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Macroporous hydrogel formed by high internal phase emulsion-templating and their applications in tissue engineering

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A thesis presented for the partial fulfillment of the Joint Degree of Doctor of Philosophy of the Nanyang Technological University and Imperial College London and the Diploma of the Imperial College

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Declaration of originality

I hereby certify that the work presented in this thesis is the results of my own investigations, except where otherwise acknowledged with all referenced works duly cited to the best of my knowledge.

Bernice Oh

Jan 2014

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ABSTRACT

This thesis reports and discusses the development of several multifunctional biopolymers to be used in a cleaner approach for producing high porosity macroporous polymers by high internal phase emulsion (HIPE) templating for application as tissue engineering scaffolds. The aim is to identify and create biocompatible polymers that can self-stabilize HIPEs, be self-crosslinkable and also act simultaneously as the matrix for the macroporous polymers after emulsification, solidification and removal of the templating phase and aqueous solvent for the polymers.

Firstly, the design of a self-emulsifying biopolymer was carried out using a chitosan based biopolymer grafted with thermoresponsive poly(*N*-isopropylacrylamide) (PNIPAM) and oligolysine (CSNLYS). Self-emulsification of HIPEs was found to be successful using this copolymer. The HIPEs could be solidified by raising the temperature above the lower critical solution temperature (LCST) of the PNIPAM component to 40°C. In addition, it was found that by changing the degree of polymerization of the grafted oligolysine, HIPEs with very different emulsion droplet sizes resulted, ranging from microemulsions with average droplet sizes of 0.13 μm , to macroemulsions with average droplet sizes of 10.5 μm . Polymerized (high internal phase macroemulsion) (polyHIPE) and polymerized (high internal phase

microemulsion) (polyHIPME) were formed by removing the liquid templating phases resulting in closed celled high porosity foams.

Chitosan-*graft*-poly(*N*-isopropylacrylamide)-*graft*-oligoproline (CSN-PRO) and chitosan-*graft*-poly(*N*-isopropylacrylamide)-*graft*-oligo(glutamic acid) (CSN-GLU) were synthesized next to produce self-stabilized HIPE. CSN-PRO was found to be able to stabilize HIPE but not CSN-GLU, forming closed pores with pore sizes ranging from 32 μm to 71 μm . Upon addition of a low concentration of the surfactant PEG(20)sorbitan monolaurate, and varying the polymer concentration and internal phase volume ratio, different polyHIPEs with pore size of up to 143 μm , porosities of up to 99%, surface areas $>300 \text{ m}^2/\text{g}$ and controlled pore interconnectivity can be formed. The CSN-PRO stabilized polyHIPEs are able to retain their thermoresponsiveness and remain intact when immersed into water at physiological temperature but dissolve below their LCST, which is useful in applications such as drug delivery and for tissue engineering scaffolds. Murine embryonic stem cells which are non-anchorage dependent were seeded to assess biocompatibility and were found to be able to survive and enter the pores of the poly(CSN-PRO)HIPE hydrogel.

To produce scaffolds suitable for the attachment of anchorage dependent cells, a polypeptide, gelatin was used to create the self-emulsifying copolymer gelatin-*graft*-PNIPAM (GN). It was found GN does self-stabilize HIPEs. Upon solidification of the HIPEs and the removal of the templating oil phase and water from the aqueous phase highly porous and interconnected tissue

engineering scaffold resulted without the use of any additional surfactant. Poly(GN)HIPEs can be formed by two different solidification mechanisms as conferred by the components of GN, gelatin and PNIPAM. By inheriting the temperature sensitivity of these two components, GNHIPEs can be solidified at either 4°C due to the gelatin component or 40°C due to the PNIPAM component. The physical properties of the resultant self-stabilized poly(GN)HIPEs can be controlled by varying the aqueous phase, emulsion phase volume ratio and solidification temperature. Because of the inherent temperature sensitivity, poly(GN)HIPE hydrogels are able to response rapidly to changes in temperature during the initial cell culturing period. Fibroblast cells seeded into the scaffold were seen to thrive, spread and proliferate in a culture period of 10 days, with a maximum depth of penetration of 360 μm . The cell-laden poly(GN)HIPE scaffold was shown to be injectable through a syringe without harming the encapsulated cells. This system provides a new strategy for the easy fabrication of safe and injectable biocompatible scaffolds for tissue engineering.

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LIST OF ABBREVIATIONS

2D	2-dimensional
3D	3-dimensional
ADC	anchorage dependent cells
ATR-FTIR	Attenuated total reflectance - Fourier Transform Infrared spectroscopy
CAD	computer assisted design
CAN	ceric ammonium nitrate
CS	chitosan
CSN	chitosan- <i>graft</i> -poly(<i>N</i> -isopropylacrylamide)
CSN-GLU	chitosan- <i>graft</i> -poly(<i>N</i> -isopropylacrylamide)- <i>graft</i> -oligo(glutamic acid)
CSNLYS	chitosan- <i>graft</i> -poly(<i>N</i> -isopropylacrylamide)- <i>graft</i> -oligolysine
CSN-PRO	chitosan- <i>graft</i> -poly(<i>N</i> -isopropylacrylamide)- <i>graft</i> -oligoproline
D ₂ O	deuterium oxide
DCI	deuterium chloride
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulphoxide
DP	degree of polymerization
DS	degree of substitution
DVB	divinylbenzene
ECM	extracellular matrix
EDC•HCl	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride

ESC	embryonic stem cells
FBS	fetal bovine serum
GAG	glycosaminoglycans
GN	gelatin- <i>graft</i> -poly(<i>N</i> -isopropylacrylamide)
GPC	gel permeation chromatography
HCL	hydrochloric acid
hESCs	human embryonic stem cells
HIPE	high internal phase emulsion
IFT	interfacial tension
LCST	lower critical solution temperature
LIF	Leukemia Inhibitory Factor
mESCs	murine embryonic stem cells
MW	molecular weight
MWCO	molecular weight cut off
NaOH	sodium hydroxide
NHS	<i>N</i> -hydroxysuccinimide
NIPAM	<i>N</i> -isopropylacrylamide
NMR	nuclear magnetic resonance
o/w	oil-in-water
PCL	poly(caprolactone)
PEG	poly(ethylene glycol)
PEO-PPO-PEO	poly(ethylene oxide)– <i>b</i> -poly(propylene oxide)– <i>b</i> -poly(ethylene oxide)

PGA	polyglycolic acid
PLA	polylactic acid
PLG	poly(lactide- <i>co</i> -glycolide)
PLGA	poly(lactic- <i>co</i> -glycolic acid)
PLLA	poly-L-lactide
PLLA-CL	poly(L-lactide- <i>co</i> - ϵ -caprolactone)
polyHIPE	poly(merized)high internal phase emulsion
polyHIPME	poly(merized)high internal phase microemulsion
Ref.	reference
RGD	Arg-Gly-Asp
SEM	scanning electron microscope/y
St	styrene
TCPS	tissue culture polystyrene
TEMED	N,N,N',N'-tetramethylethylenediamine
TIPS	Thermally induced phase separation
UV	ultraviolet
w/o	water-in-oil

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Patent application

US Provisional Patent Application No. 61/886,891 “Chitosan-functional polymer as a self-emulsifying thermoresponsive polymer for high internal phase macro-/micro-porous foam via a polymerization-free route” Chan Bee Eng Mary, Oh Hui Lin Bernice, Alexander Bismarck, filed on 14 October 2013

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3. “Chitosan-graft-PNIPAM-graft-Oligolysine as a Self-Emulsifying Thermoresponsive Polymer For Poly (High Internal Phase Macro- And Micro-Emulsion) Via A Polymerization-Free Route”, Bernice Hui Lin Oh, Alexander Bismarck, Mary B. Chan-Park (*In progress*)

4. “Solidifying Thermoresponsive Surfactant-free High Internal Phase Emulsions stabilized solely by Gelatin-g-PNIPAM with dual solidification mechanism; Solid HIPEs for Tissue Engineering”, Bernice Hui Lin Oh, Alexander Bismarck, Mary B. Chan-Park (*Submitted*)
5. “The suitability of dextran-*graft*-PNIPAM for mESCs encapsulation”, Bernice Hui Lin Oh, Alexander Bismarck, Mary B. Chan-Park (*In preparation*)

CHAPTER 1

INTRODUCTION

1.1 Background and Motivation

Looking back from the 21st century, the human species have survived and progressed leaps and bounds since the start of human history. Ingenious minds have created huge advancement in technologies and mankind are now living a better life never before envisioned, longer than ever before. Nevertheless, it is still inevitable throughout the course of history that humans have to struggle for their life against diseases. This struggle has motivated us to intensely invent and improve strategies to ensure our survival as a species in this world.

The most likely cause of mortality in modern times with our globally ageing population is end-stage organ failure, whereby the organ completely loses its function to maintain normal survival conditions, with diseases of the heart being the most common cause of deaths in the world [1-2]. The most intuitive solution would be to replace the damaged organ with a healthy one by organ transplantation. This technique, though proven to be possible, has seen limited success due to the severe lack of suitable donors and immunorejection of the guest organ by the host body [3]. This pressing need to find alternative solutions has sparked the dawn of tissue engineering techniques in which a

proactive approach is taken to try to grow the replacement parts in the laboratory prior to implantation [4]. This technique requires the integration of the patient's autologous cells into synthetic scaffolds that temporarily host and support the growth of cells into a functional tissue that can replace lost tissue or impaired organ function *in vivo* after implantation into the patient.

Hence, scaffolds for tissue engineering play a pivotal role in providing support for anchorage dependent cells to adhere to and grow in a 3-dimensional (3D) environment for their spreading and proliferation [5]. To be deemed suitable for tissue engineering, scaffolds must possess certain characteristics that facilitate cells seeding and proliferation within the scaffold. The most important consideration in scaffold design is its biocompatibility, porosity, pore interconnectivity and biodegradability, which has a direct impact on the mass transport of nutrients and cell migration [6-7]. Hence, scaffolds with high porosity and degree of pore interconnectivity will provide capacity for higher cell density and facilitating cell-cell junctions to form as well as for diffusion of nutrients into and metabolic waste out of the scaffold.

Various strategies of producing porous scaffolds commonly include porogen leaching, gas foaming and more recently rapid prototyping [8]. While porogen leaching techniques are able to create scaffolds with interconnected pores, it requires the inevitable use of organic chemicals, which if not thoroughly removed will pose a risk to the cells cultured [9]. Gas blowing on the other hand, eliminates the use of organic solvents by creating pores using

supercritical carbon dioxide. Nonetheless, this results often in practically closed-pore structures [10]. In the past 15 years, with the advent of rapid prototyping techniques, scaffolds of any dimensions and structure imaginable can now be created. The limitations presented thus far are the requirement for specialized equipment, the limited types of suitable polymers which can be processed, and the rigidity of the scaffolds produced, which confine its use mainly to bone tissue engineering [11].

To overcome these challenges, the method of high internal phase emulsion (HIPE) templating is identified as an effective and facile way to create tailor-made very high porosity, interconnected macroporous polymer foam [12-16]. HIPEs are formed by emulsifying a mixture of oil and water with an internal phase volume fraction (ϕ) greater than 74.05% using a suitable emulsifier. A poly(merized)HIPE is subsequently formed by polymerizing or solidifying the continuous emulsion phase and after removing the liquid dispersed templating phase. Nevertheless, there are only a few studies which demonstrate the use of polyHIPEs as scaffolds in tissue engineering [17-20], which mostly shows limited cell spreading within the scaffold. This is because the pore and, therefore, pore throat sizes of polyHIPEs are relatively small (Figure 1-1) and the polymer formed by polymerization of the continuous emulsion phase are mostly hydrophobic, which impedes cell spreading.

Figure 1-1 Scanning electron micrograph of a representative conventional polyHIPE. (Reproduced with permission from Ref.[21])

It has been found that the optimal pore size for tissue engineering scaffolds is recommended to be in the range of 50 μm to 300 μm depending on the cell type [22]. In recent years, hydrophilic biopolymers have been explored in the production of polyHIPEs [23-27]. However, strategies to increase the pore sizes and their interconnectivity in these cases made use of introducing salt and organic solvents such as DMSO in the emulsion to destabilize it partially or require the addition of surfactants [28]. These organic solvents and surfactants are difficult to remove completely and this can be detrimental to the cultured cells [29].

HIPE templating is the most straightforward method to produce scaffolds with well-defined and tailor-made highly porous and interconnected pore structures, but require large amounts of surfactants or stabilizing particles. Hence, it would be ideal if a multifunctional biocompatible and degradable biopolymer could be designed, which acts simultaneously as emulsifier and as the matrix for the

polyHIPE but does not require additional chemical reactions for its solidification. Thereby, risk-free scaffolds can be produced easily.

1.2 Aims and Objectives

The aim of this study was to create and develop porous injectable scaffolds for soft tissue engineering using oil in water (o/w) high internal phase emulsion (HIPE) templating, taking a ‘cleaner’ approach by developing multifunctional self-emulsifying polymers, which do simultaneously act as the polymer matrix of the scaffold. The specific objectives are as follows:

- To develop multifunctional biopolymers that act as emulsifier for o/w HIPEs, self-crosslink and also form the bulk matrix of a macroporous polymer with a high degree of pore interconnectivity
- To develop scaffolds with well-defined porous structures with appropriate pore size, high pore interconnectivity and high porosity for tissue engineering without the need of harmful surfactants.
- Fabrication of injectable 3D scaffold platforms for tissue engineering suitable for incorporation with non-anchorage and anchorage dependent cells

1.3 Thesis Outline

This thesis presents my work on the development of multifunctional biopolymers acting as emulsifiers that undergo self-crosslinking upon experiencing a temperature trigger and also form the polymer matrix of a HIPE-templated high porosity macroporous polymer that can be used as scaffold for tissue engineering. The synthesis and properties of the polymers were characterized; their ability in stabilizing HIPE was investigated as well as their suitability as tissue engineering scaffolds. Chapter 2 gives an in-depth review of the relevant literature. Chapter 3 describes the common materials and methods used throughout the work done in this thesis. Methods, which are unique to the work presented in the different chapters, are found in their respective Experimental Method section of each chapter. Chapter 4 through Chapter 6 describe in detail the research work done in this thesis. Chapter 4 describes the synthesis of a copolymer based on poly(*N*-isopropylacrylamide) and oligolysine grafted chitosan and its polymer properties are reported. Investigations on the suitability of chitosan-*graft*-poly(*N*-isopropylacrylamide)-*graft*-oligolysine as emulsifier for HIPEs, which acted as templates for polyHIPEs and their characterization in terms of the pore structure, porosity and surface area are presented and discussed. In Chapter 5, the oligo (amino acid) component of the chitosan-based copolymer was varied to oligoproline and oligo(glutamic acid) and their resultant solution properties and surface active properties were elucidated. A further study on varying the polymer concentration, internal phase

volume ratio and presence of surfactant for stabilizing HIPEs is also presented. The characterization of the properties of the resultant foams formed is shown and the feasibility of seeding murine embryonic stem cells into these scaffolds is also explored in this chapter. Chapter 6 discusses the formation of a gelatin-based thermoresponsive copolymer modified with poly(*N*-isopropylacrylamide) on the production of self-stabilized HIPE-templated foams suitable for anchorage dependent cells seeding and attachment. The possibility of solidifying these self-stabilized emulsions by two different temperature responsive mechanisms was investigated and the properties of the resultant foams were characterized in this study. A study of the performance of anchorage dependent human foreskin fibroblast cells seeded into these scaffolds is also presented and the feasibility of subjecting cell-laden porous hydrogel scaffold to injection, evaluated by the viability and morphology of the cells within the ejected hydrogel, is also elucidated in this chapter. Finally, Chapter 7 draws overall conclusions and gives recommendations for future work.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of Tissue engineering

Tissue engineering refers to the re-creation of tissues as substitutes of native tissues or the repair of damaged tissue by amalgamating biology with materials engineering and cell transplantation [4]. It aims to regenerate the functionality of damaged organs or tissue in the body as one of the main motivations of tissue engineering arises from the pressing need to replace or repair the functions of damaged or failed organs. Although organ transplantation is an available option, shortage of healthy organ donors and immunorejection presents a major setback for this technique [3]. This is a major cause of mortality in diseases in which patients have limited time until a suitable organ is found [1]. Taking for example, cardiovascular disease, according to statistics from the American Heart Association, only a mere 2,300 people out of 40,000 patients in the U.S.A. are able to receive a heart transplant [30]. Tissue engineering is envisioned as alternative that is potentially capable of overcoming these problems by expanding autologous cells *in vitro* into functional tissue that would be implanted back into the defect site.

Figure 2-1 Typical tissue engineering cycle; Cells are first isolated from the patient (autologous cells) and expanded. Thereafter, cells are retrieved and incorporated into highly porous 3D scaffolds with interconnected pores, together with growth factors. When the cells cultured in the 3D scaffolds have organized themselves into functional tissues, the tissue-matrix construct is then implanted back into the patient.

Tissue engineering approaches attempt to reproduce the native environment of the cells *in vitro*, and this typically involves the interaction of cells, scaffold and their microenvironment. The ultimate aim is to re-create tissue that functionally, as well as morphologically, mechanically and chemically, resembles that of the native tissues. This is usually done by introducing cells into a temporary scaffold, which should mimic the extracellular matrix (ECM) of the cells'

native environment. Growth factors, drugs or other nutrients can be delivered to the cells directly or released by the scaffold or suitable carriers, including the scaffold. If the environment is favorable, the cells will then settle in the scaffold and proliferate, migrate or differentiate [31-32]. When the ideal cell mass has been achieved, this engineered tissue-matrix construct is implanted into the defect site to replace lost tissue mass or aid the regeneration of the functionality of the damaged organ [33] (Figure 2-1). Examples of attempts to implant tissue engineered constructs have seen successes in repairing cartilage [34], skin [35], resulted in vascular grafts [36], and bladder constructs [37], which further reinforce the promise of tissue engineering strategies as a solution for more disease models.

2.2 Cell Types

There are generally two classes of mammalian cells that can be used in tissue engineering, namely anchorage dependent cells and suspension cells, which exhibit different behaviors and require slightly different culture conditions and have requirements on their scaffold environment.

2.2.1 Anchorage dependent cells

Most of the mammalian cells types are anchorage dependent cells (ADC). ADC require a surface that contains factors that they can recognize in order to generate intercellular signals for attachment to the surface and to spread. These factors are surface ligands that contain the cell-binding domain, peptide sequence Arg-Gly-Asp (RGD). These ligands such as fibronectin, laminin and

vitronectin are recognized by integrin on the cell surface membrane, that will then prompt them to spread out and proliferate, without which, ADC will choose to undergo apoptosis [38-43]. As a result, the morphology of proliferating ADC are usually flat and spread. Examples of ADC include fibroblasts, osteoblasts, cardiomyocytes, smooth muscle cells to name a few. Of the many materials studied, collagen in particular is found to be the best candidate that is able to provide recognition sites to support the attachment and growth of ADC [44-46].

2.2.2 Suspension cells

Suspension cells are non-anchorage dependent cells that as its name suggests, do not require an anchorage surface to survive and typically exhibit a rounded morphology in their native environment. They are able to grow without a scaffold, suspended in cell medium, forming cell aggregates as they proliferate. These cell types are mostly hemopoietic cell lines, and multi-potent cells like chondrocytes and pluripotent stem cells, which can be triggered to differentiate by environmental stimuli. Of the different types of stem cells, embryonic stem cells (ESCs) are the most attractive cell source. ESCs are isolated from the inner cell mass of the blastocyst of the embryo and being pluripotent, they are able to differentiate into all cell types [47-50]. For example, ESCs were found to be able to differentiate into all cardiac cell types [51-53] and have been shown to be able to integrate into infarcted hearts and improve myocardial function [54-56]. ESCs were also found to be able to be expanded in culture for

a long time without karyotypic changes, hence providing a good supply of cells [57-58]. Tissue engineering with suspension cells is preferably cultured in spinner vessels or rotating bioreactors, in which the gas exchange will fare better than static culture and higher number of cells can be cultured. Works done on culturing murine ESCs (mESCs) in bioreactors demonstrated good reproducibility of large scale cells expansion while maintaining their pluripotency [59-60]. The performance and control of differentiation can be further enhanced by first encapsulating the ESCs in 3-dimensional (3D) hydrogel beads and further culturing in a bioreactor [61-62].

The ultimate aim of culturing stem cells would most likely be differentiating them into functional tissues, and these differentiated cells type would most likely turn out to be ADC, which then require an attachment surface in culture to bind to continue to proliferate. Nevertheless, suspension culturing of pluripotent stem cells is still a good approach for initial cell expansion in numbers.

The real challenges are however the isolation and purification of the required cells types from the cocktail of differentiated cells due to the inhomogeneous differentiation of ESCs into the different cell types during culture, their tendency to develop teratoma [63-64] and the ethical constraints that surround the use of human ESCs.

2.3 2D versus 3D Scaffolds

The choice of tissue engineering scaffolds is of the utmost importance as it is the environment that cells are going to reside in and interact with before they proliferate and form new tissue *in vitro* or undergo apoptosis. The environment has a huge influence on shaping the cells' behavior, dictating their response and determining their viability and functionality. Indeed, it has been shown that the structural features of scaffolds does influence cell adhesion, proliferation and differentiation, and subsequently dictate the cell response [65].

2D tissue culture has been the paradigm of tissue engineering and has been shown to be able to support cell viability and proliferation [66-67]. However, it was soon realized that tissue cultured on 2D scaffolds is a far less accurate model of native tissue as it causes cells to be abnormally polarized [68]. This is because the ECM, neighboring cells and integrin binding sites are only available to a portion of the cell in contact with the 2D surface. This polarized integrin binding causes the cells to adopt an unnatural morphology which subsequently affects intracellular signaling and phenotypic regulation [68]. In addition, nutrients, soluble factors, and waste can only transverse the cells in one area, creating a non-physiological environment and causing polarized cell growth *in vitro*. Nevertheless, culturing cells in 2D still remains a convenient method for cell expansion in the laboratory, rather than as scaffold for cell transplantation.

3D scaffolds have been the focus in current tissue engineering research as they better mimic the cells' native environment, giving the cultured cells a more natural stimulus to develop characteristics that closely resemble native tissues [69]. The 3D scaffold environment allows the cells to contact the synthetic ECM in all directions and allows soluble factors to be diffusion-controlled and more homogeneously available to the cells, giving more control to the cell phenotype. Indeed, it was shown that chondrocytes seeded on 2D surfaces were observed to dedifferentiate and have a flattened morphology, whereas when grown in 3D scaffolds, the chondrocytes were able to maintain their differentiated phenotype and function [70]. In addition, cultured skin cells when challenged under cytotoxic conditions showed better resilience in a 3D culture environment than in 2D culture [71]. A study of cardiomyocytes cultured in 3D environment resulted in higher cell seeding density and better maintenance of their high metabolic activity [67]. Also, a comparison of seeding human ESCs (hESCs) in 2D and 3D culture for the purpose of osteogenesis revealed that the 3D environment enhanced the differentiation of hESCs into the desired phenotype (osteoblasts), given that the same type and amount of culture media was given to both tissue culture systems [72].

2.4 Requirements of 3D Scaffolds

Scaffolds for tissue engineering serve as a template for cultured cells to reside in and grow. They should therefore be as close a mimic to their native environment as described in the previous section. Different cells types have

slightly different requirements. To facilitate the optimum regeneration of tissue in culture, not only do the properties of the 3D scaffolds need to be biocompatible, they also have to be controlled in terms of their pore architecture, surface properties, mechanical properties and degradability, which will be described further in the following sections.

2.4.1 Porosity and pore interconnectivity

Porosity of a scaffold is an important factor for successful tissue regeneration in 3D scaffolds. The measure of porosity of a 3D scaffold is the percentage of voids or pores present in the bulk structure and is usually preferred to be greater than 90% [67, 73]. High porosity and degree of pore interconnectivity in a scaffold are required to enhance cell seeding and cells ingrowth, and also to facilitate the transport of nutrients into and waste out of the matrix [6]. Typical pore sizes of tissue engineering scaffolds are in the range of 50 μm to more than 300 μm depending on cell types, in which the lower limit is determined by the size of typical cells which is approximately 20 μm [74].

