to be a 3D porous structure with high porosity and have interconnected pores to allow cell penetration and nutrient and waste transportation within the scaffold [60]. A high surface area of scaffold is required

for sufficient cells to adhere and grow, in order to shorten the healing process [60,141,142]. In addition, the bioactive surface of scaffold should enhance cell-material interactions for improved cell attachment, proliferation and differentiation [143,144]. A scaffold should also possess suitable mechanical properties that match the particular need of each tissue engineering application [58,145]. It should be strong enough to be handled during the implantation, and also to offer sufficient mechanical support throughout the whole healing process. Last but not least, a scaffold should be easily sterilized in order to avoid contamination. In Chapters 4 to 7, the efficacy of using adipose tissue-derived ECM material and bullfrog skin-derived collagen as novel biomaterials is demonstrated.

Table 3. Some key requirements of tissue engineering scaffold [38,58-60]

Key requirements	General remarks
Biocompatible	Compatible with the host tissue
Non-toxic and non-immunogenic	Should not evoke the toxicity and immunogenic response to the host tissue
Biodegradable	Degradation profile match the new tissue regeneration and safe degradation products
3D structure	Facilitate cell growth, differentiation and new tissue formation
Adequate porosity with interconnected pores	Enable more space for cell growth and nutrient exchange
High surface area	To support high density of cells
Bioactive surface	Improve cell-material interactions
Proper mechanical property	To match the tissue mechanical property
Sterilizable	To avoid contamination

2.5 Decellularization of Adipose Tissue

2.5.1 Overview

Adipose tissue has been recognized as a rich source of ECM material [19,21]. The ECM of the adipose tissue contains abundant fibrous collagen type I, which acts as main structural proteins to support the cells. Additionally, the adipose tissue ECM is also rich in collagen type IV and laminin, which are mainly found in the basement membrane [146]. The collagen type IV together with ECM components, such as laminin and proteoglycan, form the basement membrane, and the laminin regulates the functions of cells including cell adhesion, growth, migration, proliferation and differentiation [146-150]. The adipose tissue ECM also consists of other proteins such as collagen type II, collagen type III, elastin and fibronectin [146]. Moreover, the adipose tissue ECM has been found to have various growth factors, including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and epidermal growth factor (EGF) [47,151]. Hence, various groups have studied to use adipose tissue derived ECM as a scaffolding material for adipose tissue engineering applications.

2.5.2 Decellularization methods

To extract the entire ECM material from tissue, it is necessary to process the tissue to remove the cellular components while preserving the matrix as much as possible. This process is called decellularization. Numerous studies have shown that the decellularization methods by which ECM material is extracted play a

critical role upon the biochemical and structural properties of the resultant ECM material as well as the ability of the material to support a positive tissue remodeling outcome after implantation [19,152-155]. Therefore, it is critical to find proper decellularization processing techniques that can efficiently remove all cellular and nuclear material, whilst minimizing any adverse effect on the composition, biological activity, and mechanical integrity of the remaining ECM [156].

Generally, current methods used to decellularize the ECM include a combination of various physical, chemical, and enzymatic treatments [20-22]. Logically, each treatment involved in these methods affects the structure and properties of the ECM material, and eventually alter its ability to support growth and differentiation of cells [19]. The chemical reagents, such as acids, bases, alcohols and detergents, used in the decellularization can effectively remove cellular and nuclear material, but damage the biological components within the ECM material including collagens, glycosaminoglycans (GAG), and growth factors [157]. Enzymes reported in adipose tissue decellularization protocols usually include nucleases, trypsin, and lipase. Enzymes can provide high specificity for removal of cell residues or undesirable ECM constituents [157,158]. However, complete removal of enzymes is difficult and these enzyme residues may evoke an adverse immune response or impair recellularization [157,158]. Physical treatments involving temperature, force and pressure applied directly on the adipose tissue can break up

and remove cells, but damage original ECM structure [157,158]. Due to the large amount of lipid within adipose tissue, it is challenging to preserve the original ECM structure. Considering the objective of using the adipose tissue-derived ECM as a bioactive coating material, two novel physical decellularization methods are demonstrated in Chapters 4 and 5 to that allow the preservation of more ECM components as compared to conventional methods.

2.5.3 Application of adipose tissue-derived ECM

With the development of decellularization technology, adipose tissue-derived ECM as a complete novel scaffolding biomaterial has attracted the attention of many researchers. Uriel *et al.* [159] fabricated adipose tissue extracts containing basement membrane proteins and growth factors into gel form. The study showed that adipose tissue-derived ECM hydrogels promoted rapid adipogenesis both *in vitro* and *in vivo*. A number of studies on decellularization of human adipose tissue have also been carried out by Choi's groups [160,161]. Adipose tissue-derived ECM scaffolds were successfully extracted and fabricated into a variety of macroscopic shapes such as round dishes, squares, hollow tubes and beads. Flynn *et al.* [20,162] developed methods to isolate and decellularize adipose tissue, which showed adipogenesis after directly implanted into mice. Young DA *et al.* [163] generated an injectable adipose matrix scaffold after efficiently removing both the cellular and lipid contents from human lipoaspirate. The decellularized material retained the complex composition of ECM material, various

biomolecules were found in the resulting ECM material. This hydrogel was able to support the growth of ASCs *in vitro*. Although these studies showed promising results, the disruption of the ECM structure and low yield of the ECM material limited the application of using adipose tissue-derived ECM material as a scaffolding material. To date, no study has yet to be carried out on the use of adipose tissue-derived ECM material as an enrichment biomaterial. In Chapters 5 and 6, the efficacy of using adipose tissue-derived ECM material as an enrichment biomaterial is demonstrated.

2.6 Biofunctionalization

2.6.1 Overview

Biofunctionalization is generally achieved by either surface modification or bulk modification [164,165]. Surface properties play instrumental roles in the regulation of cell-material interactions and for controlling cellular fate, whilst bulk properties contribute towards the degradation properties and overall host tissue integration [164-167].

2.6.2 Surface modification

Various techniques can be used to modify surface properties [165]. A physical method is the simplest method to modify the surface, as it is easily achieved by direct coating of cell adhesive proteins such as collagen, fibronectin and laminin.

It is one of the most commonly used methods to improve the biological property of a surface [165]. However, it has several limitations as well, including detachment, activating the immune system *in vivo*, and poor long term sustainability. Most of these limitations can be improved by using chemical methods, since the proteins and molecules are chemically bonded to the surface [168-170]. Briefly, this method starts with surface activation, which creates the functionality or reaction site on the surface, and subsequently the bioactive proteins or peptides can react to these functional sites to form a stable bioactive layer on the surface [165]. Wet chemistry, covalent immobilization and alkali hydrolysis are the most commonly used chemical modification methods [171,172]. Plasma treatment is another efficient method to create a bioactive surface without affecting the bulk property [173,174]. It simply uses high energy plasma, which includes electrons, ions, protons, free radicals and gas atoms, to bombard the surface. The energy transfer from plasma to the surface causes a series of physical and chemical changes to the surface. The resulting plasma treated surface can have improved wettability, better biocompatibility and increased cell adhesion [173-176]. The highly activated surface after plasma treatment can be further treated with various chemical reactions to grafting bioactive biomaterial on it. In Chapter 6 of this thesis, chemical methods were used to modify the surface of polymers using ECM material.

2.6.3 Bulk modification

All the surface modifications can well improve the bioactivity of the scaffolds. However, there are still limitations, one of which is the modification taking place only on the surface of the scaffold [165]. The approach of using bulk modification can eliminate this concern. As compared to surface modification, bulk modification enables the bioactive molecules, peptide or proteins to be embedded all over the bulk material rather than only on the surface [177,178]. As for scaffold fabrication, bulk modification can be achieved by both physical and chemical methods. The physical method involves the blending of two materials together, resulting the encapsulation or entrapment of the bioactive proteins within the hybrid system, whilst the chemical method is similar to that of copolymerization. Various chemical reactions can be achieved by covalent immobilization of bioactive proteins with synthetic polymers, resulting in a hybrid material exhibiting properties of both materials. Examples of bulk modification using ECM material are shown in Appendix II of this thesis.

2.6.4 Hybrid scaffold

Generally, one material solely cannot achieve all the criteria for tissue regeneration. Both synthetic and natural biomaterials have their own advantages and disadvantages when applied in tissue engineering applications. Therefore, the use of a hybrid scaffold, which combines both benefits from synthetic and natural materials by using the different strategies of biofunctionalization including surface

and bulk modification, is worth exploring. In most cases, the synthetic polymers are used as the main scaffolding material, whilst the natural biomaterial is used as an enrichment material to modify the properties [179-181]. The poor bioactivity of synthetic polymers can be improved by incorporating one or several natural biomaterials including proteins, growth factors and other ECM components [179,182-185]. Although these approaches improved the material properties for tissue engineering applications, these scaffolds could not mimic exactly the microenvironment of the cells *in vivo*, especially on the aspect of the chemical cues and biological composition. The tissue-specific ECM material is represented by a complex mixture of biological molecules, and each of these molecules can affect cell activities [128]. Therefore, the use of entire ECM material as an enrichment material could be an alternative approach to fabricating hybrid scaffolds for tissue engineering applications.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Source of adipose tissue

Freshly excised adipose tissue samples that includes lipoaspirates and fat grafts were obtained from patients were obtained from subcutaneous depot site of Chinese female patients ($n = 55$) with the average age of 42 (ranging from 28 to 54) at National University Hospital (NUH) and/or Tan Tock Seng Hospital (TTSH) (Singapore), following procedures established by the National Healthcare Group Domain Specific Review Board (DSRB 2011/01721 and DSRB 2012/00071 respectively). The adipose tissue samples were processed within 6 h of harvesting.

3.1.2 Chemical reagents

All the chemicals used in the studies described in this thesis were obtained from Sigma-Aldrich (Singapore) unless stated otherwise.

3.1.3 Cell culture reagents

All cell culture-related reagents were obtained from Life TechnologiesTM (Singapore) unless stated otherwise. All the substrates and scaffolds used for cell culture were sterilized by using ethylene oxide.

3.2 Decellularization of Adipose Tissue

3.2.1 Decellularization methods

A total of four methods were used for the decellularization of adipose tissues to extract the ECM material. These methods include three newly developed methods by the author and one previously published enzymatic method [130,186,187] for comparison purposes (Figure 3). The three novel methods developed by the author include a pure physical treatment method and a pure chemical method for isolating ECM material from fat graft, and the supercritical carbon dioxide (SC-CO₂) method for extracting ECM material from lipoaspirate. The resulting ECM material was stored at 4 °C. All the characterizations and further processing were started immediately after obtaining the dried adipose tissue-derived ECM material.

3.2.1.1 Pure physical method

Briefly, adipose tissue was rinsed with distilled (DI) water until all the blood components were completely removed. Subsequently, the adipose tissue was broken up into small pieces by using a laboratory blender (Waring® Laboratory Science, Singapore) for 5 min. A same volume of DI water was added and the adipose tissue sample was homogenized at 25,000 rpm for 5 min. The tissue suspension was centrifuged for 5 min at 5,000 rpm and the lowest cell debris layer and upper lipid layer were discarded. This homogenize-centrifuge process was

repeated until no lipid was observed. The lipid-free tissue suspension was then washed three times by addition of three volumes DI water and placed into an ultrasonic cleaner for 5 min prior to centrifuge for complete removal of cellular components. Finally, the tissue suspension was placed into the -80 °C fridge overnight and freeze-dried for 24 h prior to use. The resultant decellularized and delipidized material was renamed as ECM-P.

3.2.1.2 Chemical method

The cleaned adipose tissue was soaked in the detergent solution with 0.25% Triton X100 and 0.25% SDS, and incubated for 24 h at 37°C on an orbital shaker (Stuart SSL1, UK) at a speed of 50 rpm. Next, the tissue was washed with DI water to remove the remaining Triton X and SDS. Magnesium chloride hexahydrate solution was then added into the tissue for the nuclear digestion. The nuclear-free tissue sample was rinsed with DI water after digestion. The sample was subsequently immersed into the diethyl ether solution for 2 h and isopropanol for another 2 h with continuous shaking to remove the lipids. Finally, it was washed thoroughly with 70% ethanol and followed by rinsing with DI water. The final freeze-dried ECM product collected was renamed as ECM-C.

3.2.1.3 Enzymatic method

The enzymatic method is one of the most commonly used methods for decellularization of adipose tissue, and described in detail by several researchers. Briefly, the tissue was first disrupted by three freeze-thaw cycles (-180°C and 37°C). Next, the tissue sample was incubated in 0.25% trypsin-EDTA solution at

37°C for 16 h. Subsequently, the tissue was soaked into isopropanol with continuous shaking at room temperature for 48 h, in between the isopropanol was changed every 24 h. The tissue was then rinsed with DI water and incubated in the 0.25% trypsin-EDTA solution at 37°C for 6 h. After rinsing with DI water, the tissue sample was transferred to an enzymatic solution which contained DNase, RNase and Lipase for further removal of DNA, RNA and lipid at 37°C for 16 h. The final sample was freeze-dried after incubation in isopropanol for 8h and rinsed with 70% ethanol and DI water. The resultant ECM from this method was renamed as ECM-E.

Figure 3. Schematic of the steps and time frame involved in the enzymatic, chemical, physical and SCF methods of decellularizing adipose tissue in order to obtain ECM-E, ECM-C, ECM-P and LpECM respectively.

3.2.1.4 Decellularization of lipoaspirate using SC-CO₂ method

The lipoaspirate was decellularized in the SC-CO₂ set-up. Figure 4 illustrates the schematic diagram of the overall set-up. Firstly, the lipoaspirate was rinsed with distilled (DI) water until all the blood components were completely removed. Subsequently, the lipoaspirate was left stirring in absolute ethanol for 20 min. The pure ethanol was replaced and this procedure was repeated once again. The tissue was then loaded into the reaction vessel of the set-up (Supercritical Fluid Technologies Inc, USA) with pure ethanol as modifier. Liquid carbon dioxide was flowed into the vessel as shown in Figure 4. It was compressed by a pump first and passed through the regulator until the desired pressure (180 bar) was achieved. The temperature of the reaction vessel was maintained at 37⁰C and the final dry decellularized adipose tissue ECM was collected after 3 h. The final dry lipoaspirate-derived ECM product collected was renamed as LpECM.

Figure 4. Schematic view of the overall set-up for the SC-CO₂ decellularization method.

3.2.2 Characterization of decellularized adipose tissues

3.2.2.1 Confirmation of decellularization

The resultant decellularized ECM samples were characterized to evaluate the effectiveness of cell and lipid removal by using Hematoxylin and Eosin (H&E) and Oil Red O staining (Millipore). Fresh tissue sample was used as the control. All tissue and ECM samples were fixed with 4% paraformaldehyde solution for 24 h at 4 °C and rinsed three times with 1X phosphate buffered saline solution (PBS) prior to the further steps.

For H&E staining, paraffin-embedding of sample sections was necessary. After the fixation in 4% paraformaldehyde, samples were dehydrated by using

increasing concentration of ethanol (50, 60, 70, 80, 90, 100 %v/v in water) for 1 h each. These samples were then immersed overnight in a second 100% ethanol. The dehydrated samples were placed in 1:1 ethanol xylene mixture and pure xylene for 2 h each, followed by second xylene solution overnight. Subsequently, the samples were embedded in paraffin within an oven at 58 °C overnight. The sample sections were cut by using rotary microtome (Leica Biosystems, Singapore) and each section was 5 µm. These sections were deparaffinized, following the reverse steps of above protocol, which included 30 min in the 58 °C oven, 5 min each for xylene and ethanol steps. The hydrated sections were fixed in 80% methanol for 5 min at 4 °C followed by 100% acetone for 2 min at -20 °C. The sections were then stained using H&E to check the presence of remaining cells and cell nucleus.

For observation of lipid content, cryosections were required for Oil Red O staining. The paraformaldehyde fixed samples were transferred to aluminum foil molds and embedded in tissue freezing buffer instead of paraffin overnight at 4 °C. Subsequently, samples with the mold were snap frozen using liquid nitrogen. 60 µm cryosections of samples were cut using cryostat (Thermo Scientific™, Singapore). The cryosections were stained using Oil Red O working solution (1.8 mg Oil Red O powder in 1 mL of 60% isopropanol solution) to check the remaining lipid content within the samples.

3.2.2.2 Immunostaining

The paraffin sections were deparaffinized at 60 °C for 30 min followed by rehydration by using different gradient ethanol (50, 60, 70, 80, 90, 100 % v/v in water) for 5 min each. The rehydrated sections were fixed with pure methanol and acetone at -20 °C for 10 min each. The sections were then blocked in a humid chamber with 0.1% Triton-X 100 and 2% bovine serum albumin (BSA) for 45 min at 25 °C. Subsequently, rabbit anti-human primary antibody against Collagen I, Collagen IV, Elastin, Fibronectin and Laminin (Abcam, Singapore) was added onto the sections respectively and incubated in a humid chamber overnight at 4 °C. Next, the sections were rinsed three times in 1X PBS for 5 min each. Goat anti-rabbit secondary antibody IgG (Abcam, Singapore) was added and incubated in a humid chamber for 1 h at 4 °C. Finally, the sections were rinsed with 1X PBS and mounted using DAPI mounting medium. The presence of these structural proteins was observed under fluorescence microscope (Carl Zeiss Inc, Singapore).

3.2.2.3 SDS-PAGE and western blotting

Proteins were extracted from the samples by homogenization and resolved on Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Briefly, proteins were electrotransferred onto nitrocellulose membrane (Millipore, Singapore). Membrane was blocked in 3% milk prepared in 1X Tris Buffered Saline mixed with 0.1% Tween 20 (TBST) and subsequently incubated with in-house rabbit polyclonal anti-human cFIAF primary antibody prepared in blocking buffer. Next, the ECM was rinsed with 1X TBST. The membrane was

rinsed thrice in 1X TBST and then incubated in the secondary antibody, IRDye 800CW Goat Anti-Rabbit secondary antibody (LI-COR Biosciences, USA) in blocking buffer. Proteins were identified using the Odyssey CLx infrared imaging system (LI-COR Biosciences, USA).

3.2.2.4 Growth factor detection

The freeze-dried samples were minced and analyzed in five replicates. Briefly, 600 mg of each sample was dissolved in 9 ml of urea-heparin extraction buffer as described by Brown *et al.* [19]. The solution was incubated at 37 °C for 24 h. After incubation, the sample solutions were then centrifuged at 3000 g for 30 min and supernatant was collected. The samples were then quantified for VEGF and basic fibroblast growth factor (bFGF) using Quantikine immunoassay kit (R&D systems, Singapore). All assays were performed in triplicates following manufacturer's protocol.

3.2.2.5 Glycosaminoglycans (GAG) assay

The freeze-dried samples were analyzed in five replicates. Briefly, 50 mg of each ECM sample was dissolved in 1 mL papain extraction solution in 1.5 mL microcentrifuge tube. The suspension was then incubated at 65 °C for 24 h. Subsequently, the ECM sample solution was centrifuged at 10000 g for 10 min and the supernatant was collected. The GAG amount of each sample were then quantified using the Blyscan Sulfated Glycoaminoglycan (Biocolor, UK) Assay following the manufacturer's protocol.

