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NANYANG
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**A 3D INJECTABLE AND ENZYMATIC RESPONSIVE
HYDROGEL-BIOCERAMIC SYSTEM FOR BONE
TISSUE ENGINEERING**

CHUN YONG YAO
SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING
2016

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HYDROGEL-BIOCERAMIC SYSTEM FOR BONE
TISSUE ENGINEERING**

CHUN YONG YAO

School of Chemical and Biomedical Engineering

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Abstract

Autologous bone graft is the current gold standard for treatment of bone defect as it possesses superior osteoconductive, osteoinductive and osteogenic properties that are essential for bone healing. Recent studies have shown that the superior healing properties of the autologous bone graft are associated with the presence of the intact periosteum on the autologous bone graft. The periosteum provides the essential angiogenic and osteogenic properties for bone regeneration and acts as the extracellular matrix for the periosteal stem cells.

However, the extraction of the autologous bone graft from the patient will cause donor site morbidity and postoperative complications such as chronic pain, infection, and hematoma. Hence, this thesis aimed to develop a novel injectable scaffold inspired by the properties of periosteum for bone regeneration. The overall objective of this thesis is to develop a novel injectable periosteum-inspired scaffold targeted for bone regeneration. In order to achieve the aim of the thesis, three main studies are carried out: (1) To develop an injectable system with controllable gelation time to fill different shape and size of the bone defect and tailorable physical properties to support bone regeneration, (2) to develop a tunable porosity of the injectable system *post-fabrication* to enhance the effectiveness of bone regeneration, and (3) to enhance the mechanical and bioactive properties of the injectable system and investigate its potential as a cell carrier for bone healing.

Firstly, a phenol-conjugated gelatin hydrogel was developed as a basic component for the injectable bone scaffold. Phenol molecules, 3-(4-hydroxyphenyl)propionic acid (HPA), were conjugated onto gelatin (Gtn) through carbodiimide/active ester-mediated coupling reaction in distilled water (DI water). Gtn-HPA conjugates were crosslinked using horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2) that allow *in situ* gelation to form the Gtn-HPA hydrogel. By varying the polymer, HRP, and H_2O_2 concentration, the material properties can be finely tuned for desired application. The *in vivo* studies showed the biocompatibility, the degradability and the angiogenic properties of the Gtn-HPA hydrogel.

Subsequently, in order to achieve tunability properties of the Gtn-HPA hydrogel post-fabrication, phenol-conjugated carboxymethyl cellulose (CMC) was incorporated into the

hydrogel as the sacrificial polymer. Tyramine (Tyr) molecules were conjugated onto CMC using the same carbodiimide/active ester-mediated coupling reaction. The CMC-Tyr conjugates were mixed with Gtn-HPA conjugates prior to crosslinking to form Gtn-HPA/CMC-Tyr hybrid hydrogel. Cellulase was used to digest the CMC, which will enable post-fabrication tunability in terms of degradation rate and drug release rate. Findings showed that 5 w/v % Gtn-HPA/CMC-Tyr hybrid hydrogel fabricated with 6 mM H₂O₂ exhibited the suitable material properties for bone regeneration. In addition, the cellulase treatment demonstrated the tunable properties of the hybrid hydrogel as well as its cytocompatibility when tested using human pre-osteoblast cells.

Lastly, fish scale-derived calcium phosphate (CaP) compounds were incorporated into the Gtn-HPA/CMC-Tyr hybrid hydrogel to develop periosteum-inspired hydrogel-bioceramic composite for bone regeneration. The incorporation of CaP improved the mechanical properties and the bioactive properties of the hydrogel-bioceramic composite. *In vitro* cellular studies showed that the hydrogel-bioceramic composite was biocompatible and even suitable to be used as the cell carrier for bone tissue engineering.

Overall, the injectable periosteum-inspired hydrogel-bioceramic composite developed in this study is a potential alternative for current methods of treating bone defects. Existing autologous bone grafting techniques have shown to possess problems such as donor site morbidity and surgical complications. The proposed method of using an injectable periosteum-inspired hydrogel-bioceramic composite potentially circumvents these problems with its properties as a bone scaffold and as a periosteum-inspired layer to assist allograft and synthetic bone graft in treating large bone defect.

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Abbreviations

2D	Two-dimensional
3D	Three-dimensional
4-MU	4-methyl-umbelliferone
4-MUP	4-methyl-umbelliferyl phosphate
β -TCP	Beta-tricalcium phosphate
ABAM	Antibiotic-antimycotic
AdMSCs	Adipose-derived mesenchymal stem cells
ADSCs	Adipose-derived stem cells
ALP	Alkaline phosphatase activity
ApoE-/-	Apolipoprotein E-Deficient
BCA	Bicinchoninic acid
BMP	Bone morphogenetic protein
CaCl ₂	Calcium chloride
CaP	Calcium phosphate
CD	Cluster of differentiation
(CH ₂ OH) ₃ CNH ₂	Tris(hydroxymethyl) aminomethane
CMC	Carboxymethyl cellulose
CMC.Na	Sodium carboxymethyl cellulose
CMC-Tyr	Carboxymethyl cellulose-tyramine
DI	Deionized
D ₂ O	Deuterium oxide
DMEM	Dulbecco's Modified Eagle Medium
DMEM/F-12	Dulbecco's Modified Eagle Medium: nutrient mixture F-12
DMF	N,N-dimethylformamide
ECM	Extracellular matrix
EDC	N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide
EDC.HCl	N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride
EDTA	Ethylenediaminetetraacetic acid
EDX	Energy-dispersive X-ray spectroscopy
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum

FITC	Fluorescein isothiocyanate
FTIR	Fourier transform infrared spectroscopy
Gtn	Gelatin
Gtn-HPA	Gelatin-3-(4-hydroxyphenyl)propionic acid
Gtn-HPA/CMC-Tyr	Gelatin-3-(4-hydroxyphenyl)propionic acid/ carboxymethyl cellulose-tyramine
Gtn-HPA/CMC-Tyr/CaP	Hydrogel-bioceramic composite
hFOBs	Human fetal osteoblasts
HEK 293FT	Human kidney 293FT
HA	Hydroxyapatite
HCl	Hydrochloric acid
H & E	Hematoxylin and Eosin
H ₂ O ₂	Hydrogen peroxide
HPA	3-(4-hydroxyphenyl)propionic acid
HRP	Horseradish peroxidase
HUVECs	Human umbilical vein endothelial cells
hMSCs	Human mesenchymal stem cells
IACUC	Institutional Animal Care And Use Committee
KCl	Potassium chloride
K ₂ HPO ₄	Potassium hydrogen phosphate trihydrate
K ₂ HPO ₄ ·3H ₂ O	Potassium phosphate dibasic trihydrate
LCST	Lower critical solution temperature
LYVE-1	Anti-lymphatic vessel endothelial hyaluronic acid receptor-1
MgCl ₂ ·6H ₂ O	Magnesium chloride hexahydrate
MMPs	Matrix metalloproteinases
MSCs	Mesenchymal stem cells
NaCl	Sodium chloride
Na ₂ CO ₃	Sodium carbonate
NaHCO ₃	Sodium bicarbonate
Na ₂ SO ₄	Sodium sulfate
NHS	N-hydroxysuccinimide
NiPAAM	N-isopropylacrylamide
NMR	Nuclear magnetic resonance spectroscopy

NUS	National University of Singapore
PAA	Poly(acrylic acid)
PBS	Phosphate buffered saline
PDCs	Periosteum-derived cells
PDGF	Platelet-derived growth factor
PEG	Polyethylene glycol
PHB	Polyhydroxybutyrate
PLGA	Poly(lactic-co-glycolic acid)
PMA	Poly(methacrylic acid)
PFA	Paraformaldehyde
RGD	Arginine-glycine-aspartic
SBF	Simulated body fluid
SEM	Scanning electron microscopy
SMA	Smooth muscle actin
SV-LECs	Mouse lymphatic endothelial cells
TCP	Tissue culture plate
TGF- β	Transforming growth factor β
Tyr	Tyramine
Tyr.Cl	Tyramine chloride
UCST	Upper critical solution temperature
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
XRD	X-ray powder diffraction

Chapter 1

Introduction

This chapter includes the problem statement and hypotheses of this research, followed by its objectives. The reason and motivation for the research work found in this thesis will be fully described in this chapter. Subsequently, the dissertation overview and the novel findings of these research works will be also included in this chapter. This research finds that the need of using bone tissue engineering technique to develop a periosteum-inspired layer in assisting allograft or synthetic bone graft in treatment for large bone defects. Besides, the increase in demand to fill different shape and size of bone defects in clinical procedures necessitates the development of injectable scaffolds. Hence, this thesis will posit the notion of developing a novel hydrogel-bioceramic composite as the injectable scaffold for bone tissue engineering. To address the challenges of post-fabrication tunability, a phenol-conjugated hybrid hydrogel system that possessed the ability of induced drug release, tunable degradation rate and enhanced cell proliferation post-fabrication through enzymatic digestion was developed and studied. Finally, a bioactive component was incorporated into the phenol-conjugated hybrid hydrogel system to improve the overall mechanical properties. This hydrogel-bioceramic composite will also provide bone-like mineral forming ability for bone regeneration.

1.1 Problem Statement and Hypothesis

Autologous bone graft provides the superior healing ability for bone regeneration and it is a current gold standard in treating the bone defects. More clearly, it provides osteoconductive, osteoinductive and osteogenic properties that are essential for bone healing [1, 2]. However, the extraction of the autologous bone graft from the patient will cause donor site morbidity [3] and postoperative complications [2, 4]. Cases studies by Banwart *et al.* found that in 225 cases of harvesting iliac bone from the patient, 10% reported having major complications such as chronic pain, infection, and hematoma while 39% reported to have minor complications such as sensory deficit and drain disorder [5]. Another study by O'Malley *et al.* on 181 patients that went through harvesting of percutaneous calcaneal autograft bone, 13.8% of patients reported having minor complications even though oblique incision was performed to extract the bone graft [6]. Thus, there is the need to replace current autologous bone graft to improve treatment for bone defect using bone tissue engineering techniques. Bone tissue engineering involved using a scaffold material as the mechanical support and vehicle to delivery cells and therapeutic signals in order to facilitate bone regeneration [7]. Besides, recent studies found that the superior healing properties of the autologous bone graft are due to the presence of intact periosteum [8]. Periosteum acts as an extracellular matrix (ECM) to host periosteal stem cells and provides the suitable environment and growth factors to encourage osteogenic differentiation [9]. Periosteum also provides angiogenesis and osteogenic properties [10] that essential for bone healing [8, 11]. Inspired by the properties of periosteum, the overall objective of this thesis is to develop a novel injectable system as a bone scaffold and also as a periosteum-inspired layer to assist allograft or synthetic bone graft in treatment for large bone defects. This thesis hypothesized that the development of injectable bone scaffold with properties inspired by the periosteum and post-implantation tunability to enhance cell proliferation and remodeling at the same time as smart release vehicle will be able to enhance bone regeneration. The overall objective will be achieved through 3 methods addressing different aims that ultimately contribute to the development of this novel bone scaffold.

Firstly, most of the bone defects have complex shapes and different sizes. Certain defects such as spinal cord lesions also have very small defect sizes ($< 100 \text{ mm}^3$) that eliminate the option to use performed scaffold [12]. Moreover, current surgical

procedures also desire a less invasive method to reach certain defect sites that are buried deep within healthy tissues to minimize the damage caused to the health tissue. Therefore, less invasive techniques could minimize the patient discomfort as well as reduce the risk of infection and scar formation. Besides, clinical procedures can reduce the cost and time by using less invasive method [13]. Thus, the first need is to create an injectable scaffold that can fill different shape and size of defects without prior knowledge of the bone defect, which is also a less invasive method to treat bone defects. This thesis proposed the used of the injectable hydrogel as the bone scaffold and periosteum-inspired layer. The used of the injectable hydrogel can ensure completely filling and conformity to have good interaction and integration between host tissue and scaffold, and between bone graft and scaffold [12]. Several basic requirements are needed to design an injectable scaffold; these include biocompatible components that are easily sterile, easily injectable, possess a solidification and biodegradable mechanism, as well as desired properties to match the host tissue. In addition, the components used to design the injectable scaffold should possess the biodegradable qualities to ensure that it will not cause harm to the host tissue by releasing toxic components upon degradation. Also, the solidification mechanism should be biocompatible and ideally occurs at physiological conditions to prevent damage to the host tissue and encapsulated cells or biological agents [13, 14].

Consequently, this thesis has proposed that by using gelatin, it can fulfill the basic requirements of the injectable bone scaffold and periosteum-inspired layer with excellent angiogenic properties [15] that are essential for bone regeneration while containing arginine-glycine-aspartic (RGD) proteins that are essential for cell attachment [16]. Over the past years, injectable hydrogel based on gelatin are generally formed using chemical crosslinking such as EDC, 1,4-Butanediol diglycidyl ether and glutaraldehyde, UV-crosslinking and enzyme crosslinking such as transglutaminase. However, the use of chemical crosslinking has shown to cytotoxicity as well as triggering immune respond. The use of UV radiation as the initiator of the crosslinking process could potentially be damaging to the surrounding tissues. In addition, most of the conventional chemical and enzyme crosslinking methods are using a single crosslinker in the crosslinking process that in turn determines both the gelation rate and crosslinking density. In general, the gelation rate plays an important role in determining the injectability while the crosslinking density plays an important role in determining

the material properties. As a result, there are limited controls on both the gelation rate and the material properties using those conventional crosslinking methods. Thus, a crosslinking system that is able to tune these two properties individually is desired to have more control over the material properties of the injectable scaffold formed.

This thesis proposed to use of horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2) as the crosslinking method for the periosteum-inspired injectable scaffold. Peroxidases are important enzymes in the human body that break down H_2O_2 , which is harmful to cells, production of antibacterial factors and biosynthesis of hormones. In this thesis, gelatin is conjugated with phenol molecules through carbodiimide-mediated coupling reaction [17]. Conjugated phenol compounds find on the gelatin (Gtn-HPA) enables solidification of the gelatin through enzymatic crosslinking using HRP and H_2O_2 [18]. The use of enzymatic crosslinking allows tunable gelation rate and material properties of Gtn-HPA hydrogel independently by varying HRP and H_2O_2 concentration respectively to achieve desired properties [19]. The preceding hypothesis was derived through investigating the effect of HRP and H_2O_2 concentration on its material properties and release profile while utilizing *in vivo* studies of injectability, biocompatibility, degradability and angiogenic properties as a basis for the conclusion.

Secondly, inspired by the periosteum which acts as ECM that provides a suitable environment to host stem cells, this phenol-conjugated gelatin will be further developed to facilitate better in hosting and delivering cells. The development of new tissue is highly dependent on the cell proliferation, differentiation and tissue morphogenesis at the defect site [20]. Cell proliferation and differentiation are affected by the mechanical properties of hydrogels while the degradation behavior of hydrogel affects the effectiveness of tissue morphogenesis [21]. Aside from mechanical and degradation properties, studies also showed that the permeability and network's size of hydrogel play important roles in cell viability, proliferation, fate and ultimately the effectiveness in new tissue formation [22, 23]. Especially for bone tissue engineering, hydrogel with high mechanical strength or stiffness are usually achieved through higher crosslinking density. Hydrogel with higher stiffness will have smaller permeability and network size, which causes the problem for cell proliferation. Moreover, most of the preformed scaffold once fabricated and implant into defect site are not able to alter their properties [24]. Some of the well-studied smart hydrogels are able to alter their degradation rate

when stimulated by physical changes in the body such as temperature and pH. However, the harsh change in microenvironment in term of temperature and pH will have harmful effect on cells. Other than as a cell carrier, injectable hydrogels can be used as delivery vehicles to retain and sustained release of growth factors such as bone morphogenetic protein (BMP) and transforming growth factor β (TGF- β) to encourage bone formation while vascular endothelial growth factor (VEGF) encourage blood vessels formation that helps in bone healing [25]. Moreover, there is increasing interest in developing smart delivery system to have further control on release and on demand release leads to development of scaffold responsive to local environments such as temperature [23] and pH [24], and externally stimulates such as magnetic field [26, 27], electrical field [28] and light [29, 30]. However, more studies are needed to understand how these systems performed *in vivo* applications [31]. Thus, there is a demand on creating a smart responsive system that has tunable degradation rate and release rate, at the same time enable co-encapsulation and co-delivery of cells and signaling molecules to enhance bone tissues regeneration. Thus, the second need will be on creating a smart responsive system that has tunable degradation rate on demand. This will lead to pores enlargement on demand to facilitate cell proliferation and differentiation. At the same time, this smart responsive system should have the ability to further control on release rate and enable co-delivery of cells and signaling molecules to enhance bone healing.

This thesis proposed that incorporating carboxymethyl cellulose (CMC) into the Gtn-HPA hydrogel will enable post-fabrication alteration of scaffold properties through specific enzymatic digestion of CMC. CMC is also conjugated with phenol molecules to enable enzymatic crosslinking together with Gtn-HPA conjugates to form smart enzymatic responsive Gtn-HPA/CMC-Tyr hybrid hydrogel. Phenol-conjugated CMC component (CMC-Tyr) will act as sacrificial polymer and digested by cellulase. The Gtn-HPA/CMC-Tyr's properties in terms of degradation rate and release rate are proposed to be able to tune through cellulase digestion of CMC-Tyr polymers. Studies of cellulase treatment on Gtn-HPA/CMC-Tyr hybrid hydrogel in altering the degradation properties thus enhancing inducing drug release and bone cells' proliferation rate and will be done to prove this hypothesis.

Lastly, the novel periosteum-inspired layer should possess osteogenic properties to encourage osteogenic differentiation of stem cells. However, the Gtn-HPA/CMC-Tyr

hybrid hydrogel lacks of the osteogenic component that essential for bone regeneration. Thus, this thesis proposed that the incorporation of a bioactive component into the Gtn-HPA/CMC-Tyr hybrid hydrogel will able to improve the mechanical properties and enhance the bioactive properties of the whole system. Fish scale-derived calcium phosphate (CaP), a bioactive component, was incorporated into the Gtn-HPA/CMC-Tyr hybrid hydrogel to form a periosteum-inspired hydrogel-bioceramic composite for bone regeneration. The incorporation of CaP is proposed to be able to improve the mechanical properties of the Gtn-HPA/CMC-Tyr hydrogel system [32] and thus able to provide the mechanical environment that encourages osteogenic differentiation of stem cells. The hydrogel-bioceramic composite also potentially provides osteogenic properties and the bone forming ability for bone regeneration. CaP showed to have good bioactivity and osteogenic properties [33, 34]. Besides, CaP was also widely being studies to induce osteogenic differentiation of stem cells [35, 36]. The potential of the hydrogel-bioceramic composite as a periosteum-inspired layer in terms of material properties, drug delivery efficacy, in situ bone-like mineral formation and cytocompatibility was studied to confirm the third hypothesis in this thesis.

1.2 Objectives

The objectives of this thesis are as follows:

1. Development of injectable phenol-conjugated gelatin hydrogel

Firstly, this thesis aims to develop a phenol-conjugated gelatin hydrogel as a basic component for the injectable bone scaffold. Phenol molecules, 3-(4-hydroxyphenyl)propionic acid (HPA), were conjugated onto gelatin through carbodiimide-mediated coupling reaction in distilled water (DI water). Gtn-HPA conjugates were crosslinked using HRP and H₂O₂ and solidified *in situ* to form Gtn-HPA hydrogel. The mechanical properties, structural properties, and degradation behavior of Gtn-HPA hydrogel fabricated with different HRP and H₂O₂ concentrations were studied and understood. Finally, *in vivo* studies using hind limb of C57BL/6 mice were conducted to study the injectability, biocompatibility, degradability and angiogenic properties of the Gtn-HPA hydrogel. Besides, the ability of the Gtn-HPA hydrogel performed in small defect size (injectable volume = 10 μ m) was also evaluated.

2. Development of enzymatic responsive phenol-conjugated gelatin and carboxymethyl cellulose hybrid hydrogel.

Secondly, this thesis also aims to improve the functionality of the Gtn-HPA hydrogel through creating a hybrid hydrogel system that enables post-implantation tunability through enzymatic digestion. Tyramine (Tyr) molecules were conjugated onto CMC using the same carbodiimide-mediated coupling reaction in DI water. CMC-Tyr conjugates were mixed with Gtn-HPA conjugates and crosslinked using HRP and H₂O₂ to form Gtn-HPA/CMC-Tyr hybrid hydrogel. CMC-Tyr component will act as sacrificial polymer and degradable specifically by cellulase. The use of cellulase will enable post-implantation tunability on the degradation rate which ultimately affects the drug release rate and the cell proliferation rate. The properties of the Gtn-HPA/CMC-Tyr hybrid hydrogel in terms of mechanical properties, structural properties, and degradation behavior were studied and understood. The ability of post-implantation tunability in terms of increasing release rate, degradation rate and bone cell proliferation rate through cellulase treatment were studied to show the potential of the Gtn-HPA/CMC-Tyr hybrid hydrogel as a smart responsive scaffold.

3. Development of periosteum-inspired 3D injectable hydrogel-bioceramic composite for enhanced bone regeneration.

Finally, fish scale-derived CaP was incorporated into the Gtn-HPA/CMC-Tyr hybrid hydrogel to develop a periosteum-inspired hydrogel-bioceramic composite for bone regeneration as illustrated in **Figure 1.1**. The properties of the Gtn-HPA/CMC-Tyr hybrid hydrogel with different amount of CaP were being studied in terms of its mechanical properties and structural properties to test the hypothesis that the incorporation of CaP will improve the mechanical properties of the whole system. Besides, the potential of this injectable composite as a drug and cell delivery vehicle was also studied. Finally, the bone-like mineral forming ability of injectable hydrogel-bioceramic composite was being studied to show its potential as the periosteum-inspired injectable layer in assisting bone regeneration.

Figure 1.1 Illustration of the Gtn-HPA/CMC-Tyr/CaP composite as a periosteum-inspired layer for the bone graft.

1.3 Dissertation Overview

This thesis consists of 7 chapters as follow:

Chapter 1 discusses the rationale for the research and set up the objectives and scope to be achieved for this thesis. This chapter also includes the dissertation overview and findings and outcomes for this thesis.

Chapter 2 reviews the literature on current treatment for bone defects, and bone tissue engineering follows by focusing on periosteum and, current development and challenges of the periosteum-inspired scaffold. This chapter will also review on hydrogel to be used as the injectable scaffold and CaP in bone regeneration.

Chapter 3 provides the details of techniques, methods, parameters and statistical analysis used to characterize the properties and functions of samples. All techniques used throughout this thesis are mentioned in this chapter and being cited in others chapters when needed.

Chapter 4 elaborates the results and discussion of *Study 1* “Development of injectable phenol-conjugated gelatin hydrogel” that involve the details characterization of material properties of the Gtn-HPA hydrogel with different crosslinkers’ concentration. Next, this chapter will also discuss the results of *in vivo* studies using hind limb of C57BL/6 mice to investigate the potential of Gtn-HPA hydrogel as an injectable bone scaffold.

Chapter 5 elaborates the results and discussion of *Study 2 “Development of enzymatic responsive phenol-conjugated gelatin and carboxymethyl cellulose hybrid hydrogel system”* where material properties of Gtn-HPA/CMC-Tyr hybrid hydrogel system and its potential in inducing drug release, altering degradation rate and enhancing bone cell proliferation were being investigated.

Chapter 6 elaborates the results and discussion of *Study 3 “Development of periosteum-inspired 3D injectable hydrogel-bioceramic composite for enhanced bone regeneration”*. The effect of incorporating fish scale-derived CaP on the material properties of Gtn-HPA/CMC-Tyr hybrid hydrogel was studied. The potential of this injectable hydrogel-bioceramic composite as a drug delivery vehicle and a cell carrier were also being investigated. Finally, the bone forming ability of hydrogel-bioceramic composite was studied using biomineralization study.

Chapter 7 concludes the thesis by drawing out implications from the three studies in previous chapters. This chapter also includes a section on potential future works and discusses the various possible potential of the injectable phenol-conjugated hydrogel systems and hydrogel-bioceramic composite.

1.4 Findings and Outcomes/Originality

The studies carried out during the course of this thesis led to novel findings and outcomes as followed:

1. Detailed characterization of Gtn-HPA hydrogel especially in terms of material properties and *in vitro* degradation profiles showed that higher H₂O₂ and polymer concentration can achieve higher mechanical properties and slower degradation rate. The release rate of the Gtn-HPA hydrogel also found to be affected by H₂O₂ and polymer concentration, where the Gtn-HPA hydrogel with stiffer structure contributes to slower release rate.

2. *In vivo* studies found that the Gtn-HPA is a good injectable system with good biocompatibility and biodegradability. The Gtn-HPA hydrogel also found to perform well in the small volume (10 µl), thus suitable to be used for small defects. *In vivo*

studies also found that the Gtn-HPA hydrogel alone will encourage the growth of blood vessels *in vivo* and thus providing angiogenic properties that suitable for bone regeneration. Further *in vivo* studies using Gtn-HPA hydrogel with higher stiffness also showed that the implanted stiff Gtn-HPA hydrogel supported both angiogenesis and lymphangiogenesis, which lead to the new potential novel application of the Gtn-HPA hydrogel in lymphatic vessel regeneration.

3. Detailed characterization of Gtn-HPA/CMC-Tyr hybrid hydrogel in terms of material properties and degradation rate found that the incorporation of CMC-Tyr into Gtn-HPA hydrogel does not alter the trend of material properties and degradation rate. It is also found that the Gtn-HPA/CMC-Tyr hybrid hydrogel is suitable for supporting attachment and proliferation of bone cells, thus potentially to be used as a bone scaffold.

4. Cytotoxicity test of cellulase treatment on bone cells found that the cellulase treatment is cytocompatible and suitable for bone applications. This thesis first time demonstrates the potential of Gtn-HPA/CMC-Tyr hybrid hydrogel as the enzymatic responsive scaffold in inducing drug release through alteration of degradation rate using cellulase treatment. The cellulase treatment also found to enhance encapsulated bone cell proliferation.

5. Developed periosteum-inspired injectable hydrogel-bioceramic composite for bone regeneration with detailed material characterization related to different fabrication parameters. The incorporation of CaP into Gtn-HPA/CMC-Tyr hybrid hydrogel improves its mechanical properties and the whole hydrogel-bioceramic composite provided bone-like mineral forming ability. The injectable hydrogel-bioceramic composite also found to be a potential drug delivery vehicle and cell carrier.

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Chapter 2

Literature Review

This chapter describes in detail the literature concerning research works described in the thesis. It provides the background and current trends in the research area that is being explored in this thesis, mainly bone regeneration. This chapter will first review on bone structure, followed by current treatment of bone defects and bone tissue engineering. This chapter will then review on periosteum's structure and properties follow by current research on developing periosteum inspired technologies on bone regeneration. Subsequently, injectable hydrogel, composite, and calcium phosphate, particularly hydroxyapatite are reviewed as a potential bone scaffold and periosteum-inspired layer for bone regeneration.

2.1 Bone

2.1.1 Bone structure

Bone structure is being studied for many years to assist in understanding the bone healing process. Bone consists of 25% of collagen type I and 65% of mineral phase that made of small calcium phosphate crystals called hydroxyapatite [1]. Bone collagen provides the flexibility and toughness for bone while the hydroxyapatite provides strength and hardness for bone. Bone collagen is secreted by bone cells and it consists of three intertwined protein chains which will further interact to form long thin rods. The long thin rods of collagen will organize themselves into a larger fiber by chemical bonding. Hydroxyapatite, the mineral phase for bone is bound in an orderly manner on the bone collagen (**Figure 2.1**) [2]. Both collagen and hydroxyapatite work together to provide bone with strength and resilience to withstand impact.

Figure 2.1 Hierarchical nanostructure of the bone tissue. Adapted from: Rho, J.-Y., L. Kuhn-Spearing, and P. Zioupos, Mechanical properties and the hierarchical structure of bone. Medical Engineering & Physics, 1998. **20**(2): p. 92-102. Reproduced with permission.

Bone can be classified into compact and spongy type depending on its porosity. Bone consists of a highly dense and compact outer shell called cortical bone while a porous fine network of connecting plates and rods called spongy bone. The bone is unique in nature as the bone structure consists of a dense outer shell and a hollow core, forming

a strong yet light skeletal structure. Other than providing structural strength, cortical bone provides the attachment site for tendon and muscle while trabecular bone has a large surface area suitable for mineral exchange [3]. Bone is an essential organ that provides mobility, skeletal support, and protection for the inner organs as well as a reservoir for minerals such as calcium and phosphorous. Calcium and phosphorous are important minerals for normal cell's functionality where calcium is essential to maintain healthy body organ's function, especially for muscle and nerves. Thus, adequate minerals of bone are important to maintain the structural strength as well as ensuring the body system is well functioned. Lack of calcium and phosphorous would weaken the bone leading to the bone disorder such as osteoporosis and fracture.

Bone is constantly changing or undergoes remodeling in order to maintain the two main functions as mention above- (i) skeletal support and (ii) regulation of calcium and phosphorous minerals. According to Wolff's Law, when bone is subjected to load for sufficient period of time, the bone formation would occur. Similarly, the excessive bone loss will occur or bone will become weaker if the bone is not subjected to sufficient weight for sufficient time such as prolong exposes to weightlessness in space or prolonged bedridden. Bone consists of metabolically active tissue that continuously undergoes the process of remodeling to adapt its structure through mechanical stimulation and repair bone damage. Human bone is more or less similar to most mammals and the structure consists of four bone envelopes, which are periosteal, endocortical, trabecular and Haversian envelopes. Each envelope has different surface area-volume ratio depending on different mechanical stimulation on different envelopes and constantly being remodeled by osteoclasts and osteoblasts. According to Frostian bone modeling and remodeling, activation of osteoclasts contributes to bone resorption while activation of osteoblasts leads to bone formation. However, for bone modeling, both processes are the localized process where bone resorption and formation cannot happen mutually at the same location (**Figure 2.2**). The process of bone modeling will greatly reduce once the human skeletal maturity is reached. Besides, adult skeletal renewed remodeling will also occur when human bone is under certain disease states or significant altered mechanical loading condition. Bone remodeling is different from bone modeling in which both the bone resorption and formation of the bone mineral occurred at the same location. Bone remodeling process involves removing and replacing the specific amount of packets of bone. In bone remodeling

process, osteoclasts team that involves in bone resorption is closely followed by osteoblast team that involves in bone formation. These coupling processes are moving as one unit where the net amount of bone removed and bone formed must have a zero bone balance in order to achieve healthy bone remodeling. Negative bone balance (bone resorption is higher than formation) will cause osteoporosis, hypocalcemia, and fracture while positive bone balance (bone formation is higher than resorption) may form a tumor on bone. The remodeling process is affected by molecular signaling that mainly controls bone cell differentiation and activities and biomechanical [4, 5].

Figure 2.2 Localized process of bone resorption followed by bone formation in bone modeling and remodeling. Adapted from: Imai, Y., *et al.*, Nuclear Receptors in Bone Physiology and Diseases. *Physiological Reviews*, 2013. **93**(2): p. 481-523. Copyright link: <https://creativecommons.org/licenses/by/3.0/legalcode>

2.1.2 Current approach for bone defects

Currently, bone graft approach is still commonly used to fill bone defects and treatment for bone reconstruction. Finding a suitable and effective bone graft for bone defects still remained a significant clinical challenge where each method has its own advantages and disadvantages. Ideally, a graft material for bone healing should exhibit osteointegration, osteoconduction, osteoinduction and osteogenesis properties in order to have superior bone healing properties. The material with osteointegrative property is able to form a chemical bond with the bone surface without being encapsulated by fibrous layer. The osteoconductive material is able to support the formation of bone on the surface while the osteoinductive material is able to induce pluripotent stem cells

to differentiate to an osteoblastic phenotype [6]. Lastly, it is also very important that osteoblastic cells within the graft material to be able to form new bone, where the graft material can help in osteogenesis [7].

For the current approaches, only autogenous cancellous bone graft exhibits these four characteristics as thus it is known to be the most effective bone graft material in bone healing. However, the undesirable procedure to harvest the autogenous cancellous bone graft will cause donor site morbidity and therefore other types of substitute bone graft material is still in search as a replacement for autograft [8]. For allograft, it offers the same characteristics as autograft and plays an important role in providing large graft volume for large bone defects. However, allograft lacks of osteoinductive ability and exhibits the disadvantages of operation complications include fracture, nonunion, and infection [9].

Besides natural bone grafts, the synthetic bone graft is also being explored and studied for its potential as bone substitutes. Synthetic bone grafts are mostly made of ceramic, such as bioactive glasses, glass ionomers, aluminum oxide, calcium sulfate, beta-tricalcium phosphate (β -TCP), synthetic hydroxyapatite and coralline hydroxyapatite. However, synthetic bone graft usually consists only of osteointegration and osteoconductive properties. Besides those ideal characteristics of bone graft, the synthetic bone graft should be biocompatible as well as provides similar mechanical strength as the bone site, prevents total encapsulation caused by the fibrotic reaction, and helps in new bone formation and remodeling in order to become a potential candidate for bone graft material.