The pores are required to be large enough for the cells to enter and migrate into the scaffolds, but also not too large so that the scaffold does still provide a high specific surface area for a sufficient number of cells to attach [74]. Indeed, differences in pore sizes were found to elicit different behavior of the cells in terms of cell adhesion and cell activity [75]. The optimal pore size of a scaffold depends on the cell type as well as the type of scaffold. For example, fibroblast cells were shown to be able to attach on poly(L-lactic acid) (PLLA) scaffolds

with a wide range of pore sizes from 38 μm to 150 μm , but prefer to attach only when pore sizes are greater than 90 μm when they were cultured in a stiffer silicon nitride scaffold, whereas endothelial cells were found to attach when pore sizes were smaller than 80 μm [76-77].

2.4.2 Surface properties of scaffolds

The surface properties of a scaffold which the cells are in contact with are an important consideration for fabricating tissue engineering scaffolds as the cells are very sensitive to even nanoscale changes in their local environment [78-79]. Hydrophilic surfaces have been proven to be much more preferable for cell attachment as in the native environment cells are primarily in contact with an aqueous environment. Attachment factors are also more likely to adhere to hydrophilic surfaces [80-81]. Provision of a high surface area is also desirable especially for sufficient ADC to attach and grow [7] to achieve a critical cell mass during the culture.

2.4.3 Mechanical properties of scaffolds

Mechanical properties of scaffolds must also be tuned the cultured cells; the aim is to recreate similar mechanical properties as the cells' native ECM. Scaffold substrate stiffness were found to influence the morphology of seeded cells by affecting the actin cytoskeletal reorganization, thereby playing a major role in determining their phenotype, spreading, contractility and motility [5]. Indeed, controlling the scaffold mechanical properties was found to be able to direct the differentiation of stem cells into different cell lineage [82-83].

Different cell types require different mechanical scaffold properties. For example in bone tissue engineering, stiffer scaffolds with elastic moduli in the range of 100 GPa are required to allow the tissue engineered construct to develop the required properties of engineered bone tissue [84-85]. On the other hand, for scaffolds for soft tissue engineering such as skin tissue, require stiffness's as low as 50 Pa [86]. A delicate balance should exist as too stiff a scaffold may allow cellular survival but impedes cell proliferation and long term viability, whereas, too soft a scaffold cannot provide adequate mechanical support for the cells to spread and grow into [87]. For example, in the case of cardiomyocytes, the scaffold must be able to withstand the mechanical forces of the beating cardiomyocytes while continuing to support their viability [88].

2.4.4 Degradability of scaffolds

Degradation kinetics of a scaffold are also important as timely degradation of the scaffold is required to provide space for the cells to proliferate further [89]. The ideal degradation rate is one that matches the rate of tissue formation [90]. This is because by balancing the degradation rate with the proliferation rate of cells, the scaffold is able to provide the mechanical support required in the early stages and creates space for subsequent cell growth in the later stages of cell culture [91]. The best approach is to use biodegradable scaffolds that are able to harmonize the degradation behavior with the cell proliferation rate, in which the degradation by-products have to be non-toxic and non-inflammatory [92].

The degradation of the scaffolds occurs by different mechanisms depending on the materials from which the scaffolds were produced. Commonly used synthetic polymers such as poly(lactic-co-glycolic acid) (PLGA) or polyglycolic acid (PGA) scaffolds typically degrade by the chemical hydrolysis of ester bonds into non-toxic by-products which can be removed by the body [92-93]. On the other hand biopolymers such as proteins like collagen [94], or polysaccharides such as chitosan [95-96] or dextran [97] do degrade by the action of enzymes secreted by the cells present in the body.

2.5 Materials Used for the Fabrication of Tissue Engineering Scaffolds

Throughout the short history of tissue engineering, many materials have been tested and screened for their suitability to support cell growth [98]. Materials that have been found to be biocompatible and shown success as tissue engineering scaffolds range from metals and ceramics which are used mainly in bone implants, to polymer materials that are widely used for most other types of soft tissue engineering. Polymeric materials are broadly classified into synthetic polymers and biopolymers, which will be further discussed in the following sections.

2.5.1 Synthetic polymers for tissue engineering applications

A myriad of synthetic polymers have emerged as biocompatible materials for tissue engineering scaffolds. Synthetic polymers offer great flexibility in

controlling their (surface) properties as their hydrophilicity, molecular weight, chain length, chemistry, can be tuned depending on the application. Examples of some successful synthetic polymers and copolymers used in tissue engineering are tissue culture polystyrene (TCPS), which is most widely used in 2D expansion culture, and also PGA [92], PLGA [10, 90], poly-L-lactide (PLLA) [99], poly(L-lactide-co- ϵ -caprolactone) (PLLA-CL) [100], polyethylene glycol (PEG)–PLLA (PEG-PLLA) [101-102] for fabrication of 3D scaffolds, just to name a few.

2.5.2 Biopolymers for tissue engineering applications

Biopolymers are polymers derived from natural sources such as plants and animals, and can be classified into proteins and polysaccharides. They are more preferable as compared to synthetic polymers because they were shown to better mimic the native ECM environment of the cells, on top of being biocompatible and biodegradable [103]. It has been shown that when cells were cultured in an environment which closely resembles native conditions, they will also give a response that more closely matches their native properties [69]. The native ECM comprises of polysaccharides and proteins, namely glycosaminoglycans (GAG) of the proteoglycan of the ECM, such as chondroitin sulphate or hyaluronic acid (Figure 2-2A and B) [87] and proteins, such as laminin or collagen [104]. GAGs *per se* are not widely used due to their low molecular weight and the high cost of obtaining them [105]. Here we will

focus on some biocompatible polysaccharides and proteins that are widely used in tissue engineering.

Figure 2-2 Examples of glycosaminoglycans: A) chondroitin-6-sulphate and B) hyaluronic acid; and some examples of biomimetic polysaccharides: C) dextran and D) chitosan.

2.5.2.1 Dextran

Dextran is a high molecular weight polysaccharide, which is most commonly derived from microorganism *Leuconostoc mesenteroides*. It has chains formed by $1\alpha\rightarrow6$ glycosidic linkages and side branches attached by $1\alpha\rightarrow3$ linkages, [106-107] (Figure 2-2C). It is hydrophilic and has structures very similar to GAG and also possesses many modifiable pendant hydroxyl groups, which makes it an ideal candidate for attaching cell affinity ligands or crosslinking

functionality. Because of its ease in availability and modification, dextran has been used in numerous studies as tissue engineering scaffolds, particularly in hydrogels, and has been shown to be a good support for the growth and proliferation of cells [108-111]. For example, dextran-based hydrogels were shown to be beneficial for ESCs viability and directing specific differentiation lineage [112].

2.5.2.2 Chitosan

Chitosan is the only cationic polysaccharide consisting of β -[1 $\rightarrow$ 4]-linked 2-acetamido-2-deoxy-D-glucopyranose and 2-amino-2-deoxy-D-glucopyranose (Figure 2-2D). It is obtained by the alkaline deacetylation of chitin, which is commonly derived from the exoskeleton of crustaceans such as shrimps, prawns, crabs or lobsters [113] and can be degraded by lysozymes secreted by the cells [114-116]. It has been used in various biomedical applications for instance for the fabrication of tissue engineering scaffolds [117], as anticoagulant [118], and gene delivery vehicle [119] to name a few. Being a positively charged biopolymer gives it the added advantage of enhancing cell attachment because cell surface membranes are negatively charged [120-121]. However, most of the applications are restricted to work in acidic pH because chitosan is insoluble at physiological pH. This also greatly limits the method in which chitosan scaffolds can be formed, to mainly phase separation from acidic solutions [116, 122-123]. The potential of using chitosan can be fully harnessed if the chitosan is modified so it is soluble at physiological pH.

Strategies to solubilize chitosan

The main reason why chitosan is only soluble at low pH is the formation of hydrogen bonds between the amine groups of its glucosamine unit and the abundant hydroxyl groups on the glucosidic rings [113]. When chitosan is added into a solution with a pH below the pK_a of 6.4 of the amino groups, the amino groups become protonated and this disrupts the intermolecular hydrogen bonding between the abundant -NH₂ and -OH groups of the glucosidic rings of chitosan. Therefore, its solubility is largely dependent on the degree of deacetylation (DD) of the chitosan used as well as its molecular weight. To render chitosan soluble above pH 6.4, several strategies have been employed to disrupt this intermolecular hydrogen bonding. One of the methods includes decreasing the DD of chitosan to below 50% [124], but this will greatly reduce the amount of amine groups for post-functionalization. It was also suggested that decreasing the DD will reduce the crystallinity of chitosan and, therefore, increase solubility but at the same time will increase its biodegradation rate [125]. Reducing the molecular weight of chitosan by chemical or enzymatic hydrolysis [126-127] can also improve solubility but the lower molecular weight affects the mechanical properties and degradation rate of chitosan and limits its applications. Another method involves grafting of moieties either onto the hydroxyl group or onto glucosamine -NH₂. Examples include grafting dimethyldecylammonium [128], poly(*N*-isopropylacrylamide) (PNIPAM) [129] onto medium or low molecular weight chitosan or other commercially available chitosan copolymers, such as carboxymethyl-chitosan, *N*-phthaloylchitosan,

and chitosan-g-PEG [130]. However, it was reported that most of the modified chitosans have only have a slightly improved pH solubility of up to pH 7.4, and still do not dissolve in water over wider pH range or at higher concentrations [131].

2.5.2.3 Gelatin

Gelatin is a natural polypeptide formed by the hydrolytic degradation of collagen's triple-helix coil into random coils. It is well-known for its biocompatibility and has been used in the numerous tissue engineering applications [132-135], providing good cell attachment and excellent cell viability as compared to less- or non-gelatinized scaffolds [136-137]. Gelatin has a high percentage of NH_2 groups, which can be used in a wide range of reactions. An important feature of gelatin is the presence of cell attachment sites to allow ADC to adhere to and survive. Although commercially available RGD tripeptide by itself has been shown to allow integrin binding of cells and support cells spreading, gelatin is a more attractive option as it is very cheap and readily available. Furthermore, gelatin is biodegradable by proteases secreted by cells during their growth [138-139], which is an added advantage as timely degradation of the scaffold is imperative to provide space for the cells to further proliferate. Work done on UV photopolymerized dextran-gelatin hydrogel formulation has shown that the polymer mixture is suitable for cell encapsulation and can further support their viability and proliferation [87].

Gelatin solution typically dissolves in water at temperature above 30°C and the polypeptide chains exists as single coil in solution. When a gelatin solution is

left to cool below its gel point of 25.8°C [140-141], gelatin chains are able to partially reform the collagen triple-helix structure during the gelling process when some of the chains undergo a coil to helix transformation to form ordered junction zones held together by hydrogen bonds [142].

In consideration of this thermoresponsive property, strategies have to be developed for gelatin chains to be held together at physiological temperature of 37°C to maintain a solid structure for cell culture. Methods to do so include chemical crosslinking using glutaraldehyde or genipin as crosslinkers [143-144], attaching vinyl groups to enable radical crosslinking [28, 87, 145], enzymatic crosslinking catalyzed by microbial transglutaminase [146] and physical crosslinking methods like temperature induced physical crosslinking with grafted PNIPAM [147].

2.6 Methods to Produce 3D Scaffolds and Implantation Methods

Many different 3D scaffolds have been designed for the tissue engineering applications. These include rigid scaffolds such as meshes [148-149], porous sponges [67, 73], fibers [148, 150] (Figure 2-3) and soft scaffolds like hydrogels [151] (Figure 2-3).

Figure 2-3 Examples of some 3D scaffolds for tissue engineering; a) Meshes [152] b) porous sponge [153] c) fibers [154]. (Figures reproduced from the respective references with permission)

These scaffolds should be biocompatible and also allow the cells to adhere to, reside in and survive and proliferate as described in earlier sections. Hence, various methods were devised that aim to produce scaffolds that fulfill the requirements for cell culture, with most efforts put into creating adequate porosity and pore interconnectivity.

After successfully forming a suitable scaffold and achieving relevant tissue growth and mass, the logistics of implanting these engineered tissue-matrix constructs into an *in vivo* defect site has to be considered next. Methods of

implantations of tissue-matrix constructs will then depend greatly on the physical properties of the scaffold - whether they are rigid or flexible. For rigid scaffolds, the cultured cells will either have to be removed from the internal of the scaffolds and injected into the defect site, in which the localization of the injected cells at the defect site might be a problem, or the entire tissue-matrix construct can be implanted directly into the defect site by open surgery. This is common for bone tissue engineering in which the implanted scaffold should provide suitable mechanical support for the bone recovery process [155-156]. For soft and flexible scaffolds such as hydrogels, direct injection of the cells and scaffold mixture into the defect site can be achieved. This is more desirable for administering soft tissue as injection is minimally invasive to the patient and it can ensure that the tissue-matrix construct can fill any irregular shape of the defect site in contrast to a rigid preformed scaffold.

The next section will talk about some of the methods of production of porous scaffolds, categorizing them into rigid and soft scaffolds and their advantages and disadvantages as tissue engineering scaffolds.

2.6.1 Rigid scaffolds in tissue engineering

2.6.1.1 Porogen leaching to produce scaffolds

Figure 2-4 SEM of A) fused NaCl particulate porogens for fabrication of B) PLGA scaffold. (Reproduced with permission from Ref.[157])

The porogen leaching method is by far the most common method used in fabrication of tissue engineering scaffolds [7]. It involves the use of porogen particles, usually salt particles [158-159], which are first dispersed in a polymer solution (e.g. PLG, PLGA etc.) in which the solvent is usually organic (e.g. chloroform etc.). The solvent is then removed by evaporation and the particulate porogens are leached away, leaving behind a porous polymer matrix [9] (Figure 2-4). The porosity achieved from this method does depend on the amount of porogen used and can be greater than 93% with pore sizes of up to 500 μm [160]. However, this method uses large quantities of organic solvents may not be desirable due to the risk of incomplete removal of the template.

2.6.1.2 Gas foaming to produce scaffolds

Figure 2-5 Scaffold produced by A) gas foaming method (Reproduced with permission from Ref. [10]) and B) combination of gas foaming and salt leaching method. (Reproduced with permission from Ref. [161])

Gas foaming method for producing porous foams involves the use of gas as a porogen in a polymer solution. This method can avoid the use of organic solvents when the gas used involve the use of supercritical carbon dioxide as the blowing agent, which can be easily removed. For example, solid PLGA discs are first exposed to carbon dioxide at high pressure to saturate carbon dioxide within the solid polymer. The gas pressure is then reduced to atmospheric pressure causing thermodynamic instability which results in the nucleation and expansion of carbon dioxide bubbles within the solid but plasticized polymer, creating pores [10]. However, this method tends to result in a non-porous skin enveloping the scaffold and internal closed or partially interconnected porous structure [10] (Figure 2-5A). Attempts to improve the interconnectivity include combining the salt leaching method with gas foaming method [161-162]. The porosity achieved using this method is as high as 90%

with pore sizes in the range 200-500 μm [163] (Figure 2-5B). However, in this case organic solvents are once again required.

2.6.1.3 Electrospinning to produce scaffolds

Figure 2-6 Electrospun fibers A) prior to cell seeding and B) 1 week after cell culture. (Reproduced with permission from Ref. [154])

Electrospinning is a method for the production of nanoscale fibers. The produced fiber meshes have also been explored as tissue engineering scaffold. Here, the nanoscale fibers act collectively as a surface for cell attachment [148, 150]. The process involves passing a jet of polymer solution through a needle subjected to a high-voltage difference between the needle and a collector substrate, which causes the ejection of a liquid jet from the needle. This jet will then form a long nanosized solid thread which when the solvent evaporates on its way to a grounded collector will form a nanofiber [164] (Figure 2-6A). Cells were shown to be able to be cultured on the surface of the collected fibers and spread [154, 165] (Figure 2-6B). However, because of the nanometer mesh size of electrospun nanofiber scaffold, the cells are unable to penetrate into the

scaffold, thereby causing this type of scaffold to be only a 2D scaffold with 3D features.

2.6.1.4 Rapid prototyping method to produce 3D scaffolds

Figure 2-7 A) A 3D scaffold with interconnected channels made by 3D printing and B) its nanostructure showing deposition of hydroxyapatite granules. (Reproduced with permission from Ref. [166])

With the advent of rapid prototyping techniques, scaffolds of any desired dimensions and structure are now possible to be created using computer assisted design (CAD) systems, in which complex 3D structures can be designed and subsequently printed [167] or produced by stereolithography [168] or fused deposition modelling [152] (Figure 2-7). The limitations of such rapid prototyping approaches are the requirement for specialized equipment, the limited types of suitable polymers that can be used as “ink”, and the rigidity of the scaffolds produced. As such, the applications of the scaffolds made by rapid prototyping in tissue engineering are confined mainly to bone tissue engineering [11].

2.6.1.5 Overall assessment of rigid scaffolds

Although these abovementioned rigid scaffolds are 3D, the cells are actually attaching to two dimensional (2D) surfaces due to the rigidity of the scaffolds. These scaffolds are more aptly described as providing 2D attachment sites inside a 3D structure. In addition, one of the problems encountered is cell inhomogeneity within these rigid scaffolds; cells are typically introduced into the solid porous scaffolds by injection or some form of suction and may not fully reach the inner depths of a rigid porous scaffold [169-170]. In addition, the solid scaffolds also pose a problem if the cells are to be retrieved after expansion in these 3D scaffolds. This is because cells seeded in the tortuous internal pores may adhere too tightly onto the surface such that they are difficult to be retrieved [171]. Moreover if a cell-laden preformed solid scaffold were to be directly implanted, open surgery is required and preformed scaffolds pose a problem of spatial misfit in the *in situ* defect site, which may impede the integration of the engineered tissue into their native environment.

2.6.2 Injectable scaffolds and method to fabricate them

2.6.2.1 Hydrogels for tissue engineering

Hydrogels are 3D hydrophilic water-swollen polymer networks. In recent years, they have been the focus in tissue engineering techniques due to their excellent properties that support tissue engineering applications (Figure 2-8). Hydrogels used for 3D tissue engineering applications range from purely natural to purely synthetic materials [151]. Biopolymers that chemically resemble ECM

components such as chitosan [172], hyaluronic acid [151], dextran [139] and gelatin [87] can be chosen as the hydrogel precursors for scaffolds. The hydrogels' mechanical properties and water content can also be designed to simulate native tissues [173]. The use of polysaccharides that contain a vast number of functional groups, which can easily be modified, also provide the versatility for attachment of different ligands in varying degrees and using various chemistries. This gives flexibility in designing hydrogels with modifiable mechanical strength and degradation kinetics by altering the degree of substitution with ligands and hence the degree of crosslinking against enzymatic degradation. This tunable property of biodegradation can be harnessed to create bioresponsive scaffolds in which the cell proliferation rate and their secretion of enzymatic factors can be matched with the scaffold's biodegradation rate [91]. However, natural biopolymers typically have weaker mechanical properties than synthetic polymers [174]. Therefore, the combination of natural and synthetic polymers has been studied to harness the advantages of these different classes of materials to optimize the hydrogel's properties [175].

Figure 2-8 Smooth muscle cells (green) proliferate well in dextran-g-lysine-g-glycidyl methacrylate/gelatin hydrogels, showing good viability at day 1 (1d) day 7 (7d) and day 14 (14d). (Reproduced with permission from Ref. [87])

Advantages of hydrogels in tissue engineering

Because the hydrogel precursor is typically a liquid, cells can be mixed easily and distributed homogeneously within the precursor. The hydrogel can then be set under mild non-cytotoxic physiological conditions [176] in the presence of the cells, hence allowing for easy encapsulation of cells with a homogeneous distribution throughout the 3D network. This property also allows the cell pre-hydrogel mixture to be injected, and offers the prospect of *in-situ* polymerization for hydrogel formation at the defect site. This provides good physical proximity with the neighboring environment aiding the integration of the engineered tissues in the defect site [176]. Another boost added by hydrogels to tissue engineering in 3D is the efficient transport of soluble factors, nutrients and waste in and out of the hydrogel due to its inherent hydrophilicity and water swollen interior [173], which further mimics the highly hydrated conditions that cells reside in [151]. This property facilitates the homogeneous

introduction of soluble nutrients and the removal of waste by simply replacing the culture media.

Challenges in cell encapsulation in hydrogels

Cell encapsulation in hydrogel solutions provides the cells with an environment closer to the native 3D environment. Nevertheless, it is usually preferred for non-ADC such as mesenchymal stem cells [177], ESCs [61] chondrocytes [178]. Only a few examples demonstrating successfully the spreading of the ADC within the hydrogel have been reported [111, 179-180]. This is usually the case because it is hard for the ADC encapsulated in these highly constrained environment to spread quickly enough and prevent apoptosis [39] as they have to first overcome the physical constraint by degrading the nanoporous hydrogel matrix in order to make space for spreading [181]. On the other hand, if bigger pores of at least tens of microns were to be present in the hydrogel, i.e. a macroporous hydrogel, cell spreading would occur more easily as space is pre-created for the cells [181]. We dedicate the next section to a method of producing highly macroporous rigid polymers and hydrogels.

2.7 HIPE Templating to Create Macroporous Polymers and Hydrogels

High internal phase emulsion (HIPE) templating is a relatively facile method of fabricating porous polymers by exploiting the immiscibility of hydrophobic and hydrophilic liquids in the formation of an emulsion [14, 16, 182]. Porous

polymers can be formed from emulsion templates by subsequently polymerizing or solidifying the continuous but minority emulsion phase followed by the removal of the liquid templating phase. The pore size and degree of pore interconnectivity of the resultant macroporous polymer can be tuned by varying the composition of the emulsion and emulsification conditions. Recently, the products of the polymerization of HIPEs, polyHIPEs, have found their way as scaffold into tissue engineering [28, 183-185]. If the polyHIPEs have pores in the micropore range, they can have potential applications in drug release [186] and as drug delivery vehicles [187]. This method of fabrication of high porosity macroporous polymers is the focus of this research and will be further described in detail below.

Figure 2-9 Emulsion-templating to produce colloidal particles, porous polymers and composite materials depending on which emulsion phase is polymerized or removed. (Reproduced with permission from Ref. [188])

An emulsion is formed when two or more immiscible liquids are mixed, usually in the presence of surfactant or particulate emulsifiers [189-190]. Droplets formed within the mixture and will be dispersed within the continuous phase. The dispersed droplets phase is thus defined as internal emulsion phase while the continuous phase is defined as the external phase. The liquids used in emulsification are usually hydrophobic oils and water, thereby forming either oil in water (o/w), in which oil is the dispersed phase, or water in oil (w/o) emulsions, in which water is the dispersed phase. If the dispersed phase is polymerized, colloidal particles will form. Whereas if the continuous phase consists of polymerizable monomers or a prepolymer and the emulsion is solidifiable, a porous material can be formed after the removal of the liquid phases. If both phases are to be polymerized, then a composite material is produced [188] (Figure 2-9). For the purpose of producing porous polymers, we will focus on the polymerization of the continuous phases.

2.7.1 Typical methods of producing HIPE and polyHIPE

To qualify as a HIPE, as its name suggests, the emulsion phase volume ratio (ϕ) in the emulsion must be greater than 74.05% of the total volume of the emulsion, creating a concentrated emulsion of droplets dispersed within the continuous but minority phase [191]. If an emulsion would consist of monodispersed droplets at $\phi = 74.05\%$, the droplets should be at their

maximum possible packing fraction. When ϕ exceeds this value, the droplets will start to become deformed resulting in rhomboidal dodecahedral or tetrakaidecahedral packing [192]. On the other hand, very high internal phase volume fractions can also be achieved for emulsions with broad droplet size distributions allowing for more efficient droplet packing. The very dense droplet packing will result in very thin films of continuous phase separating the dispersed phase droplets in which the thinnest sections will rupture during post-processing procedures, leading to pore throats that interconnect the pores [193]. With sufficient surfactant and polymerization in the continuous phase, the high porosity and high degree of pore interconnectivity of the poly(merized)HIPEs is hence caused by this high internal phase close packing feature.