3.2.2.6 Collagen content

Total collagen content and acid soluble collagen content of isolated ECM material was quantified using hydroxyproline assay (Chondrex Inc, Singapore) and Sirius Red collagen detection kit (Chondrex Inc, Singapore) followed by the manufacturer's protocols respectively.

3.2.2.7 Morphology

The morphology of each sample was observed using a scanning electron microscope (SEM) (JEOL JSM-5410). Firstly, 2.5% glutaraldehyde solution was used to fix the sample at 4 °C for 24 h. Subsequently, the fixed sample was rinsed with DI water, dehydrated in a graded ethanol series and 100% hexamethyldisilazane (HMDS) before being air dried. Finally, the dry samples were sputter-coated with gold and viewed under SEM using an acceleration voltage of 5 kV.

3.3 ECM Coating Studies

3.3.1 Preparation of the ECM coating solution

ECM samples were dried, milled, and subsequently dissolved in 0.1 M HCl for 48 h at 4 °C with constant stirring. The pH of the ECM solution was adjusted to 7.4 using 0.1 M NaOH. The ECM solution was diluted to 4 mg/ml using 1X PBS solution prior to use.

3.3.2 ECM-coated tissue culture plates

0.5 ml of the ECM solution was added into 24-well plates and kept at 4 °C overnight. After which, the remaining solution was carefully pipetted out and washed with 1X PBS twice. Finally, the ECM coated tissue culture plastic (TCP) plates (TCP-ECM) was dried overnight at 4 °C.

3.3.3 Characterization of ECM-coated tissue culture plates

3.3.3.1 Contact angle analysis

The static water contact angles of the TCP and TCP-ECM surfaces were measured using a FTÅ 200 contact angle goniometer (First Ten Angstroms Inc., Portsmouth, VA) to investigate surface wettability. The sessile drop method was used to obtain the static contact angles from different locations of the surface at the ambient temperature. Briefly, the sample was placed on the platform of the equipment and a 2 µL water droplet was pushed out from the needle of 5 ml syringe. The CCD camera was used to take the picture of the water droplet dispersing on the sample surface, which was used to measure the contact angle by using the FTÅ 200 contact angle system.

3.3.3.2 Fourier transform infrared (FTIR) spectroscopy

TIR spectroscopy (Perkin Elmer, Singapore) was used to detect the vibrations of chemical functional groups in the sample to investigate the different functional groups within the sample using previously established methods. With the atmosphere as a reference, the absorption spectra of samples were then collected

and analyzed using Spectrum™ software.

3.3.3.3 Cell culture

Adipose tissue-derived mesenchymal stem cells (ASCs) were cultured in the ASCs cell culture medium consisting of DMEM/F-12, HEPES, 10% Fetal Bovine Serum (FBS), 1% L-glutamine, 1% MEM Non-Essential Amino Acids Solution 10 mM (100X), and 1% Penicillin-Streptomycin (P/S). Human umbilical vein endothelial cells (HUVECs) (American Type Culture Collection Number CRL-1730) were cultured in MCDB 131 medium supplemented with 10% FBS, 0.2% bovine brain extract, 0.1 mg/ml heparin, 1% P/S, and 0.25 mg/ml amphotericin. HaCaT cells (Division of Genetics of Skin Carcinogenesis, Germany Cancer Research Centre), which is an immortalized human keratinocyte cell line, were cultured in DMEM containing 10% FBS and 1% P/S. A free floating, non-adherent human monocyte cell line known as THP-1 (American Type Culture Collection Number TIB-202™) was maintained in RPMI-1640 medium (Sigma-Aldrich, USA) containing 10% fetal calf serum (FCS). All cell culture medium were changed every two days, and cells were harvested around 85% confluency by using a 0.25% Trypsin-EDTA solution. Cells from passage 4 or 5 were eventually used for all subsequent studies.

Prior to cell seeding, the TCP-ECM was sterilized by ethylene oxide. HaCaT, HUVECs and ASCs were trypsinized and seeded onto the TCP and TCP-ECM at densities of 15,000 per well. THP-1 cells were initially plated in 6-well plate at

densities of 1×10^6 cells per well. THP-1 monocytic differentiation was then initiated by incubating the cells in RPMI-1640 medium with 10% FCS and 50 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich P# 1585-1MG, USA) for 60 min with gentle shaking at 25 °C. PMA-treated cells were washed with 1x PBS and cultured in RPMI-1640 medium without PMA for 12 h overnight. The successful differentiation of THP-1 to macrophage-like cells was determined by the observation of cells adhered to and spreading on the bottom of the culture plate instead of remaining in a free floating state.

3.3.3.4 Cell proliferation

Cell proliferation on the normal well plates and TCP-ECM was determined using the PrestoBlue® assay (Life Technologies™, Singapore). The cell seeded wells were washed three times by 1X PBS after removing the cell culture medium. Subsequently, the PrestoBlue® solution was added to the wells and incubated at 37 °C in a 5% CO₂ atmosphere for 30 min. The optical density was evaluated with a microplate reader (Bio-Rad Laboratories, Singapore) at the excitation and emission wavelengths of 560 nm and 590 nm respectively.

3.3.3.5 Adipogenesis

ASCs were seeded and cultured in proliferation medium for seven days before supplementing the cells with StemPro® adipogenesis differentiation medium (Life Technologies™, Singapore) for 2 weeks. The medium was changed every 3 days. Oil Red O staining was carried out at the end of culture. Briefly, medium was removed. Subsequently, cells were rinsed with PBS twice and fixed with 4%

paraformaldehyde for 30min. Oil Red O working solution as described in section 3.2.2.1 was added in after the fixation and incubated at room temperature for 10 min. The image was taken using a light microscope (Carl Zeiss Primo Vert, Germany).

3.3.3.6 Real-time PCR analysis

Cellular RNA extraction was performed on THP-1 cells using the e.Z.N.A® Total RNA Kit I (Omega-Biotek, USA) followed by the manufacturer's protocol. The obtained cellular RNA was quantified using Nanodrop (Thermo Scientific) transcribed into cDNA via Reverse Transcription PCR (Polymerase Chain Reaction) using iScript™ (Bio-Rad, USA). Real time qPCR was carried out to examine TNF α and housekeeping gene (L27) mRNA expression using Bio-rad SybrGreen set-up.

3.4 ECM-containing Hybrid Material Scaffold Studies

3.4.1 Polycaprolactone (PCL) film preparation

PCL films were prepared by the solvent casting method. PCL solution was first prepared by dissolving 5 g PCL pellets (Mn=45,000 Da) in 40 ml dichloromethane, followed by casting onto a glass substrate using an automated film applicator (PA-2105, BYK). Subsequently, the solvent was removed by evaporation in air at ambient temperature for 24 h. The PCL translucent films were then obtained by drying in a vacuum oven at 30 °C for 24 h. The pristine PCL film was

subsequently cut into circular-shaped samples with a diameter of 1.5 cm.

3.4.2 Surface modification of PCL by ATRP method

In order to introduce the active free amine groups onto the PCL substrates, the pristine PCL films were placed in a solution of 10% w/w isopropanol of 1,6-hexanediamine at 40 °C (Figure 5a). The aminolysis reaction proceeded for 15 and 60 min, respectively, to produce the PCL-NH₂-1 and PCL-NH₂-2 surfaces. The resultant aminolyzed PCL films were finally washed with large amounts of isopropanol and DI water before being dried in a vacuum desiccator. To immobilize the alkyl bromide ATRP initiator onto the aminolyzed PCL surfaces, TEA-catalyzed condensation between free -NH₂ groups of the PCL-NH₂ surface and 2-bromoisobutyryl bromide (BIBB) was performed (Figure 5b). The PCL-NH₂ films were placed in 30 ml of anhydrous hexane solution with 1.0 ml (7.2 mmol) of triethylamine (TEA) and cooled in an ice bath after degassing with nitrogen for 30 min. 0.89 ml (1.65g, 7.2 mmol) of BIBB was then added dropwise using a syringe. The reaction proceeded at 0°C for 2 h and subsequently at 25°C for 12 h with gentle stirring. The resultant PCL-Br surfaces of PCL-NH₂-1 (15 min aminolysis) and the PCL-NH₂-2 (60 min aminolysis) samples were renamed PCL-Br-1 and PCL-Br-2 respectively.

For the grafting of P(MAA) brushes from the PCL-Br surfaces, surface-initiated ATRP of methacrylate sodium (MAAS) was performed in a mixture solution,

containing a [MAAS (2 g)]:[CuBr]:[CuBr₂]:[PMDETA] molar feed ratio of 100:1:0.2:2 in 4 ml of DI water in a Pyrex® tube at room temperature (Figure 5c). The PCL-Br films were then added into the mixture, followed by degassing and stirring for 30 min. The reaction tube was sealed and placed into 30 °C oil bath for 4 h. After the reaction, the resultant PCL-g-P(MAA) films were washed thoroughly with large amount of DI water, then immersed in DI water for 24 h to remove the physical adsorbed reactants or polymers,. The P(MAA)-grafted PCL films of PCL-Br-1 and PCL-Br-2 were renamed PCL-g-P(MAA)1 and PCL-g-P(MAA)2 respectively.

Rat tail collagen type I or LpECM was conjugated to the pendant carboxyl groups of the grafted P(MAA) brushes with carbodiimide chemistry so as to improve the cytocompatibility of the PCL materials (Figure 5d). The PCL-g-P(MAA) films were immersed in PBS with 10 mg/ml of EDAC and 1 mg/ml of NHS for 1 h to pre-activate the carboxyl groups. The activated films were transferred to PBS solution with 1 mg/ml of rat tail collagen type I or LpECM solution. The reaction proceeded at room temperature for 24 h to produce the PCL-g-P(MAA)-c-collagen or PCL-g-P(MAA)-c-LpECM surface. The reversibly-bound LpECM (or collagen) on the P(MAA) brushes were then desorbed in large amounts of PBS solution at room temperature for 24 h.

Figure 5. Schematic illustration of the various steps involved in the surface modification of PCL (a) aminolysis of PCL (PCL-NH₂); (b) immobilization of initiator via condensation reaction (PCL-Br); (c) ATRP of MAAS onto PCL (PCL-g-P(MAA)) and; (d) application of carbodiimide chemistry to obtain collagen and LpECM enriched PCL substrates (PCL-g-P(MAA)-c-collagen/PCL-g-P(MAA)-c-LpECM).

3.4.3 Characterization methods

3.4.3.1 Grafting Density

The grafting density of the P(MAA) brushes, collagen or LpECM on the PCL

films was evaluated by the grafting yield (GY) according to the following equation:

$$GY = \frac{W_a - W_b}{A} \quad (1)$$

Where W_b and W_a are the weights of the dry film before and after the reaction respectively, and A is the area of the film. At least three PCL films were tested for each GY measurement, and the average GY values were calculated.

3.4.3.2 Surface characterization of PCL films

The static water contact angles (WCA) were measure following the method mentioned in section 3.3.3.1. The chemical compositions of the functionalized PCL film surfaces were characterized using X-ray photoelectron spectroscopy (XPS). XPS measurements were carried out using a Kratos AXIS His spectrometer with a monochromatized Al K α X-ray source (1486.6 eV photons). The change in surface topography of functionalized PCL film surfaces was determined with atomic force microscopy (AFM). AFM images with scan size of $20 \times 20 \mu\text{m}$ in tapping mode in air were captured using a multimode scanning probe microscope equipped with a Nanoscope IIIa controller (Digital Instrument, USA). The root-mean-square roughness (R_a) was determined using the Nanoscope software.

3.4.3.3 Cell culture

ASCs were cultured in the ASCs cell culture medium which mentioned in section 3.3.3.3. ASCs were seeded on the films at a density of 5000 cell/cm². The cell

viability and proliferation of ASCS on the films was determined using the alamarBlueTM (AB) assay (Life TechnologiesTM, Singapore). Briefly, the cell seeded films were washed three times by 1X PBS after removing the cell culture medium at each time point (Day 1, 3, 5 and 7). Subsequently, the alamarBlue® solution was added to the wells and incubated at 37 °C in a 5% CO₂ atmosphere for 3 h. The optical density was evaluated with a microplate reader (Bio-Rad Laboratories, Singapore) at the excitation and emission wavelengths of 560 nm and 590 nm respectively.

3.4.3.4 Cell viability

LIVE/DEAD® viability/cytotoxicity assay was used to evaluate the *in vitro* qualitative analysis of cell coverage and viability. Briefly, 2mM Ethidium homodimer-1 and 4 mM Calcein AM dye were added to cell culture medium (1:1000 ratio) to produce a LIVE/DEAD® working solution. The cell-seeded PCL samples (after 7 days of culture) were rinsed three times with PBS prior to staining with 0.1 ml of LIVE/DEAD® working solution. After incubation at 37 °C for 30 min in a 5% CO₂ atmosphere, the samples were imaged using a fluorescence microscope (Nikon Instruments, Tokyo, Japan) equipped with the NIS-Elements Br image software.

3.4.4 Fabrication of ovalbumin (OVA) microcarriers

The OVA microcarriers were fabricated using an inverse mould-leaching method modified from Flynn *et al.* [188]. The overall schematic for fabricating OVA

microcarrier is shown in Figure 6a. Briefly, 10% (w/w) OVA solution and 1% (w/w) alginate solution was prepared separately with different volume ratios (1:1, 2:1, 1:2) to obtain the OVA/alginate mixture. OVA/alginate microcapsules were fabricated using an electrostatic microcapsule generator (Nisco Var V1, Switzerland). The gelling bath consisted of 0.1 M calcium chloride that was constantly stirred with a magnetic stirrer to prevent microcapsule coalescence [189]. The cross-linking of OVA/alginate was allowed to proceed for 30 min. After this, OVA/alginate carrier was transferred and soaked in 1% (w/w) 1,4-Butanediol diglycidyl ether (BDE) solution overnight to complete crosslinking of OVA/alginate. The microcarriers were rinsed using DI water to remove the excess crosslinker before being immersed in 2% (w/w) sodium citrate bath under gentle shaking. The sodium citrate solution was changed every 10 min for three times to ensure complete removal of the alginate. Finally, OVA microcarriers were washed with DI water and freeze-dried.

Figure 6. Schematic illustration of the various steps involved in the (a) fabrication and (b) surface modification of the porous OVA microcarriers.

3.4.5 Surface Modification of OVA microcarriers

In order to encourage cellular differentiation, LpECM was directly conjugated to the OVA-based microcarriers via carbodiimide chemistry, as shown in Figure 6b above. The dried OVA-based microcarriers were immersed in the LpECM solution with the addition of 10 mg/ml of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC) and 2 mg/ml of N-hydroxysuccinimide (NHS) for the conjugation reaction. After 6 h, LpECM-modified OVA microcarriers were washed with DI water and freeze-dried.

3.4.6 Characterization of LpECM-modified OVA microcarriers

3.4.6.1 Morphology and porosity

Physical properties such as color and structural features of the microcarriers were observed and imaged using a Canon digital camera (IXUS 950, Japan). The surface and cross-sectional characteristics of the microcarriers were further investigated using a scanning electron microscope (JEOL JSM-5410). Microcarriers were placed on carbon tape and coated with gold prior to imaging. An acceleration voltage of 5×10^3 V was used and images were obtained at different magnification. Porosity and pore sizes of scaffolds were calculated using SEM micrographs with ImageJ software installed with JPOR[®] macro, and SEMAFORE[®] software respectively. Section images were converted to binary format using ImageJ software, where the pores appeared white and the remaining parts appeared black. The percentage of the white area relative to the total microcarrier section surface area in each image was defined as the percent porosity.

3.4.6.2 Mechanical property

The compressive modulus of the microcarriers was tested using an Instron 5869 machine using a 10 N load cell. Compression tests were carried out on five replicates of each type of sample at a crosshead speed of 0.01 mm/s until maximum extension was reached. The materials were tested in wet conditions. Microcarriers were submerged in saline for 24 h at 37 °C for complete wetting of the samples. Hertz analysis describes the linear elasticity of spheres compressed

between two flat rigid surfaces for small deformations [190]. It correlates the compression force (F) with the displacement (H) as shown in equation:

$$F = \left(\frac{4R^2}{3} \right) \left(\frac{E}{1-\nu^2} \right) \left(\frac{H}{2} \right)^{\frac{3}{2}} \quad (2)$$

Where F is the force applied onto the microcarrier, R is the initial radius of the microcarrier, ν is the Poisson's ratio, E is the Young's modulus of the material and H is the displacement. The Poisson's ratio was assumed to be 0.5 in this study.

3.4.6.3 Maximum swelling ratio

Swelling ratio analysis was conducted to observe the stability of the microcarriers under physiological (pH 7.4, 37°C) conditions. Samples were soaked in saline for 24 h to determine its maximum mass gained. The initial (M_i) and final (M_f) mass of the scaffolds was determined by a precision weighing balance. The swelling ratio was determined with the equation (3).

$$\text{Swelling Ratio (\%)} = (M_f - M_i) / M_i \times 100\% \quad (3)$$

3.4.6.4 Cell viability test

Prior to cell seeding, 200 mg of sterile microcarriers were weighed and placed into each well. ASCs were seeded onto the microcarriers (10000 cells per well). The culture medium was changed every 2 days. The cell viability and proliferation of ASCs on the scaffolds was evaluated with the AB assay (Life Technologies™, Singapore) following the method described in section 3.3.3.3.

3.5 Bullfrog Skin Collagen Studies

3.5.1 Extraction of collagen from bullfrog skin

All steps of the extraction process were performed at 4 °C. Fresh bullfrog (*Rana catesbeiana*) skins were collected from a local aquatic farm (KhaiSeng Trading & Fish Farm Pte Ltd., Singapore). Firstly, the bullfrog skin sample was washed several times with distilled water DI water and ethanol to remove the impurities before obtaining the dry weight using vacuum oven. Secondly, the dry skin was cut into pieces and soaked in 1% SDS for 2 h to remove cells. Subsequently, the pretreated skin was placed in 10 volumes of 0.5 M sodium hydroxide to eliminate the non-collagenous proteins and pigments for 48 h (the solution was changed every 24 h). Next, the collagen extraction was done by immersing the skin in 0.5 M acetic acid for 48 h after being rinsed with DI water. The viscous collagen was salted out with 0.9 M of sodium chloride and left undisturbed for 24 h. The mixture was centrifuged at 12 000g for 1 h and the supernatant was removed. The protein pellet was reconstituted in a 10 volumes of 0.5 M acetic acid and dialyzed using snakeskin dialysis tubing (Piere, 10K MWCO) against 0.1 M acetic acid, followed by distilled water for 24 h each. The final purified collagen powder was collected after freeze-drying.