The common synthetic bone grafts discussed in details are bioactive glasses, β -TCP, and synthetic hydroxyapatite (HA). Sodium oxide, calcium oxide, phosphorus pentoxide and silicon dioxide are the main components for the bioactive glasses. Bioactive glasses are able to integrate with the host bone tissue well and encourage the formation of new bone. Different composition of these elements can vary different properties and resorption rate of the bioactive glasses. The silicon dioxide components of bioactive glasses will become silica-gel when exposed to physiological aqueous solutions which enable calcium ions and phosphate ions in this layer to form hydroxyapatite crystals that are similar to bone. Thus, bioactive glasses can bond

strongly with bone [10, 11]. Currently, these bioactive glasses are mainly used as bone graft expander or substitute in the applications of maxillofacial reconstruction, ossicular replacement and as osteointegration coating for metal implant [12-15]. β -TCP and synthetic HA both belong to calcium phosphate family. β -TCP has been made in the form of solid or porous granules and blocks. Since 1920 β TCP has shown to be able to improve the rate of the bone union for the bone defect by Albee and Morrison, it has been widely studied as bone graft substitute [16]. Similarly to other types of calcium phosphate, β -TCP has high mechanical properties similar to the spongy bone but failed to withstand tension and shear [17, 18]. Resorption of β TCP takes place over 6 to 18 months period through dissolution and fragmentation. However, its resorption rate is always higher than bone regeneration when β -TCP is used as bone graft substitute, making β -TCP only suitable to use as an expander or as a supplement to other bone graft materials that are less resorbable for current clinical use [19]. As for synthetic hydroxyapatite, it has the chemical component of $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$. This chemical component resembles the bone mineral component. Synthetic hydroxyapatite can be in the form of ceramic or non-ceramic. A ceramic synthetic HA means the HA crystals have undergone heat treatment and sintering at the temperature between 700°C and 1300°C. The high temperature of the sintering process will create a highly crystalline ceramic synthetic HA that resists resorption during *in vivo* implantation (resorption rate only 1 to 2% per year) [12]. As the contrast, non-ceramic synthetic HA has low crystalline and would resorb readily *in vivo*. Similar to any other types of calcium phosphate, synthetic hydroxyapatite resists compression but easily breaks under tension and shear force. Even though synthetic HA as a solid block has higher elastic modulus than bone, synthetic HA have disadvantages of difficulty in shaping and does not permit fibro-osseous ingrowth, thus limits the usage of synthetic HA. Currently, the synthetic HA is used clinically as metal implants coating and bone void fillers in the form of granule [20-22].

In general, there are problems relating to most of the grafts such as lack of bone integration with the host leading to high failure rate as well as post-implantation complications. Thus, bone tissue engineering approach has become a potential alternative approach to treating bone defects to overcome these issues and has been studied extensively for these recent years.

2.2 Bone Tissue Engineering

2.2.1 Bone scaffold

The aim of tissue engineering is to help to regenerate the damaged tissue using the combination of the multidisciplinary effect of materials, cells, engineering, biochemical and physio-chemical factors. In particular for bone tissue engineering, the purpose is to help replace and regenerate bone defects or fractures caused by traumatic injuries, skeletal defects, and tumor resections. Similar to the general concept of tissue engineering field, bone tissue engineering also involves bone scaffold, cells and growth factors that stimulate bone regeneration to have the combination effects on bone regeneration (**Figure 2.3**). In order to design a bone tissue engineered scaffold, the specific design of the material and its properties needs to be tailored to match to different types of bone defect and conditions. Nonetheless, the material has to be biocompatible and must not trigger adverse immunological responses when implanted inside the body. The material used must also have a reasonable biodegradable ability such that the material will act only as a temporary support. Ultimately, the scaffold will degrade away and be replaced by newly regenerated bone tissues. Besides, the mechanical properties, structural design and porosity, surface chemistry and osteoinductivity also play an important part in scaffold's design for bone tissue engineering. Adequate vascularization, tissue ingrowth, and efficient mass transport are essential to ensure cells survivability in thick scaffolds and the success in bone tissue restoration. All these factors are highly depended on the sufficient porosity of scaffold [23]. Scaffold with well-designed and interconnected porosity is important to improve intracellular migration and bridging, transportation of mass, and aid in the ingrowth of bone tissue [24, 25]. Studies have also shown that different architecture design of scaffold will present different stiffness even though same material is used [26, 27]. Thus, specific design and porosity of scaffold have to be optimized to cater for different types of defects and medical conditions. The overall mechanical properties of scaffold accounted for the material used, design and porosity are critical criteria for bone tissue engineering, especially for load-bearing applications. The insufficient structural strength of scaffold will result in the failure of the scaffold to withstand biomechanical forces. Meanwhile, inappropriate scaffold stiffness will cause stress shielding at the

defect site and bone resorption of host tissues, thus impairs bone formation, remodeling and repair progress [23, 28, 29].

Figure 2.3 Two current bone tissue engineering approaches for bone regeneration [30]. Adapted from: Vallet-Regí, M., M. Colilla, and B. González, Medical applications of organic–inorganic hybrid materials within the field of silica-based bioceramics. Chemical Society Reviews, 2011. **40**(2): p. 596-607. Reproduced with permission.

2.2.2 Cells

Other than scaffolding materials, cells are used in bone tissue engineering primarily to assist in the early stage of bone repair as recruitment of skeletal progenitors is inadequate at the defect site. The main purpose of using cells in bone tissue engineering is to help in the early release of osteogenic and vasculogenic growth factors to initial recruitment of host osteogenic and vasculogenic cells. The implanted cells are proposed to be able to aid in building up the bone matrix and vascularization network in the scaffold [31]. However, the major challenge of cells usage in bone tissue engineering is to identify the source of cells that are able to perform efficiently in fulfilling those proposed roles [32, 33]. Many studies are done on different types of stem cells to examine their potential to aid in bone reconstruction. Many of these undifferentiated progenitors are shown to be able to differentiate to biosynthetically mature cells [34]. Although these stem cells have the potential in aiding bone healing, other factors such as isolation and expansion efficiency, cells' ability to express osteogenic markers and long-term safety issues need to be studied before considering for their usage in clinical applications. In this report, mesenchymal stem cells (MSCs) and adipose-derived stem

cells (ADSCs) are reviewed and potentially to be used in future studies on material-induced differentiation.

MSCs show the ability to be able to differentiate to osteolineage cells and cause bone formation during the natural bone development process. Thus, MSCs has gained large attention and potential usage in bone tissue engineering. The characterization of MSCs shows that they have negative expression for cluster of differentiation (CD)34, CD45, CD12, CD11a, CD19, and HLA-DR while a positive expression for STRO-1, CD29, CD73, CD90, CD105, CD106, CD116, CD146, and CD44 [35]. MSCs are mainly isolated from adult sources such as bone marrow, dental pulp, skin, adipose tissue, and peripheral blood [36-39]. The isolation of MSCs is relatively easy but they are highly dependent on their ability to attach to culture plate [40]. Besides, MSCs also has high proliferation rate when cultured *in vitro* and they have the ability to withstand freezing conditions, which make them a potential candidate in clinical usage [36]. MSCs have been widely used in combination with scaffolds to help in bone repair. Preclinical trials for repairing critical-size femoral defects, craniomaxillofacial deformities and spinal fusion have proven that MSCs help in improving the rate of bone repair [41]. However, there are several problems that limit the potential of MSCs in clinical applications. MSCs have a limited population doubling ability of 24 - 40 before senescence-associated growth arrest occurs, which limits their numbers to be used for clinical applications. Besides, their ability in osteogenic differentiation and bone formation is significantly affected by donor age and systemic disease. Lack of understanding of the common markers to define the different sources of MSCs has also limited their amount and quality that can be used for clinical applications [35, 42]. Long-term cell expansion using artificial culture conditions also potentially lead to abnormal karyotype development and become cancerous. The artificial culture that contains fetal bovine serum (FBS) will also lead to the risk of transmitting zoonotic or prion-related diseases inducing the immunological responses [34].

For the ADSCs, ADSCs possess the ability of multi-lineage differentiation, including osteogenic and chondrogenic lineage *in vitro* as well as *in vivo* [42-47]. ADSCs also have the surface markers CD44, CD90, CD13, CD29, CD73, CD166, and CD105, while some of the markers such as STRO-1 and CD34 still need a further characterization and clarification [43, 44]. The source for ADSCs isolation usually obtained through

lipoaspirates from the arm, thigh, abdomen, and breast. Pre-differentiation of ADSCs through additional supplements in culture medium such as ascorbic acid, β -glycerophosphate, dexamethasone, and BMP-2 have shown good adhesion, proliferation rate and bone formation on scaffolds *in vitro* and *in vivo* [48-50]. ADSCs are easily accessible and abundance in human lipoaspirates, making them an attractive cell source to use in one step intra-operative bone grafting method. All the procedures from tissue harvesting, cell isolation, cell seeding on the scaffold to implantation will be done in few hours without going through cell expansion steps. As compare to MSCs, treatment requires more time as cell expansion required 4 to 6 weeks in order to have enough cells for clinical applications [51-53]. Thus, the use of ADSCs will greatly reduce cost and time at the same time still having positive results on bone repair, making ADSCs an attractive source for bone tissue engineering.

2.2.3 Growth factors

On top of scaffolding materials and cells, growth factors play a crucial role in bone healing and repair. Growth factors are cytokines that function as signaling molecules to induce cell proliferation, migration, and differentiation. Bone morphogenetic proteins (BMPs), which belong to the family of TGF growth factors, have been found to have implications in osteogenesis and widely studies in bone tissue engineering [54]. Over 30 types of BMPs have been identified to have potential in recruiting and stimulating differentiation of bone progenitor and thus help in bone formation [55-57]. For example, porous poly(lactic-co-glycolic acid) (PLGA) with high molecular weight hyaluronic acid and poly(DL-lactide-co-glycolide) have been used in delivery recombinant human BMP-2 *in vivo* to promote cortical bone formation for long bone defects [58]. Although recombinant human BMPs are commercially available, challenges such as optimization of the dosage, release dynamics and its carrier for long-term therapeutic efficacy prevent these growth factors to be used in clinical applications at the moment.

2.3 Periosteum: Its Structure, Functions, and Properties

2.3.1 Introduction of the periosteum

In our study, we are developing a periosteum-inspired layer using bone tissue engineering approach to assist in bone graft healing. Thus, the understanding of periosteum's properties and functions is crucial in developing a scaffold that has similar properties and functions like periosteum. Periosteum is a bounding envelope of the skeletal system that consists of composite material that acts as a mechanical structure and also a biological habitat for stem cells. **Figure 2.4** shows the illustration of periosteum enveloping bone structure. Since 1742 Duhamel discovered the periosteum has the ability to help in new bone formation, the following studies have shown the important of the periosteum to provide a suitable environment various types of cells. The periosteal cells are contributing in both endochondral and intramembranous ossification during the event of bone healing. The periosteum also has the superior regenerative capacity as compared to the alternative tissues in term of ability to heal large and critical-sized defects, either in long bone or flat bone [59-64]. Besides, the periosteum also shows good results in enhancing bone formation for the lumbar segmental spine fusion and the maxillofacial reconstruction [65-67].

Figure 2.4 Illustration of periosteum layer covering the bone.

2.3.2 Structure and components of the periosteum

Periosteum is a highly vascularized membrane that consists of two main layers. The first layer is called thin cambial layer (zone I) that attaches to the bone and contains

most of the progenitor cells. The thin cambial layer grows along with the bone during the development of bone in youth [68]. The second layer is a thick fibrous layer which can be further separated into two different zone layers [69]. Periosteum is anchored strongly in a pre-stressed state through Sharpey's fibers [70]. This fibrous layer is aligned and expanded in the direction of bone growth [71]. Periosteum consists of 90 % fibroblasts cells, which is in round morphology when they are closer to bone and flatter when away from bone [72, 73]. Other cells that are found in the periosteum include osteoblasts, MSCs, and pericytes [69, 74]. Fibroblasts and pericytes in the periosteum have morphologies, phenotypes, and differentiate ability that similar to MSCs, and some studies suggest that the pericytes may contribute to vascularization and promote bone formation [75-78].

2.3.3 Functions of the periosteum

Periosteum provides attachment sites called enthuses or osteotendinous or osteoligamentous junctions that anchor at certain tendons and ligaments to bone [79, 80]. Periosteum plays an important role in transporting nutrients and waste for the bones and muscles. Periosteum contains periosteal arteries that supply blood for the periosteum and one-third of the cortical bone. Above the periosteum layer, larger periosteal arteries are found whose function is to interact with the muscle arteries and supply blood for the muscle adjacent to the bone [81]. Periosteum also contains different types of nerves for specific functions. Cholinergic sympathetic nerves called vasomotor nerves in the periosteum regulate the diameter of periosteal arteries and thus control blood flow through the periosteum [82]. Sensory nerves in the periosteum are sensitive to tension and tearing, that make the bone sensitive to pain during injury. Periosteum has an essential role in bone growth and bone healing. Osteoblasts that inhabit in the inner cambial layer cause new bone formation and play an important role in bone growth and repair. During an event of bone injury or fracture, the periosteum will act as a hinge or bridge that helps two broken parts of the bone to remain intact. The progenitor cells in the cambial layer will differentiate into osteoblasts and chondroblasts, and then forms a soft periosteal callus and initiate bone healing [79].

2.3.4 Periosteum-derived cells

Recent studies have proved that stem cells have the ability to help in regeneration and restoring of the damaged bone structure. For example, MSCs, multipotent and immunoprivileged cells, are easily cultured and found in various types of tissues such as bone marrow, adipose, and muscle [83]. However, harvesting these stem cells from patients still remains the main challenge as the harvesting procedure would cause donor site morbidity. Besides, the problem of an inadequate volume of tissue to isolate MSCs is also one of the major disadvantages of using MSCs. Thus, the periosteum is more suitable to harvest MSCs as the periosteum tissue can be easily extracted from many areas of the bone [84]. Recent studies showed that periosteum-derived cells (PDCs) have the bone healing and regeneration ability comparable to bone marrow mesenchymal stem cells [85, 86]. Another advantage of PDCs is that PDCs has fast proliferation rate and able to retain their ability to differentiate during *in vitro* culture [87]. Besides biochemical inductions such as using BMP-2 [88], PDCs also respond to surface morphology and mechanical strain for cell differentiation. Periosteum is very sensitive to mechanical inductions. Mechanical loading or induction has shown to cause periosteum to have a rapid change in gene expression that stimulates DNA synthesis, cell proliferation, periosteal hypertrophy, bone formation, and bone growth [89-92]. For surface morphology, PDCs tend to become more osteogenic on a smooth surface and also increase in proliferation in a 3D-scaffold as compared to a 2D culture [93].

2.3.5 Periosteum: Bone's "smart" bounding membrane

Periosteum is an impermeable barrier membrane for bone that serves two purposes: (1) gateway for mass and cells transportation and (2) prevent fluid loss from the bone [94, 95]. In a study done by Sarah *et al.*, they reviewed the behavior of ovine periosteum's permeability when responds to different volumetric flow rate and pre-stressed condition [96]. Their experimental studies showed that periosteum's permeability is significantly dependent on flow rate. Periosteum tends to be more permeable when the fluid has higher flow rate. Periosteum from the different part of bone also showed different directional behavior in response to flow rate. At high volumetric flow rate, femoral periosteum shows significant directional dependence toward flow behavior while the tibial periosteum did not. There was also higher permeability when the higher flow rate

is used by flowing in the direction from bone to muscle. However, the thickness of periosteum has negative implications on permeability. The study hypothesized that periosteum is a smart biomaterial because it is not passive, but is highly dependent on flow rate and responds to dynamic environment conditions. Pre-stressed periosteum will also have lesser permeability than non-pre-stressed periosteum. Thus, the periosteum is hypothesized to be mechanical responsive and directionally dependent due to the fibrous layer of the periosteum acts as a mechanical valve at the tissue length scale and exhibits an anisotropic mechanical behavior.

2.3.6 Recent advances in periosteal tissue engineering

Periosteal tissue engineering mainly focuses on the improvement of allograft remodeling where the recent development is concentrated on delivery and localization of cells to the allograft's surface with the aid of scaffold. From the literature review, Hoffman *et al.* have demonstrated using a polyethylene glycol (PEG) hydrogel to provide for localization of MSCs at the allograft's surface for 12 days in order to achieve cell-mediated healing mechanism. The purpose of this system with the cell rich hydrogel is to reproduce the cellular function of periosteum, where periosteal stem cells persisted at the autograft's surface for 14 days during the healing process. They have found out by enveloping the allograft with this MSCs encapsulated in PEG hydrogel, significant enhancement in graft vascularization, endochondral bone callus formation and improvement in host-graft biochemical stability as compared to control allograft. However, the only drawback is that the healing process has significantly delayed as compared to autograft with this system and further improvement is required in terms of healing rate of bone [97]. Next, hydroxyapatite-containing the electrospun scaffold developed by Frohbergh *et al.* and Ding *et al.* have been used to induce osteogenic differentiation of the stem cells aiming to emulate the function of cambium layer of the periosteum [98, 99]. This highly mineralized scaffold has proved to be able to shift murine MSCs towards osteogenic lineage *in vitro* and thus enhanced bone integration and formation *in vivo* as compared to chitosan alone. The direct mixing of hydroxyapatite into chitosan scaffold provides the bioactive properties to the whole system enhancing bone integration *in vivo* even without delivering MSCs to the defect site. However, the main disadvantage of the electrospun scaffold is limited penetration of cells and tissue ingrowth into the scaffold due to insufficient porosity [98].

Apart from the tissue-engineered scaffold, cell sheet technology has emerged recently as a new method for tissue regeneration [100-103]. Cells such as human bone marrow stromal cells [104] and human MSCs [105] have been cultured to form cell sheets and its potential as periosteum-inspired layer in enhancing bone integration and healing is being investigated. The study by Kang *et al.* has shown to develop a cell-sheet-engineered a periosteum-inspired layer by forming an hMSCs cell sheet followed by seeding human umbilical vascular endothelial cells (HUVECs) to mimic the fibrous layer of the periosteum that consists of the microvasculature. Besides, a cell sheet of hMSCs that has been osteo-differentiated is formed to mimic the cambium layer of the periosteum. The combination of the “cambium mimicking layer” and the “fibrous mimicking layer” provided the vascularization and osteogenic properties to the bone graft. However, lack of the understanding of the mechanism of prevascularized periosteum-inspired cell sheets connects with host vasculature and the osteogenic properties of this periosteum-inspired cell sheet in enhancing bone healing efficiency are not fully investigated and understand [105]. Besides, cell source is a major problem for using this technology [106].

Lately, Shi *et al.* have shown the important of the surface topography of the periosteum in bone elongation other than studying the chemical functions and mimicking the properties of the periosteum. In their study, topographic cues were created on the surface of PLGA nanosheet using micropatterning. These topographic cues are able to guide and to align the arrangement of cells and ECM resembles periosteum. The microgrooved patterns caused the elongation of adipose-derived mesenchymal stem cells (adMSCs) that promotes the expression of osteogenic markers and that ultimately led to the osteogenic differentiation of stem cells. The nanosheet also provided the advantage with the improvement of film flexibility and physical adhesiveness when the film size decreases, which potentially adhere tightly on the bone's surface [107].

Although efforts have been done to emulate the functions and properties of the periosteum mainly targeting on angiogenic and osteogenic properties to enhance bone healing efficiency of the bone graft, to date, the bone healing efficiency is still incomparable to autograft. Thus, in order to further understand the mechanisms and the roles of native periosteum in bone healing and repair required to improve the strategies in mimicking the functions and properties of periosteum. On top of emulating the

functions of periosteum, the combination with other strategies such as specific design on the surface, architecture, porosity or structural properties of the scaffold matches with the periosteum could be a way to further enhance angiogenic and osteogenic properties of the whole system.

2.4 Hydrogels

2.4.1 Hydrogel's properties and injectability

Our main scaffold component consists of an injectable hydrogel. Thus, understanding the current in situ crosslinking hydrogel will greatly help in our design of scaffold. Hydrogels are a three-dimensional polymeric structure that is able to withhold a large amount of water [108]. A large amount of water in the hydrogel's structure causes the hydrogels to have the environment similar to ECM. Therefore, hydrogels have great biocompatibility and suitable environment for cells to inhabit and proliferation [109]. Due to the high water content, hydrogels are an excellent candidate for delivering water-soluble proteins and drugs. Previous studies have been using the synthetic polymer such as PLGA or polyhydroxybutyrate (PHB) to encapsulate protein drugs for delivery. However, the harsh procedures such as homogenization and emulsification as well as the usage of organic solvent during the microcapsules fabrication of the synthetic polymers result in the decrease in bioactivity or denaturation of proteins and drugs. Besides, degradation products from PLGA are acidic and will also cause denaturation of protein drugs [110, 111]. Thus, hydrogels that mainly involve water as the solvent and require mild cross-linking to gel have greatly improved the technique of proteins and drugs delivery.

Hydrogels are generally classified by two cross-linking methods: physical cross-linking or chemical cross-linking. The increase in the variety and designs of hydrogels has further increased the hydrogels' classification involving the nature of the material: natural polymer or synthetic polymer, types of cross-linker, types of network, degradability, and porosity. The traditional design of the hydrogels involves the control of their detailed structure to obtain desired properties. Current hydrogels design is more focusing on creating a smart and responsive biomaterial to tailor for different environments and medical conditions. The same criteria of tissue engineering applied

for hydrogels where hydrogels must have biochemical, mass transport and physical properties that able to organize cells into 3D structure in order to serve as an ECM mimic. Besides, they should also be able to stimulate cells for desired tissue formation for the specific application. The design of hydrogels must not deteriorate the encapsulated cells but to promote cell growth. For encapsulation of protein or drug, the hydrogels must not decrease the bioactivity of the protein or drug and should have the ability of controlled release for the specific application. Like a typical scaffold, the rate of degradation of the hydrogels ideally should match the rate of tissue regeneration. The design of hydrogels should allow adequate mass transport of nutrients into the hydrogels and metabolites out of the hydrogels. The effectiveness of hydrogels as the structural support for the defect site and a platform for cell migration, proliferation, and differentiation are mainly determined by their mechanical properties. Mechanical or the stiffness of the hydrogels' microenvironment also served in regulating cell functions and tissue morphogenesis [112-114].

Advances in the designs of the hydrogel system have been exploited the “injectability” properties of the hydrogels to minimize invasive surgeries and reduce patient trauma for quicker recovery. Injectable can be done through in situ polymerization strategies. Using this method, a gel precursor in a solution form is suspended with cells and bioactive agents prior to injection into the defect site. The in-situ polymerization is then initiated by using different methods of cross-linking. Some of these methods are photopolymerization, enzyme-mediated cross-linking method, and stimuli responsive polymers. The advantages of using the in-situ polymerization over the preformed hydrogels are that the liquid form of the gel precursor is able to fill any shapes of the defects and reach places that are difficult to access without an invasive surgical procedure. Besides, if the hydrogels system is biodegradable, it is also not required to have a second surgical procedure to remove the hydrogels after tissue regeneration [115].

2.4.2 Gelatin based injectable hydrogel

Gelatin, a partially hydrolyzed form of collagen, is a widely used natural polymer for tissue engineering. Gelatin consists of Arginine-Glycine-Aspartic acid (RGD) motifs that are essential for cell adhesion. In addition, gelatin also exhibits a large number of

functional groups for crosslinking and targeting ligands that essential for drug delivery [116, 117]. Gelatin is also easily available, low cost, biocompatible, biodegradable, and have low antigenicity, thus make gelatin an attractive material for biomedical applications [116]. Gelatin is potentially designed to be an injectable scaffold through crosslinking using chemical crosslinkers (EDC [118, 119]), natural crosslinkers (genipin [120, 121], nordihydroguaiarectic acid [122] and Tannin acid [123]), enzymatic crosslinkers (transglutaminase [124, 125] and tyrosinases [126]) and photocrosslinking using photoinitiators (Irgacure 2959 [127, 128] and Rose Bengal [129, 130]). Most of the chemical, natural and enzymatic crosslinkers are used to covalently link the functional groups found on the gelatin. For examples, EDC can react with the carboxyl, hydroxyl and sulfhydryl group while genipin can react with hydroxyl and carboxyl groups found on the gelatin [131]. However, most of these chemical, natural and enzymatic crosslinking methods depend on the same crosslinker concentration to control both the gelation rate and mechanical properties of the hydrogel formed. Consequently, this limited the design parameters of the injectable hydrogel formed as certain applications required very low or high stiffness at the same time reasonable gelation rate for handability as an injectable scaffold. In addition, certain chemical and natural crosslinkers such as nordihydroguaiarectic acid [132] possess toxicity issues and might cause immune responses. As for the photocrosslinking methods, the use of high energy radiation during the crosslinking process such as UV radiation will cause damage to the surrounding tissues [133]. Thus, this thesis proposed the use of HRP and H_2O_2 as the crosslinking method for the periosteum-inspired injectable system. This enzymatic crosslinking method is biocompatible and able to control the gelation rate and mechanical properties individually through using different HRP and H_2O_2 concentrations respectively. The enzymatic crosslinking system using HRP and H_2O_2 will be reviewed in **Chapter 2, Section 2.4.3**.

2.4.3 Enzyme-catalyzed crosslinkable hydrogels

In general, chemical crosslinking gels are preferable than the physical cross-linking gels as they are more stable and strong in the physiology condition with superior mechanical properties [134]. However, chemical cross-linking methods may cause damage to the surrounding tissue if the initiator or cross-linker used is toxic. Thus, the newly developed enzymatically cross-linked hydrogels have increasingly gained attention due

to their mild reaction conditions. Most of the enzymes used for cross-linking the enzymatically cross-linked hydrogels are common natural enzymes that are easily found in the catalytic reactions of our body [135-142]. Enzymes react mostly at neutral pH, moderate temperature and in an aqueous solvent. These mild crosslinking conditions have made enzymatic reaction suitable to be used in the normal physiological conditions. Besides, natural polymers that will be degraded and lose its bioactivity due to the harsh chemical can be avoided by using enzymatic cross-linking [143]. Enzymatic reactions will not produce toxic by-products as compared to other chemical cross-linking methods, such as photopolymerization. Using enzymatic cross-linking methods can also prevent the use of organic solvent, which is harmful to our body. Currently, some of the enzymes that are being explored for the enzymatic crosslinking of the natural polymers are β -lactamase and phosphatase/kinase, transglutaminases, tyrosinases, phosphopantetheinyl transferase, phosphatases, thermolysin, lysyl oxidase and plasma amine oxidase, and peroxidase [126, 135, 144-152]. However, each of these enzymatic crosslinking systems has its own advantages and disadvantages. For example, peroxidases group usually use a plant peroxidase such as horseradish peroxidase or soybean peroxidase to catalyze the hydrogen peroxide to initiate polymerization (**Scheme 2.1**) [153]. This enzymatic system has a good biocompatibility and high mechanical strength but a fast gelation rate, which is not desirable in most of the applications [136-138, 154]. Besides, to date, the *in vivo* role of the horseradish is yet fully understood and no human peroxidase has been reported to cause hydrogels formation. Considering the advantages of good biocompatibility and mechanical strength, this thesis will use peroxidase as the crosslinking method for the novel injectable hydrogel for bone applications.

Scheme 2.1 Mechanism of the enzymatic reaction using the peroxidase. Adapted from: Moreira Teixeira, L.S., *et al.*, *Enzyme-catalyzed crosslinkable hydrogels: Emerging strategies for tissue engineering*. Biomaterials, 2012. **33**(5): p. 1281-1290. Reproduced with permission.

2.4.4 Stimuli-responsive hydrogels

Stimuli-responsive hydrogels undergo volume phase transition or change in the degree of swelling when responded to an external change of environment [155]. Small changes in physical or chemical conditions such as pH, temperature, light, magnetic field or electric field will cause stimuli-responsive hydrogels to swell and deswell. Polymers such as and N-isopropylacrylamide (NiPAAM) polymers [156] that have a lower critical solution temperature (LCST) are able to form the temperature responsive hydrogels. When the temperature is below polymer's LCST, the polymer is soluble in water and able to form the precursor solution. As the surrounding temperature rises above the LCST, the temperature responsive polymer will become more hydrophobic and insoluble, and finally, become gel completely or solidify [157]. As seen in **Figure 2.5**, when temperature again decreases, the polymers with LCST will respond to the external changes through swelling, causing the release of encapsulated proteins, growth factors or drugs. Another well studied stimuli-responsive hydrogel is the pH-responsive hydrogel. This type of responsive hydrogels contains pendant acid or basic groups that enable them to accept or release of protons when responded to the change of pH. The release and accept of protons in respond to pH will cause swelling and deswelling of the pH-responsive hydrogel [157]. Some of the examples of pH-responsive hydrogels

are poly(acrylic acid (PAA) and poly(methacrylic acid) (PMA) grafted with PEG [157, 158]. Thus, stimuli-responsive hydrogels are attractive smart biomaterial, especially for delivery of drug, protein and gene, as a wound dressing and prevent tissue adhesion [159]. Inspired by the previous design of smart hydrogel, this thesis will develop a smart enzymatic responsive hybrid hydrogel that enables alteration of degradation rate and release rate for bone regeneration.

Figure 2.5 Release of encapsulated molecules due to swelling of the stimuli-sensitive hydrogel when responds to external stimuli. © 2011 Ferreira, P, Almeida, JF, Coelho, JFJ and Gil, MH. Adapted from: Ferreira, P., *et al.*, Photocrosslinkable polymers for biomedical applications. 2011: INTECH Open Access Publisher; under Creative Commons Attribution-NonCommercial-ShareAlike 3.0 Unported (CC BY-NC-SA 3.0) license. Available from: <http://www.intechopen.com/books/biomedical-engineering-frontiers-and-challenges/photocrosslinkable-polymers-for-biomedical-applications>

2.5 Hydrogel-Based Composites

As discussed in **Chapter 2, Section 2.4.1**, hydrogels show many advantages as the tissue-engineered scaffold, cell encapsulation, and protein/drug delivery application. However, most hydrogel's mechanical properties are too low for load bearing applications [112, 160]. Thus, the idea of combining both organic and inorganic component was born to achieve a composite with advantages of both good strength and biological properties. The synergetic effects of both organic and inorganic will counterbalanced the disadvantages of each component in an ideal case [161]. The composite can be formed by simple mixing of inorganic components such as CaP [162,

163], bioactive glass [164, 165] and carbonaceous materials [166, 167] into the hydrogel system prior to crosslink. The incorporation of the inorganic component will result in different interaction with polymers that lead to new structure properties such as increased strength and mechanical stability, and change in degradation rate [161]. Besides, the composite can also achieve through *in situ* mineralization of hydrogel's structure [168]. The alteration of properties, especially in terms of mechanical properties, will have benefited not only for *in vivo* applications, but also in processability that required different viscosity and flow characteristic such as bioprinting, and cell encapsulation that required different stiffness [169, 170]. In this thesis, natural fish scale-derived CaP that has similar chemical properties like hydroxyapatite will be incorporated into the novel hydrogel system to improve mechanical and bioactive properties. The properties of calcium phosphate, mainly hydroxyapatite, will be review in following section (**Chapter 2, Section 2.6**)

2.6 Hydroxyapatite – Calcium Phosphate Compounds

CaPs are compounds that are found to have similar chemical properties as bones or teeth and have been widely studied as biomaterials due to their excellent biocompatibility [171]. Among all the types of CaP, HA and TCP are mostly studied and used in the clinical trials due to the similarity in osteoconductive, crystallographic, and chemical properties as compared to the bone. The main different between these two types of the CaP is their resorbability *in vivo*. The HA is very stable and nondegradable *in vivo* while the TCP is resorbable [172, 173]. Since the first used of the TCP as a bone graft substitute in 1920, the CaP compounds became popular for usage and being studied as the bone graft in augmentation of the alveolar cleft, periodontal defects, tooth replacement and large bone defects [174-178]. Besides, the CaP compounds also being developed into the bone tissue engineering scaffold, the coating for implants to provide bone integration properties and as the injectable bone cement [179-182].

In this literature review, the HA will be emphasized to study on its properties. HA is a CaP compound that has a close calcium and phosphate ratio to the bone, enamel, and dentine (Ca/P ratio of 1.67 to 1.71, 1.63 and 1.61 respectively). HA has a hexagonal crystal structure with lattice parameters similar to the bone and the teeth mineral crystal structure (**Figure 2.6**) [183]. HA crystal structure is predicted to have a preferred

orientation and growth direction at c-axis and thus a needle-like morphology can be observed for the HA using SEM [184]. The similarity in the chemical composition and crystallographic properties of the hydroxyapatite as compared to the bone and the teeth makes it widely used in clinical applications for the bone defects (**Table 2.1**) [185].

Figure 2.6 Crystal structure of the hydroxyapatite. © 2010 Uddin, Mohammad Hafiz, Nakahira, Atsushi, Okazaki, Masayuki, Sohmura, Taiji and Matsumoto, Takuya. Adapted from: Uddin, M.H., *et al.*, Biomimetic fabrication of apatite related biomaterials. 2010: INTECH Open Access Publisher; under Creative Commons Attribution-NonCommercial-ShareAlike 3.0 Unported (CC BY-NC-SA 3.0) license. Available from: <http://www.intechopen.com/books/biomimetics-learning-from-nature/biomimetic-fabrication-of-apatite-related-biomaterials>

Table 2.1 Comparison of the chemical and crystallographic properties between the bone, the teeth, and the HA. Adapted from: Dorozhkin, S.V. and M. Epple, Biological and medical significance of calcium phosphates. *Angewandte Chemie International Edition*, 2002. **41**(17): p. 3130-3146. Reproduced with permission.

Composition	Bone	Enamel	Dentine	HA
Calcium (wt%)	34.8	36.5	35.1	39.6
Phosphorous (wt%)	15.2	17.1	16.9	18.5
Ca/P ratio	1.71	1.63	1.61	1.67
Lattice parameter, a (Å)	9.410	9.441	9.421	9.430
Lattice parameter, c (Å)	6.890	6.880	6.887	6.891

HA has osteoconductive and osteointegrative properties on top of its excellent biocompatibility, which helps in promoting rapid bone formation to have a strong integration with the host bone tissue [186]. Thus, the HA is most commonly used as the coatings for implants to promote bone integration [20]. However, the HA does not have adequate mechanical properties for load-bearing applications [187]. Generally, a dense HA has high mechanical properties with the Young modulus higher than the bone [188]. Although the HA has higher Young Modulus than the bone, the hydroxyapatite has a low fracture toughness that makes it brittle in nature and breaks easily. Fabrication of the porous HA as a scaffold will cause even lower mechanical properties of the whole structure especially in term of strength and fracture toughness. Thus, the HA is mainly used as an additive in the scaffold fabrication to improve the properties of the scaffold.

