2013

Gelatin and gellan gum based hydrogel materials: towards soft tissue scaffolds

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GELATIN AND GELLAN GUM BASED HYDROGEL MATERIALS: TOWARDS SOFT TISSUE SCAFFOLDS

By

DAMIAN MARTIN KIRCHMAJER

This thesis is presented as part of the requirements for the award of

DOCTORATE OF PHILOSOPHY

From

UNIVERSITY OF WOLLONGONG

SCHOOL OF CHEMISTRY

DECEMBER 2013
DECLARATION

I, Damian Martin Kirchmajer, declare that this thesis, submitted in fulfilment of the requirements for awarding the degree of Doctor of Philosophy, in the School of Chemistry, University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged. This document has not been submitted for qualifications at any other academic institution.

Damian Martin Kirchmajer

December, 2013
Tissue engineering is an exciting technology that may provide replacement tissue and organs for patients requiring transplantation therapy. Crucial to the preparation of engineering soft tissues is the cellular scaffold which must resemble the cells native environment and be mechanically robust. Hydrogel materials present the greatest potential as candidate materials for soft tissue scaffolds, however, it is very challenging to identify hydrogel materials that satisfy the extensive list of performance criteria required for this application. This thesis describes several hydrogel materials that are prepared from gellan gum and gelatin. The main focus of this thesis is the mechanical characterisation of these materials which is aimed towards addressing the performance criteria of soft tissue scaffold materials. The materials’ degradability, swelling behaviour and gelation behaviour were also focal points of this research.

It was found that genipin cross-linked gelatin hydrogels could be prepared to be thermally stable, self-supporting and degradable, with pH sensitive swelling behaviour that were compatible with endothelial cells. Commercial gellan gum was able to be purified of inorganic contaminants, which improved its gel-forming ability, but diminished its mechanical performance. Ionic-covalent entanglement (ICE) hydrogels of gellan gum and gelatin possessed excellent mechanical strength and a self-recovering ability when subjected to mechanical deformation. The ICE hydrogels were successfully fabricated as microspheric particles, which were subsequently demonstrated to be capable of mechanically reinforcing gelatin
hydrogels. These materials may have significance for the field of tissue engineering because of their mechanical potential to be used as soft tissue scaffold materials.
ACKNOWLEDGEMENTS

I would like to thank my supervisor, Marc in het Panhuis, for guidance and assistance in my research and in particular for providing me with fruitful research topics and allowing me to pursue topics that interest me greatly.

I would also like to thank Clare Watson, Marie Ranson, Robert Gorkin, Benedikt Steinhoff, Holly Warren and Ross Clarke for the opportunity to collaborate and produce great research together. I also thank Paul Calvert, Cameron Ferris, Kelvin Mews, Philip Whitten and Dianne Jolley for particularly useful discussions on my research. Additionally, I thank the members of my research group, associated research groups, the entire School of Chemistry and members of the Australian Centre of Excellence for Electromaterials Science who have become good friends and colleagues over the last few years.

Finally I would like to show my appreciation for my friends and family who have supported me tremendously and provided me with the work-life balance that has kept me on track during my studies. In particular I would like thank my wife Michelle for her continued support and encouragement, her motivational powers were ultimately what drove me when my enthusiasm waned and without her this thesis might never have been completed.
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KEYWORDS

AAS – Atomic absorption spectroscopy

A-GG – Acid form gellan gum

ATR – Attenuated total reflectance

AV – Arteriovascular

CAM – Chorioallantoic membrane

CATE – Computer aided tissue engineering

DI - Deionised

DN – Double network

EC – Extent of cross-linking

ECM – Extracellular matrix

FALGPA - 2-furanacryloyl-L-leucylglycyl-L-prolyl-L-alanine

FDA – United States Food and Drug Administration

FTIR – Fourier transform infrared

GG – Gellan gum

HAGG – High acyl gellan gum

HLB – Hydrophile-lipophile balance
ICE – Ionic-covalent entanglement

IPN – Interpenetrating network

LAGG – Low acyl gellan gum

LVE – Linear viscoelastic

LYS - Lysine

MMP – Matrix metalloprotease

MR-hydrogel – Microgel reinforced hydrogel

Na-GG – Sodium form gellan gum

NC – Nanocomposite

O/W – Oil-in-water

O/W/O – Oil-in-water-in-oil

PAAm – Poly(acrylamide)

PAMPS – Poly(2-acrylamido,2-methyl,1-propanesulfonic acid)

PAS – Periodic acid-Schiff

PBS – Phosphate buffered saline

PCL – Poly(caprolactone)

PEG – Poly(ethylene glycol)
PEG-DA – Poly(ethylene glycol diacrylate)

PGA – Poly(glycolic acid)

PGS – Poly(glycerol sebacate)

PLA – Poly(lactic acid)

PMA – Phorbal-12-myristate-13-acetate

PPO – Poly(propylene oxide)

PVA – Poly(vinyl alcohol)

RGD – Arginine-glycine-aspartic acid

RGDS - Arginine-glycine-aspartic acid-serine

SBF – Simulated body fluid

SD – Standard deviation

SW – Swelling ratio

µCT – Micro-computer tomography

VEGF – Vascular endothelial growth factor

W/O – Water-in-oil

W/O/W – Water-in-oil-in-water
PUBLICATIONS

Published manuscripts


Submitted manuscripts


Presentations and Posters
Damian M. Kirchmajer, Clare A. Watson, Marie Ranson, Marc in het Panhuis, *Can we grow new organs in gelatin?*, presented as an oral address and a poster at the 3rd Asia Pacific Symposium on Nanobionics (Wollongong, Australia), 2012
Damian M. Kirchmajer, Clare A. Watson, Marie Ranson, Marc in het Panhuis, *Genipin cross-linked gelatin hydrogels*, presented as a poster at the 8th International Electromaterials Science Symposium (Wollongong, Australia), **2013**

Damian M. Kirchmajer, Marc in het Panhuis, Tough and self-repairing ICE network hydrogels of gellan gum and gelatin, presented as an oral address at the 34th Australasian Polymer Symposium, **2013**
CHAPTER 1: INTRODUCTION

Damian M. Kirchmajer, a Robert Gorkin III, b and Marc in het Panhuis a

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Sections 1.2 and 1.3.3 in this chapter were submitted as a review to Journal of Materials Chemistry B.

Author statement
Damian M. Kirchmajer wrote the review. However, this work would not have been possible without the intellectual input and support from Robert Gorkin III and Marc in het Panhuis.

Primary supervisor confirmation
I, Associate professor Marc in het Panhuis (primary supervisor), support and certify the above author statement.

