



**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**THE DEVELOPMENT AND APPLICATION OF FISH
SCALE WASTE FOR TISSUE ENGINEERING
APPLICATIONS: WASTE-TO-RESOURCE
CONVERSION OF RESOURCE RESILIENT
MATERIALS**

WANG JUN KIT

**INTERDISCIPLINARY GRADUATE SCHOOL
NANYANG ENVIRONMENT AND WATER RESEARCH
INSTITUTE – RESIDUES & RESOURCE RECLAMATION
CENTRE**

2016

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– RESIDUES & RESOURCE RECLAMATION CENTRE

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Abstract

Collagen is the most abundant protein found in the human body and plays an important role in directing cellular behavior such as cell adhesion, proliferation and differentiation. Hence, collagen is one of the most studied biomaterial for tissue engineering (TE) applications. However, the current principle source of collagen is mainly from mammalian animals, which may contain some risk of zoonotic disease transmission and which may have restricted use for some religious and ethnic communities. Therefore, alternative sources of collagen such as fish-derived collagen, which has no risk of disease transmission to humans and is not associated with any religious barriers, is highly desirable. However, the potential applicability of fish-derived collagen is highly dependent on its thermal stability, which is affected by the living environment and the body temperature of the fish species. In general, collagen with high thermal stability is required to provide the necessary thermo-mechanical strength and degradation stability when implanted *in vivo*. Although studies have shown that the freshwater fish-derived collagen exhibits better thermal stability as compared to seawater fish-derived collagen, to our knowledge, limited studies have been carried out to compare the fish-derived collagen from different fish species, particular those extracted from fish scales. Moreover, fish scales also consist of hydroxyapatite (HA), which is the main component of bone with excellent osteoconductive properties to promote bone regeneration. Therefore in this thesis, fish scale-derived collagen (FSCol) and fish scale-derived HA (FSHA) were extracted from different fish species and the potential of the extracted products for various TE applications were explored. Findings from the studies demonstrated that Type I FSCol was successfully extracted using a modified acid solubilization extraction method with preserved structural quality (i.e. retention of triple helical structures) where snakehead

scale-derived collagen (SHCol) exhibited significant higher ($p < 0.05$) thermal stability as compared to other species. Meanwhile, a monophasic HA was successfully extracted using heat-sintering method. Subsequently, the extracted FSCol was conjugated onto polyvinylidene fluoride (PVDF) substrates to improve the cytocompatibility of PVDF substrates. Overall, SHCol-conjugated PVDF films demonstrated better hemocompatibility properties as compared to other fish species, and thus is a promising material to be used for blood contacting applications. The SHCol was then chemically-modified and crosslinked to produce a water-soluble collagen with improved physicochemical properties as a potential drug carrier for biomedical applications. In addition, the chemically-modified SHCol (i.e. methylated SHCol) promoted growth of blood vessels (BVs) and lymphatic vessels (LVs) during *in vivo* studies. From the histological results, the uncrosslinked methylated SHCol promoted greater cellular infiltration, thus making it most suitable for wound healing applications. On the other hand, the methylated SHCol crosslinked with 1,4-butanediol diglycidyl ether (BDE) crosslinker significantly promoted ($p < 0.05$) the formation and growth of LVs, thus making it suitable for treatment of lymphedema or other inflammatory related diseases. Lastly, a biocomposite scaffold consisting of both SHCol and snakehead scale-derived HA (SHHA) was fabricated. Based on the *in vitro* cellular studies, the presence of SHHA and SHCol significantly promoted ($p < 0.05$) the cell-material interactions of pre-osteoblast cells, and thus has potential to be used for bone TE applications. Taken together, this thesis demonstrated the potential of the extracted FSCol and FSHA as a resource resilient, cost effective source of waste-to-resource bioactive materials for various biomedical applications.

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Abbreviations

0BDE-MCol	Uncrosslinked Methylated Collagen Patch
0BDE-UCol	Uncrosslinked Unmodified Collagen Patch
15BDE-MCol	15 w/w% 1,4-Butanediol Diglycidyl Ether-crosslinked Methylated Collagen Patch
15BDE-UCol	15 w/w% 1,4-Butanediol Diglycidyl Ether-crosslinked Unmodified Collagen Patch
¹ H-NMR	Proton Nuclear Magnetic Resonance
3D	Three-dimensional
5BDE-MCol	5 w/w% 1,4-Butanediol Diglycidyl Ether-crosslinked Methylated Collagen Patch
5BDE-UCol	5 w/w% 1,4-Butanediol Diglycidyl Ether-crosslinked Unmodified Collagen Patch
A'	Absorption Spectra
ACP	Amorphous Calcium Phosphate
ADP	Adenosine Diphosphate
ALP	Alkaline Phosphatase
ATCC	American Type Culture Collection
ATR-FTIR	Attenuated Total Reflectance Fourier Transform Infrared Analysis
ATRP	Atom Transfer Radical Polymerization
BCA	Bicinchoninic Acid
BDE	1,4-Butanediol Diglycidyl Ether
BFCol	Bullfrog Skin-derived Collagen
BSE	Bovine Spongiform Encephalopathy

BVCol	Bovine Collagen
BVs	Blood Vessels
Ca	Calcium
Ca/P	Calcium-to-Phosphorus Ratio
CDI	1,1'-Carbonyldiimidazole
CMC-Tyr	Carboxymethylcellulose-Tyramine
CO ₂	Carbon Dioxide
CPP	Calcium Pyrophosphate
CPPD	Calcium Pyrophosphate Dihydrate
CS	Chondroitin Sulfate
C _t	Threshold Cycle
CuBr	Copper (I) Bromide
CuBr ₂	Copper (II) Bromide
D ₂ O	Deuterium Oxide
DCP	Dicalcium Phosphate
DCPD	Dicalcium Phosphate Dihydrate
DMEM/F-12	Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12
	Ham
DMSO	Dimethyl Sulfoxide
DSC	Differential Scanning Calorimetry
DSRB	Domain Specific Review Board
E	Eosin
ECL	Enhanced chemiluminescent
ECM	Extracellular Matrix
EDTA	Disodium Ethylenediaminetetraacetate Dihydrate

EDX	Energy Dispersive X-ray
EMA	European Medicines Evaluation Agency
eNOS	Endothelial Nitric Oxide Synthase
ePCL	Electrospun Nanofibrous Polycaprolactone Scaffold
ePCL/FSHA	Electrospun Nanofibrous Polycaprolactone/Fish Scale-derived Hydroxyapatite Scaffold
ePCL/SHHA	Electrospun Nanofibrous Polycaprolactone/Snakehead Scale-derived Hydroxyapatite Scaffold
ePCL/SynHA	Electrospun Nanofibrous Polycaprolactone/Synthetic Hydroxyapatite Scaffold
EtO	Ethylene Oxide
FACTS	Facility for Analysis, Characterization, Testing and Simulation
FAO	Food and Agriculture Organization
FBS	Fetal Bovine Serum
FDA	Fluorescein Diacetate
FITC-BSA	Fluorescein Isothiocyanate-conjugated Bovine Serum Albumin
FMD	Foot-and-Mouth Disease
FSCol	Fish Scale-derived Collagen
FSHA	Fish Scale-derived Hydroxyapatite
GA	Glutaraldehyde
Gtn-HPA	Gelatin-Hydroxyphenylpropionic Acid
H	Hematoxylin
HA	Hydroxyapatite
HCl	Hydrochloric Acid
HEK	Human Embryonic Kidney

HEMA	2-Hydroxyethyl Methacrylate
HFIP	1,1,1,3,3,3-Hexafluoro-2-Propanol
hFOB	Human Fetal Osteoblastic
HMTETA	1,1,4,7,10,10-Hexamethyltriethylenetetramine
HUVECs	Human Umbilical Vein Endothelial Cells
IACUC	Institutional Animal Care and Use Committee
ICAM-1	Intracellular Adhesion Molecule
IRB	Institutional Review Board
LVs	Lymphatic Vessels
LYVE-1	Lymphatic Vessel Endothelial Hyaluronic Acid Receptor-1
MCol	Methylated Collagen
MCPM	Monocalcium Phosphate Monohydrate
MFI	Median Fluorescence Intensity
MSE	School Of Materials Science and Engineering
MU	Methylumbelliferone
MUP	Methylumbelliferyl Phosphate
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NH ₂	Amine Group
NHG	National Healthcare Group
NTU	Nanyang Technological University
NUS	National University of Singapore
OCP	Octacalcium Phosphate
P	Phosphorus
PAI-1	Plasminogen Activator Inhibitor

PBS	Phosphate Buffered Saline
PCL	Polycaprolactone
PDF	Powder Diffraction File
pHEMA	Poly-2-Hydroxyethyl Methacrylate
PPP	Platelet-Poor Plasma
pPVDF	Pristine Polyvinylidene Fluoride
PRP	Platelet-Rich Plasma
PVDF	Polyvinylidene Fluoride
PVDF- <i>g</i> -pHEMA	Poly-2-Hydroxyethyl Methacrylate-grafted Polyvinylidene Fluoride
qPCR	Real-time Polymerase Chain Reaction
SBCol	Seabass Scale-derived Collagen
SBHA	Seabass Scale-derived HA
SBS	School of Biological Sciences
SCBE	School of Chemical and Biomedical Engineering
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Scanning Electron Microscope
SHCol	Snakehead Scale-derived Collagen
SHHA	Snakehead Scale-derived HA
SynHA	Synthetic Hydroxyapatite
TCP	Tricalcium Phosphate
T _d	Denaturation Temperature
TE	Tissue Engineering
TGA	Thermogravimetric Analysis

THF	Tetrahydrofuran
T _m	Melting Temperature
TMB	3,3',5,5'-Tetramethylbenzidine
TNBS	2,4,6-Trinitrobenzene Sulfonic Acid
TNF α	Tumour Necrosis Factor Alpha
tPA	Tissue Plasminogen Activator
TPCol	Tilapia Scale-derived Collagen
TPHA	Tilapia Scale-derived HA
TTCP	Tetracalcium Phosphate
TTSH	Tan Tock Seng Hospital
UCol	Unmodified Collagen
VCAM-1	Vascular Cell Adhesion Molecule
VEGF	Vascular Endothelial Growth Factor
vWF	von Willebrand Factor
WCA	Water Contact Angle
XRD	X-ray Powder Diffraction
YD%	Yield Percentage

Chapter 1

Introduction

This chapter contains the hypothesis and problem statement for the research work carried out, followed by the objective and scope for this thesis. Next, an overview of the dissertation of the thesis is described. Finally, the novel findings and outcomes of the research are described. Briefly, this thesis explores the utilization of the abundantly available aquaculture waste material, fish scales, as an alternative source of collagen and hydroxyapatite (HA) for biomedical applications. It is hypothesized that by using a freshwater fish scales and by using a modified acid solubilization method, collagen with high thermal stability can be easily extracted from fish scales with preserved structural quality (i.e. retention of triple helical structures) without deteriorating the biological function of collagen. Likewise, a monophasic naturally-derived HA can be easily extracted from fish scales using a heat treatment method. Subsequently, the potential biomedical applicability of these extracted products are investigated and compared to that of commercially available counterparts. Overall, this thesis demonstrates the extraction of resource resilient, cost effective sources of biomaterials for tissue engineering (TE) applications.

1.1 Hypothesis/Problem Statement

Fish and other aquatic foods are a source of protein and essential micronutrients, which is why they are recognized as key components of a balanced diet and healthy lifestyle [1, 2]. As a result, the aquaculture industry is one of the fastest growing food industries in the world [3]. According to a 2014 report by the Food and Agriculture Organization (FAO), about 158 million tonnes of fish and aquaculture products were produced in 2012, of which 86% were consumed by humans. However, during the harvesting, handling and processing of these fisheries products, about 50-70% of the original raw materials are treated as waste. These aquaculture waste material include bones, fins, heads, scales, skin, tails and viscera, which is often discarded and could potentially pose a risk to human health and the environment [4, 5]. Therefore, there has been increasing global awareness of proper waste management in order to reduce the amount of waste generated and to minimize their negative impact on the environment [5]. Several studies have shown that fish waste is rich in fish oils, proteins, enzymes and minerals [6]. However, these aquaculture waste material have commonly been used as fertilizer, feeds for agriculture and aquaculture, or production of gelatin for food and beverages industry as low value-added products [7, 8]. In fact, aquaculture waste material also contain collagen, which is an important biomaterial used in biomedical applications for tissue regeneration [9]. Therefore, there is a need to exploit the aquaculture waste material as an alternative source of collagen for biomedical applications to maximize the value of the aquaculture waste material and as an effective waste management solution.

Collagen is the most abundant protein in humans. It is the major constituent of extracellular matrix (ECM), found in all tissues of the human body. It is highly versatile, exhibits excellent biodegradability, biocompatibility and bioactivity and is

known to modulate cell attachment, proliferation and differentiation [9, 10]. Currently, the principle sources of collagen are mammalian animals such as cows, pigs or chicken. However, these mammalian-derived collagen from farm animals poses the risk of zoonotic disease transmission such as mad cow disease, bovine spongiform encephalopathy (BSE), foot-and-mouth disease (FMD), Creutzfeld-Jacob disease, avian and swine influenza, ovine and caprine scrapie, and other zoonoses, which have limited the clinical application of these mammalian-derived collagen [10-12]. Although an alternative safer source of collagen, in the form of recombinant collagen, is available. However, the recombinant technology involves complex processing steps, large amounts of enzyme and limited production. Hence, this method is considered cumbersome, rather expensive and time consuming [11-13]. In addition, there are other issues related to the current principle sources of mammalian-derived collagen such as restriction and even banning of collagen derived from cows and pigs in some regions due to the religious reasons [14]. In some cases, allergic reactions to bovine and porcine, but not fish-derived collagen were also reported [4, 15]. On the other hand, fish-derived collagen has relatively low risks of pathogen and is not associated to any religion associated barriers. Hence, it could be a comparatively safer source of collagen [11, 16].

Although fish-derived collagen may have advantages over mammalian-derived collagen, the poor thermal stability of fish-derived collagen has limited its biomedical applicability. The thermal stability of collagen plays an important role in the mechanical stability and the biodegradation rate of collagen [17]. Therefore, the poor thermal stability of the fish-derived collagen could potentially cause the implant to failure and lead to partial tissue regeneration when implanted into human body due to the poor thermo-mechanical strength and rapid *in vivo* resorption rate of the implanted

collagen [18]. However, studies have shown that the thermal stability of fish-derived collagen is highly dependent on the living environment and the body temperature of the fish species [19]. In fact, several studies have shown that the freshwater fish-derived collagen has relatively higher thermal stability as compared to seawater fish-derived collagen, thus making it a potentially safer biomaterial for biomedical applications in its native form at body temperature without any denaturation [11].

To our knowledge, limited studies have been carried out to compare collagen derived from different fish species, particularly those extracted from fish scales, which are one of the major aquaculture waste material and an untapped source of bioactive materials for biomedical applications. Therefore, we hypothesize that by using freshwater fish as a source of fish scales and by using a modified acid solubilization extraction method, collagen with high thermal stability will be easily extracted from fish scales with preserved triple helical structures and biological function for biomedical usage.

Although collagen has superior biological properties, the poor physicochemical properties of collagen have often limited its clinical applications. Therefore, crosslinking reaction is often carried out to improve the physicochemical properties of collagen. However, most of the crosslinking agents used can potentially lead to cytotoxicity issues [9]. Meanwhile, the TE approach commonly involves the use of scaffolds incorporated with biological agents to facilitate cell proliferation and tissue regeneration. In this case, unmodified collagen (UCol), which mainly dissolved in acidic solution, may not be a suitable carrier for the biological agents such as cells, drugs, genes or growth factors due to the possibility of the harsh acidic environment in denaturing the biological agents during the scaffold fabrication process [20, 21]. Therefore, we hypothesize that by using a methylation modification and an epoxy crosslinker, will be possible to produce a water-soluble methylated collagen (MCol)

with improved physicochemical properties without cytotoxic issues. In addition, this physiological friendly MCol will also improve the cell-material interactions and could further extend its potential applicability as drug carrier.

Other than collagen, fish scales also consist of high amounts of HA, which is a bioactive ceramic that resembles bone mineral [22, 23]. HA has excellent osteoinductive and osteoconductive properties and has been widely used in bone TE applications to repair bone defects and promote bone tissue regeneration [24, 25]. However, chemical synthesis of HA involves complex reactions and the chemical residues can potentially be cytotoxic, and thus making it less biocompatible [22]. Therefore, by using a heat-sintering method, a monophasic naturally-derived HA could also be easily obtained from the fish scales for biomedical applications.

1.2 Objective and Scope

The main objective of this thesis is to extract collagen and HA from fish scales, as a potential alternative resource resilient, cost effective source of waste-to-resource bioactive materials for biomedical applications. However, the potential applicability of the fish scale-derived collagen (FSCol) is highly dependent on its thermal stability, whilst the potential applicability of fish scale-derived HA (FSHA) is highly dependent on its pH stability. Hence, various physicochemical characterizations are carried out on the extracted FSCol and FSHA to investigate the physicochemical stability of the extracted products. Subsequently, in order to demonstrate the applicability of the extracted FSCol and FSHA, various types of scaffolds are fabricated using the extracted products on their own or as a composite scaffold followed by *in vitro* cellular studies and *in vivo* studies as described below:

1. Extraction and characterization of FSCol and FSHA from different fish species

Firstly, the presence of collagen and HA in the fish scales will be confirmed followed by the extraction of FSCol and FSHA using a modified acid solubilization extraction method and a heat-sintering method respectively. Subsequently, various characterizations are performed on these extracted products to identify the types of collagen and the chemical phase of HA. The applicability of FSCol is highly dependent on its thermal stability, which is affected by the conformational stability and the amount of triple helical structures within the collagen. Hence, the structural properties and the thermal properties of the FSCol extracted from different fish species are characterized and compared.

2. Application of FSCol

The potential application of the extracted FSCol will be tested by conjugating the extracted FSCol onto a bioinert synthetic polymer [i.e. polyvinylidene fluoride (PVDF)], via surface-initiated atom transfer radical polymerization (ATRP). The scope of this study included the confirmation of each successful surface modification step, cellular studies to evaluate the cell-material interactions using endothelial cells and lastly, investigating the thrombogenic properties of the cells seeded onto the collagen-conjugated PVDF films.

3. Modification of FSCol as water-soluble collagen for drug delivery applications.

In the field of TE, scaffolds are commonly used as a platform to guide the growth of new tissues to replace damaged tissues. One of the most commonly used scaffolding materials is collagen due to its superior bioactive properties with excellent cell-material interactions. However, due to collagen's poor physicochemical properties, crosslinking is often carried out to improve the physicochemical properties of collagen.

In addition, biological agents such as cells, drugs, genes or growth factors are often incorporated into the scaffolds to enhance the tissue regeneration process. However, UCol is mostly soluble under acidic conditions, which could potentially denature the biological agents during scaffold fabrication process. Hence, in order to preserve the quality of these biological agents and to improve the physicochemical properties of collagen, methylation modification will be carried out on the extracted FSCol to yield a water-soluble MCol. The scope of this particular study included the confirmation of the chemical modification and chemical crosslinking processes, assessment of the physicochemical properties, drug release studies of the chemically-modified FSCol, and *in vivo* studies to test the biocompatibility and degradability of the chemically-modified FSCol using a murine model.

4. Develop an electrospun nanofibrous polycaprolactone (PCL)/FSHA scaffolds grafted with FSCol as potential biocomposite scaffolds for bone TE applications.

Lastly, the potential applicability of FSHA will be tested by incorporating the extracted SHHA with a biopolymer, followed by grafting of SHCol onto the polymer surface to form biocomposite scaffolds. Subsequently, cellular studies are carried out to investigate the cell-material interactions with the biocomposite scaffolds using pre-osteoblast cells.

1.3 Dissertation Overview

Chapter 1 provides the rationale to utilize the abundantly available aquaculture waste material for the extraction of FSCol and FSHA as alternative safer sources of biomaterials for biomedical applications. The objective and scope of the thesis are also outlined.

Chapter 2 reviews the literature in the context of the scope of this thesis. The various topics related to TE, the role of collagen and HA in biomedical applications, the thermal stability of collagen, current sources of aquaculture-derived collagen, basic structure of fish scales and different types of extraction methods are reviewed.

Chapter 3 describes all the experiment methodologies and characterization techniques used in this thesis, and also discusses the principles underlying the characterization techniques employed in various studies.

Chapter 4 describes the characterization and comparison of the structural and physical properties of the FSCol and FSHA from different fish species. The extraction yield percentage (YD%), the types of collagen, the amount of triple helical structures and the thermal properties of the extracted FSCol as well as the chemical phase of the FSHA are compared and discussed in detailed.

Chapter 5 investigates the potential application of FSCol from different fish species by examining endothelial cell interactions with the collagen-conjugated PVDF films. Cell proliferation, thrombogenic markers and inflammatory responses by the cells seeded on different species of FSCol surfaces are reported and discussed.

Chapter 6 discusses the use of chemically-modified FSCol as a water-soluble collagen for drug delivery applications. The physicochemical properties of both the UCol and MCol are examined followed by *in vivo* biocompatibility and degradability studies using a murine model.

Chapter 7 describes the fabrication of electrospun nanofibrous PCL/FSHA scaffolds followed by surface modification for the grafting of FSCol. The cell-material interactions of the biocomposite scaffolds with pre-osteoblast cells are also reported and discussed.

Chapter 8 concludes the thesis by summarizing all the findings from the four studies as well as the implications and impacts of the findings. This chapter also includes the potential future work that could be further explored based on the findings obtained from this thesis.

1.4 Findings and Outcomes/Originality

The research carried out during the course of this thesis has led to several novel outcomes including:

1. A comprehensive characterization of fish scales from different fish species followed by the comparison of the physicochemical properties of FSCol and FSHA. The modified acid solubilization method used for the extraction of FSCol preserved the structural quality of the extracted collagen (i.e. retention of triple helical structures). To our knowledge, our studies are the first to show that collagen derived from freshwater snakehead scales is thermally most stable as compared to other fish species, and hence is a promising material for biomedical applications;
2. Functionalization of PVDF films with the extracted FSCol through ATRP. The functionalized PVDF films showed improved surface chemistry. In addition, the presence of collagen improved the cell-material interactions as well as the hemocompatibility of the PVDF films, thus potentially to be used for blood-contacting applications;
3. Successful chemical modification of the extracted FSCol using acidified methanol to produce a form of water-soluble methylated FSCol. Subsequently,

methyated FSCol was then chemically-crosslinked with 1,4-butanediol diglycidyl ether (BDE) with improved physicochemical properties of methyated FSCol. This BDE-crosslinked methyated FSCol allowed for a controlled release of drug, which makes it a promising drug carrier material. Moreover, the *in vivo* studies demonstrated that the methyated FSCol, either in the form of uncrosslinked or BDE-crosslinked, promoted better growth of blood vessels (BVs) and lymphatic vessels (LVs). Between the uncrosslinked and BDE-crosslinked methyated FSCol, greater cellular infiltration and vessels ingrowth were observed for the uncrosslinked methyated FSCol patches, thus making it suitable for wound healing applications. Meanwhile, BDE-crosslinked methyated FSCol patches significantly promoted the growth of LVs during the *in vivo* studies, thus making it suitable for applications in inflammatory related diseases. To our knowledge, our studies are the first to demonstrate the potential of methyated FSCol for various biomedical applications;

4. Improvement of the particle size distribution and pH stability of FSHA through ball-milling and 1× phosphate buffered saline (PBS) treatment. The FSHA was then incorporated into PCL solution to produce electrospun nanofibrous PCL/FSHA scaffolds. Subsequently, the electrospun nanofibrous PCL/FSHA scaffolds were chemically-modified using FSCol to improve the surface properties of PCL. Overall, the presence of SHCol and SHHA significantly improved the cell-material interactions of pre-osteoblast cells, and thus are promising scaffolding materials for bone TE applications.

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Chapter 2

Literature Review

This chapter describes in detail, the literature pertaining to the work conducted in this thesis. It provides the background and current knowledge regarding the hypotheses that are explored. Overall, this chapter is a review of the various topics related to TE such as the requirements of a scaffold in TE applications, collagen as a biomaterial for TE applications, the importance of the thermal stability of collagen and the strategies used to crosslink or to modify collagen to improve its thermal stability. Subsequently, the different types of fish scales, the basic structure of fish scales and the various types of extraction methods for both collagen and HA are also reviewed.

2.1 TE and Regeneration

TE is a multidisciplinary field that uses principles of engineering and cell biology to develop a scaffold incorporated with cells and growth factors as a biological substitute in order to replace, restore or regenerate defective tissues as summarized in **Figure 2.1** [1]. In general, the scaffold acts as a platform for cell attachment and as a structural support for tissue formation, whilst the growth factors are responsible for speeding up the regeneration process. Different fabrication methods have been used for the synthesis of scaffolds. These methods include freeze-drying, emulsion capsule formation, electrospinning, solid freeform fabrication and self-assembly methods. During scaffold fabrication, there are several requirements that need to be considered. For example, the material used for scaffold fabrication should be biocompatible with the host tissue so that it will not impede or adversely affect it. In addition, the material should have a biodegradation rate that matches the rate of new tissue formation. Also, the structure of the scaffold should have sufficient porosity in order to facilitate cell infiltration, nutrient and oxygen diffusion into the scaffold and waste removal from the scaffold [2, 3].

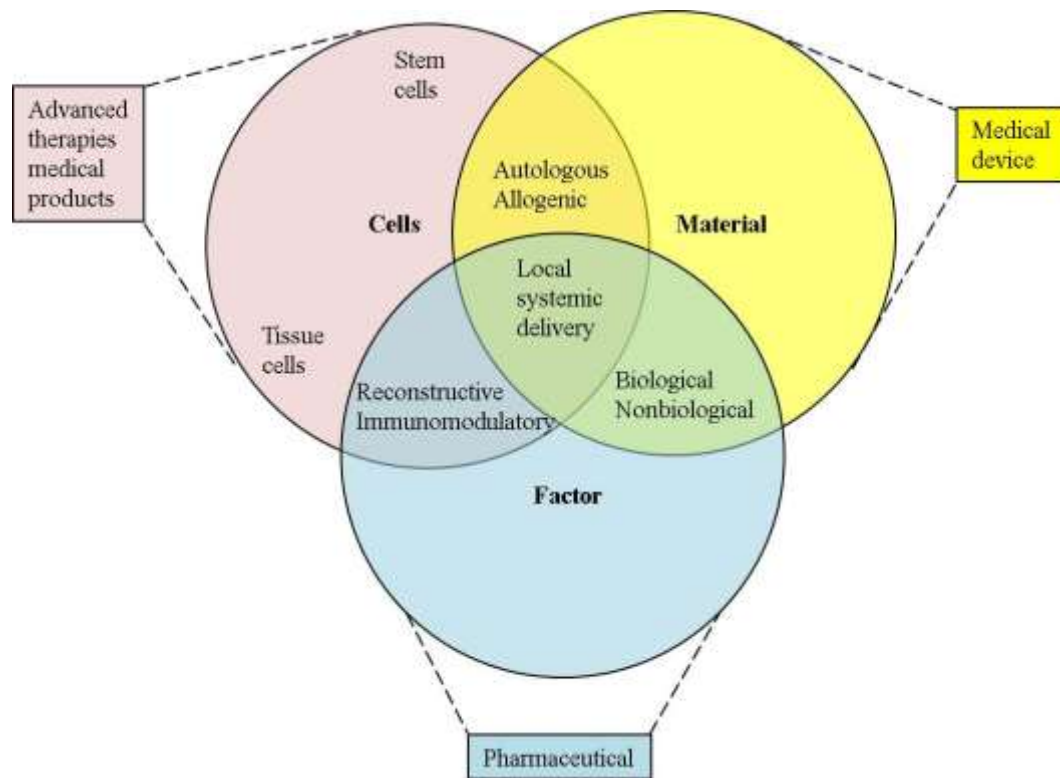


Figure 2.1 Main components of TE as adapted from: Ringe, J., G.R. Burmester, and M. Sittinger, Regenerative medicine in rheumatic disease[mdash]progress in TE. Nature Reviews Rheumatology, 2012. Volume 8. Pages. 493-498. Copyright Nature Publishing Group. Reproduced with permission.

2.2 Collagen as a Biomaterial

Amongst different types of scaffolding materials, collagen is one of the most preferred biomaterial used as it is a widely distributed class of protein in our body with superior properties such as biodegradability, excellent cell-matrix interactions, low immunogenicity and high versatility. To date, a total of 29 distinct types of collagen have been found in humans, albeit at different amounts; Type I collagen being the most abundant [4]. Type I collagen is commonly found in skin dermis, bones and other connective tissue, providing them with tensile strength and structural rigidity [5]. Type

I collagen has also been extensively used to regulate the cellular behavior in terms of migration, proliferation and differentiation [6]. Hence, it has been used for various for tissue regeneration applications such as wound dressing, ulcer treatment, surface modification, or dermal filler [4].

2.2.1 Marine Collagen

The current principle source of Type I collagen is mainly from bovine and porcine. However, due to the outbreak of zoonotic disease transmission and religious constraints associated with bovine and porcine collagen [7, 8], alternative sources of collagen (i.e. marine sources) are highly desirable. Recently, research has been focusing on extracting collagen from the marine waste products for TE applications including squid skin [9], cuttlefish [10], fish skin [11-15], fish bone [16, 17], fish scales [18], jellyfish exumbrella [19] and marine sponge [20, 21]. In terms of publications, an increasing number of research has also been published over the past few years regarding the term ‘marine collagen’ with regards to the development of potential alternative sources of collagen for biomedical applications [7]. However, the thermal stability of the marine collagen is a crucial factor to be considered for biomedical applications. In general, collagen with a higher thermal stability will have better resistance toward enzymatic degradation, and therefore it will not readily undergo dissolution during the course of implantation [22]. On the other hand, gelatin, the denatured form of collagen, is susceptible to digestion by most proteases during the course of implantation and the rapid degradation rate will result in implant to failure and lead to partial tissue regeneration [23].

2.2.2 Thermal Stability of Collagen

The thermal stability of collagen is highly dependent on the conformational stability and the amount of triple helical structures within the collagen, which could be determined by measuring the denaturation temperature of collagen. A high denaturation temperature is an indication of the high thermal stability with a stable structural organization of the collagen molecules and thus, the retention of triple helical structures [24]. Briefly, the hierarchical structure of collagen is shown in **Figure 2.2**. As seen from the figure, the primary structure of collagen consists of a polypeptide chain in the form of either Glycine(Gly)-Proline(Pro)-X sequence or Gly-X-Hydroxyproline(Hyp) sequence, in which glycine would always be the third repetitive acid. Subsequently, three polypeptide chains are twisted into a bundle to form triple helical molecules. This classic collagen triple helix structure is formed through left-handed twisting of the amino acid sequence into an α -helix secondary structure, which is in turn bundled into a left-handed helix structure in tertiary state to form procollagen. The procollagen is eventually secreted into the extracellular space where metalloproteinase enzymes cleaved the propeptides to form collagen [23]. In general, hydroxyproline, a hydroxylated form of proline, forms the hydrogen bonding between the polypeptide chains of collagen, which improves the conformational stability of the triple helical structures of collagen [25-27]. The hydroxyproline content stabilizes the triple helical structures, which determines the amount of triple helical structures within the collagen and thus, its thermal stability. Taken together, the amino acid compositions and the amount of triple helical structures of collagen are important features of the thermal stability of collagen [7]. Other than the amino acid compositions and the amount of triple helical structures of collagen, several methods

have been employed in order to tailor the thermal stability of collagen such as crosslinking or chemical modification of collagen for biomedical applications.

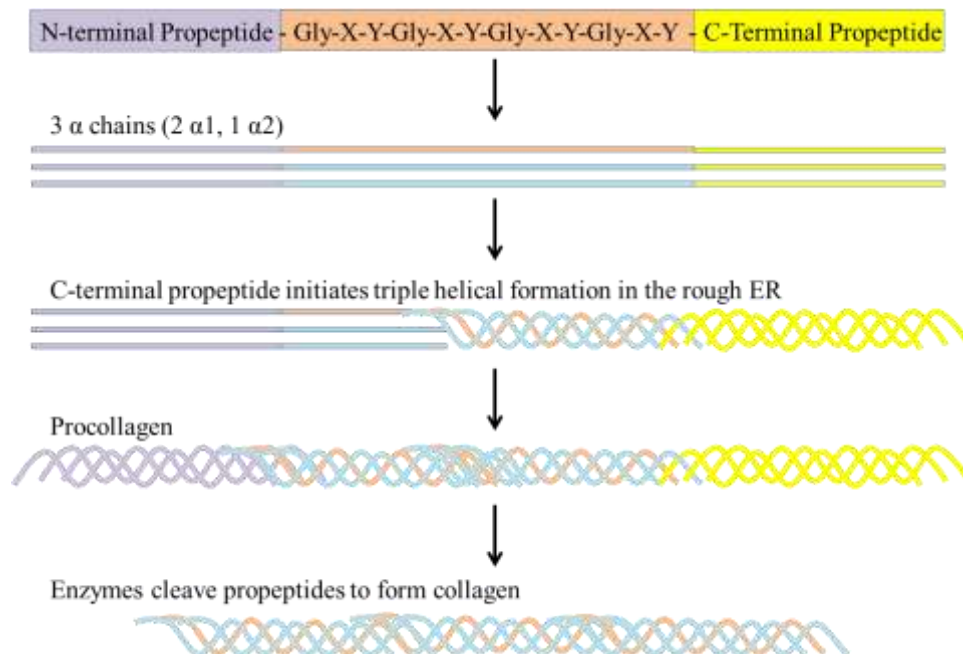


Figure 2.2 Hierarchical structure and the synthesis process of collagen as adapted from: Janna K. Mouw, Guanqing Ou, Valerie M. Weaver, Extracellular matrix assembly: a multiscale deconstruction. Nature Reviews Molecular Cell Biology, 2014. Volume 15. Pages. 771-785. Copyright Nature Publishing Group. Reproduced with permission.

2.2.3 Crosslinking of Collagen

Crosslinking has been carried out to tailor the thermal stability, the mechanical behavior, and the degradation stability of collagen [28]. There are several types of crosslinking procedures, including physical and chemical methods [29]. Physical crosslinking does not involve the use of chemical agents. Some of the commonly used physical crosslinking methods are heating, drying, irradiation such as UV or gamma

ray, dehydrothermal treatment and dye-mediated photo-oxidizing method. For the chemical crosslinking of collagen, the most commonly used chemical agents are aldehydes compounds [e.g. glutaraldehyde (GA) and formaldehyde], isocyanate compound (e.g. hexamethylene diisocyanate), carbodiimides [e.g. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/ Sulfo-(N-hydroxysulfosuccinimide)], acyl-azide compound (e.g. diphenylphosphorylazide), epoxy compounds (e.g. BDE). Other types of crosslinking reagents have also been used such as photoreactive crosslinking agents (e.g. rose Bengal and riboflavin), carbohydrates (e.g. ribose and glucose), plant extracts (e.g. genipin, and oleuropein) and biological agents (e.g. transglutaminase, tyrosinase and laccase) [23, 29]. Among different types of crosslinking methods, chemical crosslinking often result in highest degree of crosslinking and hence the thermal and degradation stability of collagen [5, 30]. In general, epoxy compounds have been shown to exhibit the lowest toxicity as compared to other chemical crosslinking agents, particularly for BDE, which is a bifunctional epoxy compound, has been successfully used to crosslink collagen [31-33], and which is used as the crosslinking agent for collagen in this thesis.

2.2.4 Chemical Modification of Collagen

UCol commonly exhibits poor physical properties both *in vitro* and *in vivo* [34]. In one particular study, it was shown that when exposed to just 0.01 % collagenase, UCol could be fully digested within 48 h [35]. Thus, chemical modification may be required to improve the thermal and degradation stability of collagen. In addition, chemical modification of collagen has also been shown to improve the cellular behavior of the material [36]. In general, the chemical modification of collagen produces a charged collagen, which in turn improves the interaction of the modified collagen with the

oppositely charged proteins on the cell membrane, as well as the hydrophilicity of the overall material. Moreover, the modified collagen is soluble at physiological pH and body temperature, which could further extend its potential application as drug delivery device by creating a friendly environment for the drug molecules. Several different types of chemical modifications have been performed on collagen such as acetylation, succinylation and methylation [37]. Acetylation modification converts the positively-charged amino groups of lysines and hydroxylysines of collagen into neutral acetyl groups using acetic anhydride to yield a negatively-charged collagen, whereas succinylation modification converts the positively-charged amino groups of lysines and hydroxylysines of collagen into carboxyl groups using succinic anhydride to yield a negatively-charged collagen. On the other hand, methylation modification converts the negatively-charged carboxylic acid groups into methyl ester ($-\text{COOCH}_3$) groups using methyl alcohol via esterification process to yield a positively-charged collagen. In this thesis, methylation modification is chosen as the method for chemical modification of collagen, due to the net positive-charge of the resulting MCol structure, which could improve the interaction with the negatively-charged proteins on the cell membrane, and therefore improving the overall cell-material interactions [38, 39]. In addition, as compared to other chemical modification methods, the methylation modification conserves the stereoelectronic effects of the hydroxyl groups presence in collagen, which could preserve the conformational stability of the triple helical structures of the modified collagen [40].

2.3 Freshwater Fish Scales as an Alternative Source of Collagen

As the world population increases, global food demand and production have also dramatically increased. Among different types of food production, fish has become more popular as it has been recognized as one of the primary sources of animal protein in most countries [41]. According to a 2014 report by the FAO (**Table 2.1**), an average annual growth rate of 6% was maintained for the aquaculture production between 2007 to 2012 [42, 43]. Similarly, data from the Agri-Food & Veterinary Authority of Singapore (**Table 2.2**) also showed that fish production in Singapore is equally important, as there is an increasing supply of fish to meet the demand of an increasing population [44].

Table 2.1 Data obtained from the 2014 State of World Fisheries and Aquaculture Report showing the increasing supply of fish to meet the demand of an increasing population [42].

	2007	2008	2009	2010	2011	2012
	(Million Tonnes)					
Production						
Capture						
Inland	10.1	10.3	10.5	11.3	11.1	11.6
Marine	80.7	79.9	79.6	77.8	82.6	79.7
Total Capture	90.8	90.1	90.1	89.1	93.7	91.3
Aquaculture						
Inland	29.9	32.4	34.3	36.8	38.7	41.9
Marine	20.0	20.5	21.4	22.3	23.3	24.7
Total Aquaculture	49.9	52.9	55.7	59.0	62.0	66.6
Total World Fisheries	140.7	143.1	145.8	148.1	155.7	158.0
Utilization						
Human Consumption	117.3	120.9	123.7	128.2	131.2	136.2
Non-food uses	23.4	22.2	22.1	19.9	24.5	21.7
Population (Billions)	6.7	6.8	6.8	6.9	7.0	7.1
Per Capita Food Fish Supply (kg)	17.6	17.9	18.1	18.5	18.7	19.2

Table 2.2 Data obtained from the Singapore Agri-Food and Veterinary Authority Annual Report 2013/2014 showing increasing supply of fish to meet the demand of an increasing population [44].

Item (kg)	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013
Chicken	33	27	30	27	31	32	31	32	33	33	32
Pork	20	21	19	20	21	20	19	20	19	20	17
Seafood	27	27	27	26	25	24	24	22	23	22	22
- Fish	18	17	18	17	16	16	16	15	16	15	16
- Other seafood	9	10	9	9	8	8	7	7	7	7	7
Vegetables	96	92	93	93	93	91	91	93	93	94	93
- Leafy vegetables	18	19	18	18	17	16	17	16	15	16	16
- Other vegetables	78	72	74	76	76	75	74	77	78	78	77
Hen eggs (pcs)	306	268	286	291	302	302	300	311	307	308	312
Fruits	88	87	85	80	74	71	71	68	67	67	70
Beef	3	3	4	3	4	4	4	4	4	3	2
Duck	4	3	3	3	3	3	3	3	3	3	3
Mutton	2	2	2	2	2	2	2	2	2	2	2

*Per capita consumption from Jan to Dec each year

In parallel to these trends, it is also observed that large amounts of waste are generated annually, which causes a big impact socially, environmentally and economically [42, 45]. In general, the majority of the fish products come from the aquaculture fish farms, where the common waste produced during the food processing steps include solid waste such as inedible fish scales, bones, as well as the liquid waste such as water discharged from the cleaning and rinsing of fish, particularly fish blood and mucous [42, 45]. Most of these waste material are either disposed by landfill, recycled as fish meal production, hydrolyzed as fertilizers, incinerated for the production of biodiesel

or biogas, used as dietetic products, utilized as a source of enzyme, or used for cosmetic applications [46, 47]. Unlike the past, these aquaculture waste material are now being recognized as a resource that can be tapped upon by recycling and recovery methods, which is why there is a surge in interdisciplinary resource recovery research. In this thesis, collagen is extracted from fish scales, which is an abundantly available aquaculture waste for biomedical applications. However, studies have shown that different environments and temperatures would affect the amino acid composition of collagen, which consequently affects the thermal stability of the collagen by altering the conformation stability and the amount of the triple helical structures within the collagen [10-13, 18, 27, 48, 49]. Therefore, scales from freshwater fish are selected for the extraction of collagen. Overall, collagen derived from freshwater fish scales exhibits the advantages of low production costs, is zoonosis free and is not associated to any religious and ethical issues as compared to commercially available mammalian-derived collagen [28, 50]. In addition, the conversion of these aquaculture waste material into secondary value-added products is also a green revolution of biotechnology as well as an effective waste management strategy.