3.5.2 Fabrication of the collagen scaffold

The freeze-dried acid-soluble collagen (AC) was ground into powder and dissolved in a 0.5 M acetic acid solution at 10 mg AC/ml. Three different

concentrations (0.125%, 0.25% and 0.5%) of the chemical crosslinker 1,4-butanediol diglycidyl ether (BDE) were added to the solution. The final viscous solution was then homogenized at 8000 rpm for 5 min, followed by the removal of air bubbles in vacuum. Subsequently, 1 ml of solution was pipetted into each well of a 24-well plate (or 100 μ L in each well of a 96-well plate). The plate was placed onto a shaker at room temperature to enable crosslinking for 12 h. The BDE-crosslinked AC gels were then frozen at -25 ° C for 12 h and subsequently freeze-dried for 2 days. Pure AC without any BDE crosslinker was used as the control group.

3.5.3 Characterization methods

3.5.3.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The AC was subjected to SDS-PAGE according to protocols established by Laemmli [191]. Briefly, acid-soluble rat tail collagen Type I (Sigma, USA) was used as a control for the extracted AC. Electrophoresis was conducted at a constant voltage of 30 mV for 2 h. Protein bands were stained using 2.5% Coomassie brilliant blue R-250 and destained in a solution of 40% methanol and 10% acetic acid.

3.5.3.2 Amino acid analysis

In order to determine the amino acid composition of AC, 10 mg of AC extracted from bullfrog skin was hydrolyzed in 25 ml of 6 N hydrochloric acid at 110 °C with nitrogen protection for 22 h. A 1 ml portion of the sample solution was

removed and dried by nitrogen at 55 °C. The dried sample was dissolved in 1 ml of 0.02 M hydrochloric acid for analysis. The hydrolysates were analyzed with the Hitachi amino acid analyzer L-8900.

3.5.3.3 Morphology

The cross-sectional characteristics of the scaffolds were investigated using a JEOL JSM-5410 scanning electron microscope (SEM) (JOEL Ltd., Japan) at 5 kV. Scaffolds were sputter coated with gold using the SPI-Module Sputter Coater System (Structure Probe Inc., USA) prior to imaging under the SEM. The average pore size and porosity were measured using the ImageJ software as described in section 3.4.6.1.

3.5.3.4 Fourier transform infrared (FTIR) spectroscopy

FTIR spectroscopy (Perkin Elmer, Singapore) was used to confirm the successful BDE crosslinking of the AC scaffolds. The AC scaffolds were milled into a fine powder and mixed with KBr powder at a ratio of 1:20 and then pelletized. The pure KBr pellet was used as a reference. The absorption spectra were then collected and analyzed using the Spectrum™ software.

3.5.3.5 Thermal properties

A Thermo-gravimetric Analyzer (TGA) (TA Instruments, USA) was used to identify the decomposition temperature (T_d) of AC scaffolds. The AC scaffolds were cut into an average sample mass of 2.5 mg and placed onto a platinum sample holder pan and heated at a rate of 5 °C/min up to 850 °C under air with a

nitrogen flow of 90 ml/min. Differential Scanning Calorimetry (DSC) (TA Instruments, USA) was carried out to determine the denaturation temperature (T_n) of the AC scaffold. Dried samples were weighed and loaded into the DSC pan prior to crimping. The results were collected by ramping the sample from -50 °C to 200 °C at 5 °C/min.

3.5.3.6 Maximum swelling ratio

Swelling ratio was measured following the same method described in section 3.4.4.2.

3.5.3.7 Mechanical property

The scaffolds were subjected to compression testing using an Instron 5869 machine fitted with a 10 N load cell. Compression tests were carried out on five samples (n=5) of each sample type at a crosshead speed of 2 mm/min until maximum extension was reached. The materials were tested under wet conditions. Scaffolds were submerged in saline for 24 h at 37 °C to achieve complete wetting of the samples. The slope of the stress versus strain curve, which represents the compressive Young's modulus, was calculated for all samples.

3.5.3.8 *In vitro* collagenase degradation study

In vitro degradation studies were carried out by immersing the scaffolds in phosphate buffered saline (PBS, pH 7.4) with 0.01 mg/ml collagenase at 37 °C to simulate the *in vivo* microenvironment. After 2 h, 0.25 M ethylenediaminetetraacetic acid (EDTA) was added to inhibit the reaction. The

samples were transferred to a fresh well-plate and washed with DI water prior to freeze-drying. The initial (M_i) and final (M_f) masses of the dry samples were measured by a precision weighing balance. The percentage degradation was determined by the following equation:

$$\text{Degradation (\%)} = (M_i - M_f) / M_i \times 100\% \quad (4)$$

3.5.3.9 Cell viability test

ASCs were cultured in ASCs cell culture medium which mentioned in section 3.1.4., and the cell viability and proliferation of ASCs on the scaffolds was determined using the AB assay described in section 3.4.3.3.

3.5.3.10 In vivo study

The biocompatibility and stability of AC scaffolds *in vivo* were studied using C57BLKS/J mice. This study was approved and performed in compliance with the regulations of the Institutional Animal Care and Use Committee of Nanyang Technological University (ARF SBS/NIE-A0174 AZ). After anaesthetizing the mice, two slits were made on the dorsal side of the skin, and the AC scaffolds (0.5 cm³) were inserted subcutaneously. Subsequently, an occlusive dressing was applied to the wound to ensure that the samples stayed in place under the skin. After 2 weeks, the mice were euthanized using CO₂ and the samples were excised along with the surrounding skin area for H&E staining. All animals were maintained in pathogen-free conditions.

3.6 Statistical Analysis

All test was carried out using five replicates (i.e. $n=5$) with data shown as mean $\pm$ standard deviation (SD) unless otherwise stated. Analysis was evaluated using a one-way analysis of variance (ANOVA) with replicates and Tukey's honest significant difference (HSD) Post Hoc Test. Data are considered to be statistically significant with a confidence level of 95% ($p<0.05$).

4. COMPARISON OF DECELLULARIZATION METHODS

4.1 Overview

As compared to the other commonly used materials for tissue engineering applications, the rich milieu of growth factors and other biological components is able to confer bioactivity to the decellularized ECM material [47,146,151]. Decellularized ECM material has been shown to induce cell infiltration, thus promoting tissue remodeling when implanted *in vivo* [192]. Apart from its outstanding biological properties, it has been shown that the ECM extracted from various tissues has good biocompatibility and minimum immune response when reimplanted [47,151,192]. The ECM is tissue-specific in terms of the composition and the distribution of different ECM proteins, which eventually contributes to the tissue architecture and function. Therefore, the adipose-derived ECM material would be the most suitable material for adipose tissue engineering applications [146,193]. Currently, adipose tissue is often discarded as a clinical waste after plastic and reconstructive surgery in the form of lipoaspirates. However, it has been recognized as a rich source of ECM material. Hence, steps could be taken to transform this waste material into a valuable biomaterial for tissue engineering applications.

Since it has been shown that for different tissues the type of decellularization method has great influence on the property of the final decellularized ECM

[19,152-155,194], it is necessary to find out a suitable method for decellularizing the ECM of adipose tissue. Unlike other tissues, adipose has a huge amount of lipid component, which makes the decellularization more challenging. For clearing immunogenic cellular contents, many groups have combined a series of enzymatic, chemical and physical agents for optimal cell and lipid removal. These methods utilize reagents including detergents such as Triton-X 100, sodium doecyl sulfate (SDS) and sodium deoxycholate, enzymatic agents such as nucleases and trypsin and other chemical agents like alcohols, acetone, calcium chloride and sodium chloride to achieve effective decellularization [19-22]. However, these reagents have been reported to disrupt the ultrastructure of ECM and remove biological components [186,195]. Furthermore, these reagents act slowly, thus extending the time required for ECM isolation, which may lead to the loss of more biological components [19,195].

In the current study, we developed a purely physical and a purely chemical method and compared them with an already established enzymatic method to decellularize fat grafts to understand the effect of these different treatments on the properties of the decellularized ECM material. The different methods were previously shown schematically in Figure 3 and discussed in detail in section 3.2.1.

4.2 Results and Discussion

4.2.1 Adipose tissue decellularization

The three methods of decellularization resulted in ECM materials of different morphologies and color (Figure 7). Both the ECM isolated via enzymatic method (ECM-E) and the ECM isolated via chemical method (ECM-C) had a sponge-like appearance that was similar to the original adipose tissue structure, whilst, the ECM isolated via physical method (ECM-P) had a paste-like structure that was very different from the original adipose tissue architecture. The difference in the morphology of ECM-P is probably due to the fact that the physical decellularization methods involved direct forces being applied to the tissue in most steps [186,195]. The steps of blending, homogenizing and ultrasonication removed cells from the tissue but also damaged the tissue structure and disrupted and fractured the resultant ECM-P. On the other hand, both ECM-E and ECM-C exhibited sponge-like structures due to less disruption of the original tissue structure during the respective decellularization methods. It was also observed that all three types of ECM material were white in color after the decellularization process as compared to the original adipose tissue, which was yellow in color as the result of the presence of lipids. The yield of ECM from each method was calculated when compared to the weight of the original freeze-dried adipose tissue. The yield was $1.42 \pm 0.19\%$, $1.70 \pm 0.08\%$ and $1.11 \pm 0.24\%$ for ECM-E, ECM-C and ECM-P, respectively. The low yield of adipose tissue-derived ECM as compared to other tissues was due to the large volume of lipids within the native tissue

[76,78]. Among these three methods, the physical method resulted in the lowest amount of ECM, which is probably due to the numerous steps involved manually separating ECM material from tissue suspensions, which caused the loss of ECM material along the way. On comparison between chemical method and enzymatic method, the lower yield of ECM-E could result due to the longer processing time and using the enzymatic reagents which could cleave many biological components including structural proteins and GAGs from ECM. Overall, the extreme low yield of adipose tissue-derived ECM as compared to other tissues was due to the large volume of lipids within the native tissue. It is therefore highly desirable to use adipose tissue-derived ECM material as a bioactive coating material rather than as a scaffolding material to maximize the usage of this valuable biomaterial for tissue engineering applications.

Figure 7. Images of the original adipose tissue and the decellularized tissue: (a) native adipose tissue, (b) ECM-E, (c) ECM-C and (d) ECM-P. Scale Bar =3mm.

4.2.2 Confirmation of decellularization

Using the three different decellularization methods, all cells and cellular components were confirmed to be removed from native tissue (Figure 8a) and the remaining DNA contents were all below 50 ng/mg (Figure 8d), which met the requirement for clinical usage based on previous studies that assessed levels of DNA remaining within commercially available ECM products [186,195]. The presence of cells is typically the main cause for activating the immune system and causing immune rejection. In addition, it is important to delipidize the adipose tissue during the ECM extraction process since the lipid would have led to adverse host response *in vivo* [195,196]. Thus, the complete removal of lipid was confirmed by Oil Red O staining (Figure 8b) and no red color staining appeared in any ECM material. This was also confirmed by the previous observation of the change of color of the native adipose tissue as compared to the decellularized ECM (Figure 7). It is important to delipidize the adipose tissue during the ECM extraction process since any remnant lipids would cause an adverse host response *in vivo*. Furthermore, SEM was used to observe the microstructures of the three different ECM as well as the native adipose tissue (Figure 8c). Despite similar efficacies for decellularization and delipidization, the different decellularization methods investigated resulted in ECM materials of differing morphologies. The images of ECM microstructures showed a denser and finer fibrous structure of ECM-P as compared to those of ECM-C and ECM-E. This could be due to the breakdown of the big fibers by the direct mechanical forces applied during the

physical process. This could justify the aforementioned paste-like appearance of the ECM-P and the spongy-like appearance of the other two ECM products. The SEM results provided evidence that no lipid droplets or cells remained in the ECM. Overall, all three methods were able to remove cells, DNA contents and lipids effectively.

Figure 8. The successful decellularization procedures were confirmed by employing staining procedures on the cross-sections. (a) H&E staining showed that all three protocols were capable of removing the cellular and nuclear content; (b) Oil Red O staining showed that the ECM-P and ECM-C were more efficient in lipid removal and (c) scanning electron microscopy images illustrating the fibrous structures of the final ECM. Scale Bar=100 μ m. (d) Residual DNA content in ng/mg dry weight in all the three ECM samples was quantified to be <50ng/mg.

4.2.3 Retention of biological components

An optimal decellularization process should preserve the biological components of the ECM as much as possible. Hence, further immunostaining was carried out and the results showed that key proteins such as collagen type I, collagen type III, collagen type IV, elastin, laminin and fibronectin remained in the LpECM material (Figure 9). The blue DAPI staining of the original adipose tissue (Figure 9i) corresponds to the nuclear material, which was absent in all the decellularized ECM structures (Figure 9ii-iv), which once again confirms the complete removal of the nuclear material. Overall, the proteins within the decellularized material were less organized as compared to those in the native tissue due to the lack of preservation of original ECM structure once the cells and lipids were removed. Unlike other types of tissues, adipose tissue contains large amount of lipid, thus resulting in a large volume reduction after decellularization. Similar to the native ECM, the most abundant protein found in the isolated LpECM was that of collagen, including collagen type I (Figure 9a) and collagen type IV (Figure 9b). In addition, elastin (Figure 9e), laminin (Figure 9c) and fibronectin (Figure 9d) were also detected in all the three ECM structures. These ECM proteins not only provide structural support but also instruct the cells to attain distinct morphology, migrate, proliferate, differentiate and develop into an appropriate tissue [128,130-133]. The most abundant and well-studied of the ECM proteins, collagens, are structural proteins that entrap, locally store and deliver growth factors to cells. Specifically, collagen type I binds decorin which indirectly

inhibits transforming growth factor beta (TGF- β), an inhibitor of adipogenesis [197,198]. The non-collagenous domains of basement membrane collagen - like collagen type IV - have also been shown to play a role in angiogenesis [199]. Laminins have been reported to bind preadipocytes via integrins and mediate their migration [200]. Fibronectin is a glycoprotein that contains an adhesive peptide argine-glycine-aspartic acid (RGD) motif. Since its discovery in 1984, tissue engineering scientists began conjugating this peptide to the scaffolds to enhance cell attachment. Apart from this peptide, fibronectin also contains a binding domain for integrins, collagens, heparin and fibrin. Therefore it is beneficial if the whole functional fibronectin molecule is retained on decellularized extracellular matrices [201]. Elastin protein plays a structural role maintaining recoil-resilience function for tissues constantly under stress and therefore its role in adipose tissue function (shock-absorber) is predictable [202]. Other than structural significance, elastin induces fibroblast migration [203], proliferation, elastin synthesis [204], and matrix metalloproteinase-1 (MMP-1) expression [205] during wound healing. Hence, the presence of all these important proteins in the final ECM products from all three methods suggests that all three methods were able to preserve these proteins. Also, there was more collagen type I, collagen type IV and elastin as compared to laminin and fibronectin in all three ECM, which may be due to the high composition of collagen type I, collagen type IV and elastin in the original tissue. However, as the immunostaining is only a qualitative analysis, the actual amount of the proteins cannot be compared. In the next section, the amount of biological

components including collagen, GAGs, VEGF and bFGF, was measured quantitatively.

Figure 9. The immunostaining images of decellularized ECM in comparison with original tissue for (a) collagen type I, (b) collagen type IV, (c) laminin, (d) fibronectin and (e) elastin. Scale Bar=50 μ m.

4.2.4 Quantification of biological components

The total and acid soluble collagen content, a main component of ECM, were measured using the hydroxyproline assay and Sirius Red method respectively. The amount of total collagen in the freeze-dried ECM material was approximately 283 $\mu\text{g}/\text{mg}$, 75 $\mu\text{g}/\text{mg}$ and 376 $\mu\text{g}/\text{mg}$ from enzymatic, chemical and physical methods of decellularization (Table 4). On the other hand, the amount of soluble collagen content post decellularization was 32 $\mu\text{g}/\text{mg}$, 15 $\mu\text{g}/\text{mg}$ and 170 $\mu\text{g}/\text{mg}$ respectively for the ECM-E, ECM-C and ECM-P material (Table 4). Among the three decellularization methods, the physical method was capable of preserving the largest amount of collagen, followed by the enzymatic and the chemical methods. The extremely low amount of collagen preserved in the chemical methods may be caused by using of diethyl ether solution, which has been reported to extract collagen. However, without using this reagent in the decellularization process, a large amount of lipid will be remained in the resulting ECM material. In order to fulfill the most important criteria of decellularization of adipose tissue, which refers to the removal of cells, DNA contents and lipids, it will sacrifice the collagen content in the pure chemical method. Another important observation is that the ECM material from the enzymatic method has the lowest soluble to total collagen ratio, which is about 11.27% as compared to 45.33% and 19.77% from physical and chemical methods respectively. This may be caused by the enzymatic reagents which altered the properties of the collagen. Overall, different treatments affected the collagen within the ECM material, and could

eventually change both the mechanical property and chemical properties of ECM. Therefore, it is important to choose the decellularization method according to the specific needs of different applications.

Table 4. Collagen content of the ECM obtained using different decellularization methods

Method	Total Collagen ($\mu\text{g}/\text{mg}$)	Soluble Collagen ($\mu\text{g}/\text{mg}$)
Enzymatic	283 ± 6	32 ± 5
Chemical	75 ± 8	15 ± 3
Physical	380 ± 20	170 ± 20

Other than the collagen content, the contents of other biological factors such as GAGs and ECM-associated growth factors were also measured. The GAG content of ECM-E, ECM-C and ECM-P was quantified to be $17 \pm 5 \mu\text{g}/\text{g}$, $300 \pm 100 \mu\text{g}/\text{g}$ and $2200 \pm 200 \mu\text{g}/\text{g}$ of dry weight ECM, respectively (Table 5). Among the three methods, it was observed that the physical method was able to conserve the highest amount of GAGs since ECM-P showed an 8.2 fold increase in the amount of GAGs as compared to that of ECM-C and a 134.8 fold higher than ECM-E in the expression of GAGs from ECM-E ($p < 0.05$). The amount of two important growth factors, VEGF and bFGF, within the final ECM derived from these three different decellularization methods was also analyzed (Table 5). The values for the VEGF content in ECM-E, ECM-C and ECM-P were $0.6 \pm 0.1 \text{ pg}/\text{mg}$, $0.37 \pm 0.02 \text{ pg}/\text{mg}$ and $8 \pm 1 \text{ pg}/\text{mg}$ of dry ECM respectively. The amount of VEGF in the

ECM-P was found to be 14.83-fold and 22.05-fold higher than ECM-E and ECM-C, respectively ($p<0.05$). The amount of bFGF was found to be 200 ± 50 pg/g of dry ECM after processing by the physical method but for enzymatic and chemical methods, the amount of bFGF was lower than the detection limit of the VEGF assay kit, which is 5 pg/mL (Table 5). Similar to the VEGF content, the amount of bFGF detected in ECM-P was significantly higher than ECM-E and ECM-C ($p<0.05$). Inherent GAGs have previously shown to load bFGF in the context of acellular pericardial tissues. These GAGs may be utilized to anchor bFGF molecules that promote vascularization [206]. The increased bFGF conservation post-physical method is probably due to the high anchoring capacity of the GAG molecules conserved during the decellularization process. In addition to GAG and bFGF, the adipose tissue also contains VEGF, which is the most important molecule necessary for angiogenesis [207]. Matrix bound VEGF promotes endothelial cell survival and neoangiogenesis [208]. bFGF upregulates VEGF and attracts pericytes which form a layer outside the new capillaries to mature them, thus protects it from degradation [209]. The high vascularity of the adipose tissue might be due to possibility of crosstalk between these molecules to induce angiogenesis [206,207]. Overall, the physical method for decellularizing adipose tissue was recommended as the highest amount of biological components, including collagen, GAGs, VEGF and bFGF, was preserved. The amount of biological components within the ECM material eventually affects the ability of the ECM to support growth and differentiation of

cells [19].