Scheme 2-1 Typical process of w/o polyHIPE formation: The aqueous internal phase of volume ratio of greater than 74.05% is added drop wise to the continuous oil phase under continuous stirring. A w/o HIPE is formed when all the internal phase has been incorporated into the emulsion. The continuous phase of the HIPE is subsequently polymerized and then the internal liquid phases are removed to produce porous polyHIPE.

A typical method of producing HIPEs is shown in Scheme 2-1. Briefly, methods to produce HIPEs typically involve the dropwise addition of the internal phase into the continuous phase, which usually consists of surfactants and polymerizable monomers. When the amount of internal phase added exceeds a total volume ratio of 0.74, a HIPE is formed. When the continuous phase of the HIPE is polymerized or solidified and the liquid phases are removed usually by freeze-drying or Soxhlet extraction, pores will be left where the dispersed droplets used to be, yielding a highly porous matrix called polyHIPE.

Myriad of different polyHIPEs were created [12-16]. The most widely studied monomer systems used for the synthesis of polyHIPEs since the advent of polyHIPEs are styrene (St) and divinylbenzene (DVB) used in or as the continuous oil phase [12, 194], but have since diversified to include many other monomers and/or prepolymers. Focusing on polyHIPEs for tissue engineering applications, poly-D-lysine coated poly(St-DVB)HIPE scaffolds were used as support for neuronal cells, which were found to attach and remain viable on the surface [195]. *In vitro* growth and maturation of osteoblast-like cells was also shown to be possible in hydroxyapatite coated poly(ST-DVB)HIPEs [196]. In addition, fibroblasts were shown to be able to be grown on the surface of poly(ϵ -caprolactone-St)HIPEs [153].

However, these scaffolds are mainly rigid solid scaffolds and cells were mainly grown on the surface and cell penetration into the scaffold was found to be

limited. Also, as the aforementioned scaffolds were made from w/o emulsion templates, polymerizable monomers have to be in the continuous oil phase. This means that the resultant porous polymers were relatively hydrophobic, which made them undesirable for the attachment of ADC [27]. This can be seen from the various studies, which indicated that an additional coating of the polyHIPEs with cell-adhesive factors was necessary. Even so, the coating of the cell-adhesive factors would not be homogenous throughout the scaffold and would most likely be concentrated on the outermost surface of the scaffold and hence the number of cells that will actually attach or penetrate into the scaffold will be meager [195]. In addition, by having the polymerization within the organic oil phase, it may be difficult to completely remove all the harmful organic solvents that may be trapped within the polymer matrix [27].

2.7.2 Porous polyHIPE hydrogels

To produce hydrophilic scaffolds, o/w emulsion templating is more desirable, and since biopolymers are mainly hydrophilic, they can be employed as a prepolymer in the aqueous continuous phase. Because hydrophilic polymers are used, the resultant polyHIPEs formed are hydrophilic too and tend to swell when placed in to an aqueous medium, forming a macroporous hydrogel which combines the benefits of an injectable hydrogel with the high macroporosity of polyHIPEs.

Recent advances have explored the use of polysaccharides and proteins in the formation of o/w polyHIPEs. These include dextran [197-198], alginate [23, 25], and gelatin [26, 28, 199], in which primary rat hepatocytes has been shown to be able to be cultured in gelatin-based polyHIPEs [199] (Figure 2-10).

Figure 2-10 Scanning electron micrograph of A) gelatin-based polyHIPE foam and with B) primary rat hepatocytes within its pores. (Reproduced with permission from Ref. [199])

However, even with the use of biopolymers, to stabilize HIPEs, a huge amount of surfactants is usually required which could pose a risk to the cells if not removed thoroughly [29]. In addition, though high porosities of more than 90% can be achieved, the pore sizes of these hydrogel polyHIPEs tend to be small in the range of 20 μm or lesser as a consequence of the presence of surfactants. This is at the lower limit of the pore sizes required to allow cells to penetrate into the scaffold and to form cell-cell interactions as described in the earlier sections. Strategies have been developed to create bigger pores by introducing various additives into the emulsion templates, such as salt or organic solvents [28]. However, these additives can be difficult to be removed completely and any residue is undesirable. Hence, it would be ideal if HIPEs could be formulated without the need for harmful stabilizers, for instance it would be preferable if the biopolymer in the continuous phase could act as matrix for polyHIPEs and simultaneously as emulsifier for the emulsion template and at the same time able to stabilize sufficiently large droplets resulting in adequate pore sizes and pore interconnectivity.

2.7.3 Self-emulsifying HIPEs for polyHIPEs

The common self-emulsifying systems available refer to the formation of self-emulsifying microemulsions in which an emulsion with droplets within the nanometer range can spontaneously form with slight agitation, usually in the presence of large amounts of surfactant and a co-surfactant [200-201]. Microemulsions are thermodynamically stable emulsions, which are for

instance used as a drug delivery system for the solubilization and delivery of oil soluble drugs [202-203].

A polymer that acts both as the matrix for polyHIPEs and emulsifier during the formation of HIPEs has not yet been described. The aim is to create hydrophilic biopolymers that can be solidified/gelled in the continuous minority emulsion phase and at the same time act as the (self-)emulsifier. The requirements for a molecule to act as an emulsifier is that it has to be amphiphilic, i.e. contains both hydrophilic and hydrophobic parts, so it prefers the interface between the oil and water phase [204]. However, when using a potentially amphiphilic polymer, such as dextran-*graft*-(glycidyl methacrylate) to stabilize HIPE it was observed that a great deal of energy was required to emulsify the oil and water phases which rapidly destabilize after the energy input is removed [197]. Hence, the design of the polymeric emulsifier should aim to reduce the oil-water interfacial tension sufficiently to allow stabilization of HIPEs without the need of high energy input or additional stabilizers.

2.7.4 Solidification/gelation mechanisms of hydrogels and emulsions

The solidification of hydrogels and emulsions is required to create a 3D structure that can support and hold the cells. Different solidification mechanisms have been utilized for the solidification of hydrogels or emulsions, such as chemical crosslinking and physical crosslinking [199, 205-210]. The crosslinking process should be mild and achievable under physiological

conditions such that it will adversely affect the encapsulated cells. Traditional methods of thermal free-radical crosslinking of hydrogels require the presence of an emulsifier and temperature of around 70°C and are hence unsuitable for *in situ* gelation of hydrogels in presence of cells in hydrogel precursors. This section will introduce some common solidification mechanisms that are suitable for use in tissue engineering scaffolds. They are broadly classified into chemical crosslinking methods and physical crosslinking methods.

2.7.4.1 Chemical crosslinking of hydrogels

Radical initiated crosslinking

Methods of radical initiated crosslinking include thermally initiated and photoinitiated radical generation. Thermal initiated free radical crosslinking is triggered by an increase in temperature usually at least 60°C, in the presence of thermal initiators [14]. For *in situ* gelation of hydrogels with encapsulated cells, the cytocompatible and water soluble thermal initiator ammonium persulfate in presence of the accelerator *N,N,N',N'*-tetramethylethylenediamine (APS/TEMED) was found to be able to cause gelation of the pre-hydrogel solution at 37°C [205].

Another method of solidifying hydrogel which can allow quick *in-situ* gelation and encapsulation of cells in room temperature by free radical crosslinking is the use of ultraviolet (UV) induced photocrosslinking. This will require the monomers or polymers involved to possess UV crosslinkable groups, such as those present in acrylates and an UV initiator [87, 111, 211]. This is a relatively

quick method in which solidification does occur within minutes at room temperature. However, exposure to UV may not be desirable for cells as it is known to cause DNA damage and other undesirable side effects to the cells [212-213]. Moreover, this method usually requires the addition of a crosslinker and initiators which if they remain in the scaffold will be able to enter the cells or react with the cell membrane, therefore affecting normal cell activity.

Enzyme mediated crosslinking of hydrogels

Enzyme mediated crosslinking requires specific enzymes that are able to catalyze the formation of specific covalent bonds between functional groups present on the polymer. For example, the enzyme horseradish peroxidase, which catalyzes the coupling of phenol or aniline groups via decomposition of hydrogen peroxide [214] can be used to catalyze the formation of hydrogels based on polysaccharides grafted with tyramine with a gelation occurring in less than 30 s or close to 600 s by controlling the concentration of polymer, enzyme and hydrogen peroxide [215-216]. Another commonly used enzyme is transglutaminase (TG), which catalyzes the formation of an amide bond between primary amine groups and the γ -carboxamide groups of peptidyl glutamine groups in a calcium ions dependent reaction [217]. An example is the use of TG for the enzymatic crosslinking of a gelatin containing emulsion [199]. However, the introduction of external enzymes may perhaps interfere with the regulation of the cells' normal activity.

Crosslinking by alginate complexation with multivalent ions

The most widely used method of *in situ* cell encapsulation is the Ca^{2+} -alginate hydrogel system (Scheme 2-2), whereby the cells are first mixed with a solution of alginate containing negatively charged carboxyl groups, which is then introduced drop wise into a solution containing with divalent cations, in this case Ca^{2+} , to form crosslinked hydrogel beads containing the encapsulated cells within [61, 218-219]. The downside of using Ca^{2+} -alginate hydrogel is that it is easily destabilized by monovalent cations and calcium ions chelating agents in physiological media, resulting in poor long-term mechanical stability [220-221]. In addition, the formation of these hydrogels are most likely to be non-homogenous with the surface of the bead more strongly crosslinked than at its core due to the hindrance of the diffusion of Ca^{2+} ions into the core [222]. Another practical problem is that cell retrieval requires the use of EDTA or citric acid derivatives [223-224] to destabilize the ionic bonds which could be detrimental to the health of the cells.

Scheme 2-2 Schematic of ions responsive alginate gelation by forming a complex with calcium ions.

2.7.4.2 Physical crosslinking

Biopolymers that are able to form gels in respond to changes in the environmental conditions, otherwise known as responsive polymers, bring a whole new dimension to formation of tissue engineering matrices. The environmental driving force, for example, could be a change in pH, or temperature. This allows for *in situ* gelation by physical methods which are gentle and less damaging to the cells than using chemical methods such as chemical crosslinking agents or initiators for polymerization. By using *in situ* physical gelation methods, cells can be easily encapsulated and released simply by changing a trigger from the change in the environmental conditions minimizing the risks posed by chemical methods. In this section, focus will be put on temperature sensitive mechanisms.

Temperature responsive gelling mechanism

The transition from solution to gel (sol-gel transition) can occur by a temperature change when the solubilized polymers possess thermoresponsive characteristics. These polymers can be synthetic or natural. For synthetic polymers, the polymers usually undergo phase separation from solution and gel above a certain critical temperature. This temperature is defined as the lower critical solution temperature (LCST), in which the polymer is soluble below LCST and becomes insoluble above LCST. This is largely due to changes in the polymer-polymer, polymer-water, and water-water interactions as function of temperature [225]. Above the LCST, the polymer-polymer interactions becomes more favorable due to entropic changes and exceed the polymer-water interactions causing it to come out of solution either forming polymeric micelles [226] or undergo coil to helix or globule transition to form a polymeric network [227] (Scheme 2-3). These associations are only physical and are reversible once the temperature stimulus is removed.

Scheme 2-3 Schematic of the reversible coil-globule transition of a copolymer containing grafted thermoresponsive pendant chains (red). Below the LCST the copolymer exists as an extended coil in solution, with water molecules bound to the polymer, above the LCST thermoresponsive grafts collapse and a water insoluble polymeric network is formed, resulting in gelation.

Many synthetic thermoresponsive polymers have been found and synthesized which include poly(ethylene oxide)-*b*-poly(propylene oxide)-*b*-poly(ethylene oxide) (PEO-PPO-PEO), PEG-block copolymers, PNIPAM [228] (Figure 2-11).

Figure 2-11 Chemical structures of some thermoresponsive polymers.

PEO-PPO-PEO is a water soluble triblock copolymer also known by its trade name Pluronics or Poloxamer. It is amphiphilic containing hydrophilic PEO blocks and hydrophobic PPO blocks. When its solution is raised above its LCST they tend to form polymeric micelles. The LCST of PEO-PPO-PEO was shown to depend on the solution conditions, such pH, chemical composition, molecular weight and polymer concentration [229]. Because of its bioinertness and high hydrophilicity of PEO, serum proteins are repelled and cell attachment is low [230]. Hence PEO-PPO-PEO copolymers are more widely used in drug or gene delivery vehicles [231-234].

Special focus is given to PNIPAM as in recent years, PNIPAM has seen a surge in its use in tissue engineering and drug delivery applications [206-210]. The most exploitable property of PNIPAM for tissue engineering is its thermoresponsiveness. It undergoes a sharp expanded-coil to compact-globule transition above its LCST of 32°C in aqueous media and vice versa [235-236]. Below the LCST, PNIPAM is soluble due to the hydrogen bonding of its amide group with water. Above the LCST, hydrophobic interactions take over, causing the chains to aggregate into globules and precipitate out of the solution [237].

The LCST of PNIPAM can be modified by the introduction of more hydrophilic (or hydrophobic) co-monomers to allow it to form a hydrogel nearer to the physiological temperature of 37°C, rendering it suitable for tissue engineering. It has been used mainly for the creation of cell sheets by culturing cells on PNIPAM coated surfaces. Cell sheets can then be retrieved by lowering the temperature below the LCST of PNIPAM in which the surface becomes more hydrophilic and the cell sheet becomes detached from the coated surface [206, 238-239]. These 2D cell sheets can then be used directly such as for skin grafts [206]. However, most other tissue engineering applications require the fabrication of functional 3D tissues, for example, blood vessels. It has been reported that these 2D cell sheets can be made into 3D by stacking these cell sheets layer by layer [240]. A more direct method of creating a 3D environment for the cells is to graft PNIPAM as a component of a copolymer to create thermoresponsive hydrogel encapsulation systems [129, 241]. Cells in

suspension can be easily encapsulated and released from the hydrogel simply by varying the temperature in the range in which the hydrogel exhibit a volume phase transition. This process is entirely safe for the encapsulated cells without introduction of chemical factors. However, it is most commonly used for non-ADCs, because as mentioned earlier, this method of cell encapsulation has its limitations due to the nanopores restraints within the hydrogel that restricts the spreading of ADCs. Hence, it will be ideal to create a 3D thermoresponsive hydrogel with macrosized interconnected pores for the ADCs, in which the HIPE-templating will be very useful in this respect.

Natural thermoresponsive polymer

Gelatin is revisited here as it is a representative of a biopolymer that exhibits thermoresponsive properties. The mechanism is different from the LCST mechanism described earlier, as it does not include the temperature dependent dissociation of water molecules to induce its gelation. A gelatin solution can be produced by dissolving it in water at temperature above 30°C. This solution can be gelled by lowering its temperature to below 25.8°C. The gelation mechanism is a temperature dependent coil to triple-helix transition bound by intermolecular hydrogen bonds when the temperature is lowered (Scheme 2-4). As ordered structure has to be formed, the time for gelatin solution to gel will be much longer than the LCST mechanism when random globules are formed. When the temperature is increased, the breaking of these hydrogen bonds will cause the chains to dissociate thereby going back to solution.

Scheme 2-4 Schematic of the thermoreversible gelation of gelatin from a random coil in solution at 40°C to the onset of gelation by the formation of ordered coil and triple helical structures during cooling. With further cooling the triple helices start to associate and a stronger gel is form.

2.8 Overall Assessment on the Fabrication of Tissue Engineering Scaffolds

In summary, tissue engineering scaffold requires high porosity and pore interconnectivity, in addition to being biocompatible, biodegradable and preferably injectable. The main problem in creating 3D tissue engineering scaffolds is the use of organic additives and small crosslinker monomers in order to produce a macroporous scaffold that is highly porous, interconnected and injectable, which may pose a risk to the cells. In addition, high energy input is often required. This research aims to explore a ‘cleaner’ method to produce highly porous tissue engineering scaffolds using minimal input of energy, materials and eliminating the use of crosslinkers monomers and harmful additives by formulating novel multifunctional biopolymers that act as emulsifier for emulsions templates, exhibit a thermoresponsive behavior allowing to gel/solidify these templates and form the matrix of a macroporous polymer, which can be used as tissue engineering scaffold. The synthesis of biopolymeric surfactants will be described, their use in stabilizing HIPEs is demonstrated, as well as their feasibility as tissue engineering scaffolds are investigated in this thesis.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Chitosan (CS, from shrimp shells, >75% deacetylated), Gelatin Type A (MW 40kDa – 50kDa), acetic acid, ceric ammonium nitrate (CAN), *N*-isopropylacrylamide (NIPAM), L-Lysine monohydrochloride, L-proline, L-Glutamic acid monosodium salt hydrate, Gelatin Type A, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC•HCl), *N*-hydroxysuccinimide (NHS), *N,N,N',N'*-tetramethylethylenediamine (TEMED), hydrochloric acid (HCl), sodium hydroxide (NaOH), *p*-xylene, 2-mercaptoethanol, ninhydrin reagent solution and Leukemia Inhibitory Factor (LIF) were purchased from Sigma Aldrich. A 10% aqueous solution of PEG(20) sorbitan monolaurate was purchased from Biorad Laboratories. Undifferentiated murine ESCs (mESCs) (E14/Tg2a cell line) was purchased from ATCC, UK. Human foreskin fibroblasts (FibroGRO™) was purchased from Merck Millipore, US. Dulbecco's Modified Eagle's Medium (DMEM), heat-inactivated fetal bovine serum (FBS), L-Glutamine, 1% penicillin/streptomycin, and 0.25% Trypsin-EDTA were purchased from Gibco.

Cell proliferation assay CCK-8 (Cell counting kit-8) was purchased from Dojindo Molecular Technologies, Japan and LIVE/DEAD® Viability/Cytotoxicity Assay Kit was purchased from Invitrogen.

3.2 Experimental Methods

3.2.1 Synthesis of Chitosan-*g*-PNIPAM (CSN)

PNIPAM was grafted to chitosan using CAN as an initiator in a redox reaction under nitrogen atmosphere. Briefly, 1 g of chitosan was dissolved in 250 ml of 0.12% acetic acid and the solution was clarified by centrifugation. 10 g of NIPAM was added to the solution and the reaction vessel was purged with nitrogen gas for 1 h. 0.8 g of CAN was dissolved in 4 ml of 1 M nitric acid, purged with nitrogen gas and added to the reaction solution. The reaction was maintained at 30°C and was allowed to proceed for 24 h in the dark. The reaction solution was then dialyzed against deionized water in dialysis tubing with MWCO of 12400 and lyophilized. A white fluffy product was obtained and the structure was confirmed by ¹H-NMR, FTIR and gel permeation chromatography (GPC).

3.2.2 Characterization of polymers

3.2.2.1 NMR and FTIR

The structure of the graft copolymers was confirmed by ^1H -NMR (Bruker Avance 300 MHz) in $\text{DCl}/\text{D}_2\text{O}$ or D_2O as the solvent. ATR-FTIR (Imaging Golden Gate, Specac, UK) was performed in the range of $4000\text{--}900\text{ cm}^{-1}$ with a spectral resolution of 8 cm^{-1} using 64 scans.

3.2.2.2 Molecular weight determination of the polymers

Molecular weights of the polymers were determined by GPC (LC-20AD, Shimadzu) relative to Shodex pullulan standards (Showa Denko, Munich, Germany) using PLAquagel-OH mixed columns (Agilent Technologies). The mobile phase was 0.3 M sodium acetate buffer, pH 4, with a flow rate of 1 ml/min and the sample injection volume was 50 μl . Detection was done with a refractive index detector (RID-10A, Shimadzu).

3.2.2.3 Solubility and thermo-reversibility properties of the polymers

To determine the water solubility range, the polymers were dissolved in deionized water at pH 7 at various concentrations. To determine the pH solubility range, the polymers were dissolved in aqueous HCl/NaOH solutions whose pH was adjusted from 1 to 14 at a concentration of 2.5% w/v. The LCST of the solution was determined by turbidity measurements, in which the temperature of the solution was increased from 25°C to 40°C and the LCST determined as the temperature where the solution first turned turbid.

3.2.2.4 Zeta potential measurements

Polymers were dissolved in aqueous HCl/NaOH solutions whose pH was adjusted from 1 to 14 at a concentration of 2.5% w/v and their ζ -potentials were measured using ZetaPALS (Brookhaven Instruments) at 25°C. The reported values are mean values of 10 measurements.

3.2.2.5 Determination of the effects of polymer solution on the air/water surface tensions and water/*p*-xylene interfacial tensions

A drop shape analysis system (FTA32, First Ten Angstroms Inc.) was used to determine the surface and interfacial tension between the aqueous polymer solution and *p*-xylene based on the pendant drop method. The polymer was first dissolved in water or PBS at 1% w/v and extruded slowly through a blunt tip needle to form a pendant drop in air and in *p*-xylene at room temperature, and its image was captured. The interfacial tension was calculated using the instruments software.

3.2.3 Characterization of polyHIPE

3.2.3.1 SEM characterization of polyHIPE

The morphology of the polyHIPEs and its respective pore and pore throat sizes were determined from images taken using a scanning electron microscope (JEOL JSM6701F, JEOL Ltd, Japan). Fractured pieces of the polyHIPEs were fixed on the sample holder using carbon black sticker and sputtered with platinum prior to imaging in the SEM. Pore and pore throat sizes were analyzed using an image processing software (ImageJ, US National Institutes of Health, Bethesda, Maryland, USA).

3.2.3.2 Surface area determination of polyHIPE

The surface area of polyHIPEs was determined from nitrogen adsorption isotherms at 77K using the Brunauer–Emmet–Teller (BET) model. The measurements were performed using a surface area analyzer (Autosorb1, Quantachrome Instruments). About 100 mg of each polyHIPE was used for the analysis. Before performing the gas adsorption experiments, the adsorbed impurities were removed via a degassing step.

3.2.3.3 Density and Porosity of polyHIPE

The envelope density of the foam, ρ_e , was determined by measuring the mass of cylindrical foams with radius of 25 mm and height of 40 mm and dividing by the cylinder volume. The true matrix density, ρ_t , of the macroporous polymers was determined by using a pycnometer (Ultrapycnometer 1000, Quantachrome Instruments). The mass, M_f , of the macroporous polymers was first measured before inserting into a sample cell of a known volume, V_c , with n number of moles of gas molecules. The system was then pressurized to a pressure (P_2) above ambient pressure (P_a). Nitrogen gas of volume V_A was then introduced through a solenoid valve and the pressure was lowered to P_3 .

The true matrix density, ρ_t , was calculated with the formula:

$$\rho_t = M_f / \left(V_c + \frac{V_A}{1 - (P_2/P_3)} \right)$$

The porosity of the foam, P , was then calculated by $\left(1 - \frac{\rho_e}{\rho_t} \right) \times 100\%$.

CHAPTER 4

CHITOSAN-*GRAFT*-PNIPAM-*GRAFT*- OLIGOLYSINE AS A SELF-EMULSIFYING THERMORESPONSIVE POLYMER FOR POLY (HIGH INTERNAL PHASE MACRO- AND MICRO-EMULSION) VIA A POLYMERIZATION-FREE ROUTE

4.1 Introduction

This chapter presents the synthesis of a water-soluble thermoresponsive chitosan-*graft*-poly(*N*-isopropylacrylamide)-*graft*-oligolysine (CSNLYS) copolymer which not only acts as the sole emulsifier in the formation of high internal phase emulsions, but can also be self-crosslinkable and be solidified to form the polymer matrix foam.

Chitosan is chosen as a representative of a charged polysaccharide that is known to be biocompatible and also possesses many amine groups for chemical modifications. Lysine is chosen to be grafted onto chitosan to improve the solubility of chitosan graft copolymer as it is a very hydrophilic amino acid [242], and being positively charged, it can contribute to the charges that the copolymer carries. This is advantageous to the formation of an emulsifier

because polyelectrolytes are known to contribute to the electrostatic repulsive forces that tend to stabilize emulsions [243]. To solidify the emulsion, PNIPAM is chosen to be grafted onto chitosan to allow the temperature-induced physical aggregation of grafted PNIPAM components at a relatively low temperature, avoiding the use of high temperatures and crosslinkers monomers. At the same time, the hydrocarbon chains in PNIPAM can confer some degree of hydrophobicity to the copolymer, giving amphiphilicity to CSNLYS which is one of the prerequisite to act as an emulsifier. The choice of these materials is envisioned to be able to meet the aim of creating a multifunctional biopolymer that can self-emulsify, self-crosslink, and form the polymer matrix simultaneously.