2.3.1 Types of Fish Scales

Fish scales are the primary protective barriers of fish from enemy attack. In general, there are three different classes of fish scales, namely placoid scales, cosmoid scales and elasmoid scales as shown in **Figure 2.3** [51]. Placoid scales are mainly found in sharks or rays, cosmoid scales are mainly found in lungfish or fossil fish, while elasmoid scales are found in the majority of bony fish (teleostei). Elasmoid scales come in two different forms, namely cycloid scales and ctenoid scales; cycloid scales

have a smooth and rounded posterior edge, while ctenoid scales have a fine teeth or comb-like ctenii at the posterior edge [52, 53]. In addition, elasmoid scales are thin, transparent and composed of three different layers such as outer limiting layer, external layer and basal plate. The outer limiting layer located at the surface of the scales does not contain any collagen. Collagen is mainly found in the external layer and basal plate. Based on biochemical analyses carried out previously by Ikoma et al., elasmoid scales consist mainly of mineralized Type I collagen [54]. Similar to bone, the minerals found in elasmoid scales are mainly composed of HA $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$, which is deposited between the interfibrillary matrix [54-56].

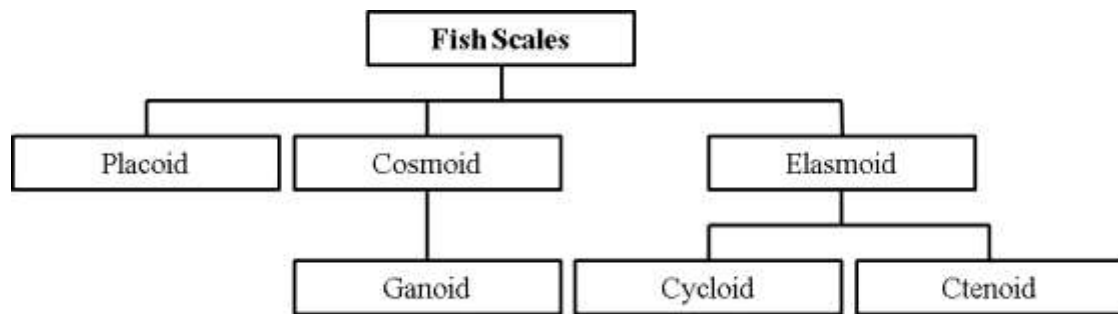


Figure 2.3 Different types of fish scales and configurations for the cartilaginous and bony fish [57].

2.3.2 HA as a Biomaterial

Human bone consists mainly (i.e. 50 – 70 %) of mineral, the majority of which is made up of HA. As a bioceramic, HA is known for its osteoconductivity, which is why it is widely used in bone TE applications [58]. Other than HA, several different types of calcium phosphate compounds are commonly used in bone TE applications. The different types of calcium phosphate compounds are listed in **Table 2.3** based on the calcium-to-phosphorus (Ca/P) ratios. The variation of calcium phosphate compound in

terms of chemical composition and structural formula has led to different solubility and stability. A Ca/P ratio of less than 1 is physiologically unstable with rapid resorption rate [59]. In contrast, a Ca/P ratio of more than 1.67 results in a drastic decrease in resorption rate. As a result, HA is an insoluble calcium phosphate compound that is stable at pH above 4.2, making it physically and thermodynamically stable under physiological pH and temperature, thus allowing it to be implanted into the body [60, 61].

Currently, the common synthetic methods used for producing HA are wet chemical precipitation [62], hydrothermal reaction, sol-gel process [63], solid-state reaction [64], or mechanosynthesis methods [65, 66]. Some of these synthetic methods require high temperatures and pressures as well as excessively long fabrication times, and the resulting HA typically has poor crystallinity and inhomogeneous composition [67]. Therefore in terms of efficiency and cost effectiveness, synthesis of HA from natural sources remain the best choice [68]. Fish scales consist of both collagen and HA, which are important biomaterials used for TE applications [48, 69]. Therefore, the extraction of HA from fish scales also exhibits benefits as compared to the commonly synthesized commercial HA. Taken together, fish scales are potentially an excellent choice of alternative sources of collagen and HA, which will be demonstrated in this thesis.

Table 2.3 Different types of calcium phosphate compounds used in hard TE applications [70].

Ca/P ratio	Formula	Chemical Name	Acronym
2.00	$\text{Ca}_4(\text{PO}_4)_2\text{O}$	Tetracalcium Phosphate	TTCP
1.67	$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$	Hydroxyapatite	HA
< 1.67	$\text{Ca}_{10-x}\text{H}_{2x}(\text{PO}_4)_6(\text{OH})_2$	Amorphous Calcium Phosphate	ACP
1.50	$\text{Ca}_3(\text{PO}_4)_2$	Tricalcium Phosphate	TCP
1.33	$\text{Ca}_8\text{H}_2(\text{PO}_4)_6 \cdot 5\text{H}_2\text{O}$	Octacalcium Phosphate	OCP
1.00	$\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$	Dicalcium Phosphate Dihydrate	DCPD
1.00	CaHPO_4	Dicalcium Phosphate	DCP
1.00	$\text{Ca}_2\text{P}_2\text{O}_7$	Calcium Pyrophosphate	CPP
1.00	$\text{Ca}_2\text{P}_2\text{O}_7 \cdot 2\text{H}_2\text{O}$	Calcium Pyrophosphate Dihydrate	CPPD
0.50	$\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$	Monocalcium Phosphate Monohydrate	MCPM

2.3.3 Methods of Collagen Extraction

Several methods, including salt solubilization, acid solubilization and enzymatic solubilization methods have been developed for the extraction of collagen from different tissues [71]. For the salt solubilization method, the tissues are treated with salt solutions such as disodium phosphate between pH 8.7 – 9.1. Subsequently, the extracted collagen is collected by adding sodium chloride to precipitate out the collagen prior to the purification steps in which a dialysis process is used. On the other hand, the acid solubilization method involves the usage of organic acid to extract the collagen from the tissues. Briefly, the tissues are immersed in acetic acid in the presence of disodium ethylenediaminetetraacetate dehydrate at a pH range of 2.5 – 3.0. Subsequently, the supernatant containing the acid soluble collagen is dialyzed against

disodium phosphate solution. The enzymatic solubilization method is in fact similar to that of the acid solubilization method, except that pepsin is added to the organic acid extraction solution at a concentration of 0.05 g per 100 g of tissue. Comparing among different types of extraction methods, the salt solubilization method has the lowest extraction yield. On the other hand, a long extraction time is required for the acid solubilization method, whilst the enzymatic solubilization method could potentially cause collagen to cleavage into monomeric subunits. As a result, the pepsin treatment of collagen will result in the loss of telopeptides and crosslinking sites of collagen [72, 73].

Unlike normal tissues, fish scales contain mineralized collagen fibers as the result of the presence of HA. Hence, additional modification steps are required prior to extraction of collagen in order to improve the purity or the yield of the extracted collagen. For this thesis, fish scales were treated with sodium hydroxide to remove non-collagen proteins from the fish scales. Subsequently, a demineralization step was carried out followed by extraction of collagen using an acid solubilization method. The acid treatments were repeated 5 times to ensure optimal removal of collagen. Next, the extracted collagen was treated with salt solution to increase the extraction yield and to improve the purity of the extracted collagen. Lastly, the collagen precipitates were re-dissolved in acid solution followed by dialysis to further purify the extracted collagen.

2.3.4 Methods of HA Extraction

Several methods have been developed for the extraction of HA from different types of natural materials. These include enzymatic hydrolysis, hydrothermal treatment and heat-sintering methods. For the enzymatic hydrolysis method, samples are treated with

protease N and flavourzyme at a specific pH and temperature before boiling to 100 °C to deactivate the enzyme. Subsequently, the hydrolysates are centrifuged, and the HA particles are collected [74]. For the hydrothermal treatment method, the samples are placed in water or other solvents in a closed vessel. Subsequently, the solution is heated above the boiling point to obtain the HA. Several natural sources have been used for the extraction of HA using this hydrothermal treatment method such as coral [75], eggshells [76], fish bone [77] and teeth [78]. For the heat-sintering method, the samples are heated to 900 °C for 1 to 2 h to obtain the HA powder [58, 69, 79]. In general, different techniques would result in different morphology and crystallinity of the HA, as well as different stoichiometric composition [80]. Comparatively, the natural conversion of HA using heat-sintering method is preferred as it is simple, time effective, economical fabrication process and safer without involving high temperature and high pressure [68, 81]. Hence, the heat-sintering method was the method chosen for extracting HA from fish scales for this thesis.

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Chapter 3

Experimental Methodology

This chapter gives a detailed description of the experimental procedures used to extract the bioactive components from the fish scales and the different scaffold fabrication techniques used for the various studies. A detailed description of the material characterization techniques, functional studies and data analysis methods used to challenge the hypotheses of this thesis are also included. Briefly, collagen and HA are extracted from fish scales using a modified acid solubilization extraction method and a heat-sintering method respectively. Subsequently, the potential application of the extracted FSCol is investigated by conjugating the extracted FSCol onto a bioinert synthetic polymer (i.e. PVDF), using ATRP process as a bioactive coating to examine the cell-material interactions. The extracted FSCol is then further modified to produce a water-soluble MCol and chemically-crosslinked, followed by studying the physicochemical properties and the in vivo biocompatibility and degradability of the chemically-modified FSCol using a murine model. Lastly, the potential application of FSHA is investigated by fabricating a biocomposite scaffold comprising of both extracted FSCol and FSHA. In vitro cellular studies are carried out on this biocomposite scaffold using pre-osteoblast cells, in order to investigate its potential application for bone TE applications.

3.1 Overview of Methodologies

Summary of the overall strategy and the methodologies used for this thesis are presented in the flow diagram shown in **Figure 3.1** below.

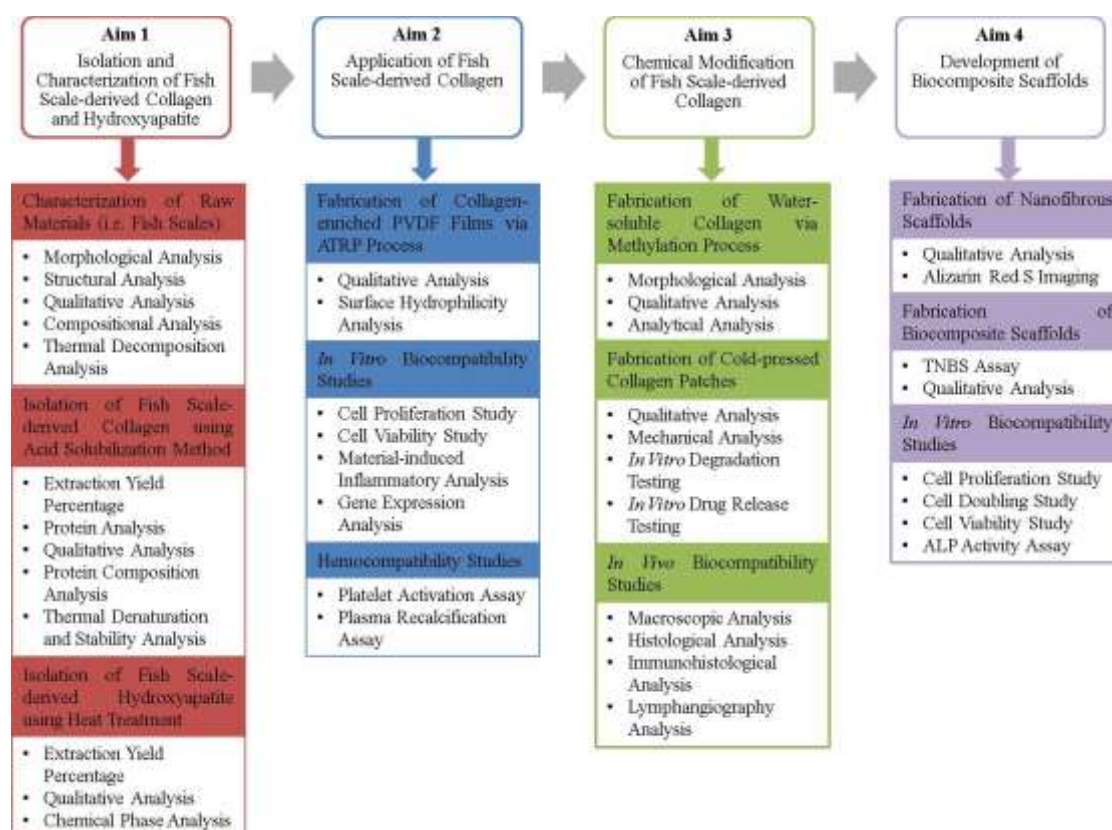


Figure 3.1 Flow diagram of the aims and the different approaches conducted for the thesis.

3.2 Materials

3.2.1 Source of FSCol and FSHA

Fish scales were kindly contributed by KhaiSeng Trading & Fish Farm Pte. Ltd., Singapore. Fish scales from different origin and living environment were collected and were used for the extraction of collagen and HA. The freshwater fish used were

snakehead (*Channa Micropeltes*) and tilapia (*Oreochromis Mossambicus*) while the seawater fish seabass (*Lates Calcarifer*) was used as control (**Figure 3.2**).



Figure 3.2 Different species of fish as a source of FSCol and FSHA and the macroscopic images of the fish scales from different fish species (Scale bars represent 10 cm for fish species and 3 mm for fish scales respectively).

3.2.2 Chemical Reagents

All chemicals mentioned in this thesis were purchased from Sigma-Aldrich (USA) and were used without further purification, unless otherwise specified.

3.2.3 Cell Culture Reagents

All cell culture reagents mentioned in this thesis were purchased from Sigma-Aldrich (USA), unless otherwise specified.

3.2.4 Cell Lines

Human umbilical vein endothelial cells (HUVECs, EndoGRO™, SCCE001) were purchased from Merck Millipore (Germany). Human fetal osteoblastic (hFOB) 1.19

cells (ATCC[®] CRL-11372[™]) were purchased from American Type Culture Collection (ATCC; USA).

3.3 Material Synthesis and Scaffold Fabrication Techniques

3.3.1 Extraction of FSCol and FSHA

The application of a waste-to-resource strategy using the abundantly available aquaculture waste material, fish scales, is an effective waste management system. Fish scales contain both the collagen and HA with low risk of disease transmission, thus it could be alternative safer and cost effective biomaterials for TE applications. In order to extract collagen from fish scales, acid solubilization followed by salt precipitation were carried out according to the previously established method [1]. All extraction procedures were performed at 4 °C to preserve the quality of the collagen. Briefly, the freshly obtained fish scales were washed thoroughly with distilled water to remove any blood and impurities. Subsequently, the fish scales were treated with 0.5 M sodium hydroxide (NaOH) at a solid-to-solvent ratio (wt/vol) of 1:10 for 48 h with a solution change every 24 h to remove non-collagenous protein on the fish scales as well as the black pigments. Next, the cleaned fish scales were treated with 0.5 M disodium ethylenediaminetetraacetate dihydrate (EDTA; Bio-Rad, USA) at pH 7.7 for 48 h with a solution changed every 24 h to decalcify the fish scales. Next, the decalcified fish scales were treated with 0.5 M acetic acid for 48 h for the extraction of collagen from the decalcified fish scales. The extracted collagen was then transferred into a beaker with the addition of 0.9 M of sodium chloride (NaCl) to induce salting of the collagen for 24 h followed by centrifugation at 10,000 $\times g$ for 1 h at 4 °C to collect the collagen salt. The collagen pellet was then reconstituted in a 5 mL of 0.5 M acetic

acid and dialyzed using 10 K MWCO SnakeSkin[®] dialysis tubing (Thermo Scientific – Pierce, USA) against 0.1 M acetic acid for 24 h followed by distilled water for another 24 h. The purified collagen was then lyophilized and kept at 4 °C until required.

In order to obtain the FSHA, fish scales were heat-sintered in order to decompose all the organic components within the fish scales according to previously established method [2]. Briefly, the freshly obtained fish scales were washed thoroughly with distilled water to remove any blood and impurities followed by drying the fish scales under room temperature. The dried fish scales were then transferred into an alumina combustion boat crucible and sent for heat treatment. The fish scales were heated at a constant rate of 10 °C/min to a temperature of 850 °C using a furnace and kept constant at this temperature for 1 h. The heat-sintered FSHA was collected and stored in a dry cabinet until required.

3.3.2 Fabrication of Collagen-enriched PVDF films via ATRP process

In order to investigate the applicability of the extracted collagen, different species of FSCol were conjugated onto the PVDF films via ATRP process according to previously established method [1]. Subsequently, the cell-material interactions of the collagen-conjugated PVDF films were evaluated using HUVECs. Commercially available bovine collagen (BVCol) was used as control. The overall reaction pathway used is illustrated in **Figure 3.3**. As seen from the figure, PVDF films were grafted with hydrophilic 2-hydroxyethyl methacrylate (HEMA) brushes via surface initiated ATRP process, followed by immobilization of 1,1'-carbonyldiimidazole (CDI) onto the PVDF-*g*-pHEMA films prior to the conjugation of collagen.

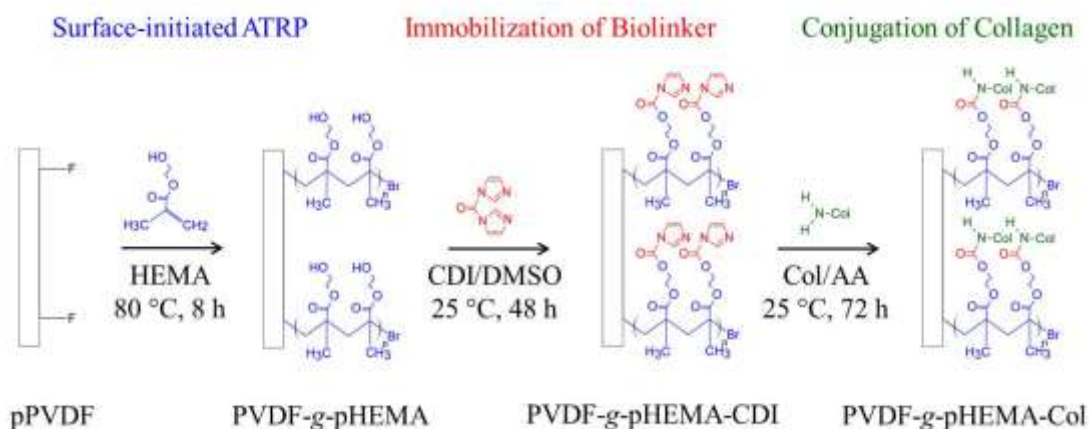


Figure 3.3 Schematic representation of the reaction pathway of the surface-initiated ATRP process used to obtain collagen-conjugated PVDF films [1].

Briefly, the PVDF films were cut into disc-shaped substrates of 1.5 cm in diameter and washed thoroughly with acetone (Fisher Scientific, UK), followed by absolute ethanol (Merck Millipore, USA) and then distilled water for 5 min each to remove grease and any organic contaminants before drying in a vacuum oven at 40 °C. The cleaned PVDF films were then added into a 50 mL round bottom flask containing 5 mL of deionized water and 12.37 mmol of HEMA solution followed by a degassing step for 30 min using pure argon gas. Subsequently, 0.12 mmol copper (I) bromide (CuBr), 0.025 mmol copper (II) bromide (CuBr₂) and 0.25 mmol 1,1,4,7,10,10-hexamethyltriethylenetetramine (HMTETA) were added into the reaction mixture making a final molar ratio of [HEMA (monomer)]:[CuBr (catalyst)]:[CuBr₂ (deactivator)]:[HMTETA (ligand)] of 500:5:1:10 in deionized water. The reaction tube was then sealed and the reaction was allowed to proceed for 8 h at 80 °C to produce PVDF-g-pHEMA films. At the end of the reaction, the PVDF-g-pHEMA films were washed thoroughly with absolute ethanol and deionized water to remove the unreacted

reactants adsorbed on the ATRP-modified PVDF films and dried in a vacuum oven at 40 °C.

Subsequently, the PVDF-*g*-pHEMA films were soaked in 5 mL of dimethyl sulfoxide (DMSO) solution containing 0.5 g of CDI for the immobilization of the biolinker. This reaction was carried out at room temperature for 48 h to produce PVDF-*g*-pHEMA-CDI films. At the end of the reaction, PVDF-*g*-pHEMA-CDI films were washed thoroughly with tetrahydrofuran (THF) and deionized water, and dried in a vacuum oven at 37 °C. Lastly, the PVDF-*g*-pHEMA-CDI films were immersed in 3 mg/mL of collagen solution dissolved in 0.5 M acetic acid and stirred at room temperature for 72 h to produce PVDF-*g*-pHEMA-Col films. The collagen-enriched PVDF films were then washed with absolute ethanol and deionized water to remove any unreacted collagen, and dried in a vacuum oven at 40 °C before storing at dry cabinet until required.

3.3.3 Fabrication of Water-soluble Collagen via Methylation Modification

FSCol was chemically-modified via methylation modification to produce a water-soluble MCol according to previously established method [3, 4]. The reaction involves the partial esterification of the carboxyl acid groups of collagen with the acidified methanol as shown in **Figure 3.4**. Briefly, the lyophilized collagen was treated with dehydrated methanol (Merck) consists of 0.1 M hydrochloric acid (HCl; Merck, Germany) for 24 h at 4 °C to produce MCol. At the end of the reaction, the MCol was dialyzed using 10 K MWCO SnakeSkin[®] dialysis tubing against distilled water for 72 h with solution changed every 24 h to remove excessive HCl. The purified MCol was then lyophilized and stored in dry cabinet until required.

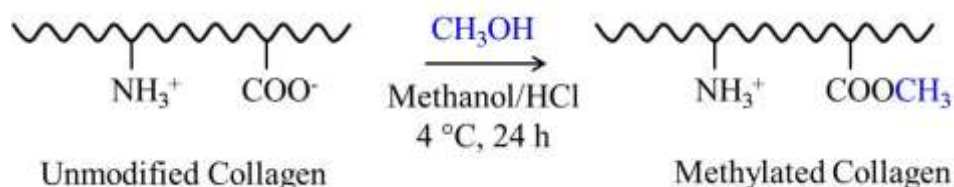


Figure 3.4 Chemical modification of UCol to produce water-soluble MCol via the methylation modification [3].

3.3.4 Fabrication of Cold-pressed Collagen Patches

In order to investigate the effect of methylation modification and BDE-crosslinking on the physicochemical properties of the collagen samples, different collagen patches were fabricated. The overall crosslinking mechanism of the BDE in both acidic and alkali conditions is illustrated in **Figure 3.5**. Briefly for the uncrosslinked UCol collagen patches, 5 mg/mL of UCol solution was prepared by dissolving lyophilized UCol in 0.5 M acetic acid. Subsequently, 0.6 mL of the UCol solution was aliquoted into 24-well plates and lyophilized to produce UCol scaffolds. The lyophilized UCol scaffolds were then cold-pressed to produce thin UCol patches using Carver Model 3889 auto C hot press machine for 10 min at 147.1 kN. For the fabrication of BDE-crosslinked UCol patches, 5 w/w% and 15 w/w% of BDE crosslinker were used. Briefly, BDE crosslinker was added to the 5 mg/mL of UCol solution and stirred for 24 h at 4 °C according to previously established method [5]. At the end of the reaction, 0.6 mL of the BDE-crosslinked UCol solutions was aliquoted into 24-well plates followed by lyophilization and cold-pressing to produce BDE-crosslinked UCol patches. For the MCol patches, similar procedures were performed, except that the MCol was dissolved in distilled water instead of 0.5 M acetic acid.

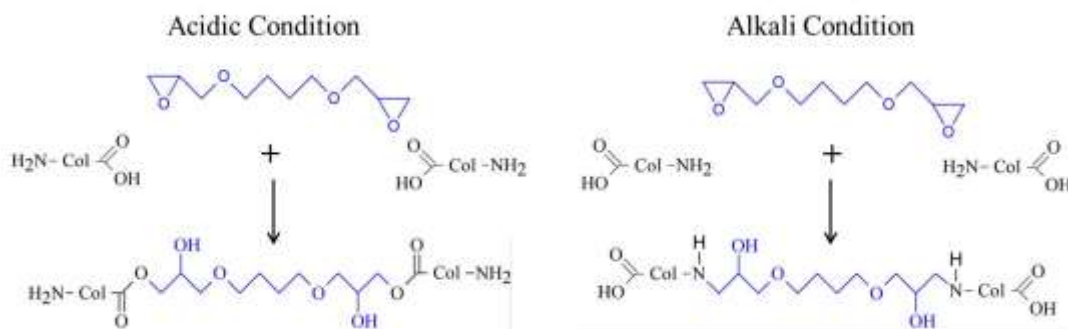


Figure 3.5 Crosslinking mechanism of BDE under different pH conditions [6].

3.3.5 Fabrication of Electrospun Nanofibrous Scaffolds

The electrospun nanofibrous PCL (ePCL) scaffolds were fabricated according to previously established method [7]. Briefly, 10 wt% of PCL was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) solution and loaded into 5 mL syringe attached to a 22 G blunt tip needle where the solution was pumped at a feeding rate of 3 mL/h using a syringe pump (Harvard Apparatus, USA) in a horizontal setup. The electrospun fibers were generated using a high-voltage power supply (Gamma High-Voltage Research, USA) at 11 kV and the fibers were collected using an aluminium foil placed at a distance of 8 cm from the tip of the needle. During each fabrication, 0.75 mL of the PCL solution was electrospun and the collected fibers were then dried in a vacuum oven overnight to produce ePCL scaffolds. For the fabrication of electrospun nanofibrous PCL/FSHA (ePCL/FSHA) scaffolds, 10 wt% of FSHA were added into the PCL solution during the electrospinning process. Commercially available synthetic HA (SynHA) was used as control to produce electrospun nanofibrous PCL/SynHA (ePCL/SynHA) scaffolds.

3.3.6 Fabrication of Biocomposite Scaffolds

In order to enhance the cytocompatibility of the PCL scaffolds, collagen was grafted onto the PCL scaffolds via an aminolysis reaction and GA coupling according to previously established method [8, 9]. Briefly, the electrospun nanofibrous scaffolds were chemically-modified via aminolysis reaction to introduce active free amine groups ($-NH_2$) onto the surface. Subsequently, a two-step crosslinking process was employed where the aminolyzed scaffolds were firstly treated with GA followed by covalent coupling of collagen onto the surface-activated scaffolds to prevent protein aggregation [9]. The overall reaction pathway is illustrated in **Figure 3.6**.

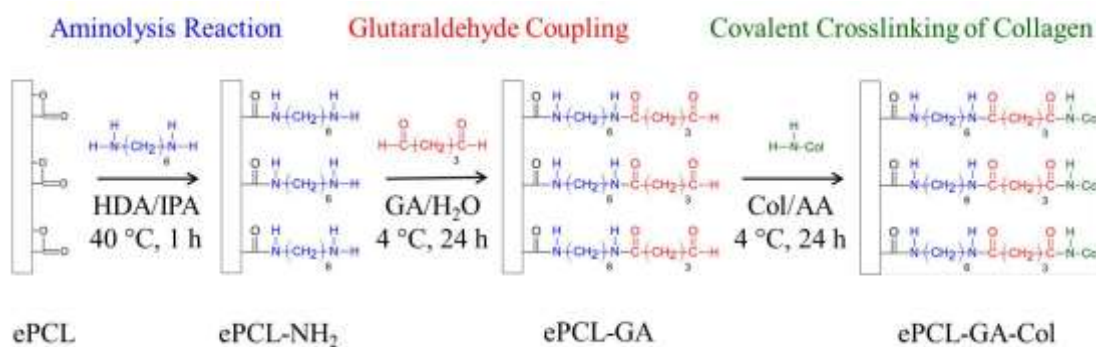


Figure 3.6 Schematic representation of the reaction pathway of the aminolysis reaction and GA coupling to immobilize collagen on electrospun nanofibrous scaffolds [8, 9].

Briefly, all the electrospun nanofibrous scaffolds, namely ePCL, ePCL/FSHA and ePCL/SynHA scaffolds, were cut into 15.6×15.6 mm square pieces and immersed in isopropanol (Fisher Scientific, UK) containing 10 w/w% of 1,6-hexanediamine at $40\text{ }^{\circ}\text{C}$ for 1 h to produce ePCL-NH₂, ePCL/FSHA-NH₂ and ePCL/SynHA-NH₂ scaffolds respectively. At the end of the reaction, all the scaffolds were washed with large

amounts of isopropanol followed by distilled water to remove unreacted 1,6-hexanediamine, and dried in a vacuum oven at 37 °C. Next, the aminolyzed electrospun nanofibrous scaffolds were immersed in 1 wt% of GA solution for 24 h at 4 °C to produce ePCL-GA, ePCL/FSHA-GA and ePCL/SynHA-GA scaffolds. At the end of the reaction, all the scaffolds were rinsed with large amounts of distilled water to remove free GA. Lastly, all the scaffolds were incubated either in 3 mg/mL of FSCol dissolved in 0.5 M acetic acid for 24 h at 4 °C to produce different biocomposite scaffolds (ePCL-GA-FSCol, ePCL/FSHA-GA-FSCol and ePCL/SynHA-GA-FSCol scaffolds). At the end of the reaction, all the samples were washed with distilled water, and dried in a vacuum oven at 37 °C before storing at dry cabinet until required.

3.4 Material Characterization

3.4.1 Extraction Yield Percentage

The extraction YD% of the collagen and HA (**Section 3.3.1**) was calculated using the ratio of the products obtained to the initial amount of air dried fish scales [10, 11]. The YD% of the collagen and HA was calculated using **equation (1)** and **equation (2)** respectively.

$$\text{YD\% (Collagen)} = M_C / M_S \times 100 \% \dots\dots\dots (1)$$

M_C = mass of the lyophilized collagen

M_S = mass of the air dried fish scales

$$\text{YD\% (HA)} = M_H / M_S \times 100 \% \dots\dots\dots (2)$$

M_H = mass of the heat-sintered HA powders

M_S = mass of the air dried fish scales

3.4.2 SEM Analysis

The morphologies of the various samples were imaged using JSM-6360LV Scanning Electron Microscope (SEM; JEOL Co., Japan) according to previously established method [1]. Briefly, the samples were coated with a thin layer of gold using SPI Module Sputter Coater (SPI Supplies Inc., USA) and the samples were imaged at 5 kV with a magnification of $\times 350$ and $\times 2000$ or 15 kV with a magnification of $\times 1000$.

3.4.3 SEM-EDX Analysis

The elemental compositions of the various samples were identified using SEM equipped with Energy Dispersive X-ray (EDX) following previously established method [2]. Briefly, the samples were sputtered coated with a thin layer of gold and imaged at 20 kV with a magnification of $\times 1000$. For the EDX analysis, the elemental spectrum was collected where the peaks were used to identify and to quantify the elements present in the sample.

3.4.4 ATR-FTIR Analysis

The chemical bonds in the various samples were identified via Attenuated Total Reflectance Fourier Transform Infrared Analysis (ATR-FTIR) using Spectrum GX (Perkin Elmer Inc., USA) according to previously established method [1]. Briefly, the

samples were placed directly onto the ATR sampling accessory with a germanium crystal at an incident angle of 45° and a sampling area of 2 mm. The samples were scanned in a range of $4000 - 650 \text{ cm}^{-1}$ with a resolution of 4 cm^{-1} over 16 – 32 scans to produce an infrared spectrum to identify the chemical bonds based on the peak number obtained. For the different species of extracted collagen (**Section 3.3.1**), further analysis was carried out using the absorption spectra where the peak intensity ratio of the A'(Amide III)/A'(1450) was calculated as an indication of the amount of triple helical structures preserved in the collagen molecules.

3.4.5 ^1H -NMR Analysis

The functional groups present in the various samples were identified using proton Nuclear Magnetic Resonance (^1H -NMR) spectroscopy following previously established method [2]. Briefly, the 4 mg of the sample was dissolved in 1 mL of the deuterium oxide (D_2O ; 99.96 atom% D) where the solution was subjected to Bruker Avance 300 MHz spectrometer (Bruker Instruments Inc., USA) to obtain a ^1H -NMR spectrum where the peak at certain chemical shift represent the functional group present in the various samples.

3.4.6 XRD Analysis

The chemical phases of the various samples were identified using X-ray Powder Diffraction (XRD) instrument equipped with $\text{CuK}\alpha$ -radiation ($\lambda = 1.5406 \text{ \AA}$) following previously established method [2]. Briefly, the powder was loaded onto the sample holder where the scanning was done using Shimadzu XRD-6000 (Shimadzu Corp.,

Japan) under theta/2theta mode and a step size of 0.05 °. To obtain the XRD profile, the powder was scanned in a range of $20 < 2\theta < 60$ with a scan speed of 1 °/min where the spectrum was compared to the database from “International Centre of Diffraction Data” according to Powder Diffraction File (PDF) to identify the different chemical phases present in the various samples.

3.4.7 Protein Analysis

3.4.7.1 SDS-PAGE Analysis

The extracted collagen (**Section 3.3.1**) was subjected to Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) to separate and to analyze the protein according to protocol established by Laemmli [12]. Briefly, 10 µg of the lyophilized collagen was added to a 1× SDS Loading Dye and heated at 95 °C for 5 min. Subsequently, the collagen was loaded in 10 % polyacrylamide gel where the migration was done with a current of 0.2 A for 1 h 45 min. After the electrophoresis process, the polyacrylamide gel was stained with Bio-Safe™ Coomassie Brilliant Blue R-250 (Bio-Rad, USA) and all the separated protein bands were viewed using an enhanced chemiluminescent (ECL) substrate on photographic film. BVCol was used as control to compare the different protein bands obtained.

3.4.7.2 Amino Acid Composition Analysis

The protein composition of the extracted collagen (**Section 3.3.1**) was determined qualitatively and quantitatively using an amino acid analyzer according to previously established method [1]. Briefly, 10 mg of the sample was dissolved in 25 mL of 6 M

HCl and hydrolyzed at 110 °C for 22 h with nitrogen protection. At the end of the reaction, 1 mL of the solution was removed and dried at 55 °C using nitrogen. Subsequently, the dried sample was dissolved in 1 mL of 0.02 M HCl and subjected to the Hitachi amino acid analyzer model L-8900 (Tokyo, Japan) to determine the different types and the amount of the amino acid present in the various samples.

3.4.8 WCA Analysis

The surface hydrophilicity of the various samples were measured by static Water Contact Angle (WCA) analysis according to previously established method [8]. Briefly, 3 μ L of the distilled water was added onto each of the sample surface using the sessile drop method to form a water droplet where the measurement was done using a FTÅ 200 contact angle goniometer (First Ten Angstroms Inc., USA) equipped with an automated dosing system and contact angle determination software to calculate the mean contact angle of the water droplet.

3.4.9 TGA Analysis

The decomposition temperatures of the fish scales from different fish species (**Section 3.2.1**) were identified using Thermogravimetric Analysis (TGA) following previously established method [13]. Briefly, the fish scales were washed thoroughly with distilled water and air dried. 5 – 10 mg of each sample was then loaded onto a flat platinum pan and heated to 900 °C using TGA Q500 (TA Instruments, USA) at a heating rate of 10 °C/min and under a nitrogen atmosphere (50 mL/min) to obtain the thermal decomposition profile of the sample.

3.4.10 DSC Analysis

The thermal stability of the extracted collagen (**Section 3.3.1**) was determined by measuring the denaturation temperature (T_d) of the extracted collagen using Differential Scanning Calorimetry (DSC) following previously established method [13]. Briefly, the extracted collagen samples were soaked in 1× PBS overnight at room temperature. Subsequently, 5 – 15 mg of each sample was blotted dried and placed in an aluminium hermetic pan before sealing. The sample was heated using DSC-Q10 Analyzer (TA Instruments, USA) at a rate of 2 °C/min from 15 – 85 °C against an empty reference pan under a nitrogen atmosphere (50 mL/min). The T_d was measured as the peak temperature of the endothermic peak.

3.4.11 Mechanical Analysis

The mechanical properties of the various samples were determined by measuring the tensile modulus of the sample following previously established method [2]. Briefly, the sample was prepared in accordance to ASTM D638V and loaded to an Instron mechanical tester Model 5567 (Instron Corp., USA). Subsequently, the sample was pulled at a rate of 10 mm/min with a load cell of 10 kN until the sample rupture to obtain a stress-strain curves where the initial gradient was measured as the tensile modulus.

3.4.12 *In Vitro* Degradation Analysis

The *in vitro* degradation profiles of the various samples were determined using the Bicinchoninic Acid (BCA) assay according to manufacturer's protocol [14]. Briefly,

different collagen patches were treated with 2 units/mL of Type I collagenase solution (Life Technologies – Gibco®, NZ). The samples were incubated at 37 °C with constant shaking at 60 rpm using an orbital shaker (Bibby Sterilin Ltd., UK) over 2 weeks. On various time points, the degraded filtrate was replaced and the amount of degraded collagen in the filtrate was quantified using BCA Protein Assay Kit (Thermo Scientific – Pierce™, USA) by measuring the colorimetric changes using a SpectraMax M2 microplate reader (Molecular Devices, USA) at 562 nm. The amount of protein was subsequently calculated using a standard curve that correlate the known concentration of protein with the obtained absorbance intensity.

3.4.13 *In Vitro* Drug Release Analysis

The *in vitro* drug release profiles of the various samples were determined using fluorescein isothiocyanate-conjugated bovine serum albumin (FITC-BSA) as drug model in accordance to previously established method [2]. Briefly, 20 µg/mL of the FITC-BSA was added into each sample prior to crosslinking and lyophilization. Subsequently, each sample was cold-pressed and immersed in 1 mL of 1× PBS solution incubated at 37 °C for 28 days. Each day, 1× PBS solution was replaced and 200 µL of the solution was scanned using a SpectraMax M2 microplate reader under fluorescence mode with an excitation wavelength of 495 nm and emission wavelength of 519 nm at 20 flashes per read to obtain a cumulative FITC-BSA release profile.

3.4.14 Alizarin Red S Imaging

The HA particles dispersed in the various samples were imaged by Alizarin Red S staining and imaging under light microscopy in accordance to previously established method [14, 15]. Briefly, a 40 mM of Alizarin Red S solution was prepared using distilled water and adjusted the pH to 4.1. Subsequently, the samples were immersed in Alizarin Red S solution for 24 h to allow the binding of Alizarin Red S onto the HA. Next, the Alizarin Red S stained samples were washed thoroughly with distilled water until the wash water was nearly clear. The cleaned samples were imaged using Primo Vert light microscope (Carl Zeiss, Germany) to visualize the HA particles.

3.4.15 TNBS Assay

The amount of free amino groups on the various samples after surface modification (**Section 3.3.6**) was determined using the 2,4,6-Trinitrobenzene Sulfonic Acid (TNBS) assay according to the manufacturer's protocol [16]. Briefly, the samples were placed in a microtubes followed by addition of 400 μL of 0.1 M sodium bicarbonate (pH 8.5) and 250 μL of 0.1 w/v% of TNBS. The mixture was incubated at 40 °C for 2 h. At the end of the incubation, 300 μL of 12 M HCl was added to the sample and incubated for another 2 h at 60 °C. At the end of the incubation, the presence of free amino groups resulted in the colorimetric changes where the intensity was measured using a SpectraMax M2 microplate reader at 335 nm.

3.5 Cellular Studies and Molecular Assays

3.5.1 Cell Culture Procedures

Two types of cells, namely HUVECs and hFOB 1.19 cells were cultured for different studies. HUVECs were cultured and expanded in 75 cm² tissue culture flasks using EndoGro-LS endothelial cell culture complete medium (Merck Millipore, Germany) supplemented with 1× antibiotic-antimycotic (Life Technologies – Gibco®, USA). The cells were cultured at 37 °C with 5 % carbon dioxide (CO₂) environment and saturated humidity. The cells were trypsinized upon reaching 80 % confluency using 0.25 % Trypsin-EDTA (Life Technologies – Gibco®, USA) where passages 4 – 6 were used for various experiments. hFOB 1.19 cells were cultured and expanded in 75 cm² tissue culture flasks using Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F-12) medium supplemented with 10% Fetal Bovine Serum (FBS; Research Instruments Pte. Ltd. - GE Hyclone™, USA), 1× antibiotic-antimycotic, 1.6 g/L sodium bicarbonate and 2.5 mM L-glutamine. The cells were cultured at 34 °C with 5 % CO₂ environment and saturated humidity. The cells were trypsinized upon reaching 80 % confluency using 0.5 % Trypsin-EDTA where passages 4 – 6 were used for various experiments.