Table 5. Concentration of GAG, bFGF and VEGF for the different decellularization methods.

Method	GAG content μg/ g	bFGF content pg/g	VEGF content pg/g
Enzymatic	17 ±5	-	0.6 ±0.1
Chemical	300 ±100	0.32 ±0.17	0.37 ±0.02
Physical	2300 ±200	200 ±50	8 ±1

4.2.5 Detection of matricellular proteins

Aside from these common biological components, Western Blot was used to detect the presence of matricellular proteins within resulting ECM isolated from the three methods. All five matricellular proteins, including SPARC, Cyr61, OPN, thrombospondin 1 (TSP 1) and tenascin C (Ten C), were detected in ECM-P (Figure 10). For the chemical decellularization method, SPARC and Cyr61 were clearly expressed; whilst TSP 1 and Ten C showed lower in ECM-C. In addition, only TSP 1 and Ten C were showed in the ECM-E extracted using enzymatic decellularization method. During the last two decades, matricellular proteins have been recognized as important components of ECM [210-212]. Instead of structurally supporting the ECM, the matricellular proteins play a role in modulating cell-matrix interactions and cellular functions [210,213-216]. These matricellular proteins were reported to have contributions to the wound healing and facilitate angiogenesis [210,213,217-220]. Once again, the physical

decellularization method appeared to be the best method for preserving the matricellular proteins.

Figure 10. Detection of key matricellular proteins including SPARC, Cyr61, OPN, TSP 1 and Ten C in the different ECM materials: Lane 1 ECM-E, Lane 2 ECM-C, Lane 3 ECM-P, and Lane 4 protein ladder.

4.3 Summary

The advantages and disadvantages of each of these three different decellularization methods are summarized in Table 6. Purely enzymatic, chemical and physical methods were compared in terms of their effectiveness of decellularization and ability to conserve specific ECM components. All three methods were successfully isolated the ECM material form adipose tissue. Due to the large volume of lipid within the adipose tissue, the yield of adipose tissue derived ECM was much lower than other tissues. Each of these methods presented a different structure and

composition of the ECM post-processing. The physical method was the most effective in preserving the biological components. It also required shortest processing time but compromised with lower yield and structure collapse. The second shortest processing time method, the chemical method, was also able to preserve the structure as well as the important growth factors. However, it led to the denaturation of the collagen due to the various chemical reagents involved. Lastly, the previously reported enzymatic method was able to remove most cellular components as well as DNA contents, but all growth factors were removed during the decellularization process which may require additional input growth factors in future applications. In general, the detection of matricellular proteins may extend the applications of adipose tissue derived ECM for other tissue engineering such as wound healing and revascularization. Hence, depending on application, different decellularization methods should be used for processing adipose tissue.

Table 6. Evaluation of the different decellularization methods for extraction of adipose tissue-derived ECM

Parameter	Enzymatic method	Chemical method	Physical method
Cost	+++	++	+
Yield	++	+++	+
Degree of Decellularization	+++	+++	+++
Degree of Delipidization	+++	+++	+++
Structure Integrity	++	++	+
Amount of Collagen	++	+	+++
Amount of GAGs	+	++	+++
Amount of Growth Factors	+	++	+++
Detection of Matricellular Proteins	+	++	+++
Environmental Friendly	+	+	+++

+ refers to low, ++ refers to moderate, +++ refers to high

Overall, ECM-P will be recommended for use as a bioactive coating material as all the important biological components are preserved. Whilst ECM-E and ECM-C could be used as scaffolds for direct implantation for adipose tissue engineering applications since the overall structure of the tissue was preserved. Other than fat graft, lipoaspirate is another form of adipose tissue. In next chapter, another physical method was developed for decellularization of lipoaspirates by using

supercritical dioxide, which does not require many manual operation steps, as well as the efficacy of using ECM material as a bioactive coating material will be shown.

5. DECELLULARIZED ADIPOSE TISSUE AS A BIOACTIVE COATING

5.1 Overview

The properties of a biomaterial surface have been shown to play instructive roles in the regulation of cell-material interactions and for controlling cellular fate functions such as adhesion, migration, proliferation and differentiation [221-224]. However, most biomaterials may have the desired bulk properties but not surface properties to meet the required bio-application needs. Hence, surface modification of the tissue-engineered material with a bioactive component, is an effective method to improve surface biofunctionality while maintaining the bulk property of the material. [180] Presently, various natural extracellular matrix (ECM)-derived proteins, such as collagen [225], laminin [226] and fibronectin [227], have been used as a surface coating material to improve the cell-material interactions. However, to our knowledge, no study that involves the direct application of the entire ECM as a coating material has yet been carried out.

Since the end of the 1970s, supercritical fluids have been used to isolate natural products, and it has been well recognized as an environmentally friendly processing technique that is clean, cheap and deemed to be safe by the FDA. [228] Hence, in this study, a “green” method using supercritical carbon dioxide (SC-CO₂) was developed to decellularize lipoaspirate material, which does not

require as many steps as compared to the physical method mentioned in the previous chapter. As the result of multiple biological components including various growth factors within the ECM material, which was discussed in detail in Chapter 4, the entire ECM material was used as a bioactive coating material to improve cell-material interactions in the current study.

5.2 Results and Discussion

5.2.1 Characterization of decellularized adipose tissue

An easily industrialized decellularization method was successfully developed to obtain lipoaspirate-derived ECM (LpECM) using the supercritical carbon dioxide (SC-CO₂) method, which was described in detail in section 3.2.1.4. The advantage of using SC-CO₂ is that it is relatively easy to achieve the supercritical condition (30.9 °C and 73.8 bar). The high diffusivity, permeability and solubility of SC-CO₂ [229] would also facilitate the extraction of cells and lipids from lipoaspirate within a much shorter processing period. Meanwhile, the SC-CO₂ remains in the gaseous state at room temperature and pressure, which enables it to evaporate immediately after the SC-CO₂ treatment and results in a solvent-free LpECM product. The concentration of each component of lipoaspirate is listed down in Table 6. For the first time, the yield of the ECM from lipoaspirate is reported. Due to the high proportion of water and lipid content (29.8% ±0.8% and 69.2% ±0.1% respectively), the final yield of dry ECM material from lipoaspirate

was only 0.9% $\pm$ 0.1%.

Table 7. The composition of adipose tissue (lipoaspirate).

CompositionAmount (%)	
Water	29.8 $\pm$ 0.8
Lipid	69.2 $\pm$ 0.1
ECM	0.9 $\pm$ 0.1
Others*	0.1

* The result was not measured directly but computed by eliminating the other components i.e. water, lipid and ECM from the total tissue content of 100%.

In order to remove the immunogenic cell components and lipid, pure ethanol was added as a modifier in the decellularization process [230]. As seen from Figure 11, without the addition of ethanol, the processed lipoaspirate material remained yellow (Figure 11b), whereas after the addition of ethanol during the SC-CO₂ treatment, the processed lipoaspirate material turned to white (Figure 11c) after the lipid content was removed from the original unprocessed lipoaspirate material (Figure 11a). The addition of ethanol could expand the extraction range to include more polar lipids such that the overall extraction efficiency is enhanced. It should also be pointed out that ethanol is the only organic solvent allowed to be used for nutraceutical and pharmaceutical purposes [231].

Figure 11. Images of lipoaspirate tissue at different stages of decellularization: (a) original unprocessed yellow color lipoaspirate, (b) slightly yellowish lipoaspirate tissue after SC-CO₂ treatment without using ethanol, and (c) white color lipoaspirate-derived ECM after SC-CO₂ treatment with ethanol as a co-solvent. Scale Bar = 5mm.

To evaluate the efficiency of cell and DNA removal, histological staining and dsDNA intercalators were used. The H&E staining results (Figures 12a and 12b) showed no evidence for visible nuclei and nuclei material, and the amount of DNA left in the dry LpECM was 43.5 ng/mg, which is below the level which is safe for clinical applications [195]. Also, the Oil Red O staining results showed complete removal of lipids from the original lipoaspirate tissue (Figure 12c) in the final LpECM product (Figure 12d). Moreover, SEM results showed significant differences between the original tissue and decellularized tissue in the microstructure, as fibrous structures were clearly observed in the ECM (Figure 12f), while the original lipoaspirate tissue had a smooth surface (Figure 12e). Overall, the cells and lipids from the ECM material were removed after the SC-CO₂ decellularization process as compared to the original unprocessed lipoaspirate tissue.

Figure 12. Confirmation of the effectiveness of SC-CO₂ decellularization method for extraction of ECM from lipoaspirate using (a, b) H&E staining, (c, d) Oil Red O staining, and (e, f) SEM imaging. Overall, no cells and nuclei was observed in any of the extracted ECM material. Scale Bar=50 μ m.

5.2.2 Evaluation of biological components in ECM

In addition to ensuring complete cell, lipid and DNA removal, it is also very important to confirm that the desired biological components were still retained after the decellularization process. Immunostaining results showed that key proteins such as collagen type I, collagen type III, collagen type IV, elastin,

laminin and fibronectin were still present in the ECM (Figure 13b). The proteins were much denser than original structure after decellularization due to the removal of the cells and lipids. Similar to the native ECM, the most abundant protein found in the isolated LpECM was that of collagen, which mainly provides structural support to the cells in the lipoaspirate tissues [132]. In addition, elastin, another important protein present in the ECM, was also detected in the extracted LpECM, which could contribute to the elasticity of the tissue [232]. Moreover, fibronectin was also found in the LpECM, which mainly help connecting the ECM proteins and direct cell-material interactions [132,233]. Laminin, influences various cell activities such as cell adhesion, migration and differentiation, was also detected in LpECM [234-237]. Detailed description of the roles of these structural proteins in decellularized ECM material can be found in section 4.2.3 of this thesis.

Figure 13. Observation of positive immunostaining for Collagen Type I, III, IV, elastin, fibronectin and laminin in (a) the original lipoaspirate, and (b) the lipoaspirate-derived ECM (LpECM) material. Scale Bar =100 μm.

Other than these main structural proteins, other biological factors were also measured. The glycosaminoglycan (GAG) content of the LpECM was 4600 ± 800 μg/g of dry LpECM. The basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) were 280 ± 10 pg/mg and 12 ± 3 pg/mg of dry

LpECM respectively. GAGs are strongly hydrophilic and typically formed proteoglycans in the ECM to keep the ECM and cells hydrated, whereas both bFGF and VEGF play important roles in promoting angiogenesis and vascularization [207,238-240]. From the current study, similar to the physical method described in previous chapter, we also found the presence of matricellular proteins, including SPARC, Cyr61, OPN, thrombospondin 1 (TSP 1) and tenascin C (Ten C) in decellularized LpECM (Figure 14). Matricellular proteins are a class of ECM proteins that regulate cellular functions including foreign body response, angiogenesis and osteogenesis [210,212,213,215,217-220], and were discussed in detail in section 4.2.5 of this thesis.

Figure 14. Key matricellular proteins including SPARC, Cyr61, OPN, TSP 1 and Ten C, were detected in the decellularized ECM material using Western Blot: Lane 1 LpECM, Lane 2 protein ladder.

5.2.3 Confirmation of ECM coating on TCP

After the ECM material was coated onto the TCP, several techniques were used to confirm the successful coating of the ECM material. Various surface characterisation methods were used to confirm the successful coating of LpECM material onto TCP (Figure 15). FTIR was used to detect the presence of characteristic peaks of LpECM in the spectrum compared to the original TCP (Figure 15a). Compared to the original TCP material, characteristic peaks for proteins were found for TCP-LpECM. The strong peak appearing at 3309 cm^{-1} was due to the function group N-H stretching of amide A and amine within the various proteins within complex LpECM material. The shallower band at 2922 cm^{-1} was linked to the asymmetrical stretch of $-\text{CH}_2$ for amide B. Besides amide A and amide B, another strong region was located in the range of $1000\text{--}1500\text{ cm}^{-1}$. The strongest peaks were observed for amide I at 1647 cm^{-1} , amide-II at about 1553 cm^{-1} , and amide III at 1239 cm^{-1} . The strongest band of LpECM, amide I, was almost entirely due to amide carboxyl $\text{C}=\text{O}$ backbone stretching with a little effect from C-N stretching [241]. The combination of the N-H in-plane bend and the C-N stretching vibration contributed to the amide II peak. The peak of amide III peptide bond generally is an indication of the presence of a complex intermolecular interaction, which in this case is mainly from the C-N stretches and the N-H bending vibrations of peptide groups within LpECM.

The static water contact angle was also measured before and after LpECM surface

coating onto TCP to evaluate changes in surface hydrophobicity (Figure 15b). Due to introduction of LpECM onto the TCP surface, a large amount of hydrophilic chemical groups including carboxylic acid groups, amine groups and hydroxyl groups were present on the LpECM-coated TCP. Therefore, a reduction of contact angle from 79.2° to 52.9° was observed, which is favorable for initial cell attachment and migration.

In addition, immunostaining was used to confirm that the main structural proteins were coated onto the TCP. As shown in Figure 15c, collagen type I, collagen type II, collagen type IV, elastin, fibronectin and laminin, all remained intact even after the LpECM coating process. In fact, the composition of each component was also similar to the immunostaining of the LpECM material alone. Taken together, these results confirmed the successful coating of LpECM material onto the TCP and the preservation of different types of useful biological proteins during the coating process.

Figure 15. Confirmation of successful coating of the entire ECM material onto TCP by (a) FTIR, where the characteristic ECM peaks were highlighted , (b) contact angle analysis, where a reduction of contact angle was observed on TCP-ECM, and (c) positive immunostaining of collagen type I, III, IV, elastin, fibronectin and laminin on the TCP-ECM surface. Scale Bar=100 μ m.

5.2.4 Immunogenicity

Optimal implantable materials should aim to reduce foreign body response and toxic effect in a specific application [242-244]. At the cellular level, the initial phase of foreign body response is similar to acute inflammation with the infiltration of neutrophils and macrophages. TNF α is one of the major inflammatory cytokines secreted by immune cells during foreign body response [245]. In the current study, THP-1 cells were used to investigate the function and regulation of monocytes and macrophages in the presence of LpECM coating material. Results showed that THP-1 differentiated macrophages interacting with TCP-LpECM exhibited lower level of TNF α production as compared to cells seeded directly onto the TCP (Figure 16a). Furthermore, there was no sign of more activated macrophages on the TCP-LpECM surface compared to the TCP surface (Figure 16b). Although an increase (3.37 fold) in the TNF α expression for THP-1 monocytes interacting with TCP-LpECM as compared to pristine TCP was observed, the TNF α expression of monocytes were much lower than the macrophages in both TCP and TCP-LpECM. Also, the morphology of the cells attached to the TCP-LpECM material was similar to that of the TCP surface. Thus, there was minimal pro-inflammatory effect from the macrophage and monocyte responses when using LpECM as a bioactive enrichment. Taken together, the LpECM did not evoke an immune response and therefore has good biocompatibility with low cytotoxicity and immunogenicity.

Figure 16. Immune response study for THP-1 cells and THP-1 induced macrophages: (a) qPCR results of TNF α expression level of monocytes and macrophages on TCP and TCP-ECM, and (b) light microscopic images of the morphology of monocytes and macrophages on TCP and TCP-ECM. Arrows pointed to the activated macrophages. Scale Bar=100 μ m.

5.2.5 Cellular studies

HaCaT cells are one of the commonly used cell types in research to study the human keratinocyte behavior in wound healing for tissue engineering applications [246,247]. Figure 16a showed that the LpECM coating significantly increased the proliferation of immortalized human keratinocyte (HaCaT) cells without changing the cell morphology. After 72 h of culturing, the HaCaT cells on TCP-ECM formed a much denser cell layer compared to the normal TCP as shown in Figure 16b.

Figure 17. Cellular results of HaCaT cell on TCP and TCP-ECM: (a) PrestoBlue® assay showed significantly improved ($p < 0.05$) HaCaT cell proliferation over a period of 72 h, and (b) light microscopic images of the HaCaT cell morphology and coverage area at time points of 12 and 72 h. Scale Bar=200 μm .

Epidermal wound healing is very important in both full-depth wounds and epidermal wounds [248,249]. In addition to cell proliferation, the migration of keratinocytes plays an equally important role in this wound healing process [250-252]. To mimic the *in vivo* keratinocyte migration during wound healing, an *in vitro* wound assay using silicone inserts (ibidi, USA) was performed to analyze keratinocyte migration. As shown in Figure 18b, the silicone insert created a defined cell-free gap of 500 μm width which acted as the *in vitro* wound. The

extent of keratinocytes migration was quantified by measuring the uncovered surface area (analogous to the *in vivo* wound gap) at each time point as compared to the original gap area at 0 h. Figure 18a shows the migration curve of HaCaT on the TCP and TCP-ECM surface. The wound gap on the TCP-ECM surface was closed between 15 h to 20 h, whereas the gap on the TCP surface remained open even after 20 h. Overall, the ECM coating material could potentially contribute towards epidermal wound healing as shown from the significant improvement in the keratinocytes migration results. The potential of LpECM as a pro-healing material would be due to the biological components within the LpECM, such as laminin (refer to Figure 15c) and matricellular proteins (refer to Figure 14), which all contribute to the wound healing process.

Figure 18. Migration study results for HaCaT cells on TCP and TCP-ECM: (a) the migration curve of HaCaT on TCP and TCP-ECM over 20 h, and (b) the representative time-lapsed microscopic images of HaCaT in a scratch-wound assay. The black dotted line demonstrated the scratch gap at the time of wounding. Scale Bar=500 μ m.

As discussed in section 2.1.3, adipose tissue-derived stem cells (ASCs) are commonly used for adipose tissue engineering studies [51-53]. After coating with LpECM, both the initial cell attachment and proliferation of ASCs were shown to be significantly improved. The LIVE/DEAD® staining of ASCs after 7 days of culture is shown in Figure 19b. A larger number of ASCs were present on the TCP-ECM surface, and negligible dead cells were detected in both TCP and TCP-LpECM surfaces. Moreover, the cells grown on the TCP-ECM surface also retained their normal cellular morphology when compared to the cells grown on the TCP. After 14 day of culturing ASCs in adipogenesis medium, ASCs were able to differentiate into adipocytes. Figure 19c shows the Oil Red O staining results of the differentiated ASCs on TCP and TCP-ECM surface. More lipid droplets were observed on the TCP-ECM when compared to the TCP surface. The improvement of ASC attachment, growth, proliferation and differentiation on TCP-ECM is an indication of potential of ECM as a bioactive coating material for soft tissue engineering applications.