HA has been used widely in combination with the synthetic polymers or metals to form a composite scaffold. By combining the HA with a biodegradable material, it could help to develop a partially biodegradable scaffold that may be useful for bone applications [189, 190]. The HA crystal particles are being used to reinforce mainly on the polymeric materials and proved to be able to strengthen the whole structure, where the amount, size, shape, and volume fraction of the HA used are the parameters to be considered [191-193]. For example, the HA particles with a smaller size will have a larger surface area and energy, thus able to have more interaction and bonding between the polymer and the hydroxyapatite, leading to higher mechanical strength [192]. To date, the highest values of the fracture strength and toughness of the composite that consist of the HA had been reported by Suchanek and Yoshimura, where 20 to 30 % of the long fiber Iron-Chromium (Fe-Cr) alloy fibers are incorporated into the HA matrix [193]. Lastly, on top of the reinforcement's method such as using particles, long fibers and partially stabilized zirconia has been studied with the aim to improve the HA's mechanical properties [188, 194-196].

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Chapter 3

Experimental Methodology

This chapter describes in detail the methods to develop the periosteum-inspired hydrogel-bioceramic composite. Phenol-conjugated gelatin and carboxymethyl cellulase hybrid hydrogel incorporated with the fish scale-derived CaP was developed as the injectable scaffold for bone applications. Next, characterization and analysis methods used to address the hypotheses and the potential of the hydrogel-bioceramic composite as the bone scaffold were described in details. This chapter will first list down all the materials used for all the experiments, followed by details steps of synthesis, fabrication, and characterization of the hydrogel-bioceramic composite for bone tissue engineering.

3.1 Methodology Overview

This chapter will describe in detail the methodology used in all the research in this thesis and has been organized into 9 main sections: (i) materials used in all the research, (ii) synthesis of the phenol-conjugated polymers, (iii) extraction of the fish scale-derived CaP, (iv) fabrication of the Gtn-HPA hydrogel, the Gtn-HPA/CMC-Tyr hybrid hydrogel and the hydrogel-bioceramic composite, (v) material characterization, (vi) functional studies, (vii) *in vitro* cell studies, (viii) *in vivo* studies and (ix) statistical analysis. **Figure 3.1** summarizes all the methodologies used to achieve objectives of the 3 main studies outlined in **Chapter 1, Section 1.2**.

Study 1	Study 2	Study 3
Development of Gtn-HPA Hydrogel	Development of Gtn-HPA/ CMC-Tyr Hybrid Hydrogel	Development of Hydrogel-Bioceramic Composite
Synthesis of Gtn-HPA Precursor	Synthesis of CMC-Tyr Precursor	Extraction of CaP
<ul style="list-style-type: none"> ¹H Nuclear Magnetic Resonance UV-visible Spectrophotometer 	<ul style="list-style-type: none"> ¹H Nuclear Magnetic Resonance 	<ul style="list-style-type: none"> X-ray powder diffraction Fourier transfer infrared spectroscopy Scanning Electron Microscope Energy-Dispersive X-ray Spectroscopy
Material Characterization	Material Characterization	Material Characterization
<ul style="list-style-type: none"> Compressing Testing Swelling Studies Degradation Studies 	<ul style="list-style-type: none"> Compressing Testing Swelling Studies Degradation Studies 	<ul style="list-style-type: none"> Scanning Electron Microscope Compressing Testing Swelling Studies Degradation Studies
Functional Studies	<i>In Vitro</i> Cellular Studies	Functional Studies
<ul style="list-style-type: none"> Release Profile Studies 	<ul style="list-style-type: none"> 2D Cell Culture Cytotoxicity Test on Cellulase 	<ul style="list-style-type: none"> Release Profile Studies Biom mineralization Studies
<i>In Vivo</i> Studies	Functional Studies	<i>In Vitro</i> Cellular Studies
<ul style="list-style-type: none"> <i>In vivo</i> Biocompatible Evaluation Histological Analysis Immunohistochemical Analysis Angiography Assay 	<ul style="list-style-type: none"> Induced Drug Release through Cellulase Treatment Enhanced Cell Proliferation through Cellulase Treatment 	<ul style="list-style-type: none"> 2D Cell Culture pH Stabilization of CaP 3D Cell Encapsulation

Figure 3.1 Overall summary of methods used for each main study.

3.2 Materials

3.2.1 Chemical reagents

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

3.2.2 Cell culture reagents

All cell culture reagents mentioned in this thesis were purchased from Sigma-Aldrich, USA unless otherwise specified. All cell culture reagents were used without any further purification.

3.2.3 Cell lines

The human fetal osteoblast (hFOB) cell line was purchased from ATCC®, USA. Human kidney 293FT (HEK 293FT) cell line was purchased from Life Technologies, USA.

3.3 Synthesis of Phenol-Conjugated Polymers

3.3.1 Conjugation of 3-(4-hydroxyphenyl)propionic acid onto gelatin structure

Gelatin-3-(4-hydroxyphenyl)propionic acid (Gtn-HPA) conjugates were synthesized according to previously established procedures [1]. Briefly, 3.32 g of 3-(4-hydroxyphenyl) propionic acid (HPA) and 3.20 g N-hydroxysuccinimide (NHS) were first dissolved in 150ml DI water mixed with 100 ml of N,N-dimethylformamide (DMF). 3.82 g of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC.HCl) was added to this solution to initialize the reaction. The pH of the mixture was adjusted and maintained at 4.7 for 5 h. Next, 6.67 w/v % Gtn (Wako Pure Chemical Industries, Japan) solution was added to this mixture and the pH was adjusted and maintained at 4.7 for 1 h. The reaction was then allowed to take place overnight at room temperature. After the reaction proceeded, the pH of the mixture was adjusted to 7.0. The mixture was then dialyzed against 0.1M sodium chloride (NaCl) followed by 25%

ethanol and deionized (DI) water in sequence for 2 days each. The dialyzed product was lyophilized to get the final dry product.

3.3.2 Conjugation of tyramine onto carboxymethyl cellulose structure

Carboxymethyl cellulose-tyramine (CMC-Tyr) conjugates were synthesized according to previously established procedures [2]. Briefly, 5 g of sodium carboxymethyl cellulose (CMC.Na) was dissolved in 250 ml of DI water. 0.8648 g of tyramine chloride (Tyr.Cl), 0.5732 g of NHS, and 0.9547 g of EDC were then added to this solution to initiate the reaction. The pH value of the mixture was adjusted and maintained at 4.7 for 5h. The reaction was then allowed to take place overnight. After the conjugation reaction was completed, the mixture was dialyzed against 0.1M NaCl, 25% ethanol, and DI water for 2 days each. The dialyzed product was lyophilized to obtain the final dry product.

3.3.3 ^1H Nuclear magnetic resonance (^1H NMR)

Nuclear magnetic resonance spectroscopy (NMR; Bruker Avance 300MHz, Bruker Instruments, Inc., USA) was used to characterize the successful of HPA and Tyr molecules conjugation onto the Gtn and the CMC respectively. Proton nuclear magnetic resonance (^1H NMR) spectra were recorded from the liquid sample with a concentration of 10 mg/ml in deuterium oxide (D_2O).

3.3.4 UV-visible spectrophotometer

The phenol content in the Gtn-HPA and CMC-Tyr conjugates was determined using NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). Briefly, the Gtn-HPA and CMC-Tyr conjugates were dissolved in 0.1 M NaHCO_3 solution to obtain 2 mg/ml sample separately. In this study, the UV-visible mode was used and 2 μl of the sample was scanned using the wavelength ranging from 230 to 350 nm. The pristine Gtn and CMC solutions were used as blank. Finally, the normalized absorbance value at 276 nm was compared with the known concentration of HPA or Tyr standards.

3.4 Extraction of Natural Fish Scale-Derived CaP

3.4.1 Extraction of CaP from snakehead scales

Fish scales from snakeheads (*Channa argus*) were obtained from Khai Seng Trading and Fish Farm Pte Ltd., Singapore. Fish scales were cleaned with DI water and heated at a constant rate of 10 °C min⁻¹ to 850 °C. The temperature was then kept at 850 °C for an hour. After the heat treatment, the heat-treated fish scales were milled evenly to obtain sintered powder.

3.4.2 Fourier transform infrared spectroscopy (FTIR)

The chemical bonds of sintered powder were identified using Fourier transform infrared spectroscopy (Frontier FTIR; PerkinElmer Instrument, USA). The CaP powder was scanned from 4000 to 650 cm⁻¹ with a scan number of 32 to obtain the FTIR spectrum. A blank reference spectrum was used for background correction.

3.4.3 X-ray powder diffraction (XRD)

The chemical phase of sintered powder was characterized using X-ray diffractometry (XRD; Shimadzu XRD-6000, Shimadzu Corp., Japan). The sintered powder was mounted on the sample holder and scanned using CuK α -radiation ($\lambda = 1.5406 \text{ \AA}$) via theta/2theta mode ($20 < 2\theta < 60$) with a step size of 0.05° at a scan speed of 1° min⁻¹.

3.4.4 Scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDX)

The morphology and the calcium-to-phosphate ratio (Ca/P ratio) of the sintered powder were characterized using scanning electron microscope (SEM; JSM-6360LV, JEOL Corp., Japan) equipped with energy-dispersive X-ray spectroscopy (EDX). The sintered powder was coated with a thin layer of gold using sputter coater (Structure Probe, Inc., USA) for 90 s and imaged using a voltage of 20 kV.

3.4.5 Optimization of the calcium phosphate's pH stability

The sintered fish scales were first ball milled for 2 h using the mass ratio of fish scale: zirconia ball of 10 w/w % to obtain white CaP powder. The pH of the CaP powder was then stabilized using $1 \times$ phosphate buffered saline (PBS) solution. The CaP powder was soaked in different volume of $1 \times$ PBS solution (200, 400 and 600 ml) and the pH of the solution was measured. The pH of $1 \times$ PBS solutions soaked with CaP powder was compared to the initial pH of $1 \times$ PBS solution. The treated CaP powder was characterized using FTIR and XRD with the same parameters as mentioned in **Chapter 3, Sections 3.4.2 and 3.4.3** respectively.

3.5 Fabrication of Phenol-Conjugated Hydrogel System

3.5.1 Fabrication of Gtn-HPA hydrogel

The Gtn-HPA hydrogel was formed through enzymatic crosslinking using HRP and H_2O_2 . The lyophilized Gtn-HPA conjugate was dissolved in $1 \times$ PBS to form a 5 w/v % or 10 w/v % hydrogel precursor. HRP (100 units/mg, Wako Pure Chemical Industries, Japan) and H_2O_2 (30 wt%, Wako Pure Chemical Industries, Japan) was then added into the hydrogel precursor to initialize the crosslinking process. The crosslinking process was allowed to proceed for 1 h to ensure complete setting. Different HRP (0.05, 0.10, 0.12 and 0.15 units/ml) and H_2O_2 (3, 6, 9, and 12 mM) concentrations were used to vary the hydrogel's properties.

3.5.2 Fabrication of Gtn-HPA/CMC-Tyr hybrid hydrogel

The Gtn-HPA/CMC-Tyr hybrid hydrogel was formed through enzymatic crosslinking using HRP and H_2O_2 . The lyophilized Gtn-HPA and CMC-Tyr conjugates were dissolved separately in $1 \times$ PBS to form 5 w/v % polymer solutions. The hydrogel precursor was formed by mixing the Gtn-HPA and the CMC-Tyr polymer solutions using the volume ratio of 4:1. The hydrogel precursor was crosslinked using 0.15 units/ml HRP and different H_2O_2 concentrations (3, 6, 9 and 12 mM) to form samples with different properties. The crosslinking process was allowed to proceed for 1 h to ensure complete setting.

3.5.3 Fabrication of the injectable hydrogel-bioceramic (Gtn-HPA/CMC-Tyr/CaP) composite

The hydrogel-bioceramic composite was formed through enzymatic crosslinking using HRP and H₂O₂. The lyophilized Gtn-HPA and CMC-Tyr conjugates were dissolved separately in 1× PBS to form 5 w/v % polymer solutions. The hydrogel precursor was formed by mixing the Gtn-HPA and the CMC-Tyr solutions at a volume ratio of 4:1. Next, different amounts (5, 10, 20 and 40 w/v %) of fish scale-derived CaP were incorporated into the hydrogel precursor. The mixture was vortexed to ensure an even dispersion of CaP prior to crosslink with HRP and H₂O₂. The precursor was then crosslinked using 0.15 units/ml HRP and 1.5 mM H₂O₂ to form the 3D injectable hydrogel-bioceramic composite.

3.6 Material Characterization

3.6.1 Gelation rate of Gtn-HPA hydrogel

The gelation rate of the Gtn-HPA hydrogel was investigated using tube-tilt test. 0.5 ml of 5 w/v % Gtn-HPA precursor was placed into a 2 ml microtube. Different HRP concentrations (0.05, 0.10, 0.12 and 0.15 units/ml) and 10 mM H₂O₂ were added into the precursor and the microtube was placed on a tube rotator (Clifton Range-Nickel-Electro Ltd, UK). The tube rotator was set to rotate at medium speed (~15 rpm) until there no apparent movement could be observed within the tube. The Gtn-HPA hydrogel was then considered polymerized and the gelation time was recorded.

3.6.2 Scanning electron microscopy (SEM)

The morphology and pore size of the lyophilized samples at the cross-sectional area were characterized by scanning electron microscope (SEM; JSM-6360LV, JEOL Corp., Japan). The lyophilized samples were coated with thin layer of gold using sputter coater (Structure Probe, Inc., USA) for 90 s. Lyophilized Gtn-HPA hydrogel, Gtn-HPA/CMC-Tyr hybrid hydrogel and hydrogel-bioceramic composite with different amount of CaP (5, 10, 20 and 40 w/v %) were imaged using an acceleration voltage of 10 kV using the same magnification.

3.6.3 Compression testing

Samples precursors were first crosslinked using HRP and H₂O₂ and cast into a mold to form a cylindrical scaffold. The mechanical properties of the samples were measured using a mechanical testing machine (Instron 5567, Instron, USA). Compression mode was used for this testing and samples were compressed to 50% of the sample height or until the sample was broken. The compression rate of 0.05 mm/s was used for all the compression testing. The compression modulus of the samples was obtained from the linear portion of the stress-strain curve. For **Chapter 4, Section 4.3.3** and **Chapter 5, Section 5.3.3**, the cylindrical scaffolds of Gtn-HPA hydrogels (5 and 10 w/v %) and Gtn-HPA/CMC-Tyr hybrid hydrogels (5 w/v %) with diameter of 13 mm and height of 13 mm were formed using (i) different HRP concentrations (0.05, 0.10, 0.12 and 0.15 units/ml) and 3 mM H₂O₂, and (ii) 0.15 units/ml HRP and different H₂O₂ concentrations (3, 6, 9 and 12 mM). The load cell of 500 N was used in these sections. For **Chapter 6, Section 6.3.3**, a cylindrical scaffold of Gtn-HPA/CMC-Tyr hybrid hydrogel and hydrogel-bioceramic composite with different amounts of CaP (5, 10, 20 and 40 w/v %) were formed using 5 w/v % polymer concentrations, 0.15 units/ml HRP, and 1.5 mM H₂O₂. The cylindrical scaffold has a diameter of 13 mm and a height of 11.5 mm. The load cell of 10 N was used for this section. Each sample group was done using n = 4.

3.6.4 Percentage of swelling

0.5 ml Gtn-HPA hydrogels, Gtn-HPA/CMC-Tyr hybrid hydrogels, and hydrogel-bioceramic composites were used to investigate their swelling ratio. 0.5 ml of sample precursor was crosslinked using HRP and H₂O₂ and cast into a 2ml microtube. The crosslinking reaction was allowed to proceed for 1 h to ensure complete setting. The samples were then immersed in 0.5 ml 1 × PBS and incubated at 37 °C. At different time points, the samples were removed from the solution, blotted dry and weighed to measure the wet weight, W_w. The samples were then lyophilized to get the dry weight, W_d. The percentage of swelling of the sample was calculated using equation (1):

$$\text{Percentage of swelling} = \frac{W_w - W_d}{W_d} \times 100 \quad \text{Equation (1)}$$

For **Chapter 4, Section 4.3.4** and **Chapter 5, Section 5.3.4**, the Gtn-HPA hydrogels (5 and 10 w/v %) and the Gtn-HPA/CMC-Tyr hybrid hydrogels (5 w/v %) were formed by adding different 0.15 units/ml HRP and different H₂O₂ concentrations (3, 6, 9 and 12 mM). The percentage of swelling of the Gtn-HPA hydrogel and Gtn-HPA/CMC-Tyr hybrid hydrogel was characterized at day 1, 3, 5 and 7. For **Chapter 6, Section 6.3.4**, the Gtn-HPA/CMC-Tyr hybrid hydrogel and hydrogel-bioceramic composites with different amounts of CaP (5, 10, 20 and 40 w/v %) were formed using 5 w/v % polymer concentration, 0.15 units/ml HRP and 1.5 mM H₂O₂. The percentage of swelling of Gtn-HPA/CMC-Tyr hydrogel and Gtn-HPA/CMC-Tyr/CaP was characterized at day 1, 4 and 7.

3.6.5 *In vitro* degradation studies

The *in vitro* degradation behavior of the Gtn-HPA hydrogels, the Gtn-HPA/CMC-Tyr hybrid hydrogels and the hydrogel-bioceramic composites were studied by immersing the samples into type I collagenase solution (Life Technologies, USA). For **Chapter 4, Section 4.3.5** and **Chapter 5, Section 5.3.5**, 0.5 ml Gtn-HPA hydrogels (5 and 10 w/v %) and Gtn-HPA/CMC-Tyr hybrid hydrogels (5 w/v %) were formed using 0.15 units/ml HRP and H₂O₂ concentrations (3, 6, 9 and 12 mM). The crosslinking reaction was allowed to proceed for 1 h to ensure complete setting. The samples were immersed into 0.5 ml 1 × PBS containing 0.50 units/ml type-I collagenase and incubated at 37 °C. Each day, the samples were removed from the solution, blotted dry and weighed. Then, the samples were immersed again in a fresh type-I collagenase solution. The degradation studies for these sessions were carried out for 21 days or until the sample was completely degraded. Each sample group was done using n = 5. For **Chapter 6, Section 6.3.5**, 2 ml Gtn-HPA/CMC-Tyr hybrid hydrogels and hydrogel-bioceramic composites with different amounts of CaP (5, 10, 20 and 40 w/v %) were formed using 5 w/v % polymer concentration, 0.15 units/ml HRP and 1.5 mM H₂O₂ on the Petri dish (diameter = 35 mm, height 10 mm). The samples were immersed into 2 ml 1 × PBS solution containing 0.70 units/ml type-I collagenase. The samples were then incubated at 37 °C and shook at 60 rpm using the orbital shaker (Bibby Sterilin Ltd., England). At each time point, the samples were removed from the solution, blotted dry and weighed. The degradation study for this session was carried out hourly for 8 h. The percentage weight change of the sample was calculated using equation (2):

$$\text{Percentage weight change} = \frac{W_o - W_t}{W_o} \times 100\% \quad \text{Equation (2)}$$

where W_o is the initial weight of the sample and W_t is the sample weight at a specific time point.

3.6.6 *In vitro* fluorescein isothiocyanate (FITC)-dextran release studies

The release profile of the Gtn-HPA hydrogels, the Gtn-HPA/CMC-Tyr hybrid hydrogels, and the hydrogel-bioceramic composites were studied using the model drug, FITC-dextran. For **Chapter 4, Section 4.3.6**, FITC-dextran with average molecular weight (mol. wt.) of 70 000 was incorporated into the Gtn-HPA precursor with a concentration of 200 $\mu\text{g/ml}$ prior to crosslink. 0.5ml 5 w/v % Gtn-HPA precursor containing FITC-dextran was then crosslinked using 0.15 units/ml HRP and different H_2O_2 concentration (3, 6, 9 and 12 mM) and cast into 24 well plates. Meanwhile, 0.5ml 10 w/v % Gtn-HPA precursor containing FITC-dextran was crosslinked using 0.15 units/ml HRP and 9 mM H_2O_2 . For **Chapter 5, Section 5.3.6**, 0.5 ml 5 w/v % Gtn-HPA/CMC-Tyr precursor containing 250 $\mu\text{g/ml}$ FITC-dextran (average mol wt. 70 000) was crosslinked using 0.15 units/ml HRP and different H_2O_2 concentrations (3 and 6 mM). Each sample group used for this section was $n = 6$. For **Chapter 6, Section 6.3.6**, FITC-dextran with average mol. wt. 20 000 was incorporated into Gtn-HPA/CMC-Tyr and hydrogel-bioceramic composite precursor using the concentration of 20 $\mu\text{g/ml}$ and then crosslinked using 0.15 units/ml and 1.5 mM H_2O_2 . The samples containing FITC-dextran were allowed to set for 2 h to form a cylindrical shape scaffold. All samples were then immersed in 1 ml of $1 \times \text{PBS}$ solution and incubated at 37°C . At a certain time point, 200 μl of $1 \times \text{PBS}$ release buffer solution from each well plate was taken out and scanned using a microplate reader (SpectraMax M2, Molecular Devices, USA) under the fluorescence mode with an excitation wavelength of 495 nm, emission wavelength of 519 nm, and emission cut-off at 515 nm. Each sample was scanned with 20 flashes per read. The fluorescence intensity of the sample at each time point was compared using a standard curve correlating to the fluorescence intensities of known FITC-dextran concentrations. For **Chapter 4, Section 4.3.6** and **Chapter 5, Section 5.3.6**, the readings were taken daily for 21 and 7 days respectively. For **Chapter 6, Section 6.3.6**, the initial FITC-dextran release test was carried out hourly for 6 h.

Subsequently, the readings were continuously taken every day until a plateau was reached for the full release study.

3.7 Functional Studies

3.7.1 Induced drug release through enzymatic digestion

For **Chapter 5, Section 5.3.6**, 5 w/v % Gtn-HPA/CMC-Tyr precursor was incorporated with 250 $\mu\text{g/ml}$ FITC-dextran (average mol wt of 70 000). 0.5 ml of Gtn-HPA/CMC-Tyr precursor containing FITC-dextran molecules was crosslinked using 0.15 units/ml HRP and H_2O_2 different (3 and 6 mM). The precursor was then cast into a 24 well plate. All samples were allowed to set for 1 h to ensure complete crosslinking. Samples were then immersed in 0.5 ml $1 \times$ PBS release buffer at day 1, followed by 0.5 ml $1 \times$ PBS containing different cellulase concentrations (0.1, 1.0 and 2.0 mg/ml). Each day, 200 μl $1 \times$ PBS release buffer solution from each well plate was taken out and scanned using microplate reader (SpectraMax M2, Molecular Devices, USA) according to **Chapter 3, Section 3.6.6**. The release profile of the Gtn-HPA/CMC-Tyr hybrid hydrogel treated with different cellulase concentrations was done for 7 days. Each sample group was done using $n = 6$.

For **Chapter 6, Section 6.3.6**, hydrogel-bioceramic precursor with 5 w/v % polymer concentration and 10 w/v % CaP was incorporated with 250 $\mu\text{g/ml}$ FITC-dextran (average mol. wt. of 70 000). 0.5 ml of sample precursor containing FITC-dextran molecules were crosslinked using 0.15 units/ml HRP and 3 mM H_2O_2 , and cast into a 24 well plate. All samples were allowed to set for 1 h to ensure complete crosslinking. Samples were then immersed with 0.5 ml $1 \times$ PBS release buffer solution at day 1, followed by 0.5 ml $1 \times$ PBS containing 1.0 mg/ml cellulase. Each day, 200 μl $1 \times$ PBS release buffer solution from each well plate was taken out and scanned using microplate reader (SpectraMax M2, Molecular Devices, USA) according to **Chapter 3, Section 3.6.6**. The release profiles of the hydrogel-bioceramic composite without and with 1.0 mg/ml cellulase treatment were studied for 7 days. Each sample group was done using $n = 6$.

3.7.2 Bicinchoninic acid (BCA) protein assay

For **Chapter 5, Section 5.3.6**, 25 μ l 1 $\times$ PBS release buffer solution was also collected each day and characterized using BCA protein assay according to the manufacturer's protocol (Pierce, Thermo Fisher Scientific, USA). The BCA protein assay was used to confirm the enzymatic digestion of Gtn-HPA/CMC-Tyr hybrid hydrogel using cellulase. Briefly, BCA reagent A (sodium carbonate, sodium bicarbonate, Bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide) was mixed with BCA reagent B (2 mg/ml bovine serum albumin and 4 % cupric sulfate in 0.9 % saline and 0.05 % sodium azide) using the ratio of 50:1. 200 μ l BCA mixture was then added to 25 μ l 1 $\times$ PBS release buffer solution. The final mixture was incubated at 37 °C for 20 min. Next, the final mixture was cooled to room temperature and scanned using a microplate reader (SpectraMax M2, Molecular Devices, USA) under the absorbance mode with a wavelength of 562 nm. Each sample group was done using n = 6.

3.7.3 Enzymatic degradation profile of the Gtn-HPA/CMC-Tyr hybrid hydrogel

Degradation behavior of the Gtn-HPA/CMC-Tyr hybrid hydrogel treated with different cellulase concentrations was investigated. 5 w/v % Gtn-HPA/CMC-Tyr hybrid hydrogels were fabricated using 5 w/v % polymer concentration, 0.15 units/ml HRP and 3 or 6 mM H₂O₂. The crosslinking reaction was allowed to proceed for 1h to ensure complete setting. The samples were immersed into 0.5 ml 1 $\times$ PBS containing different cellulase concentrations (0.1, 1.0 and 2.0 mg/ml) and incubated at 37 °C. Each day, the samples were removed from the solution, blotted dry and weighed. Then, the samples were immersed again in a fresh cellulase solution. The enzymatic degradation studies were carried out for 7 days. The percentage weight change of sample was calculated using the equation (2) as mentioned in **Chapter 3, Section 3.6.5**.

3.7.4 Biomineralization study

Non-filled Gtn-HPA/CMC-Tyr hybrid hydrogel and hydrogel-bioceramic composites were crosslinked using 0.15 units/ml HRP and 1.5mM H₂O₂ and cast in a 48 well plate using 0.5 ml of the precursor solution. The samples were pre-treated using 200 mM calcium chloride dihydrate (CaCl₂.2H₂O) and 200 mM potassium hydrogen phosphate

trihydrate (K_2HPO_4) prior to simulated body fluid (SBF) treatment. $1 \times$ SBF was prepared according to Kokubo *et al.* In brief, 7.996 g NaCl, 0.350 g sodium bicarbonate (NaHCO_3), 0.224 g potassium chloride (KCl), 0.228 g potassium phosphate dibasic trihydrate ($\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$), and 0.305 g magnesium chloride hexahydrate ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$) were dissolved in 1 L of DI water. Then, 40 ml of 1 M hydrochloric acid (HCl; 37%, Merck, Germany) was added into solution before dissolving 0.278 g CaCl_2 , 0.071 g sodium sulfate (Na_2SO_4) and 6.057 g tris(hydroxymethyl) aminomethane ($(\text{CH}_2\text{OH})_3\text{CNH}_2$) into the solution. The pH of the solution was adjusted using 10 M HCl to 7.25 and a clear solution was obtained [3]. The samples were then soaked in 0.5 ml $1 \times$ SBF, incubated at 37 °C. The $1 \times$ SBF solution was changed on a daily basis. On days 1 and 7, respectively, the samples were removed from the solution, washed with DI water three times, and lyophilized. The lyophilized samples were then characterized using SEM-EDX under the same conditions as previously described in **Chapter 3, Section 3.4.4** and a voltage of 10 kV to study its morphology and the Ca/P ratio of the mineral formed on the sample's surface. The Ca/P ratio was obtained from an average of three regions from three different samples ($n = 9$).

The bioactive properties of the hydrogel-bioceramic composite were further studied using $10 \times$ SBF. 0.7 ml Gtn-HPA/CMC-Tyr hybrid hydrogel and hydrogel-bioceramic composite were crosslinked using 0.15 units/ml HRP and 1.5 mM H_2O_2 and cast into 24 well plates. The samples were pre-treated using 200 mM CaCl_2 and 200 mM $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ prior to simulated body fluid treatment. $10 \times$ Simulated body fluid ($10 \times$ SBF) was prepared according to Mavis *et al.* In brief, 58.443 g NaCl, 0.373 g KCl, 3.675 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.016 g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 1.4196 g NaH_2PO_4 and 0.846 g of NaHCO_3 were dissolved in 1 L of DI water. The pH of the solution was adjusted using 10 M HCl to the range of 4.35 to 4.40 and a clear solution was obtained [4]. The samples were then soaked in 0.5 ml $10 \times$ SBF, incubated at 37 °C and shook at 60 rpm using the orbital shaker. The $10 \times$ SBF solution was changed on a daily basis. On days 1 and 7 respectively, the samples were removed from the solution, washed with DI water three times and lyophilized. The lyophilized samples were then characterized using SEM-EDX under the same conditions as previously described in **Chapter 3, Section 3.4.4** and a voltage of 10 kV to study its morphology and the Ca/P ratio. The Ca/P ratio was obtained from an average of three regions from three different samples ($n = 9$).

3.8 Cell Culture

3.8.1 Human fetal osteoblasts (hFOB) cell line

hFOB cells were cultured in T75 tissue culture flasks using Dulbecco's Modified Eagle Medium: nutrient mixture F-12 (DMEM/F-12) supplemented with 10 % fetal bovine serum (FBS; Hyclone, USA), 1× antibiotic-antimycotic (ABAM; Life Technologies® - Gibco®, USA) and 2.5 mM L-glutamine at 34°C and 5% CO₂. Culture medium was changed every 2 or 3 days. Upon confluence, cells were passaged using 2ml 0.25 % Trypsin-ethylenediaminetetraacetic acid (Trypsin-EDTA; Thermo Fisher Scientific, USA) and expanded.

3.8.2 Human kidney fibroblasts (HEK293FT) cell line

HEK293FT cells were cultured on T75 tissue culture flask using Dulbecco's Modified Eagle Medium (DMEM) – high glucose supplemented with 10 % FBS (Hyclone, USA) and 1× ABAM (Life Technologies® - Gibco®, USA) at 37°C and 5% CO₂. Culture medium was changed every 2 or 3 days. Upon confluence, cells were passaged using 2ml 0.25 % Trypsin-EDTA (Thermo Fisher Scientific, USA) and expanded.

3.8.3 2D cell culture studies

Lyophilized sample precursors were dissolved in culture medium and filtered through a 0.22 µm filter syringe. For **Chapter 5, Section 5.3.7**, 0.5 ml Gtn-HPA/CMC-Tyr precursor (5 w/v %) was crosslinked with 0.15 units/ml HRP and 6 mM H₂O₂ and cast into a 24 well plate. The samples were allowed to set for 1 h. Each sample was seeded with hFOBs with a seeding density of 3.0×10^4 cells/cm². For **Chapter 6, Section 6.3.8**, 0.7 ml precursor solution of Gtn-HPA/CMC-Tyr and hydrogel-bioceramic composite with different amounts of CaP (5, 10, 20 and 40 w/v %) were crosslinked with 0.15 units/ml HRP and 1.5 mM H₂O₂ and cast into a 24 well plate. The samples were allowed to set for 2 h. Each sample was seeded with HEK 293FT cells with a seeding density of 2.1×10^4 cells/cm². All samples were then topped up with 0.5 ml culture medium and incubated at 34 °C with 5 % CO₂ for **Chapter 5, Section 5.3.7**, and 37 °C with 5 % CO₂ for **Chapter 6, Section 6.3.8**.

3.8.4 3D Cell encapsulation studies

The polymer solutions containing 5w/v% Gtn-HPA or 5w/v% CMC-Tyr conjugates were prepared in DMEM together with 10% FBS (Hyclone, USA) and 1× ABAM (Life Technologies® - Gibco®, USA). The polymer solutions were then filter sterilized using a 0.22mm syringe filter. The precursor solution was prepared by mixing the Gtn-HPA and the CMC-Tyr polymer solutions using a volume ratio of 4:1. For **Chapter 6, Section 6.3.8**, the precursor solution was added with different amounts of untreated fish scale-derived CaP to form the hydrogel-bioceramic composite precursor solution. For **Chapter 6, Section 6.3.10**, PBS-treated fish scale-derived CaP was further treated with DMEM in order for the powder to stabilize at the physiological pH. Different amounts of treated CaP (5, 10 and 20 w/v %) were then mixed with the hydrogel precursor solution to form the hydrogel-bioceramic composite precursor solution. For both sections, HEK293FT cells were then mixed with the precursor solution at a cell density of 5×10^4 cells/ml. 0.5 ml Gtn-HPA/CMC-Tyr hybrid hydrogel and Gtn-HPA/CMC-Tyr/CaP composites with different amounts of CaP were then formed in 24-well plates by adding 0.15 units/ml HRP and 1.5mM H₂O₂. All samples were then topped up with 0.5 ml culture medium and incubated at 37 °C with 5 % CO₂.

3.8.5 Cell proliferation studies

Cell proliferation was measured using either the PrestoBlue® cell viability reagent (ThermoFisher Scientific, USA) or WST-1 cell proliferation reagent (Abcam®, United Kingdom) according to the manufacturer's protocol. For **Chapter 5, Section 5.3.7**, hFOB3 cells cultured on the Gtn-HPA/CMC-Tyr hybrid hydrogel's surface were incubated with 0.5 ml 10 % WTS-1 cell proliferation reagent at 34 °C and 5 % CO₂. For **Chapter 6, Section 6.3.8**, HEK 293FT cells cultured on the Gtn-HPA/CMC-Tyr hybrid hydrogel's surface were incubated with 0.5 ml 10 % PrestoBlue® cell viability reagent at 37 °C and 5 % CO₂. For **Chapter 6, Sections 6.3.8 and 6.3.10**, HEK 293FT cells encapsulated in the Gtn-HPA/CMC-Tyr hybrid hydrogel and Gtn-HPA/CMC-Tyr/CaP composite were incubated with 0.5 ml 20 % PrestoBlue® cell viability reagent at 37 °C and 5 % CO₂. After incubating the samples for 1 h for 2D cell culture in **Chapter 5, Section 5.3.7** and **Chapter 6, Section 6.3.8**, 16 h for 3D cell encapsulation in **Chapter 6, Section 6.3.8** and 24 h for 3D cell encapsulation in **Chapter 6, Section 6.3.10**, 200

µl culture medium containing reagent was transferred to a 96 well plate. The culture medium containing WTS-1 cell proliferation reagent was scanned using a plate reader (SpectraMax M2, Molecular Devices, USA). Absorbance mode with the wavelength of 420 nm and a reference wavelength of 650 nm were used and each sample was scanned with 20 flashes per read. For culture medium containing PrestoBlue® cell viability reagent, fluorescence mode with the excitation wavelength of 560 nm and the emission wavelength of 590 nm were used. The fluorescence or absorbance intensity of the sample at each time point was compared with a standard curve correlating to the fluorescence or absorbance intensities of known cell numbers. After the testing, 0.5 ml fresh culture medium was replenished on each sample and incubated until the next time point. Cell proliferation was measured at day 1, 3, 5 and 7. For **Chapter 5, Section 5.3.7**, each sample group was done using n = 5.