HIPE templating method is chosen to fabricate the porous polymers as it represents a facile method of producing very porous polymers with controlled porosity, pore morphology and interconnectivity after solidification of the continuous phase and removal of the templating dispersed phase [12, 16]. However, most of the methods to date involve polymerization to solidify the continuous phase and large amount of surfactant(s) for the stabilization of the HIPE [13-14, 244]. In tissue engineering applications, the use of surfactants poses a challenge because it is difficult to be completely removed and can potentially contaminate the foams produced. Hence, it is highly desirable to have self-emulsifying polymers, which can be used to formulate various types of emulsion templates without the need for additional surfactants, and can be

solidified into porous polymers with controlled porosity, pore size (ranging from macro- to micro-emulsions) and structure.

4.2 Experimental Methods

4.2.1 Synthesis of Chitosan-*g*-PNIPAM-*g*-oligolysine (CSNLYS)

Synthesis of CSNLYS proceeded according to Scheme 4-1. CSN was first synthesized according to Section 3.2.10. 5 g of CSN was then dissolved in 20 mM tetramethylethylenediamine/Hydrochloric acid (TEMED/HCl) pH 4.7 and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC•HCl) and *N*-hydroxysuccinimide (NHS) and 0.06 mol (CSNLYS-H) or 0.03 mol (CSNLYS-L) of L-Lysine monohydrochloride, was added at room temperature and the reaction was allowed to continue for 2 days. At the end of the reaction, the solution was neutralized with 1 M sodium hydroxide solution (NaOH) and was clarified through filtration through 0.22 µm filter unit. The filtrate was then dialyzed against deionized water in a dialysis tubing (MWCO 12400) until the conductivity of the solution remained constant and the polymer solution lyophilized. The products were characterized by ¹H NMR.

4.2.2 Formation of HIPE

Emulsions were prepared at room temperature by mixing 125 µl of either 20% w/v CSNLYS-L or CSNLYS-H in the continuous water phase at pH 7 and 375 µl of *p*-xylene as internal oil phase to give an internal phase volume fraction of 0.75. The emulsions-templated porous polymers were formed by raising the

temperature of the emulsions to 40°C, rapidly freezing them in liquid nitrogen and removing the entrapped solvents by freeze-drying (Scheme 4-2).

Scheme 4-1 Reaction scheme. Step 1: Ce^{4+} -initiated radical graft copolymerization of PNIPAM to form CSN. Step 2: Condensation of lysine monomers onto CSN to form CSNLYS.

Scheme 4-2 Schematic of the formation of the emulsions and porous foams. a) Formation of oil-in-water emulsion with CSNLYS solution and *p*-xylene with internal phase volume fraction, ϕ , = 0.75. Self-assembly of CSNLYS molecules at water and oil interface b) Solidification of emulsion by temperature-induced aggregation of PNIPAM side chains by raising the temperature to 40°C. c) Removal of water and *p*-xylene by freeze drying results in emulsion template porous foam.

4.3 Results and Discussion

Chitosan-*graft*-PNIPAM (CSN) was synthesized by graft radical copolymerization of NIPAM monomers onto a chitosan macroradical formed by ceric mediated redox initiation. Then, CSN was modified to produce CSN-*graft*-oligolysine by polycondensation of the residual amine groups in CSN with lysine monomers in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl) and *N*-hydroxy-succinimide (NHS). Adjusting the ratios of lysine monomers to the amino groups of CSN to be 2:1 and 4:1 respectively, two graft copolymers with low (CSNLYS-L) and high (CSNLYS-H) oligolysine contents were produced (Scheme 4-1). The synthesis of the copolymers was confirmed by ¹H-NMR analysis (Figure 4-1 and Table 4-1).

The weight average molecular weights of CS, CSN, CSNLYS-L and CSNLYS-H determined using GPC were 1.80, 2.25, 2.32 and 2.35 × 10⁶ Daltons, respectively (Table 4-2 and Figure 4-2). The degree of substitution (DS) (basis: 100 repeat units, m or x in Figure 4-1a) and the degree of polymerization (DP) (based on 1 glucosamine repeat unit), which represents the average chain length of the side chains, were determined from the ¹H-NMR integrals of chitosan (Calculation details in Appendix 1).

Figure 4-1 a) Structure of CSNLYS. ^1H -NMR spectra of bi) native chitosan in DCl solution 20% in D_2O , bii) Chitosan-g-PNIPAM (CSN) in D_2O , CSN-g-oligolysine (CSNLYS) with biii) lower DP (CSNLYS-L) and biv) higher DP (CSNLYS-H), in D_2O . The DS and DP were determined from the ^1H -NMR integrals of chitosan and the grafted moieties.

Table 4-1 ^1H chemical shifts of chitosan, CSN, and CSNLYS-H/-L found in the NMR spectra.

NMR	Protons	Shifts (ppm)
Chitosan		
Proton on Anomeric carbon	H1	4.65
-CH ₃ of acetylated group	H7	1.81
Proton on carbon bearing NH ₂	H2	2.84
Protons on glucosidic ring	H3-6	3.67,3.50,3.33
PNIPAM		
-CH-CH ₂	H8,H9	1.91, 1.51
-CH ₃	H10	1.08
Lysine		
-NH ₂	H15	3.02
-CH ₂ CH ₂ CH ₂ -	H12,H13,H14	2.0, 1.70, 1.45

Table 4-2 Weight average molecular weights of CS, CSN, CSNLYS-L, CSNLYS-H determined by GPC.

	Weight Average Molecular Weight (g/mol)	Polydispersity Index
CS	1.80×10^6	1.03
CSN	2.25×10^6	1.17
CSNLYS-L	2.32×10^6	1.04
CSNLYS-H	2.35×10^6	1.53

Figure 4-2 Weight average molecular weights of CS, CSN, CSNLYS-L, CSNLYS-H determined by GPC.

Table 4-3 DS and DP as determined from NMR integrals. *p+q+m+x =100 where m=DS of units grafted with NIPAM (%), x=DS of units grafted with oligolysine (%), n=DP of grafted PNIPAM and y=DP of grafted oligolysine.

Composition*			Degree of Substitution (DS)		Degree of polymerization (DP)	
	p	q	m	x	n	y
Chitosan	22	78	0	0	0	0
CSN	22	69.3	9	0	21	0
CSNLYS-H	22	65.4	9	4	21	4
CSNLYS-L	22	65.4	9	4	21	1

For the PNIPAM grafts of both CSNLYS-L and CSNLYS-H, the DS and DP are similar (m=9, n=21). For the oligolysine graft, the 2 copolymers CSNLYS-L and CSNLYS-H yielded similar DS values (x=4) while differing only in their DP (CSNLYS-H y=4, CSNLYS-L y=1) (Table 4-3). The residual unreacted –NH₂ on the graft copolymers were 65.4 mol%, which indicated that a significantly large fraction of the glucosamine monomers remained unmodified by the lysine condensation, suggesting that grafting of lysine onto the chitosan backbone may not be as favorable as the condensation of oligolysine in free solution, possibly because of steric hindrance.

Table 4-4 Physical properties: solubility, zeta potential, LCST and interfacial tension of CSNLYS-L and CSNLYS-H. All measurements were made at pH 7, the pH during emulsification. [a] Interfacial tension of aqueous sample in air. [b] Interfacial tension of aqueous sample in p-xylene.

Sample	Solubility (% w/v)	ζ (mV)	LCST (°C)	^[a] γ_{AW} (mN/m)	^[b] γ_{ow} (mN/m)
CSNLYS-L	40	0.1 ± 0.1	34	44.6 ± 0.2	12.6 ± 0.0
CSNLYS-H	40	7.5 ± 1.5	34	40.9 ± 0.8	~ 0

Figure 4-3 Zeta potential of 2.5% w/v CSNLYS-H and CSNLYS-L in deionized water over a pH range from 1 to 12. Isoelectric points of CSNLYS-H and CSNLYS-L were determined to be at pH 8.8 and 7.4, respectively.

Unlike native chitosan, which does only dissolve in water below pH 6.4 [130], both CSNLYS-L and CSNLYS-H are soluble over a wide pH range from 1-12 measured at 2.5% w/v, and have improved solubility of up to 40% w/v at pH 7. The differences between CSNLYS-L and CSNLYS-H are evident in their solution properties (solubility, zeta potential and interfacial tension) (Table 4-4). Higher ζ -potentials were measured for CSNLYS-H than for CSNLYS-L (Figure 4-3 for ζ -potentials at pH from 1-12); at pH 7, CSNLYS-H has ζ -potential of 7.5 ± 1.5 mV compared to 0.1 ± 0.1 mV for CSNLYS-L. This can be attributed to the longer oligolysine chains in CSNLYS-H as compared to that of CSNLYS-L which contribute to the charges. The interfacial tensions of aqueous solutions of the two copolymers measured against air at a concentration of 1% w/v are similar. However, the interfacial tensions measured against *p*-xylene differ significantly; CSNLYS-L exhibited oil-water interfacial tension (γ_{ow}) of 12.6 ± 0.0 mN/m while that of CSNLYS-H is zero or very close to zero as the aqueous droplet is seen solubilizing immediately in the oil phase (Not shown).

Emulsions were prepared at room temperature by mixing 125 μ l of either 20% w/v CSNLYS-L or CSNLYS-H dissolved in water at pH 7 as continuous aqueous phase and 375 μ l of *p*-xylene as internal oil phase to give an internal phase volume fraction of 0.75 (Scheme 4 - 2). When using CSNLYS-L, mixing using a vortex mixer at 2500 rpm resulted in milky emulsion (Figure 4-4A), forming HIPE. Remarkably, when CSNLYS-H was used, a clear transparent one-phase emulsion spontaneously formed when mixing the oil and water

phases (Figure 4-4B). This phenomenon is akin to the formation of microemulsions [245] whereby the diameter of the emulsion droplets is smaller than 25% of the wavelength of visible light, forming high internal phase microemulsion (HIPME).

Figure 4-4 Photographs of A) HIPE stabilized by CSNLYS-L and B) HIPME stabilized by CSNLYS-H. HIPE was seen to be C1) flowing at room temperature and C2) solidifying at 40°C. PolyHIPE was shown to retain its thermoresponsive properties by D1) staying solid in water at 70°C, D2) swelling and becoming translucent when temperature decreases to 40°C,

indicating pore interconnectedness, D3) and completely dissolving in water below the LCST at 25°C.

Figure 4-5 Drop test to determine the emulsion type. The emulsion at 25°C (left column) and 40°C (right column) was dropped into water (top row) and *p*-xylene (bottom row) using a pipette. Inset arrows show position of emulsion. When the emulsion is at 25°C, it A) dispersed completely in water while forming an oil layer at the surface, and B) remained as a droplet in *p*-xylene, indicating that it is an o/w emulsion. When the emulsion's temperature was increased to 40°C, it C) remained intact and floats in water, and D) sank to the

bottom in *p*-xylene, indicating that it remained as o/w after solidification at 40°C.

Figure 4-4A shows that CSNLYS-L stabilizes a macroemulsion with droplets' sizes in the range of $13.6 \pm 6.0 \mu\text{m}$ (Figure 4-6B1 inset) that scatter light, possibly due to the relatively higher γ_{ow} interfacial tension; energy has to be provided in the form of vortexing during the emulsification process when using CSNLYS-L as emulsifier. The emulsion, which formed, was shown to be an oil-in-water (o/w) emulsion at both room temperature and the solidification temperature (40°C) using the drop test (Figure 4-5).

The stability of the HIPEs stabilized by CSNLYS without additional surfactant can be attributed to the fact that the CSNLYS is surface active due to the hydrophilicity of the polysaccharide backbone and the grafted oligolysine side chains and the hydrophobicity of the isopropyl groups of the long PNIPAM chains. A proposition of the self-assembly of the copolymer is shown in Scheme 4 - 2. The hydrophilic oligolysine chains and polysaccharide units extend into the aqueous phase. The PNIPAM chain consists of hydrophilic and hydrophobic component, in which the relatively hydrophobic isopropyl groups of a portion of the grafted chains extend into the oil phase [246] while the remaining chains of water soluble PNIPAM are in the aqueous phase. With an increase in temperature, dissociation of water molecules around the isopropyl groups occur. At the same time, hydrogen bonds between water molecules and the amide groups weakened significantly, resulting in an aggregation of the PNIPAM component in the aqueous phase, while sufficient isopropyl groups still remained in the oil phase keeping the emulsion stabilized [247].

Furthermore as the chitosan has a high molecular weight, it increases the viscosity of the aqueous continuous phase such that diffusion of droplets slows down, thereby inhibiting Ostwald ripening [248-250].

The emulsions were then solidified by merely raising the temperature of the samples to 40°C, above the LCST of the copolymer (compare Figure 4-4C1 with Figure 4-4C2), which was determined by turbidity to be 34°C for the two copolymers (Table 4-4). The solidification occurred due to the phase separation and aggregation of thermoresponsive PNIPAM side chains above the LCST of CSNLYS [198]. In order to remove the solvents from the solidified emulsions, they were rapidly frozen in liquid N₂ and the water and *p*-xylene was removed by freeze-drying. After removing the template, i.e. the dispersed oil phase, solid polyHIPEs were obtained. As a control an aqueous solution of CSNLYS-L without *p*-xylene was treated the same way (solidified by raising the temperature and freeze drying) (CSNLYS-C). Because thermally induced solidification was employed, the resultant polyHIPEs inherited thermoresponsivity as well and retained its form above its LCST, and dissolved completely in water below its LCST (Figure 4-4D). The physical properties of the porous foams formed were then characterized, in terms of its surface area, pore size, porosity and morphology.

Table 4-5 Physical properties: Surface area, pore size and porosity of foams.

Sample	Surface area (m ² /g)	Pore size (μm)	Porosity %
CSNLYS-C	20	22.1 ± 4.8	86.8
CSNLYS-L	195	10.5 ± 6.9	88.3
CSNLYS-H	988	0.13 ± 0.3	80.5

Using CSNLYS-L, a control foam, CSNLYS-C, formed without *p*-xylene was prepared by thermally induced phase separation (TIPS). The measured surface areas of the polyHIPE and polyHIPME (CSNLYS-L & CSNLYS-H, Table 4-5) was much higher (195 and 988 m²/g) as compared to the control CSNLYS-C (20 m²/g, Table 4-5), which was simply produced by TIPS, as additional interface was created by both liquid templates (water and *p*-xylene) during emulsification, resulting in a greater surface area after removal of both oil and water templating phases by freeze-drying.

The control CSNLYS-C (Figure 4-6A) has a very different pore structure from the polyHIPEs (Figure 4-6B) - the pores of the CSNLYS-L are smaller, more ordered and spherical than CSNLYS-C. Also, the droplet size of the HIPEs stabilized by CSNLYS-L determined by optical microscopy were determined to be 13.6 ± 6.0 μm, slightly larger than the pore sizes measured by analyzing SEM micrographs as there was shrinkage of the polyHIPE of about 20% during freeze drying (Figure 4-6B1 – inset).

Figure 4-6 SEM images of A1-A2) Control (CSNLYS-C, no *p*-xylene added), B1-B2) polyHIPE (CSNLYS-L) (inset shows optical micrograph of HIPE) and C1-C2) polyHIPME (CSNLYS-H) after removal of solvents by lyophilization. *Asterisks show positions of some of the open pores. A2, B2 and C2 are higher magnification images of A1, B1 and C1 respectively.

Figure 4-7 Pore size distribution of A) macroHIPE and B) microHIPE.

When CSNLYS-H was used, the average pore size determined by analyzing their diameters on SEM images of the polyHIPME formed after solidification and freeze-drying of the HIPME was 130 nm (Figure 4-7). SEM images (Figure 4-6C) show that the pores (arising from removal of the oil phase) have dimensions of around 100 nm, which are interconnected (indicated by asterisks), contributing to the large surface area per unit volume of the polyHIPMEs as compared to CSNLYS-C and CSNLYS-L polyHIPEs (Table 4-5). When the interfacial tension is close to zero as in the case of CSNLYS-H an o/w microemulsion with nano-sized droplets forms spontaneously.

The results show that by varying the chain length of the hydrophilic oligolysine side chain of the CSNLYS copolymer, which also contains the relatively hydrophobic grafted PNIPAM pendent chains, micro- and macro HIPEs can be stabilized using this copolymer. It is postulated that the PNIPAM side chain is

compatible with the oil phase while the oligolysine side chain is compatible with the water phase and the relatively rigid polysaccharide backbone forms extended segments at the interface to minimize the total energy of the system. CSN is able to stabilize emulsion which are however, unstable without the oligolysine side chains (Figure 4-8). The improved solubility after grafting oligolysine onto CSN allow higher polymer concentration to be incorporated in the continuous phase, and the reduced oil-water interfacial tension allow more stability during emulsification, solidification and drying process.

Figure 4-8 Scanning electron micrograph of foam formed from emulsion templating with 2% w/v CSN shows irregular structure and collapsed pores.

While keeping the DS and DP of PNIPAM constant, a higher DP of the grafted oligolysine resulted in zero interfacial tension between water and *p*-xylene to allow for HIPME formation and stabilization, without the need for additional surfactants or co-surfactants, as required for most conventional macro- and microemulsions [251-253]. On the other hand, when using CSNLYS with a

lower DP of oligolysine, a typical HIPE formed. A possible reason could be the presence of the longer hydrophilic oligolysine-graft. With the amount of the hydrophobic hydrocarbon moiety being constant, a larger hydrophilic group can reduce the packing efficiency in o/w emulsions [254], thereby increasing the curvature and decreasing the size of the droplets formed. This collective effect may serve to the lower interfacial tension.

4.4 Conclusion

The synthesis of a multifunctional thermoresponsive highly water-soluble chitosan that is able to 1) stabilize emulsions, 2) allow the solidification of the emulsion template without crosslinking and 3) acts as the polyHIPE matrix was reported. By varying the DP of oligolysine grafted to PNIPAM grafted chitosan while keeping the other components constant, it was found that very different emulsion types can be formed, namely HIPEs and HIPMEs. It was shown here that the formation of very porous polyHIPEs and polyHIPMEs is possible without using additional surfactant, co-surfactant and crosslinking agents if the properties of the polymer in the continuous phase are suitably tailored. The emulsion and the subsequent emulsion-templated foam formed from this type of self-emulsifying biopolymer is found to be highly sensitive to the emulsifier used.

CHAPTER 5

HIGH INTERNAL PHASE EMULSION TEMPLATING WITH SELF-EMULSIFYING AND THERMORESPONSIVE CHITOSAN- *GRAFT*-PNIPAM-*GRAFT*-OLIGOPROLINE

5.1 Introduction

In the chapter four, the synthesis of chitosan, PNIPAM and lysine based thermoresponsive surfactants that allows self-emulsification of microHIPE and macroHIPE by tuning the chain length of the grafted oligolysine component were shown. Though a multifunctional biopolymer for self-emulsification was successfully devised, the effective pore size of the foam formed is still too small at $10.5 \pm 6.9 \mu\text{m}$ and porosity of 88.3%, and lacking in pore interconnectivity. It is deduced from the previous study that since the amino acid graft plays a major role in affecting the properties of the emulsion, changing the properties of the grafted amino acid may improve the emulsion-templated foam properties.

Here, two other charged amino acids were identified to be incorporated as part of the CSN biopolymer for emulsion stabilization - proline and glutamic acid.

Proline

Glutamic acid

Figure 5-1 Structure of amino acid proline and glutamic acid.

Proline is found as a component in a large number of proteins, such as collagen, and hence could act to enhance the biomimicry of the scaffold. It is highly water-soluble and possesses a slight positive charge at pH 7. Its pyrrolidine rings can interact and stack together by hydrophobic interaction, while the imino and carboxyl groups interact with water [255]. In its polyproline form, it forms the polyproline II helix, with three residues per turn, in which its pyrrolidine rings can also aggregate by hydrophobic interaction while the backbone amide and carbonyl groups are partially exposed to water [256], allowing it to interact with both hydrophobic and hydrophilic environments. Therefore, proline was chosen for its unique structure that gives it both hydrophilic and hydrophobic properties which show some promise in augmenting the stabilization of HIPE, and at the same time its slight hydrophobicity may act to not decrease the oil-water interfacial tension as much as lysine, therefore bigger pores may be formed.

Glutamic acid, on the other hand, possesses negative charges at pH 7 due to the presence of its carboxylate side chain. The monosodium glutamate form of glutamic acid is highly water soluble. L-glutamic acid was also found to augment the solubility of poorly soluble proteins by reducing the intermolecular interactions amongst them [257]. The structure of glutamic acid is similar to lysine, being a linear molecule, the difference being the negative charges that it possess instead. It would also be interesting to find out the effect of varying the charge of the oligo(amino acid) side chain on the self-emulsification property of the copolymer.

The aim of the work in this chapter is to improve the characteristics of the foam i.e. bigger pore sizes, without the use of organic additives, and at the same time to further uncover the effects of different amino acids on the self-emulsifying properties of the multifunctional biopolymer. Chitosan-*graft*-PNIPAM-*graft*-oligo(amino acid) was synthesized by initiating the free radical polymerization of NIPAM from chitosan to confer thermoresponsiveness. The thermoresponsive copolymer was then grafted with oligo(amino acid) (specifically oligoproline or oligo(glutamic acid)) using a condensation reaction with EDC•HCl and NHS to impart water solubility at physiological pH. These copolymers were then dissolved in aqueous solutions and emulsified with an oil (*p*-xylene) to create HIPEs stabilized solely by the copolymer. The polyHIPEs were then formed by solidifying the HIPE at 40°C, which was then freeze-dried. The properties of the copolymer were characterized, its use in the formation of a series of thermoresponsive polyHIPEs formed by varying the polymer

concentrations, internal phase volume ratios, and presence of small concentration of surfactant was investigated and their surface area and pore sizes and porosities were characterized.

5.2 Experimental Methods

5.2.1 Synthesis of Chitosan-*graft*-PNIPAM-*graft*-oligoproline (CSN-PRO) and Chitosan-*graft*-PNIPAM-*graft*-oligo(glutamic acid) (CSN-GLU)

Synthesis of CSN-PRO and CSN-GLU proceeded according to Scheme 5-1. CSN was first synthesized according to Section 3.2.10. 0.5 g of CSN was then dissolved in 20 mM TEMED/HCl at pH 4.7 for 24 h and 0.03 mol of L-proline or L-Glutamic acid monosodium salt hydrate was added at room temperature. 0.06 mol of EDC•HCl and 0.09 mol of NHS were then added and the reaction was allowed to proceed for 2 days. At the end of the reaction, the solution was neutralized with 1M NaOH and was clarified through filtration through a 0.22 μ m filter. The filtrate was then dialyzed against deionized water in a dialysis tubing (MWCO 12400) until the conductivity of the solution remained constant, after which the polymer solution was lyophilized. The products were then characterized by ^1H NMR, ATR-FTIR and GPC.

5.2.2 Determination of degree of substitution (DS) and degree of polymerization (DP)

The DS (number of rings grafted per 100 glucosidic rings) of PNIPAM and oligo(amino acids) was determined by titration of the residual amine groups of the copolymer [258]. The copolymer was first dissolved in 2 M HCl and titrated drop wise with 2 M NaOH. The DS was then calculated according to the method detailed in Appendix 3. The DP was determined by taking the integral ratio of the NMR peaks as detailed in Appendix 2 and Appendix 3.