Signature: __________________________

Date: __________________________
1.1 Tissue engineering

Every day, people are in need of organ and tissue transplants. For many people an organ transplant is their last resort and a lifesaving treatment. Transplantation therapy was acknowledged in the 2008 Australian National Clinical Taskforce’s report as the most effective treatment for end-stage renal failure and for those that would otherwise die of heart, liver or lung failure\textsuperscript{1}. Tissue transplantation is also regarded as a highly effective treatment for those requiring new skin, cardiac and musculoskeletal tissues\textsuperscript{1}. As of 2008, it is estimated at any given time in Australia there are approximately 1700 people waiting for an organ donation, approximately 50 of which are children\textsuperscript{1}. Depending on the organ, these people could be expected to wait between six months and four years before they receive a transplant, and some might die before they receive one at all\textsuperscript{1}. Generally, organs and tissue only become available when someone donates them upon their death and as a consequence of this, the supply of donated organs and tissue is vastly short of the demand for them.

In their seminal review, Robert Langer and Joseph Vacanti describe the emerging field of tissue engineering as the application of “the principles of biology and engineering to the development of functional substitutes for damaged tissue”\textsuperscript{2}. It is anticipated that tissue engineering technologies may one day provide a plentiful supply of cost efficient and highly compatible laboratory grown tissue and organs for implantation\textsuperscript{2}. Tissue engineering may also provide the opportunity to enhance our current physiology, and create spin-off technologies which could accelerate the development of new drugs and our understanding of diseases\textsuperscript{3}. Many tissue engineering materials are comparatively cheap to obtain making them potentially cost effective alternatives to donor transplantations\textsuperscript{4}. Tissue engineering materials
and cells could also be hypothetically harvested and expanded from a patient biopsy or blood sample would eliminate the problem of organ/tissue incompatibility and greatly reduce the occurrence of immunological rejection which can occur in conventional donor transplantation therapy5,6.

It is now widely accepted that all tissue engineering schemes must carefully consider and integrate three critical components – i) cells, ii) a scaffold, and iii) cellular stimulation (Figure 0-1)2. Cells, once harvested from the patient and expanded in vitro, can be incorporated into a scaffold where they will grow, proliferate, and remodel their environment until it is ready to assume its function in the body7,8. In the past, acellular therapies have been attempted but it was revealed that by relying solely on cellular intrusion from the surrounding tissue, maturation happens too slowly or not at all2. Scaffolds are 3D architectures comprised of various components that can include structural materials; biological materials like proteins, or growth factors; and in some cases even functional materials like conductors9. The properties of these scaffolds should resemble the extracellular matrix and they should also be designed to initially hold cells in place and deliver bioactive molecules, whilst maintaining space for tissue to develop10,11. The scaffold can also be designed and fabricated in such a way as to guide cellular migration and blood vessel growth which are very important12-14. Cellular stimuli, typically in the form of growth and differentiation factors (but also may include electro/mechanical stimuli and matrix stimuli), is also essential to tissue maturation15. Cells respond to stimuli by producing enzymes, matrix materials, cytokines and by undergoing cellular processes such as differentiation and proliferation16.
Engineered tissue has already been successfully used as replacement tissue for the treatment of damaged and diseased tissue as well as being used as in vitro models for the study of various tissue pathologies. Tissue engineered skin has been successfully used to treat patients with full thickness burns and skin diseases including vitiligo, melanoma, psoriasis and blistering disorders (Figure 0-2a)\textsuperscript{18} as well as being used as a model for the in vitro study of psoriasis\textsuperscript{19}. Large size blood vessels have also successfully been engineered and employed as adult vascular grafts for bypass surgeries (Figure 0-2c)\textsuperscript{20,21}. Other examples of successful engineered tissues which
have emerged recently and are used for transplantation therapy and pathological studies are heart valves (Figure 0-2c), bronchial tissue and cornea tissue\textsuperscript{22-24}. These tissue engineering successes demonstrate that the concept of tissue engineering in plausible, however there are numerous challenges in the field which are still unmet.

Of the many challenges still faced, the oldest and most prevailing challenge has been the development of good scaffolds for \textit{in vitro} tissue engineering that meet the extensive schedule of design criteria (for example biocompatibility and porosity). Another challenge has been the preparation of larger and more complex tissues and ultimately the engineering of entire organs which are ultimately too intricate and delicate to be fabricated by hand or traditional casting and moulding technologies. For these the most promising way forward here is computer aided fabrication using rapid prototyping and additive manufacturing technologies such as extrusion printing. Unfortunately, there are not many scaffold materials that are capable of being printed effectively so there is a challenge to find scaffold materials that are printable. Current successes in tissue engineering have been for relatively simple and small or thin pieces of tissue because larger and thicker pieces are harder to nourish properly. The incorporation of a vascular network within the scaffold to supply nutrients and oxygen to the cells within is a major challenge preventing the preparation of larger pieces of engineered tissue. Certain tissue types, such as cartilage, require especially tough and resilient scaffold materials to become achievable. Unfortunately, traditional hydrogel materials are weak and brittle, so the development of tougher hydrogel materials is current challenge in soft tissue
engineering. These challenges will now be discussed in further detail because they provide the context for the materials presented in this thesis.

Figure 0-2: Examples of successfully engineered tissues: a) Tissue engineered skin (reproduced from National Geographic website); b) a tissue engineering trileaflet heart valve; c) an engineered vascular graft.

1.2 Materials for tissue scaffolds
The cellular scaffold is a critical component of in vitro tissue engineering strategies. It’s primary role is to maintain a space for the tissue to develop in and to hold the cells in place. For this to happen, the scaffold must allow cells to adhere to it; possess the room required for growth and proliferation; and permit them to intrude and occupy the material all the way through. It may also incorporate a vascular
network for supplying nutrients and oxygen, and removing of metabolites and carbon dioxide throughout the construct which will ultimately integrate with the patient vasculature\textsuperscript{14,26,27}. Additionally, it is advantageous to encourage cell growth, proliferation, differentiation and function by way of topographic and biomolecular cues (loading of growth and differentiation factors)\textsuperscript{28}. The biological compatibility of the scaffold and its constituent materials are also important: the materials must not be immunogenic, cytotoxic, or in any way hinder cellular function\textsuperscript{29}. Finally, most scaffolds should be fully assimilated into the patient over time and therefore must biodegrade as it is replaced by the cells own natural extracellular matrix\textsuperscript{30}.

Scaffolds for tissue engineering can either be recycled from old or created from new (\textit{de novo}). Scaffold recycling refers to the use of a decellularised scaffold from another organism which has some distinct advantages over \textit{de novo} scaffolds as well as some major drawbacks. The main advantages of decellularised scaffolds is that they retain the complex micro- and macro-structure of the target tissue and are constructed from native tissue so are therefore inherently biocompatible (excepting where tissue from other organisms is used)\textsuperscript{31}. However the scaffolds must first undergo a decellularisation process as well as sterilisation to ensure there are no alien cells or pathogens carried forward, and these processes can damage the delicate scaffold features and leave immunogenic and cytotoxic residues\textsuperscript{32,33}.