3.8.6 Cell population doubling

The proliferation rate of cells cultured on the sample was measured using the method as described in **Chapter 3, Section 3.8.5**. The cell numbers for day 1, 3, 5 and 7 were recorded for each sample. Cell population doubling was calculated using equation (3):

$$\text{Cell population doubling} = \frac{\ln N_f - \ln N_i}{\ln 2} \quad \text{Equation (3)}$$

where N_f is the final cell number and N_i is the initial cell number. Cumulative cell population rate was plotted against different time point to obtain the cell's growth rate.

3.8.7 Cell viability

The viability of the cells cultured on or encapsulated inside the samples was assessed using LIVE/DEAD® viability/cytotoxicity kit, for mammalian cells (Life Technologies, USA) according to the manufacturer's protocol. Staining solution was created by adding 5µl calcein AM (4mM solution in DMSO) and 20 µl ethidium homodimer-1(2 mM solution in 1:4 DMSO/H₂O) into the culture medium. For 2D cell culture in **Chapter 5, Section 5.3.7** and **Chapter 6, Section 6.3.8**, the 0.5 ml staining solution were added to each sample and incubated at 34 °C and 37 °C respectively, and 5% CO₂

for 1 h. For 3D cell encapsulation in **Chapter 6, Sections 6.3.8 and 6.3.10**, samples were incubated at 37 °C and 5% CO₂ together with the 0.5 ml staining solution for 2 h. After the incubation, the staining solution was removed and the samples were washed three times with culture medium to remove the excess stains. The samples were then imaged using Zeiss Axio Observer Z1 inverted fluorescence microscope fitted with a camera (Carl Zeiss, Germany). The live cells stained with calcein AM were excited using blue laser line (488 nm) and detected using filters for Alexa Fluor® 488 (emission wavelength of 517 nm). The dead cells stained with ethidium homodimer-1 were excited using yellow-green laser line (561 nm) and detected using filters for Alexa Fluor® 594 (emission wavelength of 618 nm).

3.8.8 Cytotoxicity test for cellulase solution

hFOB cells were seeded on 24 well plates using the cell density of 1.0×10^4 cells/cm². 0.5 ml culture medium was then added to each well and samples were incubated at 34 °C with 5 % CO₂. Starting from day 1, hFOB cells were supplied with 0.5 ml culture medium containing different cellulase concentrations (0.1, 1.0 and 2.0 mg/ml). The culture medium with cellulase was changed daily until day 5. Cell proliferation and viability of hFOB cells were compared between the sample group without and with cellulase treatment. Cell proliferation was characterized using 10 % PrestoBlue® cell viability reagent according to **Chapter 3, Section 3.8.5**. The cell population doubling was calculated according to **Chapter 3, Section 3.8.6**. For cell viability study, hFOB cells were stained, incubated for 1 h and imaged according to **Chapter 3, Section 3.8.7**. Each sample group was done using n = 6.

3.8.9 Alkaline phosphatase (ALP) expression

Alkaline phosphatase expression of hFOB cells after treated with different cellulase concentrations (0, 1.0 and 2.0 mg/ml) were compared to the untreated hFOB cells at day 7. hFOB cells were lysed using 250 µl 0.2 % Nonidet P40 and three cycles of freeze-thaw. Next, 10 µl Tris buffer solution containing 0.1 % bovine serum albumin and 100 µl 4-methyl-umbelliferyl phosphate (4-MUP) working solution were added to the lysate. The mixture was then incubated at 37 °C for 30 min. After that, 100 µl 0.6 M sodium carbonate (Na₂CO₃) was added to the mixture to stop the reaction. The mixture was

then scanned using a plate reader (SpectraMax M2, Molecular Devices, USA). Fluorescence mode with the emission wavelength of 360 nm and the excitation wavelength of 450 nm were used and each sample was scanned with 20 flashes per read. The intensity of the sample was compared to the known amount of 4-methylumbelliferone (4-MU) reacted to 4-MUP. ALP expression was then normalized to the cells number obtained from cell proliferation study at day 7.

3.8.10 Pore enlargement through enzymatic digestion for enhanced cell proliferation

The polymer solutions containing 5w/v% Gtn-HPA or 5w/v% CMC-Tyr conjugates were prepared in DMEM/F-12 supplemented with 10 % FBS (Hyclone, USA), $1 \times$ ABAM (Life Technologies® - Gibco®, USA) and 2.5 mM L-glutamine. The polymer solutions were then filter sterilized using a 0.22mm filter syringe. Hydrogel precursor was prepared by mixing the Gtn-HPA and the CMC-Tyr polymer solutions at a volume ratio of 4:1. HFOB cells were then mixed with the precursor solution at a cell density of 3×10^6 cells/ml. 0.5ml Gtn-HPA/CMC-Tyr hybrid hydrogel were then formed in a 24-well plate by adding 0.15 units/ml HRP and 1.5mM H_2O_2 . All samples were then topped up with 0.5 ml culture medium and incubated at 34 °C with 5 % CO_2 . Starting at day 1, the samples were soaked with 0.5 ml medium containing different cellulase concentrations (0.1 and 1.0 mg/ml). Cell proliferation and viability at day 1, 3, 5 and 7 were characterized and compared with the samples without cellulase treatment. HFOB cells encapsulated in the Gtn-HPA/CMC-Tyr hybrid hydrogels were incubated with 10 % PrestoBlue® cell viability reagent at 34 °C and 5 % CO_2 for 4 h and characterized according to **Chapter 3, Section 3.8.5**.

3.9 In Vivo Studies

3.9.1 Subcutaneous implantation of Gtn-HPA hydrogel for *in vivo* biocompatibility evaluation

All *in vivo* studies were conducted according to the institutional guidelines and approved by National University of Singapore (NUS, Singapore) Institutional Animal Care And Use Committee (IACUC R15-0315). 6 to 8 weeks old C57/BL6 mice were

obtained from the animal breeding center of NUS and maintained under pathogen-free conditions in the satellite animal housing unit for the Center for Comparative Medicine (Singapore). Prior to the surgical procedure, C57BL/6 mice were anesthetized and shaved on the hind limb. Next, 10 μ l of 5 w/v % Gtn-HPA hydrogel crosslinked with 0.15 units/ml HRP and 6 mM H₂O₂ was injected on the incision site. Skin closure was then performed using interrupted suture technique. For the sham control group, C57BL/6 mice's hind limb was not injected with the Gtn-HPA hydrogel. After the surgery, the condition of C57BL/6 mice was monitored by injecting 0.1 mg/kg buprenorphine per day for 3 days and 85 mg/kg Enrofloxacin per day for 5 days. Post-implantation analysis was done on week 1 and week 3 for the implanted 5 w/v % Gtn-HPA hydrogel. Five mice were used for each time point and each sample group. The C57BL/6 mice were euthanized using CO₂ and the implanted 5 w/v % Gtn-HPA hydrogel along with the surrounding tissue was excised for analysis.

3.9.2 Hematoxylin and Eosin (H&E) staining

C57BL/6 mice were sacrificed to excise the implanted 5 w/v % Gtn-HPA hydrogel together with the surrounding skin. The implanted 5 w/v % Gtn-HPA hydrogel and skin were fixed using 2 w/v % paraformaldehyde (PFA) and 30 w/v % sucrose solution overnight at 4 °C. After the fixation, the implanted 5 w/v % Gtn-HPA hydrogel and skin were frozen in Tissue-Tek Optimum Cutting Temperature. The frozen block was then cryosectioned using the microtome to create 10 μ m sections and mounted on individual microscope slides. The sections were then stained with H & E according to manufacturer's protocol. The sections were first deparaffinized by treating with xylene for 2 times, each time for 10 min. The sections were then rehydrated by treating with absolute ethanol for 2 times, each time 5 min, 95 % ethanol and 70 % ethanol for 2 min each, followed by washing with DI water. Next, the sections were stained with hematoxylin solution and for 8 min and then washed with running DI water for 5 min. The sections were differentiated in 1 % HCl in 99 % ethanol for 30 s followed by washing with running DI water for 1 min. after that, the sections were treated with 0.2 % ammonia water for 1 min and washed with running DI water for 5 min. The sections were then rinsed with 95 % ethanol and counterstained with eosin Y solution for 1 min. The sections were dehydrated by treating with 95 % ethanol for 5 min and 2 times absolute ethanol for 5 min. Finally, the sections were cleared using 2 times of xylene,

with 5 min each and mounted with the xylene-based mounting medium. The stained sections were then imaged using fluorescence microscope (Axio imager.Z1, AxioCam HRM camera, Carl Zeiss, Germany).

3.9.3 Mason's trichrome staining

The implanted 5 w/v % Gtn-HPA hydrogel and the surrounding skin were harvested from C57BL/6 mice. The implanted 5 w/v % Gtn-HPA hydrogel and skin were fixed, paraffinized, cryosectioned, deparaffinized and rehydrated according to **Chapter 3, Section 3.9.2**. The sections were stained with Mason's trichrome staining according to the manufacturer's protocol. The sections were first stained with Weigert's iron hematoxylin working solution for 10 min followed by rinsing with warm DI water for 10 min to remove the yellow color. The sections were then stained with Biebrich scarlet-acid fuchsin solution for 15 min followed by washing with DI water. Next, the sections were differentiated in phosphomolybdic-phosphotungstic acid solution for 15 min. The sections were then immediately stained with aniline blue solution for 10 min, followed by washing using DI water and differentiating in 1 % acetic acid solution for 5 min. The stained sections were washed with DI water, dehydrated using 95 % ethanol and absolute ethanol, and cleared with xylene. Finally, the stained sections were mounted using resinous mounting medium and imaged using fluorescence microscope (Axio imager.Z1, AxioCam HRM camera, Carl Zeiss, Germany).

3.9.4 Immunohistochemical analysis

Implanted 5 w/v % Gtn-HPA hydrogel and surrounding skin were fixed and cryosectioned according to **Chapter 3, Section 3.9.2**. Armenian hamster anti-CD31 (Millipore, USA), rat anti-CD68 (AbD Serotec®, Germany) and rabbit anti-lymphatic vessel endothelial hyaluronic acid receptor-1 (LYVE-1) polyclonal (Abcam®, UK) antibodies were used as primary antibodies for immunohistochemical analysis. For detection, Cy3-conjugated anti-Armenian hamster, Alexa Fluor® 647-conjugated anti-rat and Cy2-conjugated anti-rabbit (Jackson ImmunoResearch Laboratories, USA) were used. Sections were counterstained with 4,6-diamidino-2-phenylindole to make cell nuclei visible. Lastly, the sections were mounted on polylysine coated glass slide for analysis. Sections were imaged using fluorescence microscope (Axio imager.Z1,

Axiocam HRM camera, Carl Zeiss, Germany) or confocal microscope (FV1200 Laser Scanning Microscopes, Olympus, Japan).

3.10 Statistical Analysis

All data are expressed in mean $\pm$ standard deviation with a replicate of $n = 3$ unless otherwise specified. The differences between the values were assessed using Kruskal – Wallis one-way analysis of variance followed by Mann–Whitney U-test, where $p < 0.05$ is considered statistically significant.

Acknowledgement

All the chemical structures were draw using ACD/ChemSketch (USA). The SEM images in this thesis were processed using ImageJ (USA). All the graphs were draw using OriginPro 8.5 (USA).

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Chapter 4

Study 1 – Development of Injectable Phenol-Conjugated Gelatin Hydrogel.

This chapter will present in detail the development of an injectable phenol-conjugated gelatin hydrogel. This chapter will include the synthesis and fabrication of an injectable Gtn-HPA hydrogel and the, material characterization of the Gtn-HPA hydrogel. The effect of different fabrication parameters on the compression modulus, swelling ratio and degradation profile were investigated. Next, the potential of the Gtn-HPA hydrogel as a delivery vehicle will be investigated using a FITC-dextran release study. Subsequently, in vivo studies involving C57BL/6 mice model to evaluate the Gtn-HPA hydrogel's potential as an injectable scaffold for bone regeneration in term of injectability, biocompatibility, degradability, angiogenic properties will be discussed.

4.1 Introduction

The overall aim of this chapter is to develop an injectable system where the based material is being inspired by the various function of the periosteum as follow: (a) inspired by the role of periosteum as an ECM for hosting stem cells while the material used should be able to provide the suitable environment as a cell carrier, (b) periosteum is highly vascularized while the material used should be able to provide superior angiogenic properties, and (c) as an injectable system, the crosslinking method should be able to tune both the gelation rate and mechanical properties individually to have further control on the design parameter.

In this chapter, gelatin is proposed to be used as the based material for this periosteum-inspired injectable system. Studies have showed that gelatin has good biocompatibility [1] and consists of RGD proteins that are essential for cell attachment [2, 3]. In addition, gelatin is also cheap, readily available and easily to process and to modify [4]. Moreover, gelatin is also widely used in combination with other tissue engineered materials to promote cell attachment and to induce angiogenesis [5]. Thus, gelatin is a good candidate to be used as the based material for the periosteum-inspired injectable system as it can potentially provide a suitable environment like periosteum to host and deliver cells. More importantly, gelatin is also potentially provides angiogenic properties for effective bone healing.

Next, an enzyme-catalyzed crosslinking mechanism using HRP and H_2O_2 is proposed to be used as the crosslinking method for the periosteum-inspired injectable system. The enzyme-catalyzed crosslinking mechanism using HRP and H_2O_2 have the great potential to be used for the biomedical application due to its biocompatibility and relatively fast gelation time (from seconds to minutes) [6]. Hence, for this study, gelatin was conjugated with phenol molecules to enable enzymatic crosslinking using HRP and H_2O_2 to form phenol-conjugated gelatin hydrogel. In addition, the material properties of phenol-conjugated gelatin hydrogel can be easily tuned using different amounts of HRP and H_2O_2 to meet different requirements [7, 8]. Hence, the gelation rate and mechanical properties can be tuned individually using HRP and H_2O_2 , while most of the other crosslinkers not able to individually control both [9]. Overall, HRP and H_2O_2

crosslinking mechanism shows to be a great potential to be used for an injectable system with more tunability for the overall properties.

In this chapter, the phenol-conjugated gelatin (Gtn-HPA) hydrogel system will be fabricated and thoroughly studied to investigate its potential in fulfilling the aims of this chapter. The material properties and release profile of the system will be investigated using different HRP and H_2O_2 to show its tunable properties that potential to be tailored for different requirements, especially for bone applications. In addition, the potential of the phenol-conjugated gelatin hydrogel to be used as an injectable bone tissue-engineered scaffold in term of biocompatible, degradability and vessel forming ability were also studied.

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

1. To synthesis and fabricate the Gtn-HPA hydrogels.
2. To perform material characterization of the Gtn-HPA hydrogels.
3. To identify Gtn-HPA hydrogel's potential as a delivery vehicle through FITC-dextran release.
4. *In vivo* evaluation of Gtn-HPA hydrogels in term of injectability, biocompatibility, degradability, and vessel forming ability.

4.2 Materials and Methods

Small phenol HPA molecules were conjugated onto gelatin as described in detail in **Chapter 3, Section 3.3.1**. The conjugation of HPA molecules onto gelatin was confirmed using 1H NMR according to **Chapter 3, Section 3.3.3** and the HPA content was quantify using UV-visible spectrophotometer according to **Chapter 3, Section 3.3.4**. Gtn-HPA hydrogel was then fabricated using different HRP and H_2O_2 concentrations according to **Chapter 3, Section 3.5.1**. The gelation rate of Gtn-HPA hydrogel related to different amounts of HRP was investigated according to **Chapter 3, Section 3.6.1**. The mechanical properties and percentage of swelling of Gtn-HPA hydrogel related to different crosslinking concentrations were characterized according to **Chapter 3, Sections 3.6.3 and 3.6.4**. *In vitro* degradation studies and release studies using the type-I collagenase and model drug, FITC-dextran respectively were then performed to understand the degradation and release profile of Gtn-HPA hydrogel with

different fabrication parameters (**Chapter 3, Sections 3.6.5 and 3.6.6**). Finally, *in vivo* studies using C57BL/6 mice were performed to test the suitability of Gtn-HPA hydrogel as an injectable bone scaffold in term of injectability, biocompatible, degradability and vessel forming ability were studied according to **Chapter 3, Sections 3.9.1, 3.9.2, 3.9.3 and 3.9.4**).

4.3 Results and Discussion

4.3.1 Synthesis of Gtn-HPA conjugates

HPA molecules were conjugated onto gelatin structure using a general carbodiimide-mediated coupling reaction using EDC according to the previously established procedures [7]. The reaction was performed in DI water. NHS was added to increase the efficiency of reaction by forming more stable amine-reactive intermediates. As shown schematically in **Scheme 4.1**, EDC will first form an unstable o-Acylisourea intermediate with HPA (**Scheme 4.1a**) before being coupled to NHS to form an amine reactive NHS-HPA ester (**Schematic 4.1b**). This NHS-HPA ester then reacts with primary amines found on the gelatin structure to form the Gtn-HPA conjugates (**Scheme 4.1c**) [10, 11].

Scheme 4.1 Carbodiimide-mediated coupling reaction scheme showing (a) the formation of unstable o-Acylisourea intermediate between HPA and EDC, followed by (b) the coupling reaction with NHS to form the amine-reactive NHS-HPA ester and finally (c) the reaction of the amine reactive NHS-HPA ester to the primary amines found on the Gtn to form the Gtn-HPA conjugates.

The successful of HPA conjugation onto the gelatin was confirmed using ^1H NMR. At the chemical shift (δ) region between 6.5 and 7.5, two extra peaks were observed at the ^1H NMR spectrum of Gtn-HPA as compared to Gtn (**Figure 4.1**). These two extra peaks correspond to the two aromatic protons found on HPA molecules [7, 8, 12]. Also, the peak at $\delta = 7.2$, which is found on both Gtn and Gtn-HPA, corresponds to the aromatic protons that are found on the phenylalanine and tyrosine residues [7]. In addition, the

HPA molecules conjugated onto the Gtn was analyzed using the UV-visible measurement at an absorbance wavelength of 276 nm. The amount of HPA molecules conjugated onto the Gtn was determined to be 4.11×10^{-7} mol/mg, which was similar with the results from the literature [13]. Overall, the results showed that the HPA molecules were successfully conjugated on to the Gtn using carbodiimide-mediated coupling reaction.

Figure 4.1 ¹H NMR spectrum at chemical shift region between 6.5 and 7.5 showing two extra peaks for the Gtn-HPA conjugates as compared to the Gtn, indicates the success HPA conjugation onto the Gtn.

4.3.2 Fabrication of Gtn-HPA hydrogel

The successful conjugation of HPA molecules to the gelatin structure allows for crosslinking to take place through peroxidase-mediated coupling reaction [7]. For Gtn-HPA hydrogel fabrication, HRP, a single-chain β -type hemoprotein, was used as catalyst and H_2O_2 as the substrate to conjugate HPA groups found on the Gtn-HPA structure. HRP will readily form complex with H_2O_2 to oxidize HPA group and caused crosslinking between *ortho*-carbon – *ortho*-carbon (C-C) or *ortho*-carbon – phenolic oxygen (C-O) of the aromatic rings. [14, 15]. The linkage between C-C and C-O bonds on the HPA group results in the formation of the Gtn-HPA hydrogel. The reaction mechanism of the peroxidase-mediated coupling reaction, as well as the macroscopic view and SEM images of the porous Gtn-HPA hydrogel were shown in **Figure 4.2**.

Figure 4.2 Fabrication of Gtn-HPA showing (a) the reaction scheme of peroxidase-mediated coupling reaction, (b) macroscopic view of Gtn-HPA hydrogel and (c) SEM image of lyophilized Gtn-HPA hydrogel [Scale bars represent (b) 1 cm and (c) 500 μ m].

In general, the amount of HRP will affect the gelation rate of Gtn-HPA hydrogel formation [16]. For this study, the gelation rate related to different HRP concentrations are shown in **Table 4.1**. For 5 w/v % Gtn-HPA hydrogel, an increase in HRP concentration from 0.10 units/ml to 0.15 units/ml results in a decrease in gelation rate from 3.1 ± 0.8 min to 0.6 ± 0.1 min. Meanwhile, the increase in polymer concentration from 5 w/v % to 10 w/v % results in slightly decreases in gelation rate when same HRP concentration was used. The increase in polymer concentration will increase the amount of phenol group. The higher amount of crosslinking site decreases the gelation rate [12]. For 10 w/v % Gtn-HPA hydrogel, the gelation was 2.6 ± 0.1 min when 0.10 units/ml HRP was used. However, the gelation rate was decreased to 0.5 ± 0.0 min when 0.15 units/ml HRP was used. Taken together, the gelation rate can be tuned to tailor for different tissue engineering applications using different HRP concentrations. In addition, the result also showed the suitability of this Gtn-HPA hydrogel to be used as injectable scaffold due to its tunable gelation rate. In general, rapid gelation rate can prevent hydrogel precursor diffuses uncontrollably to the surrounding tissue after injection to defect. Meanwhile, slower gelation rate will ensure the hydrogel precursor has enough time to completely fill the defect site [17]. Thus, a reasonable gelation rate is desirable to be used for the injectable scaffold. Overall, the HRP between 0.05 – 0.15

units/ml showed to have reasonable gelation rate (i.e. 0.5 – 3 min) to be used for injectable scaffold applications.

Table 4.1 Gelation rate of Gtn-HPA hydrogel with different HRP and polymer concentrations.

HRP Concentration (units/ml)	Gelation Rate (min)	
	5 w/v% Gtn-HPA	10 w/v% Gtn-HPA
0.05	3.1 ± 0.8	2.6 ± 0.1
0.10	1.1 ± 0.1	1.0 ± 0.0
0.12	0.9 ± 0.1	0.7 ± 0.0
0.15	0.6 ± 0.1	0.5 ± 0.0

4.3.3 Mechanical properties

The HRP concentration determined the gelation rate of the crosslinking process, but do not significantly affect the mechanical properties of the Gtn-HPA hydrogel. As showed in **Figure 4.3**, the Gtn-HPA hydrogels fabricated with different HRP concentrations showed to have similar compressive modulus.

Figure 4.3 Compression modulus of the 5 w/v % Gtn-HPA hydrogel with different HRP concentrations.

Between HRP and H_2O_2 , the mechanical properties of Gtn-HPA hydrogel are significantly affected by the H_2O_2 concentration. **Figure 4.4** shown the compressive modulus of the Gtn-HPA hydrogel fabricated with different H_2O_2 concentrations. In general, different H_2O_2 concentrations used create Gtn-HPA hydrogel with different mechanical properties. For 5 w/v% Gtn-HPA hydrogel (**Figure 4.4 a**), the compressive modulus was significantly increased ($p < 0.05$) from 34.9 ± 0.8 kPa to 74.8 ± 3.4 kPa when the H_2O_2 concentration was increased from 3 mM to 6 mM. The increase in H_2O_2 concentration from 3 mM to 6 mM resulted in increasing crosslinking density that led to the improvement in the mechanical properties of the Gtn-HPA hydrogel. However, a further increased in the H_2O_2 concentration beyond 6 mM showed a significant decreased ($p < 0.05$) in the compressive modulus. When 9 and 12 mM H_2O_2 were used, the compressive moduli of the Gtn-HPA hydrogel were 57.8 ± 2.0 kPa and 39.1 ± 0.8 kPa respectively. Recent studies have suggested that the significant decreases in modulus when the higher H_2O_2 concentration was used might be due to the excessive H_2O_2 that caused the deactivation of the HRP [7, 8, 18]. In addition, excessive H_2O_2 could potentially oxidize and partially deteriorated the gelatin matrix [19].

The compressive modulus of Gtn-HPA hydrogel with a higher polymer concentration (i.e. 10 w/v %) was further investigated. The compressive modulus of 10 w/v % Gtn-HPA hydrogel fabricated with different H_2O_2 concentrations was shown in **Figure 4.4b**. The compressive modulus of 10 w/v % Gtn-HPA hydrogel had a significant increased trend ($p < 0.05$) from 3 to 12 mM H_2O_2 . The compressive modulus was significantly increased ($p < 0.05$) from 15.7 ± 0.3 kPa to 63.9 ± 0.1 kPa when H_2O_2 concentration used was increased from 3 mM to 6 mM. The compressive modulus was further significantly increased ($p < 0.05$) to 89.5 ± 0.1 kPa and 154.1 ± 0.4 kPa when 9 and 12 mM H_2O_2 were used respectively. For 10 w/v% Gtn-HPA hydrogel, higher compressive was achieved when the higher H_2O_2 concentration was used. In general, 10 w/v % Gtn-HPA precursors contain more phenol groups as compared to 5 w/v % Gtn-HPA precursors. Hence, there is a higher number of crosslinking sites for 10 w/v % Gtn-HPA precursors as compared to 5 w/v % Gtn-HPA precursors that might outweigh the effect of excessive H_2O_2 deteriorating the gelatin matrix. As a result, an increasing trend in compressive modulus was observed for the 10 w/v % Gtn-HPA hydrogel fabricated with increasing H_2O_2 concentration.

Figure 4.4 Compression modulus of the (a) 5 w/v % and (b) 10 w/v % Gtn-HPA hydrogel with different H_2O_2 concentration (* $p < 0.05$).

The mechanical properties of scaffold play an important role in supporting the defect site and providing the right environment for cells to proliferate, differentiate and function [20]. Ideally, the mechanical properties of the scaffold should match the local defect site [21]. The mechanical properties of Gtn-HPA hydrogel showed that it was able to be tuned by different H_2O_2 and polymer concentrations. Thus, in terms of mechanical properties, the Gtn-HPA hydrogel shows its potential to be used in different tissue engineering applications. Studies show that scaffold with stiffness > 25 kPa is able to mimic bone matrix and provides a mechanical environment that encourages osteogenic differentiation of stem cells that results in promoting bone regeneration [22-24]. The Gtn-HPA hydrogel showed to have the compressive modulus of > 25 kPa, showing a great potential in providing right matrix stiffness to encourage osteogenic differentiation of stem cells. Thus, Gtn-HPA hydrogel showed to be suitable to be used as an injectable scaffold for bone regeneration.

4.3.4 Percentage of swelling

The effect of H_2O_2 concentration on the percentage of swelling of the Gtn-HPA hydrogel was studied using $1 \times$ PBS solution. The percentage of swelling of the Gtn-HPA hydrogel fabricated with different H_2O_2 concentrations were compared at day 7. For 5 w/v % Gtn-HPA (**Figure 4.5**), the percentage of swelling was significantly

decreased ($p < 0.05$) from 1517.9 ± 49.9 % to 1088.4 ± 39.1 % and 982.8 ± 110.2 % when H_2O_2 concentration was increased from 3 mM to 6 and 9 mM H_2O_2 respectively. There was no significant difference between the 5 w/v % Gtn-HPA hydrogel fabricated with 6 and 9 mM H_2O_2 . When 12 mM H_2O_2 was used, the 5 w/v % Gtn-HPA hydrogel had a percentage of swelling of 1523.3 ± 17.6 %, which was significantly higher ($p < 0.05$) as compared to the 5 w/v % Gtn-HPA hydrogel fabricated with 6 and 9 mM H_2O_2 . In general, the percentage of swelling is highly related to the stiffness of the hydrogel structure, where smaller percentage of swelling proportionate to a higher stiffer structure [1]. For the 5 w/v % Gtn-HPA hydrogel fabricated with different H_2O_2 concentration, the percentage of swelling was reflecting the compression modulus or stiffness of the 5 w/v % Gtn-HPA hydrogel. The increase in H_2O_2 concentration to 6 and 9 mM results in a higher compressive modulus and stiffer structure of the 5 w/v % Gtn-HPA hydrogel (**Chapter 4, Section 4.3.3**), thus showing a smaller percentage of swelling as compared to the 5 w/v % Gtn-HPA hydrogel fabricated with 3 mM H_2O_2 . Further increased the H_2O_2 concentration to 12 mM causes the deactivation of HRP, resulting in lower compressive modulus and a higher percentage of swelling as compared to the 5 w/v % Gtn-HPA hydrogel fabricated with 6 and 9 mM H_2O_2 .

Figure 4.5 Percentage of swelling of the 5 w/v % Gtn-HPA hydrogel with different H₂O₂ concentrations (* $p < 0.05$ as compared to the Gtn-HPA hydrogel fabricated with 3 mM H₂O₂).

For 10 w/v% Gtn-HPA hydrogel (**Figure 4.6**), the percentage of swelling had a decreasing trend with increasing H₂O₂ concentration. When 3mM H₂O₂ was used, the 10 w/v% Gtn-HPA hydrogel had a percentage of swelling of 1377.6 ± 114.3 %. The percentage of swelling was significant decreased ($p < 0.05$) to 970.2 ± 72.3 %, 788.1 ± 40.0 % and 666.5 ± 48.0 % when 6, 9 and 12 mM H₂O₂ were used respectively. Again, the percentage of swelling showed a relationship with the compressive modulus or stiffness of the Gtn-HPA hydrogel. For 10 w/v% Gtn-HPA hydrogel, increasing the H₂O₂ concentration will result in increasing compressive modulus or stiffness of hydrogel's structure, leading to decreasing percentage of swelling. Percentage of swelling measures the solute diffusion coefficient and thus effectiveness in oxygen, nutrients, and water-soluble metabolites exchange [25]. In generally, the Gtn-HPA hydrogel has a high percentage of swelling and the value of the percentage of swelling is affected by the H₂O₂ and polymer concentration. The Gtn-HPA hydrogel showed to have high permeability and potentially to support mass transport for 3D cell proliferation and function.

Figure 4.6 Percentage of swelling of the 10 w/v % Gtn-HPA hydrogel with different H₂O₂ concentrations (* $p < 0.05$ as compared to the Gtn-HPA hydrogel fabricated with 3 mM H₂O₂).

4.3.5 Degradation profile

The degradation behavior of the Gtn-HPA hydrogel fabricated with different H₂O₂ concentrations was studied using 0.5 units/ml type-I collagenase solution. Type-I collagenase belongs to the matrix metalloproteinases (MMPs) group that degrade collagen and gelatin [26]. **Figure 4.7** showed the degradation profiles of the 5 w/v % Gtn-HPA hydrogel fabricated with different H₂O₂ concentrations. For 5 w/v% Gtn-HPA hydrogel, the degradation rate was closely related to the crosslinking density of the Gtn-HPA hydrogel. When 3 mM H₂O₂ was used, the percentage weight loss of the 5 w/v % Gtn-HPA hydrogel reached ~ 90 % at day 9. The degradation rate was slower when higher H₂O₂ concentration was used. The 5 w/v % Gtn-HPA hydrogel fabricated with 6 and 9 mM reached ~ 90 % of percentage weight loss at day 21 and 19 respectively. The degradation rate was then increased again when 12 mM H₂O₂ was used, with the percentage weight loss the 5 w/v % Gtn-HPA hydrogel reached ~ 90 % at day 13. The deactivation of HRP by the excessive H₂O₂ formed the Gtn-HPA

hydrogel with lower crosslinking density, thus resulting in more susceptible to attack by type-I collagenase.

Figure 4.7 Degradation profiles for the 5 w/v % Gtn-HPA hydrogel with different H₂O₂ concentrations.

For 10 w/v% Gtn-HPA hydrogel (**Figure 4.8**), there was a decreasing trend for degradation rate. When 3 mM H₂O₂ was used, the percentages of weight loss of the 10 w/v% Gtn-HPA hydrogel reached ~ 90 % at day 9. The degradation rate of the 10 w/v% Gtn-HPA hydrogel was decreased when 6 mM H₂O₂ was used, where ~ 90 % percentage weight loss was only reached at day 15. The degradation rate was further decreased where the percentage weight loss for the 10 w/v % Gtn-HPA hydrogel fabricated with 9, 10 and 12 mM H₂O₂ reached 66.9 ± 7.1 %, 74.7 ± 1.6 % and 71.3 ± 0.2 % respectively at day 28.

Figure 4.8 Degradation profiles for the 10 w/v % Gtn-HPA hydrogel with different H_2O_2 concentrations.

The degradation rate of scaffold ideally should match the healing rate of the host bone tissue and still provide the necessary support until newly formed bone tissue have sufficient strength and function [27]. The degradation rate of the Gtn-HPA hydrogel has shown to be closely related to its crosslinking density and swelling ratio, where higher crosslinking density showing higher compressive modulus and smaller swelling ratio will have slower degradation rate. The smaller swelling ratio also means stiffer matrix and thus has lower penetration of collagenase solution, resulting in slower degradation rate. Generally, the degradation rate of the Gtn-HPA hydrogel showed to be able to alter by changing H_2O_2 and polymer concentration. Thus, the Gtn-HPA hydrogel showed to be potentially used in different applications that required different degradation rate. The Gtn-HPA hydrogel is suitable for both short term and long term applications. During the autograft healing process, the periosteal stem cells showed to prolong for ~ 2 weeks until chondrocyte calcification, matrix degradation and vascular invasion occurs [28, 29]. Thus, a scaffold with a degradation rate of 2 weeks is desired as periosteum-inspired scaffold to facilitate the proliferation and differentiation of stem cells for 2 weeks. The degradation rate of the Gtn-HPA hydrogel showed to be able to

tune to more than 2 weeks and potentially to be used as periosteum-inspired scaffold to deliver stem cells for bone regeneration.