3.5.2 Sterilization and Preparation of Scaffolds for Cellular Studies

For the collagen-enriched PVDF films (**Section 3.3.2**), the films were sterilized using ethylene oxide (EtO) gas for 12 h. The sterilized films were seeded with HUVECs with a cell density of 1.25×10^4 cells/cm². For the biocomposite scaffolds (**Section 3.3.6**), the scaffolds were sterilized using 70 % ethanol for 2 h and then washed with

1× PBS for another 2 h. The sterilized scaffolds were seeded with hFOB 1.19 cells with a cell density of 3×10^4 cells/cm².

3.5.3 Cell Proliferation Analysis

Cell proliferation was measured using PrestoBlue® cell viability reagent (Molecular Probes®, USA) following the manufacturer's protocol [1]. Briefly, the cells were incubated with 10 % PrestoBlue® reagent in cell culture medium 37 °C with 5 % CO₂ environment and saturated humidity. The collagen-enriched PVDF films (**Section 3.3.2**) and the biocomposite scaffolds (**Section 3.3.6**) were incubated for 1 h and 30 min respectively. After the reaction, 200 µL of the reagent containing medium was transferred to a 96-well plate where the fluorescence intensity was analyzed using a SpectraMax M2 microplate reader at an excitation wavelength of 560 nm and an emission wavelength of 590 nm. The number of cells was calculated using a standard curve correlating the fluorescence intensity to known number of cells. Lastly, the samples were incubated with fresh cell culture medium until the next time point.

3.5.4 Cell Population Doubling Analysis

The population doubling profile of the cells could be obtained from further analysis of the cell proliferation data using the following **equation (3)**.

$$\text{Population Doubling} = [\ln(n_f) - \ln(n_i)] / \ln(2) \dots\dots\dots (3)$$

n_f = final cell counts on respective time point

n_i = initial cell counts on previous time point

3.5.5 Cell Viability Imaging

The cell viability of the cells was assessed using fluorescence-based chemical staining and imaged under fluorescence microscope in accordance to previously established method [1]. Briefly, 20 μ M fluorescein diacetate (FDA) was prepared using cell culture medium. Subsequently, 200 μ L of the working solution was added to each well and incubated at room temperature for 5 min and the sample was viewed under Zeiss Axio Observer Z1 inverted fluorescence microscope (Carl Zeiss, Germany) fitted with a camera, where FDA with a green fluorescence was used to assess the viable live cells at excitation wavelength of 488 nm and emission wavelength of 517 nm.

3.5.6 Material-induced Inflammatory Analysis

The material-induced inflammatory responses of the various samples were investigated by observing the amount of the leukocyte adhesion molecule [i.e. intracellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1)] expressed by HUVECs seeded on different sources of collagen (**Section 3.3.2**) [1]. Briefly, HUVECs seeded on different sources of collagen were trypsinized and washed with 1 $\times$ PBS. Subsequently, 5 w/v% of BSA was dissolved in cold 1 $\times$ PBS and added to the trypsinized HUVECs for 15 min to block the non-specific antibody binding sites. After the blocking was done, 1:200 v/v% of ICAM-1 or VCAM-1 mouse IgG anti-human antibodies (Biolegend®, USA) were added to the blocking solution containing HUVECs and stained for 45 min where mouse IgG isotype control antibody (Kirkegaard & Perry Laboratorie Inc. KPL, USA) and 100 ng/mL of recombinant human tumour necrosis factor alpha (TNF α ; R&D Systems™, USA) was used as negative and positive control respectively. Next, HUVECs were

washed with 1× PBS supplemented with 5 w/v% BSA and 0.5 v/v% Tween-20 (Bio-Rad, USA) before staining with a secondary antibody, goat Alexa Fluor® 488-conjugated anti-mouse IgG (H+L) (Life Technologies, Singapore), at 1:200 v/v% for 20 min. Lastly, HUVECs were washed with 1× PBS supplemented with 5 w/v% BSA and 0.5 v/v% Tween-20 and the surface expression of ICAM-1 and VCAM-1 was measured using LSRFortessa™ X-20 flow cytometer (BD Biosciences, USA).

3.5.7 Gene Expression Analysis

The gene and protein expressed by HUVECs seeded on the various samples were examined using real-time polymerase chain reaction (qPCR) following previously established method [1]. Briefly, RNA was extracted from HUVECs seeded on different collagen-enriched PVDF films using RNeasy® Mini Kit (Qiagen, Germany) and quantified using NanoDrop™ 2000 (Thermo Scientific, USA). Subsequently, 100 ng of the RNA was used for the synthesis of cDNA using Superscript® III First-Strand Synthesis Supermix. The qPCR analysis was carried out using Bio-Rad C1000™ thermal cycle (Bio-Rad, USA) at an annealing temperature of 58 °C containing 10 µL of SsoAdvanced™ SYBR® Green supermix, 2 µL of the synthesized cDNA, 200 nM of forward primer and 200 nM of reverse primer where the final volume was brought up to 20 µL using RNase/DNase free water. The threshold cycle (C_t) values obtained were used to calculate and to compare the mRNA expression of HUVECs seeded on different collagen-enriched PVDF films using $\Delta\Delta C_t$ method. All the primers and housekeeping gene used are listed in **Table 3.1**.

Table 3.1 Sequences of primers used for gene expression studies by HUVECs [1].

Transcripts	Primer Sequence (5' to 3')
<u>Pro-thrombotic Factors</u>	
von Willebrand factor (vWF)	S: CACCATTCAGCTAAGAGGAGG A: GCCCTGGCAGTAGTGGATA
Plasminogen activator inhibitor (PAI-1)	S: TTGGTGAAGGGTCTGCTGTG A: GGCTCCTTTCCCAAGCAAGT
<u>Anti-thrombotic Factors</u>	
Tissue plasminogen activator (tPA)	S: ATGGGAAGACATGAATGCAC A: GAAAGGGGAAGGAGACTTGA
Endothelial nitric oxide synthase (eNOS)	S: AGCTGTGCTGGCATAACAGGA A: ATGGTAACATCGCCGCAGAC
Collagen IV	S: CCTGGCTTGAAAGGTGATAAG A: CCCGCTATCCCTTGATCTC
<u>Housekeeping Gene</u>	
GAPDH	S: CCCCTTCATTGACCTCAACTACA A: TTGCTGATGATCTTGAGGCTGT

3.5.8 Hemocompatibility Studies

The hemocompatibility of the various samples were investigated using platelet activation and plasma recalcification assays following previously established protocols [1]. Briefly, the citrated whole blood was collected from human volunteers according to the protocols approved by the Institutional Review Board (IRB-2014-08-002) of Nanyang Technological University (NTU), Singapore. Subsequently, citrated whole

blood was layered into individual component (i.e. platelet-rich plasma (PRP), peripheral blood mononuclear cells and red blood cells) by carefully adding 4 mL of the citrated whole blood onto 3 mL of Ficoll-PaqueTM PLUS (GE Healthcare, Sweden) followed by centrifugation at 400 ×g for 30 min at 25 °C. For the platelet activation assay, PRP layer was collected and diluted in 1× PBS at (100 times dilution). Next, 500 µL of the diluted PRP solution was added to HUVECs seeded on different sources of collagen and incubated for 2 h at 37 °C. Non-activated platelets were used as negative control while adenosine diphosphate (ADP)-activated platelets were used as positive control by incubating the platelets with 30 µM of ADP. At the end of the incubation period, the excess PRP solution was removed and the surface was washed thoroughly with 1× PBS to remove the unattached platelets. Next, 5 w/v% of BSA was dissolved in cold 1× PBS was added to block non-specific antibody binding sites for 15 min followed by the addition of 5 µL of the primary antibody (anti-CD62P IgG1) to the samples/positive control at 1:200 v/v% dilution and addition of IgG1 isotype control to the negative control. The samples were incubated for 1 h at 37 °C followed by washing with 1× PBS supplemented with 5 w/v% BSA and 0.5 v/v% Tween-20 (Bio-Rad, USA) before staining with secondary antibody, 5 µL of horseradish peroxidase-conjugated sheep anti-mouse polyclonal antibody (Kirkegaard & Perry Laboratorie Inc. KPL, USA), at 1:200 v/v% for 1 h at 37 °C. Lastly, the stained samples were washed with 1× PBS supplemented with 5 w/v% BSA and 0.5 v/v% Tween-20 followed by the addition of 200 µL of 3,3',5,5'-tetramethylbenzidine (TMB) chromogenic solution for 10 min. Lastly, the reaction was stopped by adding 400 µL of 1 M H₂SO₄ where the color of the solution was measured using SpectraMax M2 microplate reader at 450 nm in absorbance mode to obtain the optical density values.

In this case, a higher optical density value is an indication of higher number of activated platelets interacted and attached to the samples.

For the plasma recalcification assay, the PRP layer was collected and further centrifuged at $2000 \times g$ for 15 min at 25°C to obtain platelet-poor plasma (PPP). Next, 400 μL of PPP was added to HUVECs seeded on different sources of collagen and incubated for 10 min at 37°C . Tissue culture plate was used as negative control. At the end of incubation period, 100 μL of the PPP was transferred to a 96-well plate followed by the addition of 100 μL of 0.025 M calcium chloride, pre-warmed at 37°C . Subsequently, the plate was immediately loaded to a SpectraMax M2 microplate reader where the absorbance was recorded with a kinetic mode at 400 nm for 60 min at an interval of 30 s. The clot initiation time was taken as the onset point of the absorbance intensity over time curve.

3.5.9 ALP Activity Assay

The intracellular alkaline phosphatase (ALP) activity of the cells was measured using the ALP assay following previously established protocols [17]. In general, ALP reacts with 4-methylumbelliferyl phosphate (MUP) to produce a fluorescence 4-methylumbelliferone (MU) product. Hence, ALP activity of the cells can be determined by measuring the rate of 4-MU formation. Briefly, 50 μL of 0.2 % Nonidet P40 substitute was added to the cells followed by cells lysis using freeze-thaw process. Next, 10 μL of TRIS/BSA solution and 100 μL of the 4-MUP substrate working solution were added to the lysated cells and incubated for 30 min at 37°C . At the end of the reaction, 100 μL of 0.6 M sodium bicarbonate was added to stop the reaction and the fluorescence intensity of the solution was measured using SpectraMax M2

microplate reader at an excitation wavelength of 360 nm and emission wavelength of 450 nm. The amount of 4-MU produced was calculated using a standard curve that correlate the known concentration of 4-MU with the obtained fluorescence intensity. Subsequently, the ALP activity was calculated using the following **equation (4)**.

$$\text{ALP Activity} = A/V/T \text{ (mU/mL) (4)}$$

A = amount of 4-MU in nmol

V = volume of the sample in assay well (0.05 mL)

T = time of incubation (30 min)

Finally, the ALP activity was normalized against the number of cells to compare the ALP activity across different sample groups.

3.6 In Vivo Studies

3.6.1 Subcutaneous Implantation Procedures

The *in vivo* studies were carried out using C57/BL6 mice following previously established procedures [18]. The experiments were carried out in accordance to protocols approved by the Institutional Animal Care and Use Committee (IACUC, Reference Code: R15-0315) of National University of Singapore (NUS) and the mice were maintained in pathogen-free conditions. Briefly, the C57/BL6 mice were anaesthetized where the hind limb was shaved. Subsequently, the afferent lymphatic vessel was stained by directly injecting 1 w/v% Evans Blue dye through the footpad where about 2.5 mm of the afferent lymphatic vessel was excised. Next, different collagen patches with a size of 4 × 4 mm square piece was placed at the excised lymphatic vessel and secured in place using absorbable sutures prior to skin closure

using interrupted stitch. The mice were monitored with the injection of 0.1 mg/kg buprenorphine (NUS Comparative Medicine Unit) as post-operative analgesia and 85 mg/kg/day enrofloxacin (NUS Comparative Medicine Unit) as post-operative antibiotic for the first three and five days respectively. Lastly, the mice were euthanized and analyzed after 21 days of implantation. Mice with an excised lymphatic vessel without any collagen patch were used as control group. 21 days post-implantation, the mice were euthanized where the collagen patches were excised together with surrounding tissue for histological analysis.

3.6.2 H&E Staining

The tissue interaction with the various samples was analyzed by histological analysis to view the cells infiltration and the biocompatibility of various samples. Briefly, the samples were fixed using 2 w/v% of paraformaldehyde containing 30 w/v% of sucrose for 12 h at 4 °C. Subsequently, the fixed samples were washed with 1× PBS for thrice followed by immersed in Tissue-Tek® Optimum Cutting Temperature compound (Sakura Finetek, USA) and frozen at -20 °C. The samples were then cryosectioned into 10 µm sections using microtome at -20 °C where the samples were collected and adhered onto a glass slide. The samples were de-paraffinized using xylene and re-hydrated using ethanol gradient series. Subsequently, the nuclei of the samples were stained with hematoxylin (H) by dipping the glass slide with tissue sections in hematoxylin stain for 5 min and rinsed with distilled water for 30 s. The hematoxylin-stained samples were then immersed in 1 v/v% of HCl in absolute ethanol to destain and to differentiate the hematoxylin into hematein. The samples were then rinsed with distilled water for 30 s followed by dipping in 0.1 w/v% of sodium bicarbonate to

induce the color changes of hematoxylin-stained sample from purple to blue. The samples were then rinsed with distilled water for 30 s. Next, the cytoplasm and ECM of the samples were stained by dipping the samples into eosin (E) stain for 30 s and rinsed with distilled water for another 30 s to remove excessive stain. Lastly, the H&E-stained samples were dried using ethanol gradient series and mounted where samples were visualized under light microscope.

3.6.3 Masson's Trichrome Staining

In order to differentiate between the collagen and the muscle tissues, the samples were further analyzed using Masson's trichrome staining. The de-paraffinized samples were prepared using method described in **Section 3.6.2**. Subsequently, the samples were fixed in Bouin's solution for 1 h at 56 °C following by washing with distilled water. Next, the samples were soaked in Weigert's iron hematoxylin working solution for 10 min to stain the nuclei followed by rinsing with distilled water for 10 min to remove excessive stain. The samples were then soaked in Biebrich scarlet-acid fuchsin solution for 15 min to stain the cytoplasm and the muscle components followed by washing with distilled water for 10 min to remove excessive stain. In order to remove the Biebrich scarlet-acid fuchsin stain from the collagen, the samples were treated with phosphomolybdic-phosphotungstic acid solution for 15 min until the collagen turned from red color to colorless. Next, the collagen was stained with aniline blue solution for 10 min followed by rinsing with distilled water. The Masson's trichrome-stained samples were immersed in 1% acetic acid for 5 min to improve the shades of color followed by washing thoroughly with distilled water. Lastly, the samples were dried

using ethanol gradient series and mounted using resinous mounting medium where samples were visualized under light microscope.

3.6.4 Immunohistological Analysis

Immunohistological analysis was performed to further confirm the histological analyzed data and to visualize the presence of BVs and LVs according to previously established method [18]. Briefly, the collagen patches were excised together with surrounding tissue/skin area and mounted where the cell nuclei was firstly stained with 4',6-diamidino-2-phenylindole. Subsequently, the mounted sample was fixed with 2 w/v% of paraformaldehyde for 12 h at 4 °C. The fixed sample was immersed in 1× PBS supplemented with 0.5 w/v% BSA and 0.3 v/v% Triton X-100 for 12 h at 4 °C to block all the non-specific antibody binding sites. After the blocking was done, primary antibodies, such as Rabbit anti-lymphatic vessel endothelial hyaluronic acid receptor-1 (LYVE-1) polyclonal antibody (1.67 µg/mL; abcam®, USA), Armenian hamster anti-CD31 (2.5 µg/mL; Merck Millipore, Germany), rat anti-CD68 (3.33 µg/mL; AbD Serotec, UK), PE-conjugated anti-CD45 (1:500 dilution; eBioscience, USA) and Cy3-conjugated monoclonal smooth muscle actin (1.50 µg/mL; Sigma, USA), were added and incubated for 12 h at 4 °C. The primary antibodies stained sample was then washed with 1× PBS supplemented with 0.5 w/v% BSA and 0.3 v/v% Triton X-100 before staining with secondary antibodies, such as Cy2-conjugated anti-rabbit, Alexa Fluor® 647-conjugated anti-Armenian hamster, Cy3-conjugated anti-rat, Alexa Fluor® 488-conjugated anti-rat and DyLight™ 549-conjugated anti-rat (1:500 dilution; Jackson ImmunoResearch Laboratories, USA) respectively and incubated for 1.5 h at 37 °C. Lastly, the secondary antibodies stained sample was washed and viewed under

fluorescence microscope using Zeiss Axio Imager Z1 (Carl Zeiss, Germany) fitted with a AxioCam HRM camera with an excitation wavelength.

3.6.5 Lymphangiography Analysis

The spontaneous lymphatic reconnection was carried out according to previously establish protocol [19]. Briefly, mice were sedated during the lymphangiography using 100 mg/kg ketamine (NUS Comparative Medicine Unit) and 10 mg/kg xylazine (NUS Comparative Medicine Unit). Subsequently, 10 μ L of Lectin-FITC was injected into the hind footpad of the mice where the flow of the Lectin-FITC to the popliteal lymph node was examined and compared to the sham control group. Finally, the popliteal lymph nodes were isolated to examine the reconnection of the LVs by assessing the presence of fluorescence signal under fluorescence microscope.

3.7 Statistical Analysis

All experiments were carried out in triplicate ($n = 3$) and expressed as mean $\pm$ standard deviation (SD), unless otherwise specified. The statistical analysis was done using Kruskal-Wallis non-parametric one-way analysis of variance and Mann-Whitney U test where the data was considered to be statistical significant when $p < 0.05$.

Acknowledgement

All the chemical structures used in this thesis were drawn using ACD/ChemSketch (version 14.01, USA). Isosurface rendering and confocal z-stacks images were processed

using Imaris (version 5.0.3, Switzerland). All the graphs were drawn using OriginPro 8.5 (version b161, USA).

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Chapter 4

Study 1 – Extraction and Characterization of Collagen and HA from Fish Scales from Different Fish Species

This chapter describes the detailed characterization of the fish scales from different fish species, and includes the extraction of FSCol and FSHA using a modified acid solubilization extraction and heat-sintering methods respectively. After extraction, the properties of the extracted products are compared across different fish species.

4.1 Introduction

The aquaculture industry is one of the fastest growing food industries due to the increasing demand and consumption of fish products in most countries [1]. However, during food processing, large amounts of aquaculture waste material are being generated. These waste material are often discarded or being utilized as low value-added products [2-4]. In fact, aquaculture waste material in the form of fish scales, contain both collagen and HA, which are important biomaterials used in biomedical applications. In addition, fish-derived collagen is zoonosis free and is not associated to any religious or ethical issues as compared to commercially available mammalian-derived collagen [5]. Therefore, fish scales can potentially serve as low cost alternative sources of collagen and HA for biomedical usage. In general, the applicability of fish-derived collagen is highly dependent on its thermal stability, since this property of collagen will determine the overall mechanical stability and biodegradation rate of the material [6]. However, most of the fish-derived collagen are associated with poor thermal stability that could potentially cause the implant to fail or may even lead to partial tissue regeneration when implanted into human body [6]. In addition, studies have shown that the thermal stability of collagen is affected by the living habitat and the body temperature of the fish species [7, 8]. Therefore, we hypothesize that by using freshwater fish as a source of fish scales and by using a modified acid solubilization extraction method, collagen with high thermal stability will be easily extracted from fish scales with preserved triple helical structures and biological function for biomedical usage.

Overall, the specific objectives and aims of this chapter are as follows:

1. Characterization of the fish scales from different fish species
2. Extraction of the collagen and HA from fish scales from different fish species
3. Characterization of the collagen and HA from fish scales from different fish species

4.2 Materials and Methods

Fish scales were obtained from KhaiSeng Trading & Fish Farm Pte. Ltd., Singapore as mentioned in **Chapter 3, Section 3.2.1**. The freshwater fish used were snakehead (*Channa Micropeltes*) and tilapia (*Oreochromis Mossambicus*) while the seawater fish seabass (*Lates Calcarifer*) was used as the control. The fish scales from different fish species were firstly examined to identify the presence of collagen and HA using SEM (**Chapter 3, Section 3.4.2**), SEM-EDX (**Chapter 3, Section 3.4.3**), ATR-FTIR (**Chapter 3, Section 3.4.4**) and TGA (**Chapter 3, Section 3.4.9**) analyses respectively. Subsequently, FSCol and FSHA were extracted according to the methods described in **Chapter 3, Section 3.3.1**. The extracted FSCol was characterized in terms of its YD% (**Chapter 3, Section 3.4.1**), the presence of chemical bonds and functional groups (**Chapter 3, Section 3.4.4**), SDS-PAGE (**Chapter 3, Section 3.4.7.1**), protein composition (**Chapter 3, Section 3.4.7.2**) and its thermal stability (**Chapter 3, Section 3.4.10**). On the other hand, the extracted FSHA was characterized in terms of its YD% (**Chapter 3, Section 3.4.1**), the presence of chemical bonds and functional groups (**Chapter 3, Section 3.4.4**) and its phase composition (**Chapter 3, Section 3.4.6**).

4.3 Results and Discussion

4.3.1 Characterization of Fish Scales

4.3.1.1 Morphological Studies

The fish scales from the different fish species were viewed under the light microscope to identify the types of fish scale from different fish species. As mentioned in **Chapter 2, Section 2.3.1**, elasmoid scales are commonly found in the majority of the bony fish (teleost fish). In general, elasmoid scales come in two different forms, namely cycloid scales and ctenoid scales [9, 10]. Cycloid scales exhibit a smooth and circular outline at the posterior side of the scale, whereas ctenoid scales have a small projection structures (i.e. ctenii) at the posterior portion of the scale [11, 12]. As seen from **Figure 4.1**, cycloid scales were identified for freshwater snakehead and tilapia. On the other hand, ctenoid scales were identified for seawater seabass. Although freshwater fish possesses cycloid scales while seawater fish possesses ctenoid scales, both cycloid and ctenoid scales share the similar fundamental structure, which is mainly composed of Type I collagen and mineralized HA crystal [13]. The only main difference between the cycloid and ctenoid scales is the fundamental shape of the scales.

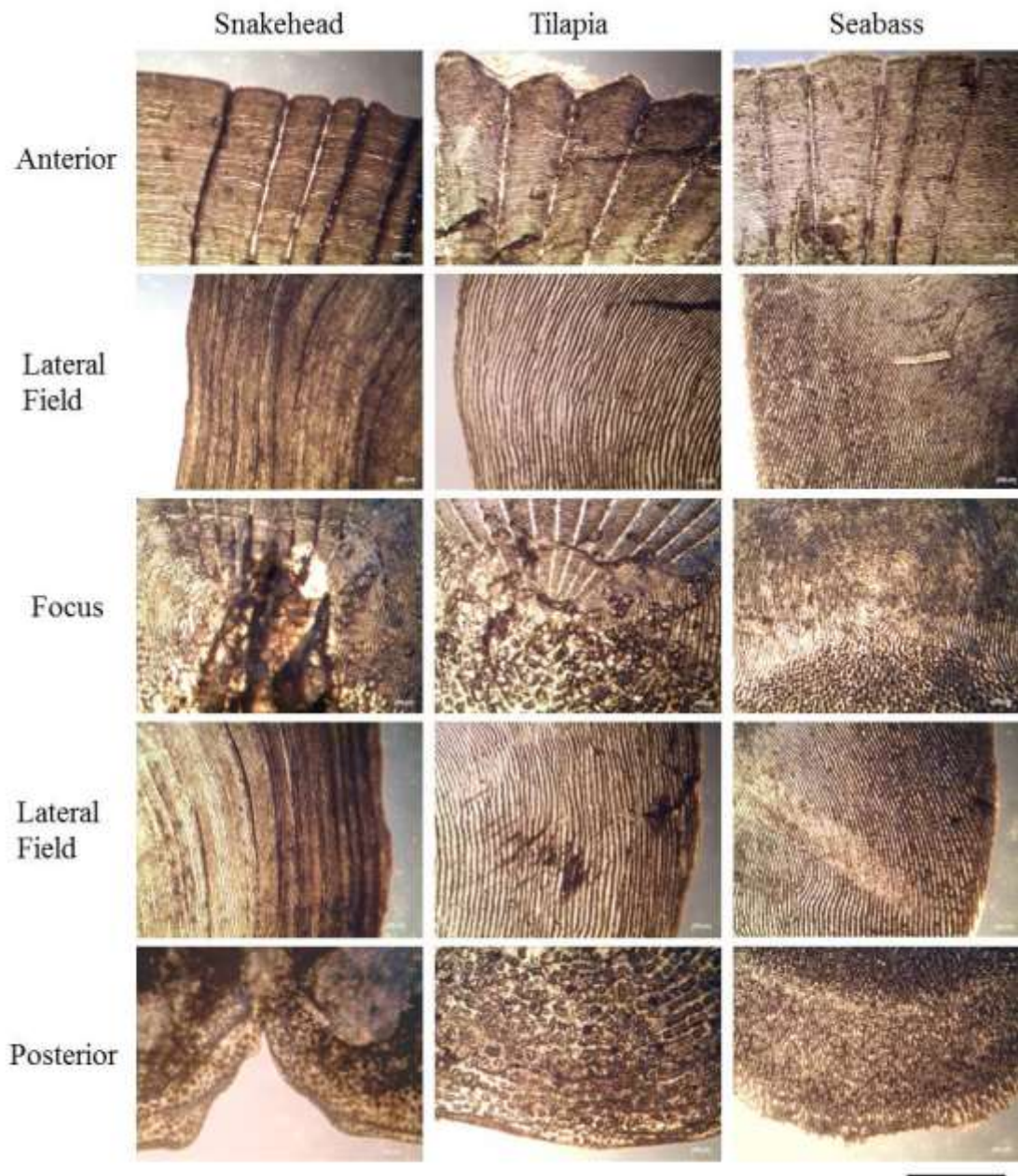


Figure 4.1 Light microscopic images for different regions of fish scales from different fish species (scale bar represents 1 cm).

4.3.1.2 Structural Analysis

The fractured section of fish scales from different fish species was imaged using the SEM as shown in **Figure 4.2**. As seen from the figure, fibrous layers of collagen were

observed in the fish scales from different fish species regardless of the living environment. In addition, the collagen layers were oriented in a plywood-like manner. Although cycloid scales were identified for freshwater fish while ctenoid scales were identified for seawater fish (**Figure 4.1**), both cycloid and ctenoid scales fall under the same classification of elasmoid scales. Hence, all the fish tested had similar structures consisting of highly oriented fibrous collagen layers in the elasmodin layer of elasmoid scales, which was also reported by others for scales from chub (*Leuciscus cephalus*) and sea bream (*Pagrus major*) [14, 15].

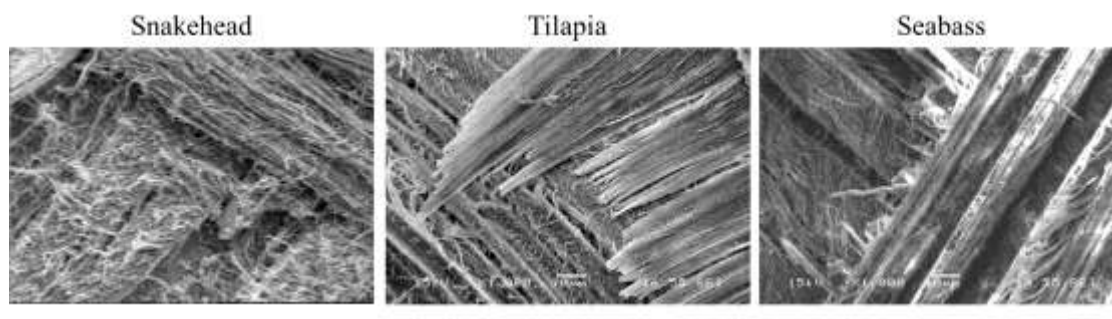


Figure 4.2 SEM images of the fractured section of fish scales from different fish species (scale bar represents 300 μm).

4.3.1.3 Chemical Analysis

The different types of chemical bonds present in the fish scales were identified using ATR-FTIR. As all the different fish species contain elasmoid scales (**Figure 4.1**), similar ATR-FTIR patterns were identified regardless of the fish species and living environment (**Figure 4.3**). In general, the characteristic amide peaks of the collagen were detected in the fish scales from all fish species tested. Amide A was detected near 3300 cm^{-1} and Amide B was detected near 2920 cm^{-1} due to N-H stretching. Other than Amide A and B, Amide I (C=O stretching), Amide II (C-N stretching and

N-H bending out of phase) and Amide III (C-N stretching and N-H bending in-phase α -helix) were also detected near 1630, 1530 and 1200 cm^{-1} respectively [16]. In addition to the collagen peaks, the characteristic phosphate peaks of HA were also detected near 960 – 1100 cm^{-1} . Taken together, the ATR-FTIR spectra showed the presence of both collagen and HA within the fish scales.

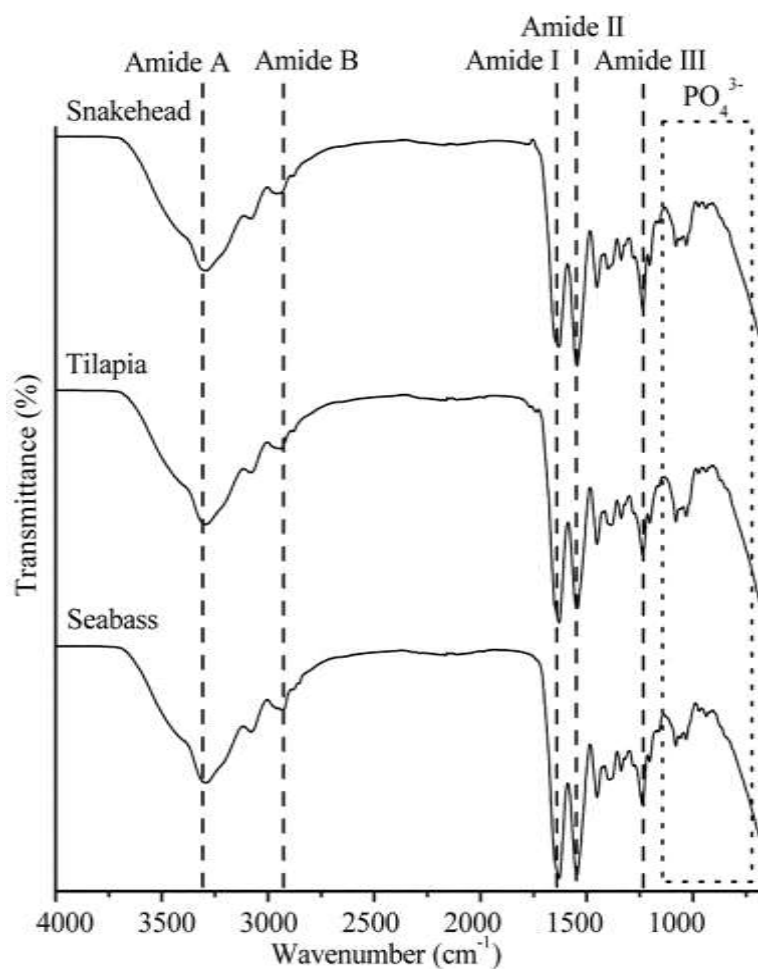


Figure 4.3 ATR-FTIR results for fish scales from different fish species (dashed lines represent amide peaks; dotted box represents phosphate peaks).

4.3.1.4 Thermal Decomposition Analysis

The decomposition profiles of the fish scales from different fish species were obtained from TGA analysis. As all the different fish species possess the same classification of fish scales (i.e. elasmoid scales as shown in **Figure 4.1**), similar TGA trends were identified as shown in **Figure 4.4**. As seen from the figure, decomposition of the fish scales were observed to have commenced at around 280 – 290 °C for all the three species of fish scales. At that point in time, decomposition of the organic substance, namely collagen, within the fish scales would have taken place, resulting in an overall decrease in the weight percentage. As the temperature continued to increase, further decrease in the weight percentage was observed until 800 °C where the weight percentage remained constant. The high percentage of residues remaining could be attributed to the presence of inorganic compounds, which is believed to be the HA component of fish scales.

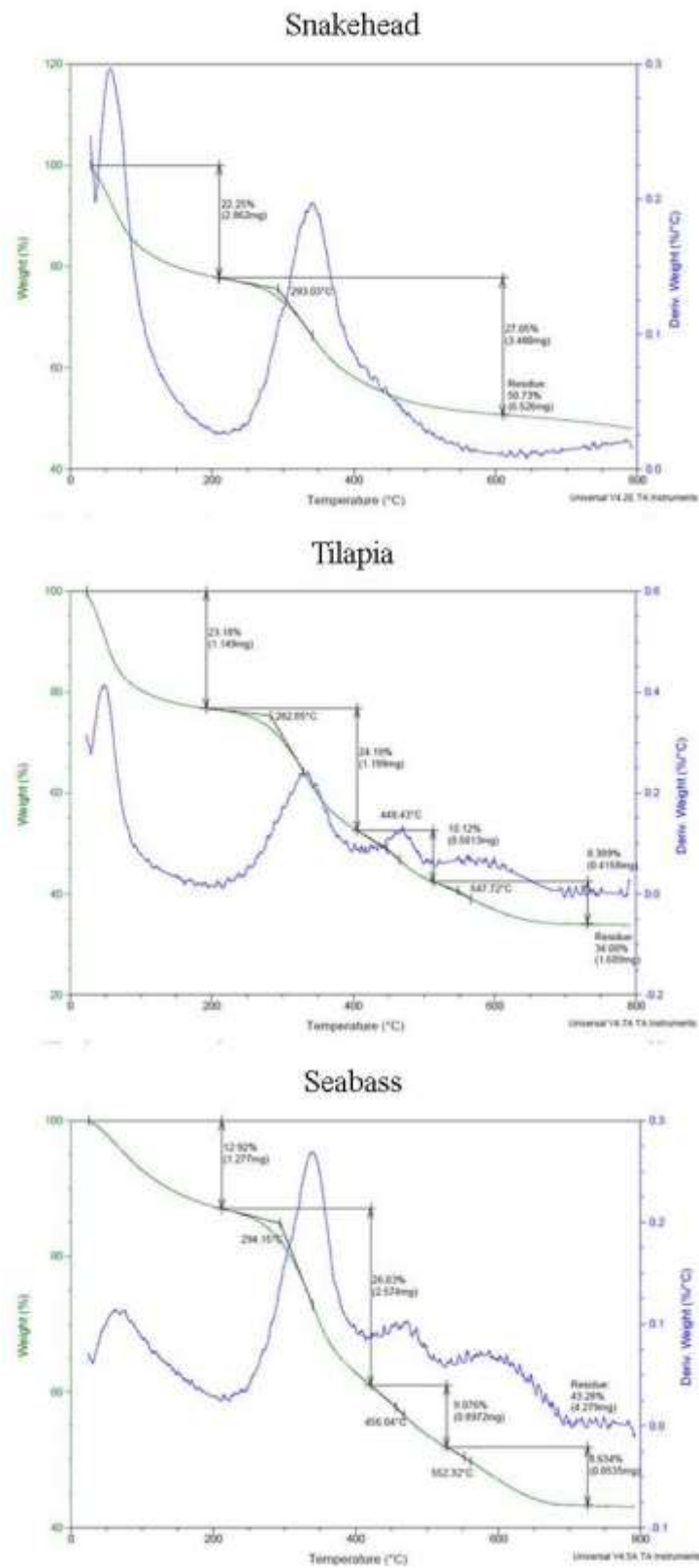


Figure 4.4 TGA results for fish scales from different fish species.

4.3.1.5 Composition Analysis

The elemental analysis of fish scales from different fish species was carried out using SEM-EDX (**Figure 4.5**). Elasmoid scales mainly consist of collagen and HA, and thus calcium (Ca) and phosphorus (P) elements were detected from all fish scales regardless of the fish species. The SEM-EDX results were further analyzed to obtain the Ca/P ratio. Based on the elemental analysis, the Ca/P ratio for snakehead, tilapia and seabass was calculated as 1.66 ± 0.22 , 1.39 ± 0.17 and 1.45 ± 0.42 respectively. However, no significant differences were observed for the Ca/P ratio among different fish species due to the similar type of scales was identified for all fish species regardless of the living environment. Overall, the elemental analysis showed the presence of the Ca and P elements within the fish scales from different fish species. In addition, the Ca/P ratio obtained was close to the stoichiometric ratio for HA (1.67) and human bone mineral (1.55-1.75) [17], which makes FSHA a suitable source of bioactive component for bone regeneration applications. Taken together, a series of analysis confirmed the presence of both collagen and HA within the fish scales from different fish species.

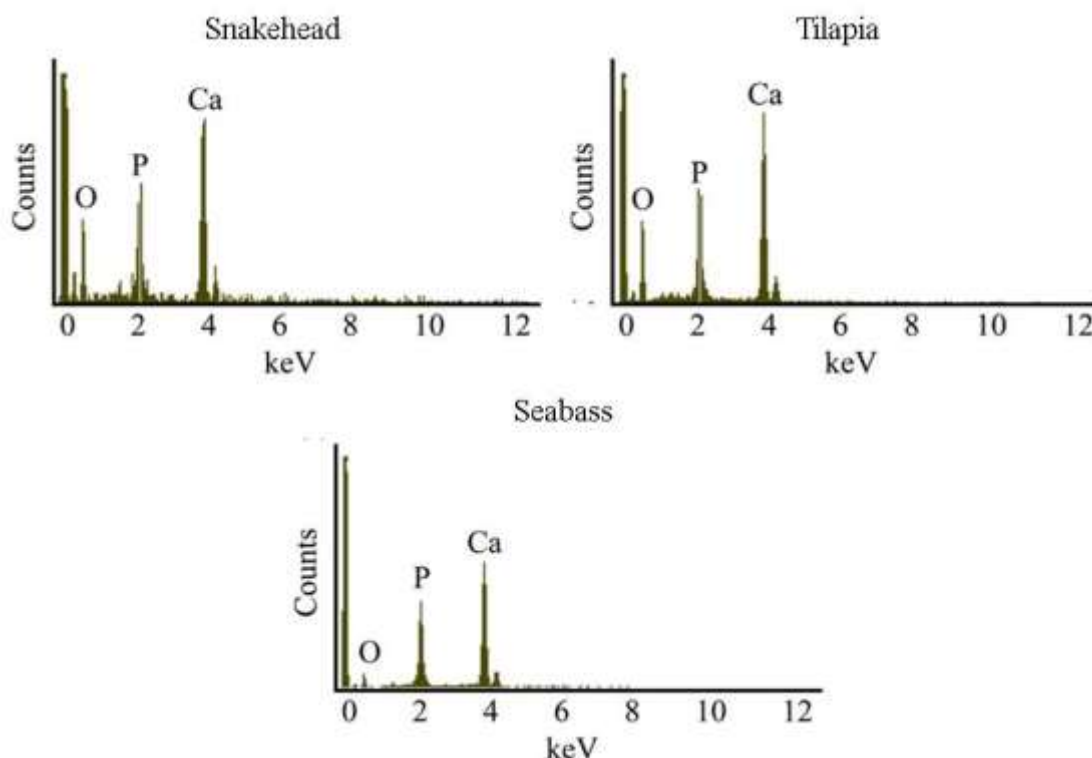


Figure 4.5 SEM-EDX results for fish scales from different fish species.

4.3.2 Characterization of FSCol

4.3.2.1 Extraction YD%

FSCol was successfully extracted from different fish species as shown in **Figure 4.6**. As seen from the figure, all the extracted FSCol from different fish species appeared white color, which is an indication that the cleaning and demineralization processes (**Chapter 3, Section 3.3.1**) used were effective in removing the black pigments from fish scales. The YD% of the extracted FSCol from different fish species was calculated based on the weight ratio of the lyophilized collagen to the air-dried fish scales and is summarized in **Table 4.1**, which was similar to that of the acid solubilized collagen from other fish species such as croceine croaker scales (*Pseudosciaena crocea*; ASC: 0.37%) [18], spotted golden goatfish scales

(*Parupeneus heptacanthus*; ASC: 0.46%) [19], silver carp scales (*Hypophthalmichthys molitrix*; ASC: 0.86%) [20] and carp scales (*Cyprinus carpio*; ASC: 1.35%) [21]. In general, the YD% of the extracted FSCol from different fish species was low (< 10%) due to fish scales consist of large portion of heavy inorganic calcium phosphate compounds. Hence, the YD% obtained in this study is considered reasonable good. Overall, no significant differences were observed for the YD% among different species of FSCol regardless of the living environment of the fish, as all the fish scales belonged to the same classification of elasmoid scales (**Figure 4.1**). Since earlier studies have shown that the thermal stability of fish-derived collagen is affected by the living habitat and the body temperature of the fish species [7, 8], further analysis of the conformational stability of the triple helical structures and the thermal stability of the extracted FSCol were carried out to investigate the effect of the fish origin and living environment on the overall properties of the extracted FSCol.