The alternative to scaffold recycling is to create a new one from scratch (\textit{de novo}) which offers the ultimate in versatility with regards to materials and design. Hydrogels are the most appropriate materials for engineered soft tissue scaffolds because, generally, they mimic and match the mechanical properties of natural soft
tissues which is essential to avoiding shearing around the implant\textsuperscript{10,34,35}. In addition to mechanical properties there are many other special properties of hydrogels that also make them suitable scaffold materials such as biological properties and degradability. Hydrogels can be produced from many different types of polymers and can be synthesised, blended, modified and processed to meet the necessary performance criteria for any type of soft tissue scaffold.

Hydrogels are materials comprised mainly of water (up to \textasciitilde 99\%) with the remainder being a hydrophilic polymer network that confines the water within its boundaries\textsuperscript{36}. Hydrogels can be processed using relatively mild conditions and aqueous chemistries. Over the past 30 years an extensive array of both naturally derived and synthetic hydrogel-forming polymers have been utilised for various soft tissue engineering objectives\textsuperscript{37}. However, hydrogel-forming polymers need to be suited for their specific application, and for tissue engineering, this means they must be prepared from biocompatible polymers using either non-toxic reagents (or in the case of toxic reagents, using those which can be completely removed after the scaffold has been fabricated)\textsuperscript{10,38}. It is also often required that hydrogel-forming polymers possess gel-forming mechanisms which allow the encapsulation of cells during processing; i.e. if cells must be integrated in the scaffold during fabrication, the gel formation process must not harm the cells\textsuperscript{39,40}. Furthermore, control of the degradability of hydrogel-forming polymers is critical as the lifetime of the material has to suit the tissue engineering application – specifically, temporary scaffolds need to degrade but permanent implants must not\textsuperscript{41}. 

10
1.2.1 *Hydrogel-forming polymers*

Hydrogel-forming polymers can be classified according to their synthetic origins, composition, electrostatic nature and gel forming mechanism (Figure 0-3). These same traits are critical considerations when selecting a hydrogel-forming polymer for specific tissue engineering applications and the ability to utilise a particular fabrication method.

Hydrogels are formed from either naturally produced polymers (also referred to as biopolymers) or synthetic polymers. Biopolymers are derived from various organisms including human, animals, plants and bacteria and are generally, more compatible and more likely to interact positively with cells\textsuperscript{42}. Human derived biopolymers such as collagen and fibrin have the greatest biological compatibility and possess proteolytic pathways of degradation (proteolysis by specific enzymes), whilst those non-human derived biopolymers such as alginate or chitosan have less compatibility and degradability\textsuperscript{43}. Notwithstanding, many human derived biopolymers are often preferentially derived from non-human sources because they are more available and cheaper to produce. For example hyaluronan, which is a glycosaminoglycan produced in humans, is much more efficiently produced from bacteria\textsuperscript{44}. Historically, plant derived biopolymers (e.g. alginate, agarose, cellulose) have been used in cell culture and are inexpensive and easy to obtain, however, because they are completely foreign molecules to humans they have at best intermediate biocompatibility and no proteolytic degradation mechanism within the body\textsuperscript{44}. There are major drawbacks with biopolymers, including significant
variations in molecular weight and structure from batch to batch and they may also present a risk of pathogen transfer from the originating organism\textsuperscript{45}.

Synthetic polymers usually possess superior mechanical properties and can be produced in large quantities, consistently, cheaply, and above all, are easy to modify to produce hydrogels with desirable properties. There is no risk of pathogens being present in a synthetic polymer hydrogel however care must be taken to ensure that there is no trace of toxic unpolymerised/uncross-linked reagents left in the hydrogel prior to use\textsuperscript{46}. Most synthetic polymers are not biocompatible; have limited biodegradability; and have poor cellular adhesion, however, many of these shortcomings have been addressed to some extent with clever processing and modification strategies\textsuperscript{15,42,47}.

A new class of synthetic hydrogel forming materials that are comprised of small discrete (organic) molecules, such as peptides is worth mentioning in this space although it will not be described in as much detail as biopolymers and synthetic polymers. This new class of material show much promise in potential application to tissue engineering scaffold materials and presents a new space to explore in hydrogel science\textsuperscript{48}. It is recommended that the intrigued reader be directed to the work of Samuel Stupp for an excellent description of the subject\textsuperscript{48}.

Polymers can also be classified based on their composition and, more specifically, the monomers/types of monomers from which they are made. Most biopolymers used for tissue engineering are either proteins/polypeptides, polysaccharides, or glycosaminoglycans while the most prevalent synthetic hydrogel-forming polymers
are polyols, polyethers or polyesters. Proteins and polypeptides are the most functional of all biopolymers because they contain peptide domains that interact directly with cells and perform specific functions (e.g., signalling and cellular adhesion), and usually there are enzymes in the body that specialise in degradation of these polymers. Conversely, proteins are also relatively expensive to mass produce and have limited lifetimes. Polysaccharides are a diverse class of biopolymers obtainable from many plant and microbial life forms which makes them a very versatile and cost effective hydrogel materials. Many polysaccharides are also polyelectrolytes that can form ionotropic hydrogels or complex coacervate hydrogels such as gellan gum and alginate. Glycosaminoglycans are a class of polysaccharides that contain amine functionality and deserve special mention because they, in combination with various proteins, form the natural extracellular matrix of human cells and consequently have excellent biocompatibility and cellular affinity. Polyols, polyethers and polyesters can be produced cheaply with consistency but are generally less degradable than biopolymers, with the exception of polyesters derived from naturally occurring α-hydroxy acids which possess greater biocompatibility and are to some extent biodegradable or excretable.

Another distinguishing feature of hydrogel-forming polymers is their electrical nature which directly relates to how a hydrogel can be formed. The electrical nature of a polymer is derived from the inherent functionality of the monomers it is constructed from. Certain functional groups are ionisable in aqueous solutions; for example the amino groups in gelatin are positively charged in acidic solution and the carboxylate groups of alginate are negatively charged in alkali solution.
Polysaccharides are often anionic in nature due to an abundance of carboxylate or sulfate containing moieties within their structure such as gellan gum which has one carboxylate containing saccharide unit in every four \(^55\). There are also polysaccharides that are cationic due to an abundance of amine containing monomer units (e.g. chitosan)\(^56\). Glycosaminoglycans, by definition, contain amino groups in their structure but also usually contain an excess of carboxylic acid functionality and hence are almost exclusively anionic in nature (e.g. hyaluronan)\(^57\). Proteinaceous biopolymers are comprised of a mixture of amino acids with many different negative and positively charged functional groups. Proteinaceous polymers are amphoteric polyelectrolytes because the population of positive and negative charges in protein molecules is mediated by the pH of the solution and its isoelectric point (pI). By no means are all biopolymers also polyelectrolytes, in fact, there are a number of neutrally charged polysaccharides such as agarose, dextran and cellulose, however, almost all polyols, polyethers and polyesters are neutral in nature\(^58-61\).