Figure 19. ASCs cell culture study on TCP and TCP-ECM: (a) PrestoBlue® assay showed significantly higher ($*p<0.05$) ASCs attachment within 24 h and proliferation rate over 7 days on TCP-ECM, (b) cell viability test using LIVE/DEAD® staining after 7 days *in vitro* culture also indicated more cells were present on TCP-ECM (Scale Bar=200 μm), and (c) Oil Red O staining of ASC adipogenesis at Day 14 (Scale Bar=50 μm).

As the vascularization is always a challenge in soft tissue engineering [59,64,65], which discussed in section 2.1.3, HUVECs is one of the most important cell types used in vascularization studies [158,253-255]. When comparing to the original TCP, TCP-ECM had 30% lower human umbilical vein endothelial cells (HUVECs)

attachment after Day 1 (Figure 20a). However, the cell proliferation rate on TCP-LpECM was much higher than on the original TCP. On Day 5, the number of HUVECs on TCP-ECM surface started to be higher than those on TCP, and the number of HUVECs on TCP-LpECM was significantly higher than those on the TCP on Day 7. To assess the overall extent of endothelialization on the different surfaces, fluorescence images of LIVE/DEAD® stained HUVECs were captured after 7 days of culture (Figure 20b). In general, the HUVECs were uniformly distributed on both surfaces. The TCP-ECM showed better endothelialization since a more confluent HUVECs layer was observed on the surface. This improvement of endothelialization was probably due to the presence of the various biological factors such as VEGF and matricellular proteins in the LpECM, which had positive effect on vascularization and angiogenesis.

Figure 20. HUVECs proliferation results on TCP and TCP-ECM: (a) PrestoBlue® assay proliferation results over 7 days showed TCP-ECM had significantly lower ($*p<0.05$) initial cell attachment rate but improved proliferation rate, and (b) LIVE/DEAD® staining after 7 days of *in vitro* culture. Scale Bar=200 μ m.

Overall, the LpECM was shown to be safe as a bioactive coating material with minimal immune response from the monocytes and macrophages. Three other types of cells (HaCaT, HUVEC and ASCs), which are commonly used in soft tissue engineering research, were showed to have positive response to the TCP-LpECM. Thus, for the first time, LpECM was demonstrated as an enrichment material for tissue engineering applications.

5.3. Summary

In summary, an alternative decellularization method for adipose tissue (lipoaspirates) that eliminates the need for harsh chemicals and allows for a shorter processing time is presented in this study. This method allowed the preservation of more vital biological components including the extracellular proteins, GAGs, matricellular proteins and growth factors as compared to previously reported decellularization methods. Instead of merely just managing the issue of clinical waste disposal, the conversion of primary waste products into useful secondary products is a better resource management solution. To show proof-of-value of the lipoaspirate waste material as an effective bioactive coating material, the isolated LpECM material was subsequently coated onto TCP. The LpECM coated surfaces improved the cell proliferation rate of various cell types (HaCaT, HUVECs and ASCs) and was shown to be good for wound healing, adipose tissue regeneration as well as for endothelialization with a minimum immune response. Hence, the current study is a stepping stone to the application of adipose tissue derived ECM material as enrichment for clinical applications.

6. ECM-CONTAINING HYBRID SCAFFOLD

6.1 Overview

Since unlike the other sources of tissues, the liposuction process destroys the original ECM structure [163], decellularized lipoaspirate material has been used mainly as a filler material [160] rather than as a scaffold for carrying cells and drugs. In our current study, we explore the use of decellularized lipoaspirate material as an enrichment material. For the first time, we were able to demonstrate the use of lipoaspirate-derived ECM (LpECM) material, obtained using a chemical-free method, as a bioactive surface coating material of both synthetic and natural polymers to enhance cell-material interactions. These polymers were fabricated in various forms (i.e. sheets and microcarriers) to demonstrate the broad range of potential applications for ECM-containing hybrid scaffolds. In addition, preliminary studies involving the use of ECM to modify the bulk properties of another polymer, in this case alginate, were also carried out (refer to Appendix A2).

6.2 Results and Discussion

6.2.1 PCL-ECM

Among the biomaterials used for biomedical applications, polyesters have been the most widely used because of their biodegradability and biocompatibility

[106,179,181]. However, their intrinsic hydrophobicity and poor cytocompatibility have greatly limited their applications in the tissue engineering field [106,179,182]. Therefore, surface modification of polyesters to improve the cell-material interactions is necessary for the polymer to act as an effective cell carrier [179,225,256]. In order to incorporate the isolated LpECM material onto PCL substrates in a well-defined manner, surface-initiated atom transfer radical polymerization (ATRP) was chosen, since this method has been previously used to prepare dense polymer brushes of tuneable length and density containing reactive pendant groups (e.g. -OH, -COOH, or epoxide groups) that provides highly reactive binding sites for functional biomolecules [144,185,257]. The ATRP method was described in more detail in section 3.4.2 of this thesis. Each step of the ATRP coating method was evaluated using water contact angle test, X-ray photoelectron spectroscopy (XPS) analysis, and atomic force microscopy (AFM) analysis. Subsequently, the effect of LpECM modification of PCL on cell attachment, proliferation and viability was investigated using adipose tissue-derived stem cells (ASCs).

In order to use the LpECM material as a coating material to modify PCL substrates, a four-stage reaction process was used (refer to Figure 5) involving: (a) aminolysis treatment for the introduction of amine groups to the PCL substrates, (b) immobilization of an alkyl bromide ATRP initiator via TEA-catalyzed condensation reaction, (c) grafting of P(MAA) brushes via surface-initiated ATRP

of methacrylic acid sodium salt, and (d) direct coupling of rat tail collagen type I or LpECM to the pendant carboxyl groups of the grafted P(MAA) brushes via carbodiimide chemistry. In the current study, rat tail collagen type I was used as a control. The results for the grafting yield, surface composition, and static water contact angles of the pristine PCL and surface-functionalized PCL surfaces are shown in Table 8.

Table 8. Grafting yield, surface composition, and static water contact angles of the pristine PCL and modified PCL surfaces.

Sample	GY ($\mu\text{g}/\text{cm}^2$)	[N]/[C] or [Br]/[C] [*]	WCA (degree)	WCA images
Pristine PCL	—	—	88 $\pm$ 2
PCL-NH ₂ -1 (15 min aminolysis)	—	4.73 $\times 10^{-3}$	78 $\pm$ 4
PCL-NH ₂ -2 (60 min aminolysis)	—	1.96 $\times 10^{-2}$	65 $\pm$ 3
PCL-Br-1 from PCL-NH ₂ -1	—	4.32 $\times 10^{-3}$	79 $\pm$ 2
PCL-Br-2 from PCL-NH ₂ -2	—	1.37 $\times 10^{-2}$	85 $\pm$ 3
PCL-g-P(MAA)1 from PCL-Br-1	3.7 $\pm$ 1.0	0.61 $\times 10^{-3}$	39 $\pm$ 3
PCL-g-P(MAA)2 from PCL-Br-2	9.8 $\pm$ 2.4	2.31 $\times 10^{-3}$	27 $\pm$ 2
PCL-g-P(MAA)1-c-collagen	1.0 $\pm$ 0.5	0.098	49 $\pm$ 4
PCL-g-P(MAA)2-c-collagen	3.8 $\pm$ 0.8	0.212	42 $\pm$ 3
PCL-g-P(MAA)1-c-ECM	1.2 $\pm$ 0.6	0.103	47 $\pm$ 3
PCL-g-P(MAA)2-c-ECM	3.7 $\pm$ 1.3	0.207	41 $\pm$ 4

^{*} Determined by the sensitivity factor-corrected element core-level spectral area ratios. The data shown for collagen/ECM modified groups is [N]/[C].

The successful introduction of amine groups onto the PCL surface was verified by the appearance of N 1s (Figure 21). Different aminolysis times were tested with the aim of demonstrating the tunability of this method. From the XPS result, the surface [N]/[C] ratios, as determined from sensitivity factor-corrected N 1s and C 1s core-level XPS spectral areas, increased from 4.73×10^{-3} for the PCL-NH₂-1 (15 min aminolysis) to 1.96×10^{-2} for the PCL-NH₂-2 surface (60 min aminolysis), suggesting that the amount of free amine groups on the PCL substrates increases with aminolysis time. This was further confirmed by a decrease in water contact angles from $88 \pm 2^\circ$ (pristine PCL surface) to $78 \pm 4^\circ$ (PCL-NH₂-1 surface) and $65 \pm 3^\circ$ (PCL-NH₂-2 surface) (Table 8). These free amine groups serve as reactive sites for further immobilization of the ATRP initiator.

The appearance of Br 3d signals confirmed the successful introduction of bromide-containing ATRP initiator (Figure 21). The water contact angle increased noticeably to $79 \pm 2^\circ$ and $85 \pm 3^\circ$ for the PCL-Br-1 surface and the PCL-Br-2 surface, respectively. Similarly, the surface [Br]/[C] ratios, as determined from the Br 3d and C 1s core-level spectral area ratio, increased from 4.32×10^{-3} for the PCL-Br-1 surface to 1.37×10^{-2} for the PCL-Br-2 surface after increased time of aminolysis treatment.

Figure 21. XPS spectra showing N 1s and Br 3d core-level spectra of the (a) PCL-NH₂-1 (15 min of aminolysis), (b) PCL-Br-1 (from PCL-NH₂-1 surface), (c) PCL-NH₂-2 (60 min of aminolysis), and (d) PCL-Br-2 (from PCL-NH₂-2 surface) surfaces. The appearance of Br signals is an indication of successful immobilization of ATRP initiator onto the PCL substrates.

In this study, the kinetics of the P(MAA) chain growth from different initiator-density surfaces was determined by the grafting yield (GY) obtained (Table 8). Overall, the grafting density of functional P(MAA) brushes was also found to be positively correlated with the density of immobilized ATRP initiator. The GY values of the PCL-g-P(MAA)1 and PCL-g-P(MAA)2 surfaces after 4h of ATRP were found to be 3.7 ± 1.0 and $9.8 \pm 2.4 \mu\text{g}/\text{cm}^2$, respectively. The significant decrease in water contact angles to $39 \pm 3^\circ$ and $27 \pm 2^\circ$, respectively, was attributed

to the large amount of hydrophilic carboxyl groups on the surface-grafted P(MAA) brushes. The C 1s core-level spectra of the PCL-g-P(MAA) surface after 4h ATRP reaction can be curve-fitted into three peak components with binding energies (BEs) at 284.6, 286.2 and 288.7 eV, attributable to C-H, C-O/C-Br and O=C-O, respectively (Figure 22b and 22d). The persistence of the Br 3d core-level signals is consistent with the fact that the “living” chain end from the ATRP process involves a dormant alkyl halide group, which can be reactivated to initiate the subsequent block copolymerization [144,185,257].

Figure 22. XPS spectra showing respective wide scans, C 1s and Br 3d core-level curve-fitted spectra of the (a, b, b') PCL-g-P(MAA)1 (from the PCL-Br-1 surface) and (c, d, d') PCL-g-P(MAA)2 (from the PCL-Br-2 surface) after 4 h of ATRP reaction. Successful ATRP of MAAS can be confirmed from the appearance of three additional peaks of C-H, C-O/C-Br and O=C-O.

As the active carboxyl groups (-COOH) of the P(MAA) brushes act as the spacers to bond with collagen, the quantity of conjugated collagen and LpECM is found to be affected by the amount of grafted P(MAA) on the PCL substrates. The concentration of covalently conjugated collagen and LpECM increased with the amount of carboxyl groups, as verified by the water contact angle and XPS results. The static water contact angles increase to $49 \pm 4^\circ$, $42 \pm 3^\circ$, $47 \pm 3^\circ$ and $41 \pm 4^\circ$ for PCL-g-P(MAA)1-*c*-collagen, PCL-g-P(MAA)2-*c*-collagen, PCL-g-P(MAA)1-*c*-LpECM and PCL-g-P(MAA)2-*c*-LpECM respectively (Table 8). The corresponding curve-fitted C 1s core-level spectra were all composed of five peak components with BEs at about 284.6, 285.5, 286.2, 288.2 and 289.1 eV, attributable to the C-H, C-N, C-O, O=CNH, and O=C-O species (Figure 23a, 23c, 23e and 23g). Moreover, the appearance of a strong N 1s signal with BE at 399.6 eV (Figures 23b, 23d, 23f and 23h), characteristic of amine species, are consistent with the fact that proteins has been covalently immobilized on the P(MAA) brushes. In particular, the [N]/[C] ratios of the PCL-g-P(MAA)1-*c*-collagen, PCL-g-P(MAA)2-*c*-collagen, PCL-g-P(MAA)1-*c*-LpECM and PCL-g-P(MAA)2-*c*-LpECM surfaces were about 0.098, 0.212, 0.103 and 0.207, respectively, which was proportional to the amount of collagen and LpECM attached to the PCL surface. The amount of proteins conjugated was 1.0 ± 0.5 , 3.8 ± 0.8 , 1.2 ± 0.6 and $3.7 \pm 1.3 \mu\text{g}/\text{cm}^2$ for the corresponding functionalized PCL substrates. Hence, the amount of proteins conjugated to the PCL surface is controllable by using ATRP method.

Figure 23. C 1s and N 1s core-level XPS spectra of (a,b) PCL-g-P(MAA)1-collagen, (c,d) PCL-g-P(MAA)2-collagen, (e,f) PCL-g-P(MAA)1-LpECM, and (g,h) PCL-g-P(MAA)2-LpECM surfaces respectively. Successful immobilization of LpECM and collagen can be deduced from the appearance of two additional peak components of C-N and O=CNH.

The surface topography was also characterized by the atomic force microscopy (AFM) to confirm the successful modification of the PCL surface (Figure 24). The noticeable increase in surface roughness by aminolysis was associated with the presence of shallow pits on the aminolyzed PCL surface, which is probably because of the aminolysis reaction taking place even at a depth of around 50 μm

[181,258]. This further increase in roughness was due to the grafting of fibril-like P(MAA) chains on the PCL film surface [259]. In addition, the collagen and LpECM immobilization broadened the P(MAA) domains on the PCL film surface, thus leading to further increase in the surface roughness. The surface roughness is reported to be an important factor that affects the cell adhesion and attachment.

Figure 24. Surface topography determined by 3D AFM images with a scan size of $20\ \mu\text{m} \times 20\ \mu\text{m}$: (a) pristine PCL, (b) PCL-NH₂-2, (c) PCL-g-P(MAA)1, (d) PCL-g-P(MAA)2, (e) PCL-g-P(MAA)1-c-collagen, (f) PCL-g-P(MAA)2-c-collagen, (g) PCL-g-P(MAA)1-c-LpECM and (h) PCL-g-P(MAA)2-c-LpECM surfaces. Surface roughness increased after each step of the modification process.

Following confirmation of the successful modification of the PCL substrate with LpECM material, we next examined whether the LpECM coating was able to modulate the bioactivity of PCL. Using the alamarBlue™ assay and LIVE/DEAD® staining, cell adhesion, proliferation and survival indexes of the cells seeded onto pristine and modified PCL substrates were studied. At 7 days post-culture, it was observed that there were more viable cells on the collagen- and LpECM-modified surfaces (Figure 25b v-viii) when compared to the pristine PCL substrates (Figure 25b i). Negligible dead cells were detected on all the surfaces. Results from the quantitative cellular studies (Figure 25a) showed that LpECM-modified and collagen-modified substrates exhibited significantly enhanced initial attachment after 1 day and increased cell proliferation over time, whilst no significant difference was observed for the aminolysed substrates. In the case for the bare P(MAA) brushes, a reverse effect to that of the LpECM-modified substrates was observed, since the least number of cells attached and subsequently proliferated on the P(MAA)-modified substrates over the 7 day culture period. The poor cytocompatibility of the P(MAA)-grafted surface is most likely due to the anionically-charged carboxyl groups. This observation is in agreement with previous studies carried out that showed that the high mobility of the hydrophilic P(MAA) brushes led to a surface that was resistant to non-specific attachment of cells and proteins [260].

When the results for the LpECM-modified and collagen-modified substrates were

compared, a more significant improvement in cell proliferation was observed for the LpECM-modified substrates, which suggests that LpECM, which is made up of different types of adhesion proteins, as opposed to collagen alone, resulted in more effective improvement of cell attachment and proliferation. In addition to collagen type I, the other types of proteins found in LpECM, such as type IV collagen and laminin, are required for effective ASCs adhesion, growth and proliferation [133], as previously discussed in detail in Chapter 4 and 5 of this thesis.

Overall, the number of cells growing on the substrates increased with increasing amounts of immobilized collagen and LpECM. After 7 days, approximately 150% and 205% more cells were attached to the collagen-modified and LpECM-modified substrates, respectively, as compared to the pristine PCL substrates. Negligible number of dead cells was detected in all surfaces. In addition, the cells grown on the collagen and LpECM-modified substrates retained their elongated morphology as compared to the cells grown on pristine PCL substrates, which were more rounded (Figure 25b).

Figure 25. LpECM-modified PCL substrates showed significantly improved cell proliferation, cell attachment and cell viability as seen from (a) alamarBlue™ cell proliferation assay and (b) LIVE/DEAD® staining after 7 days of *in vitro* culture: (i) pristine PCL; (ii) PCL-NH₂, (iii) PCL-g-P(MAA)1; (iv) PCL-g-P(MAA)2, (v) PCL-g-P(MAA)1-c-collagen, (vi) PCL-g-P(MAA)2-c-collagen, (vii) PCL-g-P(MAA)1-c-LpECM and (viii) PCL-g-P(MAA)2-c-LpECM substrates. The green colour represents the live cells while the red colour represents dead cells (Scale Bar=200 μ m). * p <0.05 and ** p <0.01 refers to statistically significant difference compared with the pristine PCL surface.

Our results support the findings from previous studies that also demonstrated that pristine PCL is unfavorable for cell attachment and growth due to its intrinsic

hydrophobic nature and lack of cell-recognition sites [258,260,261]. The slight improvement in cell growth on the aminolyzed PCL surface is the result of an improvement in surface hydrophilicity and roughness of the PCL substrates (Table 7 and Figure 24) [262]. On the other hand, the poor cytocompatibility of the P(MAA)-grafted surface is most likely due to the anionically-charged carboxyl groups, which have previously been found to be resistant to protein and cell attachment [260,261,263]. Hence, after the conjugation of collagen and LpECM onto the bare P(MAA) brushes, the cell attachment and growth on the PCL substrates significantly improved. As a matter of fact, the cell proliferation rates on the collagen-modified and LpECM-modified substrates were significantly higher than the TCP control, because the presence of collagen probably resulted in enhanced cell attachment and proliferation [20,157]. In addition, a fraction of N-containing groups of collagen (such as amine groups) might be positively charged at physiological pH because of the protonation in culture medium, which would further enhance the interaction between the modified material surface and the negatively charged cells. In the case of the LpECM, the presence of functionally active integrin ligands would also further promote cell adhesion and spreading of ASCs onto the substrate.