Figure 4.6 Macroscopic images for FSCol extracted from different fish species (scale bar represents 1 cm).

Table 4.1 YD% for FSCol extracted from different fish species.

Sample	YD%
Snakehead scale-derived collagen (SHCol)	6.33 ± 3.08
Tilapia scale-derived collagen (TPCol)	5.32 ± 3.44
Seabass scale-derived collagen (SBCol)	3.06 ± 2.86

4.3.2.2 Protein Analysis

SDS-PAGE analysis was carried out for the FSCol from different species to identify the types of collagen found in FSCol. As all the different fish species belong to the same classification of fish scales (i.e. elasmoid scales as shown in **Figure 4.1**), and thus similar SDS-PAGE patterns were observed as shown in **Figure 4.7**. In general, elasmoid scales consist of Type I collagen and mineralized HA crystal [13]. Indeed, the subunit compositions of the extracted FSCol from different fish species were similar to the commercially available Type I BVCol. Several distinct bands were observed near 400 kDa, 250 kDa, 139 kDa and 129 kDa corresponding to γ -, β -, $\alpha 1$ - and $\alpha 2$ -chains respectively. In addition, the band width ratio of the $\alpha 1$ - and $\alpha 2$ -chains was found to be 2:1 [22, 23], which further confirmed that the extracted FSCol for all three fish species were Type I collagen. These results are similar to those reported by others for elasmoid scales from sea bream (*Pagrus major*), Asian sea bass (*Lates calcarifer*) and tilapia (*Oreochromis niloticus*) [15, 24, 25].

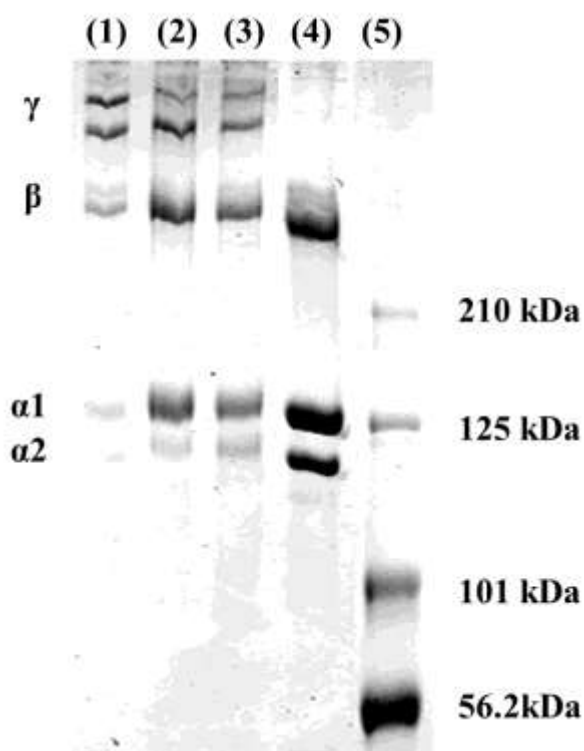


Figure 4.7 SDS-PAGE results for FSCol extracted from different fish species where commercially available BVCol was used as control [(1) SHCol; (2) TPCol; (3) SBCol; (4) BVCol and (5) protein ladder as marker].

4.3.2.3 Chemical Analysis

The different types of chemical bonds present in the extracted FSCol from different fish species were identified using ATR-FTIR. Since Type I collagen was identified for all the different species of FSCol (**Figure 4.7**), similar ATR-FTIR spectra were observed for the extracted FSCol from the different fish species as shown in **Figure 4.8a**. As seen from the figure, the ATR-FTIR spectra showed the presence of the characteristic amide peaks of the collagen, which were similar to the commercially available Type I BVCol control. In general, Amide A was detected near 3300 cm^{-1} and

Amide B was detected near 2920 cm^{-1} . Other than Amide A and B, Amide I, Amide II and Amide III were also detected near 1630 , 1530 and 1200 cm^{-1} respectively.

Although the morphological analysis showed that scales of freshwater fish and seawater fish were classified as elasmoid scales that shared the similar fundamental structure containing Type I collagen and HA (**Figure 4.1**), studies have shown that the living environment and the body temperature have a direct impact on the thermal stability of the collagen component within the fish scales [7, 8]. As mentioned in **Chapter 2, Section 2.2.2**, the thermal stability of collagen is highly dependent on the conformational stability and the amount of triple helical structures within the collagen. The amount of the triple helical structures preserved within the collagen was quantified by measuring the absorbance peak intensity ratio of Amide III to 1450 cm^{-1} [26]. In general, 1450 cm^{-1} peak corresponded to the vibration of pyrrolidine ring of proline and hydroxyproline, which is an invariant peak in relative to the denaturation extent of the collagen [27]. Hence, it is used as an internal reference for the quantitative analysis [28]. On the other hand, Amide III associated with the C-N stretching and N-H bending vibration from the amide linkages. In addition, Amide III is also assigned to the vibration of CH_2 groups in the glycine backbone and proline side chains that is crucial in bonding with the hydroxyl group of the pyrrolidine ring and the stability of the triple helical structure [29, 30]. As a result, a peak intensity ratio between the Amide III and $\text{A}'(1450)$ of close to unity (i.e. ~ 1.0) is used as an indication to evaluate the preservation of the triple helical structure and the structural conformation of the collagen [26, 29, 31, 32]. As seen from **Figure 4.8b**, the $\text{A}'(\text{Amide III})/\text{A}'(1450)$ for all the extracted FSCol from different fish species was significantly lower ($p < 0.05$) than BVCol due to the higher body temperature of mammalian animals as compared to aquatic animals. Hence, the higher amounts of

triple helical structures within BVCol suggest that the BVCol exhibits higher thermal stability as compared to the extracted FSCol. This observation is similar to those reported by others [33-35]. On the other hand, no significant differences were observed in $A'(\text{Amide III})/A'(1450)$ values for the extracted FSCol from different fish species regardless of the living environment, which could possibly due to the limitation of the modified acid solubilization extraction method used. Nevertheless, all the $A'(\text{Amide III})/A'(1450)$ ratios for the extracted FSCol were close to 1.0, which suggest that the majority of the triple helical structures within the extracted FSCol was well-preserved following the modified acid solubilization extraction method used regardless of the fish species [26, 28, 29, 31, 32, 36]. Subsequently, amino acid composition analysis was carried out to further investigate the conformational stability of the preserved triple helical structures.

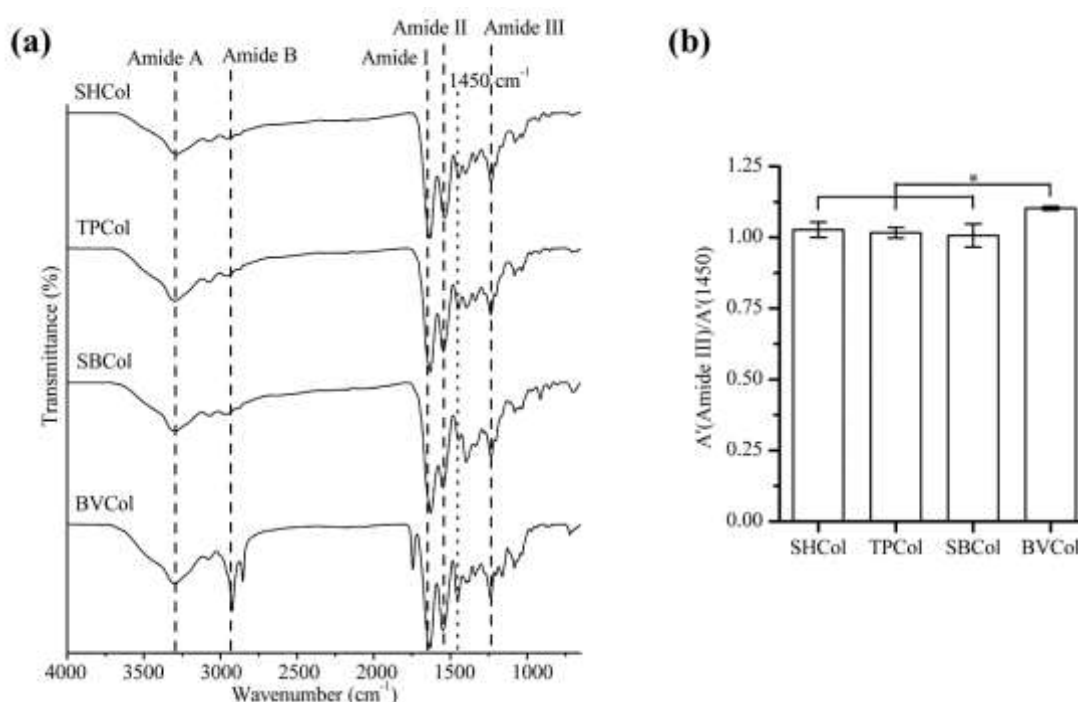


Figure 4.8 Chemical analysis showing (a) ATR-FTIR spectra for the extracted FSCol where commercially available BVCol was used as control and (b) quantitative analysis

of the amount of triple helical structures within different sources of collagen (dashed lines represent amide peaks; dotted line represents 1450 cm^{-1} peak; $*p < 0.05$).

4.3.2.4 Protein Composition Analysis

The amino acid composition of the extracted FSCol from different fish species are summarized in **Table 4.2**. Overall, the amino acid distribution for the extracted FSCol from different fish species was found to be similar to that of the commercially available Type I BVCol. In general, glycine was found to be the most abundant amino acid for all the different sources of collagen, which is typical for collagen and in accordance the collagen extracted from spotted golden goatfish scales (*Parupeneus heptacanthus*) and carp scales (*Cyprinus carpio*) [37]. When the amino acid compositions among different sources of collagen were compared, BVCol exhibited the highest amount of proline followed by freshwater fish (i.e. SHCol and TPCol) while seawater SBCol had the lowest amount of proline. In general, hydroxylation of proline has been shown to be involved in the folding of triple helical structures of collagen and the conformational stability of collagen [38, 39]. Hence, this suggests that BVCol had the highest conformational stability for the triple helical structures, and thus better thermal stability followed by freshwater SHCol and TPCol while seawater SBCol had the lowest thermal stability. Taken together, the living environment and the body temperature appear to have a direct impact on the amino acid composition of collagen and thus, the thermal stability and the potential applicability of FSCol for biomedical usage.

Table 4.2 Amino acid composition of the extracted FSCol where commercially available BVCol was used as control.

Types of Amino Acid	SHCol (residues/1000)	TPCol (residues/1000)	SBCol (residues/1000)	BVCol (residues/1000)
Aspartic Acid	41.40	43.37	42.85	48.45
Threonine	26.01	26.24	25.66	18.41
Serine	36.77	37.04	30.01	33.93
Glutamic Acid	70.48	73.70	68.00	74.00
Glycine	383.70	372.17	374.18	367.72
Alanine	134.42	134.97	138.04	122.17
Valine	18.04	21.42	18.26	19.30
Methionine	5.11	8.09	6.46	0.00
Isoleucine	10.35	9.36	10.73	12.02
Leucine	20.41	20.88	21.29	30.68
Phenylalanine	11.36	13.76	11.47	12.12
Lysine	24.69	23.15	24.92	23.43
Ammonia	30.40	30.77	44.81	41.70
Histidine	7.10	7.86	7.58	5.20
Arginine	49.10	48.68	50.10	54.28
Proline	130.65	128.54	125.65	136.59

4.3.2.5 Thermal Stability Analysis

The thermal stability of the extracted FSCol from different fish species was determined by measuring the T_d of the extracted FSCol using DSC analysis (**Figure 4.9**). During the heating process, the collagen fibrils underwent conformation changes caused by the breaking of crosslinked bonds between the intermolecular chains and the shrinkage of collagen fibrils [40]. The thermal shrinkage transition of the collagen is accompanied by the absorption of heat that resulted in an endothermic peak on the DSC curve [41]. This endothermic shrinkage temperature is often defined as the T_d of the collagen. Once again, BVCol exhibited the highest T_d ($p < 0.05$), followed by freshwater SHCol and TPCol while seawater SBCol had the lowest T_d , which is in agreement with the ATR-FTIR (**Figure 4.8**) and amino acid composition (**Table 4.2**) results. In general, T_d is highly dependent on the fish species and the habitat temperature, and thus collagen obtained from freshwater fish species exhibits higher thermal stability, similar to those reported by others [19]. Nevertheless, all the T_d of the extracted FSCol from different fish species were higher than the human body temperature of 37 °C, suggesting that the extracted collagen will not easily undergo dissolution when applied implanted *in vivo*. Interestingly, among different fish species, SHCol exhibited significantly higher ($p < 0.05$) T_d as compared to TPCol and SBCol. Overall, the results suggest that collagen extracted from fish scales using a modified acid solubilization extraction method can potentially be an alternative safe source of biomaterials for biomedical usage.

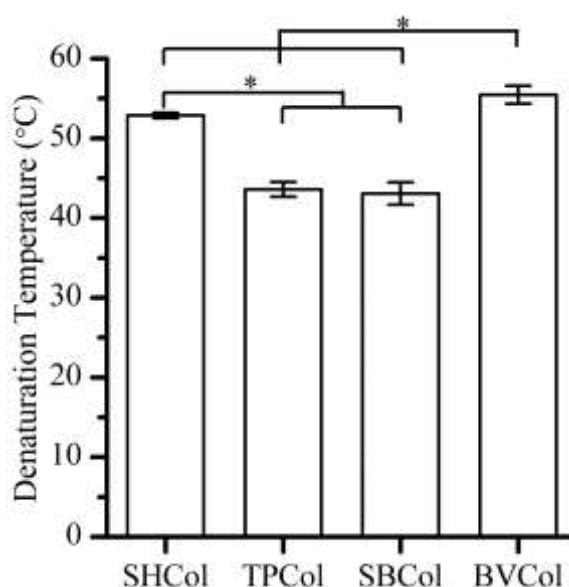


Figure 4.9 DSC results for the extracted FSCol from different fish species where commercially available BVCol was used as control (* $p < 0.05$).

4.3.3 Characterization of FSHA

4.3.3.1 Extraction YD%

FSHA was successfully extracted from different fish species using heat-sintering method as shown in **Figure 4.10**. The YD% of the extracted FSHA from different fish species was calculated based on the weight ratio of the heat-sintered FSHA to the air-dried fish scales and summarized in **Table 4.3**. Overall, scales from freshwater fish exhibited higher YD% as compared to seawater fish. Although both cycloid and ctenoid scales shared the same fundamental structure, the HA content of the cycloid scales from freshwater fish is higher than the ctenoid scales from seawater fish, which could possibly due to the effect of living environment in affecting the composition of the fish scales. In general, fish scales are the important site of ions storage. Hence, the differences in the chemical composition and the salinity between freshwater and seawater may have caused different ions exchange between the fish scales from

freshwater and seawater fish with the surrounding water [42], and thus resulted in different HA content. On the other hand, no significant differences were observed for the YD% among different species of freshwater fish as the same types of cycloid scales were identified for both snakehead and tilapia. Hence, further analysis was carried out to identify the chemical bonds and the chemical phases of the extracted FSHA.



Figure 4.10 Macroscopic images for FSHA extracted from different fish species (scale bar represents 1 cm).

Table 4.3 YD% for FSHA extracted from different fish species.

Sample	YD%
Snakehead scale-derived HA (SHHA)	41.93 ± 5.33
Tilapia scale-derived HA (TPHA)	37.80 ± 4.68
Seabass scale-derived HA (SBHA)	35.95 ± 0.67

4.3.3.2 Chemical Analysis

The different types of chemical bonds present in the extracted FSHA from different fish species were identified using ATR-FTIR (**Figure 4.11**). As all the different fish species had elasmoid type scales (**Figure 4.1**), which mainly composed of Type I collagen and HA, all the ATR-FTIR spectra for the extracted FSHA were similar to that of the commercially available SynHA regardless of the fish species and living environment. In general, the characteristic phosphate peaks were detected near 960 – 1100 cm^{-1} .

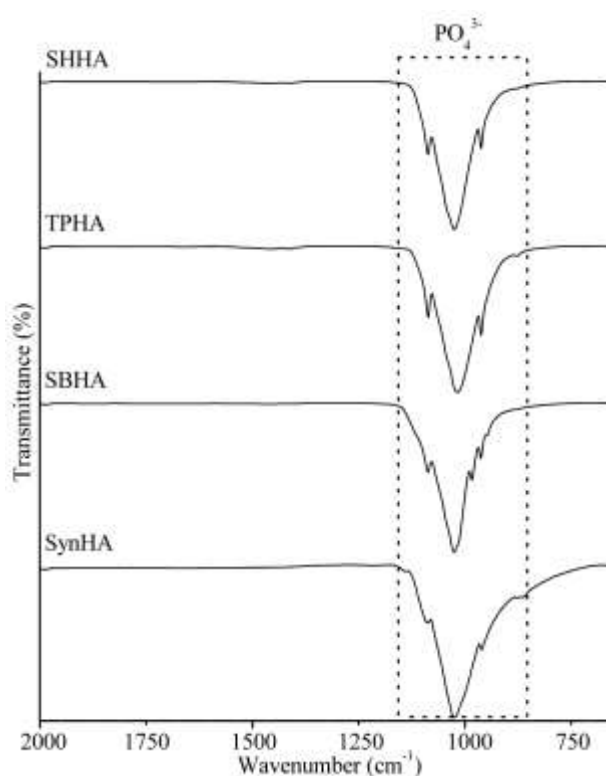


Figure 4.11 ATR-FTIR results for the extracted FSHA from different fish species where commercial available SynHA was used as control (dotted box represents phosphate peaks).

4.3.3.3 Chemical Phase Analysis

The XRD analysis of the extracted FSHA from different fish species was carried out to identify the chemical phases of the extracted FSHA as shown in **Figure 4.12**. Once again, the XRD spectra of the extracted FSHA from different fish species were similar to that of the SynHA due to the similar type of scales was identified for the different fish species (i.e. elasmoid scales as shown in **Figure 4.1**). In addition, the XRD spectra obtained matched that of the monophase HA pattern from PDF database. In general, high sintering temperature could potentially cause phase transformation of the inorganic compounds, in this case, the potential induction of the HA to β -TCP [17]. Hence, the presence of monophase HA was an indication that the heat-sintering temperature used allowed for the preservation of the crystal phase of the inorganic component without causing the phase transformation of the FSHA. Overall, HA was successfully extracted from fish scales, which is in accordance to those reported by others from tilapia scales (*Oreochromis sp.*) [43], Nile tilapia (*Oreochromis niloticus*) [44] and roho labeo (*Labeo rohita*) [45].

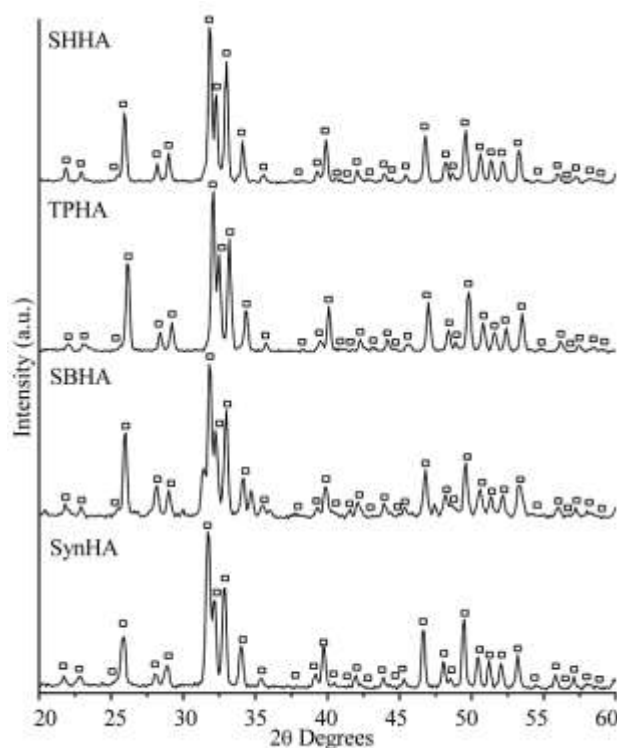


Figure 4.12 XRD results for the extracted FSHA from different fish species where commercial available SynHA was used as control (square boxes represent the monophase XRD profile from PDF database).

4.4 Summary

In this chapter, fish scales from different fish species were identified as elasmoid scales, where the presence of collagen and HA was detected through imaging and a series of chemical analysis. Subsequently, Type I collagen was successfully extracted from fish scales from different fish species using a modified acid solubilization extraction method with preserved amount of triple helical structures. When the conformational stability of the triple helical structures and the thermal stability of the extracted FSCol were compared, freshwater FSCol exhibited higher amounts of proline and a higher T_d as compared to seawater FSCol, with SHCol showing the

highest thermal stability, which is necessary for biomedical applications. In addition, a monophase HA was successfully extracted from fish scales from different fish species using a heat-sintering method with a heat-sintering temperature of 850 °C. Taken together, Type I collagen and monophase HA were successfully extracted from fish scales, which could be a potential alternative resource resilient, cost effective source of waste-to-resource bioactive materials for biomedical applications.

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Chapter 5

Study 2 – Application of FSCol

This chapter describes the detailed fabrication and characterization of PVDF films conjugated with FSCol extracted from different fish species using ATRP reaction. Subsequently, the cell-material interactions of different species of FSCol-conjugated PVDF films are evaluated using endothelial cells (i.e. HUVECs). Lastly, the in vitro hemocompatibility properties of different species of FSCol-conjugated PVDF films are assessed by investigating the interaction of different blood components with the FSCol-conjugated PVDF films.

5.1 Introduction

Type I collagen, the most abundant protein found in connective tissue, exhibits a superior biocompatibility and multiple cell binding sites in directing cellular behavior such as cell adhesion, proliferation and differentiation [1-4]. In addition, studies have shown that Type I collagen supports rapid endothelial cell attachment and proliferation, and thus it has been widely used as stent-grafting material for vascular applications [5, 6]. Hence, in this chapter, the potential of the extracted FSCol for blood-contacting applications was investigated. Briefly, the extracted collagen was conjugated onto a bioinert polymer (i.e. PVDF) via ATRP process, where the cell-material interactions and hemocompatibility of the collagen-conjugated PVDF films were assessed. PVDF polymer was chosen due to its excellent chemical stability, degradation resistance properties, excellent mechanical strength and non-toxic *in vivo*, which has been used as a membrane for hemodialysis, as a suture for cardiovascular surgery or other blood-contacting applications [7-10]. PVDF polymer is also chemically hydrophobic, which could potentially cause protein and platelets adsorption that are undesirable in blood-contacting applications [11, 12]. In addition, random protein adsorption could occur with the domination of non-adhesion proteins that could lead to poor cell adhesion [13]. Moreover, the low surface energy of the PVDF results in chemical inertness, which further limits its practical applications [14, 15]. Hence, surface modification was often carried out to improve both the surface properties and the cytocompatibility of the PVDF polymer [9].

In this particular study, the surface of the PVDF films was grafted with hydrophilic polymers via ATRP reaction followed by the conjugation of the extracted FSCol onto the chemically-modified PVDF films [16]. The selective conjugation of adhesion proteins (i.e. collagen) that contain high cell-binding domains onto the PVDF polymer

could ensure the subsequent cell-material interactions. In addition, ATRP reaction is a well-controlled radical polymerization method and its versatile and robust chemical reaction is applicable for wide range of polymers or functional groups [17]. In general, the ATRP reaction relies on the reversible redox activation between the dormant alkyl halide terminal of the polymer and the copper-based metal complex with a ligand. During the ATRP process, the halogen transfer to the transition metal complex produces the alkyl radical and oxidized transition metal-halide complex, where the hydrophilic monomer subunits were added onto the alkyl radical end [18, 19]. In the current study, with the pre-existing secondary fluorine atoms at the backbone of PVDF polymer, the direct surface-initiated ATRP reaction could be facilitated without any pre-treatment process required [19, 20]. Subsequently, a biolinker was crosslinked onto the hydrophilic polymer followed by the immobilization of collagen onto the chemically-modified PVDF films. Overall, the ATRP reaction could ensure a stronger chemical bonding between the collagen and the PVDF films as compared to physisorption, and thus improving the surface properties and the cytocompatibility of PVDF films.

Finally, the cell-material interactions and the hemocompatibility of different species of FSCol-conjugated PVDF films were investigated and compared to commercially available BVCol. The cell-material interactions were carried out using endothelial cells (i.e. HUVECs), where the cell proliferation, the inflammatory response and the gene expression of HUVECs seeded on the different species of FSCol-conjugated PVDF films were evaluated. The hemocompatibility of the collagen-conjugated PVDF films were carried out using different blood components to examine the platelet activation and plasma recalcification of different endothelialized collagen surfaces.

Overall, the specific objectives and aims of this chapter are as follows:

1. Fabrication and characterization of FSCol-conjugated PVDF films
2. Investigation of the *in vitro* cytocompatibility of FSCol-conjugated PVDF films using endothelial cells
3. Evaluation of the *in vitro* hemocompatibility of FSCol-conjugated PVDF films using different blood components

5.2 Materials and Methods

PVDF films were purchased from Goodfellow Cambridge Ltd. (UK). The PVDF films were firstly grafted with poly-HEMA (pHEMA) brushes via ATRP reaction as described in **Chapter 3, Section 3.3.2**. Subsequently, the surface-modified PVDF films were immobilized with CDI to facilitate the conjugation of different sources of collagen. The successful modification of the PVDF films was confirmed using ATR-FTIR (**Chapter 3, Section 3.4.4**) and static WCA (**Chapter 3, Section 3.4.8**) analyses respectively. Subsequently, the collagen-conjugated PVDF films were seeded with endothelial cells, HUVECs, and the cell-material interactions such as cell proliferation (**Chapter 3, Section 3.5.3**), cell viability (**Chapter 3, Section 3.5.5**), material-induced inflammatory (**Chapter 3, Section 3.5.6**) and gene expression (**Chapter 3, Section 3.5.7**) studies were carried out. Lastly, the *in vitro* hemocompatibility of the endothelialized collagen surface were evaluated by the platelet activation and plasma recalcification (**Chapter 3, Section 3.5.8**) assays.

5.3 Results and Discussion

5.3.1 Characterization of Collagen-conjugated PVDF Films

5.3.1.1 Chemical Analysis

The successful surface modification of the PVDF films was confirmed using ATR-FTIR to identify the changes in the chemical bonds at each modification step (**Chapter 3, Figure 3.3**). As seen from **Figure 5.1**, the characteristic carbon-fluorine peak was observed for the pristine PVDF (pPVDF) films near 1177.91 cm^{-1} . After the ATRP reaction, the successful grafting of the pHEMA brushes onto the pPVDF films was confirmed with the additional hydroxyl peak (3396.68 cm^{-1}) and carbonyl peak (1718.35 cm^{-1}) for the pHEMA-grafted PVDF (PVDF-*g*-pHEMA) films, which were absent in the pPVDF films. Subsequently, a biolinker was crosslinked onto the PVDF-*g*-pHEMA films to facilitate the conjugation of different sources of collagen onto the ATRP-modified PVDF films. The successful immobilization of the CDI onto the PVDF-*g*-pHEMA films was identified by the splitting of the carbonyl peak into two different carbonyl peaks, which correspond to the acrylate (1762.53 cm^{-1}) and imidazole (1724.85 cm^{-1}) groups of the CDI. In addition, a reduction in the hydroxyl peak intensity was observed, which is also an indication of the successful immobilization of CDI onto the hydroxyl site of pHEMA. The successful conjugation of different sources of collagen was confirmed with the presence of different characteristic amide peaks of the collagen namely amide B with a N-H stretching (2958.86 cm^{-1}), amide I with a C=O stretching (1661.26 cm^{-1}) and amide II with a C-N stretching and N-H bending out-of-phase (1555.42 cm^{-1}).

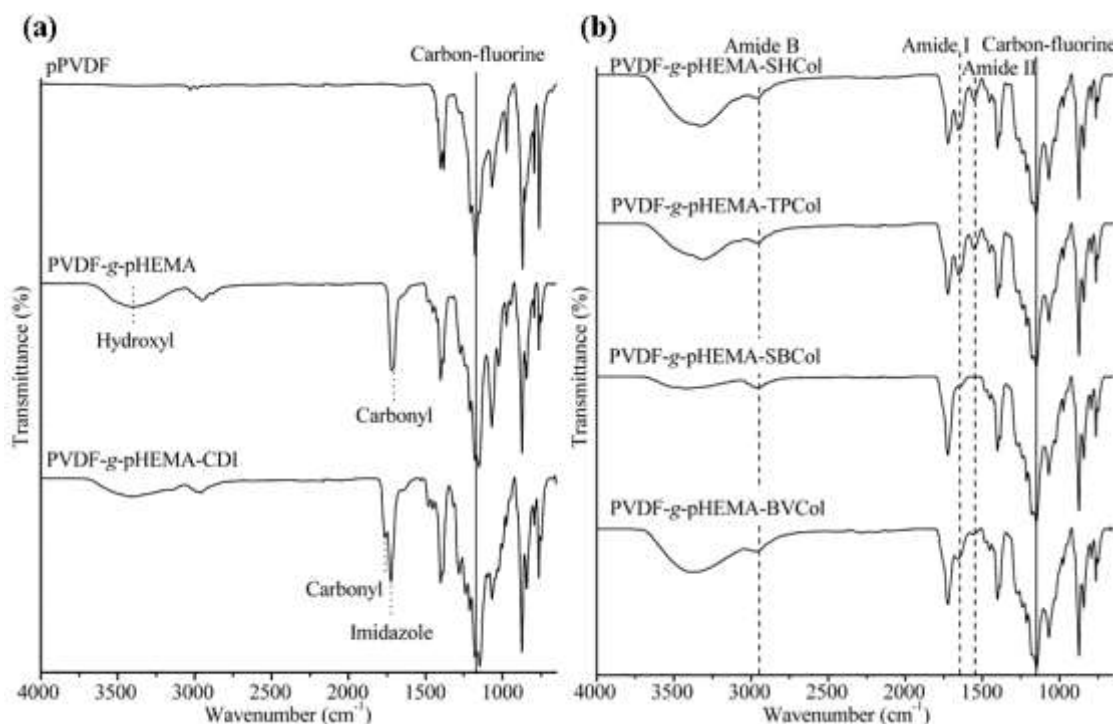


Figure 5.1 Chemical analysis showing (a) the ATR-FTIR spectra for the ATRP-modified PVDF films and (b) the ATR-FTIR spectra of the collagen-conjugated PVDF films (solid line represents the characteristic carbon-fluorine peak of PVDF; dotted lines represent the characteristic peaks resulted after each ATRP step; dashed lines represent characteristic amide peaks of collagen).

5.3.1.2 Surface Hydrophilicity Analysis

The surface hydrophilicity changes of different chemically-modified PVDF films at each modification step were measured using static WCA as shown in **Figure 5.2**. PVDF polymer is hydrophobic in nature. Hence, a high WCA value of $74.23 \pm 3.05^\circ$ was observed for pPVDF films. In general, a surface that is hydrophilic (i.e. low WCA value) is more conducive for cell attachment, whilst a surface that is hydrophobic (i.e. high WCA value) is often associated with poor affinity for cells [21]. In addition, a

hydrophobic surface is also often associated with protein and platelets adsorption that could cause thrombus formation [9], which is undesirable for blood-contacting applications. Hence, surface modification of PVDF is required in order to improve the cytocompatibility and hemocompatibility of pPVDF films. After the ATRP reaction, the WCA decreased significantly ($p < 0.05$) to a value of $45.00 \pm 1.62^\circ$ with improved surface hydrophilicity upon successful grafting of the hydrophilic pHEMA brushes. In order to facilitate the conjugation of collagen onto the ATRP-treated PVDF films, CDI was used as a biolinker and crosslinked onto the chemically-modified PVDF films. However, the crosslinking of CDI resulted in an increase in WCA to a value of $59.47 \pm 3.72^\circ$ due to the reduction of the number of hydrophilic hydroxyl groups after CDI was reacted onto the hydrophilic pHEMA chains. Finally, different sources of collagen were conjugated onto PVDF-*g*-pHEMA-CDI films. The presence of collagen led to a significant reduced ($p < 0.05$) the WCA to a range of $30 - 50^\circ$ as compared to PVDF-*g*-pHEMA-CDI films, and thus significantly improved on the overall surface hydrophilicity of the samples. In a separate study (**Appendix 1**), collagen was physically adsorbed onto the PVDF films and the surface hydrophilicity changes were also recorded as a comparison to ATRP process. As shown in **Appendix 1, Figure A1.2**, the WCA values for the different collagen-coated PVDF films remained similar to the pPVDF films, presumably because of the weak interaction between the collagen and the PVDF films that failed to improve on the surface properties of the PVDF films [7]. Hence, the ATRP process plays an important role to ensure a stronger chemical bonding between the collagen and the PVDF films, and subsequently improving the surface properties of the PVDF films.

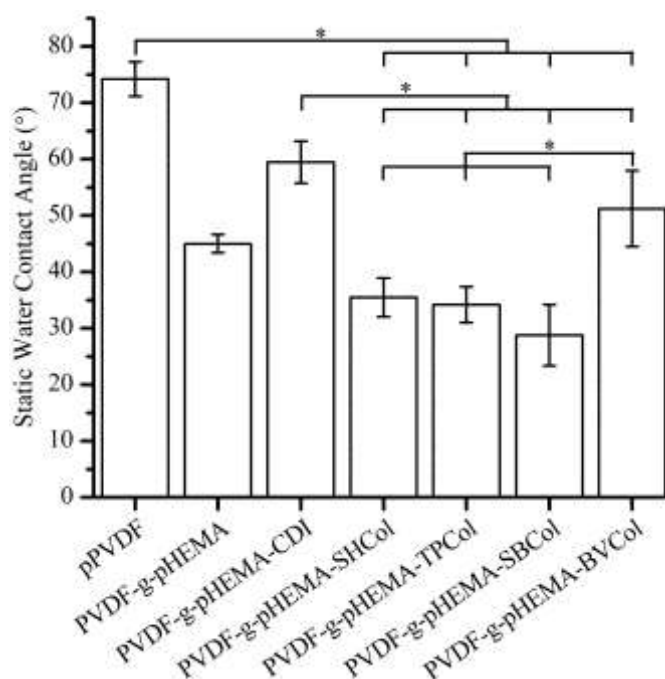


Figure 5.2 Static WCA results for the pPVDF and the different chemically-modified PVDF films (* $p < 0.05$).

5.3.2 *In Vitro* Cellular Studies of Collagen-conjugated PVDF Films

5.3.2.1 Cell Proliferation Analysis

The number of cells over 7 days of culture was measured by PrestoBlue® assay (**Figure 5.3**). For the pPVDF films, poor cell numbers were observed over the 7 days of culture due to the hydrophobic nature of PVDF polymer (**Figure 5.2**), which consequently led to poor cell adhesion and cell proliferation. For the PVDF-*g*-pHEMA films, even though surface hydrophilicity was significantly improved with the grafting of hydrophilic pHEMA brushes (**Figure 5.2**), a low number of cells measured over the 7 days of culture similar to those reported by others where pHEMA surface is unfavorable for the attachment of cells [22, 23]. Hence, this suggests that the lack of biological motif on the ATRP-modified PVDF films resulted in the overall poor cell-

material interactions. Likewise, poor cell numbers were observed on the PVDF-*g*-pHEMA-CDI films. In contrast, significantly improvement ($p < 0.05$) in the cell-material interactions was observed for all the different sources of collagen-conjugated PVDF films. Hence, the presence of bioactive collagen motif is required to enhance the cell-material interactions of the bioinert substrate, which is in accordance to those reported by others using Type I calf skin collagen-conjugated PCL substrate [24], Type I rat tail collagen-conjugated PCL substrate [25] and Type I calf skin collagen-conjugated silicon wafer [22]. However, no significant differences were observed for the different collagen-conjugated PVDF films, which could be due to the fact that Type I collagen was identified for all the different extracted FSCol, similar to that of the BVCol (**Chapter 4, Figure 4.7**). In a separate study (**Appendix 1**), the interactions of HUVECs with the physically-coated collagen on PVDF films were carried out as a comparison to the ATRP reaction. Overall, poor cell numbers were observed for all the collagen-coated PVDF films (**Appendix 1, Figure A1.3**), due to the fact that collagen is mainly absorbed through weak hydrophobic forces that could easily be desorbed over the duration [21, 25, 26]. Hence, this suggests that the ATRP modification of PVDF films was crucial to improve the surface properties (**Figure 5.2**) and the retention of collagen to ensure better cell attachment and proliferation over 7 days of culture. Taken together, the number of cells observed for the different sources of FSCol-conjugated PVDF films over the 7 days of culture was comparable to that of the BVCol and tissue culture plate control. This suggests that the FSCol could potentially be used as an alternative source of collagen to BVCol for biomedical applications, particularly for blood-contacting applications.

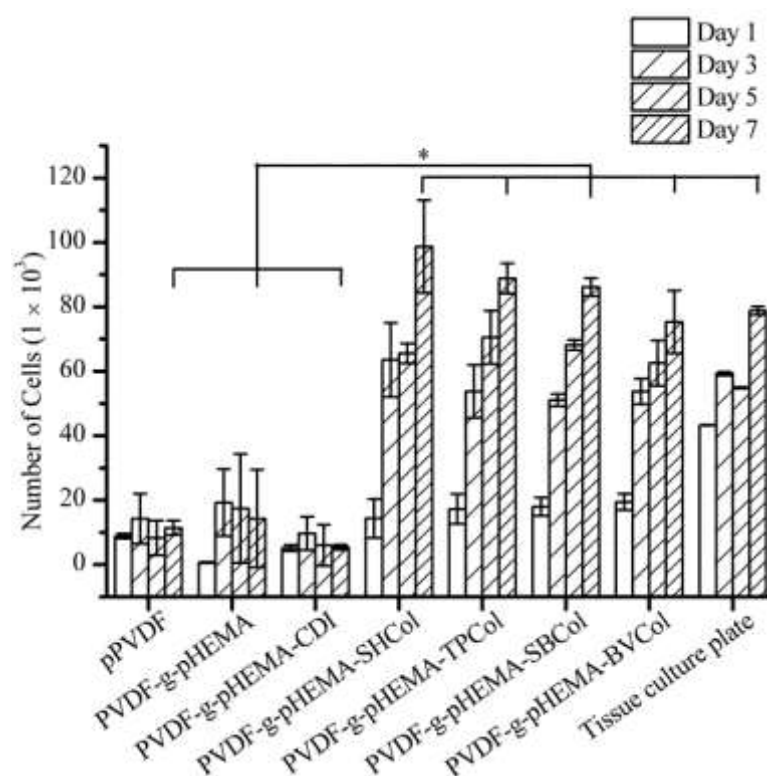


Figure 5.3 Cell proliferation results for HUVECs seeded on different chemically-modified PVDF films (* $p < 0.05$).

5.3.2.2 Cell Viability Imaging

The viability of HUVECs cultured on different chemically-modified PVDF films at day 7 was determined by fluorescence staining. As seen from **Figure 5.4**, a lower number of viable cells was observed on pPVDF films as compared to the modified surfaces, which is in agreement with the cell proliferation results (**Figure 5.3**). For the PVDF-*g*-pHEMA and PVDF-*g*-pHEMA-CDI films, even though there was significant improvement in the surface hydrophilicity (**Figure 5.2**), fewer cells were observed due to the absence of the collagen coating. On the other hand, similar to that of the tissue culture plate control, a confluent layer of cells was observed for all the PVDF films conjugated with different sources of collagen, which is an indication that the presence

of collagen improved the HUVECs endothelialization similar to those reported by others [25]. Taken together, ATRP modification of PVDF films with different sources of collagen resulted in improved surface hydrophilicity and cell-material interactions of the PVDF films. In addition, the cell proliferation results for all the extracted FSCol were similar to that of the BVCol, suggesting that the FSCol could be a promising source of collagen for biomedical applications because of its superior cell-material interactions.