![Diagram](image.png)

Figure 0-3: Hydrogel-forming polymers can be classified by origin (blue), composition (red) and electrical nature (green). To some extent, the origin, composition and electrical nature are related (arrows).
1.2.2 Hydrogel formation mechanisms

The mechanism by which a hydrogel forms has a direct impact on the methods used to fabricate the hydrogel component for tissue engineering. In general, certain gel forming processes lend themselves to rapid prototyping fabrication methods while others require more time to develop into robust hydrogels and are suited to slower fabrication techniques such as porogen leaching\textsuperscript{62,63}. All hydrogels possess some level of physical attraction between macromers as a result of hydrogen bonding and entanglements amongst one another\textsuperscript{40}. Often these physical interactions are strong enough to form a weak gel but these are seldom strong enough for tissue engineering applications or layer upon layer fabrication. Usually a hydrogel intended for tissue engineering applications must be strengthened through additional electrostatic interactions or chemical cross-linking (Figure 0-4)\textsuperscript{64}.

Ionotropic hydrogels are those formed as a result of electrostatic interactions between polyanions and cations or polycations and anions. For example, alginate is a polyanionic polymer comprised of mannuronic and glucaronic acid residues which forms a firm ionotropic hydrogel upon addition of calcium ions\textsuperscript{65}, and chitosan is a polycationic polymer containing glucosamine residues, which are positively charged above its isoelectric point and will form a firm ionotropic hydrogel with phosphate ions\textsuperscript{66}. Ionotropic hydrogels are usually able to form a firm hydrogel upon cooling and are therefore particularly useful for \textit{in situ} tissue engineering or for use in rapid prototyping fabrication techniques (e.g. extrusion printing)\textsuperscript{65,67}. Ionic cross-links are also able to be repaired if broken which can be beneficial\textsuperscript{68}. Hydrogels intended for
use as cartilage tissue scaffolds comprised of gellan gum and epoxy amine polymers have been demonstrated to recover after physical deformation\textsuperscript{69}.

Complex coacervate hydrogels, also sometimes referred to as polyion complexes or polyelectrolyte complexes, are formed upon mixing of a polyanion and a polycation with one another such as alginate and poly(L-lysine) or sometimes also with an amphoteric polymer such as chondroitin sulfate and gelatin\textsuperscript{40,70}.

Almost all hydrogels are also able to be directly cross-linked with various chemical cross-linkers, or at least be pre-functionalised so that they can be subsequently cross-linked\textsuperscript{71}. The variety of cross-linking methods and reagents are large and many cross-linking reagents are toxic and must be fully removed from the hydrogel before they come into contact with cells or a body\textsuperscript{66}. It is also possible to incorporate proteolytically degradable sequences using covalent cross-linking chemistries to improve the degradability of otherwise non-degradable polymers\textsuperscript{72}, such as the inclusion of proteolytically degradable sequences into Poly(ethylene glycol) hydrogels demonstrated by Baldwin and Kiick and Patterson and Hubbel (Figure 0-5a)\textsuperscript{42,72}. 
1.2.3 Degradation behaviour and biocompatibility

It is often advantageous for a hydrogel-forming polymer to be degradable via a natural process whose degradation rate matches the rate of the production of new extracellular matrix\(^{37}\) (but in some cases a non-permanent implant is desirable\(^{12}\)). Hydrogel-forming polymers are generally degraded by either proteolysis or by hydrolysis\(^{73}\). Proteolysis occurs when an enzyme that is produced by the cells in or around the implant are able to recognize a degradable peptide sequence in the polymer which it can sever\(^ {7,72}\). Collagen for example, is a proteinaceous biopolymer which can be degraded through the action of matrix metalloproteases called collagenases\(^ {74}\). The main advantage of proteolytically degradable polymers are that
they will be degraded at a rate that more closely matches that of cellular growth because the cells are programmed to produce these enzymes to make room for themselves to grow into. Many hydrogel polymers are also able to be hydrolysed without the aid of an enzyme under physiological conditions but at a significantly slower rate which can be advantageous if a longer lasting implant is desired.

Synthetic polyesters of α-hydroxy acids are the only synthetic polymers that can be degraded in a natural way into their naturally occurring monomers and subsequently consumed in the tricarboxylic acid cycle. Poly(lactic acid) and poly(glycolic acid) are examples of α-hydroxy acid based polymers which have been used extensively in biomedical engineering as degradable stents, sutures and wound dressings. Most other synthetic polymers used in tissue engineering are generally non-degradable and are often either selected for use in applications that require more persistent materials or limited to low molecular weight analogues (< 5,000 Da) which are able to be removed via the renal system. Alternatively, non-degradable hydrogel-forming polymers may have degradable regions built into their structure to impart finely controlled degradability. Poly(ethylene glycol) is a prevalent example of a synthetic polymer with poor inherent degradability which can and has been modified to include enzyme-cleavable domains and improve its degradability.

Materials for use in tissue engineering must be compatible with the body of the intended patient. Further, the interaction between cells and biomaterials as well as biomaterials and the body needs to be carefully considered when selecting hydrogel-forming polymers for tissue engineering applications. The term biocompatibility is often used to describe this concept, which is an ambiguous term which has evolved
and changed meaning in line with the evolution of our understanding of the interaction between biomaterials and the body\textsuperscript{83}. In this thesis, a biocompatible material is defined as one which does not incite a foreign body reaction on its own; is non-inflammatory or otherwise immunogenic; and is non-cytotoxic. The foreign body response is a reaction to the inclusion of a foreign material such as a tissue engineered construct which can be detrimental to the function of the implant\textsuperscript{84}. Often a hydrogel scaffold on its own can be responsible for a foreign body reaction, but cells and other inclusions in an implant may also contribute\textsuperscript{84}. Certain hydrogel-forming polymers have been observed to stimulate particularly strong foreign body reactions such as carrageenan which is frequently used to test the efficacy of anti-inflammatory reagents in animal models\textsuperscript{85,86}.