5.2.3 Formation of emulsions and emulsion-templated macroporous polymers

CSN, CSN-PRO and CSN-GLU were dissolved in 10-40% w/v deionized water containing either 0% or 1% v/v PEG(20)sorbitan monolaurate in the aqueous phase according to Table 5-1. *P*-xylene was then added in a volume fraction of 0.60 to 0.90 with respect to the total volume. The mixture was emulsified by mixing it in an analog vortex mixer (Fisher Scientific) at 2500 rpm. The emulsion type was determined using the drop test method at 25°C and 40°C. Macroporous polymers were formed by solidifying the emulsion at 40°C, freezing in liquid N₂, and then removing the solvents by freeze drying.

Table 5-1 HIPE compositions of samples containing CSN, CSN-GLU and CSN-PRO (polymer concentration x % w/v in the aqueous phase water) with internal phase volume fractions ϕ are denoted as Cx- ϕ , Gx- ϕ and Px- ϕ , respectively.

Sample	Polymer % w/v in aqueous phase	PEG(20)sorbitan monolaurate %v/v in aqueous phase	ϕ
Series 1			
C2.5-0.75	2.5	0	0.75
G20-0.75	20	0	0.75
P20-0.75	20	0	0.75
Series 2			
P20-0.75-T1	20	1	0.75
P20-0.85-T1	20	1	0.85
P10-0.60-T1	10	1	0.60
P10-0.75-T1	10	1	0.75
P10-0.85-T1	10	1	0.85
P10-0.90-T1	10	1	0.90
P40-0.75-T1	40	1	0.75

5.2.4 Culture of mESCs in poly(CSN-PRO)HIPE

The scaffolds were first cut into pieces with dimensions 5 mm in diameter and 2 mm thickness and sterilized in their dry state under UV exposure ($\sim 40 \mu\text{W}/\text{cm}^2$) for 1 hour and thereafter maintained under aseptic conditions. The cells used in this study was undifferentiated murine embryonic stem cells (mESCs). Expansion of mESCs were performed in complete DMEM supplemented with 10% heat-inactivated FBS, 2 mM L-Glutamine, 1% penicillin/streptomycin, 0.1 mM 2-mercaptoethanol, and 1000 U/ml Leukemia Inhibitory Factor and incubated at 37°C, 5% CO₂. When the cells culture reached 80% confluence, cells were detached with 0.25% trypsin-EDTA, centrifuged and re-suspended in complete DMEM at 2×10^6 cells/ml and at 37°C and seeded carefully on top of the scaffolds placed in each well of a 96-wells plate. The seeded scaffolds were incubated at 37°C for 5 hours to allow the cells to penetrate the pores and thereafter 200 μl of complete DMEM was added. The cells were then cultured in the scaffolds for 4 days.

5.2.5 SEM imaging of mESCs in polyHIPE scaffolds

To prepare the sample for SEM imaging, the cell-laden hydrogels were first fixed with 4% glutaraldehyde for 24 h and then washed with PBS. Thereafter, the sample is rapidly frozen in liquid nitrogen and freeze-dried using controlled temperature to obtain dry samples.

5.2.6 Cell viability assay of mESCs in polyHIPE scaffolds

LIVE/DEAD[®] Viability/Cytotoxicity Assay Kit (Invitrogen) was used to assess the viability of the encapsulated cells. The cell-laden hydrogels were incubated in “Live/Dead” solution containing 2 mM calcein AM and 4 mM EthD-1 in PBS for about 45 min at 37°C. Cells were observed and imaged using a Zeiss Axiovert 200M microscope (Carl Zeiss, Germany) and analyzed using AnalySIS[®] imaging software (Soft Image System GmbH).

5.3 Results and Discussion

5.3.1 Thermoresponsive chitosan-*graft*-PNIPAM-*graft*-oligo(amino acid)

Chitosan-*graft*-PNIPAM-*graft*-oligo(amino acid) was first prepared with either proline (CSN-PRO) or glutamic acid (CSN-GLU) as the amino acid component. CSN-PRO and CSN-GLU were synthesized in a two-step procedure (Scheme 5-1).

Scheme 5-1 Reaction scheme. Step 1: Ceric ammonium nitrate initiated radical graft copolymerization of PNIPAM onto CS to form CSN. Step 2: Condensation of L-proline or L-monosodium Glutamic acid monomers onto CSN to form CSN-PRO or CSN-GLU.

Firstly, PNIPAM was grafted from the amine group of the chitosan (CS) backbone [259-261] to form CS-*graft*-PNIPAM (CSN) by radical polymerization of NIPAM monomer initiated using ceric ammonium nitrate as radical redox initiator. Then the oligo(amino acid) was polymerized from the residual amine groups in CSN to form CSN-PRO or CSN-GLU by condensation of L-proline or L-glutamic acid monosodium salt hydrate in the presence of EDC•HCl and NHS.

The synthesis of CSN, CSN-PRO and CSN-GLU was confirmed using ^1H -NMR. The copolymer composition (a, b, c, d) (refer to copolymer structure in Figure 5-2a) was determined by amine titration, and the degree of polymerization of PNIPAM and oligo(amino acid) (x and y, respectively) was determined from NMR integrals. The NMR peaks of CS were identified (Figure 5-2b(i)) [262-263] and the degree of deacetylation determined from the ratio of H7 and H2-6 to be 82.5%.

When PNIPAM was grafted onto CS to obtain CSN (Figure 5-2b(ii)), additional peaks at $\delta 1.1$ ppm, $\delta 3.8$ ppm, $\delta 2.0$ ppm and $\delta 1.5$ ppm, corresponding to H12, H11, H8 and H9, respectively, appeared, confirming the grafting of PNIPAM onto chitosan [264-265]. Some of the peaks in spectra bii to biv (H1-7) shifted downfield because the solvent was changed from DCl in i) to D_2O in ii) to iv). The proportion of amine groups consumed by the grafting of PNIPAM was determined by amine titration and DS (column a in Table 5-2) was found to be

69%. The DP of PNIPAM (x) was determined from the ratios of the integral areas of the NMR peaks (H12:H7) and was found to be 26.

With CSN-PRO (Figure 5-2b(iii)), a new peak (H23) at δ 4.25ppm, from the pyrrolidine unit of proline, was seen. Additional peaks (H20 and H21/H22) due to the methylene bridge protons of proline were seen at δ 3-3.5ppm and δ 1.85-2.54ppm [266]. With CSN-GLU (Figure 5-2b (iv)), new peaks (H13, H15) at δ 2.4ppm and δ 3.3ppm were seen. Furthermore, H14 at δ 2.0ppm, which represents the methylene protons adjacent to glutamic acid's α -carbon, overlaps with the H8 and H7 peaks [267-268]. The degree of substitution (column b in Table 5-2) was determined from titration of residual amine groups and found to be 5 and 3 for CSN-PRO and CSN-GLU, respectively. The degree of polymerization of proline or glutamic acid (y) was calculated from the ratio of the NMR peaks (H23:H7 and H13:H7) and found to be 5 for both CSN-PRO and CSN-GLU.

Figure 5-2 a) Structure of chitosan-graft-PNIPAM-graft-oligoproline (CSN-PRO) or chitosan-graft-PNIPAM-graft-oligo(glutamic acid) (CSN-GLU). b) ^1H -NMR (300MHz, 20% w/w DCl/D $_2\text{O}$, 25°C) spectra of i) chitosan and ^1H -NMR (300MHz, D $_2\text{O}$, 25°C) spectra of ii) CSN, iii) CSN-PRO and iv) CSN-GLU.

Table 5-2 DS and DP as determined from titration and NMR integrals. *a + b + c + d =100%, where a, b, c, d, refers to the %composition of each type of unit in Figure 5-2a.

			Degree of Substitution (DS)		Degree of polymerization (DP)	
Composition*	c	d	a	b	x	y
CS	17.5	82.5	0	0	0	0
CSN	17.5	13.8	69	0	26	0
CSN-PRO	17.5	8.80	69	5	26	5
CSN-GLU	17.5	10.8	69	3	26	5

Figure 5-3 GPC elution peaks for CS, CSN, CSN-PRO and CSN-GLU.

Table 5-3 Weight average molecular weights and polydispersity index for CS,

Weight Average Molecular Weight (g/mol)	Polydispersity Index
--	-------------------------

CSN, CSN-PRO and CSN-GLU.

CS	1.80×10^6	1.03
CSN	2.25×10^6	1.17
CSN-PRO	2.36×10^6	1.15
CSN-GLU	2.27×10^6	1.25

The weight average molecular weights of CS, CSN, CSN-PRO and CSN-GLU determined using GPC against pullulan standards were 1.80, 2.25, 2.32 and 2.27×10^6 Daltons, respectively (Table 5-3 and Figure 5-3). The increase in molecular weights of the modified polymers corresponds to the trend seen in Table 5-2 whereby a greater increase in MW after the grafting of PNIPAM onto chitosan was due to a much higher DS and DP as compared to the smaller increase in MW after the further grafting of amino acid side chains, which resulted in a lower DS and DP as 83.3% of the glucosamine units present in the input CS are already occupied by the grafted PNIPAM side chains.

Figure 5-4 ATR-FTIR spectra of i) CS, ii) CSN, iii) CSN-PRO and iv) CSN-GLU.

The grafting of PNIPAM and oligoproline/oligo(glutamic acid) was further confirmed by ATR-FTIR. All the FTIR spectra shown in Figure 5-4 exhibit the characteristic signature of the chitosan backbone; an adsorption band due to the β ,1-4 glucosidic bond C-O-C at 1100 cm^{-1} [262]. In the spectra for CSN, CSN-PRO and CSN-GLU (Figure 5-4(ii-iv)), the enhanced features in the region 3100 cm^{-1} to 3500 cm^{-1} , due to N-H stretch, indicate the increased abundance of N-H groups from the grafted PNIPAM. The presence of bands at 1375 cm^{-1} and 1348 cm^{-1} (methyl $-\text{CH}_3$ groups bending in PNIPAM), 2970 cm^{-1} ($-\text{CH}$ stretching of PNIPAM), and strengthening of bands at 1640 cm^{-1} (Amide I), 1545 cm^{-1} (Amide II), and 1455 cm^{-1} (Amide III), indicate that PNIPAM was indeed grafted onto chitosan [269].

In the spectra for CSN-PRO and CSN-GLU (Figure 5-4iii-iv), the band at 1600 cm^{-1} , belonging to primary NH_2 bending in chitosan, disappeared due to the conjugation with CS of PNIPAM and proline or glutamic acid, once again indicating that the grafting of PNIPAM and amino acids occurred on the glucosamine $-\text{NH}_2$ [270]. Also, the spectra of CSN-PRO and CSN-GLU are similar to each other and to that of CSN due to substantial similarities in the amino acids spectra and overlap of PRO and GLU features with the strong amide I and amide II bands of the grafted PNIPAM (refer to Table 5-4).

Table 5-4 FTIR peaks showing the expected peaks of CSN, CSN-PRO and CSNGLU and their overlapping peaks.

	Wavenumber cm^{-1}
PNIPAM	
-CH stretch	2970
Amide I	1640
Amide II	1545
Amide III	1455
-CH ₃	1375
Proline and Glutamic acid	
-CH stretch	2970
COO- of CSN-GLU	1600-1700
-C=O stretch	1623
-C-H bending	1375, 1455
C-O stretch, OH bend of COOH, CH ₂ twisting and in-plane bending	1330-1170

5.3.2 Solution properties of CSN, CSN-PRO and CSN-GLU

Table 5-5 summarizes the important solution properties of CS, CSN, CSN-PRO and CSN-GLU in terms of their maximum solubility, ζ -potentials, LCSTs, as well as the interfacial tensions between water and *p*-xylene at pH 7, the pH during emulsification.

Table 5-5 Solution physical properties of CS, CSN, CSN-PRO and CSN-GLU: Maximum solubility, pH range of solubility, zeta potential (ζ), LCST and interfacial tensions.

Sample	Max Solubility [% w/v] ^{a)}	pH range of solubility ^{b)}	ζ [mV] ^{a),b)}	LCST [°C] ^{a),c)}	γ_{AW} [mN/m] ^{a),d)}	γ_{OW} [mN/m] ^{a),e)}
CS	1	1 - 6	0.4	-	-	-
CSN	2.5	1 - 7	0.8	35	44.0 ± 0.4	11.7 ± 0.2
CSN-PRO	40	1 - 12	1.5	34	39.0 ± 0.7	8.9 ± 0.0
CSN-GLU	20	1 - 12	-0.5	35	46.4 ± 0.8	19.6 ± 0.2

^{a)}Measurements made at pH 7, the pH during emulsification, except at pH 6 for CS; ^{b)}Measurements made at 2.5% w/v, except for CS at 1% w/v; ^{c)}As determined by turbidimetry; ^{d)} Interfacial tension of aqueous sample in air; ^{e)} Interfacial tension of aqueous sample in *p*-xylene

CS is soluble in the pH range of 1-6.4 with maximum solubility of just 1% w/v at pH 6.4 while CSN is soluble in the pH range from 1 to 7, with a maximum solubility at pH 7 of 2.5% w/v. In contrast, CSN-PRO and CSN-GLU exhibited solubility over a pH range from 1-12 at 2.5% w/v with a much improved solubility at pH 7 of up to 40% w/v and 20% w/v, respectively (Table 5-5). This improvement in solubility can be attributed to the disruption of the formation of

intermolecular hydrogen bonding between the abundant -NH₂ and -OH groups of the glucosidic rings of chitosan, due to the grafting of PNIPAM and amino acid onto glucosamine -NH₂ [113]. The further grafting of oligoproline greatly improves the solubility of CSN as its pyrrolidine ring is also an effective structure disruptor [271], as compared to glutamic acid which is a linear chain.

It is expected that these three polymers should exhibit LCSTs in the region of 32°C due to the grafted PNIPAM component. This was checked by turbidity measurements to determine their LCSTs as the temperature at which their solutions first turned cloudy when the temperature was increased from 25°C. Their LCSTs were measured to be between 34°C and 35°C (Table 5-5), which are slightly higher than the LCST of pristine PNIPAM (32°C) due to the increased hydrophilicity of the copolymers [237, 272-273].

Figure 5-5 A) ζ -potential of CSN, CSN-PRO and CSN-GLU in the pH range from 1 to 12 in pH adjusted aqueous HCl/NaOH solutions. B) LCST of CSN, CSN-PRO and CSN-GLU solution in the range of pH 1-12 in aqueous HCl/NaOH determined by turbidity method. CSN-PRO and CSN-GLU are not soluble at pH 14. CSN is not soluble above pH7.

The possible effects of varying pH on the ζ -potential and LCST of these three polymers were also investigated. Their ζ -potentials were shown to decrease with increasing pH. The isoelectric points were found to be ~7, 7.6 and 5.0 for CSN, CSN-PRO and CSN-GLU, respectively (Figure 5-5A). Their LCST were unaffected by pH, except at pH 1 and pH 12, as seen in Figure 5-5B. At the two extreme pHs, the LCSTs were lower (32°C), which is possibly due to the lower solubility of the copolymers at these extreme pHs. The observed invariability of LCST as function of pH is expected since the thermoresponsiveness is dominated by the PNIPAM grafts, which has been shown to be insensitive to environmental changes, except temperature [237].

The oil/water interfacial tension (γ_{ow}) plays a significant role in the stabilization of HIPE. CSN and CSN-PRO significantly lower the p -

xylene/water interfacial tension γ_{ow} to 11.7 ± 0.2 mN/m and 8.9 ± 0.0 mN/m, respectively, (Table 5-5), which are much lower than γ_{ow} between *p*-xylene and pure water (36.8 ± 0.0 mN/m). With CSN-GLU, γ_{ow} was reduced, but to a much lesser extent, to 19.6 ± 0.2 mN/m. The amphiphilic nature of the derivatives leads to significant lowering of the γ_{ow} . For CSN, this is conferred by the hydrophilic chitosan backbone [274], and the hydrophobic hydrocarbon chains on PNIPAM [275]. With CSN-PRO, in which the grafted oligoproline has certain degrees of interaction with water and hydrophobicity [256] (the pyrrolidine rings aggregate by hydrophobic interaction, and the short oligoproline chain can still interact with water as the amide and carbonyl groups are partially exposed to water), the γ_{ow} is further reduced.

5.3.3 Stabilization of high internal phase emulsions solely by CSN or CSN-PRO

Since the copolymers are amphiphilic and could act as an emulsifier, CSN, CSN-PRO and CSN-GLU were tested as emulsifier for o/w HIPEs with deionized water as the aqueous continuous phase and *p*-xylene as the internal oil phase. Solutions of CSN, CSN-PRO and CSN-GLU in deionized water were prepared and then enough *p*-xylene was added to result in an internal phase volume ratio (ϕ) of 0.75 (Series 1, Table 5-1).

Scheme 5-2 Fabrication of polyHIPEs. Step 1: CSN-PRO dissolved in water as the continuous phase and *p*-xylene as the dispersed oil phase at $\phi > 0.74$ was mixed together by vortexing to obtain an o/w HIPE. Step 2: Solidification of the HIPE by raising the temperature to 40°C. Step 3: Freeze drying of the solidified HIPE to remove the aqueous and oil phase.

The emulsions were created using a vortex mixer (Step 1, Scheme 5-2). The emulsions containing CSN, CSN-GLU and CSN-PRO (polymer concentration x % w/v in the aqueous phase water) with internal phase volume fractions ϕ hereafter denoted as Cx- ϕ , Gx- ϕ and Px- ϕ , respectively (Table 5-1).

Figure 5-6A1 and Figure 5-6B1 show that for C2.5-0.75 and P20-0.75, a white emulsion with no visible excess internal phase was formed, indicating that all the *p*-xylene had been internalized and a stable HIPE formed. The HIPEs formed from CSN and CSN-PRO were also seen to flow at room temperature (Figure 5-6A2 and Figure 5-6B2). The type of HIPE formed was determined by the emulsion drop test. An emulsion drop was placed into water or oil (*p*-xylene) at 25°C (Figure 5-7). When a drop of the emulsion was introduced into a large excess of water at 25°C (Figure 5-7A), the droplet dispersed into the

surrounding water but was remained in droplet form in *p*-xylene (Figure 5-7B). This suggests that the continuous phase of the droplet is similar to the aqueous medium (Figure 5-8), confirming that the emulsion formed is of oil-in-water (o/w) type [276]. For the G20-0.75 emulsion, excess *p*-xylene not incorporated into the homogenous emulsion was seen (Figure 5-6C), indicating that a HIPE cannot be formed with CSN-GLU. Although an emulsion is formed with CSN-GLU, its higher γ_{ow} does not enable the self-stabilization of HIPEs and excess *p*-xylene remains on top of the emulsion.

Figure 5-6 Photographs of HIPEs formed with C2.5-0.75 (A1) and P20-0.75 (B1) at room temperature which shows all *p*-xylene was incorporated. C) Emulsion made with G20-0.75 does not incorporate all *p*-xylene. C2.5-0.75 (A2) and P20-0.75 (B2) emulsion was seen to flow at 25°C and solidification of the emulsion of both C2.5-0.75 (A3) and P20-0.75 (B3) occurred after heating it to 40°C.

5.3.4 Enhanced gel/foam properties with CSN-PRO compared to CSN

Figure 5-7 The emulsion type is determined by the drop test method. A drop of emulsion formed with CSN-PRO at 25°C, was dropped into A) water and B) *p*-xylene. The emulsion droplet was seen to disperse into deionized water but remained as a droplet in *p*-xylene. At 40°C, the droplet remained intact in both medium, floating in C) water, while sinking in D) *p*-xylene. Arrows indicates location of droplets.

Figure 5-8 Schematic of the drop test: A drop of emulsion made with CSN-PRO was added into a vial with A) water at 25°C, B) *p*-xylene at 25°C, C) water at 40°C and D) *p*-xylene at 40°C. A: Emulsion droplet dispersed completely into surrounding water medium at 25°C; B: Emulsion droplet did not disperse in *p*-xylene at 25°C. C: Emulsion droplet remained intact in water at 40°C due to PNIPAM aggregation holding the droplet together. The droplet is floating due to the lower density of *p*-xylene which volume takes up more than 75% of the total droplet volume. D: Emulsion droplet also did not disperse in *p*-xylene at 40°C.

The HIPEs formed were then solidified by raising the temperature to 40°C to obtain polyHIPEs (Step 2, Scheme 5-2) [24]. When the temperature of C2.5-0.75 and P20-0.75 HIPEs was raised to 40°C, which is above their LCSTs, the emulsions solidified (Figure 5-6A3 and Figure 5-6B3), exhibiting thermoresponsive behavior. Although both CSN and CSN-PRO are found to be able to stabilize HIPEs, the maximum polymer concentration of CSN was limited because of its low water solubility (2.5% w/v, Table 5-5), compared to that of CSN-PRO (40% w/v, Table 5-5). The much higher solubility of CSN-PRO results in a gelled solidified HIPE with superior handle-ability. As it would be essential to know also if raising the temperature above its LCST to 40°C will have any effect on the emulsion type, the drop test was repeated for both water and *p*-xylene medium at 40°C (Figure 5-7C and Figure 5-7D). Irrespectively in which medium the emulsion droplet was introduced into at 40°C, the droplet did not disperse neither in water nor in *p*-xylene because of the gelation of the CSN-PRO in the continuous aqueous phase. At this elevated temperature, above the LCST, the PNIPAM component of CSN-PRO was physically aggregated, gelling the aqueous phase. The droplet floated on the surface of the water (Figure 5-7C) but sank in the *p*-xylene medium (Figure 5-7D) because the average density of the o/w emulsion droplet is lower than water but higher than *p*-xylene.

Porous polyHIPEs were then obtained from the gelled HIPEs after removal of water and *p*-xylene by freeze-drying (Step 3, Scheme 5-2). With CSN, the resulting foam structure (Figure 5-9A1, SEM) was seen to be irregular with many large pores, which could be the result of emulsion destabilization during the freeze-drying process. The low solubility of CSN only allows a low concentration of polymer to form the bulk structure of the foam, which tends to collapse during freeze-drying.

Figure 5-9 Scanning electron micrographs of emulsion-templated macroporous polymers made by solidifying and freeze drying HIPEs stabilized solely by A1) CSN (C2.5-0.75) A2) CSN-PRO (P20-0.75) and A3) CSN-GLU (G20-0.75). A4) A representative conventional polyHIPE (Reproduced from Ref [21] with permission of The Royal Society of Chemistry).

Table 5-6 Physical properties of polyHIPEs: Surface area, pore size, pore throat size and porosity.

No.	Sample	Surface area [m ² /g]	Pore size [μm]	Pore throat size [μm]	Porosity %
1	P20-0.75	123	32 - 71	-	73.8
2i	P20-0.75-T1	125	5 – 20	2 – 5	72.5
2ii	P20-0.85-T1	145	34 - 59	11 – 17	84.8
3i	P10-0.60-T1	245	8 – 22	2 – 9	98.1
3ii	P10-0.75-T1	290	6 – 24	2 – 6	99.7
3iii	P10-0.85-T1	322	25 – 78	6 – 21	98.5
3iv	P10-0.90-T1	333	28 - 143	7 – 46	99.2
4	P40-0.75-T1	69	48 - 58	5 – 14	71.4
	Conventional polyHIPE ^{a)}	5 - 20	1 - 20	0.2 - 10	<95

a) Conventional polyHIPE formed by Styrene-DVB ^[2c, 31c]

With CSN-PRO, a very well-defined macroporous structure (Figure 5-9A2) was obtained, with pore sizes ranging from 32 to 71 μm, a porosity of 73.8% and a surface area of 123 m²/g measured by BET (P20-0.75, Table 5-6). Poly(CSN-PRO)HIPEs (Figure 5-9A2) exhibited a rougher surface than conventional polyHIPEs (Figure 5-9A4) [21]. The surface area measured by BET of poly(CSN-PRO)HIPE is about 20 times larger than that of traditional poly(styrene-*co*-DVB)HIPEs [12, 14, 277-279]. With CSN-GLU, the solid structure formed contained few pores (Figure 5-9A3) because of its higher γ_{ow} , and appeared to have no defined pore structure.

The polyHIPE made from CSN-PRO (Figure 5-9A2) resembles that of a conventional polyHIPE (Figure 5-9A4), which has to be made with surfactant. The stark difference is the appearance of closed-pores in poly(CSN-PRO)HIPEs, which is hypothesized to be the effect of a polymer self-stabilized emulsion in which the polymer simultaneously forms the bulk polymer structure.