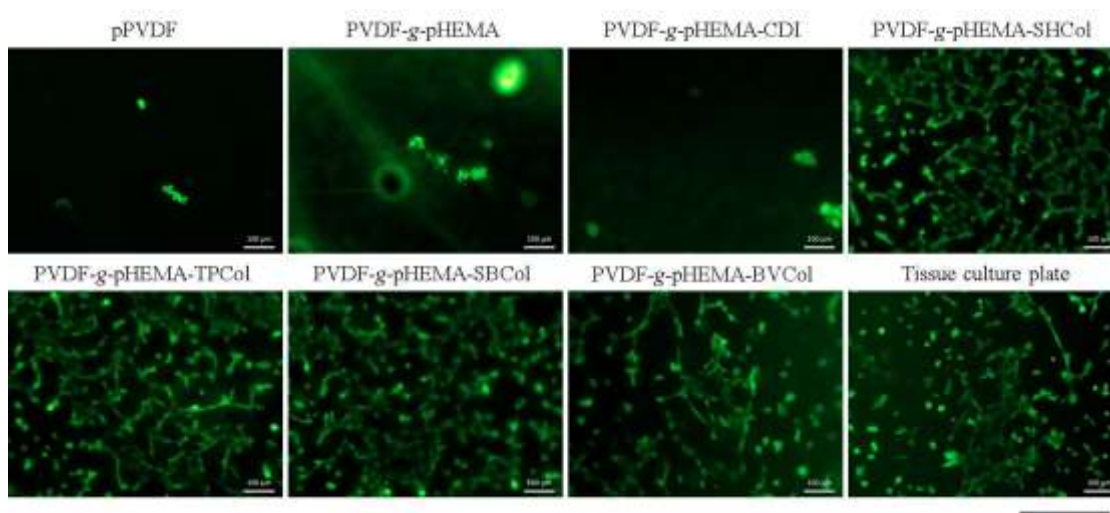


Figure 5.4 Cell viability results for HUVECs seeded on different chemically-modified PVDF films (green fluorescence represents live cells; scale bar represents 300 μm).

5.3.2.3 Material-induced Inflammatory Response Analysis

The material-induced inflammatory response of HUVECs cultured on different sources of collagen-conjugated PVDF films was assessed by the expression of leukocyte adhesion molecules. The leukocyte adhesion molecules are cytokine-regulated cell surface molecules that mediate the migration and adhesion of leukocyte during the vascular injury and inflammation. Hence, leukocyte adhesion molecules,

such as ICAM-1 and VCAM-1, could be used as markers to measure the activation of HUVECs as well as an indication of the pro-inflammatory response of HUVECs interacted with the different sources of collagen [27]. As seen from **Figure 5.5**, HUVECs cultured on different sources of collagen exhibited lower expression levels of ICAM-1 as compared to the positive control (i.e. TNF α -stimulated HUVECs). When the expression of VCAM-1 was compared, HUVECs cultured on different sources of collagen remained negative, similar to that of the isotype control [28]. Taken together, HUVECs cultured on different sources of collagen were not activated and the cells remained in a non-inflammatory state. When the different sources of collagen were compared, SHCol exhibited the lowest peak intensity with a median fluorescence intensity (MFI) of 344 as compared to TPCol, SBCol and BVCol with MFI values of 428, 448 and 377 respectively. This suggests that SHCol evoked the least pro-inflammatory response as compared to the other sources of collagen, which meant that the extracted SHCol could potentially be a good scaffolding material for blood-contacting applications.

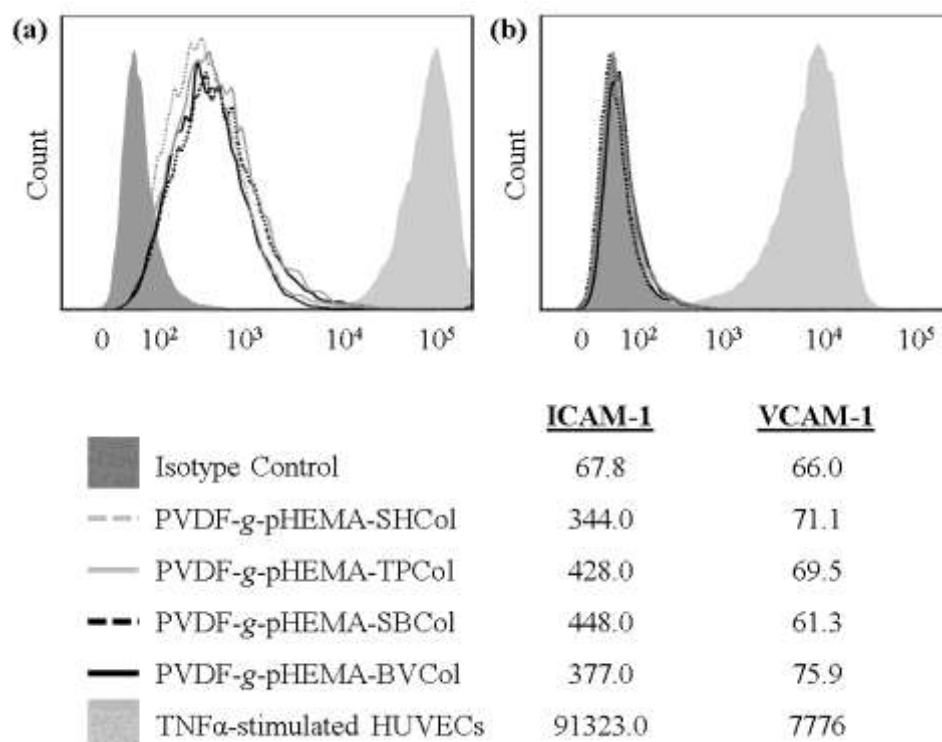


Figure 5.5 FACS analysis showing (a) ICAM-1 expression and (b) VCAM-1 expression for HUVECs seeded on different sources of collagen-conjugated PVDF films.

5.3.2.4 Gene Expression Analysis

The gene expression levels of HUVECs cultured on different sources of collagen was investigated by qPCR (**Figure 5.6**). In general, the pro-thrombotic (i.e. vWF and PAI-1) and anti-thrombotic (i.e. tPA and eNOS) markers of HUVECs cultured on different sources of collagen were measured. In addition, the Type I and Type IV collagen expression levels of HUVECs cultured on different sources of collagen were also compared. As seen from **Figure 5.6a**, higher vWF and PAI-1 expression levels were observed for HUVECs cultured on FSCol-conjugated PVDF films as compared to BVCol-conjugated PVDF films. On the other hand, higher tPA expression levels were

observed for HUVECs cultured on FSCol-conjugated PVDF films as compared to BVCol-conjugated PVDF films. In general, tPA is a class of key enzymes that promote fibrinolytic activity of the blood plasma while PAI-1 is the inhibitor of the plasminogen activators [29]. Therefore, the tPA/PAI-1 amount in blood plasma is commonly used as an indication of the thrombotic behavior of the blood plasma [30]. In addition, studies have shown that high levels of PAI-1 (i.e. low tPA/PAI-1 ratio) resulted in thrombus formation while low levels of PAI (i.e. high tPA/PAI-1 ratio) resulted in enhanced thrombolysis observed [29, 31, 32]. Hence, further analysis was carried out to determine the tPA/PAI-1 ratio for different collagen-conjugated PVDF films as shown in **Figure 5.6b**. As seen from the figure, the tPA/PAI-1 ratio for SHCol, TPCol, SBCol and BVCol was calculated as 1.06, 1.04, 0.85 and 1.00 respectively, with HUVECs cultured on SHCol exhibiting the highest anti-thrombogenic behavior as compared to the control (i.e. BVCol) as well as the other fish species. When the expression of Type IV collagen was compared, HUVECs cultured on all the FSCol from different fish species exhibited significantly higher ($p < 0.05$) levels of Type IV collagen as compared to BVCol. Type IV collagen is the most abundant type of collagen in the subendothelial basement membrane of the BVs. In addition, the presence of Type IV collagen is required to promote angiogenesis [33, 34]. Therefore, the current results suggest that HUVECs cultured on SHCol exhibited better anti-thrombogenic profile and secreted significant higher amounts of Type IV collagen as compared to BVCol. As such, SHCol could be an excellent alternative source of mammalian-derived collagen for TE applications particularly for blood-contacting applications. Taken together, cells growing on SHCol exhibited higher amounts of proline (**Chapter 4, Table 4.2**), better thermal stability (**Chapter 4, Figure 4.9**), least inflammatory response (**Figure 5.5**) and improved anti-thrombogenic behavior

(Figure 5.6), which is why for this thesis SHCol was used for further functional studies involving different blood components.

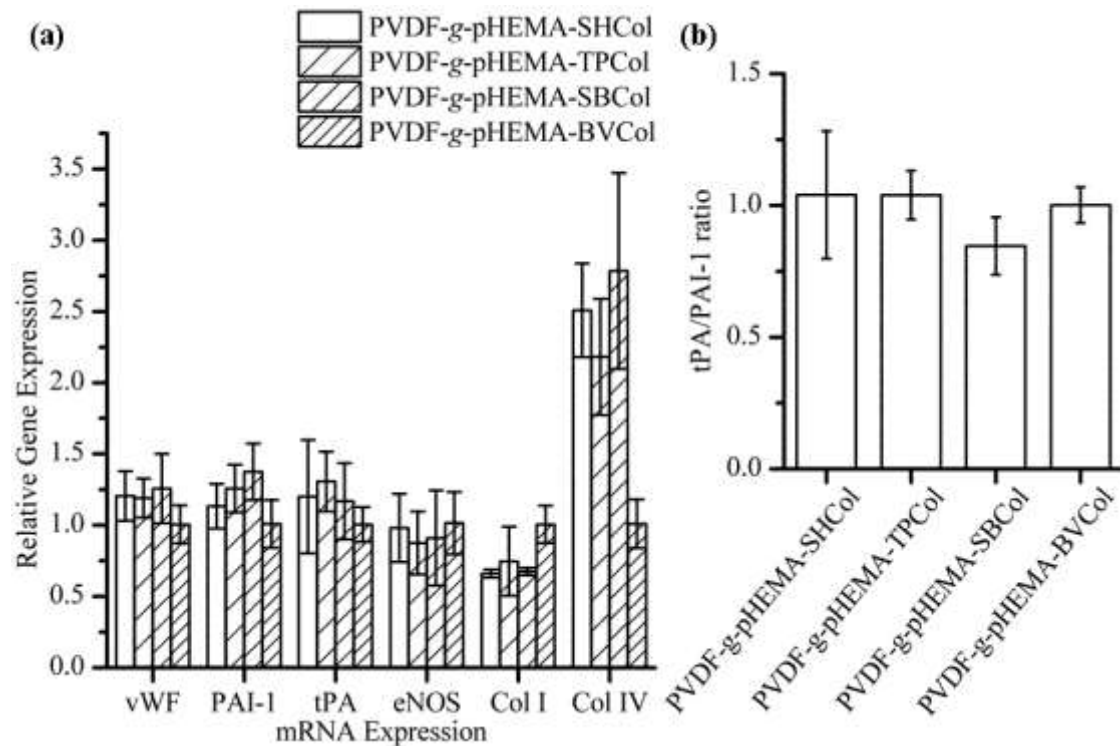


Figure 5.6 Gene expression analysis results showing (a) relative mRNA expression results and (b) the tPA/PAI-1 ratios of HUVECs seeded on different sources of collagen-conjugated PVDF films.

5.3.3 *In Vitro* Hemocompatibility Studies of Collagen-conjugated PVDF Films

5.3.3.1 Platelet Activation Assay

The thrombogenicity behavior of the endothelialized SHCol and BVCol surfaces was investigated using PRP by quantifying the P-selection expression levels. P-selectin is a cell adhesion molecules expressed by the activated platelets where the aggregation of the activated platelets could lead to the formation of thrombus [21, 35]. Hence, the number of activated platelets attached to the endothelialized SHCol and BVCol

surfaces and the interaction of the platelets with the endothelialized SHCol and BVCol surfaces could be used an indication of the thrombogenic properties of the endothelialized SHCol and BVCol surfaces. As seen from **Figure 5.7**, the P-selectin expression of the activated platelets interacting with the endothelialized SHCol and BVCol surfaces was significantly lower ($p < 0.05$) than the control (i.e. ADP-activated platelets), which is an indication that the cells cultured on the endothelialized SHCol and BVCol surfaces exhibited a good hemocompatibility properties. Also, no significant differences were observed between the endothelialized SHCol and BVCol surfaces, and thus suggesting that SHCol could be a suitable source of alternative collagen to that of BVCol.

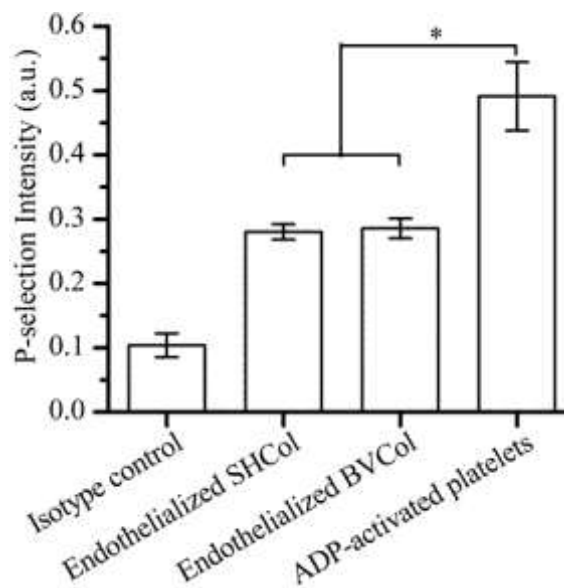


Figure 5.7 P-selectin expression results for the activated platelets interacted with different sources of collagen-conjugated PVDF films (* $p < 0.05$).

5.3.3.2 Plasma Recalcification Assay

The coagulation response of the recalcified plasma interacting with endothelialized SHCol and BVCol surfaces was measured to further confirm the hemocompatibility of different sources of collagen. As the recalcified plasma was added to the endothelialized SHCol and BVCol surfaces, the fibrin clot initiation time was significantly longer ($p < 0.05$) for the endothelialized SHCol and BVCol surfaces as compared to pPVDF films and tissue culture plate controls (**Figure 5.8**). Hence, the prolonged clot formation times suggested that the endothelialized SHCol and BVCol surfaces exhibited better anti-coagulation responses. Once again, no significant differences were observed for the clot initiation time between the endothelialized SHCol and BVCol surfaces, which is a further demonstration of the potential of SHCol as an alternative source of collagen for blood-contacting applications.

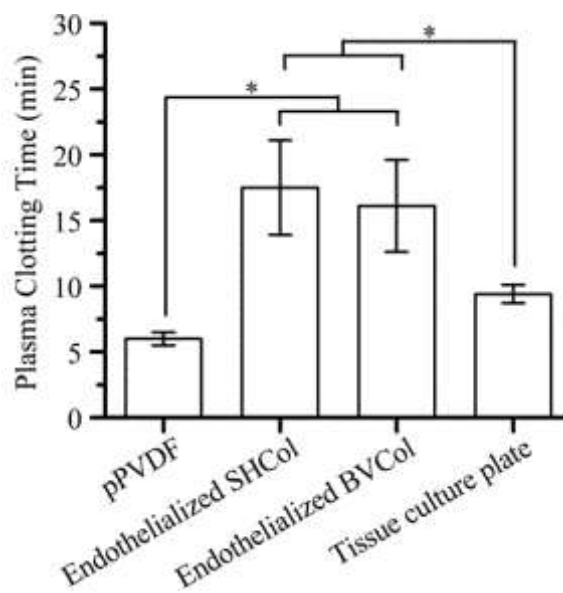


Figure 5.8 Plasma recalcification results of the recalcified plasma interacted with different sources of collagen-conjugated PVDF films ($*p < 0.05$).

5.4 Summary

In this chapter, the different sources of collagen were successfully conjugated onto the PVDF films using the ATRP process, which resulted in improved surface hydrophilicity as compared to pPVDF films. In addition, the presence of collagen significantly improved the interactions of HUVECs with the collagen-conjugated PVDF films with significantly higher number of cells proliferating on the collagen-conjugated PVDF films over 7 days of culture. Moreover, HUVECs cultured on the different sources of collagen-conjugated PVDF films remained in a non-inflammatory state. Interestingly, HUVECs cultured on the FSCol from different fish species exhibited better anti-thrombogenic and pro-angiogenic properties as compared to BVCol, and thus demonstrated the potential of the FSCol as an alternative safer source of collagen particularly for blood-contacting applications. Among the FSCol from different fish species, SHCol exhibited the best anti-thrombogenic behavior as compared to TPCol and SBCol. In addition, SHCol exhibited a good hemocompatibility with significantly lower activated platelets plated on the endothelialized SHCol surface as compared to ADP-activated platelets control. Moreover, SHCol exhibited higher amounts of proline and better thermal stability as compared to TPCol and SBCol. Hence, SHCol was chosen as the source of collagen for subsequent studies.

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Chapter 6

Study 3 – Chemical Modification and Chemical Crosslinking of FSCol

This chapter describes the detailed fabrication and characterization of the water-soluble MCol. In addition, both the UCol and MCol are crosslinked using BDE followed by lyophilization and cold-pressing to produce different collagen patches. The physicochemical properties of the UCol and MCol, with and without BDE crosslinking are also assessed. Lastly, the in vivo biocompatibility and degradability of different collagen patches are evaluated using a murine model.

6.1 Introduction

Collagen, the most abundant protein in ECM, has been widely used as scaffolding material for wound regeneration applications [1]. In general, a wound healing process involves hemostasis, tissue formation and tissue remodeling, in which the whole healing process can take up to 16 days or even longer time [2, 3]. However, studies have shown that UCol can be fully degraded within 2 days following *in vivo* implantation due to the poor physicochemical properties of UCol [4-6]. Hence, further chemical modification and chemical crosslinking steps are often carried out to improve the physicochemical properties of collagen [7].

In this chapter, SHCol was chemically-modified using methylation modification to improve its physicochemical properties. Using this modification method, the carboxylic acid groups of the collagen are partially esterified by acidified methanol to produce a positively-charged MCol [8]. The net positive-charge of MCol improves the solubility of MCol at physiological conditions, which could be useful in biomedical applications. In general, UCol is commonly dissolved in acidic solutions, which can potentially cause denaturation of biological agents such as cells, drugs, genes or growth factors that are exposed to it during scaffold fabrication process [4, 9]. In contrast, the water-soluble MCol provides a friendly environment for biological agents, and thus further extend its potential as a suitable drug carrier. Moreover, studies have shown that the net positive-charge of the MCol improves the cell-material interactions of the material by attracting negatively-charged proteins on the cell membrane, which would eventually improve wound healing and tissue regeneration [10, 11]. Overall, the methylation modification of collagen improves the water-solubility and the cell-material interactions [12], which are highly favorable properties for biomedical applications.

However, to our knowledge, limited studies have been carried out on using FSCol either in the form of UCol or MCol for biomedical applications, particularly for wound healing applications. Hence, for this particularly study, the potential of UCol and MCol as scaffolding materials for wound healing applications was investigated. Briefly, UCol and MCol were crosslinked using BDE, lyophilized and then cold-pressed to form collagen patches. Chemical crosslinking method was chosen instead of physical crosslinking due to the higher strength and degradation stability rendered by the chemical crosslinking process [13, 14]. BDE was chosen as the crosslinking agents in this particular study due to the lower toxicity of epoxy compounds as compared to other crosslinkers such as GA [15-20]. In addition, BDE could work effectively at various physiological conditions such as low molarity and low temperature environment, which could enhance the crosslinking reaction. Moreover, BDE is also approved by the Food and Drug Administration as well as the European Medicines Evaluation Agency (EMA) [21].

The mechanical properties and degradation stability of different collagen patches were firstly assessed. The drug release potential of the various MCol patches was also investigated. Subsequently, the *in vivo* biocompatibility and the degradability of the different collagen patches were evaluated using a murine model. During the wound healing process, angiogenesis is important to provide nutrients and oxygen to the fibroblasts and epithelial cells while lymphangiogenesis is important to maintain the tissue fluid homeostasis as well as for the transportation of immune cells [22]. Hence, the growth of BVs and LVs on different collagen patches were examined.

Overall, the specific objectives and aims of this chapter are as follows:

1. Modification and characterization of FSCol
2. Fabrication and characterization of different collagen patches
3. Investigation of the *in vivo* biocompatibility and the degradability of different collagen patches
4. Quantification of the growth of BVs and LVs on different collagen patches

6.2 Materials and Methods

UCol was chemically-modified via methylation modification to yield water-soluble MCol followed by crosslinking to produce different collagen patches as described in detail in **Chapter 3, Section 3.3.3**. The successful modification and crosslinking of collagen were confirmed using SEM (**Chapter 3, Section 3.4.2**), ATR-FTIR (**Chapter 3, Section 3.4.4**) and $^1\text{H-NMR}$ (**Chapter 3, Section 3.4.5**) analyses respectively. The physicochemical properties changes of different collagen patches were assessed by comparing the tensile modulus (**Chapter 3, Section 3.4.11**) and the degradation stability (**Chapter 3, Section 3.4.12**) of different collagen patches. In addition, the potential of the MCol as drug carrier was carried out to investigate the drug release behavior using FITC-BSA as drug model (**Chapter 3, Section 3.4.13**). Lastly, the potential applications of different collagen patches were evaluated by *in vivo* studies using a murine model (**Chapter 3, Section 3.6.1**). The post-implanted collagen patches were assessed using histological (**Chapter 3, Sections 3.6.2 and 3.6.3**), immunohistological staining (**Chapter 3, Section 3.6.4**) and lymphangiography analysis (**Chapter 3, Section 3.6.5**).

6.3 Results and Discussion

6.3.1 Fabrication of Water-soluble Collagen via Methylation Modification

6.3.1.1 Morphological Analysis

The morphology of the UCol and MCol was imaged using SEM as shown in **Figure 6.1**. As seen from the figure, a fibrous-like structure was observed for UCol. On the other hand, a sheet-like appearance was observed for MCol. The difference in morphologies is probably due to the partial esterification process, which produced a net positive-charge on the MCol that affected the electrostatic interactions of the collagen fibers. Consequently, the molecules of the MCol failed to undergo the self-assembly process to form fibrils during the fibrillogenesis and thus, resulted in a sheet-like appearance [23].

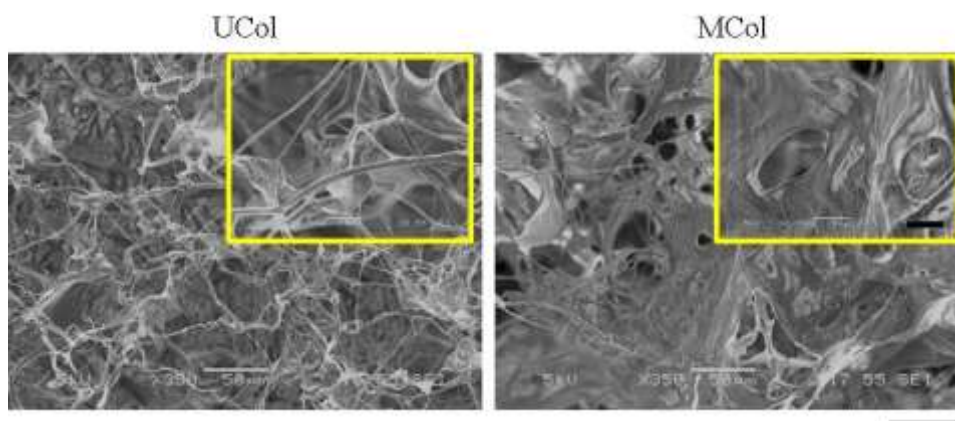


Figure 6.1 SEM images for UCol and MCol at $\times 350$ and $\times 2000$ (inset) magnifications (scale bar represents 50 μm ; inset scale bar represents 10 μm).

6.3.1.2 Chemical Analysis

The changes in chemical bonds on UCol and MCol were determined by ATR-FTIR as shown in **Figure 6.2**. The different characteristic amide peaks (dashed lines) of the

collagen were observed for both UCol and MCol. Amide A and B were detected near 3300 and 2920 cm^{-1} due to N-H stretching while Amide I (C=O stretching), Amide II (C-N stretching and N-H bending out of phase) and Amide III (C-N stretching and N-H bending in-phase α -helix) were detected near 1630, 1530 and 1200 cm^{-1} . The amount of the triple helical structures within the UCol and MCol was further quantified by calculating the $A'(\text{Amide III})/A'(1450)$ ratio [24-26]. From the ATR-FTIR spectra, the $A'(\text{Amide III})/A'(1450)$ ratio for both the UCol and MCol was calculated as 1.30 and 1.11 respectively. Although there was a slight decrease in the amount of triple helical structures, the peak intensity ratio was still ~ 1.0 , which is an indication that the majority of the triple helical structures was still well preserved even after the methylation modification.

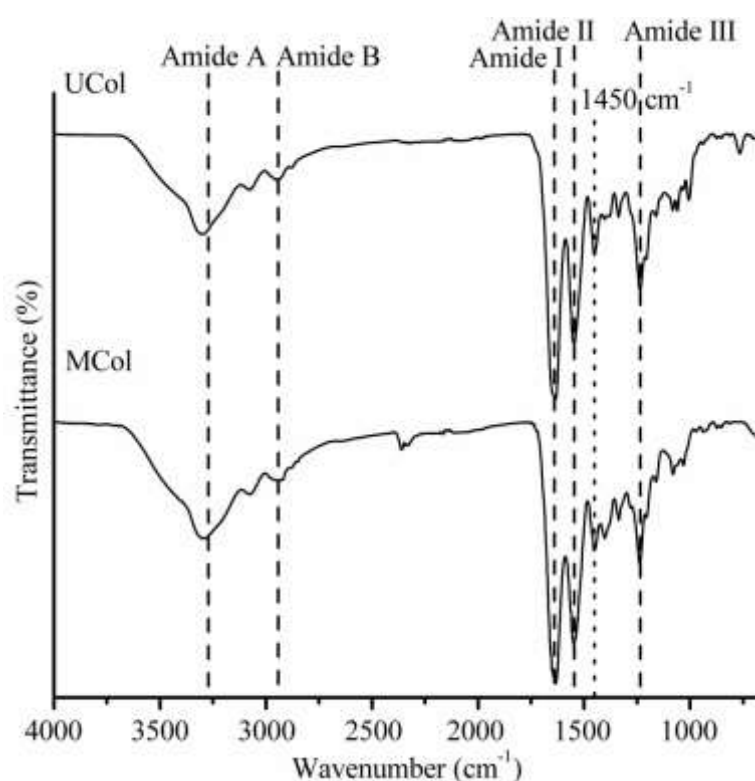


Figure 6.2 ATR-FTIR results for UCol and MCol (dashed lines represent amide peaks; dotted line represents 1450 cm^{-1} peak).

6.3.1.3 Analytical Analysis

The successful chemical modification of collagen by methylation was confirmed using ^1H -NMR analysis. As seen from **Figure 6.3**, ^1H -NMR results showed an increase in the peak intensity at 3.664 ppm attributable for the $-\text{COOCH}_3$ groups. Methylation modification involves the replacement of the hydrogen atom of the carboxyl acid groups in UCol with methyl groups during the partial esterification reaction to form a polycationic MCol [12]. Hence, the presence of the sharp peak near the 3.664 ppm, which was absent in the UCol, is an indication of the successful methylation modification of the UCol to yield MCol.

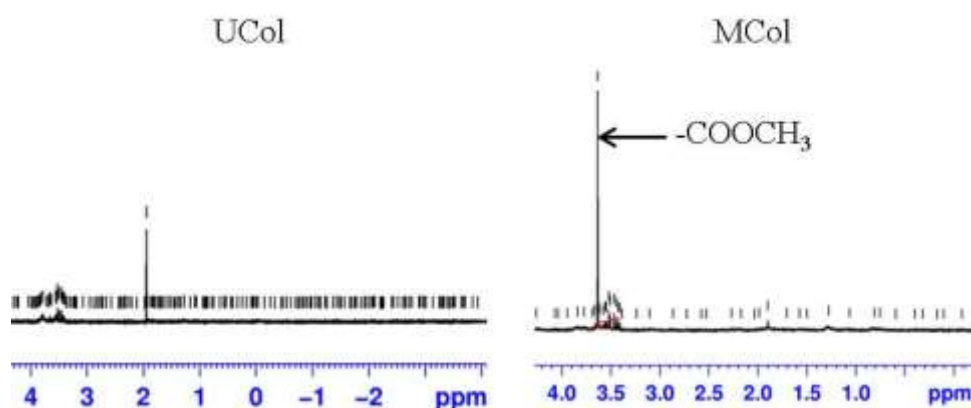


Figure 6.3 ^1H -NMR results for UCol and MCol.

6.3.2 Fabrication of Cold-pressed Collagen Patches

6.3.2.1 Chemical Analysis

In order to fabricate collagen patches, UCol and MCol were crosslinked using BDE, lyophilized and later cold-pressed. After cold-pressing, the collagen patches did not exhibit a porous 3D structure (**Appendix 2, Figure A2.1**). The successful crosslinking of different collagen patches was confirmed using ATR-FTIR. From the ATR-FTIR

results (**Figure 6.4**), the characteristic amide peaks (dashed lines) were observed for all the different collagen patches. Apart from the characteristic amide peaks, higher peak intensity was observed around 1080 cm^{-1} after the crosslinking process as compared to uncrosslinked collagen patches. BDE consists of two epoxy terminals together with two ether bonds at the backbone of the structure [15]. The two epoxy terminals act as the reaction sites by crosslinking with the free amine groups of the collagen, while the backbone with the two epoxy groups formed the linkage between different collagen chains. Hence, the higher peak intensity near the 1080 cm^{-1} as compared to uncrosslinked collagen patches is attributable to successful BDE crosslinking due to the formation of ether linkages between the collagen chains. In addition, it was observed that as crosslinking density increases, a higher peak intensity was observed for both UCol and MCol samples. The successful crosslinking of the collagen patches was also determined by TNBS assay where the free amine groups of the uncrosslinked and BDE-crosslinked collagen patches were quantified to estimate the degree of crosslinking of the collagen patches. Similarly, as the BDE concentration increases, a higher degree of crosslinking was observed indicating that higher number of successful ether linkages were formed between the collagen chains (representative data of MCol crosslinked by different concentrations of BDE is shown in **Appendix 3, Figure A3.1**), which is in agreement with the ATR-FTIR results (**Figure 6.4**).

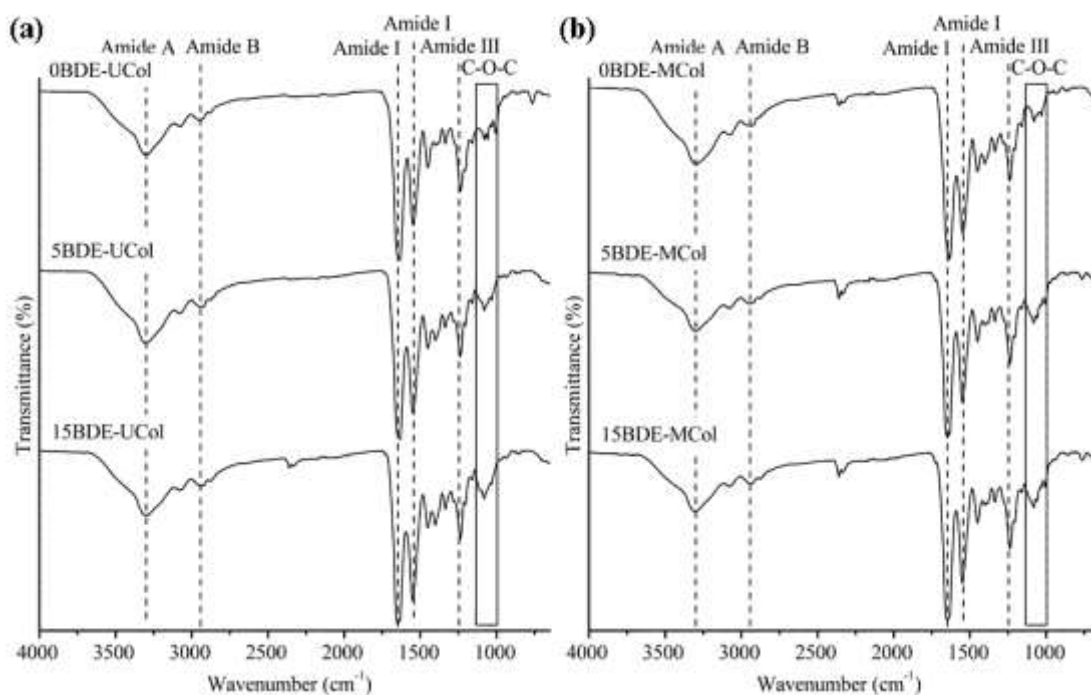


Figure 6.4 ATR-FTIR results for different collagen patches (dashed lines represent amide peaks; solid box represent ether peaks).

6.3.2.2 Mechanical Analysis

The mechanical properties of different collagen patches were compared by measuring the tensile modulus of the samples. As seen from **Figure 6.5**, the tensile moduli for all the MCol samples were significantly higher ($p < 0.05$) as compared to the UCol samples. In general, all the MCol samples exhibited a tensile modulus of between 15.5 – 20.8 MPa, whereas all the UCol samples exhibited a tensile modulus of between 1.9 – 12.9 MPa. Methylation modification affects not only the morphological properties of collagen (**Figure 6.1**) but also the stiffness of the collagen, since an improvement in mechanical properties was observed. In addition, an increase in tensile modulus was observed with increasing crosslinking density. For example, the average tensile modulus of uncrosslinked UCol patch (0BDE-UCol) increased significantly from 1.9

MPa to 3.1 MPa and 12.9 MPa after crosslinking with 5 w/w% (5BDE-UCol) and 15 w/w% (15BDE-UCol) of BDE crosslinker respectively. When higher amounts of BDE crosslinker was added to the UCol and MCol solution, an increasing number of ether linkage were formed between the collagen chains, which then resulted in improved mechanical properties of the collagen patches. Overall, methylation modification and chemical crosslinking processes successfully improved the mechanical properties of collagen. In general, the mechanical properties of skin vary for different studies depending on the age and the testing method used. Studies have shown that the tensile modulus of skin varies between 4.6 MPa to 20 MPa [27, 28]. In this study, the tensile modulus measured for all the different collagen patches range from 1.9 – 20.8 MPa. This suggests that the different collagen patches could be used as a suitable scaffolding material for wound healing applications.

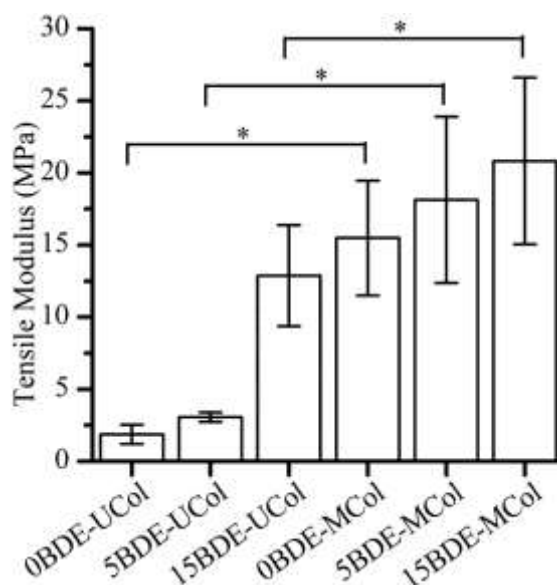


Figure 6.5 Mechanical testing results for different collagen patches (* $p < 0.05$).

6.3.2.3 *In Vitro* Degradation Studies

The degradation stabilities of different collagen patches were determined by comparing the *in vitro* degradation profile of different collagen patches treated with 2.0 unit/mL of Type I collagenase solution. As seen from **Figure 6.6**, MCol samples exhibited a slower degradation rate as compared to UCol samples, which could possibly due to the changes in the structural (**Figure 6.1**) and stiffness (**Figure 6.5**) properties of the MCol in slower degradation rate [29]. On the other hand, between the uncrosslinked and BDE-crosslinked collagen patches, the degradation resistance of the BDE-crosslinked collagen patches improved significantly over the course of 2 weeks as compared to the uncrosslinked collagen. In addition, an increase in degradation resistance was observed with increasing crosslinking density. However, between the 5 w/w% and 15 w/w% BDE-crosslinked collagen patches, 15 w/w% BDE-crosslinked collagen patches were hardly degraded by *in vitro* collagenase treatment. This extremely slow degradation rate of the 15 w/w% BDE-crosslinked collagen patches may inhibit subsequent tissue remodeling processes during wound healing, and thus the 15 w/w% crosslinking density may limit the usefulness of the collagen [30, 31]. Nevertheless, the results demonstrated that methylation modification and BDE crosslinking processes successfully improved the physicochemical properties of UCol and MCol.

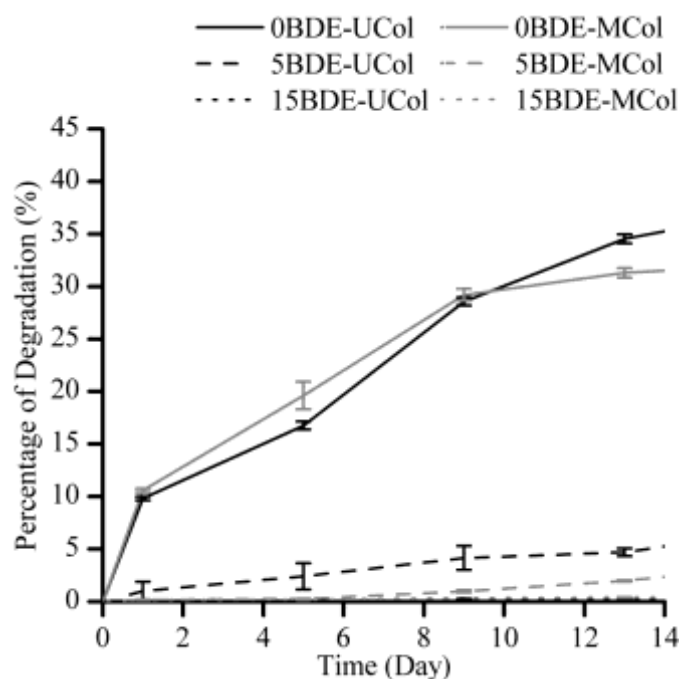


Figure 6.6 *In vitro* degradation results for different collagen patches exposed to 2.0 unit/mL of Type I collagenase solution.

6.3.2.4 *In Vitro* Drug Release Studies

In biomedical applications, various types of biological agents such as cells, drugs, genes or growth factors have been incorporated into scaffolds to enhance the tissue regeneration process. However, UCol is commonly soluble under acidic conditions [32]. During the scaffold fabrication process, this harsh acidic environment could potentially cause denaturation of biological agents, and thus limited its potential for drug delivery applications. Hence, in a separate study (**Appendix 4**), the possible denaturation of drugs under the harsh acidic environment was first carried out prior to drug release studies. As seen from **Appendix 4, Figure A4.1**, NanoDSC results showed that the heat-induced unfolding of BSA that was being dissolved in 1× PBS with a melting temperature (T_m) of 63.67 °C being detected. In contrast, the melting

temperature was not detected for the BSA dissolved under the acidic conditions, which is an indication that BSA was probably denatured and hence no enthalpy change was detected during the testing [33, 34]. Hence, UCol samples were not included in this study, since the methylation modification of UCol to produce a water-soluble MCol is required in order to provide a friendly environment for the biological agents targeting for drug delivery applications. The drug release studies for the MCol were carried out by looking at the release profile of the model drug (i.e. FITC-BSA). Prior to the drug release studies, the macroscopic images of the FITC-BSA loaded MCol patches showed that the FITC-BSA was distributed evenly across the collagen patches (**Appendix 4, Figure A4.2**). Based on the FITC-BSA release profile (**Figure 6.7**), a consistent drug release rate of FITC-BSA was observed for all the MCol patches. However, between the uncrosslinked and BDE-crosslinked collagen patches, 0BDE-MCol patches exhibited a more rapid release rate with a higher gradient which is probably due to the rapid degradation rate of the uncrosslinked MCol patches causing the FITC-BSA to release into the 1× PBS at a higher rate as compared to BDE-crosslinked collagen samples. On the other hand, for the BDE-crosslinked collagen samples, the degradation rate was slow. Thus, loaded FITC-BSA was slowly released from the samples through diffusion with at a more constant rate. In contrast, an initial burst release followed by a lower release rate of FITC-BSA was observed for the 5BDE-MCol and 15BDE-MCol patches. In addition, it was observed that as crosslinking density increases, a higher burst release was detected followed by a consistent release of FITC-BSA. The burst release observed is probably due to the changes in the net charges of the MCol patches. In general, methylation modification converts the negatively-charged carboxylic acid groups into methyl ester groups to yield a positively-charged collagen (i.e. free amine groups) [8]. On the other hand,

FITC-BSA is a negatively-charged protein molecule [35]. However, during the BDE crosslinking process, BDE crosslinker reacted with the free amine sites to form the ether linkages (**Chapter 3, Figure 3.5**). As a result, the number of free amine groups of the MCol decreased, and thus affected the net charges of the MCol patches and the subsequent FITC-BSA interaction. Hence, as crosslinking increases, a more significant burst release was observed for the MCol patches. Nevertheless, the initial burst release of the 5BDE-MCol and 15BDE-MCol patches could easily be prevented by pre-soaking the collagen patch in $1\times$ PBS for two days prior to implantation to stabilize the drug-loaded collagen patch (**Figure 6.7**). Taken together, BDE-crosslinked MCol patches with improved physicochemical properties and a controllable drug release profile could potentially be used as a drug carrier for biomedical applications.