The ability of cells to adhere to the scaffold is also an important aspect of hydrogel-forming polymers to consider. The adhesion of cells to their hydrogel scaffold has been demonstrated to provide important stimulation to the cells and directs their differentiation and activity\textsuperscript{87,88}. Lack of cellular adhesion can also result in anoikis (apoptosis induced by inadequate cell-matrix interactions)\textsuperscript{89}. Cells can adhere to a scaffold through specific “lock and key” type interactions such as the integrin and heparin binding domains in cells and extracellular matrix proteins\textsuperscript{90}. Many hydrogel-forming polymers do not inherently possess specific cellular adhesion regions but instead may be modified to do so by immobilising proteins onto the polymer\textsuperscript{91}. By far the most prevalent strategy to improvement of cellular adhesion is the tethering of the integrin binding RGD domain to the polymer backbone (Figure 0-5b)\textsuperscript{45,92–95}. The
non-adherent polysaccharide gellan gum has been demonstrated to have significantly improved cellular adhesion when the RGD peptide sequence has been tethered to it.\textsuperscript{96}

Figure 0-5: a) A schematic representation of star-PEG modified with MMP-cleavable domains and heparin which was used to prepare a degradable synthetic hydrogel that releases pro-angiogenic vascular endothelial growth factor (VEGF).\textsuperscript{97} b) A confocal micrograph of a selectively RGDS-modified star-PEG hydrogel (green colour) with fibroblasts adhered and guided in this region (pink and blue).\textsuperscript{95}

Table 0-1 presents the most prevalent hydrogel-forming polymers used in tissue engineering with a summation of their most pertinent characteristics to their utility as cellular scaffolds (polymer class, functionality, degradability, biological response, activity and compatibility, and gel formation mechanism). These polymers include familiar polymers which have been used in biomedical devices, formulations and in cell culture protocols for decades such as gelatin and collagen, and also include more recently investigated materials like carrageenans and gellan gum. From the schedule of hydrogel-forming polymers presented in Table 0-1, gellan gum and gelatin were
selected as the main materials to be used throughout the research presented in this thesis. The polymers will now be described in greater detail in the following sections.

1.2.4 Gelatin and genipin

Gelatin is a proteinaceous biopolymer produced from the controlled hydrolysis of collagen, which is the primary constituent of the extracellular matrix in all connective tissues in the human body\textsuperscript{54}. Commercially, gelatin is produced from collagen by either alkali or acid hydrolysis, however, gelatin with a variety of isoelectric points have also been produced from the enzymatic degradation of collagen\textsuperscript{98}. The source of the collagen and the method of production affect the molecular weight distribution, isoelectric point, colour, transparency and related properties\textsuperscript{54}. “Type A” gelatin is prepared from acid treated porcine skin collagen and is the most frequently used gelatin in biomedical applications on account of its gelation properties, low cost and optically transparency\textsuperscript{98}. 
Table 0-1: Description of polymers used in tissue engineering categorised by class; biological response, activity and biocompatibility; degradability; and hydrogel-forming mechanism.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Description and functionality</th>
<th>Class</th>
<th>Biological response, activity and compatibility</th>
<th>Degradability</th>
<th>Hydrogel-forming mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose</td>
<td>Polymer comprised of D-galactose and 3,6-anhydro-L-galactose with ether functionality</td>
<td>Neutral, polysaccharide biopolymer</td>
<td>Not biocompatible(^4)(^9)</td>
<td>Non degradable(^10)</td>
<td>Physical gel formation below 36°C(^9)</td>
</tr>
<tr>
<td>Alginate</td>
<td>Polyelectrolyte comprised of D-mannuronic acid and L-guluronic acid with carboxylate and hydroxyl functionality</td>
<td>Anionic, polysaccharide biopolymer</td>
<td>Varying biocompatibility, high L-guluronic acid content alginates are more immunogenic(^4)</td>
<td>Hydrolysis(^4) Ion exchange/chelation(^5)</td>
<td>Ionotropic gel formation with divalent cations(^4)</td>
</tr>
<tr>
<td>(\kappa)-Carrageenan</td>
<td>Polyelectrolyte comprised of D-galactose and 3,6-anhydro-D-galactose with hydroxyl and sulfate functionality</td>
<td>Inflammation inducing(^4), but also demonstrated anti-tumoral(^4)</td>
<td>Hydrolysis(^10)</td>
<td>Ionotropic gel formation with monovalent cations(^4,10)</td>
<td></td>
</tr>
<tr>
<td>Chitosan</td>
<td>Polyelectrolyte comprised of D-glucosamine and N-acetyl-D-glucosamine with amine and hydroxyl functionality</td>
<td>Cationic, polysaccharide biopolymer</td>
<td>Biocompatible, non-cytotoxic, anti-bacterial, anti-fungal, anti-tumoral(^9)(^10)</td>
<td>Hydrolysis(^10) Proteolysis (lysozyme)(^10)</td>
<td>Chemical gel formation via cross-linking of amino groups(^8)(^10)</td>
</tr>
<tr>
<td>Chondroitin Sulfate</td>
<td>Polyelectrolyte comprised of N-acetyl-D-galactosamine and D-glucuronic acid with amide, carboxylate, hydroxyl and sulfate functionality</td>
<td>Anionic glycosaminoglycan biopolymer</td>
<td>Biocompatible(^4)</td>
<td>Proteolysis (chondroitinase)(^10)</td>
<td>Complex coacervate gel formation with cationic polyelectrolytes(^4)</td>
</tr>
<tr>
<td>Collagen</td>
<td>Polyelectrolyte comprised of various amino acids with amine, carboxylate and hydroxyl functionality</td>
<td>Amphoteric, proteinaceous biopolymer</td>
<td>Bio compatible, non-toxic, with good cellular adhesion but potentially immunogenic(^10)</td>
<td>Proteolysis (collagenase)(^74)</td>
<td>Physical self-assembling gel formation and chemical gel formation via cross-linking of amino or carboxylate groups(^9)(^10)</td>
</tr>
<tr>
<td>Dextran</td>
<td>Polymer comprised of D-Glucopyranose with hydroxyl functionality</td>
<td>Neutral, polysaccharide biopolymer</td>
<td>Biocompatible but has poor protein and cellular adhesion(^10), and is potentially immunogenic(^10)</td>
<td>Hydrolysis(^10)</td>
<td>Ionotropic gel formation in the presence of (K^+)(^9,10)</td>
</tr>
<tr>
<td>Elastin</td>
<td>Polyelectrolyte comprised of various amino acids with amine and carboxylate functionality</td>
<td>Amphoteric, proteinaceous biopolymer</td>
<td>Biocompatible, but is hydrophobic and insoluble(^8,11)</td>
<td>Proteolysis (elastase)(^11)</td>
<td>Covalent (self-assembling)(^11)</td>
</tr>
<tr>
<td>Fibrin</td>
<td>Polyelectrolyte comprised of various amino acids with amine, carboxylate and hydroxyl functionality</td>
<td>Amphoteric, proteinaceous biopolymer</td>
<td>Biocompatible with excellent protein and cellular adhesion(^10), thrombogenic(^11)</td>
<td>Proteolysis(^10)</td>
<td>Covalent (self-assembling)(^11)</td>
</tr>
<tr>
<td>Gelatin</td>
<td>Polyelectrolyte comprised of various amino acids with amine,</td>
<td>Amphoteric, proteinaceous</td>
<td>Biocompatible(^10) with good cellular adhesion(^11)</td>
<td>Proteolysis (collagenase)(^11)</td>
<td>Physical gel formation below 27°C and chemical gel formation via cross-</td>
</tr>
<tr>
<td>Polymer</td>
<td>Composition</td>
<td>Biocompatibility and Degradability</td>
<td>Linking of Amino or Carboxylate Groups</td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------------------------</td>
<td>-----------------------------------------------------------------------------</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Gellan gum</td>
<td>Polyelectrolyte comprised of D-glucose, D-glucuronic acid and L-rhamnose with carboxylate and hydroxyl functionality</td>
<td>Anionic, polysaccharide biopolymer</td>
<td>Biocompatible, non-cytotoxic but has poor cellular adhesion</td>
<td>Hydrolysis, Ion-exchange/chelation</td>
<td>Ionotropic gel formation with cations</td>
</tr>
<tr>
<td>Hyaluronan</td>
<td>Polyelectrolyte comprised of glucuronic acid and N-acetyl-D-glucosamine with amide, carboxylate and hydroxyl functionality</td>
<td>Anionic, glycosaminoglycan biopolymer</td>
<td>Biocompatible, with good cellular adhesion</td>
<td>Proteolysis (hyaluronidase)</td>
<td>Ionotropic formation with cations</td>
</tr>
<tr>
<td>Methylcellulose</td>
<td>Polymer comprised of D-glucose with hydroxyl functionality</td>
<td>Neutral, polysaccharide biopolymer</td>
<td>Biocompatible</td>
<td>Non degradable</td>
<td>Physical gel formation at a temperature dependant on the degree of methylation</td>
</tr>
<tr>
<td>Poly(acrylamide)</td>
<td>Polymer comprised of acrylamide with amide functionality</td>
<td>Neutral, synthetic polymer</td>
<td>Non-toxic polymer, but monomer is neurotoxic</td>
<td>Non degradable</td>
<td>Covalent</td>
</tr>
<tr>
<td>Poly(caprolactone)</td>
<td>Polymer comprised of ε-caprolactone with ether functionality</td>
<td>Neutral, synthetic polyester</td>
<td>Biocompatible</td>
<td>Hydrolysis</td>
<td>Covalent</td>
</tr>
<tr>
<td>Poly(ethylene glycol)</td>
<td>Polymer comprised of ethylene oxide with ether functionality</td>
<td>Neutral, synthetic polyether</td>
<td>Biocompatible, but with poor protein and cellular adsorption</td>
<td>Non degradable</td>
<td>Covalent</td>
</tr>
<tr>
<td>Poly(glycolic acid)</td>
<td>Polymer comprised of glycolic acid with ester functionality</td>
<td>Neutral, synthetic polyether</td>
<td>Intermediate biocompatibility, mildly immunogenic</td>
<td>Hydrolysis</td>
<td>Covalent</td>
</tr>
<tr>
<td>Poly(glycerol sebacate)</td>
<td>Polymer comprised of glycerol and sebacic acid with ester and hydroxyl functionality</td>
<td>Neutral, synthetic polyether</td>
<td>Biocompatible, non-cytotoxic</td>
<td>Hydrolysis</td>
<td>Covalent</td>
</tr>
<tr>
<td>Poly(2-hydroxyethyl methacrylate)</td>
<td>Polymer comprised of 2-hydroxyethyl methacrylate with ester and hydroxyl functionality</td>
<td>Neutral, synthetic polyether</td>
<td>Intermediate biocompatibility, mildly immunogenic</td>
<td>Non degradable</td>
<td>Covalent</td>
</tr>
<tr>
<td>Poly(lactic acid)</td>
<td>Polymer comprised of lactic acid with ester functionality</td>
<td>Neutral, synthetic polyether</td>
<td>Intermediate biocompatibility, mildly immunogenic</td>
<td>Hydrolysis</td>
<td>Covalent</td>
</tr>
<tr>
<td>Poly(propylene fumarate)</td>
<td>Polymer comprised of propylene fumarate with ester and vinyl functionality</td>
<td>Neutral, synthetic polyether</td>
<td>Inflammation causing material</td>
<td>Hydrolysis</td>
<td>Covalent</td>
</tr>
<tr>
<td>Poly(vinyl alcohol)</td>
<td>Polymer comprised of vinyl alcohol with hydroxyl functionality</td>
<td>Neutral, synthetic polyol</td>
<td>Biocompatible, but with poor protein and cellular adhesion</td>
<td>Non degradable</td>
<td>Covalent</td>
</tr>
</tbody>
</table>
Gelatin shares many of collagens inherent properties, including complete biocompatibility, non-toxicity, non-carcinogenicity and biodegradability via collagenase proteolysis\textsuperscript{39,140,141}. However, there are some distinct advantages for gelatin in comparison to collagen. The denatured structure resulting from the conversion of collagen to gelatin significantly decrease its antigenicity and reduces the risk of transferral of pathogenic species from the original animal\textsuperscript{142}. Gelatin is also generally cheaper than collagen, and materials produced from gelatin are less prone to batch-to-batch variation\textsuperscript{143}. Another important distinguishing feature is gelatin’s superior solubility – collagen self assembles into insoluble fibrils but gelatin doesn’t, which makes gelatin easier to process and modify using solution based chemistries\textsuperscript{143}.