5.3.5 Thermoresponsive poly(CSN-PRO)HIPE

Figure 5-10 Thermoreversibility of poly(CSN-PRO)HIPEs was demonstrated where it was first placed in A) water at 40°C, and then B) left to cool at room temperature where the polyHIPE was seen to dissolve. C) Complete dissolution occurred within 5 minutes.

As thermoresponsive PNIPAM was incorporated, it would be interesting to see if the macroporous solid HIPE inherits the property of temperature sensitivity. To evaluate this, the poly(CSN-PRO)HIPE was first immersed in water at 40°C (Figure 5-10A). Thereafter, it was allowed to cool to room temperature. As the temperature of the bath dropped below 25°C, which is below the LCST of CSN-PRO (Figure 5-10B-C), it dissolved. This immediate response to LCST temperature is useful for applications that require a quick change when the environmental temperature stimulus changes, *e.g.* temperature stimulated

release of encapsulated drugs/cells [186] or as an injectable system for tissue engineering applications [18, 24-25].

5.3.6 Effect of surfactant, ϕ , and polymer concentration on CSN-PRO polyHIPE

Using CSN-PRO, the effect of the presence of small amount of biocompatible surfactant (0.1% v/v of aqueous phase), the internal phase volume ratio ϕ and polymer concentration in the minority continuous aqueous phase on the properties of the resulting polyHIPE (Series 2, Table 5-1) were investigated. The polyHIPEs were formed by first solidifying the HIPEs by raising the temperature to 40°C, followed by locking in the emulsion template by rapidly freezing it in liquid nitrogen and subsequently removing the water and oil phases by freeze drying (Step 3, Scheme 5-2). PEG(20)sorbitan monolaurate was chosen as the surfactant as it has been shown to be compatible at low concentration in some tissue engineering applications [280] and also as it is non-ionic and it does not form complex with CSN-PRO.

When PEG(20)sorbitan monolaurate surfactant was used in the formulation of the HIPE while keeping other variables constant (Sample #2i, P20-0.75-T1, Table 5-6), the pore sizes of the resulting polyHIPE decreased to the range of 5 – 20 μm (Figure 5-11Ai), in comparison with P20-0.75 (Sample #1, Figure 5-9A2), while the interconnectivity of the foam greatly improved by the formation of numerous pore throats in the size range of 2 – 5 μm (Figure 5-11Ai and Table 5-6). These results show that the addition of a surfactant to

the HIPE effectively opens up pore throats and increases the interconnectivity of the polyHIPE, which corroborated previous studies showing that surfactant plays the major role in determining degree of interconnectivity of the resulting polyHIPE [279, 281-282].

When ϕ was increased from 0.75 (Sample #2i, P20-0.75-T1) to 0.85 (Sample #2ii, P20-0.85-T1) (see Figure 5-11Ai and Figure 5-11Aii respectively), as expected the porosity (84.8%) increased and so did the average pore (34 – 59 μm) and pore throat size (11 – 17 μm) (Table 5-6).

At P=40% w/v, PolyHIPE with $\phi > 0.75$ cannot be formed as viscosity becomes too high

Figure 5-11 SEM of poly(CSN-PRO)HIPEs formed with PEG(20)sorbitan monolaurate with A) 20% w/v CSN-PRO i) $\phi=0.75$ (P20-0.75-T1); ii) $\phi=0.85$ (P20-0.85-T1); B) 10% w/v CSN-PRO i) $\phi=0.60$ (P10-T1-0.60), ii) $\phi=0.75$ (P10-0.75-T1), iii) $\phi=0.85$ (P10-0.85-T1), iv) $\phi=0.90$ (P10-0.90-T1) and C) 40% w/v CSN-PRO, $\phi=0.75$ (P40-0.75-T1). Inset arrow points to a pore throat.

Using a polymer concentration of 10% w/v, ϕ was varied from 0.60 to 0.90 (Samples #3i-3iv, Table 5-6). A higher porosity, increased surface area, larger pore size and pore throat size of the polyHIPEs were observed as compared to polyHIPEs formed from HIPEs containing only 20% w/v CSN-PRO. The surface area measured using BET increased by about 2.3 times (to 290 m²/g for Sample #3ii from 125 m²/g for Sample #2i) and porosity increased to 99.7%. Also, the maximum amount of internal phase that can be incorporated when 10% w/v CSN-PRO was used was found to be as high as 0.90, as compared to 0.85 when 20% w/v CSN-PRO was used, with the porosity, pore and pore throat sizes increasing with ϕ . The reduction in polymer concentration also led to a reduction of the viscosity of the continuous emulsion phase, enabling smaller droplets to be formed with the same energy input as it becomes easier to break up droplets. When the polymer concentration of the continuous phase was increased to 40% w/v (Sample #4, P40-0.75-T1, Figure 5-11C), the average measured pore size of the polyHIPE was indeed much larger (pore size: 48 – 58 μ m; pore throat size: 5 – 14 μ m, Table 5-6) because of the higher viscosity of the continuous phase.

5.3.7 Seeding of mouse embryonic stem cells (mESCs) into poly(CSN-PRO)HIPE hydrogel

Figure 5-12 mESCs A) attaching on the surface of poly(CSN-PRO)HIPE and B) infiltrating into the pores of the matrix after 4 days. Inset arrows point to representative mESCs colonies. C) Live/Dead analysis of encapsulated cells; Green: Live; Red: Dead.

To assess the biocompatibility of poly(CSN-PRO)HIPE in culturing cells, P10-0.80-T1 is chosen here because its larger pores size, higher porosity, pore interconnectedness and high surface area best meet the requirements of tissue engineering scaffold. Although P10-0.90-T1 exhibit slightly better properties, the low polymer content made the scaffold too soft to handle. Figure 5-12A shows mESCs attaching to the surface of the poly(CSN-PRO)HIPE scaffold and also penetrating and residing within the pores of the scaffold after four days (Figure 5-12B). Also the incorporation of this small amount of surfactant can

open up pore throat greatly and still do not harm cells, as seen from the live cell colonies (green) in the live/dead assay of the cells encapsulated within poly(CSN-PRO)HIPE hydrogel (Figure 5-12C). However, it did not seem to have substantial depth of cellular penetration within the scaffold as deduced from the SEM figures as the pore sizes may still need to be larger.

5.4 Conclusion

It is shown that chitosan grafted with PNIPAM and oligoproline (CSN-PRO) has a solubility of up to 40% w/v in water at pH 7. Moreover, it lowers the interfacial tension between water and *p*-xylene significantly to act as a suitable emulsifier for o/w HIPEs. The thermoresponsiveness of CSN-PRO also permits low-temperature and crosslinker-free solidification of HIPE templates to form closed-pore polyHIPEs with defined pore structures. With the incorporation of additional surfactant PEG(20)sorbitan monolaurate, pore throats were effectively created in the polyHIPEs, improving the interconnectivity of the pores, and at the same time reducing the pore size and increasing the surface area of the resulting poly(CSN-PRO)HIPEs, to achieve pore size of up to 143 μm and 99.2% porosity. These polyHIPEs also exhibited thermoresponsiveness in which its gelation in aqueous medium can be controlled by a change in temperature. mESCs seeded onto poly(CSN-PRO)HIPE were shown to be able to attach themselves to the scaffold and penetrate into the pores.

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CHAPTER 6

SOLIDIFYING THERMORESPONSIVE HIGH INTERNAL PHASE EMULSIONS STABILIZED SOLELY BY POLY (*N*- ISOPROPYLACRYLAMIDE)-*GRAFT*-GELATIN WITH A DUAL SOLIDIFICATION MECHANISM; SOLID HIPES FOR TISSUE ENGINEERING

6.1 Introduction

In Chapter 4 a multifunctional biopolymer based on chitosan, PNIPAM and oligolysine was shown to be possible in self-stabilizing HIPE, self-crosslink and act as the matrix of the porous polymer simultaneously. The pore sizes however should be bigger for the application of tissue engineering scaffolds for effective cell seeding and penetration. It was deduced from the study that the oligo(amino acid) side chain of the self-emulsifier plays a significant role in affecting the properties of the emulsion formed. Hence in Chapter 5, we show that by using oligoproline as the oligo(amino acid) side chain, highly interconnected foams with larger pores of up to 143 μm and more than 99% porosity can be formed, with the addition of just a very low concentration of

biocompatible surfactant. Non-anchorage dependent cells, mESCs seeded into the scaffolds were shown to be viable and able to penetrate into the pores of the matrix.

In this chapter, a gelatin-based polyHIPE that is suitable for anchorage dependent cells culture is presented. Gelatin is chosen because it is a polypeptide that possesses attachment sites for ADC to adhere to and proliferate [136-137]. The copolymer presented in this chapter can also self-emulsify HIPE and produce highly porous emulsion-templated foams. It was discovered that by using this copolymer, highly interconnected pores can be formed without the incorporation of additional surfactant, and the average pore sizes were also found to be bigger as compared to that in Chapter 4 and Chapter 5. The solidification of the continuous phase of self-stabilized Gelatin-*graft*-PNIPAM HIPE (GNHIPE) is done without the need for crosslinker monomers by taking advantage of the thermoresponsive properties of the copolymer using two different mechanisms conferred by the components of gelatin and PNIPAM. The poly(GN)HIPE formed by these two solidification methods with either water or PBS as the aqueous continuous phase were characterized by their pore sizes, porosity, surface area and morphologies observed by scanning electron microscopy. Human foreskin fibroblasts cells were then seeded into the poly(GN)HIPE scaffolds and the effect of using this dual solidification mechanism for cell seeding and proliferation was investigated by CCK-8 cell proliferation test, Live/Dead imaging, and the depth of cell penetration was elucidated by confocal microscopy. The feasibility of using cell-laden

poly(GN)HIPE hydrogel for injection was also investigated and the viability of the cells after extrusion from a needle was assessed using live/dead fluorescence imaging.

6.2 Experimental Methods

6.2.1 Synthesis of Gelatin-g-PNIPAM (GN)

1 g of Gelatin Type A was dissolved in 250 ml of deionized water in a round bottomed flask set in a water bath at 30°C. NIPAM (2.7 g), ceric ammonium nitrate (0.274 g) and 1 M nitric acid (10 ml) was added. Nitrogen atmosphere was maintained throughout the whole reaction for 24 h, and the reaction stopped by addition of 1M sodium hydroxide solution. The reaction product was transferred to a dialysis tubing (MWCO 12000 Da) and dialyzed against deionized water for 3 days, changing water frequently. A white fluffy product was obtained after lyophilization, and the grafting confirmed by ATR-FTIR (Imaging Golden Gate, Specac, UK) done in the range of 4000–900 cm^{-1} with a spectral resolution of 8 cm^{-1} using 64 scans.

6.2.2 Polymer characterization

6.2.2.1 Degree of grafting and degree of polymerization of PNIPAM

The ninhydrin colorimetric assay was used to determine the degree of grafting (DG). Briefly, ninhydrin reagent solution was added to solution of GN and unmodified gelatin and the relative absorbance values at 570 nm were read against a glycine standard curve to determine the amount of NH_2 that is present

before and after grafting of PNIPAM. The difference in the values obtained is the degree of grafting. Elemental analysis was done using Elementar Vario CHNS elemental analyzer (Elementar, Germany) to determine the DP by comparing C/N ratio of gelatin, GN and NIPAM. Details of DG and DP determination can be found in Appendix 4 and Appendix 5.

6.2.2.2 Dynamic rheological measurements and LCST

The variations in the intrinsic viscosity with temperature of gelatin and the GN polymer dissolved in either water or PBS were measured using a Thermo Haake Rheostress 600 Rheometer (Thermo Haake), with 20 mm plate to plate geometry at a constant strain of 0.5% and a constant angular frequency of 2 Hz, using a temperature ramp from 20-40°C at a rate of 0.1°C/min. The LCST was determined at the hairpin turn of the curve.

6.2.3 Formation of GNHIPEs and poly(GN)HIPEs

The different HIPEs were prepared according to the formulations in Table 6 - 1. The polymer was first dissolved in deionized water (W) or PBS (P) to give a solution with a concentration of 10% w/v. *P*-xylene was then added in a volume fraction ϕ of 0.75 to 0.90 with respect to the total volume. The mixture was emulsified using a vortex mixer (Fisher Scientific) operating at 2500 rpm. The emulsion type was determined using the drop test method. The polyHIPEs were formed by solidifying HIPEs at either 4°C (C, cooling) or 40°C (H, heating), followed by freezing the solidified HIPEs rapidly in liquid N₂, and then removing the liquid phases by freeze-drying. The notation for describing the

polyHIPE formulation is in the format: aqueous phase (deionized water, W or PBS, P) - ϕ - gelation method (C or H). As control (denoted Gelatin75C), a 10% w/v solution of pure gelatin in water was used as the aqueous phase of the HIPE as a comparison. Again *p*-xylene was used as the oil phase at $\phi=0.75$.

Table 6-1 Formulations of emulsions.

Sample	*Aqueous phase	#Volume fraction of internal phase ϕ	Solidification method	Polymer content in total emulsion volume % w/v
W75C	Water	0.75	^Cooling	2.5
W80C	Water	0.80	Cooling	2
W90C	Water	0.90	Cooling	1
W75H	Water	0.75	Heating	2.5
W80H	Water	0.80	Heating	2
W90H	Water	0.90	Heating	1
P75C	PBS	0.75	Cooling	2.5
P80C	PBS	0.80	Cooling	2
P90C	PBS	0.90	Cooling	1
P75H	PBS	0.75	Heating	2.5
P80H	PBS	0.80	Heating	2
P90H	PBS	0.90	Heating	1

* Polymer concentration in aqueous phase was in all formulations 10% w/v. #*P*-xylene was used as the internal oil phase. ^Only cooling was allowed for the solidification of Gelatin75C.

6.2.4 Mechanical properties of polyHIPEs

A Thermo Haake Rheostress 600 Rheometer (Thermo Haake) in parallel-plate configuration as described before was used to determine the storage (G') and loss moduli (G'') of poly(GN)HIPE hydrogels. To simulate the cell-seeding process, prior to testing, PBS at 25°C was added to the scaffolds (typically, of diameter of about 20 mm and thickness about 2 mm) ensuring that they were completely wetted, and then placed onto the stage immediately. The linear viscoelastic region was first determined by a series of amplitude sweeps followed by a frequency sweep. Mechanical spectrometry was carried out using dynamic temperature sweep using a temperature ramp at 0.1°C/min from 25°C to 40°C, frequency of 0.16 Hz and at a strain amplitude of 1%, which was performed in the linear viscoelastic region. Both auto-tension and auto-strain adjustments were applied.

6.2.5 Cell culture in polyHIPE scaffolds and determination of injectability

6.2.5.1 Cell seeding into polyHIPEs

The scaffolds were first cut into pieces with dimensions 5 mm in diameter and 2 mm thickness and sterilized in their dry state by UV exposure ($\sim 40 \mu\text{W}/\text{cm}^2$) for 1 hour and thereafter stored in aseptic conditions. 2D Expansion of FibroGROTM cells was performed in complete DMEM with 10% FBS and 1% penicillin/streptomycin at 37°C, 5% CO₂. When the cell culture reached 80% confluence, cells were detached with 2% trypsin-EDTA and re-suspended to a

concentration of 10^5 cells in 10 μ l of complete DMEM according to the method adapted from Mandal et al.[284] The concentrated cell suspension was then seeded carefully on top of the scaffolds placed in each well of a 96-well plate. The seeded scaffolds were incubated at 37°C for 5 hours to allow the cells to penetrate the pores of the polyHIPE and thereafter 200 μ l of complete DMEM was added. The cells were then cultured in the scaffolds for up to 10 days changing the medium every 2 days. Cells seeded at 2×10^4 cells/well in 96-well tissue culture polystyrene (TCPS) plate were used as a 2D control.

6.2.5.2 Cell viability

LIVE/DEAD[®] Viability/Cytotoxicity Assay Kit (Invitrogen) was used to assess the viability of the encapsulated cells. The cell-laden hydrogels were incubated in “Live/Dead” solution containing 2 mM calcein AM and 4 mM EthD-1 in PBS for about 45 min at 37°C. Cells were observed and imaged using a Zeiss Axiovert 200M microscope (Carl Zeiss, Germany) and analyzed using AnalySIS[®] imaging software (Soft Image System GmbH). Confocal microscopy using Zeiss LSM 780 (Carl Zeiss, Germany) was used to obtain the z-stack of the cell-laden scaffold at day 10 under controlled conditions of 37°C and 5% CO₂. The images were analyzed using the software ZEN 2012 SP (Carl Zeiss, Germany) to determine the cell penetration depth. To prepare the samples for SEM imaging, the cell-laden hydrogels on day 10 were first fixed with 4% glutaraldehyde for 24h and then washed with PBS. Thereafter, the sample was rapidly frozen in liquid nitrogen and freeze-dried using controlled temperature to obtain dry samples.

6.2.5.3 Cell Proliferation studies

Cell proliferation was assessed after culturing the cells in the scaffolds on day 1, 4, 7 and 10. On the day of measurement, the cell medium was replaced with 100 μ l of fresh culture medium with 10 μ l of CCK-8 reagent and the plates were incubated at 37°C for 4 hours. The absorbance was then measured on a microplate reader at 450 nm. The absorbance values were corrected by subtracting the background reading of the negative control (DMEM and CCK8 reagent only). The experiment was conducted in triplicates. Statistical analysis was performed using one-way ANOVA.

6.2.5.4 Injectability

Scaffolds, which are cultured with cells for 10 days, were placed into a 1 ml syringe with an 18G needle and extruded through the needle to assess the injectability of the polyHIPE hydrogel. The viability of the cells after injection was determined using Live/Dead analysis.

6.3 Results

6.3.1 Synthesis of Gelatin-*g*-PNIPAM (GN)

Scheme 6-1 Synthesis of gelatin -*graft*- PNIPAM (GN).

GN was synthesized by the radical graft copolymerization of PNIPAM onto gelatin, using CAN as an initiator (Scheme 6-1). Figure 6-1a shows the FTIR spectra of unmodified gelatin which displayed typical absorption bands for proteins; the N-H stretch at 3291 cm^{-1} , C=O stretching of peptide bonds at 1635 cm^{-1} (amide I), in-plane N-H bending at $1454\text{--}1530\text{ cm}^{-1}$ (amide II) and N-H bending at 1236 cm^{-1} (amide III) [285]. The FTIR spectra of GN (Figure 6-1b) shows additional peaks at 2972 cm^{-1} which can be assigned to the C-H

stretching of grafted PNIPAM and peaks at 1454 cm^{-1} , 1386 cm^{-1} and 1366 cm^{-1} corresponding to symmetric $-\text{C}(\text{CH}_3)_2$ bending of PNIPAM [286]. The DG and DP of PNIPAM were determined by the ninhydrin assay and elemental analysis respectively and found to be 82.6% and 31.5. A detailed calculation for DG and DP can be found in Appendix 4 and Appendix 5.

Figure 6-1 FTIR spectra of a) unmodified gelatin and b) GN.

6.3.2 Properties of GN

The properties of 1% w/v GN aqueous solutions determined were the surface tensions between aqueous solutions of GN and air, the interfacial tensions between aqueous solution of GN and *p*-xylene, and the LCSTs of GN in water and PBS (Table 6-2). The use of PBS as the aqueous continuous phase was investigated as it simulates the pH and ionic environment of the cell culture medium.

Table 6-2 Physical properties of Gelatin and GN in water and PBS; interfacial tension against air and *p*-xylene and LCST at a concentration of 1% w/v at 25°C.

	#Interfacial tension (mN/m)		#LCST (°C)
	Air (γ_{WA})	<i>P</i> -Xylene (γ_{OW})	
Water	72.8 $\pm$ 0.0	36.8 $\pm$ 0.0	-
Gelatin_{Water}	71.6 $\pm$ 1.1	25.5 $\pm$ 0.7	-
GN_{Water}	43.9 $\pm$ 0.4	9.9 $\pm$ 0.1	36.7
GN_{PBS}	46.1 $\pm$ 0.2	12.3 $\pm$ 0.1	32.2

Gelatin dissolved in water only marginally decreased the water/air surface tension γ_{WA} from 72.8 $\pm$ 0.0 mN/m to 71.5 $\pm$ 1.1 mN/m and water/*p*-xylene interfacial tension γ_{WO} from 36.8 $\pm$ 0.0 mN/m to 25.5 $\pm$ 0.7 mN/m (Table 6-2). However, when PNIPAM was grafted to gelatin, γ_{WA} dropped to 43.9 $\pm$ 0.4 mN/m and γ_{WO} to 9.9 $\pm$ 0.1 mN/m. When GN was dissolved in PBS its γ_{WA} and γ_{WO} was slightly higher at 46.1 $\pm$ 0.2 mN/m and 12.3 $\pm$ 0.1 mN/m, respectively.

The viscosity of unmodified gelatin and GN solutions in water and PBS as function of temperature was determined from 20°C to 40°C (Figure 6-2). For the unmodified gelatin the viscosity decreased rapidly with increasing temperature from 20°C as a result of the helix to coil transition of gelatin chains. The viscosity continued to decrease until 26°C after which it remained constant with further increasing temperature. Whereas for GN, up to 34.3°C for aqueous GN solutions and 29.5°C for GN in PBS solutions, the viscosity remained constant and then decreased rapidly due to the helix to coil transition of the gelatin component of GN chains as the temperature increased. Thereafter, a

sharp hairpin turn was observed at 36.7°C for GN in water and 32.2°C in PBS, which corresponds to the LCST of GN in water and PBS, respectively. At these temperatures the PNIPAM chains started to aggregate thereby causing the viscosity to increase again. The LCST was lower when measured in PBS than in water due to the salting out effect [272, 287-288].

Figure 6-2 Intrinsic viscosity of Gelatin and GN in water and PBS measured against a temperature ramp, with the LCST determined at the turning point of the curve.

6.3.3 Formation of HIPEs and gelled solid HIPEs by cooling or heating

Scheme 6-2 Formation of poly(GN)HIPEs. Step 1: Mix the aqueous phase and oil phase at $\phi > 0.74$ by vortexing at 25°C. Step 2: Solidification of the oil-in-water (o/w) HIPE templates at A) 4°C or B) 40°C. Step 3: poly(GN)HIPEs were obtained after freeze-drying of solidified HIPEs.

A series of HIPEs was formed by emulsifying GN dissolved in water or PBS, serving as the minority but continuous emulsion phase, and *p*-xylene as the dispersed oil phase (Scheme 6-2, Step 1). The HIPE compositions are listed in Table 6-1. In this study, two different methods were employed to gel the HIPEs; 1) by reducing the temperature to 4°C (Scheme 6-2, Step 2A) to induce the coil-helix transition of gelatin chains and 2) by increasing the temperature above the LCST of GN to 40°C (Scheme 6-2, Step 2B) to induce the phase separation and

aggregation of grafted PNIPAM moieties. As a control, unmodified gelatin dissolved in water was emulsified with *p*-xylene to yield HIPEs with $\phi=0.75$. The HIPE that formed could only be gelled at 4°C (sample name: Gelatin75C).

Figure 6-3 A) Photographs of GN-HIPEs formed with water or PBS as solvent for GN as aqueous phase. B) Flow test of the different GN-HIPEs at the respective temperatures of 4°C, 25°C and 40°C. C) Photographs of control HIPE formed from emulsification of unmodified gelatin dissolved in water as the aqueous continuous phase with *p*-xylene as internal oil phase at $\phi = 0.75$, showing the HIPE formed at Day 1, and the destabilization of the emulsion at Day 2.