4.3.6 Drug release profile

The effect of H_2O_2 and polymer concentration on the release rate of the Gtn-HPA hydrogel was thoroughly studied in using a model drug, FITC-dextran [30, 31]. **Figure 4.9** showed the release profiles for the 5 w/v % Gtn-HPA hydrogel fabricated with different H_2O_2 concentrations. The 5 w/v % Gtn-HPA hydrogel fabricated with 3 mM H_2O_2 had a burst release of 31.9 ± 1.6 % at day 1. When the higher H_2O_2 concentration was used, the burst release was reduced. The 5 w/v % Gtn-HPA hydrogel fabricated with 6, 9 and 12 mM H_2O_2 had a similar burst release of 25.8 ± 1.1 %, 23.4 ± 0.9 % and 23.7 ± 1.1 % respectively. In addition, the 5 w/v % Gtn-HPA hydrogel fabricated with 3 mM H_2O_2 also showed to have higher release rate of the FITC-dextran as compared to the 5 w/v % Gtn-HPA hydrogel fabricated with 6, 9 and 12 mM H_2O_2 . The total release of the FITC-dextran reached 68.4 ± 0.1 % at day 21 when 3 mM H_2O_2 was used. Among the 5 w/v % Gtn-HPA hydrogel fabricated with 6, 9 and 12 mM H_2O_2 , they had similar release trend. The total release of the FITC-dextran reached 52.1 ± 0.0 % and 50.4 ± 0.0 % respectively at day 21 when 6 and 9 mM H_2O_2 were used respectively. When 12 mM H_2O_2 were used, the 5 w/v % Gtn-HPA hydrogel has slightly higher release rate as compared to 6 and 9 mM H_2O_2 . The total release of the FITC-dextran reached 55.7 ± 0.1 % at day 21 for the Gtn-HPA hydrogel fabricated with 12 mM H_2O_2 .

Figure 4.9 FITC-dextran release profiles for the 5 w/v % Gtn-HPA hydrogel with different H₂O₂ concentration.

When comparing the FITC-dextran release profile for the Gtn-HPA hydrogel with different polymer concentration (**Figure 4.10**), 10 w/v % Gtn-HPA hydrogel that had higher crosslinking density exhibited slower release rate as compared to the 5 w/v % Gtn-HPA. The total release of FITC-dextran only reached 39.2 ± 0.1 % at day 21 for 10 w/v % Gtn-HPA. The release profiles of the Gtn-HPA hydrogel showed to behave differently with different H₂O₂ and polymer concentration. The FITC-dextran release rate of Gtn-HPA hydrogel also showed to closely relate to its material properties. The Gtn-HPA hydrogel with higher crosslinking density, thus higher stiffness and smaller swelling ratio, exhibits slower release rate and vice versa. The drug release from the Gtn-HPA hydrogel was proposed via diffusion and swelling [32]. The FITC-dextran release studies showed that the release rate of the Gtn-HPA hydrogel can be tuned by varying the H₂O₂ and polymer concentration. Thus, the Gtn-HPA hydrogel demonstrates its potential to be a drug delivery vehicle for short term or long term release.

Figure 4.10 FITC-dextran release profiles for the Gtn-HPA hydrogel with different polymer concentrations.

4.3.7 *In vivo* biocompatibility and degradability

The *in vivo* biocompatibility and degradability studies were performed by injecting small sample volume of 10 μ l of Gtn-HPA hydrogel onto the hind limb of C57BL/6 mice. A small sample volume of 10 μ l was used in this study to investigate the ability of the Gtn-HPA hydrogel in filling small defect sites, which possessed problem for performed scaffold especially in term of porosity [33]. At 1 and 3 weeks post-implantation, the implanted 5 w/v % Gtn-HPA hydrogel together with the surrounding tissue was harvested from the C57BL/6 mice followed by the fixation and histological analysis to evaluate its *in vivo* biocompatibility and degradability. As seen in **Figure 4.11**, the sections stained with H & E showed that the implanted 5 w/v % Gtn-HPA hydrogel was still present at 1 week post-implantation. Cellular infiltration was observed for the implanted 5 w/v % Gtn-HPA hydrogel. In addition, blood vessels can be also observed forming around the implanted 5 w/v % Gtn-HPA hydrogel. At 3 weeks post-implantation, the implanted 5 w/v % Gtn-HPA hydrogel was completely degraded

and the skin retained its normal histological structure similar to the sham control. Thus, the results indicated that the Gtn-HPA hydrogel was biocompatible and biodegradable.

Figure 4.11 H & E staining of the histological sections of the skin at a magnification of (a) $10\times$ and (b) $20\times$ for the implanted 5 w/v % Gtn-HPA hydrogel with the surrounding tissue at 1 and 3 weeks post-implantation as compared to the sham control [Scale bars represent (a) $200\ \mu\text{m}$ and (b) $100\ \mu\text{m}$. The red dotted line represents 5 w/v % Gtn-HPA hydrogel's outline. The red arrow represents blood vessel].

The sections were further stained with Masson's Trichrome to further confirm the cellular infiltration and formation of blood vessels around the 5 w/v % Gtn-HPA hydrogel. As seen in **Figure 4.12**, the Masson's Trichrome staining images further confirmed the presence of the implanted 5 w/v % Gtn-HPA hydrogel and cellular infiltration at 1 week post-implantation. In addition, blood vessels were also confirmed to form around the implanted 5 w/v % Gtn-HPA hydrogel. The implanted 5 w/v % Gtn-HPA hydrogel was also confirmed to completely degrade and the skin retained its normal histological structure similar to the sham control at 3 weeks post-implantation. Thus, the results from Masson's trichrome staining agreed with the H & E staining,

which demonstrate the biocompatibility and biodegradability of the 5 w/v % Gtn-HPA hydrogel.

Figure 4.12 Masson's trichrome staining of the cross section of the skin at the magnification of (a) $10\times$ and (b) $20\times$ for the implanted 5 w/v % Gtn-HPA hydrogel with the surrounding tissue at 1 and 3 weeks post-implantation as compared to the sham control [Scale bars represent (a) $200\ \mu\text{m}$ and (b) $100\ \mu\text{m}$. The green dotted line represents 5 w/v % Gtn-HPA hydrogel's outline. The green arrow represents blood vessel].

Overall, the *in vivo* studies showed the injectability, biocompatibility and biodegradability of the Gtn-HPA hydrogel, thus potentially to be used as injectable scaffold for tissue engineering applications. In addition, cells were able to infiltrate into the implanted Gtn-HPA hydrogel (**Figures 4.11 and 4.12**). The implanted 5 w/v % Gtn-HPA hydrogel were proposed mostly infiltrated by the macrophages which can secrete MMPs and caused the degradation of the implanted 5 w/v % Gtn-HPA hydrogel. This assumption will be further investigated through immunofluorescent staining using CD68 to show the presence of macrophages surrounded and into the implanted 5 w/v % Gtn-HPA hydrogel in the next section (**Section 4.3.8**). Cellular filtration plays an important role in wound healing. The breaking down of the Gtn-HPA hydrogel is

important to allow more space for cellular migration, growth, proliferation and matrix remodeling that ultimately leads to effective tissue regeneration [34-36].

4.3.8 Cellular infiltration and vessel forming ability

Immunofluorescent staining was carried out on the implanted 5 w/v % Gtn-HPA hydrogel with surrounding skin at 1 and 3 weeks post-implantation as compared to the sham control to investigate the cellular infiltration and vessel forming ability. As seen in **Figure 4.13a**, immunofluorescent staining using CD68 was carried out on the implanted 5 w/v % Gtn-HPA hydrogel with surrounding tissue to investigate the presence of the macrophages and the cellular infiltration. Immunofluorescent staining using CD68 showed that the macrophages were surrounded and encapsulated inside the implanted 5 w/v % Gtn-HPA hydrogel at 1 week post-implantation. The immunofluorescent staining using CD68 confirmed the cellular infiltration of the macrophages into the implanted 5 w/v % Gtn-HPA hydrogel. As discussed in **Chapter 4, Section 4.3.7**, the presence of macrophages can secrete MMPs that can cause the degradation and matrix remodeling of the implanted Gtn-HPA hydrogel that is essential for tissue regeneration. In addition, macrophages might also involve in the production of VEGF that promote angiogenesis [37, 38]. At 1 week post-implantation, immunofluorescent staining using CD31 (**Figure 4.13b**) showed that the presence of the blood vessels in proximity to the saphenous artery was growth around the implanted 5 w/v % Gtn-HPA hydrogel. However, there was no lymphatic vessel observed growing around the implanted 5 w/v % Gtn-HPA when stained with LYVE-1 at 1 week post-implantation (**Figure 4.13c**). The implanted 5 w/v % Gtn-HPA hydrogel showed to completely degrade at 3 weeks post-implantation and the implanted site was completely healed with blood and lymphatic vessels. Vascularization plays an important role for tissue engineered scaffold and particularly for bone tissue engineering, where sufficient blood vessels formed in scaffold will allow effective bone regeneration. Besides, adequate vascularization will provide efficient transportation of nutrients into tissue engineered scaffold to ensure healthy cell growth and tissue regeneration [39, 40]. Overall, 5 w/v % Gtn-HPA hydrogel demonstrated to support angiogenesis, which is the formation of blood vessels and potentially to support bone regeneration.

Recent studies have shown that the substrate's stiffness will greatly affect the proliferation, function and network formation of endothelial cells [41-44]. Thus, this

thesis will further propose that a stiffer Gtn-HPA hydrogel will be able to support not only for angiogenesis but also for lymphangiogenesis. The success of the stiffer Gtn-HPA hydrogel to be a compliance matrix in supporting lymphatic cells migration, proliferation and formation network will open up a new novel application for Gtn-HPA hydrogel in lymphatic regeneration. 10 w/v % Gtn-HPA hydrogel, which can achieve higher compressive modulus than 5 w/v % Gtn-HPA hydrogel (**Chapter 4, Section 4.3.3**), was used for further preliminary *in vivo* studies to investigate the ability of stiffer Gtn-HPA hydrogel in supporting lymphangiogenesis (**Appendix 1**). In this study, lymphatic vessels in the hind limb of C57BL/6 mice were dissected to investigate the ability of Gtn-HPA hydrogel in lymphatic regeneration. Immunofluorescent analysis on the sectional area of the implanted 10 w/v % Gtn-HPA hydrogel with surrounding tissue further confirmed the growth of blood vessels (**Figure A1.4b**) and lymphatic vessels (**Figure A1.4b**) surrounding and into the implanted 10 w/v % Gtn-HPA hydrogel at 2 and 3 weeks post-implantation. As seen from **Figure A1.5**, the whole mount immunofluorescent staining using CD31 and LYVE-1 showed that the blood and lymphatic vessels were observed to grow around and within the implanted 10 w/v % Gtn-HPA hydrogel at 2 weeks post-implantation. Thus, the results showed that stiffer Gtn-HPA hydrogel was able to support both angiogenesis and lymphangiogenesis. The ability to support lymphangiogenesis showed the potential of Gtn-HPA hydrogel to be used in lymphatic tissue engineering applications. The novel application of Gtn-HPA hydrogel in lymphatic regeneration will be further discussed in detailed in **Chapter 7, Section 7.4.1**.

Figure 4.13 Immunofluorescent staining using (a) CD68 (red), (b) CD31 (red), (c) LYVE-1 (green) and DAPI (blue) showed the presence of macrophages, blood vessels, lymphatic vessels and cells respectively for the implanted 5 w/v % Gtn-HPA hydrogel with the surrounding tissue after 1 and 3 weeks of implantation as compared to the sham control (Scale bar represents 100 μm. The white dotted line represents 5 w/v % Gtn-HPA hydrogel's outline).

4.4 Conclusion

In this chapter, small phenol molecules, HPA, were successfully conjugated onto the gelatin structure and to form the Gtn-HPA conjugates. The Gtn-HPA conjugates were subsequently crosslinked using HRP and H_2O_2 to form the injectable phenol-conjugated gelatin (Gtn-HPA) hydrogel. The gelation rate of this hydrogel can be varied using different HRP concentrations to match different surgical needs and handleability. The material properties of Gtn-HPA hydrogel in terms of mechanical properties, swelling ratio, degradability can also be tuned using different H_2O_2 and polymer concentrations. The compressive modulus of the Gtn-HPA hydrogel was able to reach > 25 kPa, thus

the Gtn-HPA hydrogel able to achieve the mechanical environment that potentially induces osteogenic differentiation of stem cells. The Gtn-HPA hydrogel also showed to be potentially used as a delivery vehicle, and since the release rate is closely related its material properties, the release rate of the Gtn-HPA hydrogel can be tuned by tuning its material properties. Lastly, *in vivo* studies showed the injectability and biocompatibility of the Gtn-HPA hydrogel as well as its degradability. In particular, 5 w/v % Gtn-HPA hydrogel demonstrated to support angiogenesis. Overall, the Gtn-HPA hydrogel demonstrated its potential to be used as injectable scaffold for bone applications by providing the mechanical properties, drug delivery ability, injectability, biocompatibility, biodegradability and angiogenic properties for bone healing.

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Chapter 5

Study 2 – Development of Enzymatic Responsive Phenol-conjugated Gelatin and Carboxymethyl Cellulose Hybrid Hydrogel.

This chapter discusses in details on the development of an enzymatic responsive phenol conjugated gelatin and carboxymethyl cellulose hybrid hydrogel. This chapter will include the synthesis of phenol conjugated carboxymethyl cellulose and the fabrication of Gtn-HPA/CMC-Tyr hybrid hydrogel. In addition, the effect of H₂O₂ concentration on the material properties of Gtn-HPA/CMC-Tyr hybrid hydrogel in terms of compressive modulus, swelling ratio and degradation behavior will be further investigated. The effect of pore enlargement caused by cellulose digestion on induced drug release was also investigated. In addition, the cytocompatibility of the Gtn-HPA/CMC-Tyr hybrid hydrogel was studied through 2D cell culture of hFOB. Lastly, the potential of Gtn-HPA/CMC-Tyr hybrid hydrogel as a cell carrier for delivery of bone cells for bone regeneration and its ability to enhance proliferation through cellulose treatment was investigated.

5.1 Introduction

The microenvironments of the hydrogel such as the permeability and its network's size play important roles in determining the cellular behavior and subsequently the new tissue formation [1, 2]. On the other hand, the degradation rate of the hydrogel is equally important to provide space for effective tissue formation at the defect site [3-5]. However, most of the hydrogel are not tunable post-implantation especially in terms of the degradation rate that could result in ineffective the tissue healing. In addition, among the tunable hydrogel, the tunability of the hydrogel is highly dependent on the conventional mechanism by changing the pH and temperature of the environment, which could potentially be harmful as cell carrier applications [3]. Hence, this chapter aimed to (a) develop a smart enzymatic responsive hybrid hydrogel that has post-implantation tunability in terms of degradation rate to facilitate new bone tissue formation and (b) to investigate the potential of the smart enzymatic responsive hybrid hydrogel drug carrier that could “release on demand” upon external stimulation. The use of enzyme as external stimuli is also proposed to be more biocompatible and potentially enable the periosteum-inspired injectable system to be the co-smart release of both cells and drugs to enhance bone healing.

In this chapter, the based material for the periosteum-inspired injectable system, Gtn-HPA hydrogel, is proposed to be incorporated with carboxymethyl cellulose (CMC) to form the smart enzymatic responsive hybrid hydrogel. Carboxymethyl cellulose will act as the sacrificial component to be digested by the enzyme, cellulase [6]. The digestion of the CMC that found in the Gtn-HPA hydrogel is proposed to cause the alteration of the degradation rate post-implantation that will create more space for cell proliferation. The alteration of the degradation rate will also affect the release rate of the whole system. Cellulase from *Trichoderma longibrachiatum* is proposed to be used in this study due to it potentially works well in physiological conditions, thus show its potential to be used in bone tissue engineering [7]. To date, there is no report on using the CMC and cellulase treatment as the smart enzymatic system and mechanism respectively for tissue engineering.

Carboxymethyl cellulose (CMC) will be conjugated with the phenol molecules, Tyr, to enable it to crosslink together with Gtn-HPA conjugates using HRP and H₂O₂. The

enzymatic crosslinking between these the Gtn-HPA and CMC-Tyr polymers will form the Gtn-HPA/CMC Tyr hybrid hydrogel. The compressive modulus, swelling ratio and degradation behavior of Gtn-HPA/CMC-Tyr hybrid hydrogel were thoroughly studied to understand its material properties. The cytocompatibility of the Gtn-HPA/CMC-Tyr hybrid hydrogel was studied through 2D cell culture using hFOB3. Through this study, the suitability of the Gtn-HPA/CMC-Tyr hybrid hydrogel in supporting the proliferation of bone cells and ultimately assists in bone regeneration was investigated. In order to assess the Gtn-HPA/CMC-Tyr hybrid hydrogel as the smart enzymatic responsive hybrid hydrogel, the samples will subject to different cellulase concentrations to investigate its ability in altering degradation rate post-implantation, inducing drug release and enhance cell proliferation through pore enlargement. Ultimately, the potential of the Gtn-HPA/CMC-Tyr hybrid hydrogel as the smart periosteum-inspired injectable system that enables co-smart release of both cells and drugs and its potential in bone regeneration will be investigated in this chapter.

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

1. To synthesis of CMC-Tyr conjugates and fabrication of Gtn-HPA/CMC-Tyr hybrid hydrogels.
2. To perform material characterization of the Gtn-HPA/CMC-Tyr hybrid hydrogels.
3. To investigate the cytocompatibility of the Gtn-HPA/CMC-Tyr hybrid hydrogels and cellulase treatment.
4. To perform functional studies for the Gtn-HPA/CMC-Tyr hybrid hydrogels in terms of induced release of drugs and enhances bone cell proliferation.

5.2 Materials and Methods

Small phenol molecules of Tyr were conjugated onto CMC as described in **Chapter 3, Section 3.3.2**. The conjugation of Tyr molecules onto the CMC was confirmed using ^1H NMR (**Chapter 3, Section 3.3.3**) and the Tyr content was quantify using UV-visible spectrophotometer according to **Chapter 3, Section 3.3.4**. Gtn-HPA/CMC-Tyr hybrid hydrogel was then fabricated using different H_2O_2 concentrations according to **Chapter 3, Section 3.5.2**. The mechanical properties and swelling ratio of the Gtn-HPA/CMC-Tyr hybrid hydrogel fabricated with different H_2O_2 concentrations were characterized

according to **Chapter 3, Sections 3.6.3 and 3.6.4**. *In vitro* degradation behavior of the Gtn-HPA/CMC-Tyr hybrid hydrogel was studied using type-I collagenase solution according to **Chapter 3, Section 3.6.5**. Drug release profile of the Gtn-HPA/CMC-Tyr hybrid hydrogel was investigated according to **Chapter 3, Section 3.6.6**. The induced release of drugs through alteration of the Gtn-HPA/CMC-Tyr hybrid hydrogel's degradation rate using cellulase treatment was studied as described in details in **Chapter 3, Section 3.7.1**. The degradation behavior of the Gtn-HPA/CMC-Tyr hybrid hydrogel treated with different cellulase concentrations was also investigated according to **Chapter 3, Sections 3.7.2 and 3.7.3**. The cytocompatibility of the Gtn-HPA/CMC-Tyr hybrid hydrogel was studied through 2D cell culture of hFOB's (**Chapter 3, Sections 3.8.3, 3.8.5, 3.8.6, and 3.8.7**). Cytotoxicity of cellulase was also investigated using hFOB's to test its suitability in bone applications (**Chapter 3, Sections 3.8.8**). Finally, a study on the cellulase treatment in enhancing bone cells proliferation was investigated according to **Chapter 3, Section 3.8.10**.

5.3 Results and Discussion

5.3.1 Synthesis of CMC-Tyr conjugates

General carbodiimide-mediated coupling reaction was used to conjugate Tyr molecules onto the CMC structure according to previously established procedures [8]. Similar to the synthesis of Gtn-HPA conjugates, NHS and EDC were used and the reaction was performed in DI water. As shown in **Scheme 5.1**, EDC will couple NHS to CMC to form the NHS-CMC ester. The NHS-CMC ester reacted with the primary amines found on the Tyr molecules to form the CMC-Tyr conjugates. ^1H NMR was used to confirm the success of the Tyr molecules conjugated onto the CMC structure.

Scheme 5.1 Carbodiimide-mediated coupling reaction scheme showing (a) the formation of unstable o-Acylisourea intermediate between CMC and EDC, followed by (b) the coupling reaction with NHS to form the amine-reactive NHS-CMC ester and finally (c) the reaction of amine reactive NHS-CMC ester to the primary amines found on the Tyr to form the CMC-Tyr conjugates.

The ^1H NMR spectrum of the CMC-Tyr conjugates (**Figure 5.1**) showed to have two extra peaks at the chemical shift (δ) region between 6.5 and 7.5 as compared to the CMC. There is no peak found on the chemical shift (δ) region between 6.5 and 7.5 for the CMC. The two extra peaks for CMC-Tyr conjugates corresponded to the two aromatic protons found on Tyr molecules, thus confirmed the success of Tyr conjugation [6, 9]. In addition, UV-vis measurement was used to quantify the amount of Tyr conjugated onto the CMC. The total Tyr content conjugated onto the CMC was determined to be 1.43×10^{-7} mol/mg. Overall, the results showed that the Tyr molecules

were successfully conjugated onto the CMC using the carbodiimide-mediated coupling reaction.

Figure 5.1 ¹H NMR spectrum of the CMC and the CMC-Tyr conjugates at chemical shift region between 6.5 and 7.5.

5.3.2 Fabrication of Gtn-HPA/CMC-Tyr hybrid hydrogel

The successful conjugation of the Tyr molecules to the CMC structure allowed the crosslinking of the CMC using peroxidase-mediated coupling reaction. Similar with the crosslinking of the Gtn-HPA conjugates, HRP and H₂O₂ will oxidize the Tyr group and cause crosslinking between *ortho*-carbon – *ortho*-carbon (C-C) or *ortho*-carbon – phenolic oxygen (C-O) of the aromatic rings [6, 10]. Both Gtn-HPA conjugates and CMC-Tyr conjugates can mix well with each other and formed a solution-like precursor that enable to be injected into the bone defects with different size and shape before solidification. Together with Gtn-HPA conjugates, both phenol-conjugated polymers can polymerize using HRP and H₂O₂ and formed the Gtn-HPA/CMC-Tyr hybrid hydrogel as seen in **Scheme 5.2**.

Scheme 5.2 Reaction scheme of the formation of the Gtn-HPA/CMC-Tyr hybrid hydrogel.

In general, CMC is more hydrophilic as compared to the Gtn, which consists of both hydrophilic and hydrophobic peptides [11]. Due to the differences in the hydrophilicity between Gtn and CMC, phase separation occurs during the polymerization process [12, 13]. As seen in **Figure 5.2a**, phase separation occurred during the crosslinking process caused the Gtn-HPA/CMC-Tyr hybrid hydrogel to be optically opaque as compared to Gtn-HPA hydrogel with clear and transparent appearance (refer to **Figure 4.2**) [14, 15]. On the other hand, the microstructure of the Gtn-HPA/CMC-Tyr hybrid hydrogel as seen in **Figure 5.2b** is similar to that of the Gtn-HPA hydrogel with of large pores and highly porous. The incorporation of CMC-Tyr polymers into the Gtn-HPA hydrogel did not alter the microstructure of the whole system. Nonetheless, the highly porous Gtn-HPA/CMC-Tyr hybrid hydrogel could potentially be a carrier for bone tissue engineering as providing sufficient space for cell proliferation and high permeability for effective nutrients exchanges.

Figure 5.2 Fabrication of the Gtn-HPA/CMC-Tyr hybrid hydrogel showing (a) the macroscopic image and (b) SEM image of the Gtn-HPA/CMC-Tyr hybrid hydrogel [Scale bars represent (a) 1 cm and (b) 200 μm].

5.3.3 Mechanical properties

The effect of H_2O_2 concentration on the compressive modulus of the Gtn-HPA/CMC-Tyr hybrid hydrogel was studied and the results are showed in **Figure 5.3**. Gtn-HPA/CMC-Tyr hybrid hydrogel fabricated with 3 mM H_2O_2 had the compressive modulus of 22.7 ± 3.0 kPa. The compressive modulus was significantly increased ($p < 0.05$) to 43.2 ± 4.2 kPa when 6 mM H_2O_2 was used. Increased the H_2O_2 concentration will increase the crosslinking density of the Gtn-HPA/CMC-Tyr hybrid hydrogel, thus improving its mechanical properties. However, further increased the H_2O_2 concentration to 9 and 12 mM significantly decreased ($p < 0.05$) the compressive modulus as compared to Gtn-HPA/CMC-Tyr hybrid hydrogel fabricated with 6 mM H_2O_2 . Both Gtn-HPA/CMC-Tyr hybrid hydrogels fabricated with 9 and 12 mM H_2O_2 had the compressive modulus of 19.3 ± 2.7 kPa and 13.9 ± 0.6 kPa respectively. Similar with the Gtn-HPA hydrogel, the significant decrease ($p < 0.05$) in the compressive modulus when higher H_2O_2 concentrations were used might be due to the deactivation of HRP [16] or deterioration of gelatin by excessive H_2O_2 [17].

Figure 5.3 Compressive modulus of the Gtn-HPA/CMC-Tyr with different H₂O₂ concentrations (* $p < 0.05$).

The compressive modulus of the Gtn-HPA/CMC-Tyr hybrid hydrogel with different H₂O₂ concentrations showed a similar trend with the Gtn-HPA hydrogel (**Chapter 4, Section 4.3.3**). The incorporation of the CMC-Tyr conjugates into the Gtn-HPA hydrogel's structure will not shift the effect of H₂O₂ concentration on the mechanical properties of the whole system. Thus, the mechanical properties of the Gtn-HPA/CMC-Tyr hybrid hydrogel potentially can be further improved and tuned by changing the polymer concentration to have more phenol groups and thus, higher crosslinking density can be achieved. Overall, the Gtn-HPA/CMC-Tyr hybrid hydrogel fabricated with 6 mM H₂O₂ had the modulus between > 25 kPa, which potentially able to provide a suitable mechanical environment to induced osteogenic differentiation of stem cells [18]. Thus, 5 w/v % Gtn-HPA/CMC-Tyr hybrid hydrogel with 6 mM H₂O₂ was the optimum parameter in terms of mechanical properties for bone tissue engineering applications.

5.3.4 Percentage of swelling

The percentage of swelling of the Gtn-HPA/CMC-Tyr hybrid hydrogel fabricated with different H_2O_2 concentrations was studied and the results are showed in **Figure 5.4**. The swelling study was done for 7 days to ensure the Gtn-HPA/CMC-Tyr hybrid hydrogel reached its equilibrium swelling. At day 7, the Gtn-HPA/CMC-Tyr hybrid hydrogel fabricated with 3 mM H_2O_2 had the percentage of swelling of $1729.5 \pm 40.9 \%$. The percentage of swelling was significantly decreased ($p < 0.05$) to $1317.1 \pm 8.7 \%$ when 6 mM H_2O_2 was used. The percentage of swelling reflected the stiffness of the Gtn-HPA/CMC-Tyr hybrid hydrogel, where higher crosslinking density is able to achieve when 6 mM H_2O_2 is used, showing a smaller swelling ratio as compared to 3 mM H_2O_2 . However, the swelling ratio was significantly increased ($p < 0.05$) to $1666.2 \pm 113.6 \%$ and $1596.6 \pm 65.1 \%$ when 9 and 12 mM H_2O_2 were used respectively as compared to the Gtn-HPA/CMC-Tyr hybrid hydrogel fabricated with 6 mM H_2O_2 . The deactivation of HRP or the deterioration of gelatin might occur when in the presence of excessive H_2O_2 , thus forming a Gtn-HPA/CMC-Tyr hybrid hydrogel with lower stiffness and larger swelling ratio.

Figure 5.4 Percentage of swelling of the Gtn-HPA/CMC-Tyr hybrid hydrogel with different H_2O_2 concentrations (* $p < 0.05$ as compared to the Gtn-HPA/CMC-Tyr hybrid hydrogel fabricated with 3 mM H_2O_2).

Generally, the Gtn-HPA/CMC-Tyr hybrid hydrogel had a larger percentage of swelling as compared to the Gtn-HPA hydrogel. Thus, the Gtn-HPA/CMC-Tyr hybrid hydrogel showed to have greater permeability and potentially greater effectiveness in mass and nutrients transportation. The larger percentage of swelling of the Gtn-HPA/CMC-Tyr hybrid hydrogel might be due to the pore formation through the phase separation during the fabrication process. The Gtn-HPA/CMC-Tyr hybrid hydrogel with a more porous structure will be able to uptake larger amount of water, thus showing a larger swelling ratio [19]. In addition, the presence of the CMC-Tyr conjugates, which is more hydrophilic, enable the whole structure to attract more water molecules. Incorporation of the CMC-Tyr conjugates into the Gtn-HPA hydrogel structure increased the percentage of swelling, thus improves the permeability of the whole system.

5.3.5 Degradation profile

The degradation behavior of Gtn-HPA/CMC-Tyr hybrid hydrogel with different H_2O_2 concentrations was studied using 0.5 units/ml type-I collagenase solution. The degradation profiles of the Gtn-HPA/CMC-Tyr hybrid hydrogel fabricated with fixed different H_2O_2 concentrations are showed in **Figure 5.5**. When 3 mM H_2O_2 was used, the percentage weight loss of the Gtn-HPA/CMC-Tyr hybrid hydrogel reached ~ 90 % at day 13. Slower degradation rate was able to achieve when 6 mM H_2O_2 was used. The Gtn-HPA/CMC-Tyr hybrid hydrogel fabricated with 6 mM H_2O_2 reached ~ 90 % percentage weight loss at day 16. Higher crosslinking was able to achieve when 6 mM H_2O_2 was used, thus showing slower degradation rate. When 9 and 12 mM H_2O_2 was used for the fabrication of Gtn-HPA/CMC-Tyr hybrid hydrogel, the percentage weight loss reached ~ 90 % at day 13 and day 12 respectively. Both Gtn-HPA/CMC-Tyr hybrid hydrogels fabricated with 9 and 12 mM H_2O_2 showed a similar degradation profile with 3 mM H_2O_2 .

Figure 5.5 Degradation profiles for the Gtn-HPA/CMC-Tyr hybrid hydrogel with different H_2O_2 concentrations.

Similar with the Gtn-HPA hydrogel, the degradation rate of the Gtn-HPA/CMC-Tyr hybrid hydrogel is also closely related to its structural properties. The Gtn-HPA/CMC-Tyr hybrid hydrogel with higher crosslinking density, thus higher stiffness and smaller swelling ratio, will have slower degradation rate. The Gtn-HPA/CMC-Tyr hybrid hydrogel with smaller swelling ratio will have smaller permeability and thus less susceptible to be degraded by type-I collagenase. In this study, the Gtn-HPA/CMC-Tyr hybrid hydrogel showed to be able to tune its degradation rate by altering its structural properties using different H_2O_2 concentrations. Overall, the Gtn-HPA/CMC-Tyr hybrid hydrogel fabricated with 6 mM H_2O_2 shows its potential to be used as the periosteum-inspired layer, where it showed to have a degradation period of ~ 2 weeks, which is desired for the bone healing process [20].

5.3.6 FITC-dextran release profile and enzymatic-induced drug release

FITC-dextran molecules, as a model drug [21], were encapsulated in the Gtn-HPA/CMC-Tyr hybrid hydrogel and the release profile was studied using $1\times$ PBS release buffer solution. **Figure 5.6** showed the FITC-dextran release profiles comparing the release rate of the Gtn-HPA/CMC-Tyr hybrid hydrogel fabricated with different H_2O_2 concentrations. Generally, the Gtn-HPA/CMC-Tyr hybrid hydrogel with higher stiffness (6 mM H_2O_2) had a slower release rate as compared to low stiffness (3 mM H_2O_2). The Gtn-HPA/CMC-Tyr hybrid hydrogel fabricated with 3 mM H_2O_2 had a burst release of $22.4 \pm 1.8\%$ at day 1 and the total release reached $59.3 \pm 0.3\%$ at day 7. When 6 mM H_2O_2 was used, the burst release of the Gtn-HPA/CMC-Tyr hybrid hydrogel reduced to $14.5 \pm 1.2\%$ and the total release reached $41.4 \pm 0.2\%$ at day 7. The Gtn-HPA/CMC-Tyr hybrid hydrogel with higher stiffer and thus lower permeability reduced the burst release and the release rate. The stiffness and permeability of the Gtn-HPA/CMC-Tyr hybrid hydrogel showed to be controlled by the H_2O_2 concentration used (**Chapter 5, Sections 5.3.5 and 5.3.4**), which will affect the release rate of the whole system. When the release profile of the Gtn-HPA hydrogel/CMC-Tyr hybrid hydrogel was compared to the Gtn-HPA hydrogel under the same H_2O_2 concentration (**Appendix 3**), Gtn-HPA/CMC-Tyr hybrid hydrogel exhibited a slower release rate as compared to the Gtn-HPA hydrogel. This might be due to the present of the hydrophilic CMC-Tyr conjugates resulted in better retention of water-soluble drugs.

Figure 5.6 FITC-dextran release profiles for the Gtn-HPA/CMC-Tyr hybrid hydrogel with different H₂O₂ concentrations.

Next, the Gtn-HPA/CMC-Tyr hybrid hydrogels were treated with 1× PBS containing different cellulase concentrations to investigate the enzymatic-induced drug release. As illustrated in **Figure 5.7**, cellulase treatment on the Gtn-HPA/CMC-Tyr hybrid hydrogel encapsulated with drugs or cells was suggested to be able to induce drugs or cells release through the digestion of the CMC by cellulase. In this study, Gtn-HPA/CMC-Tyr with high stiffness (e.g. 6 mM H₂O₂) was used as Gtn-HPA/CMC-Tyr with moderate stiffness (e.g. 3 mM H₂O₂) did not show a significant difference between the release profiles of samples with and without the cellulase treatment (**Appendix 4**). The small increase in the release rate was observed for the Gtn-HPA/CMC-Tyr hybrid hydrogel fabricated with 3 mM H₂O₂ is mainly due to its weak physical properties as compared to the Gtn-HPA/CMC-Tyr hybrid hydrogel fabricated with 6 mM H₂O₂. The sample's structure was too weak where the FITC-dextran can easily diffuse out of the matrix at the swollen state even without the cellulase treatment.