6.2.2 OVA-ECM

Microcarriers are commonly used in tissue engineering applications as they provide a large surface area for cell attachment [264,265]. However, the efficacy

of microcarriers as cell carriers is largely dependent on the surface property of the material used. Many types of natural materials have been developed into microcarrier form [266-270]. However, limited research has been done on ovalbumin (OVA), which is a relatively cheap protein found in avian egg white. Hence, in our current study OVA was fabricated into porous microcarriers and the effect of different OVA to alginate ratios on the properties of OVA microcarriers was investigated. Subsequently, in order to further improve cell-material interactions, extracellular matrix (ECM) material isolated from human lipoaspirate material was conjugated onto the porous OVA microcarriers using carbodiimide chemistry.

FTIR results confirmed successful crosslinking of OVA solution to form OVA microcarriers and the coupling of LpECM to OVA microcarriers from the characteristic peaks. Figure 26 shows the FTIR spectra of (a) raw OVA powder, (b) OVA microcarrier and (c) LpECM-modified OVA microcarrier. The strong peak appearing at 3293 cm^{-1} was due to the functional group N-H stretching of amide A and amine within the OVA structure. The shallower band at 2961 cm^{-1} was linked to the asymmetrical stretch of $-\text{CH}_2$ for amide B. Besides amide A and amide B, another strong region was located in the range of $1000\text{-}1700\text{ cm}^{-1}$: strongest peak for amide I at 1649 cm^{-1} , amide II at about 1535 cm^{-1} , and amide III caused two peaks around 1212 cm^{-1} and 1238 cm^{-1} . The strongest band of OVA, amide I, was almost entirely due to amide carboxyl C=O backbone stretching with a little effect

from C-N stretching [241,271]. The combination of the N-H in-plane bend and the C-N stretching vibration made the amide II peak [241]. The peak of amide III peptide bond of OVA was generally responsible for a complex intermolecular interaction of OVA mainly from the C-N stretches and the N-H bending vibrations of peptide groups [272]. The observation of the peak at 1450 cm^{-1} , indicated that the α -helical secondary structure only exists in OVA powder [273]. The main difference between OVA powder and OVA microcarrier was the absence of amide II and amide III peaks and the appearance of two strong peaks at 1408 cm^{-1} and 1029 cm^{-1} . Another minor difference was the decrease of the peak intensity at 3271 cm^{-1} . The BDE crosslinking mechanism in the current study is shown schematically in Figure 27. Due to the reaction of free amine groups ($-\text{NH}_2$) of OVA and epoxide groups at the two ends of BDE, more hydroxyl functional groups and C-N bonds were formed with the further consumption of amine and amide groups. As such, a reduction in the characteristic peak of amide A and the disappearance of amide II was observed. The peak at 1408 cm^{-1} was mainly due to the O-H in-plane bending and the one at 1029 cm^{-1} was due to the C-N stretching and the C-O stretching from alcohol. The loss of amide III might be caused by the breaking down of the secondary structure of OVA during the reaction. With the LpECM-modified microcarriers, the amide III peak re-appeared in the spectrum, which was due to the introduction of the complex mixture of proteins within LpECM. The additional peak at 1704 cm^{-1} was due to the C=O stretching of carboxylic group within the LpECM proteins. The appearance of amide I at a lower

region was also observed. This was because the OVA microcarrier was further crosslinked by EDAC/NHS during the modification step, as described in detail in section 3.4.3 of this thesis.

Figure 26. FTIR spectra of the (a) raw OVA powder, (b) OVA microcarrier and (c) OVA-LpECM hybrid microcarriers. Successful crosslinking and surface modification during the fabrication process was confirmed by the presence of characteristic peaks (red dotted boxes).

Figure 27. Schematic illustration of the BDE-crosslinking mechanism under acidic conditions used in the fabrication of the OVA microcarriers. During this process, the epoxide groups of BDE bond (circled) with the carboxyl groups of the collagen to form carboxylate ester groups.

In general, the mean diameter of OVA microcarriers was $910 \pm 60 \mu\text{m}$ (Figure 28a) and the size distribution indicated that 74% of OVA microcarriers ($n=100$) ranged between 850-950 μm . After modification with LpECM, the average size, and size distribution of microcarriers have no significant differences (Figure 28e and 28i). An interconnected network of random-sized pores could be observed under the SEM (Figure 28b-d for OVA microcarriers and Figure 28f-h for OVA-LpECM microcarriers). The porosity of OVA microcarriers was not able to be measured by using conventional methods such as mercury porosimetry due to their weak mechanical property. Hence, imaging techniques were employed for porosity measurements, which allowed rapid and easy measurement. With the aid of ImageJ software, the pore size range and porosity of scaffolds was determined (Table 9). With an increase in alginate proportion, the porosity of resulting OVA microcarriers increased from 34% for 1:1 (OVA:alginate) weight ratio to 57% for 1:2 (OVA:alginate). After LpECM coupling, the porosity was reduced slightly for all the microcarrier groups. In addition, there was no significant difference of the mean pore size and pore size range of OVA and OVA-LpECM microcarriers with different OVA to alginate ratios.

In general, the porosity of the OVA microcarriers was shown to be tunable by altering the amount of alginate (Table 9), since the alginate acts as a porogen during the inverse moulding process. As seen from the microcarrier fabrication process (refer to Figure 6), alginate acts as a sacrificial material, which is why a

higher amount of alginate would lead to increased porosity in the resulting OVA material. Thus, with the increase in alginate content, the porosity of the microcarriers also increased. As such, 1:2 (OVA:alginate) microcarriers had significantly higher porosity than 2:1 (OVA:alginate) microcarriers. The porosity of the OVA-LpECM microcarriers was slightly reduced after LpECM coupling due to two main reasons. The first reason is that after the modification process, all the surfaces of the internal pore structure were coated with LpECM material, which consequently led to a decrease in the overall pore volume. The other reason, as seen from the decrease in amide A peak after LpECM modification (refer to Figure 26), is due to the strengthening of the microcarriers and reduction in porosity after LpECM modification as the result of the OVA reaction with EDAC/NHS itself during the modification process. In general, the porosity of the microcarriers plays an important role in affecting cell proliferation, migration, and vascularization. Hence, the ability to tune the porosity of the microcarriers, as demonstrated in this study, allows for the development of porous microcarriers for tissue engineering applications.

Figure 28. Morphology of the OVA microcarriers: (a) Image of the pure OVA microcarrier after freeze drying (Scale Bar=300µm), (b-d) SEM images of OVA microcarriers with OVA : alginate ratios 2:1, 1:1 and 1:2 respectively (Scale Bar=300µm), (e) image of the OVA-LpECM microcarrier (Scale Bar=300µm), (f-h) cross-sectional SEM images of OVA-LpECM microcarriers with OVA-alginate ratios 2:1, 1:1 and 1:2 respectively (Scale Bar=50µm), and (i) the size distribution of one hundred OVA and OVA-LpECM microcarriers (n=100). The different amounts of alginate used in the fabrication process results in different pore architectures and porosity, and both OVA and OVA-LpECM microcarriers have interconnected pores.

Table 9. Comparison of the porosities, pore size ranges and mean pore sizes of OVA and OVA-LpECM scaffolds.

Samples	Ratio (OVA : Alginate)	Porosity (%)	Pore size range (μm)	Mean pore size (μm)
OVA	2:1	34 $\pm$ 6	28-163	120 $\pm$ 40
	1:1	42 $\pm$ 5	26-159	120 $\pm$ 40
	1:2	57 $\pm$ 4	29-178	140 $\pm$ 40
OVA-LpECM	2:1	30 $\pm$ 3	23-187	120 $\pm$ 40
	1:1	38 $\pm$ 4	26-176	130 $\pm$ 40
	1:2	49 $\pm$ 2	32-197	140 $\pm$ 40

In addition, compression testing was carried out on the microcarriers until 40% strain was reached. Both OVA and OVA-LpECM microcarriers were observed to recover within a short period of time (i.e. $\sim$ 15s). This suggests that the microcarriers had a “shape memory effect” as they quickly recovered their spherical shapes after mechanical deformation. This property would be useful when injecting the microcarriers to specific sites *in vivo*, since these microcarriers can be compacted into an area that is a small fraction of their original size [274].

With the increase in alginate to OVA ratio, a decrease in compressive moduli was observed for both OVA and OVA-LpECM microcarriers (Figure 29). Also, when compared to the pure OVA microcarriers, the OVA-LpECM microcarriers exhibited significantly improved mechanical strength. The conjugation of LpECM to OVA microcarriers led to improved mechanical strength of the OVA-LpECM microcarriers as compared to uncoated OVA microcarriers since the additional crosslinking took place during the coating process.

Figure 29. Mechanical testing results for the OVA and OVA-LpECM microcarriers of different OVA:alginate ratios under compression. OVA-LpECM microcarriers had significantly better ($*p<0.05$) mechanical strength than pure OVA microcarriers with the same corresponding OVA to alginate ratio. With decreasing OVA to alginate ratio, an increase in compressive moduli was observed for both OVA and OVA-LpECM microcarriers.

In order to investigate the water absorbing capacity of the different groups of microcarriers, the microcarriers were left in saline and their swelling ratios were measured. It was found that with increasing alginate content, the swelling ratio for the microcarriers also increased. In general, the increase in the swelling ratio was probably due to the increase in porosity with increasing alginate content, which enabled more fluid to be absorbed into the microcarrier. However, after the LpECM modification, the swelling ratio was shown to decrease for the two groups with OVA to alginate ratios of 2:1 and 1:1, and increase for the OVA to alginate ratio 1:2 group (Figure 30). This phenomenon could be due to the balance between the

decrease of the porosity after the modification (Table 9), which led to a decrease in water uptake by the microcarriers, and the introduction of more hydrophilic groups from the LpECM material, which led to an increase in water adsorption by the microcarriers. In general, the swelling capability affects the *in vivo* stability of the microcarriers, since it plays an important role in nutrient/metabolite diffusion and transportation, which are important for the maintenance of long-term cell viability. A high swelling ratio will ultimately reduce the stability of the microcarriers during application [270,275], but will facilitate cell seeding onto the microcarriers as greater porosity will be present [275]. Hence, an ideal cell carrier material will have to have the right balance between the two properties.

Figure 30. Graph showing the swelling behavior of OVA and OVA-LpECM microcarriers. Increasing alginate to OVA ratios, led to significantly higher (* $p < 0.05$) swelling ratios for both OVA and OVA-LpECM microcarriers.

The efficacy of OVA and OVA-LpECM microcarriers for supporting the growth and proliferation of ASCs was evaluated. There is no significant difference on cell study results for microcarriers with the different OVA to alginate ratios. Therefore, the cell study results of the microcarriers with OVA to alginate ratio of 1:1 were shown in Figure 31. The initial cell attachment was significantly ($*p<0.05$) increased after the pure OVA microcarrier was modified with LpECM. In addition, after a culture period of 7 days, almost twice the number of cells was observed on the OVA-LpECM microcarriers as compared to the pure OVA microcarriers (Figure 31). The LIVE/DEAD staining of ASCs after 1 day and 7 days culture on OVA and OVA-LpECM microcarriers was shown in Figure 31b-e. OVA-LpECM microcarriers showed much better cell attachment and proliferation. For OVA microcarriers, the attached ASCs appeared to have a more rounded cell morphology, as compared to the ASCs on LpECM modified OVA microcarrier. Hence, our current results showed that the addition of LpECM to the OVA microcarriers significantly improved ($*p<0.05$) the cell-material interactions, resulting in an enhanced cell proliferation profile for the LpECM-containing microcarriers. As LpECM was directly derived from adipose tissue, it retained various biological components from original native microenvironment. Thus, more cells were attached onto the OVA-LpECM microcarriers due to the incorporation of cell adhesion receptors from LpECM onto OVA, and a higher cell proliferation rate was also observed for the LpECM-enriched OVA microcarriers. Therefore, the enrichment of OVA with ECM addresses the lack of cell adhesion

receptors on OVA, which is one of the main challenges when using OVA for tissue engineering applications [276]. In this study, the efficacy of the porous microcarriers is largely dependent on the surface property, rather than the porosity and pore size. Overall, similar to the PCL substrates (section 6.2), ECM enrichment of OVA microcarriers led to improvements in cell-material interactions and facilitated the cell growth and proliferation.

Figure 31. Cellular results for OVA and OVA-LpECM microcarriers, (a) the addition of LpECM material to the OVA microcarriers significantly improved ($*p<0.05$) cellular attachment and proliferation, and (b-e) LIVE/DEAD® staining after 1 day and 7 days of *in vitro* culture on (b,d) OVA microcarriers and (c,e) OVA-LpECM microcarriers respectively

6.3 Summary

In summary, for the first time, the isolated LpECM material was used as enrichment material for both synthetic and natural biomaterials by using chemical conjugation to improve the cell-material interactions. In the case of PCL films, surface modification was carried out by means of chemical conjugation via the highly versatile and controllable ATRP method. Thus, the amount of conjugated LpECM could be readily modulated by varying the grafting yield of P(MAA) brushes. Overall, the LpECM-modified substrates exhibited excellent cell attachment, proliferation and survival rate as compared to the unmodified PCL, as well as the collagen-modified PCL substrates and TCP controls. On the other hand, porous OVA microcarriers were successfully fabricated by an inverse mold-leaching process. In order to improve the bioactivity of the OVA material, ECM material was conjugated onto OVA, thus resulting in the development of novel hybrid microcarriers. LpECM enrichment, using LpECM material that has been physically instead of chemically treated, was able to provide the necessary cell adhesion receptors and other essential biological factors for improving cell-material interactions could be presented. Hence, these bioactivated protein based microcarriers with tunable mechanical properties could potentially be used as injectable fillers for either soft tissue or bone tissue engineering applications.

Taken together, the studies in this chapter demonstrate the novel concept of conjugating LpECM to improve cell-material interactions on both synthetic and

natural materials of different forms. It also shows how a clinical waste material could be transformed into a useful bioactive coating material for tissue engineering and regenerative medicine applications; and is a stepping-stone towards the development of cell-instructive and hence patient-specific scaffolds and implants. Further studies involving the use of ECM material to modify the bulk properties of alginate scaffold is shown in Appendix II.

7. BULLFROG SKIN-DERIVED COLLAGEN SCAFFOLDS

7.1 Overview

Collagen is one of the main extracellular matrix (ECM) components and is an ideal scaffold material due to its low cytotoxicity and immunogenicity [23,24]. Collagen also has good biocompatibility and is able to degrade into physiologically well-tolerated compounds [27,277]. As such, collagen has been widely used for cosmetic and medical purposes such as creams, facial masks, wound dressings, and drug delivery carriers [112,113,278,279]. Currently, many commercially available collagen-based products are extracted from non-human sources such as cows, pigs and sheep [24,280]. Consequently, clinical application of these materials has been limited due to the risks of zoonotic disease transmittance such as foot and mouth disease (FMD), bovine spongiform encephalopathy (BSE) and transmissible spongiform encephalopathy (TSE) in farm animals [27,116]. Hence, an alternative safer source of collagen is highly desirable.

The bullfrog skin can be utilized as a source of collagen because it is a rich source of collagen and poses no threat of BSE, TSE or FMD [27]. Although both pepsin-soluble collagen (PC) and acid-soluble collagen (AC) extracted from bullfrog skin has been partially characterized by Li *et al.* [27], no study has yet to examine bullfrog skin-derived collagen as a scaffolding material for tissue engineering applications. Since studies have shown that some telopeptides are

cleaved or partially denatured when pepsin is used in the PC extraction process [24], AC may have an advantage over PC for use as a scaffolding material for tissue engineering due to the preservation of the crosslinking sites. Therefore, the present study was carried out to explore the possibility of fabricating bullfrog skin-derived AC scaffolds using chemical crosslinking method. Meanwhile, to establish the methodology for the extraction and characterization of AC from bullfrog skin (refer to sections 3.5.1 and 3.5.3) and to characterize AC-based 3D scaffolds (refer to sections 3.5.2 and 3.5.3) for use in tissue engineering applications. Overall, this study illustrates the potential of bullfrog skin, which is currently a waste product in the food industry, as a new source of AC and the development of AC-based scaffolds for tissue engineering applications.

7.2 Results and Discussion

7.2.1 Extraction and characterization of AC from bullfrog skin

Acid-soluble collagen (AC) was successfully extracted from bullfrog skin (Figure 32a) and freeze-dried into a white solid (Figure 32b) that was easily milled into powder. The powder was then dissolved in acetic acid and analyzed by SDS-PAGE (Figure 32c). The pattern of AC (lane II) was observed to be similar to that of rat tail collagen type I (lane III). Two α chains were observed, α_1 with a molecular weight of ~145 kDa and α_2 with a molecular weight of ~ 135 kDa. When the widths of the two bands were compared, the ratio of the α_1 to α_2 chain

of AC was approximately 2, typical for a collagen type I structure. This suggested that the AC from bullfrog skin was most likely to be collagen type I, a triple helix structural protein consisting of two $\alpha 1(I)$ -chains and one $\alpha 2(I)$ -chain [273]. In addition, the β -chain and γ -chain were also clearly observed in the control (lane III). The presence of β -chain and γ -chain is an indication that a high portion of AC was either inter- or intra- molecularly crosslinked [281,282].

Figure 32. The extraction of AC from bullfrog skin: (a) unprocessed bullfrog skin, (b) freeze-dried AC, and (c) SDS-PAGE results of protein ladder (lane I), AC (lane II), rat tail collagen type I control (lane III). AC was successfully extracted from bullfrog skin and freeze-dried into a white solid. SDS-PAGE results of AC showed that it had a similar pattern to that of rat tail collagen type I.

On the other hand, in a previous study on PC extracted from bullfrog skin, only a single $\alpha 1(I)$ chain band was observed with a much lower molecular weight [27]. Similar results were observed for AC and PC extracted from other sources, such as the skin of porcine and bovine [112,113], the scales of spotted golden goatfish [283] and the skin of different species of fish [116,284]. This phenomenon was probably due to the cleavage of the telopeptide region in PC, which results in the denaturation of collagen by the pepsin used in the extraction process, yielding a single $\alpha 1(I)$ chain with a much lower molecular weight.

Table 10 summarizes the amino acid composition of bullfrog skin-derived AC. The results showed that glycine was the most abundant amino acid in AC, accounting for approximately 40% of the total amino acids. In fact, the amount of glycine was much higher than the PC isolated from bullfrog and other sources [27,116,281-284]. The proline content was reported to be related to the thermal stability of collagen [284]. In this case, the second largest group in AC was proline, which accounted for approximately 15% of the total amino acid content. The presence of aspartic and glutamic acid (~12.4%) in the AC would also provide sufficient number of carboxylic acid groups as reaction sites for crosslinking to occur. Overall, the AC from bullfrog skin was comparable to the collagen isolated from other skin sources.