The emulsions formed at room temperature were shown to fully incorporate the high volume fractions of *p*-xylene, forming true HIPEs (Figure 6-3A, 25 °C). The HIPEs formed were milky white and stable after mixing was stopped. The HIPEs were liquids and were also seen to be flowing at 25°C (Figure 6-3B). When the emulsions were cooled to 4°C, all HIPEs gelled, did not flow and remained stable (Figure 6-3A, 4°C). Also the HIPE stabilized by gelatin (Gelatin75C) was a milky white emulsion and gelled upon cooling at 4°C (Figure 6-3C). The HIPE stabilized by unmodified gelatin required longer time to form than those stabilized by GN, given the same energy input. Gelatin75C was also seen to destabilize in 2 days, unlike the GN-stabilized HIPEs, which still remained stable when observed after 1 week (not shown). However, when the HIPEs stabilized by GN were heated above the LCST to induce thermal PNIPAM aggregation, a different phenomenon was observed (Figure 6-3A, 40°C); while all the W series remained stable (Figure 6-3A, W75H to W90H), emulsions made using PBS (Figure 6-3A, P80H and P90H) phase separated, with the exception of P70H, which remained stable.

The drop test was used to determine the emulsion type of the HIPEs at 25°C just after they were prepared and also of the gelled HIPEs prepared at the two gelation temperatures 4°C and 40°C. At 25°C, the emulsion droplets were seen to disperse in water (Figure 6-4A1) but remained intact in *p*-xylene (Figure 6-4A2), which showed that these were o/w emulsions. At the gelation

temperatures of 4°C and 40°C, it was found that the gelled emulsion droplets did not disperse in both media, instead it was floating in water (Figure 6-4B1 and 4C1) and sinking in *p*-xylene (Figure 6-4B2 and 4C2) due to the difference in densities.

Figure 6-4 Drop test was used to determine the emulsion type. A HIPE droplet was dropped into A1) water and A2) *p*-xylene at 25°C. The emulsion droplet was seen to disperse into deionized water but remained a droplet in *p*-xylene. At the solidification temperature of 4°C, the droplet remained intact in both media, floating in B1) water, while sinking in B2) *p*-xylene. The same phenomenon was seen at 40°C with droplet remaining intact in C1) water and C2) *p*-xylene. Arrows indicate the location of the droplets.

6.3.4 Formation of polyHIPEs

PolyHIPEs were subsequently formed by rapidly freezing the gelled HIPEs in liquid nitrogen to lock in the structure of the emulsion template. The liquid phases were then subsequently removed by freeze-drying (Scheme 6-2, Step 3). The structure of the polyHIPE was characterized in terms of the arithmetic mean pore $\langle D \rangle$ and pore throat size $\langle d \rangle$, surface area A and porosity. All polyHIPEs had porosities of up to 99% (Table 6-3).

Table 6-3 Mean pore $\langle D \rangle$ and pore throat size $\langle d \rangle$, surface area A and porosity of poly(GN)HIPEs formed with water or PBS in the continuous phase.

Sample	$\langle D \rangle$ (μm)	$\langle d \rangle$ (μm)	A (m^2/g)	Porosity %
Gelatin75C	112.7 ± 43.5	-	145	98
W75C	56.3 ± 15.8	12.6 ± 6.5	200	93
W80C	70.2 ± 48.1	15.6 ± 10.9	233	95
W90C	20.3 ± 8.0	8.2 ± 1.9	729	98
W75H	53.9 ± 18.0	22.5 ± 11.5	262	97
W80H	79.6 ± 43.9	23.4 ± 8.4	1489	96
W90H	9.7 ± 2.2	3.5 ± 1.0	823	97
P75C	160.4 ± 87.2	-	3084	98
P80C	74.7 ± 43.8	-	1227	97
P90C	31.7 ± 13.0	9.0 ± 3.9	625	99
P75H	96.3 ± 19.2	21.5 ± 6.0	508	88

When water was used in the aqueous phase, the polyHIPEs when made from HIPEs with $\phi = 0.75$, gelled at 4°C and 40°C , had pores with $\langle D \rangle$ of $56.3 \pm 15.8 \mu\text{m}$ and $53.9 \pm 18.0 \mu\text{m}$, respectively, interconnected by pore throats with $\langle d \rangle$ of $12.6 \pm 6.5 \mu\text{m}$ and $22.5 \pm 11.5 \mu\text{m}$, respectively (Sample W75C and W75H, Table 6-3 and Figure 6-5A1 and 5B1). Pore sizes of polyHIPEs made from HIPEs with $\phi = 0.80$ were larger than those of polyHIPEs made from

HIPEs with $\phi = 0.75$ (Table 6-3, Figure 6-5A2 and Figure 6-5B2), which is unexpected and could perhaps be a consequence of droplet coalescence, which occurred for HIPEs at this internal phase volume ratio as can be seen in the SEM images Figure 6-5A2 and 5B2. Probably for this system, the rate of coalescence tends to increase as ϕ increases, which is counteracted by the increase in emulsion viscosity with higher ϕ [289-290] so that the HIPEs did not phase separate. W80H had a massively higher surface area ($A = 1489 \text{ m}^2/\text{g}$) than W80C ($A = 233 \text{ m}^2/\text{g}$), although they were prepared from HIPEs with the same ϕ . This may be due to the much higher number of small secondary pores that formed in the pore walls of W80H upon freeze-drying (see SEM Figure 6-5B2) as compared to W80C (Figure 6-5A2). When HIPEs with $\phi = 0.90$ were used as template for polyHIPEs, a drastic reduction in $\langle D \rangle$ to $20.3 \pm 8.0 \text{ }\mu\text{m}$ was observed for W90C and to $9.7 \pm 2.2 \text{ }\mu\text{m}$ for W90H, with pore throats sizes of $8.2 \pm 1.9 \text{ }\mu\text{m}$ and $3.5 \pm 1.0 \text{ }\mu\text{m}$, respectively (Sample W90C and W90H, Table 6-3 and Figure 6-5A3 and 5B3). At $\phi = 0.90$ the emulsion droplets formed tend to be much smaller and closer together to accommodate the increase in ϕ . This increased the emulsion viscosity further, which worked to strongly inhibit coalescence rate, resulting in smaller average pore sizes.

In general, the pore sizes of polyHIPEs obtained from HIPE templates formed using PBS as solvent for GN were bigger than in polyHIPEs obtained when water was used in the template. For the polyHIPEs made from HIPEs with PBS as aqueous phase, gelled by cooling to 4°C , $\langle D \rangle$ decreased with increasing ϕ , from $160.4 \pm 87.2 \text{ }\mu\text{m}$ to $31.7 \pm 13.0 \text{ }\mu\text{m}$ (Samples P- ϕ -C, Table 6-3). However,

pore throats only formed when HIPEs with $\phi = 0.90$ were used as template. A decrease in surface area from 3084 m²/g to 625 m²/g was also observed, again due to the reduction in secondary porosity in the polymer walls surrounding the large pores by the removal of the internal droplet template phase (compare Figure 6-5C1 to 5C3). When the HIPEs were gelled by heating the emulsion to 40°C instead, comparing P75C with P75H, the pore size decreased to 96.3 ± 19.2 μm , and pore throat size to 21.5 ± 6.0 μm in diameter. Also in this case a reduction in surface area to 508 m²/g was observed. In comparison, the control poly(gelatin)HIPE (Gelatin75C) had bigger pore sizes than poly(GN)HIPEs, and they were also found to be closed pores. The surface area was measured to be only 145 m²/g.

Figure 6-5 SEM images of poly(GN)HIPEs formed with water as solvent for GN as the aqueous phase, HIPEs were solidified by cooling to 4°C for A1) $\phi = 0.75$ A2) $\phi = 0.80$ and A3) $\phi = 0.90$; or solidified by heating to 40°C for B1) $\phi = 0.75$ B2) $\phi = 0.80$ and B3) $\phi = 0.90$. SEM images of polyHIPEs formed with PBS as solvent for GN as the aqueous phase, solidified by cooling shown in C1) with $\phi = 0.75$, C2) $\phi = 0.80$ and C3) $\phi = 0.90$; and solidified by heating to 40°C in D) with $\phi = 0.75$. E) SEM of control poly(gelatin)HIPE made by solidification of a HIPE stabilized by unmodified gelatin dissolved in water with an internal volume fraction of $\phi=0.75$ and solidified at 4°C.

6.3.5 Mechanical properties of the poly(GN)HIPEs

Figure 6-6 Mechanical properties of poly(GN)HIPEs. Dynamic storage (G' , solid symbols) and loss (G'' , hollow symbols) moduli of swollen poly(GN)HIPEs W80C and W80H measured as function of temperature at 0.16 Hz, 1% strain, 0.5°C/min.

To simulate how changes in temperature affected the mechanical properties of the polyHIPE hydrogels in their hydrated, swollen form during the cell seeding process at 25°C, followed by an increase in temperature to 37°C during the cell culturing, the dynamic mechanical properties of PBS-swollen polyHIPE hydrogels were measured as function of temperature from 25°C to 40°C. Two polyHIPEs, W80C and W80H, were chosen because their physical properties (pore size, high porosity, and high surface area) were suitable for cell culture. PBS at 25°C was added to the dry polyHIPEs (W80C and W80H) simulating

the cell seeding procedure, and the mechanical properties of the swollen porous hydrogels were then measured immediately as a function of temperature. Figure 6-6 shows an initial G' plateau of 2.6×10^4 kPa for W80C and a lower G' plateau of 1.2×10^4 kPa for W80H at 25°C (Stage 1). After which, a significant decrease in the storage and loss modulus from 32°C followed (Stage 2). The moduli continued to decrease with increasing temperature until 36.4°C after which the moduli remained almost constant at about 5.6×10^2 kPa for W80C and 7.2×10^2 kPa for W80H, even with increasing temperature (Stage 3).

6.3.6 Poly(GN)HIPE hydrogels for tissue engineering

To investigate the effect of the different manufacturing routes of poly(GN)HIPE hydrogels on cell seeding and culturing, two polyHIPEs W80C and W80H were chosen that had sufficiently large pores and pore throats. Cells cultured on 2D tissue culture polystyrene (TCPS) were used as control. The viability, proliferation and spreading of the fibroblast cells within the poly(GN)HIPEs were determined using the CCK-8 proliferation assay and Live/Dead analysis. Moreover, the prospect of ejecting the cell-laden hydrogel scaffolds through a hypodermic needle was also investigated.

Figure 6-7 shows that both W80H and W80C had a significantly higher cell seeding density than the 2D control and hence had a higher cell proliferation rate because of the greater surface area where the cells can attach to and survive. Proliferation rate in both W80C and W80H showed no statistical difference for the 10 days of culture.

Figure 6-7 Cell proliferation of seeded human foreskin fibroblast cells in poly(GN)HIPE hydrogels and 2D control with TCPS after 1, 4, 7 and 10 days. Comparisons were made between pairs by statistical analysis by the one-way ANOVA at the level of $P < 0.05$. * Significantly different from that of 2D TCPS on their respective days.

Live/Dead imaging (Figure 6-8) showed that on day 1 the cells appeared aggregated when initially seeded and rested within the pores, as seen from the large clusters of cells at different depth of the scaffold (Figure 6-8A1 and 8A2), unlike the 2D control which showed spread cells (Figure 6-8A3). From day 4 to day 7 the cells in the scaffolds became more abundant and were seen to be spreading from the clusters into individual cells with spindle-like morphology (Figure 6-8B1 and 8B2). On day 10, clusters were smaller and the cells were seen to have colonized the bulk of the scaffold, spreading extensively and extending throughout and into the scaffold (Figure 6-8C1 and 8C2, Figure 6-9). This is also seen from the SEM image of the cell-laden scaffold on day 10 (Figure 6-8D); cells attached to the scaffold, spread and extended their filopodia

into the scaffold. The pore size at the end of day 10 was determined to be $91.4 \pm 27.7 \mu\text{m}$ as seen from Figure 6-8E.

Figure 6-8 Live/Dead fluorescence imaging of fibroblasts seeded in W80C and W80H and control plated on 2D TCPS on A1-A3) Day 1, B1-B3) Day 7, C1-C3) Day 10; Green=Live cells, D) SEM of fibroblasts in the poly(GN)HIPE scaffold on Day 10. Inset arrows locate some filopodia of spread fibroblast cells. *Big cuboidal structure are residual salt crystals from PBS. E) Optical micrograph of scaffold after cell culture on Day 10.

The depth of penetration of the cells into W80C and W80H on day 10 was 360 μm and 244 μm into the 2 mm scaffolds from the top surface, respectively (Figure 6-9).

Figure 6-9 3D Depth of penetration of human foreskin fibroblast cells into A) W80C and B) W80H measured by z stack function in confocal microscopy.

6.3.7 Injectability of cell-laden poly(GN)HIPE hydrogel

Figure 6-10 A) A cell-laden poly(GN)HIPE hydrogel in DMEM, B) Cell-laden poly(GN)HIPE hydrogel loaded into a 1 ml syringe. Arrow indicates position of loaded gel. C) Cell-laden hydrogel after extrusion through the needle, D) Fluorescence imaging of cells in the poly(GN)HIPE hydrogel after extrusion, with green fluorescence indicating the live cells.

Injectability of the cell-laden polyHIPE hydrogels was tested by placing cell-laden hydrogels at day 10 (Figure 6-10A) into a syringe (Figure 6-10B) and extruding them through an 18G needle. The cell-laden hydrogels broke up into thin elongated pieces, but still retained their bulk structure (Figure 6-10C). The viability of the cells in the extruded hydrogel was determined by conducting again a Live/Dead assay. This preliminary study showed that cells did survive in the poly(GN)HIPE hydrogel even after extrusion through a hypodermic

needle (Figure 6-10D: Green=Live cells) and retained their spindle-shaped morphology.

6.4 Discussion

This work was motivated by the need to find a clean method, i.e. a method without the need for additional porogens or surfactants, for producing injectable scaffolds for tissue engineering. The HIPE-templating method is preferred over other methods such as gas foaming or porogen leaching as these methods often results in scaffolds lacking or possessing only poor pore interconnectivity, and do not allow for controlling the pore morphology [8]. Also, the processing of HIPEs is simple and high porosity foams can be formed by removal of the templating phase. Conventional methods of producing polyHIPEs usually require large amounts of surfactants and produce foams with pore sizes typically in the range of 5-20 μm , [14, 279] which might be too small for tissue engineering scaffolds. Methods to increase the pore size of polyHIPEs and their interconnectivity by controlled destabilization using additives (surfactants, salt, organic solvents etc.) have been reported [28, 281, 291-292], but these additives may be difficult to be removed completely and any residue will be detrimental to the cells [29]. This was overcome by using GN, which acts both as emulsifier and the matrix for the scaffold. Thus, emulsion-templated macroporous polymers were able to be produced with tailored pore sizes and a high degree of pore interconnectivity of the foams, but without the use of extra addition of surfactants or additives, thereby avoiding the risk of scaffold contamination. At

the same time, the use of dual gelation mechanisms provides the scaffold with another level of flexibility to control the properties of the resulting polyHIPEs and their response to temperature changes during cell seeding, as compared to other rigid scaffolds. This section discusses the mechanisms of the HIPE stabilization and how the different HIPE gelation methods affect the properties of the poly(GN)HIPEs as well as the influence of the temperature sensitivity of poly(GN)HIPE on cell seeding.

6.4.1 Stabilization of HIPEs by GN

GN was able to stabilize HIPEs over a wide range of emulsion phase volume ratios as it does significantly lower γ_{wo} between *p*-xylene and water (Table 6-2). This is due to the improved amphiphilicity of the copolymer, which adsorbs more strongly at the oil and water interface. GN is a branched copolymer consisting of PNIPAM chains grafted from a gelatin backbone. The gelatin component of GN is very hydrophilic, forming many hydrogen bonds with water. On the other hand, the grafted PNIPAM component consists of isopropyl chains, which confers a small degree of hydrophobicity and hence is able to interact with the organic phase [293]. It is postulated that larger pores in the poly(GN)HIPEs formed because this amphiphilic branched GN copolymer did stabilize larger droplets, which were in close contact (Scheme 6-3A), as compared to conventional surfactant-stabilized HIPEs which tend to contain smaller droplets (Scheme 6-3B). At the same time, because an amphiphilic comb-type copolymer is used, acting both as emulsifier and matrix for the

polyHIPEs, high porosities can be achieved as the polymer forms entanglements and physical associations in the aqueous phase, which results in a second degree of porosity within the pore walls of the polyHIPEs after the removal of the template internal phase and water or aqueous PBS serving as solvent for GN in the continuous phase, as seen in SEM images (Figure 6-5) and illustrated in Scheme 6-3A. This does also explain the improved interconnectivity of the poly(GN)HIPEs formed and the resultant very porous pore walls, which explains the high surface areas measured, achieved without the addition of other porogens or surfactant(s) to open up pore throats.

Scheme 6-3 Stabilization of o/w emulsion by A) GN as compared to B) conventional surfactant.

6.4.2 Effect of salt on GN-stabilized HIPEs

It is known that the presence of salt in the continuous phase affects the surfactant's activity at the water/oil interface [199, 294]. PBS contains an abundance of Na^+ and PO_4^{2-} ions, and a smaller concentration of K^+ ions (ionic strength of PBS = 212.7 mM). As cell culture media contain many ions with compositions similar to PBS, the effect of salt on GN-stabilized HIPEs was investigated. It was observed that average pore sizes of the poly(GN)HIPEs produced from the P-HIPE series were in general larger than those obtained from the W- series (Table 6-3 and Figure 6-5). These effects are attributed to the reduction in stability of the HIPEs, which was caused by the lower surface activity of GN when dissolved in PBS as compared to GN in water. The ions dissolved in PBS are kosmotropic, i.e. they are strongly hydrated and tend to be depleted from the interface causing the γ_{wo} of the electrolyte solutions to be higher and therefore, lower the emulsion stability [295-296]. Also, the presence of salt tends to cause the protein-based GN to undergo conformational changes, which may affect its organization at the oil-water interface, which in this case, lowered its surface activity [297].

6.4.3 The two gelation mechanisms of GN stabilized HIPEs

The HIPEs stabilized by dissolved GN can be gelled at two different temperatures utilizing two different gelling mechanisms provided by the

inherent properties of GN. This was achieved by combining a component with a low gelation temperature (gelatin) and LCST (PNIPAM) in the copolymer, which allows for HIPEs stabilized by such a copolymer to be gelled at two different temperatures. These two gelation mechanisms can be distinguished because the gel point of the gelatin component of GN at temperatures below 25.8°C does not overlap with the LCST of GN, which is occurring at above 32°C. At 4°C, the PNIPAM components do not aggregate, while at 40°C, the gelatin component will tend to be in relaxed coils in solution, so the gelation is solely due to the association or aggregation of each component at the respective temperatures.

6.4.3.1 Effect of gelation temperature on stability and droplet size of HIPEs

Temperature is known to have a significant influence on the stability of emulsions and hence their properties [298-299]. Increasing the temperature of emulsions from 25°C to 40°C should increase Brownian motion of the molecules and decrease the viscosity of the emulsion, causing the rate of droplet coalescence to increase [300]. However, with the use of GN the coalescence rate was quickly curbed by reaching the LCST of GN (36.7°C for GN in water and 32.2°C for GN in PBS) at which point the viscosity increased sharply (Figure 6-2) and the emulsion structure was locked in by the aggregation of the PNIPAM component of the copolymer [300-301]. At and above the LCST of GN, the hydrophobic aggregation of parts of the PNIPAM in water, which did not participate in stabilizing the oil-water interface can affect the organization

of GN molecules at the o/w interface too. This could affect the emulsion stability, but not enough to break the emulsion as no phase separation was observed after the gelation of the HIPEs at 40°C (Figure 6-3). This is with the exception of P80H and P90H, which destabilized and phase separated after heating to 40°C (Figure 6-3, P80H and P90H) caused by the lower surface activity in PBS as described earlier. GN dissolved in PBS was unable to stabilize HIPEs with internal volume fractions exceeding $\phi = 0.75$ at 40°C. On the other hand, gelling HIPEs by cooling them from 25°C to 4°C should result in an increased emulsion viscosity (Figure 6-2) since the gelatin chains start to reform partially the triple helix through formation of inter- and intra- molecular hydrogen bonds, slowing down droplet coalescence [289-290]. However, the coil to helix transition of the gelatin component is much slower than the PNIPAM aggregation and so it will take a longer time for the HIPEs to gel. Therefore, it gives the emulsion time to destabilize during cooling to create bigger droplets, while still not allowing phase separation to occur. This effect of gelation temperature of HIPEs on the final pore size of poly(GN)HIPEs was especially significant when the solvent used for GN to form the aqueous continuous phase was PBS instead of water, resulting in much larger pores when cooling to 4°C than heating to 40°C. This is because the HIPEs formed with PBS were less stable. Hence, depending on the applications, by varying the composition of the aqueous continuous HIPE phase, the effect of temperature on the physical properties of the emulsion-templated macroporous polymers can be controlled. Also it was shown that the stability of HIPE

became more sensitive to temperature changes in the presence of salt, the emulsion can be made or broken on demand for certain industrial applications by varying these two parameters [302].

6.4.3.2 Effect of gelation temperature on mechanical properties of poly(GN)HIPEs

As the two HIPE-gelation mechanisms are quite different, it was interesting to investigate if they affect the mechanical properties of the poly(GN)HIPEs formed. By choosing W80C and W80H, it was shown that the W80C was stiffer than W80H initially (Figure 6-6). With the only variable being the gelation method of the HIPEs, this demonstrated that the formation of ordered triple-helices in the gelatin component during cooling [303] tends to be much stronger than the random amorphous physical aggregation of PNIPAM globules generated during gelation by heating. This is interesting as the mechanical properties of the poly(GN)HIPEs can also be tuned by simply by varying the gelation temperature.

During the heating process, three distinct stages were observed with increasing temperature with similar trends seen for both W80C and W80H (Figure 6-6).

Stage 1 (25 – 32°C): The initial plateau of G' for both swollen W80C and W80H was caused by hydrogen bonds between the gelatin chains that formed during the freeze-drying process [304-305] and additional triple-helix bonding in W80C, which remained intact until sufficient energy was provided to break them when the temperature was increased in the presence of water. The downwards slope of G'' for W80H may be attributed to the weakening of

immobilized aggregated PNIPAM in W80H during the start of the temperature ramp at 25°C. Because of the prior rapid cooling of the emulsion from 40°C to -196°C in liquid nitrogen, the PNIPAM component of GN did not have sufficient time to relax and hence was frozen in its globule form in the poly(GN)HIPEs, and upon addition of water at 25°C, the globule to coil transition resumed [306]. The cell-seeding process will take place in this stage.

Stage 2 (32 – 36.7°C): When the temperature reached 32°C, the effect of the helix to coil transition of the gelatin component and the globule to coil transition of the PNIPAM component were observed in the hydrogel by the downwards slope of the moduli, indicating that the chains are now starting to relax, and the hydrogel network was becoming increasingly looser, allowing the cells to penetrate deeper into the hydrogel. Despite the polymer relaxation in this region, the hydrogel was still held together as evidenced by the fact that the G' and G'' curves approached each other for both samples but did not crossover, indicating that the hydrogel was still elastic and did not dissolve. This means that not all of the intra- and intermolecular polymer interactions have been broken. The reason could be that although most of the secondary helical structures may be broken during the helix to coil transition, still some helical structures remained intact and hydrogen bonds persisted as the gel-sol transition being a kinetic process, is time-dependent and requires longer time and energy for total gel-sol transition to occur [307-308]. Also, in this temperature range, the onset of PNIPAM chains aggregation was induced, allowing some parts of the chains to interact again.

In stage 3 (36.7 – 40 °C), at 36.7°C the bonding in the hydrogel was dominated by PNIPAM aggregation and, therefore, the hydrogel continued to remain intact, though lower shear moduli were measured. The triggering of the aggregation of the grafted PNIPAM component occurs between 32.2°C and 36.7°C but because of the hydrogel network structure which reduces the mobility of the polymer chains, and effects such as steric hindrance, the association and dissociation of bonds occurred much slower such that there was no significant increase in the moduli when the temperature increased beyond the LCST, in contrast to the solution behavior of the polymer. A plateau however signifies that the PNIPAM aggregation was doing its part in keeping the modulus constant when the temperature continued to increase, without which, the modulus would have decreased until the hydrogel completely dissolved. This will be the hydrogels' properties during the cell culturing period.