Figure 5.7 Image showing the induced drugs or cells release from the Gtn-HPA/CMC-Tyr hybrid hydrogel through cellulase treatment.

The release profile of Gtn-HPA/CMC-Tyr hybrid hydrogel fabricated with 6 mM H_2O_2 treated with different cellulase concentrations was shown in **Figure 5.8**. Before the cellulase treatment, all the Gtn-HPA/CMC-Tyr hybrid hydrogels exhibited similar burst release at day 1, which was $\sim 14.5\%$. In general, the Gtn-HPA/CMC-Tyr hybrid hydrogel treated with cellulase had higher release rate as compared to Gtn-HPA/CMC-Tyr hybrid hydrogel without treatment. At day 7, the total release reached $41.4 \pm 0.2\%$ for the Gtn-HPA/CMC-Tyr hybrid hydrogel without treatment. Meanwhile the Gtn-HPA/CMC-Tyr hybrid hydrogels treated with 0.1, 1.0 and 2.0 mg/ml had total release of $46.9 \pm 0.3\%$, $52.7 \pm 0.3\%$ and $51.2 \pm 0.3\%$ respectively. The Gtn-HPA/CMC-Tyr hybrid hydrogels treated with 1.0 and 2.0 mg/ml cellulase had a similar release profile. The higher release rate of the Gtn-HPA/CMC-Tyr hybrid hydrogel treated with cellulase was suggested due to the alteration of Gtn-HPA/CMC-Tyr hybrid hydrogel's degradation rate of through the digestion of CMC.

Figure 5.8 FITC-dextran release profiles for the Gtn-HPA/CMC-Tyr hybrid hydrogel fabricated with 6 mM H_2O_2 and treated with different cellulase concentrations.

Enzymatic degradation studies using cellulase were carried out to further investigate the effect of cellulase concentrations in altering the degradation rate of the Gtn-HPA/CMC-Tyr hybrid hydrogel. As seen in **Figure 5.9a**, BCA assay showed the constant release of proteins from the Gtn-HPA/CMC-Tyr hybrid hydrogel treated with cellulase. The proteins detected from the release buffer solution confirmed the digestion of the Gtn-HPA/CMC-Tyr hybrid hydrogel by cellulase. When higher cellulase concentration was used, more proteins were detected from the release buffer solution. Thus, increasing the cellulase concentration is able to increase the degradation rate of the Gtn-HPA/CMC-Tyr hybrid hydrogel. As seen in **Figure 5.9b**, percentage weight change of the Gtn-HPA/CMC-Tyr hybrid hydrogel treated with different cellulase concentrations further confirm the alteration of the degradation rate by cellulase digestion. The percentage weight change at day 7 (**Figure 5.9b**) showed that the Gtn-HPA/CMC-Tyr hybrid hydrogel without treatment and treated with 0.1, 1.0 and 2.0 mg/ml cellulase had the remaining percentage weight of $86.0 \pm 1.2 \%$, $87.4 \pm 1.3 \%$, $82.3 \pm 5.3 \%$ and $17.2 \pm 0.8 \%$ respectively. The 2.0 mg/ml cellulase observed to cause rapid degradation of the Gtn-HPA/CMC-Tyr hybrid hydrogel. In general, the cellulase

treatment showed to be able to alter the degradation rate of the Gtn-HPA/CMC-Tyr hybrid hydrogel that resulting in the alteration of its release rate.

Figure 5.9 Enzymatic degradation of the Gtn-HPA/CMC-Tyr hybrid hydrogel fabricated with 6 mM H₂O₂ treated with different cellulase concentrations showing the (a) BCA protein assay and (b) percentage weight change.

Cellulase is an enzyme that produced mainly by bacteria, fungi, and protozoans to break down the cellulose or its derivatives. The breaking down of the cellulose molecules to beta-glucose involved the hydrolysis of 1, 4-beta-D-glycosidic linkages in cellulose [22]. Thus, cellulase will be only specifically hydrolyzed CMC-Tyr polymers in the Gtn-HPA/CMC-Tyr hybrid hydrogel, but not the Gtn-HPA polymer [6]. The Gtn-HPA/CMC-Tyr hybrid hydrogel showed to have a sustained release profile and potentially to be used as a drug delivery vehicles for bone applications. The release rate of the Gtn-HPA/CMC-Tyr hybrid hydrogel also related to its stiffness, where higher stiffness showed to have slower release rate. In addition, the Gtn-HPA/CMC-Tyr hybrid hydrogel also showed to be an enzymatic responsive scaffold that able to induce higher drug release on demand through the cellulase treatment. Thus, the ability of the Gtn-HPA/CMC-Tyr hybrid hydrogel in inducing drug release on demand is potentially able to enhance bone regeneration. In addition, cellulase treatment using 1.0 and 2.0 mg/ml showed to have the similar effect on the release profile of the Gtn-HPA/CMC-Tyr hybrid hydrogel. The used of 2.0 mg/ml cellulase also caused rapid degradation of

the Gtn-HPA/CMC-Tyr hybrid hydrogel, which is undesirable. Thus, 2.0 mg/ml cellulase will be excluded from the further functional studies in this chapter.

5.3.7 Cytocompatibility studies

The cytocompatibility of the Gtn-HPA/CMC-Tyr hybrid hydrogel was investigated through 2D cell culture of hFOB. **Figure 5.10** showed the proliferation profiles for the hFOB cultured on the Gtn-HPA/CMC-Tyr hybrid hydrogel's surface for 7 days as compared to the TCP. hFOB cultured on Gtn-HPA/CMC-Tyr hybrid hydrogel's surface showed a positive proliferation rate throughout 7 days of culturing. The number of cells found on the Gtn-HPA/CMC-Tyr hybrid hydrogel's surface at day 1 was 15522 ± 1323 and was increased to 77797 ± 6457 at day 3. The number of cells was further increased to 156723 ± 18144 and 193899 ± 12984 at day 5 and 7 respectively. Both Gtn-HPA/CMC-Tyr hybrid hydrogel and TCP demonstrated a similar 2D hFOB proliferation rate.

Figure 5.10 Cell proliferation profiles for the hFOB cultured on the Gtn-HPA/CMC-Tyr hybrid hydrogel as compared to the TCP.

When comparing the cell population doubling (Figure 5.11), the hFOB cells cultured on the Gtn-HPA/CMC-Tyr hybrid hydrogel's surface had significantly higher ($p < 0.05$) cell population doubling rate at day 3, 5 and 7 as compared to the TCP. hFOB cells cultured on the Gtn-HPA/CMC-Tyr hybrid hydrogel had the cell population doubling rate of 2.33 ± 0.13 , 1.00 ± 0.13 and 0.32 ± 0.12 at day 3, 5 and 7 respectively. Meanwhile, hFOB cells cultured on the TCP had the cell doubling rate of 0.76 ± 0.13 , 0.38 ± 0.06 and 0.10 ± 0.12 at day 3, 5 and 7 respectively. Thus, the growth rate of hFOB cells is faster when culturing on the Gtn-HPA/CMC-Tyr hybrid hydrogel as compared to the TCP.

Figure 5.11 Cumulative cell population doubling rate of the hFOB cells cultured on the Gtn-HPA/CMC-Tyr hybrid hydrogel as compared to the TCP (* $p < 0.05$).

LIVE/DEAD® images showed in Figure 5.12 confirmed the viability of the hFOB cells cultured on both Gtn-HPA/CMC-Tyr hybrid hydrogel and TCP's surfaces. The hFOB cells were observed to increase in number from day 1 to day 7. The 2D cell culture study using hFOB cells showed that the Gtn-HPA/CMC-Tyr hybrid hydrogel has a good cytocompatibility to be used as the scaffolding material. This study also demonstrated that the Gtn-HPA/CMC-Tyr hybrid hydrogel are able to support bone cells attachment and proliferation and showing its potential to be used in bone applications.

Figure 5.12 LIVE/DEAD® images of the hFOBs cultured on (a) the Gtn-HPA/CMC-Tyr hybrid hydrogel and (b) the TCP at day 1, 3, 5 and 7 (Scale bar represents 100 μm . The green fluorescence represents live cells and the red fluorescence represents dead cells).

5.3.8 Cytotoxicity studies of cellulase treatment

The cytocompatibility of the cellulase treatment was assessed by treating the hFOBs with different cellulase concentrations throughout the 7 days of 2D cell culture. As seen in **Figure 5.13a**, the cellulase treatment did not appear to adversely affect the hFOBs, since the cells continued to proliferate throughout the cell culture period regardless of cellulase treatment used. All the sample groups showed similar proliferation rate throughout the 7 days of culture. In fact, as seen from **Figure 5.13b**, the hFOBs had same cell population doubling rate regardless of cellulase concentrations. Thus, the results of cell population doubling rate further confirmed that the cellulase treatment would not affect the proliferation of hFOBs.

Figure 5.13 Cytotoxicity studies of cellulase treatment showing (a) the cell proliferation profiles and (b) the cumulative cell population doubling rate of the hFOB cells treated with different cellulase concentrations for 7 days.

LIVE/DEAD® images in **Figure 5.14** also confirmed the viability of the hFOB cells cultured with culture medium supplied with 0.1, 1.0 and 2.0 mg/ml cellulase throughout the 7 days, where the live cells (green fluorescence) were excessively more than the dead cells (red fluorescence). The LIVE/DEAD® images also showed that the hFOB cells cultured with normal culture medium (no treatment) or medium supplied with different cellulase were increased in number throughout the 7 days. Thus, the results further confirmed the cellulase treatment is cytocompatible and will not affect the proliferation of the hFOB cells.

Figure 5.14 LIVE/DEAD® images of the hFOB cells with (a) no treatment, (b) 0.1, (c) 1.0 and (d) 2.0 mg/ml cellulase treatment at day 1, 3, 5 and 7 (Scale bar represents 200 μ m. Green fluorescence represents live cells and red fluorescence represents dead cells).

Alkaline phosphatase (ALP) expression was then studied to investigate the effect of cellulase on the bone cell's function. As seen in **Figure 5.15**, the ALP expression of the hFOB cells cultured with different cellulase concentrations had the similar ALP expression as compared to the hFOB cells cultured on normal culture medium (no treatment), except for the hFOB cells cultured with 2 mg/ml cellulase. The ALP expression of the hFOB cells cultured with 2 mg/ml cellulase was significantly lower ($p < 0.05$) as compared to the other sample groups. Thus, the use of cellulase concentration less than 1.0 mg/ml will not affect the function of the hFOB cells.

Figure 5.15 ALP expression of the hFOB cells treated with different cellulase concentrations at day 7 (* $p < 0.05$).

Cellulase used in this study was derived from *Trichoderma longibrachiatum* and has been labeled as “Generally Recognized As Safe” (GRAS) by the US Food and Drug Administration (FDA) [23]. Cellulase also has been widely used in food processing industry [24]. In this study, the cellulase showed to be cytocompatible to bone cells thus showed its suitability to be used for bone applications. However, 2.0 mg/ml cellulase will be excluded in the latter section due to this concentration is affecting the cell’s function as well as causing rapid degradation of the Gtn-HPA/CMC-Tyr hybrid hydrogel (**Chapter 5, Section 5.3.6**).

5.3.9 Enzymatic digestion to enhance cell proliferation

The potential of the Gtn-HPA/CMC-Tyr hybrid hydrogel as bone cell carrier and the ability of cellulase treatment in enhancing cell proliferation were studied through 3D encapsulation of hFOB cells. The Gtn-HPA/CMC-Tyr hybrid hydrogel was treated with cellulase to enlarge its pore size (**Appendix 2**). As seen in **Figure A2.1**, the representative images showed the increasing in pore enlargement of the Gtn-

HPA/CMC-Tyr hybrid hydrogel when treated with increasing cellulase concentration. **Figure 5.16** showed the proliferation profiles for the hFOB3 cells encapsulated in the Gtn-HPA/CMC-Tyr hybrid hydrogel treated with different cellulase concentrations. For the Gtn-HPA/CMC-Tyr hybrid hydrogel without cellulase treatment, the cell number observed to be decreased from day 1 to day 3 and then increased again from day 3 to day 5. Finally, the cell number was again decreased at day 7. For the Gtn-HPA/CMC-Tyr hybrid hydrogel treated with 0.1 mg/ml cellulase, the cell number observed to decrease from day 1 to day 3, followed by increasing cell number from day 3 to 7. For the Gtn-HPA/CMC-Tyr hybrid hydrogel treated with 1.0 mg/ml cellulase, the cell number observed to increase throughout the 7 days of culture. Thus, cellulase treatment showed to be able to digest the Gtn-HPA/CMC-Tyr hybrid hydrogel and create more space for cell proliferation in a 3D environment. However, it was observed that the cellulase treatment in culture medium environment had a lesser effect on digesting the Gtn-HPA/CMC-Tyr hybrid hydrogel. This might be due to the high glucose concentration in the culture medium inhibited certain activity of the cellulase [25].

Figure 5.16 Proliferation profiles for the hFOB3 cells encapsulated in the Gtn-HPA/CMC-Tyr hybrid hydrogel treated with different cellulase concentrations.

The Gtn-HPA/CMC-Tyr hybrid hydrogel showed to be able to provide a cytocompatible 3D environment for hFOB cells and showed potential to be used as bone cells' carrier. In addition, cellulase treatment showed to be cytocompatible and able to enhance bone cells' proliferation rate through digesting the CMC. Thus, Gtn-HPA/CMC-Tyr hybrid hydrogel showed to be a smart enzymatic responsive bone scaffold that able to enhance bone cell proliferation and thus potentially enhance bone healing. In general, the Gtn-HPA/CMC-Tyr hybrid hydrogel showed its post-implantation tunability in terms of pore enlargement and promotes bone cells proliferation.

5.4 Conclusion

In this chapter, Tyr molecules were successfully conjugated onto the CMC structure and formed the CMC-Tyr conjugates. Phase separation occurred during the crosslinking between the Gtn-HPA conjugates and CMC-Tyr conjugates using HRP and H_2O_2 , forming a Gtn-HPA/CMC-Tyr hybrid hydrogel with macroporous structure. The material properties of the Gtn-HPA/CMC-Tyr hybrid hydrogel in terms mechanical properties, swelling ratio, and degradation rate can be tuned using different H_2O_2 concentrations. In fact, 5 w/v % Gtn-HPA/CMC-Tyr hybrid hydrogel fabricated with 6 mM H_2O_2 had the compressive modulus > 25 kPa and thus potentially able to provide a suitable mechanical environment for osteogenic differentiation of stem cells. In addition, the Gtn-HPA/CMC-Tyr hybrid hydrogel was able to provide higher permeability than the Gtn-HPA hydrogel. Together with its degradation rate, the Gtn-HPA/CMC-Tyr hybrid hydrogel is suitable to be used as a periosteum-inspired layer to deliver stem cells for bone regeneration. The Gtn-HPA/CMC-Tyr hybrid hydrogel also showed its potential as a drug delivery vehicle and a bone cell carrier for bone applications. Finally, the Gtn-HPA/CMC-Tyr hybrid hydrogel showed to be an enzymatic responsive scaffold that able to induce higher release of drugs and enhance cell proliferation through cellulase treatment. Overall, the Gtn-HPA/CMC-Tyr hybrid hydrogel showed its potential as a periosteum-inspired layer in terms of material properties and drug or cell delivery vehicle, and a smart enzymatic responsive scaffold for bone applications.

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Chapter 6

Study 3 – Development of Periosteum Inspired 3D Injectable Hydrogel-Bioceramic Composite for Enhanced Bone Regeneration.

This chapter will discuss in detail on Study 3 on the development of periosteum-inspired 3D injectable hydrogel-bioceramic composite for bone applications. This chapter will first discuss on the extraction of the calcium phosphate from fish scales, followed by studies on the chemical properties of the fish scale-derived calcium phosphate. After that, calcium phosphate was incorporated into the Gtn-HPA/CMC-Tyr hybrid hydrogel to form the hydrogel-bioceramic composite. The compressive modulus, swelling ratio and degradation behavior of the hydrogel-bioceramic composite were studied. The potential of the hydrogel-bioceramic composite as a drug delivery vehicle was investigated through FITC-dextran release and its ability to enzymatic-induced release of drugs was also studied. The bone-like mineral forming ability of the hydrogel-bioceramic composite was studied through SBF treatment. The cytocompatibility of the hydrogel-bioceramic composite was studied through 2D cell culture of HEK 293FT cells. Finally, 3D cell encapsulation studies using HEK 293FT cells were done to investigate the potential of the hydrogel-bioceramic composite as a cell carrier.

6.1 Introduction

Recently, stem cells encapsulated hydrogel and cell sheet technology had been reported to be studied as the periosteum-inspired membrane. However, the stem cells encapsulated hydrogel is lacked osteogenic components that caused a slower healing rate as compared to the standard autologous bone graft [1]. Meanwhile, the use of cell sheet technology to support bone regeneration is yet to be fully understood and the cell source is also one of the major problems for this technology [2, 3]. Hence, the overall aim of this chapter is to further develop the smart enzymatic responsive Gtn-HPA/CMC-Tyr hybrid hydrogel to provide factors in terms of (a) improved mechanical environment and (b) osteogenic properties that could help in bone regeneration.

Inspired by the ECM and angiogenic properties of periosteum, series of investigation have been carried in **Chapter 4** and **Chapter 5** and the results had shown that (a) the based material, Gtn-HPA, was able to encourage the formation of blood vessels and thus able to provide angiogenic properties for bone regeneration and (b) that Gtn-HPA/CMC-Tyr hybrid hydrogel was a smart enzymatic responsive scaffold that greatly supported the attachment and proliferation of bone cells. In this chapter, it is proposed that bioactive CaP was incorporated into the Gtn-HPA/CMC-Tyr hybrid hydrogel to develop an injectable hydrogel-bioceramic composite consist of the 3 major properties inspired by the periosteum for bone healing. It is proposed that by incorporating the bioactive calcium phosphate, it will improve the mechanical properties of the Gtn-HPA/CMC-Tyr hybrid hydrogel. The improvement in mechanical properties will provide the required environment in term of stiffness to induce osteogenic differentiation of stem cells [4, 5]. The incorporation of bioactive CaP also proposed to be able to improve osteogenic properties of the whole composite [6].

In this chapter, CaP derived from fish scales (*Channa argus*) is proposed to be used as the bioactive component to be incorporated into the Gtn-HPA/CMC-Tyr hybrid hydrogel. CaP derived from the fish scales is abundant in source and the extraction can be simple and effective [7]. In addition, CaP derived from fish scale is biocompatible [8, 9] and provide good osteogenic properties [9-11]. A series of characterization especially on the hydrogel-bioceramic composite's mechanical properties and bone-

like mineral forming ability will be done to investigate its potential as the periosteum-inspired layer for bone healing.

Overall, the potential of the hydrogel-bioceramic composite to be used as a periosteum-inspired layer will be studied in this chapter according to the aims as follows:

1. To extract and characterize the fish scale-derived calcium phosphate.
2. To fabricate the hydrogel-bioceramic composites.
3. To perform material characterization of the hydrogel-bioceramic composites.
4. To investigate the functional properties of the hydrogel-bioceramic composite in terms of drug release and bone-like mineral forming ability.
5. To investigate the cytocompatibility of the hydrogel-bioceramic composite and its ability as a cell carrier.

6.2 Materials and Methods

Calcium phosphate was extracted from the fish scales as described in **Chapter 3, Section 3.4.1** and characterized according to **Chapter 3, Sections 3.4.2, 3.4.3 and 3.4.4** to identify its chemical properties. The calcium phosphate was then used to fabricate the hydrogel-bioceramic composite as described in **Chapter 3, Section 3.5.3** and the morphology of the hydrogel-bioceramic composite was studied according to **Chapter, Section 3.6.2**. The mechanical properties and swelling ratio of the hydrogel-bioceramic composite with different amounts of CaP were characterized according to **Chapter 3, Sections 3.6.3 and 3.6.4** respectively. *In vitro* degradation behavior of the hydrogel-bioceramic composite with different amounts of CaP was studied according to **Chapter 3, Section 3.6.5**. The functional properties of the hydrogel-bioceramic composite in terms of its potential as a drug delivery vehicle, the induced release of drugs using cellulase treatment and *in situ* bone-like mineral formation through SBF treatment were investigated according to **Chapter 3, Sections 3.7.1, and 3.7.4** respectively. The cytocompatibility of the hydrogel-bioceramic composite was studied using HEK 293FT cells as described in **Chapter 3, Sections 3.8.3, 3.8.4, 3.8.5, 3.8.6 and 3.8.7**. Finally, the CaP powder was treated with PBS solution according to **Chapter 3, Section 3.4.5** and used for the 3D cell encapsulation studies as described in **Chapter 3, Sections 3.8.4, 3.8.5, 3.8.6 and 3.8.7** to investigate the potential of the hydrogel-bioceramic composite as a cell carrier for bone tissue engineering.

6.3 Results and Discussion

6.3.1 Extraction and characterization of fish scale-derived CaP

Snakehead scales were sintered at 850 °C to remove all the organic components and the sintered powder was collected for further analysis. The sintered temperature was kept at 850 °C to prevent the phase transformation of hydroxyapatite to β -tricalcium phosphate (β -TCP) when a temperature higher than 850 °C was used [12]. **Figure 6.1** showed the macroscopic view of fish scales before and after the sintering. The sintered fish scales were milled to get the white sintered powder.

Figure 6.1 Macroscopic view of fish scales before and after sintering (Scale bar represents 2 cm).

The chemical properties of sintered powder were investigated using FTIR, XRD, and SEM-EDX. **Figure 6.2** showed the FTIR spectrum of the sintered powder. There was a strong characteristic peak located at $\sim 1000\text{ cm}^{-1}$, which was the characteristic peak of the phosphate group (PO_4^{3-}), indicated the presence of CaP compound in the sintered powder. Organic components, such as collagen that consists of amide A ($\sim 3300\text{ cm}^{-1}$), amide B ($\sim 3100\text{ cm}^{-1}$), amide I (between 1600 and 1700 cm^{-1}), amide II (between 1510 and 1580 cm^{-1}) and amide III (between 1300 and 1180 cm^{-1}) characteristic peaks [13, 14] were absence in the FTIR spectrum. The absence of amide groups indicated that all the organic components were successfully removed from fish scales through sintering.

Figure 6.2 ATR-FTIR spectrum of the fish scales-derived calcium phosphate showing the characteristic peaks of the hydroxyl group (blue solid line) and phosphate group (blue dotted box). (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.)

XRD analysis was used to further confirm the presence of CaP in the sintered fish scale's powder. **Figure 6.3** showed the XRD spectrum of sintered fish scale's powder. When compared to the "Intentional Centre of Diffraction Data" according to Powder Diffraction File, the XRD spectrum of powder closely matched with the XRD spectrum of hydroxyapatite. The XRD analysis showed that the sintered powder was monophasic CaP compound resembled hydroxyapatite. The XRD analysis also confirmed that the sintering temperature used in this study will not cause phase transformation from hydroxyapatite to β -TCP.

Figure 6.3 Diffraction peaks of fish scale-derived calcium phosphate from XRD corresponding to hydroxyapatite (blue boxes). (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.)

Elemental analysis using SEM-EDX (**Figure 6.4**) showed that the fish scale-derived CaP had a Ca/P ratio of 1.75 ± 0.04 , which was similar to CaP ratio of human bone mineral (1.55 – 1.75) [15]. In this study, CaP was successfully extracted from the fish scales through the sintering process. The fish scale-derived CaP had similar chemical properties when compared to the human bone. Thus, the fish scale-derived CaP showed great potential as a low-cost source and method to obtain CaP and provides osteogenic properties for bone applications.

Figure 6.4 SEM-EDX analyses showing (a) the representative morphology of fish scale-derived calcium phosphate and (b) its chemical compositions (Scale bar represents 20 μm). (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.)

6.3.2 Fabrication and morphology of hydrogel-bioceramic composite

CaP powder was mixed with the Gtn-HPA and CMC-Tyr polymer solutions to have solution-like precursor and crosslinked using HRP and H_2O_2 to form the hydrogel-bioceramic composite [16]. The enzymatic crosslinking is suitable to be used as the injectable scaffold for bone applications because the solution-like precursor is able to fill different size and shape of the bone defects and solidify rapidly to form the bone scaffold with the bioactive component. The reaction can be carried out under the physiological conditions, which is favorable for bone tissue engineering [17]. As illustrated in **Figure 6.5 (a)**, the CaP powder was immobilized throughout the Gtn-HPA/CMC-Tyr hybrid hydrogel simultaneously during the crosslinking process. The macroscopic view of the hydrogel-bioceramic composite was showed in **Figure 6.5 (b)**.

Figure 6.5 Fabrication of the hydrogel-bioceramic composites showing (a) the CaP powder was immobilized throughout the Gtn-HPA/CMC-Tyr hybrid hydrogel simultaneously during the crosslinking process (b) the macroscopic view of hydrogel-bioceramic composite (Scale bar represents 1 cm).

The lyophilized hydrogel-bioceramic composite was visualized under SEM to have a better understanding of CaP powder distributed across the scaffold. As seen in **Figure 6.6**, the Gtn-HPA/CMC-Tyr hybrid hydrogel had a smooth surface while the hydrogel-bioceramic composite regardless of the amounts of CaP was observed to have a rough surface. The Gtn-HPA/CMC-Tyr hybrid hydrogel also observed to possess large pores while incorporation of CaP into the Gtn-HPA/CMC-Tyr hybrid hydrogel observed to decrease its pore sizes. Overall, the CaP powder was confirmed to distribute across the Gtn-HPA/CMC-Tyr hybrid hydrogel and contributed to the increase in surface roughness, which potentially promotes bone cells attachment, proliferation and spreading [18].

Figure 6.6 SEM images of the lyophilized (a) Gtn-HPA/CMC-Tyr as compared to the hybrid hydrogel hydrogel-bioceramic composite with (b) 5, (c) 10, (d) 20 and (e) 40 w/v % CaP at the cross-sectional area (Scale bar represents 100 μm). (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.)

6.3.3 Mechanical properties

The effect of the amount of CaP on the mechanical properties of the hydrogel-bioceramic composite was studied using compression test. **Figure 6.7** showed the compressive modulus related to the hydrogel-bioceramic composite with different amounts of CaP. In general, an increasing trend in compressive modulus can be observed for the hydrogel-bioceramic composite with increasing amount of CaP. The non-filled Gtn-HPA/CMC-Tyr hybrid hydrogel had the compressive modulus of 1.6 ± 0.2 kPa. Incorporated 5, 10 and 20 w/v % CaP into the Gtn-HPA/CMC-Tyr hybrid hydrogel significantly increased ($p < 0.05$) the compressive modulus to 3.2 ± 0.8 kPa, 3.1 ± 0.4 kPa and 2.9 ± 0.3 kPa respectively. However, there are no significant differences between the compressive modulus of the hydrogel-bioceramic composite with 5, 10 and 20 w/v % CaP. Further incorporated 40 w/v % CaP into Gtn-HPA/CMC-Tyr hybrid hydrogel further significantly increased ($p < 0.05$) the compressive modulus to 4.3 ± 0.5 kPa.

Figure 6.7 Compressive modulus of the hydrogel-bioceramic composite with different amounts of CaP as compared to the Gtn-HPA/CMC-Tyr hybrid hydrogel as control (* $p < 0.05$). (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.)

Generally, the CaP powder incorporated into the Gtn-HPA/CMC-Tyr hybrid hydrogel acts as the fillers to form the particle-reinforced composite that leads to the improvement of the mechanical properties. Physical interaction might be formed between calcium cation (Ca^{2+}) and the free reactive sites (mainly $-\text{COOH}$ group) found on the Gtn-HPA and CMC-Tyr polymers. Besides, a theoretical study by Reddy *et al.* showed that Ca^{2+} is able to form strong cation- π interaction with aromatic groups. [19]. The aromatic groups of HPA, phenylalanine, and tyrosine residue found on the Gtn-HPA polymers and Tyr found on CMC-Tyr polymers can potentially interact with Ca^{2+} ions. These two interactions with Ca^{2+} ions might contribute to the forming of physical interactions between different strands of Gtn and CMC polymers [20]. Thus, the filler-matrix interaction improves the overall mechanical performance of the hydrogel-bioceramic composite. It is also observed that there is an exponential relationship between the amount of CaP and compressive modulus of the hydrogel-bioceramic composite. The filler interaction might be weak when a small amount of CaP and thus might not be able to transit the whole matrix from a gel-like material to a rigid material.

When the critical CaP amount of 40 w/v % was used, the further increased in filler-matrix interaction is able to significantly transit the whole matrix to solid-like behavior. The increased amount of CaP might also cause reducing the dangling polymers and the crosslinking distance, thus reducing the motion of gel [21]. Thus, the exponential increase can be observed in the overall mechanical performance of hydrogel-bioceramic composite with different amounts of CaP.

The mechanical properties of a scaffold, especially in terms of stiffness or modulus, play an important role in providing the mechanical environment to direct stem cell into specific lineage for tissue regeneration [22, 23]. A study by Sun *et al.*, which showed the improvement of gelatin's mechanical properties from ~ 0.6 kPa to ~ 2.5 kPa through EDC crosslinking was able to promote stem cells differentiated to chondrogenic lineage at the early stage, followed by chondrogenic and osteogenic differentiation at the later stage. The whole process is similar to the endochondral ossification of the bone healing process, which involves the formation of bone callus at the early stage [24]. Thus, the improved mechanical properties of the scaffold are able to direct the fate of stem cells for tissue regeneration. Overall, the incorporation of CaP into the Gtn-HPA/CMC-Tyr hybrid hydrogel exhibits improvement in the mechanical properties. Thus, the hydrogel-bioceramic composite potentially provides the right mechanical environment for osteogenic differentiation of stem cells and ultimately to be used as a periosteum-inspired layer to assist in bone regeneration.

6.3.4 Percentage of swelling

The swelling behavior of the hydrogel-bioceramic composite with different amounts of CaP was investigated using $1 \times$ PBS solution. **Figure 6.8** showed the percentage of swelling of the hydrogel-bioceramic composite with different amounts of CaP. When compared the percentage of swelling at day 7, there was a decreasing trend in the percentage of swelling when increasing the amount of CaP. The Gtn-HPA/CMC-Tyr hybrid hydrogel had the swelling ratio of 2838.9 ± 245.3 %. The percentage of swelling was significantly decreased ($p < 0.05$) to 1154.0 ± 23.1 % when 5 w/v % CaP was incorporated into the Gtn-HPA/CMC-Tyr hybrid hydrogel. The percentage of swelling was further significantly decreased ($p < 0.05$) to 936.0 ± 132.8 %, 497.2 ± 110.1 % and 388.2 ± 27.2 % for hydrogel-bioceramic composite with 10, 20 and 40 w/v % CaP. The

decreasing in the percentage of swelling with increasing amount of CaP confirmed the higher interaction between different polymer strands and thus stiffer network as tested in **Chapter 6, Section 6.3.3** [20].

Figure 6.8 Percentage of swelling of the hydrogel-bioceramic composite with different amounts of CaP as compared to the Gtn-HPA/CMC-Tyr hybrid hydrogel as control [$* p < 0.05$ as compared to the control (0 w/v % CaP)]. (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.)

Hydrogel's swelling ability is generally an indication of its structure's permeability [25]. The permeability of the hydrogel affects the efficiency of nutrient and waste exchanges that essential for cell encapsulation [26]. Thus, optimal CaP amount is required to obtain scaffold with desired mechanical properties and effective nutrient and waste transportation for bone tissue engineering. In general, the hydrogel-bioceramic composite with the amount of CaP up to 40 w/v % has a large percentage of swelling and exhibited "hydrogel" like behavior in water content, thus indicates that it has good permeability.

6.3.5 Degradation profile

The degradation studies on the hydrogel-bioceramic composite with different amounts of CaP was conducted using type-I collagenase solution. The hydrogel-bioceramic composite mainly consisted of gelatin and thus are susceptible to proteolysis by matrix metalloproteinases [27, 28]. **Figure 6.9** showed the degradation profiles for the hydrogel-bioceramic composite with different amounts of CaP as compared to the Gtn-HPA/CMC-Tyr hybrid hydrogel. In general, there was an increasing trend in the degradation rate with increasing amount of CaP. After exposing to the type-I collagenase solution for 8 h, the Gtn-HPA/CMC-Tyr hybrid hydrogel had percentage weight loss of 23.4 ± 3.7 %. Hydrogel-bioceramic composite with 5 w/v % CaP had a percentage weight loss of 31.9 ± 9.2 %, which similar to the Gtn-HPA/CMC-Tyr hybrid hydrogel. However, further increased the amount of CaP to 10, 20 and 40 w/v % demonstrated significantly higher ($p < 0.05$) degradation rate and reached percentages weight loss of 45.0 ± 14.1 , 68.9 and 89.0 ± 2.0 % respectively at 8 h.

Figure 6.9 Degradation profiles for the hydrogel-bioceramic composites with (a) 5, (b) 10, (c) 20 and (d) 40 w/v % CaP as compared to the Gtn-HPA/CMC-Tyr hybrid hydrogel as control [$*p < 0.05$ as compared to the control (0 w/v % CaP)]. (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.)