Gelatin has been used prolifically in the pharmaceuticals industry, primarily as a soft or hard capsule to encapsulate drugs\textsuperscript{144}. It has also been used in surgical applications such as wound dressings, surgical sponges, sealants and glues\textsuperscript{145–148}. Gelatin has also been historically used as a coating on cell culture plates for the culture of endothelial cells, muscle cells and embryonic stem cells\textsuperscript{149}. Furthermore, gelatin has been studied for use as a scaffold for tissue engineering applications, in particular as a component in bone composite materials\textsuperscript{141,150,151}, articular cartilage\textsuperscript{152,153}, as guiding conduits for nerve regeneration\textsuperscript{154–156} and as a non-thrombogenic material for vascular tissue engineering\textsuperscript{157}.

Ordinarily, gelatin based materials swell extensively, rapidly dissolve and degrade at elevated temperatures (above the gel transition point ~29°C), and lose their strength and rigidity rapidly\textsuperscript{54,158,159}. Cross-linking of gelatin based materials is an effective
method to address these disadvantages and even allows for the tuning of the material properties and the opportunity to impart additional functionality\textsuperscript{160,161}. Non-chemical based cross-linking methods include dehydrothermal, freeze-thaw cycling, ultraviolet irradiation and gamma irradiation\textsuperscript{115}. Commonly used chemical cross-linking reagents include gluteraldehyde, diisocyanates, polyepoxy compounds, acyl azides, carbodiimides and genipin\textsuperscript{71,162}. Gluteraldehyde cross-linking is by far the most frequently utilised cross-linker but the toxicity of gluteraldehyde makes it troublesome to use in tissue engineering applications. In fact the toxicity of most chemical cross-linkers, and/or severity of the cross-linking reaction conditions, prohibits their use in many tissue engineering applications.