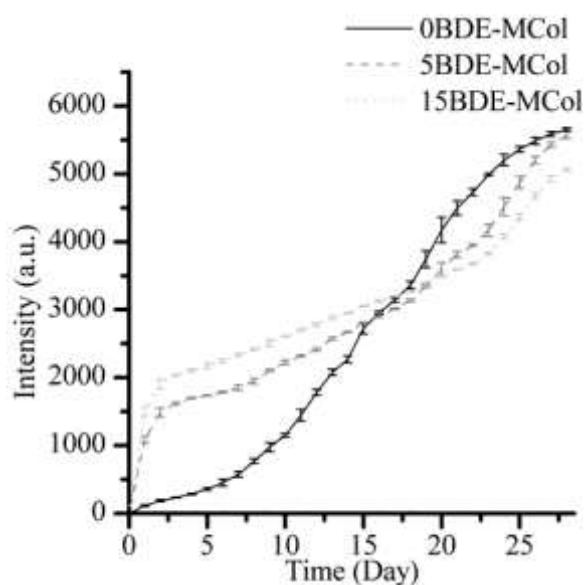


Figure 6.7 *In vitro* drug release profiles for different MCol patches.

6.3.3 *In Vivo* Biocompatibility and Degradability Studies of Collagen Patches

6.3.3.1 Macroscopic Analysis

The *in vivo* biocompatibility and degradability of different collagen patches were evaluated by implanting the collagen patches subcutaneous in mouse hind limbs. The *in vivo* study was carried out over 21 days and at the end of the implantation period, the macroscopic appearance of the collagen patches was assessed visually prior to microscopic analysis. In general, it was observed that most of the 15 w/w% BDE-crosslinked collagen patches remained intact even after 21 days of implantation (representative data of MCol crosslinked by different concentrations of BDE is shown in **Appendix 5, Figure A5.1**), which is in agreement with the *in vitro* degradation studies (**Figure 6.6**). The slow *in vivo* degradation rate (i.e. over-crosslinking) of 15 w/w% BDE-crosslinked collagen patches could potentially inhibit the tissue remodeling processes, and thus further analyses were not carried out on the 15 w/w% BDE-crosslinked collagen patches [30, 31].

Overall, at 21 days post-implantation, the mouse skin was observed to have healed completely with no infection or inflammation. In addition, the implanted collagen patches integrated well with the tissue with no significant local tissue remodeling effect observed macroscopically as shown in **Figure 6.8**. As seen from the figure, most of the uncrosslinked collagen patches were degraded whereas the BDE-crosslinked collagen patches were partially degraded, which is an indication that methylation modification and BDE crosslinking successfully improved the *in vivo* degradation stability of UCol and MCol. In addition, the macroscopic images also showed that vascularization was observed in all the different collagen patches.

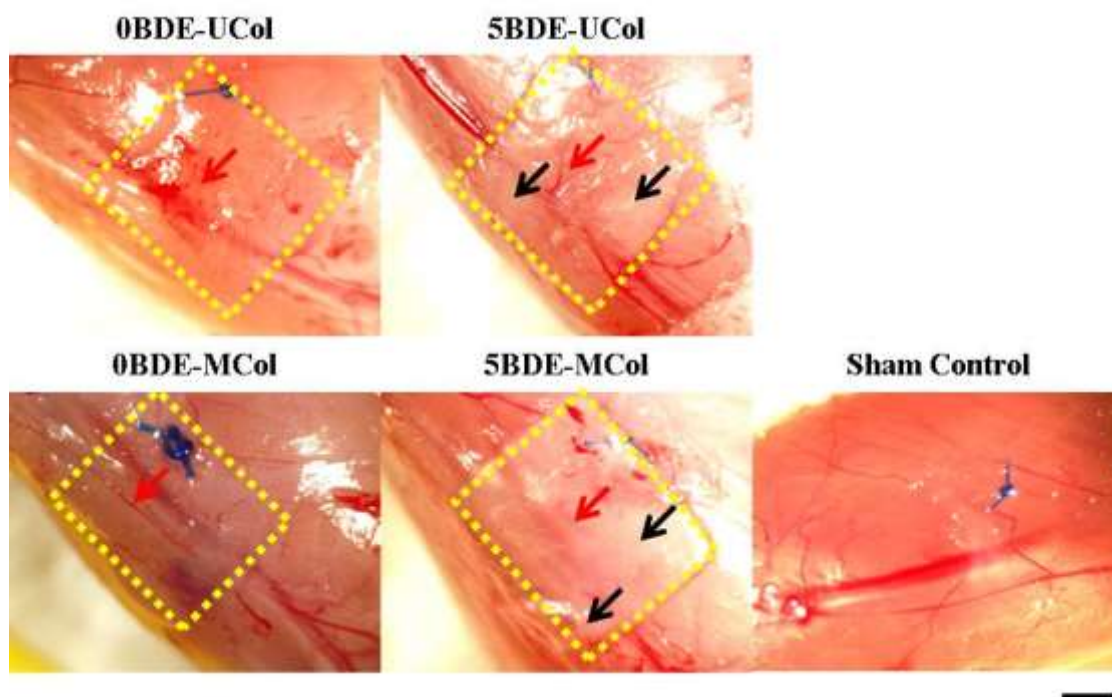


Figure 6.8 Macroscopic images for different collagen patches at 21 days post-implantation (yellow dotted box outlines the area of the implanted collagen patch; black arrows represent the partially degraded collagen patch; red arrow represents vascularization; scale bar represents 1 mm).

6.3.3.2 Histological Analysis

The histology of different implanted samples was evaluated by H&E staining. As seen from **Figure 6.9a**, the H&E stained images of different collagen patches were similar to the sham control, which is an indication that the skin retained its normal histological structure with no unfavorable inflammatory response. This suggests that the methylation modification and the BDE crosslinker used were biocompatible. In addition, cellular infiltration was observed for different collagen patches with predominant cell type to be mononuclear cells, which is consistent with those reported by others [36-38]. However, no significant microscopic difference was observed

between the UCol and MCol samples. On the other hand, between the uncrosslinked and BDE-crosslinked collagen patches, higher number of cellular infiltration was observed as compared to BDE-crosslinked collagen patches, due to the weaker physicochemical properties of the uncrosslinked collagen patches (**Figures 6.5 and 6.6**) that facilitated the cellular migration and proliferation into the uncrosslinked collagen patches.

The histological of different implanted samples was further evaluated using Masson's trichrome staining. As seen from **Figure 6.9b**, once again, no significant microscopic difference was observed between the UCol and MCol samples. On the other hand, between the uncrosslinked and BDE-crosslinked collagen patches, BDE-crosslinked collagen patches were partially degraded after 21 days of implantation with a lesser number of cellular infiltrations was observed as compared to uncrosslinked collagen patches, which is in agreement with the macroscopic (**Figure 6.8**) and H&E stained (**Figure 6.9a**) images. Overall, histological results demonstrated the *in vivo* biocompatibility and the degradability of different collagen patches. In addition, BDE crosslinking significantly improved the physicochemical properties of the collagen (**Figures 6.5 and 6.6**).

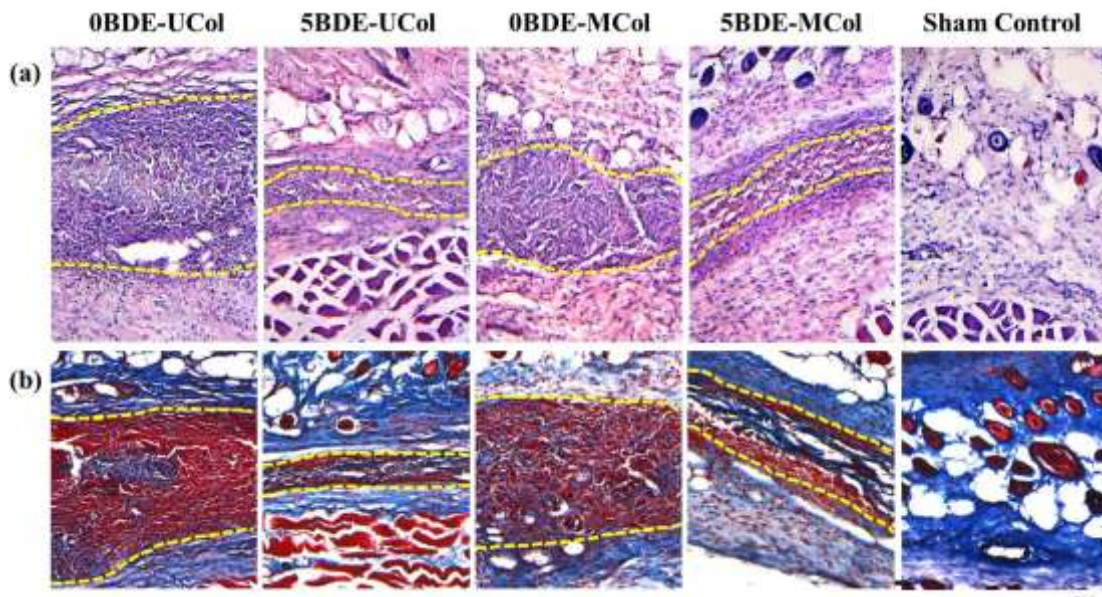


Figure 6.9 Histological results showing (a) H&E staining and (b) Masson's trichrome staining for different collagen patches at 21 days post-implantation (yellow dashed lines outline collagen patch; scale bar represents 100 μ m).

6.3.3.3 Immunohistological Analysis

The growth of BVs and LVs on different collagen patches were examined by immunohistological staining as shown in **Figure 6.10**. As seen from the figure, BVs and LVs were present in all the different collagen patches. Between the UCol and MCol samples, all the MCol samples promoted better growth of BVs and LVs similar to those reported by others [36, 39-41]. In general, BVs and LVs form at the area with high number of cells such as macrophages due to its ability to secrete growth factors that promote vessels formation [42-45]. As such, MCol with a net positive-charge enhanced the cell-material interactions [12], which in turn promoted better formation and growth of BVs and LVs. Between the uncrosslinked and BDE-crosslinked collagen patches, ingrowth of BVs and LVs were mostly observed for the

uncrosslinked collagen patches could possibly due to the weaker physicochemical properties of the uncrosslinked collagen patches (**Figures 6.5 and 6.6**) that facilitated cellular infiltration (**Figure 6.9**), and thus the vessels ingrowth. On the other hand, the BVs and LVs were mostly formed surrounding the BDE-crosslinked collagen patches, due to the stiffer material properties and greater number of cells accumulated around the BDE-crosslinked collagen patches. Nevertheless, growth of BVs and LVs were observed at different collagen patches and further analysis was carried out to quantify the number of BVs and LVs growth.

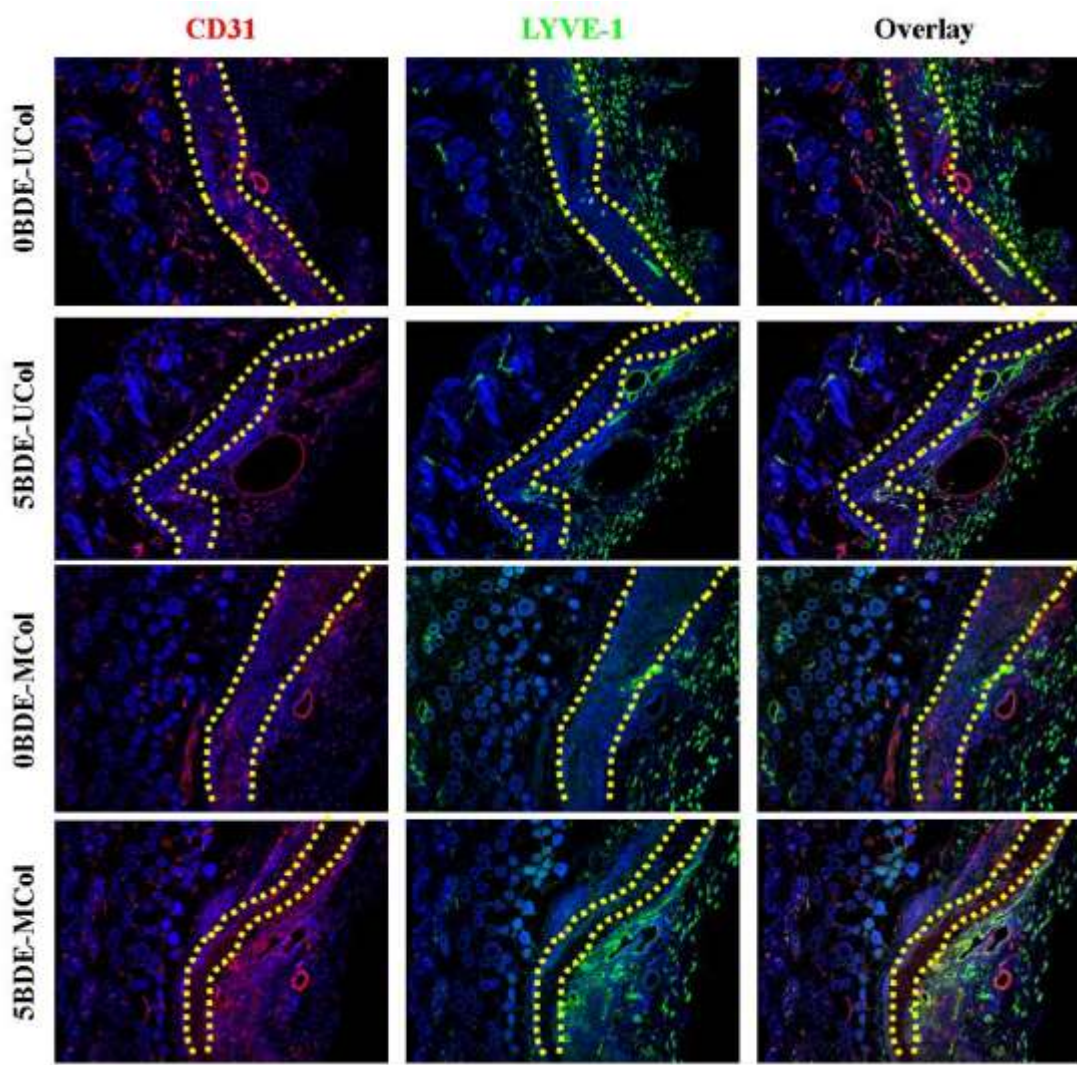


Figure 6.10 Immunohistological results for different collagen patches at 21 days post-implantation (yellow dashed lines outline collagen patch; CD31 represents BVs; LYVE-1 represents LVs; scale bar represents 200 μm).

6.3.3.4 Quantification of BVs and LVs

As seen from **Figure 6.11**, all the MCol samples exhibited higher number of BVs and LVs as compared to UCol samples. Hence, MCol samples are more suitable to be used for wound healing applications. Between the uncrosslinked and BDE-crosslinked collagen patches, BDE crosslinking supported better formation and growth of BVs and

LVs with higher number of BVs and LVs measured on the BDE-crosslinked collagen patches as compared to uncrosslinked collagen patches. Interestingly, significantly higher ($p < 0.01$) number of the LVs was observed for the 5BDE-MCol patches as compared to other collagen patches. Hence, further study was carried out using this particular group of collagen to investigate the potential of 5BDE-MCol patch for lymphatic regeneration applications.

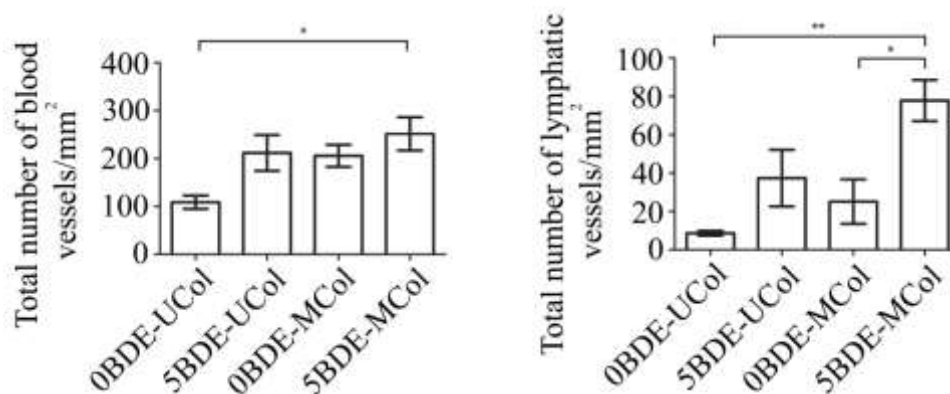


Figure 6.11 Quantification of BVs and LVs for different collagen patches at 21 days post-implantation (* $p < 0.05$; ** $p < 0.01$).

6.3.3.5 Lymphangiography Analysis

The potential of the 5BDE-MCol patch for lymphatic regeneration was investigated by evaluating the spontaneous lymphatic reconnection and restoration of lymphatic flow in the presence of 5BDE-MCol patch. As seen from **Figure 6.12a**, the flow of Lectin-FITC was perturbed in the sham control group, which is an indication that the LVs were not reconnected. On the other hand, Lectin-FITC was able to successfully flow through the newly grown and reconnected LVs all the way to the popliteal lymph node in the case of the 5BDE-MCol implanted sample group (**Figure 6.12b**). In addition, when the popliteal lymph node was isolated, the fluorescence signals were also

detected for the 5BDE-MCol implanted sample group but not the sham control group. Taken together, the presence of the 5BDE-MCol patch facilitated the reconnection of LVs, and thus can potentially be used for inflammatory related applications.

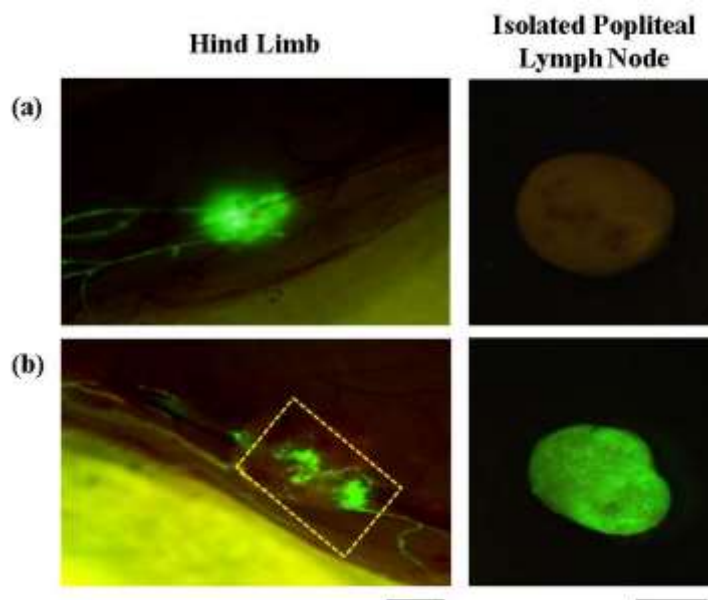


Figure 6.12 Lymphangiography results for (a) sham control and (b) 5BDE-MCol patch at 21 days post-implantation (yellow dotted box outlines the area of the implanted collagen patch; scale bars represent 2 mm for hind limb and 1 mm for isolated popliteal lymph node respectively).

6.4 Summary

In this chapter, UCol was successfully modified to yield a water-soluble MCol using a methylation modification method. Both the UCol and MCol were then crosslinked, lyophilized and then cold-pressed to produce different collagen patches. In general, the methylation modification and crosslinking process significantly improved the physicochemical properties of collagen. In addition, all the MCol patches showed a consistent drug release rate as well as solubility at physiological conditions, thus

making it a promising biomaterial for drug delivery applications. The *in vivo* results showed that the net positive-charge of MCol promoted better cell-material interactions and growth of BVs and LVs as compared to UCol, hence making it a more suitable material for wound healing applications. Between the uncrosslinked and BDE-crosslinked MCol patches, higher cellular infiltration was observed for the uncrosslinked MCol patches as compared to BDE-crosslinked MCol patches due to the weaker physicochemical properties of the uncrosslinked MCol patches that facilitated the cell proliferation and migration into the material, and thus it can potentially be used for wound healing applications. On the other hand, BDE-crosslinked MCol patches showed good integration with the surrounding tissues and supported significantly higher growth of LVs, and thus it can potentially be used for treatment of lymphedema or other inflammatory related diseases. In addition, MCol is a water-soluble collagen, which means that different types of drugs or growth factors could potentially be loaded to the MCol patches to further improve the effectiveness of the wound healing process.

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Chapter 7

Study 4 – Development of Biocomposite Scaffold

This chapter describes the detailed fabrication and characterization of biocomposite scaffolds containing both SHHA and SHCol. Subsequently, the cell-material interactions of the biocomposite scaffolds are evaluated using pre-osteoblast cells (i.e. hFOB 1.19 cells).

7.1 Introduction

HA is a type of bioceramic that possesses excellent biocompatibility, bioaffinity and osteoconductivity [1]. Due to its excellent bone integration ability and a close similarity to human bone, extensive research has been carried out on HA for bone TE applications. Currently, HA can either be chemically synthesized or extracted from natural sources. However, with the increasing need for non-toxic and clean HA, naturally-derived HA is preferred [1-3]. In addition, the HA extraction process is relatively simple and less time-consuming than the process required to synthesize HA [4]. Moreover, the extraction of HA from the natural sources particularly from the aquaculture waste material is environmentally friendly [5]. In this study, HA was extracted from snakehead scales (*Channa Micropeltes*), which is an abundantly available aquaculture waste, and the potential of the extracted SHHA was evaluated by incorporating it with a biopolymer and SHCol to form a biocomposite scaffold. In earlier studies (**Chapter 4**), collagen and HA were extracted from fish scales from different fish species. Among different fish species, SHCol exhibited the highest amount of proline (**Chapter 4, Table 4.2**) and the highest thermal stability (**Chapter 4, Figure 4.9**) as compared to TPCol and SBCol, whilst SHHA exhibited the highest YD% as compared to TPHA and SBHA (**Chapter 4, Table 4.3**). Hence, snakehead was chosen as the source of collagen and HA for this particular study.

Prior to the fabrication of the biocomposite scaffold, the SHHA was ball-milled using zirconia balls followed by soaking in 1× PBS solution to improve the particle size distribution and the pH stability of SHHA. As seen from **Appendix 6, Figure A6.1**, the mean particle size of 10 w/w% SHHA-to-zirconia balls was significantly reduced to produce small SHHA particles of < 10 µm after 2 h of ball-milling time, and thus improving the dispersion and the distribution of SHHA in the biocomposite scaffold

[6]. The ball-milled SHHA was then soaked in 1× PBS solution, which in turn significantly improved ($p < 0.05$) the pH stability of SHHA (**Appendix 6, Figure A6.2**). In addition, the fundamental cytocompatibility of SHHA was also carried out using human embryonic kidney (HEK) fibroblasts cells (i.e. HEK 293FT cells), where the cell-material interactions were assessed (**Appendix 7**). Briefly, various amounts of SHHA was incorporated into a cell encapsulating hydrogel system where the proliferation of HEK 293FT cells was characterized. As seen from **Appendix 7, Figure A7.1**, HEK 293FT cells survived and proliferated over the course of 7 days of culture regardless of the amount of HA added.

In order to further investigate the potential of SHHA for bone regeneration applications, pre-osteoblast cells (i.e. hFOB 1.19 cells) were used in this study, and the effect of the materials on cell proliferation and ALP activity were assessed and compared to commercially available SynHA controls. Briefly, SHHA was incorporated into ePCL scaffold and grafted with SHCol to produce a biocomposite scaffold. The base substrate of the biocomposite scaffold was made of ePCL scaffold, which has previously been used successfully for bone TE applications [7-9]. Electrospinning is a well-known method for the fabrication of porous scaffolds with a large surface area, which is conducive to tissue ingrowth [10]. The high porosity and the spatial interconnectivity in the structure of the electrospun nanofibrous scaffolds are crucial for tissue regeneration [11-13]. In addition, the morphology of the electrospun nanofibrous scaffolds are similar to that of the ECM of human tissues, which could potentially facilitate cell-material interactions such as cell attachment and anchorage [14]. PCL was chosen as the base material of the biocomposite scaffold due to PCL is a Food and Drug Administration approved biomaterial with excellent

biocompatibility, flexibility and long-term degradation stability that has been used clinically for bone TE applications [15-18].

However, the hydrophobic nature of PCL has resulted in poor cell attachment and proliferation [19, 20]. Hence, in order to further improve cell-material interactions, SHCol was grafted onto the electrospun nanofibrous PCL/SHHA scaffold. SHCol was not added directly to the electrospinning solution as the addition of SHCol to the electrospinning solution could result in its denaturation due to the harsh organic solvent conditions. Also, the high voltage during the electrospinning process could lead to a loss of the triple helical structures within SHCol or may even lead to the formation of gelatin instead [21-23]. Hence, a two-step reaction process was carried out in order to preserve the quality and the bioactive properties of SHCol [19, 24]. PCL was first aminolyzed to introduce free amine groups onto the PCL surface, and SHCol was then grafted onto the aminolyzed PCL surface using GA as a crosslinker. Subsequently, the effect of successful incorporation of SHHA and grafting of SHCol on the scaffold's overall cytocompatibility was evaluated using hFOB 1.19 cells.

Overall, the specific objectives and aims of this chapter are as follows:

1. Fabrication and characterization of biocomposite scaffolds containing both SHCol and SHHA
2. Investigation of the *in vitro* cytocompatibility and potential applicability of the biocomposite scaffolds for bone TE applications

7.2 Materials and Methods

SHHA was incorporated with a PCL solution in order to fabricate ePCL/SHHA scaffolds. Subsequently, SHCol was grafted onto the electrospun nanofibrous scaffold to produce a biocomposite scaffold following the method described in **Chapter 3, Sections 3.3.5 and 3.3.6**. Commercially available SynHA was used as a control. The successful incorporation of SHHA was confirmed using ATR-FTIR (**Chapter 3, Section 3.4.4**) and the distribution of SHHA across the electrospun nanofibrous scaffold was determined by staining with Alizarin Red S (**Chapter 3, Section 3.4.14**). On the other hand, the successful surface modification and grafting of SHCol were characterized using TNBS assay (**Chapter 3, Section 3.4.15**) and ATR-FTIR (**Chapter 3, Section 3.4.4**). Subsequently, the *in vitro* cytocompatibility and the potential applicability of the biocomposite scaffold were assessed using hFOB 1.19 cells (**Chapter 3, Section 3.5.1**). Various cell-material interactions were evaluated, including cell proliferation (**Chapter 3, Section 3.5.3**), cell population doubling (**Chapter 3, Section 3.5.4**), cell viability (**Chapter 3, Section 3.5.5**) and ALP activity (**Chapter 3, Section 3.5.9**) studies were carried out.

7.3 Results and Discussion

7.3.1 Fabrication of Electrospun Nanofibrous Scaffolds

7.3.1.1 Chemical Analysis

The successful incorporation of the HA into the electrospun nanofibrous scaffolds was confirmed using ATR-FTIR to identify the additional phosphate peaks of ePCL/SHHA and ePCL/SynHA scaffolds as compared to ePCL scaffolds. As seen from **Figure 7.1a**, the characteristic carbonyl peak of the PCL polymer was observed near 1726 cm^{-1} for

all the samples. Likewise, the common aliphatic compounds such as asymmetrical and symmetrical stretching of the hydrocarbon peaks of PCL polymer were also detected near 2947 and 2865 cm^{-1} for all the samples. On the other hand, the presence of the HA in the ePCL/SHHA and ePCL/SynHA scaffolds was confirmed by the presence of the characteristic phosphate peaks (solid box) of HA near 1086, 1018 and 962 cm^{-1} , which were absent in ePCL scaffolds. The presence of the HA within the electrospun nanofibrous scaffolds was further determined by staining with Alizarin Red S. As seen from **Figure 7.1b**, uniform distribution of HA was observed for the ePCL/SHHA and ePCL/SynHA scaffolds (yellow arrows), but not in the ePCL scaffolds. Overall, the results showed the successful and uniform incorporation of HA with PCL scaffolds.

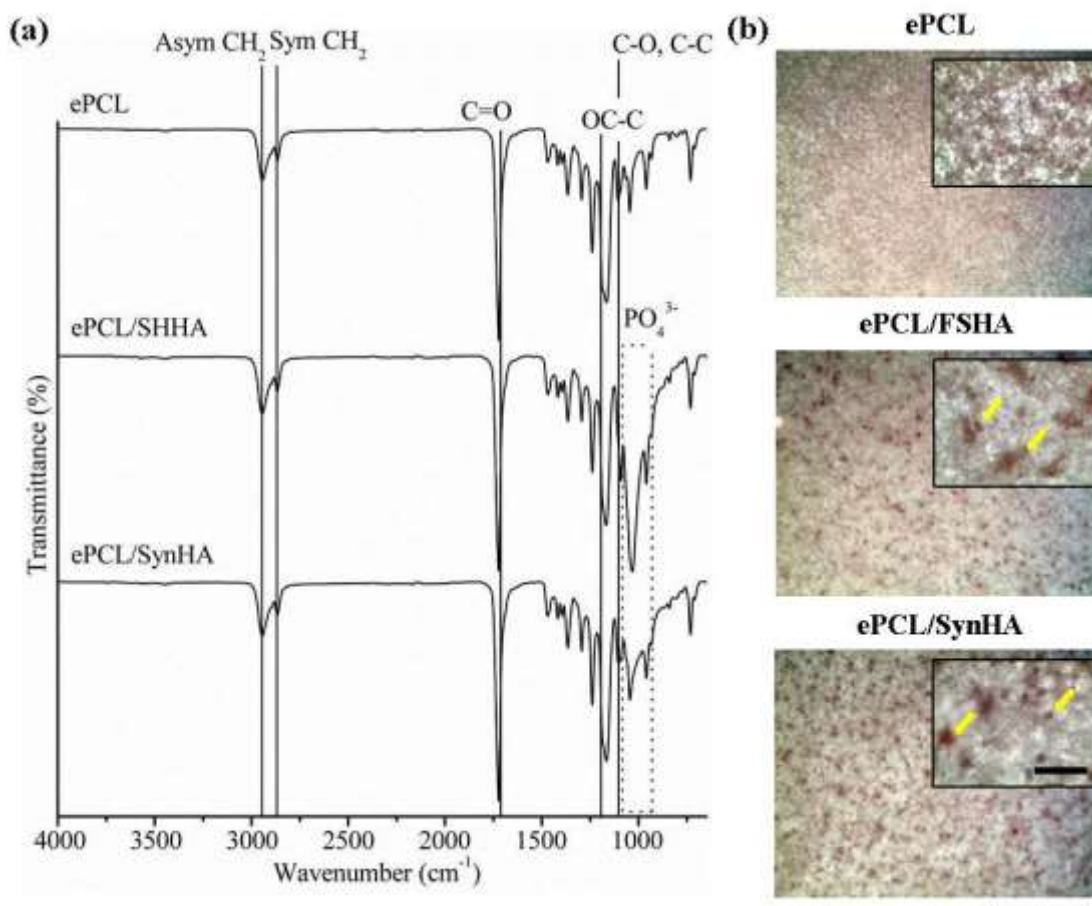


Figure 7.1 Chemical analysis showing (a) ATR-FTIR results and (b) light microscopic images of for different electrospun nanofibrous scaffolds at 4× and 40× (inset) magnifications (solid lines represent the characteristic peaks of PCL; dotted box represents characteristic phosphate peaks of HA; yellow arrows pointing to the HA; scale bar represents 1 mm; inset scale bar represents 100 μm).

7.3.2 Fabrication of Biocomposite Scaffolds

7.3.2.1 Quantification of Free Amine Groups

The successful modification of different electrospun nanofibrous scaffolds was confirmed using TNBS assay to quantify the presence of the free amine groups after both aminolysis reaction and GA-coupling. As seen from **Figure 7.2a**, a significant

higher ($p < 0.05$) color intensity of the TNBS solution was detected for all the NH_2 -containing samples, which is an indication that the scaffolds have been successfully aminolyzed with the presence of the free amine groups on the surface. Subsequently, the aminolyzed electrospun nanofibrous scaffolds were coupled with GA prior to the grafting of SHCol to prevent protein aggregation when both GA and SHCol were added together to the aminolyzed electrospun nanofibrous scaffolds [24]. The concentration of the GA used was kept at 1 w/w% to prevent any toxicity issues related to GA [25]. As seen from **Figure 7.2b**, a significant decrease ($p < 0.05$) in the color intensity was observed for GA-crosslinked samples, which is an indication that the GA was successfully coupled onto the free amine groups.

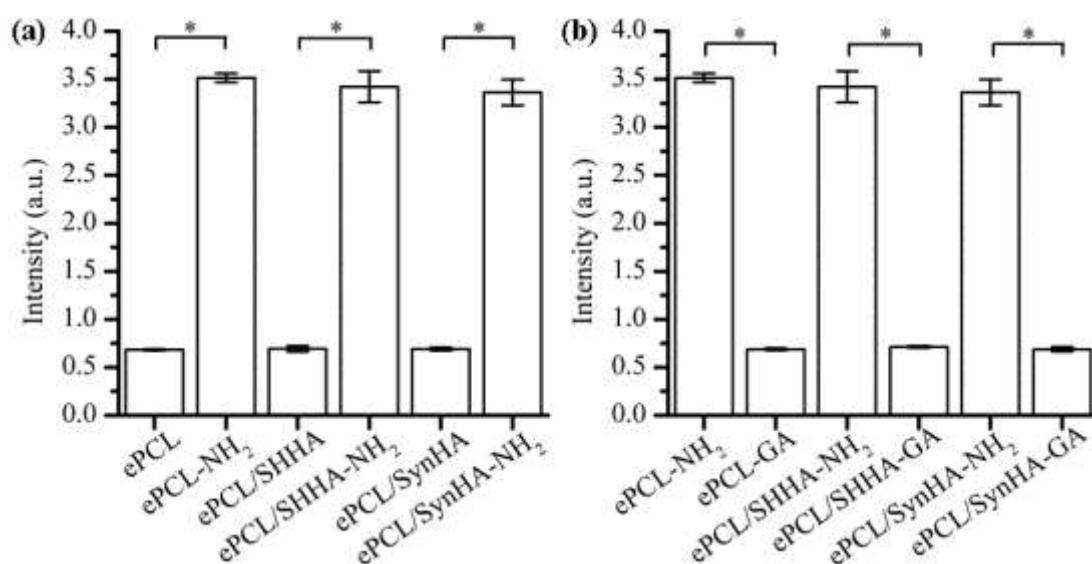


Figure 7.2 Free amine groups quantification results after (a) aminolysis reaction and (b) GA-coupling for different electrospun nanofibrous scaffolds (* $p < 0.05$).

7.3.2.2 Chemical Analysis

The successful grafting of the SHCol onto the surface-modified electrospun nanofibrous scaffolds was determined using ATR-FTIR. As seen from **Figure 7.3**, the

characteristic carbonyl peak, asymmetrical and symmetrical stretching of the hydrocarbon peaks of PCL polymer were detected for all the samples (solid lines). Likewise, the characteristic phosphate peaks of HA were also detected for the ePCL/SHHA and ePCL/SynHA samples (dotted box). Other than the characteristic carbonyl and phosphate peaks, several amide peaks were also detected, which is an indication of the successful grafting of the SHCol onto the different electrospun nanofibrous scaffolds to produce the biocomposite scaffolds. In general, the characteristic Amide A was detected near 3300 cm^{-1} due to N-H stretching while the presence of the Amide I (C=O stretching) and Amide II (C-N stretching and N-H bending out of phase) were detected near 1630 and 1530 cm^{-1} respectively.

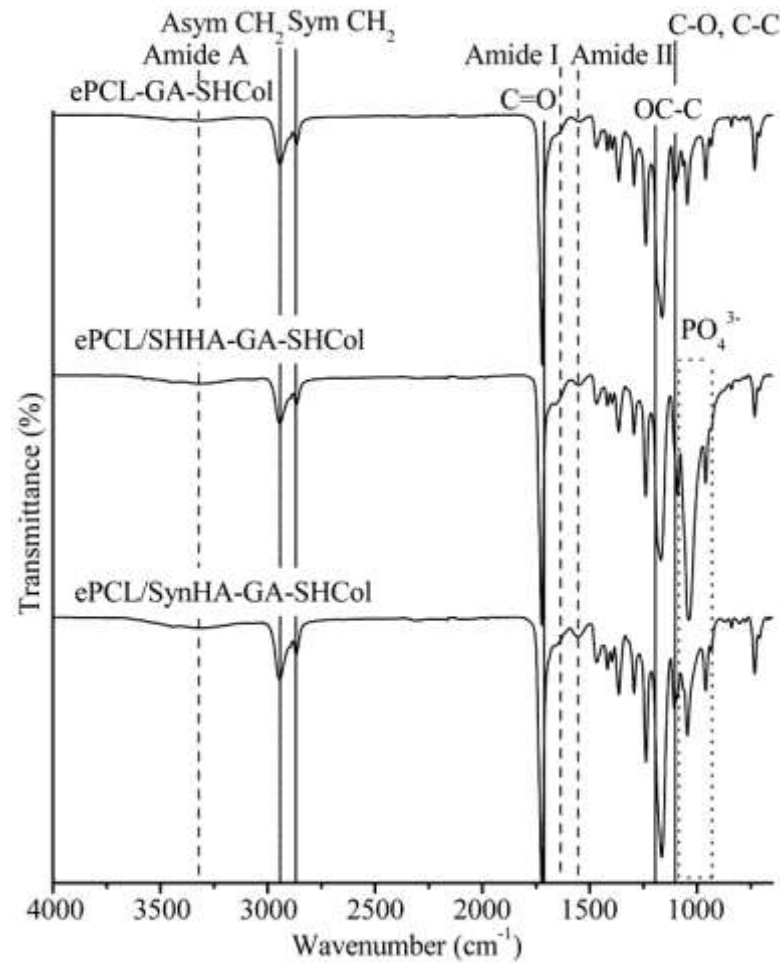


Figure 7.3 ATR-FTIR results for different biocomposite scaffolds (solid lines represent the characteristic peaks of PCL; dotted box represents characteristic phosphate peaks of HA; dashed lines represent characteristic amide peaks of collagen).

7.3.3 *In Vitro* Cellular Studies

7.3.3.1 Cell Proliferation Analysis

The number of hFOB 1.19 cells over 7 days of culture was measured by PrestoBlue® assay. As seen from **Figure 7.4a**, the number of cells attached onto the samples was similar. However, with an increasing culturing time, different samples groups exhibited different cell proliferation profiles. Among different sample groups, low

number of cells was observed on ePCL scaffolds throughout 7 days of culture, due to inherent hydrophobic properties of PCL as well as lack of bioactive sites [19]. Even though HA was incorporated into the electrospun nanofibrous scaffolds, the proliferation of hFOB 1.19 cells remained poor, which could possibly due to the fact that the hFOB 1.19 cells were mainly in contact with the surrounding hydrophobic surface of PCL as shown in **Figure 7.1b**. On the other hand, a significant improvement ($p < 0.05$) in the cell-material interactions was observed when the scaffolds were chemically-grafted with SHCol, which is an indication that SHCol was effectively grafted onto the scaffolds to improve the cytocompatibility of PCL. When the cell population doubling of hFOB cells was analyzed (**Figure 7.4b**), all the SHCol-grafted samples exhibited higher cell population doubling rate, which is in agreement in the cell proliferation results. Among different collagen-grafted biocomposite scaffolds, ePCL/SHHA-GA-SHCol and ePCL/SynHA-GA-SHCol scaffolds exhibited significantly higher ($p < 0.05$) number of cells at day 7 as compared to ePCL-GA-SHCol, which is an indication that HA and collagen on their own failed to improve the interaction of hFOB 1.19 cells with PCL. Hence, the presence of both HA and Col are equally important to improve the cell-material interactions of pre-osteoblast cells. Furthermore, ePCL/SHHA-GA-SHCol scaffolds exhibited significantly higher ($p < 0.05$) number of cells as compared to ePCL/Syn-GA-SHCol scaffolds, which could be due to the better biocompatibility of naturally-derived SHHA as compared to chemically-synthesized SynHA [26].