Besides acts as fillers to improve the mechanical properties, the presence of CaP could also facilitate as defect sites for the hydrogel-bioceramic composite. Thus, the excessive amount of CaP will lead to higher degradation rate. It is also suggested that the excessive amount of CaP could also disrupt the covalent crosslinking of the whole system and thus will be more susceptible to disintegration. CaP amount of 5 w/v % was

not effectively in significantly disrupted the crosslinking between the hydrogel's networks. Thus, hydrogel-bioceramic composite with 5 w/v % CaP showed a similar degradation profile like Gtn-HPA/CMC-Tyr hybrid hydrogel. Further increased the amount of CaP, especially to 20 and 40 w/v %, showed higher degradation rate and increasingly wider standard deviation. This indicates that the higher amount of CaP significantly disrupted the crosslinking between the polymer networks, thus compromises the integrity of whole structure [29]. In addition, the hydrogel-bioceramic composite with 40 w/v % CaP has very rapid degradation where a massive loss in material and structural integrity can be observed when treated with type-I collagenase solution. Thus, this sample group will be excluded from further functional and cellular studies in the latter sections because it is not suitable to be used as the implantable scaffold. Overall, the optimal amount of CaP incorporated into the Gtn-HPA/CMC-Tyr hybrid hydrogel is crucial to have optimal performance in terms of degradation rate and other properties.

6.3.6 Drug release profile

FITC-dextran, which is the model drug, was again used to assess the drug delivery profile of the hydrogel-bioceramic composite [30]. **Figure 6.10** showed the drug delivery profiles for the hydrogel-bioceramic composites with different amounts of CaP as compared to the Gtn-HPA/CMC-Tyr hybrid hydrogel. All the sample groups regardless of the amount of CaP showed a zero-order release profile. The hydrogel-bioceramic composite had a diffusion-controlled release process like the Gtn-HPA/CMC-Tyr hybrid hydrogel. The hydrogel-bioceramic composite with 5 and 10 w/v % CaP had comparable release kinetics with the Gtn-HPA/CMC-Tyr hybrid hydrogel. However, further incorporated 20 and 40 w/v % CaP into the Gtn-HPA/CMC-Tyr hybrid hydrogel caused higher burst release and release rate.

Figure 6.10 FITC-dextran release profiles for the hydrogel-bioceramic composites with (a) 5, (b) 10, (c) 20 and (d) 40 w/v % CaP as compared to the Gtn-HPA/CMC-Tyr hybrid hydrogel as control. (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.)

When exposed to the physiological solution, drug release from the hydrogel-bioceramic composite and degradation of the hydrogel-bioceramic composite are occurs simultaneously. The increases in burst release and release rate with increasing amount of CaP might be associated with the higher degradation rate as discussed in **Chapter 6, Section 6.6.5**. Particularly for the hydrogel-bioceramic composite with 40 w/v % CaP, the release percentage showed to have a larger standard deviation, indicated higher

burst release and unstable release. Overall, the higher amount of CaP, especially at 20 and 40 w/v %, will result in a sudden release of the drug due to higher degradation rate that might cause overdose issues. The hydrogel-bioceramic composites with 5 and 10 w/v % had a similar release profile as compared to the Gtn-HPA/CMC-Tyr hybrid hydrogel, thus more suitable to be used as the delivery vehicle and as the periosteum-inspired layer.

Hydrogel-bioceramic composite with 10 w/v % CaP was then used to study induced drug release with cellulase treatment. The FITC-dextran release profile of hydrogel-bioceramic with 10 w/v % CaP without and with 1.0 mg/ml cellulase treatment is shown in **Figure 6.11**. The result showed that the hydrogel-bioceramic composite with cellulase treatment had a slightly higher drug release rate as compared to the sample without treatment. The incorporation of CaP into the Gtn-HPA/CMC-Tyr hybrid hydrogel does not hinder its ability to respond to cellulase treatment. Thus, further optimization is needed to investigate the cellulase responsive behavior of the hydrogel-bioceramic composite (refer to **Chapter 7, Section 7.3.2**).

Figure 6.11 FITC-dextran release profiles for the hydrogel-bioceramic composite without and with 1.0 mg/ml cellulase treatment.

6.3.7 Biomineralization study

The osteocompatibility and the bioactivity of the hydrogel-bioceramic composite were studied through biomineralization study. Samples were exposed to $1 \times$ SBF solution that contained calcium and phosphate ions for 1 and 7 days. **Figure 6.12** showed the SEM images of CaP particles precipitated and distributed across the sample's surface after treated with $1 \times$ SBF for 1 and 7 days while **Figure 6.13** showed the Ca/P ratio of those particles. Fine CaP particles with a Ca/P ratio < 1.00 was observed to deposited on all the samples after the pretreatment process. When treated with $1 \times$ SBF for 1 day, these deposited CaP particles were undergoing nucleation and growth to a Ca/P ratio of 1.13 ± 0.04 , 1.12 ± 0.04 , 1.07 ± 0.04 and 1.07 ± 0.04 for Gtn-HPA/CMC-Tyr hybrid hydrogel and hydrogel-bioceramic composite with 5, 10 and 20 w/v % CaP respectively. Further treated the samples with $1 \times$ SBF until day 7 caused further nucleation and growth of CaP particles to the Ca/P ratio of 1.19 ± 0.07 , 1.18 ± 0.06 , 1.22 ± 0.02 and 1.17 ± 0.02 for the Gtn-HPA/CMC-Tyr hybrid hydrogel and the hydrogel-bioceramic composite with 5, 10 and 20 w/v % CaP respectively. All the samples had similar nucleation and growth rate of bone-like mineral on the sample's surface throughout the whole study using $1 \times$ SBF solution. The biomineralization study using $1 \times$ SBF solution showed that all the samples encouraged the nucleation and growth of CaP particles, which demonstrated the positive bioactive properties in terms of encouraging bone-like minerals formation. Thus, hydrogel-bioceramic composite showed its potential to be able to integrate with the host bone tissue and suitable to be used as the bioactive scaffold for bone regeneration.

Figure 6.12 SEM images showing the surface of lyophilized hydrogel-bioceramic composite with (a) 0, (b) 5, (c) 10 and (d) 20 w/v % CaP before and after $1 \times$ SBF treatment (Scale bar represents 50 μm). (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.)

Figure 6.13 Ca/P ratios of the bone-like minerals formed on the hydrogel-bioceramic composite with different amounts of CaP after treated with $1 \times$ SBF. (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.)

Next, further studied on the formation of the bone-like minerals on the hydrogel-bioceramic composite was conducted using $10 \times$ SBF. Highly concentrated SBF ($10 \times$) was used to increase the nucleation and formation of bone-like minerals [31]. **Figure 6.14** showed the SEM images of sample's surface after treated with $10 \times$ SBF for 1 day and 7 days while **Figure 6.15** showed the Ca/P ratio of bone-like minerals formed on the sample after treatment. Similarly, fine CaP particles with Ca/P ratio < 1.00 were observed to form at all the sample's surface after pre-treatment. After treated with $10 \times$ SBF for 1 day, the fine CaP particles formed on Gtn-HPA/CMC-Tyr hybrid hydrogel did not observe growth and remained at Ca/P ratio of < 1.00 . As for hydrogel-bioceramic composite, regardless the amount of CaP, the fine CaP particles observed to continue to grow to Ca/P ratio ~ 1.20 after treated with $10 \times$ SBF for 1 day. The CaP particles formed on hydrogel-bioceramic composite continue to growth to Ca/P ratio of

~ 1.40 after treated with $10 \times$ SBF for 7 days. However, the CaP particles formed on the Gtn-HPA/CMC-Tyr hybrid hydrogel were completely dissolved after treated $10 \times$ SBF for 7 days.

Figure 6.14 SEM images showing the surface of lyophilized hydrogel-bioceramic composite with (a) 0, (b) 5, (c) 10 and (d) 20 w/v % CaP before and after $10 \times$ SBF treatment (Scale bar represents 50 μm).

Figure 6.15 Ca/P ratios of bone-like minerals formed on the hydrogel-bioceramic composite with different amounts of CaP after treated with $10 \times$ SBF.

As showed in **Figure 6.16**, only sodium chloride salts were found to deposit on the Gtn-HPA/CMC-Tyr hybrid hydrogel's surface at day 7. The acidic environment of $10 \times$ SBF (pH 4.35 to 4.40) [32] caused the CaP particles to dissolve [33]. However, the incorporation of fish scale-derived CaP into the Gtn-HPA/CMC-Tyr hybrid hydrogel can provide the local alkaline environment that encourages nucleation and growth [34]. In conclusion, the incorporation of fish scale-derived CaP into the Gtn-HPA/CMC-Tyr showed to enhance the bioactivity of the whole scaffold and provide a suitable environment for the formation and growth of bone-like minerals.

Figure 6.16 SEM-EDX analysis of the surface of the lyophilized Gtn-HPA/CMC-Tyr hybrid hydrogel after treated with $10 \times$ SBF for 7 days (Scale bar represents 20 μm).

6.3.8 Cytocompatible study using 2D cell culture and 3D cell encapsulation studies

The cytocompatibility of the hydrogel-bioceramic composite was first studied through 2D cell culture using HEK 293FT cells. **Figure 6.17** showed the proliferation profile of HEK 293FT cells culture on the surface of the hydrogel-bioceramic composite with different amounts of CaP. All the samples showed positive cell proliferation rate throughout the 7 days of culture. However, when comparing the cell number at day 7, a decreasing trend can be observed with increasing amount of CaP. At day 7, the Gtn-HPA/CMC-Tyr hybrid hydrogel had the cell number of 232368 ± 27484 . The cell number was significantly decreased ($p < 0.05$) to 153539 ± 21874 , 90327 ± 23368 and 21145 ± 14042 at day 7 for the hydrogel-bioceramic composite with 5, 10 and 20 w/v % CaP.

Figure 6.17 Cell proliferation profiles for the HEK 293FT cells cultured on the hydrogel-bioceramic composite with different amounts of CaP as compared to the Gtn-HPA/CMC-Tyr hybrid hydrogel (0 w/v % CaP) and the TCP (* $p < 0.05$).

The LIVE/DEAD® images as seen in **Figure 6.18** confirmed the viability of HEK 293FT cells cultured on hydrogel-bioceramic composite's surface at day 7. When comparing the cell population doubling rate as shown in **Figure 6.19**, all the sample groups exhibited similar positive growth rate comparable to TCP. Thus, the decreasing cell number with increasing amount of CaP might be due to the incorporation of CaP affected the initial cell attachment that ultimately leads to lesser cell number at day 7.

Figure 6.18 LIVE/DEAD® images of HEK 293FT cells cultured on the hydrogel-bioceramic with (a) 5, (b) 10 and (c) 20 w/v % CaP as compared to (d) the Gtn-HPA/CMC-Tyr hybrid hydrogel and (e) the TCP (Scale bar represents 200 μm).

Figure 6.19 Cumulative cell population doubling rate of the HEK 293FT cells cultured on the hydrogel-bioceramic composite with different amounts of CaP as compared to the Gtn-HPA/CMC-Tyr hybrid hydrogel (0 w/v % CaP) and the TCP.

3D cell encapsulation using HEK 293FT cells was done to further study the cytocompatibility of the hydrogel-bioceramic composite and its ability as the cell carrier. **Figure 6.20** showed the proliferation profiles for the encapsulated HEK 293 FT cells in the hydrogel-bioceramic composite with different amounts of CaP. The 3D cell encapsulation had similar cell proliferation profile with 2D cell culture study. The encapsulated HEK 293FT cells had positive proliferation rate in the hydrogel-bioceramic composite with different amounts of CaP. Similar to the 2D cell culture study, cell number was observed to significantly decrease ($p < 0.05$) with increasing amount of CaP at day 7. At day 7, the Gtn-HPA/CMC-Tyr hybrid hydrogel had the cell number of 76215 ± 6494 while the hydrogel-bioceramic composite with 5, 10 and 20 w/v % CaP had the cell number of 26088 ± 2789 , 14511 ± 5819 and 1718 ± 2299 respectively.

Figure 6.20 Cells proliferation profiles for HEK 293FT cells encapsulated in the hydrogel-bioceramic composite with different amounts of CaP as compared to the Gtn-HPA/CMC-Tyr hybrid hydrogel (0 w/v % CaP) (* $p < 0.05$).

LIVE/DEAD® images in **Figure 6.21** visually confirmed the viability of HEK 293FT cells encapsulated in the hydrogel-bioceramic composite. Again, the decreasing cell

number with increasing amount of CaP at day 7 might be due to the incorporation of CaP affected the initial cell attachment. Both 2D cell culture and 3D cell encapsulation studies showed the cytocompatibility of hydrogel-bioceramic composite with a positive cell proliferation rate. However, further optimization is needed to improve the cell attachment and proliferation on hydrogel-bioceramic before being used as the cell carrier.

Figure 6.21 LIVE/DEAD® images of the HEK 293FT cells encapsulated in the (a) Gtn-HPA/CMC-Tyr hybrid hydrogel and the hydrogel-bioceramic with (b) 5, (c) 10 and (d) 20 w/v % CaP at day 7 (Scale bar represents 200 μ m).

Initial cell attachment study on the hydrogel-bioceramic composites with different amounts of CaP was done to confirm the assumption that the incorporation of CaP will affect the initial cell attachment. **Figure 6.22** showed the number of HEK 293FT cells attached on the surface of the hydrogel-bioceramic composite with different amounts of CaP after 6 h of seeding. After 6 h of cell seeding, the cell number attached on the Gtn-HPA/CMC-Tyr hybrid hydrogel was 21235 ± 4448 . For hydrogel-bioceramic composites with 5, 10, and 20 w/v %, the number of cell attached after 6 h of cell seeding were 12881 ± 2872 , 9254 ± 1358 , and 12230 ± 1038 , which is significantly lower ($p < 0.05$) as compared to the Gtn-HPA/CMC-Tyr hybrid hydrogel. Thus, this confirmed that the incorporation of CaP decreased the initial cell attachment.

Figure 6.22 Initial attachment profiles for the HEK 293FT cells on the hydrogel-bioceramic composite with different amounts of CaP as compared to the Gtn-HPA/CMC-Tyr hybrid hydrogel (0 w/v % CaP) and the TCP (* $p < 0.05$).

In addition, the color of the culture medium as shown in **Figure 6.23** was observed to change to the alkaline region (from light red to pink) when CaP came in contact with the culture medium. As shown in **Table 6.1**, the initial pH of the medium was 7.41 ± 0.02 and the pH value increased to 8.01 ± 0.04 when the CaP powder was added into the medium. As compared to the commercial HA as control, the culture medium only has slightly increased in pH value after soaked with the commercial HA. Thus, the leaching of ions might occur when the fish scale-derived CaP comes in contact with the physiological solutions or the culture medium [35-38] and causes the elevation of pH that discouraged cell attachment [39, 40]. Thus, further stabilization of the fish scale-derived CaP is required to improve the initial cell attachment of the hydrogel-bioceramic composite.

Figure 6.23 Image showing the color of (a) culture medium, (b) culture medium with fish scale-derived CaP and (c) commercial HA (Scale bar represents 2 cm).

Table 6.1 pH values of the culture medium before and after soaking with the fish scale-derived CaP and the commercial HA.

Sample	Initial pH	Final pH
Fish scale-derived CaP	7.41 ± 0.02	8.01 ± 0.04
Commercial HA	7.44 ± 0.02	7.54 ± 0.03

6.3.9 pH stabilization of fish scale-derived CaP

After the sintering process, the inorganic part of the fish scale was ball milled for 2 h using the mass ratio of fish scale: zirconia ball of 10 w/w % to get the white sintered powder. The CaP powder was then soaked with different volume of $1 \times$ PBS to study the optimal volume of physiological solution in stabilizing the pH of the fish scale-derived CaP. As seen in **Figure 6.24**, the initial pH of $1 \times$ PBS was 7.40 ± 0.01 and the pH was elevated to 7.57 ± 0.02 when 10 g of fish scale-derived CaP was added into the 200 ml $1 \times$ PBS. When 400 ml and 600 ml $1 \times$ PBS were used, the pH only elevated to 7.53 ± 0.01 and 7.50 ± 0.01 respectively. The elevation of pH was significantly smaller ($p < 0.05$) when 600 ml $1 \times$ PBS was used as compared to 200 ml. Thus, treatment with 600 ml $1 \times$ PBS or more is able to stabilize the pH of fish scale-derived CaP.

Figure 6.24 pH of $1 \times$ PBS with different volume after soaked with the fish scale-derived CaP.

The chemical properties of the fish scale-derived CaP before and after the $1 \times$ PBS treatment were then studied using SEM-EDX, FTIR, and XRD. As seen in **Figure 6.25**, the Ca/P ratio of fish scale-derived CaP before and after PBS treatment were 1.75 ± 0.04 and 1.61 ± 0.13 .

Figure 6.25 Ca/P ratio of the fish scale-derived CaP before and after $1 \times$ PBS treatment.

When analysis using FTIR as shown in **Figure 6.26**, there was no changed in the chemical structure of treated CaP where before and after the treatment had a similar

phosphate ($\sim 1000\text{ cm}^{-1}$) peak's intensity and slightly decreased of carbonate ($\sim 2400\text{ cm}^{-1}$) peak's intensity.

Figure 6.26 FTIR spectra of fish scale-derived CaP before and after $1 \times$ PBS treatment.

Analysis using XRD as shown in **Figure 6.27** further confirmed that the treatment does not alter the chemical phase of fish scale-derived CaP and it remained as hydroxyapatite phase. Overall, the pH of fish scale-derived CaP was successfully stabilized using a large volume of $1 \times$ PBS solution without altering its chemical properties. The treated CaP powder was then further used for cellular studies.

Figure 6.27 XRD spectra of the fish scale-derived CaP before and after $1 \times$ PBS treatment.

6.3.10 3D cell encapsulation studies using Gtn-HPA/CMC-Tyr hybrid hydrogel incorporated with treated CaP

Fish scale-derived CaP powder treated with $1 \times$ PBS solution was further treated with DMEM to ensure it reached the stable physiological pH. The treated CaP powder was then used to fabricate hydrogel-bioceramic composite. The cytocompatibility of hydrogel-bioceramic composite fabricated using treated CaP was then studied through the 3D cell encapsulation using HEK 293FT cells. **Figure 6.28** showed the proliferation profiles for the encapsulated HEK 293FT cells in the samples with different amounts of treated CaP. HEK 293FT cells remained highly viable after the encapsulation for all the sample groups. Positive proliferate rate was observed for all the sample groups for

7 days of 3D cultured. When compared at day 7, the hydrogel-bioceramic composite with 5 and 20 w/v % treated CaP had similar cell number as compared to the Gtn-HPA/CMC-Tyr hybrid hydrogel. Meanwhile, the hydrogel-bioceramic composite with 10 w/v % treated CaP had significantly higher ($p < 0.05$) cell number as compared to the Gtn-HPA/CMC-Tyr hybrid hydrogel at day 7.

Figure 6.28 Cell proliferation profiles for the HEK 293FT cells encapsulated in the hydrogel-bioceramic composite with different amounts of treated CaP (* $p < 0.05$). (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.)

Cell population doubling rate as seen in **Figure 6.29** further confirmed the positive growth rate for all the sample groups and showed that the hydrogen-bioceramic composite had higher cell proliferation rate as compared to the Gtn-HPA/CMC-Tyr hybrid hydrogel.

Figure 6.29 Cumulative cell population doubling rate of the HEK 293FT cells encapsulated in the hydrogel-bioceramic composite with different amounts of treated CaP. (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.)

LIVE/DEAD® images in **Figure 6.30** showed higher viable cells (green fluorescence) as compared to dead cells (red fluorescence) in all the sample groups at day 7. Thus, the results confirmed the cytocompatibility of the treated CaP and the hydrogel-bioceramic composite.

Figure 6.30 LIVE/DEAD® images of the HEK 293FT cells encapsulated in the (a) Gtn-HPA/CMC-Tyr hybrid hydrogel and the hydrogel-bioceramic with (b) 5, (c) 10 and (d) 20 w/v % treated CaP (Scale bar represents 200 μm). (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.)

The hydrogel-bioceramic composite was able to provide a viable 3D environment for cell attachment and proliferation. In addition, the proliferation result showed that the hydrogel-bioceramic composite with 10 w/v % treated CaP was the optimal conditions to be used as a cell carrier. The hydrogel-bioceramic composite, particularly with 10 w/v % treated CaP, with a favorable degradation rate (**Chapter 6, Section 6.3.5**) was able to provide sufficient space for cell proliferation. Overall, the 3D cell encapsulation studies proved that the pH of fish scale-derived CaP was successfully stabilized and it is able to improve the cytocompatibility for the whole system by preventing the leaching of ions. In addition, the hydrogel-bioceramic composite with 10 w/v % treated CaP showed to be optimal system and potentially to be used as the periosteum-inspired layer and the cell carrier for bone regeneration.

6.4 Conclusion

In this chapter, CaP was successfully extracted from the fish scales through sintering. The hydrogel-bioceramic composite was successfully fabricated through incorporating the fish scale-derived CaP into the Gtn-HPA/CMC-Tyr hybrid hydrogel. The incorporation of CaP was able to improve the mechanical properties of the whole system, favorable for bone regeneration. A series of systematic investigations showed that the hydrogel-bioceramic with 10 w/v % CaP was the optimal system in terms of enhanced mechanical properties, sufficient permeability for mass transportation, degradation rate and as an effective drug delivery vehicle. The biomineralization study using SBF showed that the hydrogel-bioceramic composite has positive bioactive properties that encourage the formation of bone-like minerals. The pH of the fish scale-derived CaP was successfully stabilized using $1 \times$ PBS solution and culture medium and proved to be cytocompatible. The 3D cell encapsulation study showed that the hydrogel-bioceramic composite was cytocompatible and suitable to be used as a cell carrier. Taken together, the hydrogel-bioceramic composite showed potentially be used as a periosteum-inspired injectable system to enhance bone regeneration and healing.

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Chapter 7

Conclusions and Future Works

This chapter will conclude all the studies described in the previous chapters and draw out the implications and impacts of these studies. Next, future studies on the current works and the proposed future works will be discussed and supported by the preliminary results.

7.1 Overview of Research Findings

In this thesis, 3 main studies in **Chapter 4, 5 and 6** were carried out to fulfill the overall objective of developing a 3D injectable periosteum-inspired hydrogel-bioceramic composite for bone regeneration. The successful in developing the periosteum-inspired layer is able to provide a treatment with healing properties comparable to the autograft and thus greatly supports bone regeneration. The replacement of the current autograft treatment can help to prevent the problem such as donor site morbidity.

In line with the overall objectives of this thesis, the following 3 hypothesis were tested:

(i) That the enzymatic crosslinking phenol-conjugated gelatin is a suitable injectable system which can provide angiogenic properties for bone regeneration, (ii) that the smart enzymatic responsive scaffold would be able to further assist in bone regeneration as it has the ability of induced release and enhance encapsulated bone cell proliferation, and (iii) that the incorporation of the fish scale-derived CaP is able to improve the mechanical properties and the whole system provides bioactive properties for bone regeneration.

Overall, the main findings of this thesis were as follows:

1. The enzymatic crosslinked Gtn-HPA hydrogel showed to be a good injectable scaffold with its solution-like precursor for injectability and tunable gelation rate. This thesis first demonstrated the characterization of the Gtn-HPA hydrogel in detail. Firstly, it demonstrated the detailed materials properties of the Gtn-HPA hydrogel in terms of compressive modulus, swelling ratio, degradation rate and release rate fabricated with different HRP and H_2O_2 concentrations. Secondly, it showed that the increase in polymer concentration would greatly affect all the properties mentioned above; it was found that the increase in polymer concentration could achieve higher mechanical properties and slower degradation rate that were suitable for bone applications. The Gtn-HPA hydrogel also showed to be a potential injectable system with tunable properties that are not confined solely to bone applications, but also for different applications that required different properties (**Study 1, Chapter 4**).

2. Through the *in vivo* studies using C57BL/6 mice, the biocompatibility and degradability of the Gtn-HPA hydrogel were demonstrated in great detail. In addition, these studies also demonstrated the injectability of the Gtn-HPA hydrogel as well as its ability to perform well even with irregular shape and small size (volume = 10 μ l) defects. The Gtn-HPA hydrogel was also shown to support vascularization, as blood vessels observed to form around and into the Gtn-HPA hydrogel. Thus, the results demonstrated its potential as the injectable scaffold for bone regeneration. Further preliminary *in vivo* studies using stiffer Gtn-HPA hydrogel showed to support both angiogenesis and lymphangiogenesis, which open up novel applications in lymphatic regeneration (**Study 1, Chapter 4**).

3. The properties of the Gtn-HPA/CMC-Tyr hybrid hydrogel, especially in terms of mechanical properties and degradation rate, were showed to affect by different H_2O_2 concentrations. It was also found that the effect of H_2O_2 concentration on the Gtn-HPA/CMC-Tyr hybrid hydrogel's properties have the similar trend as compared to the Gtn-HPA hydrogel, where higher H_2O_2 concentration leads to higher crosslinking density, thus higher mechanical strength and slower degradation rate. In addition, *in vitro* cellular studies using hFOB cells showed good cytocompatibility of the Gtn-HPA/CMC-Tyr hybrid hydrogel as it supported the proliferation of bone cells. Thus, the Gtn-HPA/CMC-Tyr hybrid hydrogel also showed great potential in working as bone scaffolds to support the growth of bone tissues (**Study 2, Chapter 5**).

4. The cellulase treatment up to 1 mg/ml demonstrated its cytocompatibility for bone applications, as it would not affect the viability, proliferation, and function of the bone cells. This was proven by a cytotoxicity test of cellulase which demonstrated the cytocompatibility of 0.1 to 1 mg/ml cellulase treatment on the hFOB cells. Cellulase was used to specifically digest CMC-Tyr to alter the properties of the Gtn-HPA/CMC-Tyr hybrid hydrogel post-implantation. The enzymatic digestion of the Gtn-HPA/CMC-Tyr hybrid hydrogel using cellulase demonstrated the ability to alter the degradation rate and the drug release rate post-implantation. The cellulase treatment also demonstrated to be able to open up more space to enhance encapsulated bone cells proliferation, which potentially enhances effectiveness in bone healing (**Study 2, Chapter 5**).

5. CaP was successfully extracted from the fish scales through sintering. The fish scale-derived CaP was successfully used to fabricate the hydrogel-bioceramic composite. The detailed studies on the properties of the hydrogel-bioceramic composite with different amounts of CaP were first demonstrated in this thesis. The incorporation of CaP demonstrated to enhance the mechanical properties of the whole system and create a mechanical environment potentially for bone regeneration. The amount of CaP also showed to affect the swelling ratio and degradation behavior. Thus, the optimal amount of CaP was determined to be 10 w/v % CaP, which formed the hydrogel-bioceramic composite with desired properties as the periosteum-inspired layer (**Study 3, Chapter 6**).

6. The incorporation of CaP showed to be not affecting the release profile for the hydrogel-bioceramic composite. The incorporation of CaP also did not inhibit the cellulase treatment on the hydrogel-bioceramic composite. Cellulase treatment on hydrogel-bioceramic composite showed to induced higher drug release rate. The hydrogel bioceramic composite showed to provide bioactive properties and encourage the formation of bone-like minerals. The presence of CaP also demonstrated in providing a local environment that further enhances the bioactive properties of the whole system. 3D cell encapsulation studies showed the cytocompatibility of hydrogel-bioceramic composite, thus potentially to be used as a cell carrier for bone regeneration (**Study 3, Chapter 6**).

7.2 Implications of Research Findings

This thesis showed that the injectable periosteum-inspired hydrogel-bioceramic composite is a potential viable alternative for current methods of treating bone defects. Current methods of autologous bone grafting have proven to possess problems such as donor site morbidity and surgical complications. The proposed method of using an injectable periosteum-inspired hydrogel-bioceramic composite potentially circumvents these problems with its properties as a bone scaffold and as a periosteum-inspired layer to assist allograft and synthetic bone graft in treating large bone defect.

First, this thesis showed the details material characterization of the Gtn-HPA hydrogel, the Gtn-HPA/CMC-Tyr hybrid hydrogel, and the hydrogel-bioceramic composite in

terms of mechanical properties, swelling ratio and degradation studies. These detailed studies built up the understanding of properties related to different HRP and H₂O₂ concentrations, thus as a reference for different potential applications that require different properties. Firstly, as discussed in previous chapters (**Chapter 4, Section 4.3.3, Chapter 5, Section 5.3.3, and Chapter 6, Section 6.3.3**), the mechanical properties or matrix stiffness of the scaffold play an important role in regulating the fate and functions of stem cells. Recent studies also showed that different stiffness will direct stem cell's fate in differentiating into different cell lineage [1-3]. Studies showed that matrix with low stiffness (~ 0.1 to 1.0 kPa) favor neuronal differentiation while matrix with medium stiffness (~ 8 to 15 kPa) favors myogenic differentiation. Lastly, a matrix with high stiffness (> 25 kPa) that close to developing bone tissue will direct stem cells towards osteogenic lineage [4, 5]. Matrix with high stiffness will activate the integrin-focal adhesion signaling that encourages cell attachment, spreading and formation of the actin cytoskeleton. The increase in cell spreading and formation of more actin cytoskeleton will encourage the stem cells to favor towards osteogenic differentiation [6]. Particularly for bone applications, the mechanical properties of the Gtn-HPA hydrogel and the Gtn-HPA/CMC-Tyr hybrid hydrogel showed to be able to achieve > 25 kPa through using > 5 w/v % polymer concentration and higher H₂O₂ concentration. These scaffolds showed their potential in creating a mechanical environment that encourages osteogenic differentiation of stem cells. Besides, the present of the fish scale-derived showed to enhance mechanical of the Gtn-HPA/CMC-Tyr hybrid hydrogel and the whole system provided the bioactive properties for bone healing. Thus, the further optimization of mechanical properties or matrix stiffness of the hydrogel-bioceramic composite through synergy effect between degrees of phenol conjugation, polymer concentration, crosslinker concentration and the present of fish scale-derived CaP is proposed to potentially achieve stiffness-stimulated osteodifferentiation of stem cells. This is to further mimic the osteogenic properties and environment of the periosteum in inducing osteodifferentiation of stem cells during bone healing process [7]. The mechanical properties studies in this thesis laid out the foundation for the future studies to further investigate the potential of the hydrogel-bioceramic composite as the periosteum-inspired layer for bone regeneration.

Secondly, the degradation studies on the Gtn-HPA hydrogel and the Gtn-HPA/CMC-Tyr hybrid hydrogel (**Chapter 4, Section 4.3.5 and Chapter 5, Section 5.3.5**) showed

to have tunable degradation properties mainly using different polymer and crosslinker concentrations during the fabrication process. Thus, the degradation rate of these scaffolds can be tuned to match different healing rate for different applications. The incorporation of CMC-Tyr polymers into the Gtn-HPA hydrogel also showed to enable tunability of the degradation rate post-implantation, thus it is a smart enzymatic responsive scaffold that able to match different healing rate on demand. The cellulase treatment showed to degrade the Gtn-HPA/CMC-Tyr hybrid hydrogel and thus able to induced drug release (**Chapter 5, Section 5.3.6 and Chapter 6, Section 6.3.6**) and open up more space to enhance bone cell proliferation (**Chapter 5, Section 5.3.9**). Together with biocompatible studies of the Gtn-HPA hydrogel, and cytocompatible studies of the Gtn-HPA/CMC-Tyr hybrid hydrogel and the hydrogel-bioceramic composite (**Chapter 4, Section 4.3.7, Chapter 5, Section 5.3.7, Chapter 6, Sections 6.3.8 and 6.3.10**), theses scaffolds showed to have great potential as cell carrier to delivery cells and provide degradability for desired healing rate. Therefore, the degradation studies of the Gtn-HPA hydrogel, the Gtn-HPA/CMC-Tyr hybrid hydrogel and the hydrogel-bioceramic composite in this thesis laid out essential information for proposed future works in other applications. Particular to develop a periosteum-inspired layer, scaffold with degradation rate ~2 weeks is desired for the stem cells to prolong for ~ 14 days until chondrocyte calcification during the bone healing process. The understandings on the factors affecting the degradation rate of the Gtn-HPA hydrogel, Gtn-HPA/CMC-Tyr hybrid hydrogel and the hydrogel-bioceramic composite is proposed to able to help in further optimizing the hydrogel-bioceramic to achieve the desired degradation properties as a periosteum-inspired layer.

In addition, studies using the FITC-dextran as the model drug in this thesis (**Chapter 6, Section 6.3.6**) had shown that hydrogel-bioceramic composite is potential drug delivery vehicle. This might due to the hydrogel-bioceramic still possess its water retention ability even though the inorganic component was incorporated in the whole system (**Chapter 6, Section 6.3.4**). The hydrogel-like environment of the hydrogel-bioceramic composite will help in preserving and releasing of signaling molecules. The hydrogel-bioceramic composite is also a smart enzymatic responsive system, where cellulase treatment will induce higher release rate (**Chapter 6, Section 6.3.6**). A responsive system that enables induced slightly higher release of the therapeutic proteins or drugs on the defect site on demand is potentially able to further improve the

healing efficiency. The release studies of this thesis will lead to the further studies of the hydrogel-bioceramic composite as a delivery vehicle for BMP-2. BMP-2 is widely used and studied as a therapeutic growth factor that encourages bone regeneration [8, 9]. Thus, BMP-2 is proposed to be used for the future studies on the sustained release to enhance bone regeneration. Together with the post-implantation tunability of the hydrogel-bioceramic composite through cellulase treatment, co-encapsulation of the adipose-derived stem cells and BMP-2 is proposed to be done using the hydrogel-bioceramic composite. The combined effect of CaP, BMP-2 and cellulase treatment in inducing BMP-2 release rate on the osteogenic differentiation of ADSCs are proposed to thoroughly investigate. The combining effects of bioactive CaP, induced release of BMP-2 and pore enlargement are suggested will be greatly improved the ADSC proliferation and osteogenic differentiation and ultimately enhance bone regeneration.