Genipin (Figure 0-6) is the aglycone of geniposide, an iridoid glycoside that is the major component of the fruit of the gardenia plant, \textit{Gardenia jasminoides} Ellis\textsuperscript{163}. It has recently emerged as a popular cross-linker for proteinaceous biopolymers such as gelatin because it is equally effective as other popular cross-linkers but is also non-cytotoxic, non-genotoxic and an anti-inflammatory agent\textsuperscript{46,164–166} which are virtuous properties for a component of a tissue engineered scaffold material.

\[
\begin{align*}
&\text{H}_3\text{CO} - \\
&\text{H} - \\
&\text{H} - \\
&\text{OH} \quad \text{OH}
\end{align*}
\]

Figure 0-6: Structural diagram of genipin.
Genipin forms chemical cross-links between the primary amino groups of either the ε-amino groups of lysine and hydroxylysine residues, or the guanidinium group of arginine residues in gelatin. With respect to gelatin, it has been reported that 85-90% of the free epsilon amino groups can be cross-linked with genipin. The cross-linking mechanism is a two-step process (Figure 0-7). In the first step the ε-amino group of lysine (or hydroxylysine) from gelatin is incorporated into the dihydropyran ring resulting in formation of a tertiary amine link and loss of 2H2O. In the second step, another ε-amino group from a different lysine residue replaces the methyl ester group forming an amide bond to the genipin molecule and producing CH3OH.

![Figure 0-7: Cross-linking mechanism for genipin and gelatin (R = an ε-amino group of gelatin).](image)

One of the most distinct properties of genipin cross-linked polymers is the development of a deep blue colour. This occurs because genipin, after reacting with a primary amine group creates intermediate species that are able to undergo radical polymerization with other genipin molecules initiated by oxygen radicals in the air. The intermediate species and oligomers of genipin can then undergo dehydrogenation to produce conjugated systems that absorb photons of around 575 nm wavelength, producing the strong blue colour. These blue pigments are
commonly known as the edible blue food dye “gardenia blue”. Fortunately, the polymerisation of the genipin and the formation of the intermediates do not inhibit the ability to cross-link gelatin\textsuperscript{163,169}.

Genipin cross-linked gelatins have been used for various biomedical applications over the last 10 years. In 2003, a study of genipin cross-linked gelatin wound dressings were compared with gluteraldehyde cross-linked wound dressings where it was demonstrated that both were effective as wound dressings, but the healing rate for the genipin cross-linked dressing was significantly faster and inflammatory reaction significantly lower than the gluteraldehyde cross-linked counterpart\textsuperscript{147}. Microspheres prepared from genipin cross-linked gelatin have also been used as intramuscular drug delivery vessels where it was shown that inflammatory response was minimal, and that swelling, drug retention and release and degradation could be controlled by the degree of cross-linking\textsuperscript{171}. Genipin cross-linked gelatin materials have also been explored as a component of nerve-guiding conduits for peripheral nerve gap regeneration in rat models\textsuperscript{154–156}, a component of bone composites for bone tissue scaffolds\textsuperscript{141,150}; and as a scaffold material for articular cartilage tissue engineering\textsuperscript{152,153}. Finally, genipin cross-linked gelatin microspheres have been used as cellular micro-carriers of hepatocytes which were used in the regeneration of liver tissue\textsuperscript{172–174}.

1.2.5  \textit{Gellan gum}

Gellan gum is a polysaccharide biopolymer produced from the bacteria \textit{Pseudomonas elodea} and is commercially marketed under the trade names Gelrite\textsuperscript{TM} and Kelcogel\textsuperscript{TM} \textsuperscript{175,176}. The molecular structure is a repeating tetra saccharide unit
consisting of: one α-L-rhamnose, one β-D-glucuronic acid and two β-D-glucose residues (Figure 0-8 a-b)\textsuperscript{175,176}. Native gellan gum (high acyl gellan gum or HAGG) has one acetyl substituent bound to every tetramer. The degree of acylation can be decreased (hereafter referred to as low acyl gellan gum or LAGG) with alkali treatment and is often performed to modify the gel properties\textsuperscript{177,178}. HAGG forms opaque, soft and more flexible hydrogels, whilst LAGG forms transparent, hard and more brittle hydrogels (Figure 0-8c)\textsuperscript{178–180}. For example, 2\% (w/v) HAGG with 5 mM Ca\textsuperscript{2+} possesses a compressive modulus of 87 ± 3 kPa and compressive failure stress of 50 ± 8 kPa, compared to 2\% (w/v) LAGG with 5 mM Ca\textsuperscript{2+} which has a compressive modulus of 540 ± 20 kPa and a compressive failure stress of 130 ± 5 kPa\textsuperscript{180}.

![Structural diagrams of gellan gum](image)

Figure 0-8: The structural diagrams of a) high acyl gellan gum and b) low acyl gellan gum\textsuperscript{177}; c) a photograph of typical gellan gum hydrogels prepared with low acyl gellan gum, high acyl gellan gum and a 1:1 blend\textsuperscript{180}.
Gellan gum solutions form hydrogels upon cooling to below the sol-gel transition temperature which lies between 30°C and 50°C\textsuperscript{51}. Upon cooling, random coils of gellan gum molecules change to a double helix conformation which aggregate together to form weak hydrogels in the absence of cations (Figure 0-9)\textsuperscript{119,181}. The presence of cations in the gel solution affects the gelation of gellan gum because they are able to interact with charged carboxylate groups on the polymer backbone. When monovalent cations are present, the cations are able to shield opposing negatively charged carboxylate groups so that the double helical aggregates can form firmer junction zones and create stronger hydrogels (Figure 0-9)\textsuperscript{182}. When divalent cations are present, even stronger gels are formed because the divalent cation acts as a cross-link between carboxylate groups to form even stronger junction zones (Figure 0-9). Gellan gum hydrogels which have been cross-linked with divalent cations will not return to the solution state upon reheating to above the sol-gel transition temperature\textsuperscript{178}.

![Figure 0-9: Schematic representation of conformation of gellan gum in its random coiled state, weak gel state and strong gel state through temperature changes. Adapted from reference\textsuperscript{120}.

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Calcium cross-linked gellan gum is an attractive material for tissue engineering because of its inherent gelation properties and its resemblance to native glycosaminoglycan ECM materials. The cross-linking mechanism for gellan gum is particularly amenable to certain tissue engineering techniques such as in situ/injectable cellular therapies\textsuperscript{183,184}. Calcium ion cross-linking in particular produces very firm hydrogels and is a comparatively non-toxic cross-linking alternative to many other chemical cross-linkers\textsuperscript{55}. The hydrogels are also degradable via ion exchange process where calcium ions are swapped for sodium ions\textsuperscript{180}. Gellan gum is also one of the few polysaccharides that are commercially available in endotoxin free form and approved for use in pharmaceuticals by the FDA\textsuperscript{177}. However, gellan gum has also been known to produce a mild inflammatory response \textit{in vivo}\textsuperscript{184}.