6.4.4 Tissue engineering with poly(GN)HIPE hydrogels

The use of poly(GN)HIPE as scaffolds for the culture of human foreskin fibroblast cells as a model of anchorage dependent cells (ADC) was then investigated. ADC have stricter requirements in terms of scaffold hydrophilicity and provision of cell-adhesion surface sites as compared to non-adherent cells. These cells require a biocompatible surface to attach to in order to avoid apoptosis [41, 309]. Once attached, cytoskeletal reorganization occurs, which causes the cells to spread and initiate proliferation [310]. The use of gelatin as one component of the scaffold provided cell attachment sites and imparted

biodegradability to provide more space for the proliferation of cells as time proceeds [311]. However, gelatin needs to be modified as its gelation point is too low to be used as a gel at physiological temperature [300]. PNIPAM on the other hand is known to be biocompatible and non-toxic [312] and has been used widely in tissue engineering applications, supporting the attachment and proliferation of cells [206-210]. In this chapter, PNIPAM was grafted onto gelatin to allow for the temperature-induced formation of physical crosslinking points at physiological temperature. The results showed that the cells thrived and were able to spread and proliferate well in poly(GN)HIPE scaffolds for a culture period of at least 10 days (Figure 6-8). The combination of gelatin and PNIPAM in a copolymer was shown already [313] to be able to support cell attachment and spreading of human umbilical vein endothelial cells (HUVECs) on the surface of the hydrogel. However, this surface does not provide a truly biomimetic environment for the cells as it was a 2-dimensional (2D) culture, in which only a portion of the cells are contacting the surface of the hydrogel, resulting in polarized growth. On the other hand, the macroporous poly(GN)HIPE hydrogel provides a more realistic 3D environment that facilitates cell spreading in 3D. Indeed, the human foreskin fibroblasts cells seeded in poly(GN)HIPE macroporous hydrogel were seen to spread within 7 days as shown in Figure 6-8B. In addition, the pore size of poly(GN)HIPE hydrogel increased towards the end of culture period on day 10 (Figure 6-8E), which could probably be due to the degradation of the polymer or digestion by enzymes secreted by the cells [314].

The high porosity and degree of interconnectivity between the pores in the scaffold are also important requirements for cell penetration and migration to avoid overcrowding and tissue necrosis, in addition to the transport of nutrients into and metabolic waste out of the scaffold. PolyHIPE scaffolds possess high porosities and high degree of interconnectivity suitable for use in tissue engineering, however there are limited reports on the depth of penetration of cells into biopolymer based polyHIPE scaffolds [26, 28, 153, 199]. Using non-degradable poly(styrene-*co*-DVB)HIPEs scaffolds with pore sizes around 100 μm , Akay et al. [185] reported that osteoblasts were able to penetrate only 20 – 30 μm into the scaffolds in 14 days, unless the scaffolds were modified with hydroxyapatite, which showed an improved average cellular penetration depth of 400 μm after 14 days in 3 mm thick scaffolds, with only few cells penetrating deeper than 1mm. As hydroxyapatite is known to be osteoconductive and also hydrophilic, it could aid in osteoblasts' cellular penetration [315], but may not be suitable for all cell types. Moreover, the use of a non-degradable and non-injectable scaffold material renders the cultured cells inaccessible for further use as they are difficult to be retrieved from the internal part of the rigid and tortuous scaffold.

For poly(GN)HIPE scaffolds, the maximum depth of penetration of fibroblast cells measured after 10 days was 360 μm in a 2 mm thick scaffold, which was attributed mainly to this dual temperature sensitivity of poly(GN)HIPE hydrogel that created the three stages seen in Figure 6-6, which aided cell seeding and penetration as compared to rigid polyHIPEs solidified by covalent

crosslinks during the free radical polymerization of the continuous emulsion phase. This dual-temperature sensitive mechanism allowed the warm culture medium to break the associations between gelatin chains slowly when the cells were initially seeded (Stage 1), which widened the pores and loosened the hydrogel network for the cells to penetrate deeper into the scaffold (Stage 2). However, the aggregation of the grafted PNIPAM chains occurring when temperature increased beyond their LCST to 37.2°C in the incubator (Stage 3) held the scaffolds in place. With this mechanism and the use of dynamic seeding [316], instead of static seeding which was used in this study, deeper penetration of cells into the scaffold should be possible.

6.4.5 Injectability of cell-laden poly(GN)HIPE hydrogels

Injection is a preferred method of introducing engineered cells into the body as it is minimally invasive and ensures spatial fit into the defect site [317-319]. Injectability of polyHIPE hydrogels has been previously shown to be possible while maintaining the interconnected pore morphology and integrity of the scaffold [25]. This chapter now show that injectability of a cell-laden poly(GN)HIPE hydrogel is also feasible. Moreover, it did not affect the cells; they were seen to be still alive after being extruded through a hypodermic needle and still retained their spindle-shaped morphology (Figure 6-10D). This ability to inject the entire biodegradable cell-laden tissue-scaffold construct into the defect site offers significant advantages over other types of rigid scaffolds that require open-surgery for their implantation. This strategy will eliminate

other complications that may accompany invasive surgery or a potential misfit of the scaffold in the defect site. In addition, as compared to encapsulating cells and placing them immediately into the defect site, allowing the cells to grow into a critical cell mass before insertion reduces the time taken for recovery to occur and exposure of the hydrogel to the *in vivo* environment where it might be degraded faster [320-321]. A possible disadvantage of such injectable hydrogel is that since it is thermoresponsive, it has to be handled quickly as the mechanical properties are changing with temperature. When applied to a defect site with plenty of blood supply, such as near a blood vessel, the injected hydrogel will possibly face the fate of being carried along with the blood flow away from the defect site. Therefore, it will be more suitable to be used in applications where the defect site is far away from rapid blood flow, such as for skin grafts etc.

6.5 Conclusions

A surfactant-free biodegradable HIPE stabilized solely by GN was produced. It was demonstrated that such HIPEs could be easily solidified by gelling, which is highly desirable for tissue engineering applications as it removes the need for additional emulsifiers, porogens and crosslinkers that may be detrimental to cultured cells. The poly(GN)HIPEs formed possessed high porosity, a very high degree of pore interconnectivity and high surface area, which is ideal for tissue engineering scaffolds. By varying the aqueous continuous phase, the internal phase volume ratio and HIPE gelation temperature, the properties of

poly(GN)HIPEs can be tailored. The effect of gelation temperature on the properties of poly(GN)HIPEs was more pronounced when PBS was used as solvent for GN and as aqueous continuous minority phase rather than water. Poly(GN)HIPE hydrogels provide a dual-temperature sensitive mechanism, which aids cell penetration during seeding into the 3D scaffolds, which is usually a challenge. Fibroblast cells were seen to thrive in and spread into the poly(GN)HIPE hydrogel scaffolds. Furthermore, the cell-laden poly(GN)HIPE hydrogel was demonstrated to be injectable through a hypodermic needle and still retained its bulk form after extrusion through the needle while the encapsulated cells survived the process and maintained their morphology. The work presented here provides a strategy of producing non-toxic tissue engineering scaffolds with tunable properties and that changes dynamically with temperature aiding the cell seeding process while providing injectability as a safe route of administering the engineered cells to a potential defect site.

CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

In summary, a series of multifunctional copolymers were developed which do act as emulsifiers for o/w high internal phase emulsions, allow the aqueous emulsion phase to be gelled by a temperature trigger, and do form the polymer matrix of macroporous polyHIPEs produced by the removal of the templating oil (droplet) phase and the water from the aqueous phase. It was shown that the properties (pore size, pore interconnectivity, porosity, surface area) of the resultant polyHIPEs formed from these multifunctional copolymers depends significantly on the type of copolymer, their solution properties in the aqueous continuous phase, especially polymer solubility and surface activity, and also emulsification conditions (polymer concentration, ϕ , temperature). The ability to solidify at physiological temperature without the need for chemical crosslinking provides a feasible route for the co-encapsulation of cells without the risk of residual monomers affecting the cells. The copolymers described in this thesis for the formation of polyHIPEs for tissue engineering scaffolds

provides a clean, facile and low energy method thus moving beyond the current state-of-the-art of producing scaffolds with relevant pore sizes and interconnectivity for soft tissue engineering.

The overall evaluation and contribution of this work lies in the creation and understanding of self-stabilized HIPE which can be used to create interconnected macroporous hydrogels that are suitable for use in tissue engineering. Previous works done on the fabrication of HIPE required high amounts of stabilizers and crosslinkers to form the highly porous and interconnected structure. The additions of these extraneous factors pose a risk if they were to be used for bio-applications and further represent considerable waste in terms of additional organic crosslinker monomers and solvents. This can all be rendered unnecessary with the design of a self-stabilizing polymer which is also able to allow stabilization of HIPEs, and also self-solidify with a temperature trigger. The use of HIPE-templating with the designed polymers allows the creation of highly porous scaffolds that have well-defined porosity and properties which can be easily tunable. In addition, the biocompatibility of these polymers are demonstrated which prescribed their suitability in bio-applications such as tissue engineering, in which highly porous thermoresponsive hydrogels with macroporosity produced by HIPE-templating could act to enhance the seeding and spreading of ADCs. This thesis thus reveals the design considerations for synthesizing such multifunctional polymers with detailed conclusions drawn in the following sections:

7.1 On the Synthesis of Highly Water Soluble High MW Chitosan Derivatives

Native CS is only poorly soluble in aqueous solutions of pH lower than 6.4, which greatly restricts its applications. This is due to the abundant intermolecular and intramolecular hydrogen bonding formed between the amine and hydroxyl groups. It was shown in this study that the grafting of PNIPAM from CS allows some degree of improvement in solubilizing high MW CS. CSN was made to be soluble from pH 1 to pH 7, but still with low maximum solubility of just 2.5% w/v measured at pH 7. Further grafting of oligo(amino acids) greatly improved the solubility of CS over a wide range of pH from 1-12 with maximum solubility concentration measured at pH 7 of 40% w/v for CSNLYS and CSN-PRO and 20% w/v for CSN-GLU.

7.2 On the Design of Modified Chitosan-Based HIPE Emulsifiers and PolyHIPEs

Different oligo(amino acids) grafted onto CSN were shown to greatly affect the properties of the emulsion stabilized by them. Without the grafting of oligo(amino acid), CSN is unable produce polyHIPE with sufficient mechanical properties and stability due to its low solubility in the aqueous continuous phase and low surface activity.

It was subsequently found that the type of oligo(amino acids) and the DP of the oligo(amino acids) side chains does significantly affect the properties of HIPEs formed. This is due to the difference in the respective copolymer solution properties, especially their solubility in the aqueous continuous phase and their surface activity, which affected their ability to stabilize HIPEs. It was shown that by tuning the DP of the grafted oligolysine, the type of emulsion produced can be tuned, namely macroemulsion (HIPE) and microemulsion (HIPME) can be stabilized. The properties of the chitosan-based polyHIPE and polyHIPME produced from the HIP(M)Es of their respective self-stabilized emulsions are starkly different in terms of their average pore sizes which range from 130 nm (Chapter 4) to 73.8 μm (Chapter 5), depending on the type of the grafted oligo(amino acid) side chains. With oligoproline grafts, stable HIPEs can be formed whereas with oligo(glutamic acid) only a low internal phase emulsion could be stabilized, because its surface activity was insufficient to incorporate a higher ratio of internal phase.

The pores formed from self-emulsifying chitosan based copolymer for polyHIPEs were found to be closed pores with pore sizes from 0.03-71 μm . Nevertheless, pore throats could be introduced into the polyHIPEs by the addition of a small concentration (0.1% v/v in the aqueous phase) of a non-ionic biocompatible surfactant. The pore size can be tuned by varying the polymer concentration and internal phase volume ratio of the emulsion templates. mESCs seeded onto the polyHIPE hydrogel scaffold were able to penetrate into the scaffold due to the interconnected pores. Moreover, the presence of the

small amount of biocompatible surfactant used during the formation of the polyHIPE did not seem to affect the viability of the cells. However, due to the relatively small pore size, cell penetrated only the scaffold close to the surface of the polyHIPE.

While it was not aimed to create polymers that self-stabilize microemulsions, the discovery of such a polymer opens new opportunities of producing microemulsions without the need of substantially large amount of surfactants, co-surfactants and organic templates. Though for the aim of seeding cells in 3D, the nanoscale pore sizes may not be ideal, it nevertheless, opens up several possibilities for applications such as in the field of drug delivery for the solubilization of oil soluble drugs in microemulsions.

7.3 HIPEs with Dual Solidification Temperatures

A highly porous and interconnected poly(gelatin-*graft*-PNIPAM)HIPE made without the need for any other additional emulsifiers was successfully prepared. Poly(GN)HIPE can subsequently be formed by two different solidification mechanisms as imparted to the copolymer by the components of GN, gelatin and PNIPAM. By inheriting the temperature sensitivity of these two components, GNHIPEs can be solidified at low temperature due to the presence of the gelatin component or temperatures above the LCST of the copolymer caused by the PNIPAM component. The physical properties of the resultant poly(GN)HIPEs can be controlled by varying the aqueous phase composition, ϕ , and solidification temperature. Poly(GN)HIPEs were found to be suitable for

the seeding and spreading of ADCs. Fibroblast cells seeded into the scaffold were seen to thrive, spread and proliferate over a culture period of 10 days. Because of the inherent temperature sensitivity, poly(GN)HIPE hydrogels are able to respond dynamically to changes in temperature during the initial cell culturing period, and hence the depth of cellular penetration was found to be improved reaching a maximum depth of penetration of 360 μm , comparing this dual solidification mechanisms discussed in Chapter 6 to the solidification method that was imparted by only the presence of PNIPAM in a copolymer as described in Chapter 5. The cell-laden poly(GN)HIPE scaffolds were also shown to be injectable using a syringe through a needle without harming the encapsulated cells. This system provides a new strategy for the easy fabrication of safe and injectable biocompatible scaffolds for tissue engineering.

7.4 Summary of Requirements for Designing “Self-Stabilizing” HIPEs for Tissue Engineering Scaffolds

The properties of the self-emulsifying copolymers described in this thesis and the properties of their respective polyHIPEs with $\phi = 0.75$ are summarized in Table 7-1. The relationships between these properties and the formation and properties of polyHIPEs are briefly summarized as follows:

- To self-stabilize o/w HIPEs, specifically a mixture of water and *p*-xylene, the copolymer acting as emulsifier should be able to sufficiently

lower the γ_{ow} to below 19.6 ± 0.2 mN/m, with the highest γ_{ow} recorded in this work to be 12.6 ± 0.0 mN/m.

- The surface activity of the self-emulsifying copolymer in the aqueous phase does affect the pore size of the polyHIPEs formed; smaller pores in polyHIPEs resulted when the copolymer produced the lower γ_{ow} . Other factors that affect the pore sizes of the polyHIPEs obtained from self-stabilized HIPE are the temperature during emulsification and solidification, the presence of salt, polymer concentration in the continuous phase and the type of polymer.
- To maintain the pore structure and structural integrity of the self-stabilized polyHIPEs after removal of the solvent and organic phase, the polymer content in total emulsion volume is required to be at least 2.5% w/v.
- Interconnected pores in polyHIPEs made from HIPEs stabilized by modified chitosan can only be produced when a small amount of surfactant was added to the emulsion template. Whereas for GN self-emulsifier, it was found to be dependent on whether ions are present in the aqueous continuous phase and the solidification temperature.

Table 7-1 Summary table of the properties of the self-emulsifying copolymers and the properties of the respective self-stabilized polyHIPEs formed with $\phi = 0.75$.

Self -emulsifier	γ_{ow} (mN/m)	X % w/v	High internal phase incorporated?	Pore size (μm)	Interconnectivity
CSN	11.7 ± 0.2	0.63	YES, pore collapsed during drying	-	-
CSNLYS-L	12.6 ± 0.0	5	YES	1-20	Closed pores
CSNLYS-H	~ 0	5	microHIPME	0.03 -0.2	-
CSNGLU	19.6 ± 0.2	5	NO	-	-
CSNPRO	8.9 ± 0.0	5	YES	32-71	Closed pores
GN _{Water} , 4°C	9.9 ± 0.1	2.5	YES	56.3 ± 15.8	Open porous
GN _{Water} , 40°C		2.5	YES	53.9 ± 18.0	Open porous
GN _{PBS} , 4°C	12.3 ± 0.1	2.5	YES	160.4 ± 87.2	Closed pores
GN _{PBS} , 40°C		2.5	YES	96.3 ± 19.2	Open porous

X: Polymer content in total emulsion volume % w/v

7.5 Recommendations for Future Work

In this work, the creation of a clean and energy efficient method of producing scaffolds for tissue engineering is elucidated. Some recommendations for future works are as follows:

- Drugs or growth factors could be incorporated into the polyHIPE (from the continuous phase) during the emulsification process and be entrapped within the polymer matrix. Controlled release of such drugs or growth factors from the polymer matrix during tissue culture might help to enhance the growth of the cultured cells or attempt to direct their differentiation in the case of stem cells.
- To further improve the cell penetration depth into the polyHIPE hydrogels, dynamic seeding could be used in tandem with the temperature sensitive mechanism to allow cells to penetrate deeper into the scaffold during the cell seeding process.
- Simultaneous emulsification and cell encapsulation could be explored to allow *in situ* gelation and to improve the cell seeding efficiency within the scaffold. This can be achieved by replacing *p*-xylene with a biocompatible oil, e.g. soybean oil.
- Cell-laden polyHIPE hydrogels should be assessed in animal models to determine its actual performance *in vivo*.

APPENDIX

Appendix 1 Calculation of DS and DP of CSN and CSNLYS

Calculation of Degree of Substitution and Degree of Polymerization

Degree of deacetylation (DD) of chitosan, degree of substitution (DS) and degree of polymerization (DP) of PNIPAM and lysine were determined by NMR[322].

Degree of Deacetylation determined by NMR

$$\text{DDA of chitosan} = [1 - (I_{H7}/3)/(I_{H2-6}/6)] \times 100\% = 78\%$$

Degree of substitution of PNIPAM grafted

Since the reaction occurs on the NH_2 of deacetylated groups, the reduction in NH_2 signal will represent the number of rings that have initiated the polymerization.

Taking the protons from the glucosidic ring as the base, NH_2 in chitosan = 0.18

NH_2 in CSN = 0.16

$$\% \text{ of deacetylated (NH}_2\text{) rings grafted} = ((0.18 - 0.16)/0.18) \times 100\% = 11.1\%$$

Therefore, ratio of units grafted onto, $m = 0.11 \times 78\% = 0.087$

$$\begin{aligned} \text{DP of PNIPAM per unit} &= [(I_{H10}/6)/((I_{H3-6}/5) \times 0.087)] \times 100\% = \\ &[(2.91/6)/((1/5) \times 0.087)] = 21.4 \end{aligned}$$

DS and DP of oligolysine

Ratio of NH₂ deacetylated rings used for conjugating lysine = $[(I_{H3-H6})/5]*0.69-$

$$I_{H2} = (1/5)*0.69-0.10=0.039$$

$$\text{DP of CSNLYS-H} = (I_{H15} / 2) / 0.039 = 0.30 / 2 / 0.039 = 3.89$$

$$\text{DP of CSNLYS-H} = (I_{H15} / 2) / 0.06625 = 0.1 / 2 / 0.039 = 1.29$$

I_{H7} , I_{H2-6} , I_{H10} represents the integral signals of the –CH₃ protons on acetylated groups, anomeric protons on the glucosidic unit, –CH₃ protons on NIPAM units respectively.

Appendix 2 Calculation of NMR integrals for CSN-PRO and CSN-GLU

Subtraction of integrals of CSN from CSN-PRO

Subtraction of NMR integrals to determine ratio/proton of oligoproline

Representative peaks	Integral values		Result	Number of protons represented	Integral/proton	Ratio/proton
	CSN-PRO	CSN	Subtraction			
CSN-PRO(H7+H21+H22)-CSN(H7)	5400.3	8507.4	5332.7	4	1333.2	0.97
H23	1368.8	0	1368.8	1	1368.8	1

H12 integrals representing -CH₃ groups from PNIPAM was used as the base since there is no overlapping in this region. Normalizing the integrals of CSN and CSN-PRO by taking the ratio of the integrals of H12, (R_{H12}), from CSN-PRO (3511.5) and CSN (441365.5), the equation for subtraction is as follows:

$$\text{Integral}_{\text{CSN-PRO}} - (3511.5/441365.5) * \text{Integral}_{\text{CSN}} = \text{Integral}_{\text{Proline}}$$

Subtraction of integrals of CSN from CSN-GLU

Subtraction of NMR integrals to determine ratio/proton of oligo(glutamic acid)

Representative peaks	Integral values		Result	Number of protons represented	Integral/proton	Ratio/proton
	CSN-GLU	CSN	Subtraction			
CSN-GLU(H7+H8+H14)-CSN(H7+H8)	131513.4	100903.8	45922.7	2	22961.34	0.935602
H13	49083.58	0	49083.58	2	24541.79	1

Similarly, normalizing the integrals of CSN and CSN-GLU by taking the ratio of the integrals of H12, (R_{H12}), from CSN-GLU (374384.4) and CSN (441365.5), the equation for subtraction is as follows: $\text{Integral}_{\text{CSN-GLU}} - (374384.4/441365.5) * \text{Integral}_{\text{CSN}} = \text{Integral}_{\text{Glutamic acid}}$

Appendix 3 Calculation of DS and DP of CSN, CSN-PRO and CSN-GLU

Calculation of DS and DP determined by a combination of titration study and NMR

Degree of Deacetylation (DD) of CS determined by NMR

$$\text{DD of chitosan} = [1 - (I_{H7/3}) / (I_{H2-6/6})] \times 100\% = 82.5\%$$

Degree of substitution determined by titration study

The amount of -NH_2 present on modified chitosan was determined by using titration with 2M NaOH, using the modified equation [258] :

$$\text{Degree of deacetylation, DD} = 16.1 (X) f/w$$

X= inflexion point, deprotonation of available NH_3^+ groups

f=molarity of NaOH solution

W=weight of chitosan

Titration curve of CSN, CSN-PRO and CSN-GLU with 2M NaOH

Degree of Deacetylation of CSN, CSN-PRO and CSN-GLU

Where,

X= 750 μ l for CSN, $DD_{CSN} = 13.85$.

X= 750 μ l for CSN-PRO, $DD_{CSN-PRO}$ of CSN-PRO = 8.80

X= 750 μ l for CSN-GLU, $DD_{CSN-GLU} = 10.82$

Degree of Substitution of CSN-PRO and CSN-GLU is calculated as follows

$$DS = DD_{CS} - DD_{CSN} - DD_{CSN-oligo(amino\ acid)}$$

Degree of Polymerization, DP, determined by NMR

$$DP \text{ of PNIPAM} = [(I_{H12}/6)/(I_{H7}/3)] \times 100\% = [(441365.5/6)/(8507.4/3)] \times 100\% \\ = 25.9\%$$

$$DP \text{ of oligoproline} = [(I_{H23})/(I_{H7}/3)] \times 100\% = [(1368.83)/(876.58/3)] \times 100\% = \\ 4.68\%$$

$$DP \text{ of oligo(glutamic acid)} = [(I_{H13}/2)/(I_{H7}/3)] \times 100\% = \\ [(15460.42/2)/(876.58/3)] \times 100\% = 4.76\%$$

Appendix 4 Degree of substitution determined by Ninhydrin assay with glycine standard curve

Glycine standard curve for ninhydrin assay

Concentration of NH₂ present for unmodified gelatin = 1.37 μmol/g

Concentration of NH₂ present for GN = 0.24 μmol/g

Degree of substitution = $100 \times (1 - 0.24/1.37) = 82.6\%$

Appendix 5 Degree of polymerization determined by Elemental analysis

Composition of C, H and N of gelatin, GN and NIPAM obtained from elemental analysis

EA	C%	H%	N%
Gelatin	42.15	7.973	15.45
GN	50.82	10.51	11.63
NIPAM	64.79	11.28	12.42

$$\text{Degree of Polymerisation} = \left[\frac{\left(\frac{C}{N}\right)_{GN} - \left(\frac{C}{N}\right)_G}{\left(\frac{C}{N}\right)_{NIPAM}} \right] \times 100\% = 31.5\%$$

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