7.3 Future Studies on Current Work

7.3.1 Stiffness-stimulated stem cells differentiation

Further studies will be focusing on further optimizing the mechanical strength of hydrogel-bioceramic composite and study the proliferation and differentiation of ADSCs cultured on the hydrogel-bioceramic composite. The combined effect of polymer concentration, crosslinker concentration and amount of CaP on the mechanical properties of the hydrogel-bioceramic composite will be thoroughly studied. Besides, the effect of these three parameters on the other properties of the hydrogel-bioceramic composite such as swelling ratio and degradation behavior will be also studied concurrently to find the optimal conditions. Besides, further increase the phenol molecules conjugated onto the Gtn and CMC structure will allow higher crosslinking density, thus higher mechanical strength. A study by Wang *et al.* showed that the second conjugation of phenol molecules proposed can be done on the Gtn-HPA conjugates through exploiting the COOH group (**Scheme 7.1**) [10]. Meanwhile, a higher amount of phenol molecules is also proposed to be used during the synthesis of Gtn-HPA and CMC-Tyr conjugates to increase the degree of conjugation that potentially increases the crosslinking sites. Thus, increasing the crosslinking sites is proposed to be done for future works to potentially allow further enhancement of the mechanical properties of the whole system.

Next, ADSCs are proposed to be cultured on the optimized hydrogel-bioceramic composite and ability to induce osteogenic differentiation of ADSCs will be studied. The optimized hydrogel-bioceramic composite will be compared with Gtn-HPA/CMC-Tyr hybrid hydrogel and hydrogel-bioceramic composite with lower modulus as the control. The bone marrow stem cells cultured on the sample for 14 days. The cell proliferation will be monitored and measured throughout the 14 days using PrestoBlue® Cell Viability Reagent. At day 7, alkaline phosphatase (ALP) activity, the early osteogenic transcription and protein expression, will be measured to compare the degree of osteogenic differentiation. At day 14, osteopontin and osteocalcin, the late osteogenic transcriptions and protein expressions, will be measured to further compare the degree of osteogenic differentiation [11, 12]. Besides, Alizarin Red will be used to have the qualitative and quantitative measurement of the calcium compound. The increased amount of calcium will indicate more ADSCs are matured to bone cells. The mature bone cells will lay down calcium minerals and initiate bone healing process [13]. The success of the optimized hydrogel-bioceramic composite to induce osteogenic differentiation of stem cells will greatly show the potential of this system as an injectable periosteum-inspired layer.

Scheme 7.1 Second conjugation of the small phenol molecule, Tyr, onto the Gtn-HPA conjugates. Adapted from: Wang, L.-S., *et al.*, Enzymatically cross-linked gelatin-phenol hydrogels with a broader stiffness range for osteogenic differentiation of human mesenchymal stem cells. *Acta Biomaterialia*, 2012. **8**(5): p. 1826-1837. Reproduced with permission.

7.3.2 The combined effect of cellulase treatment, BMP-2 release and CaP in inducing osteogenic differentiation of stem cells

Further studies will be also further optimized and investigated the hydrogel-bioceramic composite as a delivery vehicle for BMP-2. First, the BMP-2 release profile will be studied to first known the release behavior of BMP-2 using the hydrogel-bioceramic composite. The amount of BMP-2 released into the release buffer solution will be measured using BMP-2 ELISA kit. Next, the hydrogel-bioceramic composite encapsulated with BMP-2 will be treated with cellulase to investigate the enzymatically induced release profile. Different cellulase concentrations and cellulase treatment at different time point will be thoroughly studied to have the full understanding of enzymatic responsive release behavior of the hydrogel-bioceramic composite.

Next, the cellulase treatment in enhancing cell proliferation will be further studied using the hydrogel-bioceramic composite. The potential of the hydrogel-bioceramic composite as stem cell's carrier and a smart enzymatic responsive system will be investigated in this future work. The effect of cellulase treatment on the encapsulated ADSC viability, proliferation and differentiation will be studied. As mentioned in **Chapter 7, Section 7.3.1**, the ADSC proliferation will be monitored and measured for 14 days. At day 7, alkaline phosphatase (ALP) activity, the early osteogenic transcription and protein expression, will be measured to compare the degree of osteogenic differentiation. At day 14, osteopontin and osteocalcin, the late osteogenic transcriptions and protein expressions, will be measured to further compare the degree of osteogenic differentiation between different sample groups. The potential of the hydrogel-bioceramic composite as a stem cell carrier and a smart enzymatic responsive system will be investigated in this future work. Finally, the combined effects of CaP, BMP-2 and cellulase treatment on osteogenic differentiation of stem cells will be thoroughly studied to investigate the potential of hydrogel-bioceramic properties as a periosteum-inspired layer.

7.3.3 Detailed investigation of biomineralization using the hydrogel-bioceramic composite.

The biomineralization study in **Chapter 7, Section 6.3.7** showed the formation of nucleation sites with the deposition of calcium phosphate particles on the hydrogel-bioceramic hydrogel when treated with $1 \times$ or $10 \times$ SBF over 7 days. The formation of calcium phosphate on the hydrogel-bioceramic composite's surface shows its potential bioactive properties and its ability to bond with bone. To further investigate the bioactive properties of the hydrogel-bioceramic composite, the biomineralization study using hydrogel-bioceramic composite will be treated with SBF of different ion strength ($1 \times$, $1.5 \times$, $5 \times$ and $10 \times$) and a longer mineralization time point (day 7, 14 and 28) will be characterized. The crystal morphology, composition, and phase of the calcium phosphate formed with different mineralize conditions will be studied in details. The morphology and Ca/P ratio of the calcium phosphate formed will be characterized using SEM-EDX while its crystal phase will be characterized using XRD. The optimum conditions to form a calcium phosphate on the hydrogel-bioceramic composite with morphology, Ca/P ratio and crystallinity similar to hydroxyapatite will be also investigated in this future study. The detailed biomineralization study using hydrogel-bioceramic composite will develop a further understand the osteogenic properties of the hydrogel-bioceramic composite and its potential in bone regeneration.

7.3.4 *In vivo* studies to investigate the biocompatibility and bone healing ability of hydrogel-bioceramic composite.

In vivo studies are proposed to investigate the biocompatibility and bone healing ability of the hydrogel-bioceramic composite. Animal model developed by Baldwin *et al.* is proposed to be used in this study [14]. Briefly, a 15 mm skin incision is made along the lateral side of the femur to expose the murine's femoral cortical. The bone is then separated from the muscle using blunt dissection. 0.5 mm femoral cortical window is created using a 27 gauge needle and the bone marrow compartment is exposed. The hydrogel-bioceramic composite containing 1.0×10^7 cells/ml ADSCs is then crosslinked using HRP and H_2O_2 and injected onto the defect site, wrapping around the bone. Next, the wound is closed up using sutures and metal clips. Fish scale-derived CaP and Gtn-HPA/CMC-Tyr hybrid hydrogel will be implanted as controls. The

biocompatibility and the immune response of the fish scale-derived calcium phosphate are also evaluated in this study. At week 2 and 4 post-implantation, the samples are harvested from the implanted site and evaluated using H & E staining and immunohistochemistry. The samples will be cryosectioned and subsequently stained with H & E staining to evaluate the biocompatibility, the biodegradability of the sample, and the bone growth at the defect site. To study the potential immune response against the hydrogel-bioceramic composite, Gtn-HPA/CMC-Tyr hybrid hydrogel and fish scale-derived CaP. Sample groups will be cryosectioned and being evaluated through identification of the amount of multinucleated giant cells using H & E staining and immunohistochemically staining for M1 macrophage (IRF5) and M2 macrophage (CD206 and CD163) markers.

7.4 Proposed Future Works for Different Applications

7.4.1 Lymphatic vessels regeneration for atherosclerosis regression

Atherosclerosis occurs when plaques formed by cholesterol and leukocytes on the wall of arteries. Atherosclerosis will lead to cardiovascular disease, which is the number one cause of death in the world [15, 16]. In 2012, World Health Organization (WHO) estimated that 17.5 million people have died from cardiovascular disease, which included 6.7 million deaths caused by stroke [17]. Thus, there is a need to find a solution to reverse the atherosclerosis process to prevent cardiovascular disease. Recent studies showed that the lymphatic vessel plays an important role in atherosclerosis regression through mediating reverse cholesterol transport [18, 19]. A study by Lim *et al.* showed that restoring lymphatic drainage improved the cholesterol clearance while blocking of lymphatic vessels impaired it [20]. Besides, studies also showed that lymphangiogenesis was absence at the atherosclerotic, which suggested to impaired the reserved cholesterol transport process [21]. Thus, restoring lymphatic vessels using a biomaterial as delivery vehicle proposed to be able to assists in atherosclerosis regression in the aorta.

Studies in **Chapter 4** showed that the Gtn-HPA hydrogel is a good injectable system with tunable properties. Gtn-HPA hydrogel has the potential to reach small gap or deeply buried site of the aorta. Gtn-HPA hydrogel also showed to be a good delivery

vehicle with different release rate depends on the fabrication parameters (**Chapter 4, Section 4.3.6**). In this future work, the Gtn-HPA hydrogel is proposed to be used as delivery vehicles for sustained release of VEGF-C156S to encourage lymphangiogenesis at the aorta site for atherosclerosis regression. VEGF-C is a growth factor belongs to the platelet-derived/vascular endothelial growth factor family, which is well known to encourage both lymphangiogenesis and angiogenesis [22, 23]. Thus, VEGF-C156S, a mutant form of VEGF-C, is proposed to be used to specifically target lymphangiogenesis. The cysteine residue (156) of VEGF-C is being replaced by serine residue, causing the VEGF-C losing the R2 binding affinity towards blood vessels [23, 24]. For this future work, the Gtn-HPA hydrogel encapsulated with VEGF-C156S will be injected to the aorta of apolipoprotein E-Deficient (ApoE^{-/-}) mice to investigate the lymphatic restoration process. ApoE plays the important role in metabolize lipoprotein, thus involved in reserved cholesterol transport and local redistribution of cholesterol [25]. The absence of ApoE will cause mice to have hypercholesterolemia when fed with high-fat diet [26, 27]. The sustained release of VEGF-C156S at aorta of ApoE^{-/-} mice for 3 weeks is proposed to able to encourage lymphatic vessels regeneration and promotes cholesterol clearance. The *in vitro* degradation studies using type-I collagenase (**Chapter 4, Section 4.3.5**) showed that the degradation rate of 10 w/v % Gtn-HPA hydrogel is able to achieved for 3 weeks, thus potentially to be used for the long-term delivery vehicle. Immunofluorescent staining using CD31 and LYVE-1 at 2 weeks post-implantation (**Appendix 1**) showed that the implanted 10 w/v % Gtn-HPA hydrogel with stiffer structure supported both angiogenesis and lymphangiogenesis. Thus, the injectability, degradability and lymphangiogenic properties of the 10 w/v % Gtn-HPA hydrogel showed great potential to be used in this novel lymphatic regeneration application. Ultimately, the success of restoring lymphatic vessels is proposed to cause atherosclerosis regression and prevent cardiovascular disease.

Preliminary studies were done to show that the Gtn-HPA hydrogel is a good support for the growth of the lymphatic cells. **Figure 7.1 and 7.2** showed that the mouse lymphatic endothelial cells (SV-LECs) cultured on the 5 w/v % Gtn-HPA hydrogel fabricated with 0.15 units/ml HRP and 6 mM H₂O₂ had a positive proliferation profile and cumulative cell population doubling rate. The LIVE/DEAD® image the SV-LECs cultured on the Gtn-HPA hydrogel confirm the viability and proliferation of SV-LECs, where increasing in live cells (green fluorescence) can be observed throughout 7 days

of culture. The results showed that the SV-LECs were able to growth well on the Gtn-HPA hydrogel, thus Gtn-HPA hydrogel has a great potential in supporting lymphatic vessel regeneration.

Preliminary *in vivo* biocompatibility and degradability studies were then performed using 10 w/v % Gtn-HPA hydrogel crosslinked with 0.12 units/ml HRP and 10 mM H₂O₂ on the hind limb of C57BL/6 mice (**Appendix 1**). H & E staining images (**Figure A1.1**) showed that the sample was still present at 2 weeks post-implantation and almost completely degraded at 3 weeks post-implantation. Blood vessels can be observed to form around the implanted 10 w/v % Gtn-HPA hydrogel at 2 and 3 weeks post-implantation. In addition, cellular infiltration can be also observed at 2 and 3 weeks post-implantation. **Figure A1.2** showed that neutrophil and macrophages were observed to infiltrate into the Gtn-HPA hydrogel at 2 and 3 weeks post-implantation. Masson's Trichrome staining in **Figure A1.3** further confirmed the present of the implanted 10 w/v % Gtn-HPA hydrogel and blood vessels formed around the sample at 2 and 3 weeks post-implantation. Immunofluorescent staining using CD68 as seen in **Figure A1.5a** confirmed the cellular infiltration by macrophages at 2 and 3 weeks post-implantation. Thus, 10 w/v % Gtn-HPA hydrogel showed to be biocompatible and had slower degradation rate as compared to the 5 w/v % Gtn-HPA hydrogel (**Chapter 4, Section 4.3.7**). The *in vivo* biocompatibility and degradability studies of 10 w/v % Gtn-HPA hydrogel showed its potential to be used as a long-term sustained release vehicle for lymphatic vessels regeneration. Further studies will be investigated on the effectiveness of the Gtn-HPA hydrogel in delivering the VEGF-156S and restoring the lymphatic vessels at aorta of the ApoE^{-/-} mice.

Figure 7.1 Proliferation profiles for the SV-LECs cultured on the Gtn-HPA hydrogel as compared to the TCP (n=5).

Figure 7.2 Cumulative cell population doubling rate of the SV-LECs cultured on the Gtn-HPA hydrogel as compared to the TCP (n = 5).

Figure 7.3 LIVE/DEAD® images of the SV-LECs cultured on (a) the Gtn-HPA hydrogel and (b) the TCP at day 1, 3, 5 and 7 (The green fluorescence represents live cells and the red fluorescence represents dead cells. Scale bar represents 100 μm).

7.4.2 Responds of endothelial cells on the Gtn-HPA/CMC-Tyr hybrid hydrogel with different stiffness for vascular tissue engineering

Vascularized tissue engineered scaffold plays an important role in the effective transportation of nutrients and metabolites to all the encapsulated cells [28]. Cells that are far away from a vascular channel will be lacking in oxygen and nutrients that lead to cell death and tissue necrosis [29]. The sufficient vascularization of the scaffold also will ensure effective integration between the implanted scaffold and host vasculature [30]. One of the strategies to improve vascularization is through endothelialization of tissue engineered scaffold [31, 32]. The presence of the endothelial cells will undergo angiogenesis process to grow and assemble into functional blood vessels [33]. Recent studies had shown that the mechanical properties of the scaffold and chemical factors affect the proliferation, elongation and sprouting [34, 35]. In term of matrix stiffness, endothelial cell proliferation increases [36] while network formation decreases [33, 37] with increasing stiffness. Thus, a compliant matrix needs to achieve to have optimal endothelial cells proliferation and network formation.

A preliminary study was done on 2D cell culture of human umbilical vein endothelial cells (HUVECs) on the Gtn-HPA/CMC-Tyr hybrid hydrogel with different stiffness to investigate the effect of its matrix properties and its potential in supporting angiogenesis. The Gtn-HPA/CMC-Tyr hybrid hydrogels were fabricated using 0.15 units/ml HRP and different H_2O_2 concentrations (1.5, 3 and 6 mM) to achieve different stiffness.

Figure 7.4 showed the cell proliferation profiles for the HUVECs cultured on the Gtn-HPA/CMC-Tyr hybrid hydrogel fabricated with different H_2O_2 concentrations, thus different stiffness, as compared to the TCP. HUVECs cultured on the Gtn-HPA/CMC-Tyr hybrid hydrogel fabricated with 1.5 mM H_2O_2 showed to have slightly increased in the cell number from day 1 to day 3 and the cell number stagnant for day 5 and day 7. Fluorescence images showed that most of the HUVECs were already elongated at day 1 (**Figure 7.5**). Thus, the results showed that sample fabricated with 1.5 mM H_2O_2 possessed a very soft surface that caused the decreases in endothelial cell proliferation but increases in the elongation of the HUVECs. HUVECs cultured the Gtn-HPA/CMC-Tyr hybrid hydrogel fabricated with 3 and 6 mM H_2O_2 showed to have increased in cell number from day 1 to day 3 follow by a slight decrease at day 5 and day 7 (**Figure 7.4**). Fluorescence images at **Figure 7.5** showed that the HUVECs cultured on the Gtn-HPA/CMC-Tyr hybrid hydrogel with 3 and 6 mM H_2O_2 had two distinguished populations: (1) cells with a spheroid shape that resembles those HUVECs that cultured on the TCP and (2) HUVECs with the elongated shape. Thus, the Gtn-HPA/CMC-Tyr hybrid hydrogel fabricated with 3 and 6 mM H_2O_2 showed to have suitable stiffness in encouraging HUVEC proliferation and elongation, which potentially encourage the formation of the vascular network. The slightly decreased in cell number at day 5 and 7 might be due to the HUVECs started to commit themselves to form the vascular network, thus decrease in proliferation rate. The cell number on the Gtn-HPA/CMC-Tyr hybrid hydrogel fabricated with 3 and 6 mM H_2O_2 were significantly higher ($p < 0.05$) as compared to the Gtn-HPA/CMC-Tyr hybrid hydrogel fabricated with 3 mM H_2O_2 at day 7. Meanwhile, HUVECs cultured on the TCP (stiffest surface in this study) showed to have positive proliferation over 7 days of culture (**Figure 7.4**) and the fluorescence images (**Figure 7.5**) showed the HUVECs had a spheroid shape. As seen in **Figure 7.6**, the HUVECs cultured on the Gtn-HPA/CMC-Tyr hybrid hydrogel regardless of H_2O_2 concentration had higher average aspect ratio at day 7 as compared to the TCP. Therefore, Gtn-HPA/CMC-Tyr hybrid hydrogel showed to support the proliferation and elongation of HUVECs and potentially support angiogenesis. In this future work, further optimization of the Gtn-HPA/CMC-Tyr hybrid hydrogel's stiffness and seeding density of HUVECs will be done to achieve a compliant stiffness for HUVECs to completely form a vascular network. The success of achieving a compliant matrix will show the potential of the Gtn-HPA/CMC-Tyr hybrid hydrogel to be used in vascular applications. In addition, enzymatically induced degradation is proposed to be

able to open up more space post-fabrication to potentially enhance the formation of the network within the 3D environment of the Gtn-HPA/CMC-Tyr hybrid hydrogel.

Figure 7.4 Cell proliferation profiles for the HUVECs cultured on the Gtn-HPA/CMC-Tyr hybrid hydrogel fabricated with different H₂O₂ concentrations, thus different stiffness, as compared to the TCP (* $p < 0.05$).

Figure 7.5 Fluorescence images of the calcein AM-stained HUVECs cultured on the Gtn-HPA/CMC-Tyr hybrid hydrogel fabricated with different H_2O_2 concentrations and the TCP at day 1 and 7 (The red arrows represent elongation of HUVECs. Scale bar represents 200 μm).

Figure 7.6 Average aspect ratio of the HUVECs cultured on the Gtn-HPA/CMC-Tyr hybrid hydrogel fabricated with different H₂O₂ concentrations and the TCP at day 7 (n = 30).

7.4.3 UV-degradable hydrogel for smart release

Photolabile linkers are commonly used to link a molecule of interest with a solid support [38, 39]. Upon photoirradiation, the molecule of interest is able to release out from the solid support. In biomedical application, the photolabile linkers are commonly used to release peptide upon photoirradiation [40]. One of the examples will be using *ortho*-nitrobenzyloxy groups in releasing peptides when activated using the wavelength of 350 nm [41]. The used of comparable long wavelength ($\lambda > 300$ nm) to decompose photolabile linkers is able to minimize the damage to the tissue and suitable for biomedical application [42, 43]. Thus, the *ortho*-nitrobenzyloxy group is proposed to link with the Gtn as the solid support and HPA as the releasing molecules to create a novel UV-degradable hydrogel. The releasing of crosslinking site through photoirradiation at $\lambda = 350$ nm is proposed to induced degradation post-implantation of this novel UV-degradable hydrogel and potentially to be used as cells and drug releasing vehicles [44, 45]. The proposed mechanism of the synthesizing UV-degradable precursor is as shown in **Scheme 7.2**, which involves nucleophilic substitution of 3-nitro-4-bromomethylbenzoic acid's bromine molecule with the amine, followed by carbodiimide crosslinking of HPA molecules with the photolabile linker. Finally, the photolabile linker-HPA molecules will be conjugated onto Gtn using the same carbodiimide crosslinking reaction. As seen in **Scheme 7.3**, the precursor is

proposed to be able to crosslink using the HRP and H₂O₂. The crosslinking site is then able to break through photoirradiation using the wavelength of 350 nm.

(a) Nucleophilic substitution

Photolabile linker unit

(b) Carbodiimide crosslinking

(c) Photolabile linker-HPA molecules conjugated onto polymer structure

Scheme 7.2 Proposed mechanisms of three steps synthesis of the UV-degradable hydrogel precursor: (a) nucleophilic substitution of 3-nitro-4-bromomethylbenzoic acid's bromine molecule with amine, (b) carbodiimide crosslinking of HPA molecules with the photolabile linker and (c) carbodiimide crosslinking of the photolabile linker-HPA molecules with the Gtn.

(a) Formation of hydrogel (Peroxidase reaction) **(b) Photo-induced degradation**

Scheme 7.3 Proposed mechanisms of (a) the peroxidase crosslinking of the UV-degradable hydrogel precursor and (b) photo-induced degradation of the UV-degradable hydrogel.

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PUBLICATION LIST

Papers Published

- **Chun, Y.Y.**, Wang, J.K., Tan, N.S., Chan, P.P.Y, Tan, T.T.Y and Chong, C. (2015) A periosteum-inspired 3D hydrogel-bioceramic composite for enhanced bone regeneration. *Macromolecular Bioscience*. DOI: 10.1002/mabi.201500258. (IF: 3.851)

Papers Submitting

- Wang, J.K., Yeo, K.P., **Chun, Y.Y.**, Tan, T.T.Y., Tan, N.S., Angeli, V. and Choong, C. (2016) Fish scale-derived collagen patch promotes functional angiogenesis and lymphangiogenesis in growth factor-free conditions. *To be submitted*.
- Wang, J.K., Yang, Y., **Chun, Y.Y.**, Luo, B., Loh, Q.L., Choo, C.C., Tan, T.T.Y., Tan, N.S. and Choong, C. (2016) Bullfrog skin-derived collagen as potential scaffolds for tissue engineering applications. *To be submitted*.

Papers in Preparation

- **Chun, Y.Y.**, Yeo, K.P., Wang, J.K., Tan, N.S., Tan, T.T.Y., Angeli, V. and Choong, C. (2016) Long term sustained release of VEGF-C156S proteins using injectable phenol-conjugated gelatin hydrogel to promote lymphatic regeneration for atherosclerosis regression. *In preparation*.
- **Chun, Y.Y.**, Yip, D.X.Y., Choong, C. and Tan, T.T.Y. (2016) Enzymatic responsive phenol-conjugated gelatin/carboxymethyl cellulose hybrid hydrogel with tunable drug release rate and its application in bone tissue engineering. *In preparation*.

Conference Presentation

Poster

- **Chun, Y.Y.**, Wang, J.K., Choong, C. and Tan, T.T.Y. (2013) Development of Calcium Phosphate-Hydrogel Hybrid Microcarriers as Bone Filler for Dental Applications. *International Bone-Tissue-Engineering Congress (bone-tec)*. Singapore. December 2013.

Appendices

Appendix 1 *In Vivo* Studies of 10 w/v % Gtn-HPA Hydrogel

In vivo studies was performed using the hind limb of C57BL/6 mice to investigated the biocompatibility, degradability, cellular infiltration and blood and lymphatic vessels formation of the 10 w/v % Gtn-HPA hydrogel. Lymphatic vessels in the hind limb of C57BL/6 mice were dissected to investigate the ability of Gtn-HPA hydrogel in lymphatic regeneration. 10 μ l 10 w/v % Gtn-HPA hydrogel was crosslinked using 0.12 units/ml and 10 mM H₂O₂ and injected onto the hind limb of C57BL6 mice. The *in vivo* biocompatibility and degradability was then carried out as described in **Chapter 3, Section 3.9.1**. At 2 and 3 weeks post-implantation, the implanted 5 w/v % Gtn-HPA hydrogel together with the surrounding tissue were harvested from the C57BL/6 mice followed by the fixation and histological analysis according to **Chapter 3, Section 3.9.2 and 3.9.3**. **Figure A1.1 and A1.2** showed the H & E staining of the implanted 10 w/v % Gtn-HPA hydrogel with surrounding tissue, where the implanted 10 w/v % Gtn-HPA hydrogel was almost completely degraded at 3 weeks post-implantation and cell infiltration can be observed. **Figure A1.3** showed the Masson's trichrome staining of the implanted 10 w/v % Gtn-HPA hydrogel with surrounding tissue, confirming the presence and degradation of the implanted 10 w/v % Gtn-HPA hydrogel. Further immunohistochemical analysis using CD68, CD 31, LYVE-1 and DAPI was done according to **Chapter 3, Section 3.9.4**. Immunofluorescent staining as seen in Figure A1.4 confirmed the cellular infiltration by macrophages and the presences of blood and lymphatic vessels surrounding and within the implanted 10 w/v % Gtn-HPA hydrogel at 2 and 3 weeks post-implantation. For whole-mount immunohistochemical analysis, implanted 10 w/v % Gtn-HPA hydrogels with surrounding tissue were harvested from C57BL/6 mice and fixed overnight with 2 w/v % PFA at 4°C. The fixed patches were then incubated overnight in 0.5 w/v% bovine serum albumin and 0.3 w/v% Triton X-100 in 1× PBS blocking solution at 4 °C. Next, the samples were incubated with anti-LYVE-1 and anti-CD31 antibodies followed by Cy2-conjugated anti-rabbit and Alexa 647-conjugated. The whole-mount implanted 10 w/v % Gtn-HPA hydrogel with surrounding tissue was imaged using fluorescence microscope (Axio imager.Z1, Axiocam HRM camera, Carl Zeiss, Germany) or confocal microscope (FV1200 Laser

Scanning Microscopes, Olympus, Japan). Isosurface rendering and confocal z-stacks were processed using Imaris (Bitplane, UK). The whole mount images as seen in **Figure 4.15** confirmed the growth of blood and lymphatic vessels around and within the implanted 10 w/v % Gtn-HPA hydrogel at 2 weeks post-implantation. Overall, the 10 w/v % Gtn-HPA hydrogel showed to have slower degradation rate and supported both angiogenesis and lymphangiogenesis.

Figure A1.1 H & E staining of the histological section of skin at the magnification of (a) $10\times$ and (b) $20\times$ for the implanted 10 w/v % Gtn-HPA hydrogel at 2 and 3 weeks post-implantation as compared to the sham control [Scale bars represent (a) $200\ \mu\text{m}$ and (b) $100\ \mu\text{m}$. The red dotted line represents the implanted 10 w/v % Gtn-HPA hydrogel's outline. The red arrow represents blood vessel].

Figure A1.2 H & E staining of the histological section of skin at a magnification of $100\times$ for the implanted 10 w/v % Gtn-HPA hydrogel at (a) 2 and (b) 3 weeks post-implantation showing infiltration of the neutrophil and the macrophages into the implanted 10 w/v % Gtn-HPA hydrogel (Scale bar represents $20\text{ }\mu\text{m}$. The yellow arrow represents neutrophil. The red arrow represents macrophage).

Figure A1.3 Masson's trichrome staining of the cross section of skin at the magnification of $10\times$ for the implanted 10 w/v % Gtn-HPA hydrogel at 2 weeks and 3 weeks post-implantation as compared to sham control. [Scale bars represent (a) $200\text{ }\mu\text{m}$ and (b) $100\text{ }\mu\text{m}$. The green dotted line represents the implanted 10 w/v % Gtn-HPA hydrogel's outline. The green arrow represents blood vessel].

Figure A1.4 Immunofluorescent staining using (a) CD68 (red), (b) CD31 (red), (c) LYVE-1 (green) and DAPI (blue) to show the presence of macrophages, blood vessels, lymphatic vessels and cell nuclei respectively for the implanted 10 w/v % Gtn-HPA hydrogel with surrounding tissue at 2 and 3 weeks post-implantation [Scale bar represents 100 μm . The white dotted line represents the implanted 10 w/v % Gtn-HPA hydrogel's outline].

Figure A1.5 Whole mount immunofluorescent staining showing the presence of blood vessels (CD31) and the lymphatic vessels (Lyve-1) growing around and within the implanted 10 w/v % Gtn-HPA hydrogel at 2 weeks post-implantation. The images showed were at a magnification of (a) $5\times$ and (b) $10\times$ [Scale bars represent (a) $400\ \mu\text{m}$ and (b) $200\ \mu\text{m}$. The white dotted line represents 10 w/v % Gtn-HPA hydrogel's outline. POP LN represents popliteal lymph nodes].

Appendix 2 Pore Enlargement of Gtn-HPA/CMC-Tyr Hybrid Hydrogel using Cellulase

2ml Gtn-HPA/CMC-Tyr hybrid hydrogels were fabricated according to **Chapter 3, Section 3.5.2** using 0.15 units/ml HRP and 3 mM H_2O_2 . The Gtn-HPA/CMC-Tyr hybrid hydrogels were treated with different cellulase concentrations (0.1, 1.0 and 2.0 mg/ml) for 1 day. The Gtn-HPA/CMC-Tyr hybrid hydrogels were then lyophilized and view under a microscope (Primo Vert, Carl Zeiss, Germany) to investigate their pore sizes. As seen in **Figure A2.1**, cellulase treatment was able to enlarge the pore size of the Gtn-HPA/CMC-Tyr hybrid hydrogel.

Figure A2.1 Representative macroscopic images of the Gtn-HPA/CMC-Tyr hybrid hydrogels (a) no treatment and treated with (b) 0.1, (c) 1.0 and (d) 2.0 mg/ml cellulose (Scale bar represents 500 μm).

Appendix 3 FITC-dextran Release Profile of Gtn-HPA/CMC-Tyr Hybrid Hydrogels versus Gtn-HPA Hydrogels

Figure A3.1 FITC-dextran release profiles for the Gtn-HPA/CMC-Tyr hybrid hydrogel with different H₂O₂ concentrations as compared to Gtn-HPA hydrogel (n = 3).

Appendix 4 Enzymatic-induced FITC-dextran Release

0.5 ml Gtn-HPA/CMC-Tyr precursor containing 250 µg/ml FITC-dextran (average mol wt of 70 000) were crosslinked using 0.15 units/ml HRP and 3 mM H₂O₂ and cast into 24 well plate. The enzymatic-induced FITC-dextran release study was done according to **Chapter 3, Section 3.7.1**. **Figure A4.1** showed the Gtn-HPA/CMC-Tyr hybrid hydrogel fabricated with 3 mM H₂O₂ and treated with different cellulase concentrations. Before the cellulase treatment, all the Gtn-HPA/CMC-Tyr hybrid hydrogels exhibited similar burst release at day 1, which was ~20 %. In general, the Gtn-HPA/CMC-Tyr hybrid hydrogel treated with cellulase had slightly higher release rate as compared to the Gtn-HPA/CMC-Tyr hybrid hydrogel without treatment. At day 4, the total release reached 50.8 ± 0.4 % for the Gtn-HPA/CMC-Tyr hybrid hydrogel without treatment. Meanwhile the Gtn-HPA/CMC-Tyr hybrid hydrogels treated with 0.1, 1.0 and 2.0 mg/ml had total release of 52.1 ± 0.3 %, 55.1 ± 0.6 % and 54.3 ± 0.9 % respectively.

The Gtn-HPA/CMC-Tyr hybrid hydrogels treated with 1.0 and 2.0 mg/ml cellulase had a similar release profile. The slightly increase in the release rate of the Gtn-HPA/CMC-Tyr hybrid hydrogel treated with cellulase was suggested due to the alteration of Gtn-HPA/CMC-Tyr hybrid hydrogel's degradation rate of through the digestion of CMC. However, the change in the release rate of the Gtn-HPA/CMC-Tyr hybrid hydrogel treated with cellulase is very small as compared to without treatment.

Figure A4.1 FITC-dextran release profiles for the Gtn-HPA/CMC-Tyr hybrid hydrogel fabricated with 3 mM H₂O₂ and treated with different cellulase concentrations.

Enzymatic degradation studies using cellulase were done to have a further understanding on the effect of cellulase concentrations in altering the degradation rate of the Gtn-HPA/CMC-Tyr hybrid hydrogel. As seen in **Figure A4.2a**, BCA assay showed the constant release of proteins from the Gtn-HPA/CMC-Tyr hybrid hydrogel treated with cellulase. The proteins detected from the release buffer solution confirmed the digestion of the Gtn-HPA/CMC-Tyr hybrid hydrogel by cellulase. When higher cellulase concentration was used, more proteins were detected from the release buffer solution. Thus, increasing the cellulase concentration is able to increase the degradation rate of the Gtn-HPA/CMC-Tyr hybrid hydrogel. As seen in **Figure A4.2b**, percentage

weight change of the Gtn-HPA/CMC-Tyr hybrid hydrogel treated with different cellulase concentrations further confirm the alteration of the degradation rate by cellulase digestion. Increasing the cellulase concentrations will increase the percentage weight loss of the Gtn-HPA/CMC-Tyr hybrid hydrogel. In addition, Gtn-HPA/CMC-Tyr hybrid hydrogels treated with 1.0 and 2.0 mg/ml cellulase was able to completely dissolve after 7 and 6 days of cellulase treatment respectively. Hence, when a high cellulase concentration (i.e. 1.0 and 2.0 mg/ml) was used, the CMC-Tyr polymer was significantly being digested and subsequently deteriorating the whole structural integrity of the hybrid system. In addition, as the gelatin debris dissolved in the enzymatic solution, it also resulted in pH changes to an acidic range that rendered the whole system to be acidolysis. As a result, a rapid degradation of the whole system was observed when higher cellulase concentration was used. Overall, the cellulase treatment showed to be able to alter the degradation rate of the Gtn-HPA/CMC-Tyr hybrid hydrogel fabricated with 3 mM H_2O_2 that resulting in the slightly alteration of its release rate.

Figure A4.2 Enzymatic degradation of the Gtn-HPA/CMC-Tyr hybrid hydrogel fabricated with 3mM H_2O_2 treated with different cellulase concentrations showing the (a) BCA protein assay and (b) percentage weight change.