Table 10. Amino acid compositions of the AC from bullfrog skin.

Amino acid	AC (residues/1000)
Aspartic acid	49
Threonine	21
Serine	50
Glutamic acid	75
Glycine	394
Alanine	122
Valine	12
Isoleucine	8
Leucine	21
Phenylalanine	11
Lysine	28
Histidine	6
Arginine	56
Proline	146

7.2.2 Microstructure of AC scaffold

White cylindrical 3D AC scaffolds were fabricated with a diameter of 15.5 mm and a thickness of 5 mm (Figure 33a), and the microstructures of the scaffolds were analyzed by SEM. The cross-sections of the scaffolds (Figure 33b-e) showed that different microstructures were observed when the BDE concentration was varied. As BDE concentration was increased, more sheet-like layers appeared (Figure 33e) and these structures were observed to replace the original fibrous structure (Figure 33b). The freeze-drying method used in the fabrication process led to the formation of random pores in the scaffold, which is a common feature of freeze-dried scaffolds [285]. These pores appeared to be interconnected (Fig 2b-e), which is a necessary feature in order to enable the effective exchange of nutrients and waste to support cell growth and tissue formation [138,286-289]. An

interconnected porous architecture was observed in each SEM image of AC scaffolds (Figure 33b-e), where small pores were observed within the big pores of scaffolds. In addition, the results in Table 11 showed that increasing BDE concentration led to a narrower mean pore size for the AC scaffolds and an overall decrease in porosity.

As a comparison, PC-derived from the bullfrog skin was crosslinked using BDE and fabricated into scaffolds as well. However, unlike the AC scaffolds, the PC scaffolds disintegrated within half an hour when soaked in DI water due to the weak structure of the scaffold. This could be attributed to the removal of the telopeptides during the pepsin digestion stage, which led to the removal of the necessary crosslinking sites required for interaction with the BDE crosslinker. As such, no further studies were carried out using the unstable PC scaffolds.

Figure 33. Morphology of AC scaffolds: (a) image of the white color AC 3D scaffold (Scale Bar=5mm) and SEM images (Scale Bar=100 μ m) of the cross-sections of (b) untreated, (c) 0.125% BDE-crosslinked, (d) 0.25% BDE-crosslinked and (e) 0.5% BDE-crosslinked AC scaffolds. AC scaffolds were porous with interconnected pores. Both the pore size and the porosity of the scaffolds decreased with increasing BDE crosslinking concentration.

7.2.3 Confirmation of crosslinking

Figure 34 shows the FTIR spectra of untreated AC scaffold and BDE-crosslinked AC scaffold. The FTIR spectra of AC from bullfrog collagen appear to be relatively similar to the AC and PC extracted from other sources including scales and skins of different species of fish [27,28,116,281-284,290-291]. The strong IR absorption band centered at approximately 3315 cm^{-1} can be attributed to amide A, which is associated with the N-H stretching vibration involved in hydrogen bonding. The shallower bands at 3074 cm^{-1} can be attributed to amide B, which is represented by the asymmetrical stretching of $-\text{CH}_2$. The strongest signal observed at 1648 cm^{-1} belonged to amide I, which is mainly associated with backbone $\text{C}=\text{O}$ stretching. An amide II signal was observed at 1547 cm^{-1} , which represents the combination of the N-H in-plane bending and the C-N stretching vibration. Weaker amide III bands were also observed in the region of 1237 cm^{-1} , which is generally responsible for complex intermolecular interactions of collagen [290,291]. The presence of the peak at 1450 cm^{-1} indicates the presence of the triple-helical structure of collagen in the AC scaffold [292]. The relatively higher peak intensity of amide III to the peak at 1450 cm^{-1} suggests that a high proportion of triple helix structure was present in the AC structure.

After crosslinking with BDE, several smaller peaks were observed at approximately 756 cm^{-1} , 854 cm^{-1} , 909 cm^{-1} and 940 cm^{-1} for the BDE cross-linked AC scaffolds. The intensities of these peaks amplified as BDE concentration

increased due to the enhanced of -CH bending due to the addition of BDE molecules. A very strong peak with a similar trend appeared at the 1098 cm^{-1} region that was caused by C-O-C stretching between the carboxyl groups as they reacted with the BDE cross-linker. One mechanism by which BDE crosslinks collagen in acidic solution is presented in Figure 35, where the two epoxy groups of BDE react with the carboxyl groups on AC molecules forming carboxylate esters. A narrow shift of all the peaks (amide I, II, III, A, B) to the left could be caused by a decrease in all of the secondary structure elements induced by the decrease in hydrogen bonding during the crosslinking reaction [292].

Figure 34. FTIR spectra of untreated and BDE-crosslinked AC scaffolds illustrating the effect of changing BDE crosslinking concentrations on the characteristic AC peak changes.

Figure 35. Schematic illustration of the BDE crosslinking mechanism under acidic conditions, where the epoxide groups of BDE bond with the carboxyl groups of the collagen to form carboxylate esters.

7.2.4 Material properties of AC scaffolds

Three dimensional (3D) scaffolds are typically porous and are made of biocompatible materials that provide suitable microenvironments (i.e., mechanical support, physical, and biochemical stimuli) to support cellular growth and functions for tissue engineering applications [285]. Therefore, the material properties of the AC scaffolds are important as they are capable of directing cellular behavior during biomedical application [24,112,113,278,279]. Table 11 summarizes the main properties of AC scaffolds. Overall, as the BDE crosslinking concentration increased, the scaffold structure became denser, as the porosity decreased when compared to the untreated AC (i.e., uncrosslinked). Hence, significant differences in mechanical properties between the untreated and

BDE-crosslinked AC scaffolds were observed. The compressive modulus of the scaffolds was shown to be 71%, 90% and 134% higher after crosslinking with 0.125%, 0.25% and 0.5% BDE, respectively. In addition to an improvement in compressive moduli, BDE-crosslinked scaffolds were also able to return to their initial shapes and sizes after a short period of time following physical compression, while the untreated AC scaffolds remained deformed. This suggests that the use of BDE crosslinker led to the elastic response of the AC scaffolds to the compression and therefore able to return to the original shape.

Thermal analyses of the scaffolds showed that raising the concentration of crosslinker increased the denaturation temperature (T_n) but decreased the decomposition temperature (T_d). Overall, the initial T_n of AC was much higher than reported for PC from bullfrog [27], which may be due to the large portion of triple helical structures remaining in the AC, which were absent from PC. As expected, the T_n increased after BDE crosslinking, which is similar to the results for other examples of collagen crosslinking [293,294]. The increase in T_n is due to the reaction of the carboxyl group of collagen with the crosslinker, yielding a stable ester (refer to Figure 35), thus leading to an improvement in thermal stability. As for the decrease in T_d with increasing crosslinking concentration, this could be due to the damage of the secondary structure of collagen during the crosslinking process, which led to the breakdown of the hydrogen bonds within the collagen structure. Results for thermal analysis of AC correspond with the FTIR results showing

narrow shifts for all the peaks of amide I, II, III, A, and B (refer to Figure 34).

In addition, the water binding capacity of the scaffolds is an important factor for tissue engineering applications, as it indicates better cell invasion and nutrient supply [295]. The addition of crosslinking agent greatly decreased the swelling ratio of the scaffolds from 39.28% for untreated AC scaffolds to 0.125% for BDE-crosslinked scaffolds, and up to 51.39% for 0.5% BDE-crosslinked scaffolds compared to the untreated AC scaffold. The crosslinking treatment is known to reduce the number of hydrophilic groups, in this case carboxylic groups (Figure 35), thus making the scaffolds more hydrophobic [296].

Table 11. Material properties of untreated, 0.125%, 0.25% and 0.5% BDE-crosslinked AC scaffolds.

Property	Untreated AC	0.125 BDE	0.25 BDE	0.5 BDE
Pore size range (μm)	20 – 500	15 – 450	20 – 260	20 – 140
Mean pore size (μm)	310 ± 70	250 ± 40	160 ± 20	97 ± 6
Porosity (%)	93 ± 6	79 ± 7	69 ± 4	62 ± 4
Denaturation Temperature (°C)	42 ± 1	46 ± 2	47 ± 3	60 ± 2
Decomposition Temperature (°C)	279 ± 3	262 ± 1	260 ± 1	252 ± 1
Compressive modulus (kPa)	1.2 ± 0.1	2.1 ± 0.1	2.3 ± 0.1	2.8 ± 0.1
Swelling ratio (%)	120 ± 10	71 ± 4	64 ± 10	57 ± 1

7.2.5 *In vitro* biodegradation properties

The degradation behavior of the AC scaffolds was studied by exposing the scaffolds to collagenase solution for 2 h at 37 °C. As shown in Figure 36a, 92.64% of the pure AC degraded within that 2h period, while the use of the BDE crosslinker led to a significant improvement in degradation stability. The significant difference in the size of the AC scaffolds following degradation can be clearly seen in Figure 36b. In general, the crosslinking process prevented the AC from being digested by collagenase due to the protection of the cleavage sites, which were mainly located at the carboxyl-telopeptides terminal of collagen (refer to Figure 35). Enzymatic degradation of AC is related to the helical integrity, degree of crosslinking as well as the availability of cleavage sites [297]. Thus, crosslinking enhanced the stability of AC against enzymatic degradation as the formation of crosslinks with BDE protected the collagen's carboxyl groups, which would have been the original sites of enzymatic cleavage.

Figure 36. The stability of AC scaffolds in the presence of collagenase: (a) percentage degradation of untreated and BDE-crosslinked AC scaffolds, and (b) comparison of the differences in scaffold morphologies before and after the degradation study (Scale Bar=5mm). BDE-crosslinked AC scaffolds exhibited significantly greater ($*p<0.05$) stability against degradation than untreated AC scaffolds. In addition, the AC scaffold was significantly smaller after the degradation test, while the BDE-crosslinked AC scaffolds only appeared slightly smaller.

7.2.6 Cell proliferation

The efficacy of the untreated and BDE-crosslinked scaffolds in supporting the proliferation of ASCs was investigated as an initial step towards the exploration of the use of bullfrog skin-derived collagen scaffolds for tissue engineering

applications. Compared to untreated AC scaffolds, crosslinking of scaffolds with BDE resulted in reduced cell numbers at each time point (Figure 37). In addition, a lower initial cell attachment was observed for BDE-crosslinked scaffolds, which is mainly due to the increase in surface hydrophobicity as compared to the untreated AC scaffold [296]. Hence, a decrease in retention of the volume of cell suspension within the scaffolds was observed during the seeding process. Over a period of 7 days, the cell number was observed to increase for all types of scaffolds, but the greatest increase was observed for the untreated AC scaffolds. This may be due to the presence of higher porosity and greater mean pore size of untreated AC scaffolds (Table 11), which enhanced the cell proliferation rate of ASCs [285]. The combinatorial effect of the higher porosity, combined with increased mean pore size may have facilitated more efficient exchange of essential nutrients, oxygen and waste products for the untreated AC scaffolds [285,298]. Thus, depending on the application, the choice of using uncrosslinked AC scaffold or BDE-crosslinked AC scaffold has to be considered, as the improvement in mechanical strength and biodegradation properties of the BDE-crosslinked AC scaffolds is offset by the lower efficacy for supporting cell proliferation. Even though studies have been carried out showing BDE is biocompatible, future studies will be carried out to investigate the toxicity and biocompatibility of using BDE crosslinker.

Figure 37. Cell proliferation results of the untreated and BDE-crosslinked AC scaffolds over a period of 7 days. Untreated AC scaffolds supported significantly greater ($*p<0.05$) cell proliferation compared to the BDE-crosslinked AC scaffolds. The greater porosity and larger pore size of untreated AC scaffolds facilitated the cell growth and migration of ASCs. The initial lower cell attachment of BDE-crosslinked scaffolds compared to the untreated AC scaffold was mainly due to the greater hydrophobicity of the crosslinked scaffolds.

7.2.7 *In vivo* study

To study the biocompatibility and stability of the AC scaffolds *in vivo*, the AC scaffolds were subcutaneously implanted into mice for 2 weeks. After 2 weeks, all samples were harvested with the surrounding tissue and skin. H&E staining of the AC scaffolds and the surrounding tissues (Figure 38) showed the absence of macrophages, suggesting good biocompatibility of the AC scaffolds and limited inflammatory responses at the 2-week time point. The AC scaffolds were encapsulated by fibrotic collagen in all cases, but no significant difference was observed in the thickness of these fibrous encapsulation layers. However, more

cells were observed to migrate into the untreated AC scaffolds than BDE-crosslinked AC scaffolds (Figure 38), as seen from the presence of nucleus within AC scaffolds, which also correspond to the results observed from the *in vitro* study (refer to Figure 37). As the BDE concentration increases, fewer cells were found inside the scaffolds. However, similar to the *in vitro* degradation study (refer to Figure 36), the stability of the AC scaffolds *in vivo* was improved by BDE crosslinking; since the majority of the AC scaffold was degraded and absorbed by the body (Figure 38a), while the BDE-crosslinked AC scaffolds (Figure 38b-d) were still present at the end of the *in vivo* study.

Figure 38. H&E staining of the implanted AC scaffolds after 2 weeks: (a) untreated, (b) 0.125% BDE-crosslinked, (c) 0.25% BDE-crosslinked and (d) 0.5% BDE-crosslinked AC scaffolds. All implants exhibited good biocompatibility without any observations of macrophages. The red arrows indicate the direction of the host cells infiltration into scaffolds. Scale Bar=200 μ m.

7.3 Summary

Currently, the main sources of collagen for clinical applications have been bovine and porcine sources. However, the outbreak of diseases such as bovine spongiform encephalopathy (BSE) and foot-and-mouth disease (FMD) have resulted in anxiety among users of collagen and collagen-derived products from these animals [23-29]. These diseases, which became abundant in some countries during the mid-1980s, are increasingly discussed as potentially transmittable to humans [29]. Bullfrog is an amphibian that does not present a threat of prion diseases [27, 29] and can therefore represent a safer source of collagen. AC was successfully extracted from bullfrog skin, which is currently treated as a waste product of the food processing industry in Asia. The waste-to-resource biomaterial was characterized and successfully fabricated into 3D scaffolds for tissue engineering applications. From the results, AC from bullfrog skin was found to be similar to that of rat tail collagen type I, which consisted of $\alpha 1(I)$ -, $\alpha 2(I)$ -, β - and γ -chains. Therefore, the AC isolated from bullfrog skin is alike the collagen from other species. Conversely, a previous study showed that pepsin treatment used in the extraction of PC from bullfrog skin resulted in the removal of non-helical terminals (C- and N- telopeptides), which resulted in greater denaturation whilst hindering subsequent chemical crosslinking. In this study, the effect of crosslinking on scaffold properties was evaluated by further crosslinking AC with BDE. In comparison to the uncrosslinked AC scaffolds, BDE-crosslinked AC scaffolds had different morphologies and material. The BDE crosslinker resulted

in the decrease of mean pore size, porosity and swelling. The denaturation temperatures and compressive moduli of AC scaffolds were observed to increase with increasing BDE concentrations. Meanwhile, cell culture studies showed that the uncrosslinked AC scaffolds were better in supporting the cell proliferation of ASCs than the BDE-crosslinked AC scaffolds. However, in terms of degradation stability, the BDE-crosslinked AC scaffolds were superior to uncrosslinked AC scaffolds for both *in vitro* and *in vivo*, where the stability was enhanced with increasing of BDE concentrations. Therefore, it is necessary to find a balance between the superior material strength and stability of BDE-crosslinked AC scaffolds and the better efficacy of the untreated AC scaffolds for cell proliferation. In an ideal situation, the degradation of AC scaffolds should match the tissue, whilst the physical support from AC scaffolds remained until the engineered tissue was strong enough to support itself. In general, all AC scaffolds showed good biocompatibility in the two-week *in vivo* implantation study. Overall, the study showed the feasibility of using bullfrog skin-derived AC as an alternative source for the fabrication of tunable collagen scaffolds. The material properties and cell proliferation capabilities of the AC scaffolds can be further tuned using different concentrations of crosslinker to suit various tissue engineering applications.

8. CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

8.1 Conclusions

The current strategy of fabricating scaffolds from natural materials containing biofactors is a promising approach for tissue or organ repair. In general, cells are incorporated into these bioactive scaffolds and implanted in the body to promote tissue repair and the formation of new functional tissues. In this thesis, two novel natural materials, namely adipose tissue-derived ECM and bullfrog skin-derived collagen, were explored.

Adipose tissue-derived ECM is a promising candidate material for soft tissue engineering as it contains bioactive factors necessary for cell-material interactions, wound healing and vascularization. Two new decellularization methods were established during this thesis for the isolation of ECM material from adipose tissue without the need of any harsh chemicals. The ECM material isolated using these two methods were able to preserve more biological components including growth factors, matricellular proteins and structural proteins, as compared to previously established decellularization methods. *In vitro* and *in vivo* (refer to Appendix I) tests showed that the ECM materials exhibited good biocompatibility and involved minimal immune reaction. Moreover, these ECM materials were successfully used as a bioactive coating material to enrich other biomaterials. The

ECM-modified PCL and OVA materials, showed improved cell-material interactions including better cell attachment and proliferation profiles. In addition, the detection of matricellular proteins extends the application of adipose tissue derived ECM material beyond adipose tissue regeneration. *In vitro* studies showed that ECM-coated TCP improved the keratinocytes proliferation and migration, and also enhanced the proliferation rate of endothelial cells. These observations suggest that the ECM material is potentially capable of improving wound healing and vascularization.

Bullfrog skin-derived AC is an alternative source of collagen which can be used for the fabrication of tunable AC scaffolds for soft tissue engineering purposes. The material properties and cell proliferation capabilities of the AC scaffolds can be further tuned using different concentrations of crosslinker to suit various tissue engineering applications. Both *in vitro* and *in vivo* degradation rates were slower with increasing crosslinker concentration.

Taken together, the waste-to-resource strategies can be applied to obtain materials that are suitable for biomedical applications, since adipose tissue-derived ECM material and bullfrog skin-derived AC were demonstrated to be promising materials for soft tissue engineering applications. From our understanding of the composition and structure of the adipose tissue, adipose tissue-derived ECM material should be used as surface enrichment material to confer the bioactivity

rather than as a main scaffolding material because of the high lipid content within the tissue. On the other hand, AC can be used as the main scaffolding material since collagen is the main structural protein within the tissue. Overall, taking inspiration from the nature adipose tissue itself, the development of ECM-containing hybrid scaffold as an acellular scaffold with cellular benefits is a great stepping-stone towards the development of engineered adipose tissue replacement for clinical therapy. Preliminary studies involving bulk modification of alginate with ECM are shown in Appendix II.