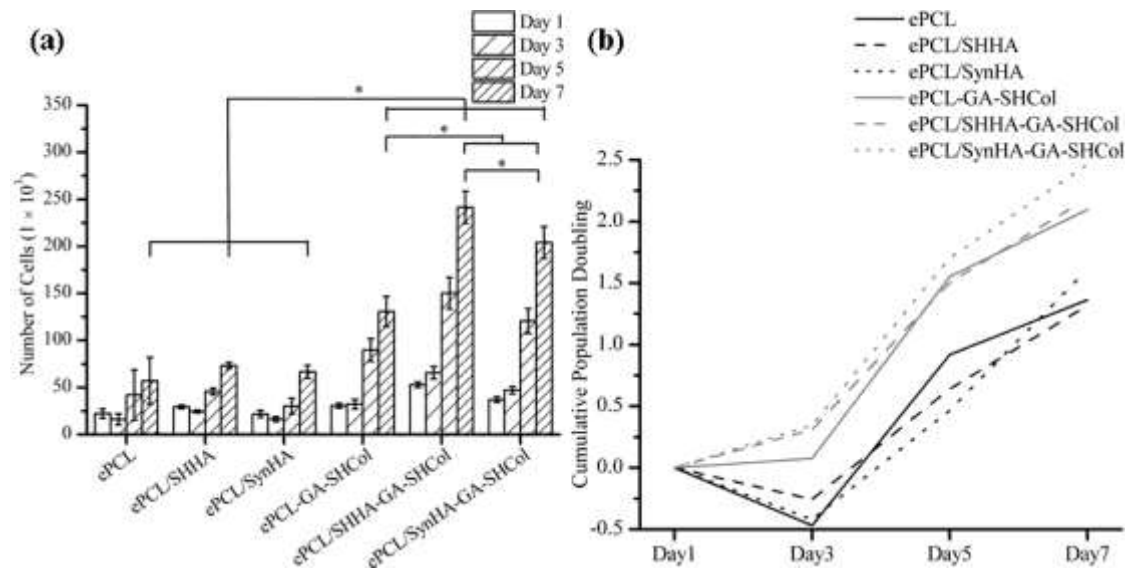


Figure 7.4 *In vitro* cellular studies showing (a) cell proliferation and (b) cumulative population doubling results for hFOB 1.19 cells seeded on different biocomposite scaffolds (* $p < 0.05$).

The hFOB 1.19 cellular coverage and the viability of hFOB 1.19 cells on different surfaces of SHCol-grafted biocomposite scaffolds at day 7 were determined by fluorescence staining. As seen from **Figure 7.5**, the inherent hydrophobic properties of PCL resulted in poor cell-material interactions, which is why there were hardly any cells being detected. Conversely, a dense coverage of hFOB 1.19 cells on the SHCol-grafted biocomposite scaffolds was observed, similar to the cell proliferation results (**Figure 7.4**). When comparing the collagen-grafted biocomposite scaffolds, the ePCL/SHHA-GA-SHCol scaffolds resulted in the most confluent layer of cells. Taken together, the presence of both SHHA and SHCol significantly improved ($p < 0.05$) the cell-material interactions of pre-osteoblast cells, which suggest that the SHHA and SHCol can potentially be used as scaffolding materials for bone TE applications.

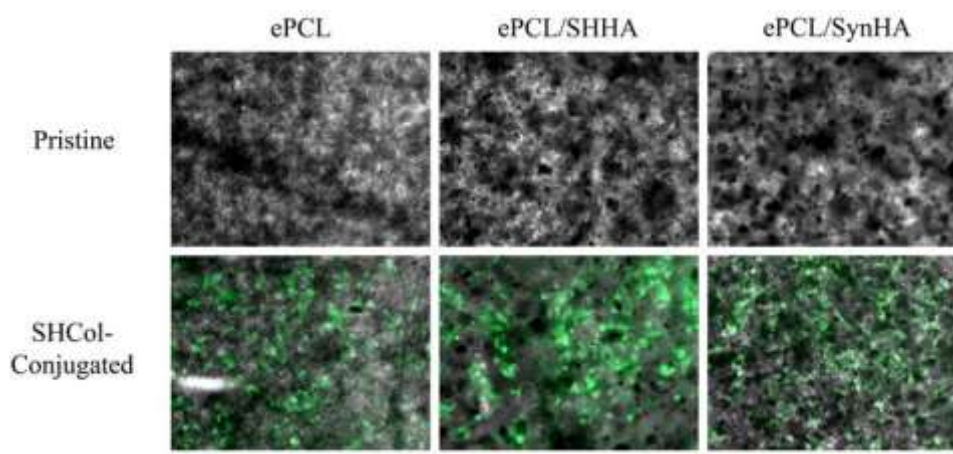


Figure 7.5 Cell viability results for hFOB 1.19 cells seeded on different biocomposite scaffolds (green fluorescence represents live cells; scale bar represents 500 μm).

7.3.3.2 ALP Activity Assay

The ALP expressed by the hFOB 1.19 cells cultured on different biocomposite scaffolds was quantified. ALP is a metalloenzyme located at the outer plasma membrane of osteoblast cells [27, 28]. The function of ALP is to catalyze the hydrolysis of phosphate reaction from phosphomonoesters to produce inorganic phosphate where the increase in the local inorganic phosphate will subsequently promote the bone mineralization process. When the total ALP activity expressed by the hFOB 1.19 cells cultured on the different biocomposite scaffolds was compared, higher total ALP activity was observed for all the HA-enriched biocomposite scaffolds, which is an indication that the presence of HA causes a significant increase ($p < 0.05$) in the ALP activity (**Figure 7.6a**), and thus the function of hFOB 1.19 cells similar to those reported by others using chitosan/ β -1,3-glucan/hydroxyapatite bone scaffold, bioactive rosette nanotube–hydroxyapatite nanocomposites and polymer-ceramic spiral structured scaffolds where the presence of HA significantly enhanced the pre-

osteoblast functions and induced osteoblastic cell differentiation [8, 29, 30]. Between the SHHA-enriched and SynHA-enriched biocomposite scaffolds, SHHA-enriched biocomposite scaffolds exhibited significantly higher ($p < 0.05$) total ALP activity as compared to SynHA-enriched biocomposite scaffolds, which is an indication that the naturally-derived SHHA led to better cell-material interactions as compared to SynHA. On the other hand, significantly higher ($p < 0.05$) total ALP activity was observed for the SHCol-grafted biocomposite scaffolds due to the presence of SHCol significantly improved ($p < 0.05$) the cell-material interactions of the biocomposite scaffolds (**Figure 7.4**). Further analysis was carried out to compare the normalized ALP activity of hFOB 1.19 cells against the number of cells. As seen from **Figure 7.6b**, ePCL/SHHA-GA-SHCol scaffolds expressed the highest normalized ALP activity as compared to the rest of the sample groups, which suggest that the presence of both SHHA and SHCol significantly improved ($p < 0.05$) the interactions of hFOB 1.19 cells with the biocomposite scaffolds. Overall, better cell proliferation and ALP expression were observed for pre-osteoblast cells cultured on PCL/SHHA-GA-SHCol scaffolds, and thus the current results demonstrated the preserved bioactive properties of the SHHA and SHCol as potential scaffolding materials for bone TE applications.

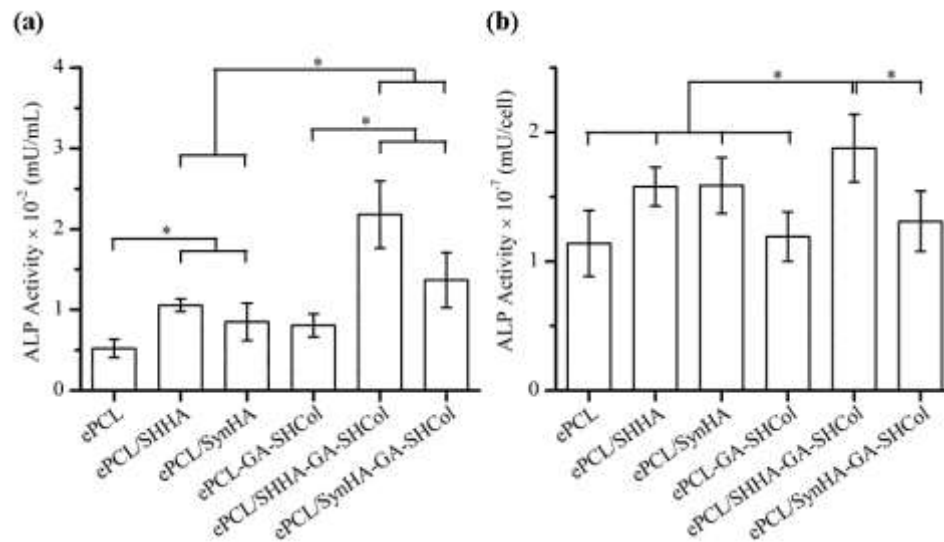


Figure 7.6 ALP expression results showing (a) total ALP activity and (b) normalized ALP activity of hFOB 1.19 cells seeded on different biocomposite scaffolds at day 7 (* $p < 0.05$).

7.4 Summary

In this chapter, SHHA was incorporated with PCL solution for the fabrication of ePCL/SHHA scaffolds followed by grafting of SHCol to produce a biocomposite scaffold. The surface modification of ePCL scaffolds with SHCol significantly improved ($p < 0.05$) the cell-material interactions using hFOB 1.19 cells. On the other hand, the presence of HA could potentially triggered the differentiation of hFOB 1.19 cells, and thus significantly improved ($p < 0.05$) the ALP expression of hFOB 1.19 cells. Between SHHA and SynHA sample groups, significantly higher ($p < 0.05$) proliferation of hFOB 1.19 cells and ALP activity were observed for ePCL/SHHA-GA-SHCol as compared to ePCL/SynHA-GA-SHCol, which is an indication that naturally-derived SHHA resulted in significant better ($p < 0.05$) cell-material interactions as compared to chemically-synthesized SynHA. Overall, the presence of

both SHHA and SHCol significantly improved cell-material interactions of pre-osteoblast cells, and thus demonstrated the potential of the resulting biocomposite scaffold for bone TE applications.

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Chapter 8

Conclusions and Future Work

This chapter summarizes all the work described in this thesis. Overall, the thesis demonstrates the development and application of the FSCol and FSHA for TE applications. This chapter also describes the implications of the research findings as well as the proposed future work with some preliminary results to support the suggested future work.

8.1 Overview of Research Findings

Fish scales, an abundantly available aquaculture waste, are rich in bioactive components such as collagen and HA, which are important biomaterials used for tissue regeneration [1, 2]. In general, fish-derived collagen has relatively no risk of zoonotic disease transmission and no religious barriers as compared to the current principle sources of mammalian-derived collagen. On the other hand, naturally-derived HA is more biocompatible and non-toxic as compared to chemically-synthesized HA [3, 4]. Hence, in this thesis, collagen and HA were extracted from fish scales from different fish species (i.e. snakehead, tilapia and seabass) and their potential applications were explored. Firstly, the structural and the physical properties of FSCol and FSHA were compared. Subsequently, the potential application of FSCol from different fish species was investigated by conjugating the extracted FSCol onto a biopolymer (i.e. PVDF) to assess the cell-material interactions using endothelial cells. Among the different fish species, the most thermally stable FSCol was selected for further studies including chemical modification and chemical crosslinking to tailor the physicochemical and the degradation stability of the FSCol. Finally, the potential application of the extracted FSHA was investigated by incorporating the extracted FSHA with a biopolymer, followed by surface modification with FSCol to form a biocomposite scaffold and the cell-material interactions was subsequently investigated using pre-osteoblast cells.

The main findings from this thesis are as follows:

1. Type I collagen was successfully extracted from fish scales using a modified acid solubilization extraction method with preserved structural quality (i.e. retention of triple helical structures) of the extracted collagen. On the other hand, a monophasic HA

was also successfully extracted from fish scales using heat-sintering method. Among different fish species, SHCol was found to exhibit the highest thermal stability, which meant that the SHCol will not degrade easily when implanted into human body. This particular property of SHCol could potentially be used as alternative source of collagen for biomedical applications (**Chapter 4**).

2. FSCol was successfully conjugated onto PVDF films via ATRP process to confer bioactive properties to the PVDF polymer. The presence of FSCol significantly improved ($p < 0.05$) the surface properties and the cytocompatibility of PVDF films, since an improvement in the cell-material interactions was observed. Among different fish species, SHCol demonstrated the best hemocompatibility behavior as compared to TPCol and SBCol. Hence, SHCol was chosen for subsequent studies (**Chapter 5**).

3. SHCol was successfully chemically-modified via partial esterification process to produce a water-soluble MCol. Subsequently, both the UCol and MCol were successfully crosslinked using BDE, lyophilized and cold-pressed to produce different collagen patches. Overall, both the chemical modification and chemical crosslinking processes significantly improved ($p < 0.05$) the physicochemical properties and the degradation stability of collagen. *In vivo* studies using a murine model showed the biocompatibility and degradability of different collagen patches. Among different collagen patches, MCol patches supported better growth of BVs and LVs. In addition, *in vitro* drug release studies also showed the potential of MCol as a drug carrier with a consistent drug release rate observed. Hence, different types of drugs or growth factors could be incorporated into MCol patches to improve the wound healing process (**Chapter 6**).

4. SHHA was successfully incorporated into PCL solution to produce ePCL/SHHA scaffold, followed by surface modification with SHCol to form a biocomposite scaffold. The presence of both SHHA and SHCol significantly improved ($p < 0.05$) the cell-material interactions of hFOB 1.19 cells with significant higher cell proliferation rate and significant higher ALP activity were observed, and thus demonstrated the potential of SHHA and SHCol as alternative bioactive materials for bone TE applications (**Chapter 7**).

8.2 Implications of Research Findings

1. Effectiveness of waste-to-resources strategy in producing cost-effective collagen

Over the years, the production of aquaculture products has been growing rapidly to meet the demand of human consumption worldwide. However during food processing, large amounts of waste material are generated such as fish skin, bone and scales, which are unavoidable [5]. In fact, fish scales are rich in collagen and HA, which are widely used for various TE applications. In general, the current principle source of collagen is mainly derived from mammalian animals, which may contain the risk of zoonotic disease transmission, may have religious restrictions in some countries and may also involve complex and high cost extraction processes [3, 6, 7]. On the other hand, fish-derived collagen is considered as a safe source of collagen without the risk of zoonotic infectious disease transmission from mammalian-derived collagen such as BSE, FMD or avian influenza virus as well as allergic issues [8-10]. Also, fish-derived collagen is not prohibited for any cultural or religious issues, and is acceptable to all global cultures [10, 11]. Hence, these aquaculture waste material particularly in the form of fish scales can be considered as an easily accessible alternative resource

resilient, cost effective source of waste-to-resource bioactive material for biomedical applications [10, 11]. In addition, utilization of fish scales for the extraction of collagen and HA is also a green revolution of biotechnology and an effective waste management system for environmental protection and sustainability [12].

2. Potential of FSCol with high thermal stability for biomedical applications

Although fish-derived collagen bears no risk of disease transmission and has no religious barriers associated with it, the low thermal stability of fish-derived collagen has limited its potential biomedical applications [11]. In general, the thermal stability of collagen is highly dependent on the living environment and body temperature of the species, which in turn affects the amino acid composition, conformational stability of triple helical structures and the thermal stability of collagen. Interestingly, collagen derived from freshwater fish origin exhibited better physicochemical properties as compared to seawater fish [13]. Hence in this thesis, fish scales from freshwater fish were used for the extraction of collagen as compared to seawater fish, and its potential application for biomedical usage was also further explored. Findings from **Chapter 4** demonstrated that the Type I collagen was successfully extracted from fish scales from different fish species using a modified acid solubilization method (**Chapter 4, Figure 4.7**). In addition, the ATR-FTIR analysis showed the presence of triple helical structures within the extracted FSCol with a $A'(Amide\ III)/A'(1450)$ of ~ 1.0 (**Chapter 4, Figure 4.8**), suggesting that the triple helical structures of the extracted collagen was well preserved using a modified acid solubilization extraction method, and thus the quality of collagen. When the amino acid composition of the extracted collagen was compared, SHCol exhibited the highest amount of proline as compared to tilapia and seabass (**Chapter 4, Table 4.2**). When the thermal analysis was carried out, once again, SHCol exhibited the highest T_d (52.9 ± 0.3 °C), significantly higher ($p < 0.05$)

than tilapia and seabass (**Chapter 4, Figure 4.9**). Overall, the T_d of SHCol is higher than that of the human body temperature of 37 °C, which suggests that SHCol will not dissolve or degrade easily during the course of implantation, and thus could be a promising safer alternative source of collagen for biomedical applications.

3. Potential of SHCol for blood-contacting applications

The potential of the extracted FSCol as a biomaterial in blood-contacting applications was also investigated. Briefly, the extracted FSCol was conjugated onto the synthetic polymer (i.e. PVDF), where the cell-material interactions were assessed using HUVECs (**Chapter 5**). The ATRP process was used for the conjugation of collagen to ensure more uniform distribution and stronger adhesion of FSCol onto the PVDF films. Overall, the presence of FSCol significantly improved ($p < 0.05$) the surface chemistry (**Chapter 5, Figure 5.2**) and the cytocompatibility (**Chapter 5, Figures 5.3 and 5.4**) of PVDF films. In addition, when the functional and hemocompatibility properties of different species of FSCol were compared, SHCol elicited the least pro-inflammatory response (**Chapter 5, Figure 5.5**) and enhanced anti-thrombogenicity behavior (**Chapter 5, Figures 5.6, 5.7 and 5.8**). Hence, findings from this study demonstrated the potential of SHCol as an alternative source of collagen for blood-contacting applications.

4. Potential of MCol for wound healing applications

SHCol was chemically-modified via partial esterification process using acidified methanol to produce water-soluble MCol. Subsequently, both the collagen in the forms of UCol and MCol were chemically-crosslinked using bi-epoxy agent (i.e. BDE), lyophilized and then cold-pressed to produce different collagen patches. Finally, the physicochemical properties and the *in vivo* biocompatibility as well as the

degradability of different collagen patches were evaluated (**Chapter 6**). Based on the physicochemical analysis, both the methylation modification and the BDE crosslinking processes significantly improved ($p < 0.05$) the stiffness and the degradation stability of collagen patches (**Chapter 6, Figures 6.5 and 6.6**). When the *in vivo* studies were carried out, histological results showed the biocompatibility of the methylation modification with no significant differences observed between the UCol and MCol (**Chapter 6, Figure 6.9**). In addition, MCol supported better growth of BVs and LVs as compared to UCol (**Chapter 6, Figure 6.11**). Overall, MCol, particularly in the uncrosslinked form, encouraged significantly greater cellular infiltration and ingrowth of vessels and is therefore suitable to be used for wound healing applications (**Chapter 6, Figures 6.9 and 6.10**). On the other hand, a significantly higher number of LVs was also found growing within and around the BDE-crosslinked MCol patches, thus suggesting that MCol could be used for treatment of lymphedema or other inflammatory related diseases (**Chapter 6, Figures 6.11 and 6.12**). Finally, MCol is a water-soluble collagen and the *in vitro* drug release studies showed that all the MCol patches exhibited a consistent drug release behavior (**Chapter 6, Figure 6.7**). Hence, it could also be further used as a drug carrier containing growth factors to enhance the regeneration process.

5. Potential of biocomposite scaffold for bone TE applications

A monophasic SHHA was successfully extracted from fish scales using a heat-sintering method. Subsequently, the potential biomedical applications of the extracted SHHA was investigated by incorporating the extracted SHHA with PCL solution to fabricate an electrospun nanofibrous scaffold, followed by surface modification using SHCol to form a biocomposite scaffold (**Chapter 7**). *In vitro* cellular studies showed that the presence of both SHHA and SHCol supported the growth and proliferation of pre-

osteoblast cells as well as the functionality of hFOB 1.19 cells (**Chapter 7, Figures 7.4, 7.5 and 7.6**), and thus demonstrated the potential of the biocomposite scaffold consisting of waste-to-resource bioactive materials, namely SHCol and SHHA, for bone TE applications.

8.3 Future Work

8.3.1 Fabrication of VEGF-C156S-loaded Methylated Collagen Patches

Methylation modification of collagen produced a positively-charged MCol, which improved its solubility at physiological conditions that broaden its potential as a delivery system as shown in **Chapter 6, Section 6.3.2.4** using a simple protein model (i.e. FITC-BSA). Further studies will also be carried out to optimize and to investigate the methylated collagen patches as a delivery vehicle for growth factor such as vascular endothelial growth factor C (VEGF-C). In general, VEGF-C is well known to encourage both angiogenesis and lymphangiogenesis, which are crucial for wound healing process [14, 15]. First, the VEGF-C release profile will be studied in order to investigate the release behavior of VEGF-C using the methylated collagen patches. The loading efficiency, recovery and the amount of VEGF-C released into the release buffer solution (i.e. $1\times$ PBS) will be measured using VEGF-C enzyme-linked immunosorbent assay (ELISA) kit over 28 days. In general, the crosslinking density and the amount of drug loaded into the methylated collagen patches will be optimized in order to obtain a localized and sustained release of VEGF-C in the range of ~ 100 ng/day [16, 17]. Subsequently, the *in vivo* sustained release of VEGF-C will be carried out and the presence of VEGF-C in promoting wound healing particularly for the chronic wound will be investigated. For this future work, the methylated collagen

patches loaded with VEGF-C will be implanted in high-fat and high-fructose diet-induced diabetes mouse model to evaluate the healing and inflammatory responses. In this case, pure methylated collagen patches will be used as controls. Other than wound healing, the potential application of the methylated collagen patches in inflammatory related diseases such as lymphedema and atherosclerosis will also be carried out. In this case, VEGF-C156S will be used instead of VEGF-C. In general, VEGF-C156S is a mutated form of VEGF-C, which is specifically used to promote lymphangiogenesis where the cysteine residue (156) of the VEGF-C is being replaced by serine residue that resulted in losing its binding affinity towards blood vessels [15, 18]. The release behavior of VEGF-C156S will be investigated prior to *in vivo* studies using ELISA kit. Subsequently, the methylated collagen patches loaded with VEGF-C156S will be implanted in apolipoprotein E-deficient (ApoE^{-/-}) mouse model to evaluate the lymphatic growth and restoration.

8.3.2 Fabrication of FSCol Hydrogels

Three-dimensional (3D) scaffolds play a vital role for biomedical applications by providing a 3D cellular environment for tissue regeneration by mimicking the physiological and the geometrical architecture of the damaged or defective tissues [19]. Hence, 3D scaffolds have been widely used for biomedical applications, particularly in the form of hydrogel due to its superior high water content that could be used for cells encapsulation as well as facilitate the transportation of nutrients and metabolic waste [20]. In general, various scaffold fabrication techniques have been used to create a 3D hydrogel for biomedical applications such as chemical crosslinking of the hydrogel polymeric precursors (free radical polymerization or irradiation crosslinking of the

hydrogel polymeric precursors) or physical crosslinking of the hydrogel polymeric precursors (ionic interaction, hydrogen bonding or hydrophobic association interaction between polymer chains) [21]. Among different types of fabrication techniques, bioprinting technique is the most favorable method due to its ability to design and to fabricate scaffolds with complex geometries for TE applications [22, 23].

In general, the printability of a hydrogel is highly dependent on the viscosity of the hydrogel solution, the shear-thinning property and the sol-gel responsive time [22]. A short responsive time is required to prevent the deformation of the hydrogel, which in turn highly dependent on the gelation rate of the material. Hence, in order to investigate the potential of the extracted SHCol as scaffolding material for 3D printing, the gelation rate (i.e. fibrillogenesis) of the extracted SHCol was investigated according to previously established protocol [24]. Briefly, 10 mg/mL of SHCol solution was prepared by dissolving the lyophilized SHCol in 0.5 M of acetic acid. Subsequently, 10 v/v% of 1× PBS was added into the SHCol solution and the pH was adjusted to 7.4. Finally, the gelation was carried out by heating the sample to 37 °C. As seen from **Figure 8.1a**, the macroscopic images showed the formation of SHCol hydrogel with a slow gelation time of approximately 10 min. However, the current SHCol hydrogel is too weak to be handled with a forceps (**Figure 8.1b**). Hence, further optimization is required in terms of the gelation rate and the physical properties of SHCol hydrogel.

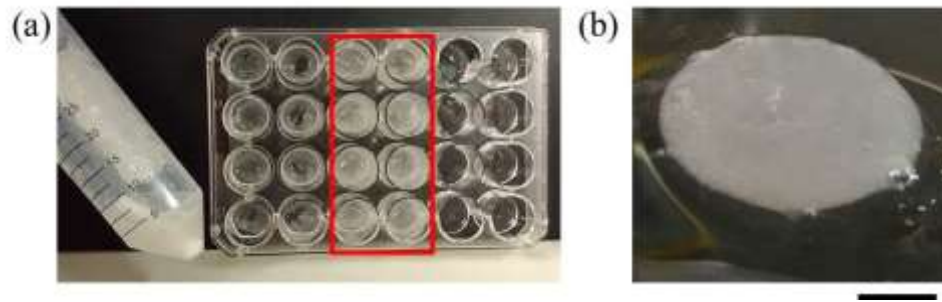


Figure 8.1 Macroscopic images showing (a) the SHCol hydrogels in 24-well plates and (b) the appearance of stable SHCol hydrogel after 10 min of gelation (scale bar represents 5 mm).

In general, the gelation rate and the physical properties of SHCol hydrogel can be tailored by incorporating different types of additives or chemical agents. The gelation rate, which is the fibrillogenesis of collagen, is highly dependent on the electrostatic interactions of the peptide chains [25]. Hence, chondroitin sulfate (CS) can be used as the additive to improve the gelation rate of SHCol by altering the charge distributions and water binding efficacy of collagen [25-27]. In addition, CS has been shown to improve the proliferation of keratinocytes, and thus making it suitable for biomedical applications [28]. On the other hand, in order to improve the physical properties of SHCol hydrogel, chemical crosslinking agents such as BDE or GA can be introduced into the SHCol hydrogel to improve the structural properties of the SHCol hydrogel in subsequent experiments.

8.3.3 Fabrication of Collagen/ECM Biocomposite Scaffolds

The ECM is a complex environment, consisting of Type I collagen as well as other biological components such as glycoproteins, glycosaminoglycans and proteoglycans

that act together to regulate cell behavior, cell differentiation, and binding of growth factors and cytokines [26, 29-31]. Hence, a single component scaffold that is made up of Type I collagen might not be able to mimic the functional and biochemical aspects of the complex tissue as compared to a multi-component scaffold. Thus, keeping to the theme of waste-to-resources, further studies involving the extraction of ECM from human adipose tissue, a surgical waste product, and subsequently incorporating the extracted ECM with SHCol to improve cellular interactions and differentiation are carried out. In addition, one particular study has also shown that by incorporating different amounts of ECM into a scaffold, it could also improve the overall physicochemical properties of the scaffold [32]. Fresh adipose tissue samples were collected from Tan Tock Seng Hospital (TTSH), Singapore using procedures approved by National Healthcare Group Domain Specific Review Board (NHG DSRB 2012/00071). The overall extraction process is illustrated in **Figure 8.2** according to the previously established method [33]. Briefly, the adipose tissue samples were cut into small pieces and cleaned using 1× PBS solution, followed by freeze-thaw process at -80 °C to lyse the cells within the tissue samples. Subsequently, the adipose tissue was homogenized at 20,000 rpm for 1 min on ice followed by centrifugation at 5,300 rpm for 5 min to collect the ECM pellet at the bottom of the centrifuge tube. The supernatant was then subjected to another 4 rounds of homogenization-centrifugation steps to maximize the collection of ECM. Finally, the homogenized ECM was treated with DNase, followed by lyophilized and storage at 4 °C until required.

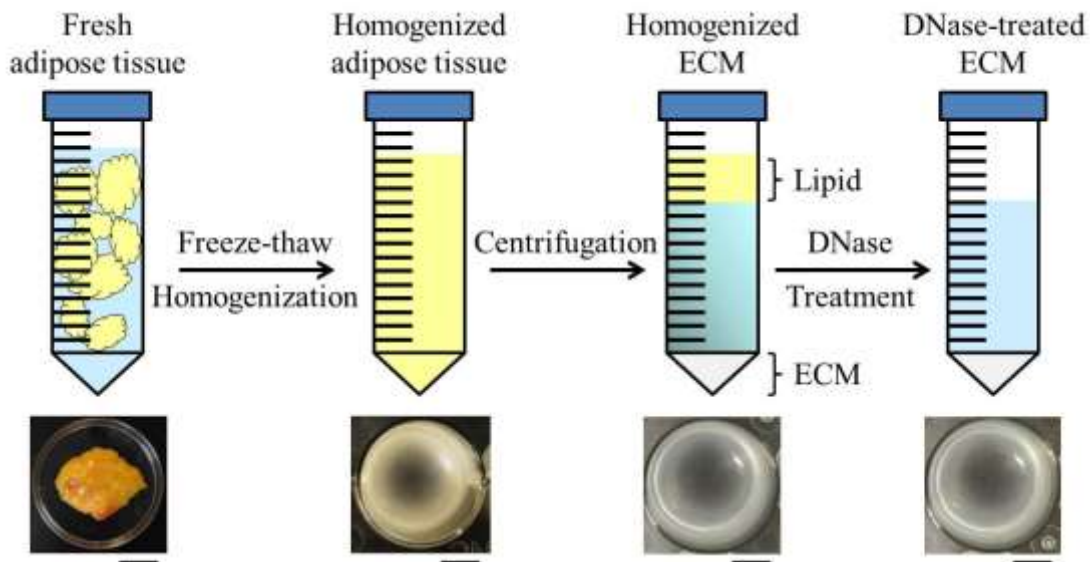


Figure 8.2 Schematic representation of the steps involved in the extraction of decellularized ECM from fresh adipose tissue using the physical homogenization method and the representative images of the adipose tissue at each stages (scale bar represents 1 cm).

The successful removal of lipids from the adipose tissue was confirmed by macroscopic imaging and Oil Red O staining. As seen from **Figure 8.2**, the homogenized adipose tissue appear yellowish in color, due to the presence of the lipid component in the homogenized solution. On the other hand, both the homogenized ECM and DNase-treated ECM appear white color, which is an indication of the successful removal of the lipid component after the centrifugation step. When Oil Red O staining was carried out (**Figure 8.3**), lipid droplets were observed on the homogenized adipose tissue. Once again, the lipids droplets were not visible for the homogenized ECM and DNase-treated ECM samples after the centrifugation process. Taken together, the homogenization and the centrifugation processes were successful in removing the lipid components from the ECM material.

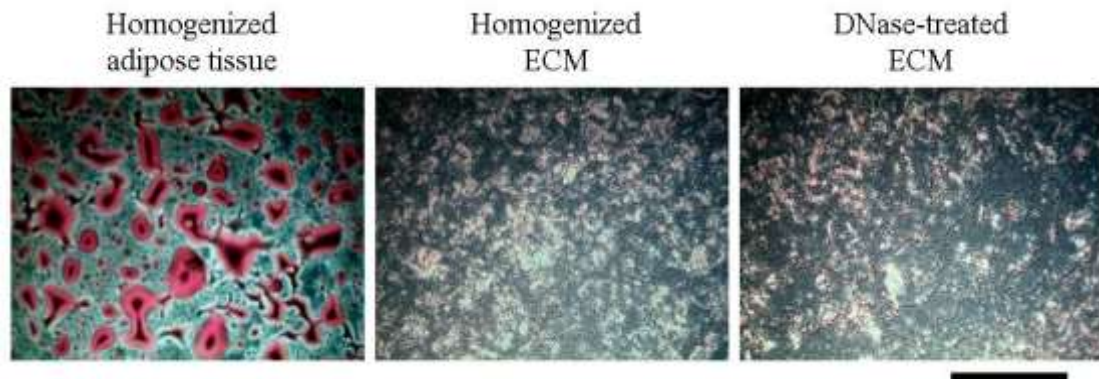


Figure 8.3 Oil Red O staining results for different adipose-derived ECM samples (red stain represents lipid droplets; scale bar represents 400 μm).

Subsequently, the effectiveness of the physical decellularization method in removing the cellular components was determined by quantifying the DNA content within different samples using Quant-iT PicoGreen dsDNA (Invitrogen, USA). DNase-treated ECM was used as control. As seen from **Figure 8.4**, no significant difference was observed between the homogenized ECM and the DNase-treated ECM. In addition, the DNA content for both samples were within the threshold value stipulated for the clinical applications of decellularized material of 50 ng/mg [34], which is an indication of the effectiveness of the physical decellularization method for the removal of cells and the cellular components from the homogenized ECM. Hence, homogenized ECM sample was used for further cellular studies.

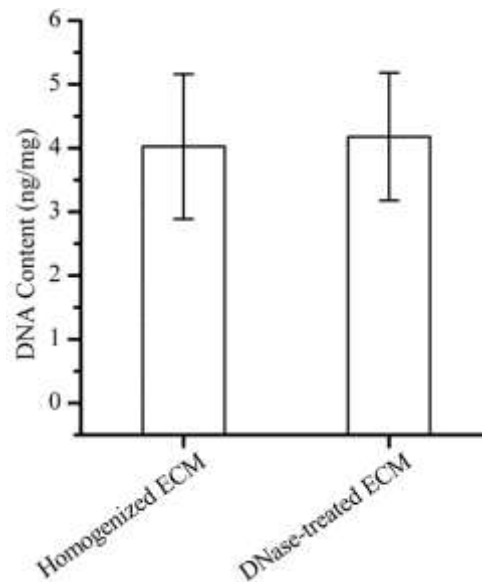


Figure 8.4 DNA quantification results for homogenized ECM and DNase-treated ECM.

Finally, the cell-material interactions of the homogenized ECM were investigated using human-derived keratinocyte HaCaT cells. SHCol was used as a control. As seen from **Figure 8.5a**, HaCaT cultured on homogenized ECM exhibited positive cell-material interactions with increasing number of cells observed over the 7 days of culture. In addition, no significant difference was observed for the cell proliferation rate between the homogenized ECM and SHCol (**Figure 8.5b**). After 7 days of culture, a dense coverage of HaCaT cells was observed on the surfaces of both the homogenized ECM and the SHCol samples(**Figure 8.5c**), which is an indication that the physical decellularization process used to extract ECM did not cause the deterioration of the biological components of the homogenized ECM. Taken together, ECM was successfully extracted from adipose tissue samples using a physical decellularization method. Thus, further studies involving the incorporation of

homogenized ECM with SHCol will allow for the fabrication of biocomposite scaffold for biomedical applications.

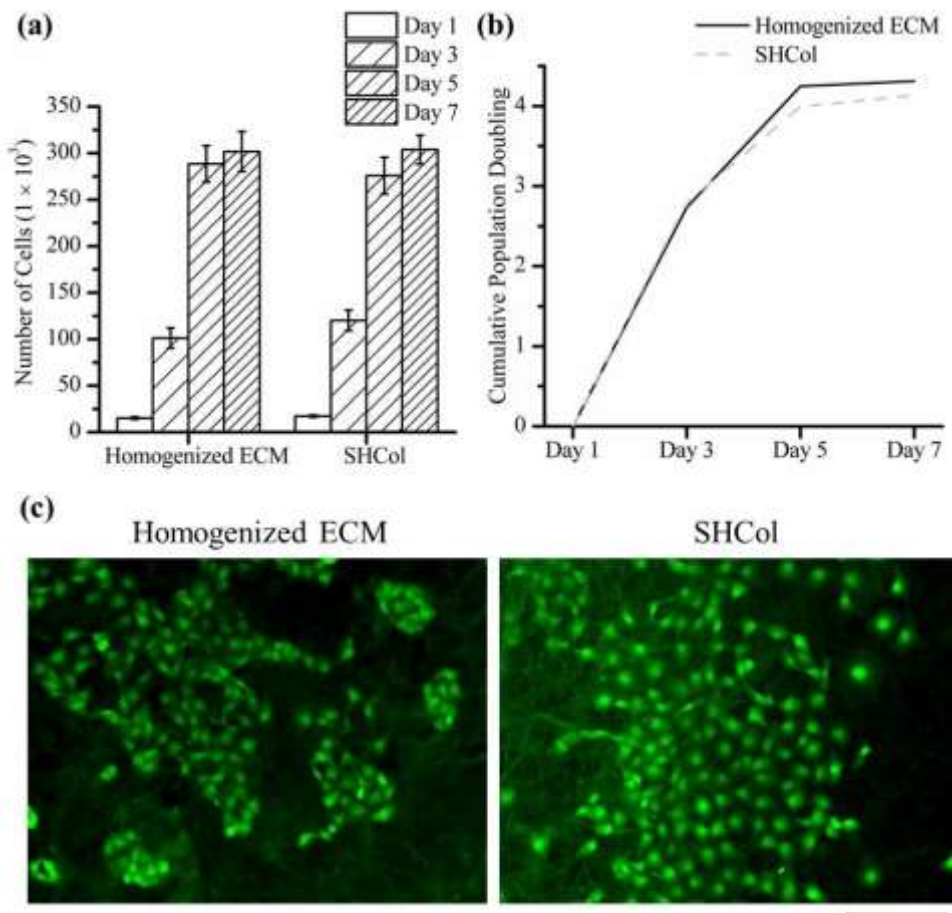


Figure 8.5 Cellular studies showing (a) cell proliferation results, (b) cell population doubling results and (c) cell viability results for HaCaT seeded on surfaces of homogenized ECM and SHCol (green fluorescence represents live cells; scale bar represents 200 μm).

8.3.4 Bullfrog Skin-derived Collagen

Within the aquaculture industry, other than the increasing demand for fish, the supply of bullfrog has also increased drastically over the past years across the world [35]. In general, the flesh of the bullfrog is consumed while the skin is generally discarded as

waste. However, similar to fish scales, bullfrog skin also contains high amounts of collagen. In addition, bullfrog is an amphibian animal and carries no threat of disease transmission [3, 36, 37]. Hence, bullfrog skin can also be used as another alternative source of collagen for biomedical applications. In this study, bullfrog skin was obtained from KhaiSeng Trading & Fish Farm Pte. Ltd., Singapore as shown in **Figure 8.6a**. Prior to the extraction process, the presence of collagen within the bullfrog skin was firstly confirmed using SEM imaging. As seen from **Figure 8.6b**, random collagen fibres were observed within the bullfrog skin.

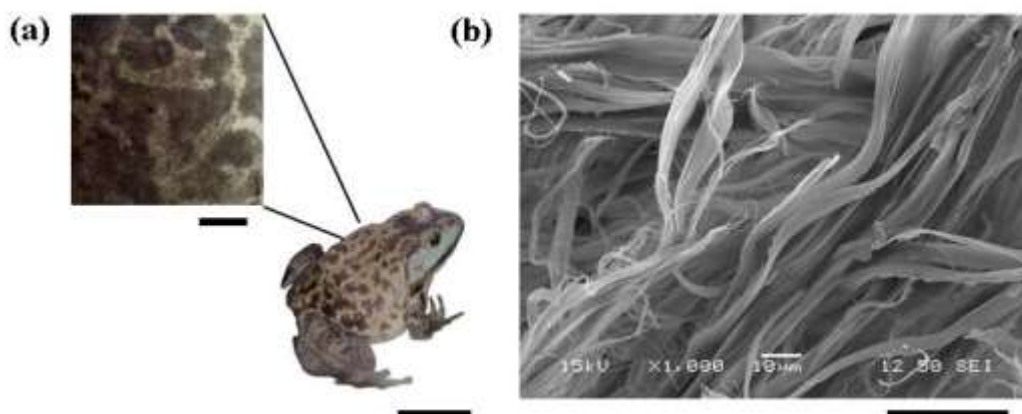


Figure 8.6 Alternative source of collagen in the form of bullfrog skin showing (a) macroscopic image of American bullfrog (*Rana Catesbeiana*) species and (b) SEM image of collagen fibres within the bullfrog skin (scale bars represent 1 cm for bullfrog skin, 5 cm for bullfrog and 30 μm for the SEM image respectively).

Subsequently, bullfrog skin-derived collagen (BFCol) was extracted using a similar modified acid solubilization method as described in **Chapter 3, Section 3.3.1**, except with the omission of the demineralization step. The different chemical bonds present in the extracted BFCol were identified using ATR-FTIR. As seen from **Figure 8.7a**, different characteristic amide peaks of collagen were identified, similar to that of

FSCol and commercially available BVCol. In general, Amide A (N-H stretching), Amide B (N-H stretching), Amide I (C=O stretching), Amide II (C-N stretching and N-H bending out of phase) and Amide III (C-N stretching and N-H bending in-phase α -helix) were detected near 3300, 2920, 1630, 1530 and 1200 cm^{-1} respectively [38]. The subunit compositions of the BFCol were further analyzed using SDS-PAGE analysis as shown in **Figure 8.7b**. As seen from the figure, a typical Type I collagen bands were detected near 400, 250, 139 and 129 kDa corresponding to the γ -, β -, α 1- and α 2-chains respectively, similar to that of the FSCol and BVCol. In addition, the width ratio of the α 1- and α 2-chains for the BFCol was close to 2:1, which suggests that the extracted BFCol was indeed Type I collagen [39].