8.2 Summary of Main Findings

This thesis mainly focuses on the development of tissue-inspired scaffolds, and the overall main contributions towards the soft tissue engineering field are as follows:

1. Developed two novel decellularization methods that do not require the use of any harsh chemicals. The resulting ECM material was thoroughly characterized, and matricellular proteins were detected in the resulting ECM material for the first time.
2. Compared pure enzymatic, chemical and physical decellularization methods in terms of their effectiveness of decellularization and ability to conserve specific ECM components. Different processing methods used to obtain the adipose tissue-derived ECM material can result in various structures that can

be used for different biomedical applications. ECM-P was recommended as a bioactive coating material, whilst ECM-E and ECM-C could be used as scaffolds for adipose tissue engineering.

3. Successfully applied adipose tissue-derived ECM material as a novel enrichment material to confer the bioactivity. Both *in vitro* and *in vivo* biocompatibility tests demonstrated minimal immune reaction from the ECM material.
4. Successfully demonstrated a waste-to-resource strategy for isolating AC from bullfrog skin. Tunable AC scaffold was fabricated using different concentrations of chemical crosslinker. It was also demonstrated to have slower degradation rates with increasing crosslinker concentration both *in vitro* and *in vivo*. Overall, bullfrog skin-derived collagen can be used as an alternative source of scaffolding material for tissue engineering applications.

8.3 Recommendations for Future Work

8.3.1 Future work on ECM material

It was proven in Chapters 4 and 5 that the pure physical treatments were able to decellularize adipose tissue. The most distinct advantage of using these two novel physical methods as compared to other methods was preserving more biological components within the resulting ECM material. It will be of great interest to investigate the complete list of the biological components as well as the

composition of each component.

The novel application of adipose-tissue derived ECM material as an enrichment material was shown in Chapters 5 and 6. Different strategies, including direct coating or chemical modification, were carried out to incorporate the ECM material onto both synthetic and natural materials. Some studies showed that the chemical crosslinking could affect the biological properties of ECM material and thus may evoke an adverse immune response or impair recellularization [299]. Therefore, further investigation on the effect of different methods of incorporation of ECM material will be necessary for tissue engineering and biomedical applications.

The biocompatibility of ECM coating and effect of the coating on different types of cells (HaCaT cells, HUVECs and ASCs) were analyzed *in vitro* (Refer to Chapter 5). The improvement of ASC attachment rates and proliferation profiles on ECM-containing hybrid scaffolds were also demonstrated *in vitro* (Refer to Chapter 6). More comprehensive studies *in vivo* should be conducted in order to demonstrate the efficacy of the ECM coating material for adipogenesis, revascularization and wound healing. Preliminary *in vivo* biocompatibility studies for the ECM material itself are shown in Appendix I.

Taken together, some recommended future studies are listed as follows:

1. To investigate the complete list of the biological components as well as their composition present in the decellularized ECM material.
2. To test the ECM-containing hybrid scaffold for biocompatibility, adipose tissue regeneration, revascularization and wound healing *in vivo*.
3. To investigate the best way of incorporating the ECM material into other scaffolds, such as physical absorption, chemical crosslinking and physical crosslinking, whilst maintaining the properties of ECM material (Figure 39).

Figure 39. Schematic view of the different possible methods for incorporation of ECM: (a) chemical crosslinking (b) physical crosslinking and (c) direct coating.

8.3.2 Future work on bullfrog skin-derived AC

Bullfrog is an amphibian that does not present a threat of prion diseases [28] and can therefore represent a safer source of collagen. The bullfrog skin-derived AC was successfully fabricated into the scaffold and the degradation profile can be altered by using chemical crosslinking. Both *in vitro* and *in vivo* studies proved the possibility of using bullfrog skin-derived AC as a biomaterial for tissue engineering applications. However, the *in vitro* results did show the adverse effect on ASCs attachment and proliferation by using chemical crosslinking. Thus, it will be necessary to analyze the toxicity effects of using different types as well as concentrations of chemical crosslinkers.

It has been reported that the bullfrog skin-derived collagen has antioxidant properties. Therefore, it will be interesting to measure the antioxidant property of AC scaffold [29]. Most of the commercially available collagen products are for wound healing purposes [24,120]. The only *in vitro* cell study carried out in this thesis for AC scaffold was using ASCs. Future studies can be carried out by evaluating AC-based scaffolds for wound healing and adipose tissue regeneration both *in vitro* and *in vivo*. Moreover, taking advantages from both the bullfrog skin-derived AC and adipose tissue-derived ECM, a hybrid ECM-containing AC scaffold can be developed for adipose tissue regeneration and wound healing applications.

Taken together, some recommended future studies are listed as follows:

1. To investigate the toxicity effects of different types and concentrations of chemical crosslinkers.
2. To explore the use of AC for wound healing applications, including the measurement of the antioxidant property and fabrication of AC wound dressing using pure physical crosslinking methods are proposed.
3. To carry out both *in vitro* and *in vivo* studies of AC-based scaffolds for wound healing and adipose tissue regeneration.
4. To develop ECM-containing hybrid AC scaffolds for clinical usage.

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LIST OF PUBLICATIONS

Journal publications as first author

1. **Luo, B.**, Yuan, S., Foo, S.E.M., Wong, M.T.C., Lim, T.C., Tan, N.S., Choong, C. (2014) From Flab to Fab: Transforming Surgical Waste into an Effective Bioactive Coating Material. *Advanced Healthcare Materials* DOI: 10.1002/adhm.201400514 (*I.F.*=4.88)
2. **Luo, B.**, Loh, Q.L., Wong, M.T.C., Tan, N.S., Choong, C. (2014) Bioactivated Protein-Based Porous Microcarriers for Tissue Engineering Applications. *Journal of Materials Chemistry B* 2014(2): 7795-7803
3. **Luo, B.**, Choong, C. (2014) Porous Ovalbumin Scaffolds with Tunable Properties: A Resource-Efficient Biodegradable Material for Tissue Engineering Applications. *Journal of Biomaterials Applications* 2015 29(6):903-911. (*I.F.*=2.640)
4. He, F*., **Luo, B.***, Yuan, S., Liang, B., Choong, C. and Pehkonen, S.O. (2014) PVDF Film Tethered with RGD-click-Poly(Glycidyl Methacrylate) Brushes by Combination of Direct Surface-Initiated ATRP and Click Chemistry for Improved Cytocompatibility. *RSC Advances* 2014(4):105-117 (*equal contribution, *I.F.*=2.562)

Publications as co-author

1. Chong H.C. , Chan J.S.K. , Goh C.Q. , Gounko N.V., **Luo B.** , Wang X. , Foo S. , Wong M.T.C. , Choong C. , Kersten S. and Tan N.S., Angiopoietin-like 4 stimulates STAT3-mediated iNOS expression and enhances angiogenesis to accelerate wound healing in diabetic mice. *Molecular Therapy* DOI: 10.1038/mt.2014.102 (*I.F.*=7.041)
2. Tan, C.K., Tan, E.H., **Luo, B.**, Huang, C.L., Loo, J.S., Choong, C. and Tan, N.S. (2013) SMAD3 Deficiency Promotes Inflammatory Aortic Aneurysms in Angiotensin II-Infused Mice Via Activation of iNOS. *J Am Heart Assoc.* 2(3):e000269.
3. Choong, C., Foord, J.S., Griffiths, J-P., Parker, E.M., **Luo, B.**, Bora, M. and Moloney M.G. (2012) Post-polymerisation Modification of Surface Chemical Functionality and Its Effect on Protein Binding. *New Journal of Chemistry* 36(5): 1187-1200. (*I.F.*=2.562)

In preparation

1. **Luo, B.**, Loh, Q.L., Wang, J.K., Chong, C.C., Foo, S., Tan, N.S., Chong, C. (2014) Bullfrog Skin-Derived Collagen Scaffolds for Tissue Engineering Applications. To be submitted to *Biomacromolecules*. (*I.F.*=5.788)
2. **Luo, B.**, Foo, S., Wong, M.T.C., Lim, T.C., Tan, N.S., Choong, C. (2014) Effect of Processing Conditions on The Properties of Adipose Tissue-Derived Extracellular Matrix Material. To be submitted to *Regenerative Medicine* (*I.F.*=3.873)

3. **Luo, B.**, Loh, Q.L., Li, L., Foo, S., Dai, Y., Wong, M.T.C., Tan, T.T.Y., Tan, N.S., Choong, C. (2014) The Exploration of Using Decellularized Adipose Tissue as A Bioactive Coating: The Advantage of Using Supercritical Carbon Dioxide. To be submitted to *ACS Applied Materials & Interfaces* (*I.F.*=5.900)

Conference presentations

1. **Luo, B.**, Wang, J.K., Chong, C.C., Tan, N.S., Choong, C. (2014) Bullfrog Skin-derived Acid-soluble Collagen: A Promising Biomaterial for Tissue Engineering Applications. 2014 MRS Spring Meeting & Exhibit. San Francisco, USA, 21st-25th April, 2014.
2. **Luo, B.**, Wong, T.C., Tan, T.T.Y., Tan, N.S., Choong, C. (2014) Decellularized Adipose Tissue-Derived Extracellular Matrix as a Bioactive Coating Material. 2014 Annual Meeting of the Society For Biomaterials. Denver, USA, 16th-19th April, 2014.
3. Shanbhag S.S., **Luo, B.**, Foo, S., Tan, N.S., Wong, T.C., Choong, C. (2014) Comparative study of different types of decellularization methods for adipose tissue. 2014 Annual Meeting of the Society For Biomaterials. Denver, USA, 16th-19th April, 2014.
4. **Luo, B.**, Loh, Q.L., Tan, N.S., Wong, T.C., Choong, C. (2013) Extracellular Matrix-Based Biohybrid Skin Substitutes. Singapore Health and Biomedical Congress 2013 (SHBC 2013). Singapore, 27th-28th September, 2013.
5. **Luo, B.**, Loh, Q.L., Tan, N.S., Wong, T.C., Choong, C. (2013) Development of ECM-based Biohybrid Skin Substitutes. International Investigative Dermatology (IID). Edinburgh, Scotland, 8th-11th May, 2013.
6. **Luo, B.**, Lim, T.C., and Choong, C. (2013) Development of Tissue-inspired Scaffolds: The Use of Adipose Tissue-Derived Extracellular Matrix Material for Improving Cell-Material Interactions. Third International Conference on Multifunctional, Hybrid and Nanomaterials (Hybrid Materials 2013). Sorrento, Italy, 3rd-7th March, 2013.
7. **Luo, B.**, Lim, T.C. and Choong, C. (2012) The use of adipose tissue-derived extracellular matrix material for improving cell-material interactions: implications for host integration & tissue regeneration. The Third World Congress for Plastic Surgeons of Chinese Descent. Xian, China, October 12th-14th, 2012.
8. **Luo, B.**, Yuan S., Lim, T.C. and Choong, C. (2012) Biofunctionalization of polycaprolactone substrates using adipose tissue-derived extracellular matrix material. Tissue Engineering & Regenerative Medicine International Society (TERMIS) World Congress. Vienna, Austria, 5th-8th September.
9. **Luo, B.**, Lim, T.C. and Choong, C. (2012) 2012 The use of adipose tissue-derived extracellular matrix material for improving cell-material

interactions: implications for host integration & tissue regeneration. International Symposium of Materials on Regenerative Medicine (2012 ISOMRM) Taipei, 29th-31st August, 2012. The Young Investigator Award (Best Oral Presentation)

10. **Luo, B.**, Chen W. and Choong, C. (2012) Optimization of 3D porous ovalbumin (OVA) scaffolds for tissue engineering applications: a study of the effect of different cross-linkers on scaffold properties. International Conference of Young Researchers on Advanced Materials (ICYRAM). Singapore, 1st-6th July.
11. Loh, Q.L., **Luo, B.**, Dong, Y. and Choong, C. (2012) Development of a novel fabrication system for 3D alginate scaffolds. ICYRAM. Singapore, 1st – 6th July.
12. **Luo, B.**, Chen W. and Choong, C. (2012) The Effect of Different Crosslinkers on the Properties of Ovalbumin-Based Porous Scaffolds. 9th World Biomaterials Congress. Chengdu, China, 1st - 5th June.
13. Wong JM, Luo, B., Lim T.C. and Choong, C. (2012) Adipose tissue: from clinical waste to biologically useful material. 1st Congress of the International Society of Plastic Regenerative Surgery (ISPRES). Rome, Italy, 9th-11th March.
14. **Luo, B.**, Xue, B. and Choong, C. (2011) The Effect of Fabrication Method and Cross-linker Concentration on Ovalbumin Scaffolds. Tissue Engineering & Regenerative Medicine International Society (TERMIS) Asia Pacific Meeting. Singapore, 3rd-5th August.

List of submitted technology disclosures

- a) Choong, C., **Luo, B.**, December 2013 “Adipose tissue-derived extracellular matrix material as a bioactive coating material for biomedical applications”, NIEO Ref: TD/317/13
- b) Choong, C., **Luo, B.**, August 2013, “Bullfrog skin-derived acid-soluble collagen”, NIEO Ref: TD/212/13
- c) Choong, C., **Luo B.**, Lim T. C., March 2012, “Adipose tissue-derived extracellular matrix material as a bioactive coating material for biomedical applications: From Clinical Waste to Useful Resource”, NIEO Ref: TD/050/12
- d) Choong, C., **Luo, B.**, December 2011, “A novel method of fabricating ovalbumin (OVA) microcarriers as minimally invasive cell delivery vehicles: Applications to soft tissue engineering”, NIEO Ref: TD/193/11

Appendix

Appendix I ECM *In Vivo* Studies (Murine Model)

Using the physical decellularization method (refer to section 3.2.1.1), we obtained decellularized ECM from white adipose tissue (WAT) of obese mice, which were later implanted subcutaneously into lean wildtype C57B16 mice (Figure A1). This study was approved and performed in compliance with the regulations of the Institutional Animal Care and Use Committee of Nanyang Technological University (ARF SBS/NIE-A0174 AZ). At both 7 days and 14 days time points post-implantation, histology results showed resorption of implants, endothelial cell infiltration into the implant space and no adverse immunological response. The findings from the *in vivo* studies not only showed host integration but also provides further evidence of the effectiveness of the decellularization method since the deleterious *in vivo* effects often observed as the results of residual autologous cellular material was not observed. Taken together, the decellularized material obtained by our method could potentially be used in allogeneic transfers as well.

Figure A1. Histology images (a) 7 days and (b) 14 days after subcutaneous implantation of decellularized white adipose tissue (WAT) from obese mice into lean wildtype C57B16 mice (i) H&E staining of explants showed gradual resorption of implants with (ii) endothelial cell infiltration (CD31 staining) and (iii) no evidence of adverse inflammatory response (F4/80 staining)

Appendix II Preliminary Study of Alginate-ECM

Alginates are natural polysaccharides, consisting of (1→4) linked β -D-mannuronic acid (M) and α -L-guluronic acid (G) monomers, that are derived from brown seaweeds. In general, they have good biocompatibility and biodegradability properties that are suitable for tissue engineering applications. As a biomaterial, alginate can be easily made into various forms, such as soft gels, fibers, foams and scaffolds. Furthermore, it can be tailored by cross-linking with divalent ions in physiological conditions which allows the preservation of cell function and viability. Generally, Ca^{2+} ions are widely used to crosslink the alginate for tissue engineering applications due to their low toxicity. However, the alginate is generally not considered as bioactive due to the highly negative charge surface and the hydrophilicity, which prevent the protein adsorption, cell attachment and proliferation. Thus in order to improve the cell-material interactions of alginate, extracellular matrix (ECM) material isolated from human lipoaspirate material was conjugated to the alginate monomers via carbodiimide chemistry (Figure A2), to form a biohybrid material that can be used for scaffold fabrication.

Figure A2. Schematic illustration of the carbodiimide chemical reaction involved in the formation of the hybrid (Alginate-c-ECM) material.

FTIR results confirmed successful coupling of ECM material to alginate from the characteristic peaks. Figure A3 shows the FTIR spectra for alginate and alginate-ECM scaffolds, where the additional peaks highlighted at 1704 cm^{-1} and 1238 cm^{-1} (red dotted boxes) for alginate-ECM indicate successful coupling of ECM. The additional peak at 1704 cm^{-1} was due to the C=O stretching of carboxylic group within the ECM proteins. The peak at 1238 cm^{-1} was generally responsible for a complex intermolecular interaction of ECM mainly from the C-N stretches and the N-H bending vibrations of peptide groups.

Figure A3. FTIR spectra of the alginate scaffold and alginate-ECM hybrid scaffold. Successful crosslinking and surface modification during the fabrication process was confirmed by the presence of characteristic peaks (red dotted boxes).

Cylindrical alginate and alginate-ECM scaffolds were successfully fabricated after crosslinked using calcium carbonate (CaCO_3) and D-glucono- δ -lactone (GDL).

Briefly 2% alginate/alginate-ECM solution was added into a well (24-well plate), followed by the addition of 0.1 M CaCO_3 solution and finally 0.2 M GDL solution. The mixture was then gently vortexed for 1 min to form a uniform suspension. The suspension was frozen at -80°C overnight before freeze-dried. The final porous microstructure of scaffolds are shown in Figure A4. The properties of alginate and alginate-ECM are summarized in Table A1. The average pore size and porosity of scaffolds was measured using the Image J software. As compared to the pure alginate scaffold, the alginate-ECM scaffold had a larger average pore size and higher porosity. This increase is due to the consumption of carboxyl groups from alginate during the ECM conjugation process (Figure A2), which resulted in a decrease in carboxyl group crosslinked by calcium ions. In addition, a significantly higher swelling ratio and lower compressive modulus ($*p<0.05$) of alginate-ECM scaffold were also observed as compared to the alginate scaffold.

Figure A4. Morphology of alginate and alginate-ECM scaffolds: (a) alginate scaffold, (b) SEM image of cross section of alginate scaffold, (c) alginate-ECM scaffold, and (d) SEM image of cross section of alginate-ECM scaffold.

Table A1. Summary of the material property of alginate and alginate-ECM scaffold

Properties	Alginate scaffold	Alginate-ECM scaffold
Mean pore size (μm)	321 ± 8	500 ± 20
Porosity (%)	36 ± 3	50 ± 3
Compressive modulus (Pa)	1020 ± 60	790 ± 60
Maximum swelling ratio (%)	6200 ± 500	8400 ± 600

Overall, the preliminary results showed the possibility of using ECM material to modify alginate by using bulk modification method (refer to section 2.4.3). The resultant alginate-*c*-ECM hybrid material was successfully fabricated into scaffold

using calcium ions. The bulk modified alginate scaffold showed higher swelling ratio but lower compressive modulus. Future study will be carried out to investigate the cell-material interactions of this novel hybrid material.

Appendix III Standard Curves

VEGF content standard curve

GAG assay standard curve

bFGF content standard curve

Hydroxyproline assay standard curve

ASCs standard curve

HUVECs standard curve

Sirius Red Standard Curve