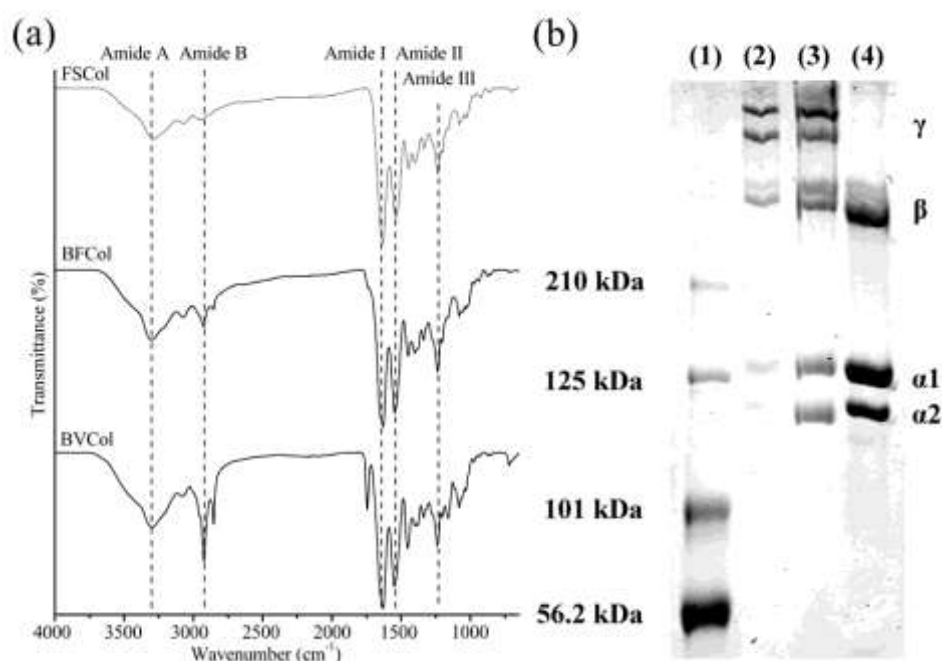


Figure 8.7 Chemical and protein analyses showing (a) ATR-FTIR spectra and (b) SDS-PAGE results of BFCol [dashes lines represent characteristic amide peaks; (1) Protein ladder as marker; (2) FSCol; (3) BFCol; (4) BVCol].

Finally, the thermal stability of the extracted BFCol was analyzed using DSC to measure the T_d of BFCol. As seen from **Table 8.1**, BFCol exhibited a T_d of 42.95 ± 0.55 °C, which is higher than the normal human body temperature. This suggests that the BFCol will not dissolve easily when it is used as scaffolding material for TE applications. Taken together, BFCol can also be a potential alternative safer source of Type I collagen with good thermal stability for biomedical applications. Hence, subsequent studies will involve the study of the physicochemical properties and the cell-material interactions between BFCol and other sources of collagen (i.e. FSCol and BVCol) for various biomedical applications.

Table 8.1 DSC results for the different sources of collagen.

Sample	T_d (°C)
FSCol	52.82 ± 0.36
BFCol	42.95 ± 0.55
BVCol	55.47 ± 1.13

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Appendices

Appendix 1: Physically-coated Collagen on PVDF Films Study

Physical adsorption of different sources of collagen onto PVDF films was carried out as a comparison to the ATRP reaction used in **Chapter 5**. Briefly, the PVDF films were immersed directly in 3 mg/mL of collagen solution dissolved in 0.5 M acetic acid and stirred for 72 h at room temperature to produce collagen-coated PVDF films. The collagen-coated PVDF films were then rinsed with deionized water, and dried in a vacuum oven at 37 °C before storing at dry cabinet until required.

The successful coating of collagen onto pPVDF was confirmed by the presence of the characteristic amide peaks of collagen as showed in **Figure A1.1**. As seen from the figure, the characteristic carbon-fluorine peak was observed for the pPVDF near 1177.91 cm^{-1} . For the collagen-coated PVDF films, several characteristic amide peaks were observed near 2958.86 cm^{-1} (carbon-hydrogen stretching of amide B), 1661.26 cm^{-1} (carbonyl stretching of amide I) and 1555.42 cm^{-1} (nitrogen-hydrogen bending of amide II), which were absent in the spectrum of pPVDF. As compared to ATRP process, the physical adsorption process is relatively uncontrollable, thus resulting in broad and inconsistent absorption peaks, due to protein agglomeration during the physical adsorption process.

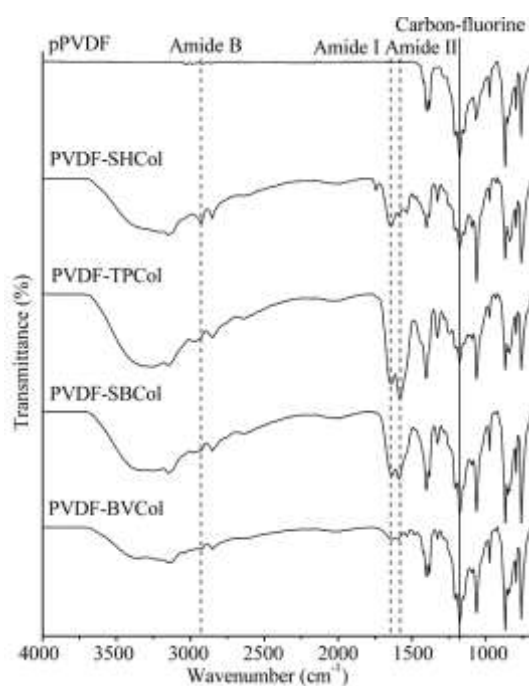


Figure A1.1 ATR-FTIR results for the different collagen-coated PVDF films (solid line represents the characteristic carbon-fluorine peak of PVDF; dashed lines represent characteristic amide peaks of collagen).

The surface hydrophilicity changes of different collagen-coated PVDF films were also measured as a comparison to the chemically-modified PVDF films. As seen from **Figure A1.2**, the WCA values for the different collagen-coated PVDF films were similar to the pPVDF. This suggests that the direct coating of collagen onto the PVDF films did not improve the surface hydrophilicity of PVDF films, unlike the chemically-modified PVDF films.

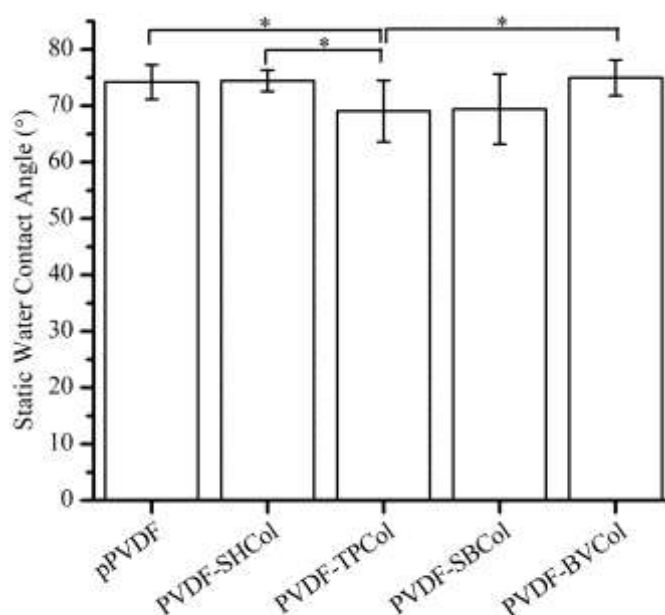


Figure A1.2 Static WCA results for the different collagen-coated PVDF films ($*p < 0.05$).

The cell-material interactions of HUVECs cultured on the different collagen-coated PVDF films over 7 days were also evaluated. As seen from **Figure A1.3**, poor cell numbers were observed for all the different collagen-coated PVDF films. In fact, the number of cells was significantly lower ($p < 0.05$) than the tissue culture plate control, which could be due to the hydrophobic nature of the surfaces of different collagen-coated PVDF films that inhibited the adherence and proliferation of cells.

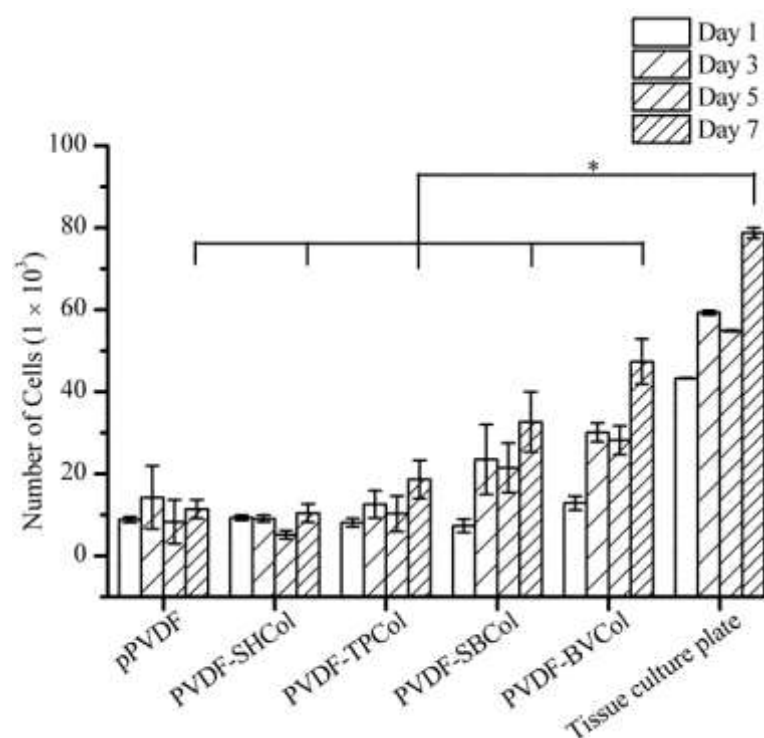


Figure A1.3 Cell proliferation results for HUVECs seeded on different collagen-coated PVDF films (* $p < 0.05$).

The viability of HUVECs cultured on different collagen-coated PVDF films at 7 days was also determined using fluorescence staining. As seen from **Figure A1.4**, all the different collagen-coated PVDF films had low number of cells as compared to the tissue culture plate control, which is in agreement with the cell proliferation results (**Figure A1.3**). Taken together, direct physical coating of collagen onto the pPVDF films did not improve the overall surface hydrophilicity and the subsequent cell-material interactions of the PVDF films.

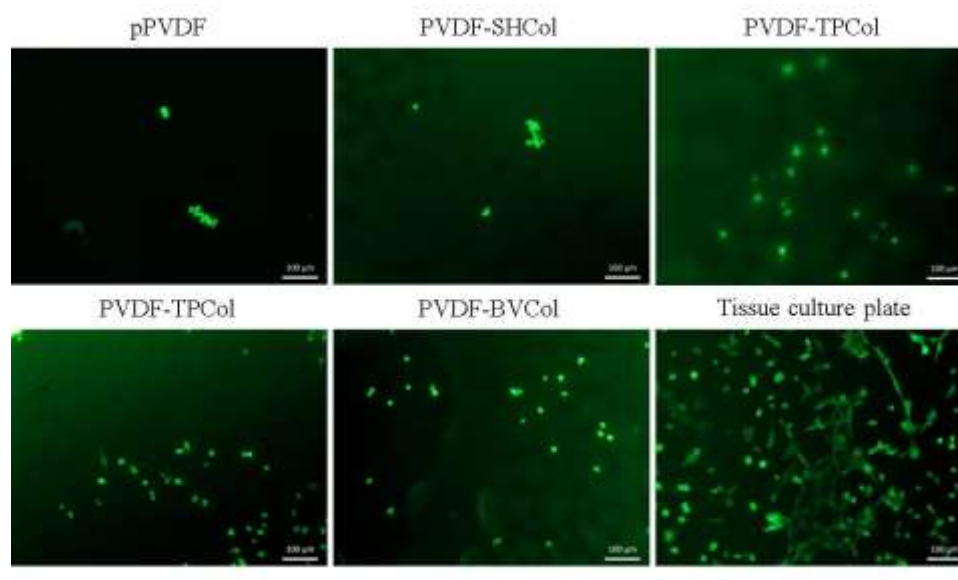


Figure A1.4 Cell viability results for HUVECs seeded on different collagen-coated PVDF films (green fluorescence represents live cells; scale bar represents 300 µm).

Appendix 2: Morphological analysis of cold-pressed collagen patches

The morphology of the cold-pressed UCol and MCol patches was imaged using SEM as shown in **Figure A2.1**. After cold-pressing, the cold-pressed collagen patches did not exhibit a porous 3D structure as seen from the figure.

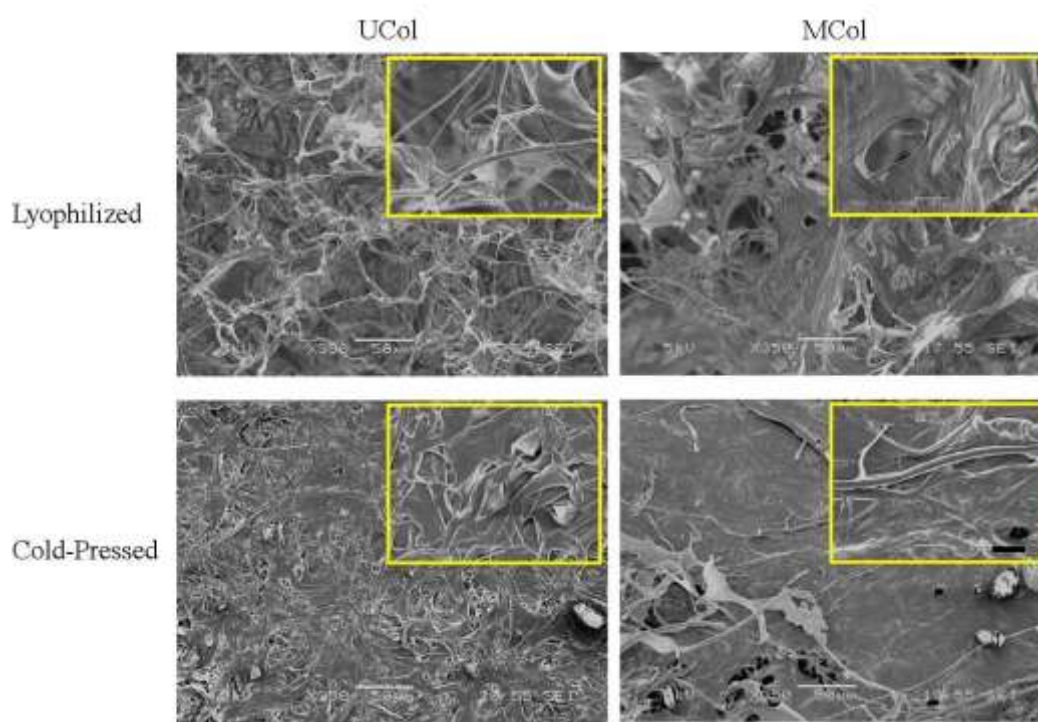


Figure A2.1 SEM images for cold-pressed UCol and MCol at ×350 and ×2000 (inset) magnifications (scale bar represents 50 μm; inset scale bar represents 10 μm).

Appendix 3: Degree of crosslinking of the collagen patches

The degree of crosslinking of the BDE-crosslinked MCol patches was evaluated using TNBS assay. In general, the amount of free amine groups of the uncrosslinked and BDE-crosslinked MCol patches were measured according to the method as described in **Chapter 3, Section 3.4.15**. Subsequently, the degree of crosslinking was estimated by comparing the free amine groups of the BDE-crosslinked MCol patches to the initial free amine groups of the uncrosslinked MCol patches according to **equation (5)** and **equation (6)** [1, 2]:

$$\frac{\text{moles(Lys)}}{g(\text{collagen})} = \frac{2 \times \text{Abs}(335\text{nm}) \times 0.02}{1.4 \times 10^4 \times b \times x} \dots\dots\dots (5)$$

$$\text{Degree of crosslinking} = \left(1 - \frac{\text{moles(Lys)}_{\text{crosslinked}}}{\text{moles(Lys)}_{\text{collagen}}} \right) \times 100 \dots\dots\dots (6)$$

Abs(335nm) = absorbance value at 335 nm

1.4×10^4 = molar absorption coefficient for 2,4,6-trinitrophenyl lysine (in $\text{L/mol} \cdot \text{cm}^{-1}$)

b = cell path length (1 cm)

x = sample weight

$\text{moles}(\text{Lys})_{\text{crosslinked}}$ = lysine molar content in crosslinked collagen

$\text{moles}(\text{Lys})_{\text{collagen}}$ = lysine molar content in uncrosslinked collagen

As seen from **Figure A3.1**, as the crosslinker concentration increases, a higher degree of crosslinking was observed, which indicated that higher number of successful ether linkages were formed between the collagen chains. As a result, the increases in the crosslinking density contributed to the improvement in the physicochemical properties of the MCol patches.

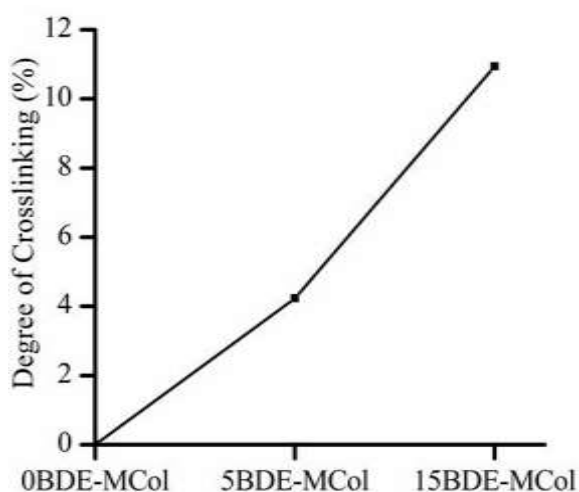


Figure A3.1 Degree of crosslinking of MCol with different BDE crosslinker concentrations.

Appendix 4: Effect of pH on denaturation of BSA Study

Collagen has been widely used as scaffolding material for TE applications. Meanwhile, the TE approach commonly involves the use of scaffolds incorporated with biological agents to facilitate cell proliferation and tissue regeneration. However, during the

scaffold fabrication process, UCol is mainly dissolved in acidic condition and this harsh acidic environment could potentially cause denaturation of biological agents such as cells, drugs, genes or growth factors, and thus limited its potential application as drug carrier. In this particular study, the possible denaturation of drugs under the harsh acidic environment was investigated by using BSA as model drug. Briefly, 1 mg/mL of BSA was prepared by dissolving lyophilized BSA in either 0.5 M acetic acid or 1× PBS. Subsequently, the BSA solution was subjected to NanoDSC (TA Instruments, USA) to measure the T_m of BSA by heating at a rate of 1 °C/min from 50 – 80 °C against the solvent used respectively. The T_m was measured as the peak temperature of the enthalpy peak. As seen from **Figure A4.1**, the NanoDSC results showed the detection of the T_m of the BSA that was being dissolved in the 1× PBS solution due to the heat-induced unfolding of BSA. In contrast, the T_m was not detected for the BSA dissolved in the acidic solution, which is an indication of the possible denaturation of BSA, since no enthalpy change was detected. Therefore, the methylation modification of UCol to produce a water-soluble MCol is necessary in order to provide a friendly environment for the biological agents used for drug delivery applications.

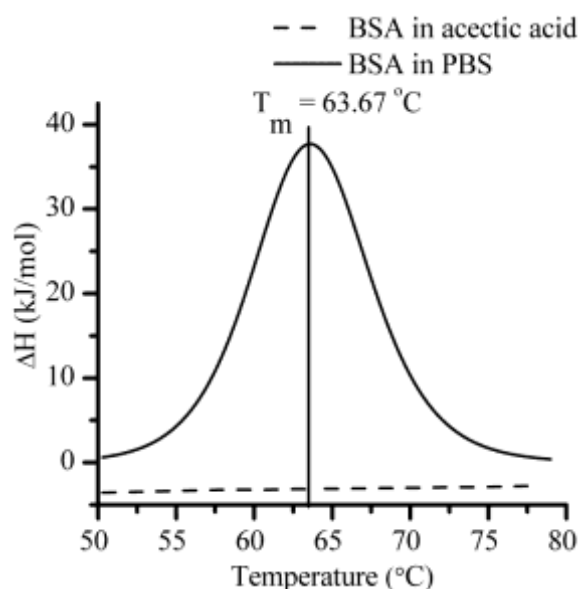


Figure A4.1 NanoDSC results for BSA dissolved under acidic and buffer conditions.

For the *in vitro* drug release studies, FITC-BSA, a water-soluble protein, was used as a protein model to show the potential of methylated collagen as a delivery system [3]. The macroscopic images of the different FITC-BSA loaded collagen patches were shown in **Figure A4.2**. As seen from the figure, the FITC-BSA loaded collagen patches exhibited light orange/yellow appearance throughout the scaffolds, which is an indication that the FITC-BSA was successfully distributed evenly across the collagen patches.

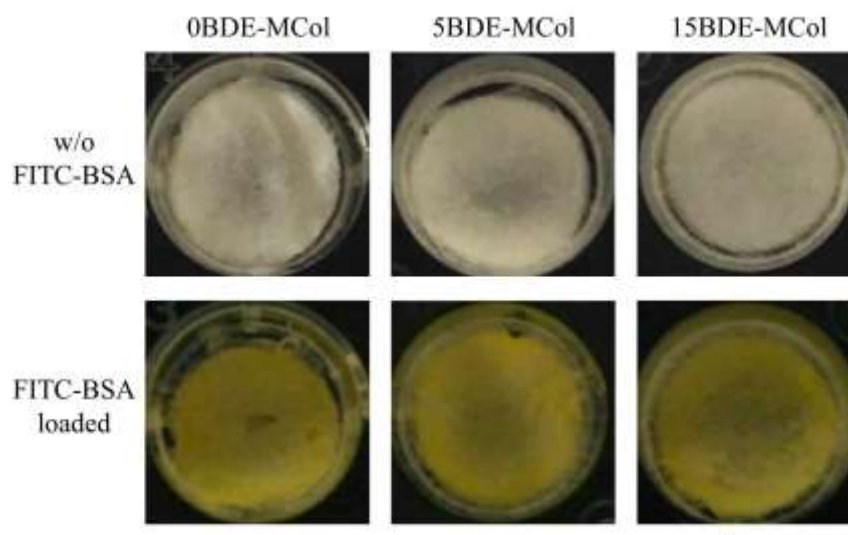


Figure A4.2 Macroscopic images of the FITC-BSA loaded collagen patches (scale bar represents 1 cm).

Appendix 5: *In vivo* Studies of Collagen Patches

In vivo degradability of different collagen patches were carried out using a murine model. Briefly, the collagen patches were implanted in mouse hind limbs subcutaneously. After 21 days of implantation, the macroscopic appearance of different collagen patches was assessed. As seen from **Figure A5.1**, the skin was healed completely with no signs of infection and inflammation after 21 days of implantation. In addition, 0BDE-MCol patch was mostly degraded, whilst 5BDE-MCol patch was partially degraded. However, 15BDE-MCol patch remained intact even after 21 days of implantation, which could possibly due to the over-crosslinking of MCol that resulted in slow degradation rate of MCol. This fairly slow degradation rate of 15BDE-MCol patch may retard or inhibit the subsequent tissue remodeling processes, and is therefore not desirable [4, 5].

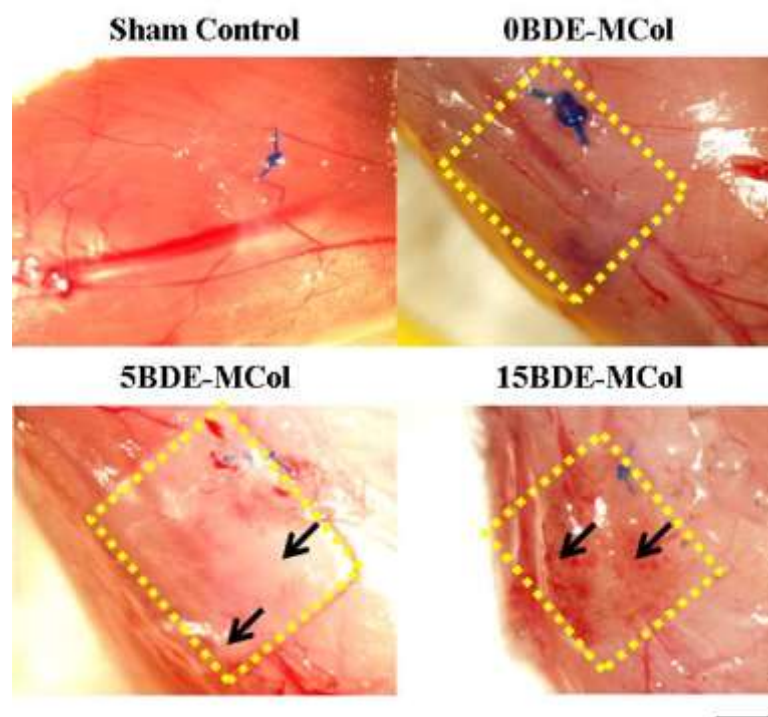


Figure A5.1 Macroscopic images for different MCol patches at 21 days post-implantation (yellow dotted box outlines the area of the implanted collagen patch; black arrows represent the partially degraded collagen patch; scale bar represents 1 mm).

Appendix 6: Optimization of SHHA Study

The heat-sintered SHHA was ball-milled and subsequently subjected to a 1× PBS treatment process, in order to improve the particle size distribution and the pH stability of SHHA. For the ball-milling process, zirconia balls with 5 mm in diameter were used. Briefly, 10 w/w%, 20 w/w% and 40 w/w% of SHHA were added to zirconia balls and the samples were ball-milled for different duration (i.e. 0.5, 1 and 2 h) to optimize the particle size distribution of SHHA particles. The particle size distributions of the various samples were measured using Analysette 22 Compact particle size analyzer (Fritsch GmbH, Germany) according to previously established method [6]. Briefly, 200 mg of the samples were added into 5 mL water. Subsequently,

the solution was transferred to a particle analyzer to obtain a particle size distribution profile and the mean particle size of each sample. As seen from **Figure A6.1**, 10 w/w% of SHHA-to-zirconia balls with 2 h of ball-milling time was most effective for reducing the mean particle size of the SHHA particles. Hence, further optimization was carried out on this particular sample group, namely 10 w/w% of SHHA-to-zirconia balls with 2 h of ball-milling time, to improve its pH stability.

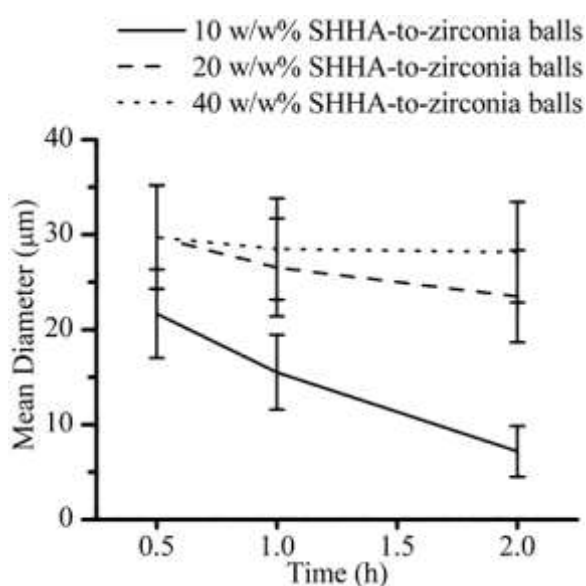


Figure A6.1 Particle size analysis of the SHHA ball-milled at different SHHA-to-zirconia balls ratios at different ball-milling times showing the reduction in the median particle with increasing ball-milling time.

The ball-milled SHHA were treated with 1× PBS solution to improve the pH stability of the SHHA. Briefly, 1.5 g of the ball-milled SHHA was soaked with either 200, 400 or 600 mL of 1× PBS where the final pH of the solution was measured. As seen from **Figure A6.2a**, the pH of the 1× PBS increased from 7.4 to 7.57 ± 0.02 when SHHA was soaked in 200 mL of 1× PBS solution. In general, naturally-derived HA contains beneficial ions such as sodium, potassium or magnesium as well as some carbonate

ions, which can be easily removed during the heat-sintering process [7-9]. Hence, the pH changes observed could be due to the trace amounts of ions, particularly that of carbonate ions that remained in SHHA. In addition, Ca ions could also be released from SHHA on its own when being exposed to a Ca-deficient buffered solution, which will also result in pH changes [10, 11]. Hence, the 1× PBS treatment is important as it allows the removal of trace amounts of unstable ions, which will lead to an improvement in the pH stability of SHHA prior to scaffold fabrication. In addition, as the volume of 1× PBS increases, it enhanced the ion-exchange process that resulted in lesser pH changes.

The successful removal of carbonate ions from SHHA was further confirmed using ATR-FTIR analysis. As seen from **Figure A6.2b-i**, the characteristic phosphate peaks (dotted box) of HA were observed near 1086, 1018 and 962 cm^{-1} for both pre and post-PBS treated SHHA. Other than the characteristic phosphate peaks, an absorption peak was detected near 2360 cm^{-1} before the 1×PBS treatment, which is attributable to the carbonate groups (solid box) or other trace ions in the SHHA. After the 1× PBS treatment, a reduction in the carbonate peak intensity was observed (**Figure A6.2b-ii**). This suggests that the carbonate ions were successfully leached out from SHHA with an overall improvement in pH stability of SHHA was observed (**Figure A6.2a**). Overall, pre-soaking SHHA in 600 mL of 1× PBS significantly improved the pH stability of SHHA, and thus the cytocompatibility of SHHA for TE applications.

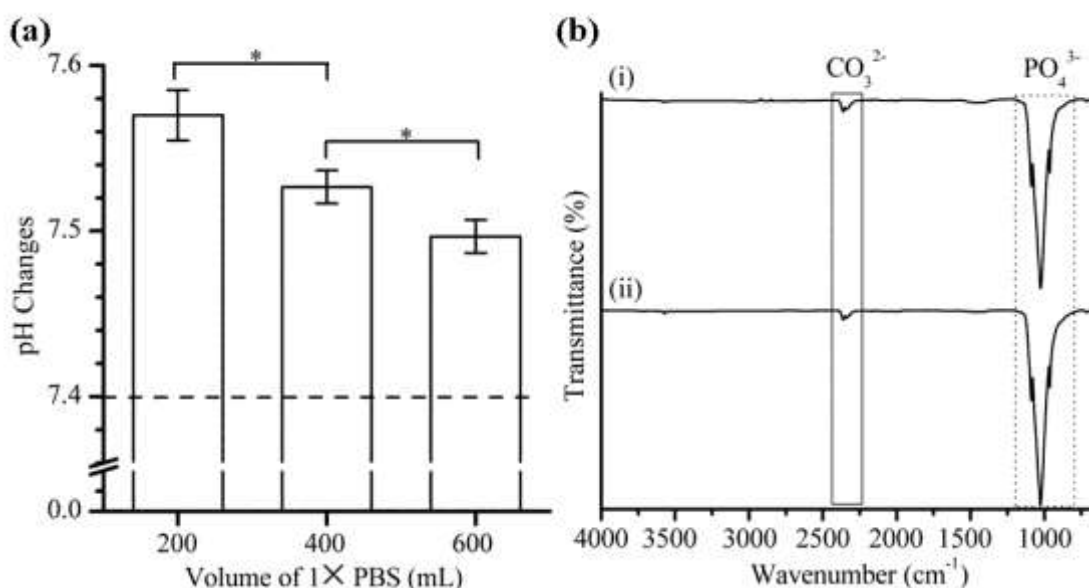


Figure A6.2 pH stability studies showing (a) pH measurement results and (b) ATR-FTIR results for SHHA (i) before and (ii) after soaking in 1× PBS [dashed line represents the initial pH (7.4) of 1× PBS; solid box represents carbonate peaks of trace ions; dotted box represents characteristic phosphate peaks of HA].

Appendix 7: Gtn-HPA/CMC-Tyr/SHHA Biocomposite Hydrogel Study [12]

The cytocompatibility of heat-sintered SHHA was investigated by incorporating the extracted SHHA into a cell encapsulating gelatin-hydroxyphenylpropionic acid/carboxymethylcellulose-tyramine (Gtn-HPA/CMC-Tyr) hydrogel system where the cell-material interactions were assessed using HEK fibroblast cells (i.e. HEK 293FT cells). HEK 293FT cells were cultured and expanded in 75 cm² tissue culture flasks using high glucose DMEM/F-12 medium supplemented with 10% FBS, 1× antibiotic-antimycotic and 1.6 g/L sodium bicarbonate. The cells were cultured at 37 °C with 5 % CO₂ environment and saturated humidity. For the fabrication of the hydrogel, 5 w/v% of Gtn-HPA and 5 w/v% of CMC-Tyr solutions were dissolved separately in DMEM solution to produce the hydrogel precursors. Subsequently, the

hydrogel precursors were mixed at a ratio of 4:1 for Gtn-HPA and CMC-Tyr, followed by the incorporation of HEK 293FT cells at a seeding density of 5×10^4 cells/mL. Next, 0.5 mL of the cell-containing hydrogel precursors was transferred into 24-well plates and crosslinked by carbodiimide reaction to produce non-filled Gtn-HPA/CMC-Tyr hydrogel (i.e. 0 wt% SHHA hydrogel). For the fabrication of biocomposite hydrogel, various wt% of SHHA was added into the cell-containing hydrogel precursors prior to the crosslinking reaction. Finally, the HEK 293FT cell proliferation and viability were assessed over 7 days of culture. As seen from **Figure A7.1**, HEK 293FT proliferated over the course of 7 days of culture and remained viable, which is an indication that the extracted SHHA was cytocompatible, and thus can potentially be used for biomedical applications.

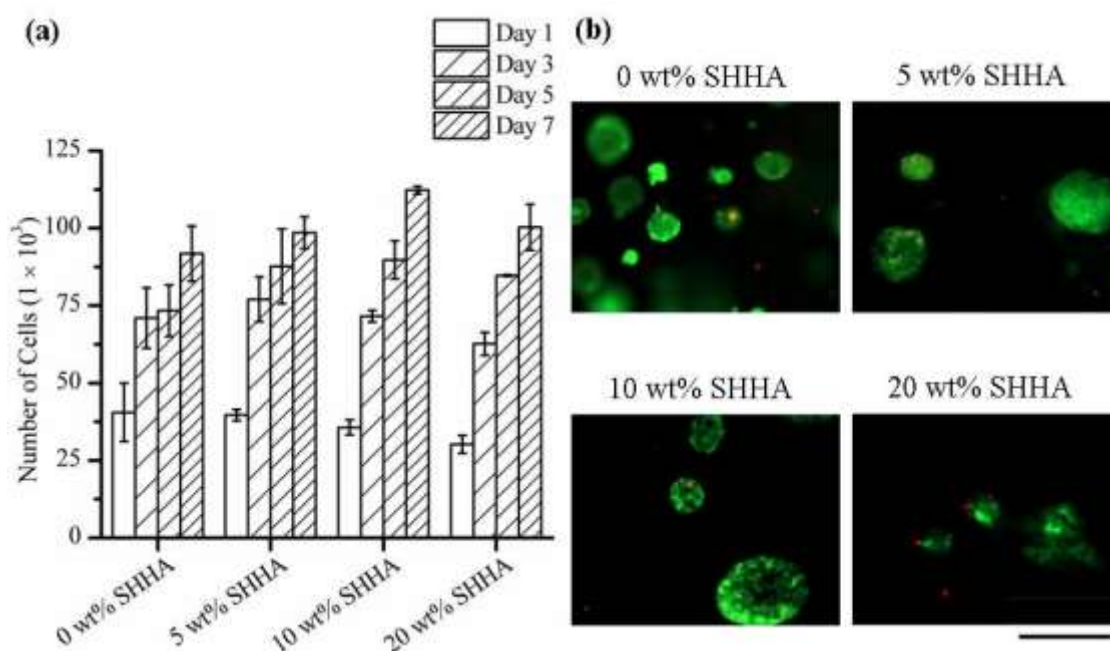


Figure A7.1 3D cell encapsulation studies showing (a) cell proliferation results and (b) cell viability results for HEK 293FT cells encapsulated within biocomposite hydrogels containing different amounts of SHHA (green fluorescence represents live cells; Red fluorescence represents dead cells; scale bar represents 2mm; as adapted from: Chun Y. Y., Wang J.K., Tan N.S., Chan P.P.Y., Tan T.T.Y., and Choong C: A

periosteum-inspired 3D hydrogel-bioceramic composite for enhanced bone regeneration. *Macromolecular Bioscience*, 2015. Volume 16. Pages 276-287. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission).

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Publication List

Publications as First Author

- [1] **Wang, J. K.**, Xiong, G. M., Luo, B., Choo, C. C., Yuan, S., Tan, N. S., and Choong, C. (2016) Surface modification of PVDF using non-mammalian sources of collagen for enhancement of endothelial cell functionality, *Journal of Materials Science: Materials in Medicine* **27**, 1-15. (IF = 2.272)
- [2] **Wang, J. K.***, Xiong, G. M.*, Zhu, M., Özyilmaz, B., Castro Neto, A. H., Tan, N. S., and Choong, C. (2015) Polymer-enriched 3D graphene foams for biomedical applications, *ACS Applied Materials & Interfaces* **7**, 8275-8283. (IF = 7.145)
- [3] **Wang, J.K.***, Luo, B.*, Guneta, V., Li, L., Dai, Y., Tan, T.Y.Y., Tan, N.S., Choong, C., and Wong, M.T.C. (2017) Supercritical carbon dioxide extracted extracellular matrix material from adipose tissue, *Materials Science and Engineering: C* **75**, 349-358.(IF = 3.420)

*Co-first authors

Publications as Co-authors

- [1] Chun, Y. Y., **Wang, J. K.**, Tan, N. S., Chan, P. P. Y., Tan, T. T. Y., and Choong, C. (2016) A periosteum-inspired 3D hydrogel-bioceramic composite for enhanced bone regeneration, *Macromolecular Bioscience* **16**, 276-287. (IF = 3.850)
- [2] Xiong, G. M., Yuan, S., **Wang, J. K.**, Do, A. T., Tan, N. S., Yeo, K. S., and Choong, C. (2015) Imparting electroactivity to polycaprolactone fibers with heparin-doped polypyrrole: Modulation of hemocompatibility and inflammatory responses, *Acta Biomaterialia* **23**, 240-249. (IF = 6.025)

- [3] Xiong, G. M., Do, A. T., **Wang, J. K.**, Yeoh, C. L., Yeo, K. S., and Choong, C. (2015) Development of a miniaturized stimulation device for electrical stimulation of cells, *Journal of biological engineering* **9**, 1-10. (IF = 3.000)
- [4] Xiong, G. M., Yuan, S., Tan, C. K., **Wang, J. K.**, Liu, Y., Yang Tan, T. T., Tan, N. S., and Choong, C. (2014) Endothelial cell thrombogenicity is reduced by ATRP-mediated grafting of gelatin onto PCL surfaces, *Journal of Materials Chemistry B* **2**, 485-493. (IF = 4.872)
- [5] Guneta, V., **Wang, J. K.**, Maleksaeedi, S., He, Z. M., Wong, M. T. C., and Choong, C. (2014) Three dimensional printing of titanium for bone tissue engineering applications: A preliminary study, *Journal of Biomimetics, Biomaterials and Biomedical Engineering* **21**, 101-115. (New Journal)
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Papers in Preparation

- [1] **Wang, J. K.**, Yeo, K. P., Chun, Y. Y., Tan, T. Y. Y., V, Angeli, Tan, N. S., and Choong, C. (2017) Fish scale-derived collagen patch promotes growth of blood and lymphatic vessels *in vivo*, *Acta Biomaterialia*. (IF = 6.025)
- [2] **Wang, J. K.**, Yang, Y., Chun, Y. Y., Luo, B., Loh, Q. L., Choo, C. C., Tan, T. Y., Tan, N. S., and Choong, C (2017). Bullfrog skin-derived collagen scaffolds for tissue engineering applications, *Journal of Biomedical Materials Research Part B: Applied Biomaterials*. (IF = 2.308)

- [3] Chun, Y. Y., Yeo, K. P., **Wang, J. K.**, Tan, T. Y. Y., V, Angeli, Tan, N. S., and Choong, C. (2017) Long term sustained release of VEGF-C156S proteins using injectable phenol conjugated gelatin hydrogel to promote efficient lymphatic drainage to induce atherosclerosis regression, *Biomaterials*. (IF = 8.387)

List of Technology Disclosure

- [1] Choong, C., Yeo, K. S., Xiong, G. M., **Wang, J. K.**, and Do, A. T. “A high throughput electrical cell stimulation array with integrated electronics for charge-balancing and recording”. NIEO Ref: TD/125/13

Conference Oral Presentation

- [1] **Wang, J. K.**, Xiong, G. M., Liu, Y., Yuan, S. J., Tan, T. T. Y, Tan, N. S. and Choong, C. (2014) Enhancement of Biocompatibility on PVDF via Collagen Conjugation. *Materials Research Society (MRS) Spring Meeting and Exhibit, San Francisco, USA*. 21st – 25th April 2014.

Conference Poster Presentation

- [1] Guneta, V., **Wang, J. K.**, He, Z. M., Maleksaeedi, S., and Choong, C. (2012) Biocompatibility of 3D Printed Titanium Scaffolds for Bone Tissue Engineering. *International Conference of Young Researchers on Advanced Materials (ICYRAM), Singapore*. 1st – 6th July 2012.