Gellan gum has been used in various biomedical applications and as an agar alternative for plant cell culture\textsuperscript{120}. Gellan gum has also been intensively researched as a controlled release/drug delivered vehicle\textsuperscript{183,185,186}. It has been used in ophthalmic therapeutics to retain, localize and slowly release eye medicines (the gellan gum solution gels upon contact with tear fluid and stops the medicine from running straight out of the eye)\textsuperscript{187}. It has also be used as an in \textit{situ} gelling solution for oral drug delivery, where a gellan solution containing the drug of interest gels spontaneously in the highly acidic stomach environment and subsequently releases the drug slowly over time\textsuperscript{183}. As far as tissue engineering applications are concerned, gellan gum has been used mostly as a scaffold for the engineering of articular cartilage tissue\textsuperscript{119,184} and intervertebral discs\textsuperscript{188}. Bolus injection of gellan with human
chondrocytes has been demonstrated as a potential *in situ* tissue engineering strategy toward cartilage regeneration. Recently, gellan gum has also been used to prepare cellular inks for use in bio-plotter technology for tissue engineering.

1.3 **Large and complex tissue engineering**

Most successfully engineered tissue to date has been on relatively simple, small and/or thin tissue pieces such as skin. However, ultimately it would be desirable to create all tissue types and even whole organs. Several ideas and technologies have evolved which are crucial steps towards achieving whole organ tissue engineering objectives. One paradigm shift that has worked towards this end is the transition from attempting to engineer new tissue inside the body (*in vivo*), to engineering tissue outside of the body (*in vitro*). Two other technologies which have also contributed greatly and will continue to contribute whole organ engineering are the implementation of computer aided tissue engineering and additive fabrication technologies such as extrusion printing. These concepts have a direct and indirect relationship to the materials that are compatible with them and hence will now be discussed in further detail.

1.3.1 **In vivo and in vitro tissue engineering**

The conventional *in vivo* tissue engineering strategy involves an injection of cells within a polymer matrix at the site of tissue damage/loss and is sometimes simply referred to as “cellular therapy”. The polymer matrix acts as a scaffold and creates a space which fills cavities in the damaged tissue, providing an environment for new cell and tissue growth to occur. A benefit of this strategy is that the injection is a minimally invasive surgery which greatly reduces the potential for post-operative
infection\textsuperscript{192}. Unfortunately this treatment is not appropriate for large or complex tissue repairs.

A very different but noteworthy \textit{in vivo} tissue engineering strategy makes use of an arteriovascular loop (AV loop) to culture new tissue\textsuperscript{193}. It is possible to surgically implant a scaffold within an AV loop so that the component will be invaded by fine blood vessels over time to enable nutrients to be supplied directly from the bloodstream\textsuperscript{194}. The scaffold can be slightly more complex compared to other \textit{in vivo} scaffolds and will naturally grow a vasculature over time, however the maximum size of the component that can be grown is limited by the size of the AV loop chamber, and it will of course require two additional surgeries – explantation and re-implantation\textsuperscript{195}.

Alternatively, engineered tissue can be designed and constructed outside of the body \textit{(in vitro)}. Cells from a patient biopsy can be expanded \textit{in vitro} to a useable quantity in 2D or 3D cell culture techniques\textsuperscript{139}. The scaffold can be carefully designed and fabricated so as to have complex shapes and internal structures conducive to tissue development\textsuperscript{196}. Cells and cellular stimuli can be spatially distributed throughout the scaffold during fabrication\textsuperscript{197}. In short, the \textit{in vitro} tissue engineering strategies offers an overall more versatile and scalable method for creating replacement tissue and organs\textsuperscript{198,199}. The \textit{in vitro} engineered scaffolds are hypothetically ready for use from the moment of implantation and can be tailored to the patient whilst in the laboratory.

Adopting the \textit{in vitro} strategy is the most logical step towards the creation of larger and more complex types of tissue and is surely essential for the fabrication of entire
new organs. More than ever before, the scaffold is of critical importance to the \textit{in vitro} approach as support structures for the tissue would be completely absent otherwise.

1.3.2 \textit{Computer aided tissue engineering}

The ultimate destination of the tissue engineering field is the fabrication of whole organs, however the structure and composition of organs is extremely complex and would be practically impossible without the assistance of computers and additive manufacturing technologies. Computer aided tissue engineering (CATE) is a concept that builds upon the idea of \textit{in vitro} tissue engineering and may one day facilitate the concept of whole organ printing\textsuperscript{200,201}. CATE, as the name suggests, utilises computer aided technologies to improve upon classical laboratory methods, it incorporates 3D medical imaging to measure and diagnose defective tissue/organs; 3D computer modelling and computer aided design software to prepare the blueprint for the replacement tissue/organ to be created; and additive manufacturing equipment such as 3D extrusion printers to precisely control the spatial placement of cells, scaffold material and cellular stimuli (an example of this approach is depicted in Figure 0-10).

Through use of the CATE methods, complex tissue and hopefully whole organs could one day be produced which are tailored specifically to individual patient needs in an “on-demand” fashion\textsuperscript{202,203}. The hypothetical scenario would go something like this: A patient with damaged kidneys would have the organ scanned to determine the shape and size required for a replacement; computer modelling software would be used to design the blue print for a healthy kidney based on the scans; and then the
healthy kidney would be manufactured, potentially all in the hospital on the day of diagnosis\textsuperscript{200}.

Figure 0-10: An example of a human tooth being engineered using CATE technology\textsuperscript{201}. A 3D model was prepared from micro-computer tomography scans and used as the blueprint for the fabrication of a tooth shaped scaffold using projection-based micro-stereolithography\textsuperscript{201}.

1.3.3 Extrusion printing of hydrogel scaffolds

Extrusion printing is a technique based on building structures by driving material out of a nozzle and onto a stage. The extruded material is either directed by moving the nozzle above the stage or by moving the stage underneath the nozzle; irrespectively, 3D structures are created through continuously depositing material layer-upon-layer. In order to successfully build 3D structures in this manner, the first layer needs have structural integrity before the second layer is deposited. Consequently, parameters such as polymer rheology and the gel forming mechanism are critically important; polymer solutions must be either viscous or viscoelastic initially, and then become
self-supporting gels before additional layers are deposited. Temporal control of gelation is crucial to avoid premature gelation of the polymer solution while it is still in the printer. To this end, several strategies for printing hydrogel-forming polymers are presented below.

Polymers which form hydrogels mainly through physical associations tend to possess a gel transition temperature below which the solution gels, such as agarose, methylcellulose, gelatin and collagen. Hot solutions of these polymers can be printed onto a cooled stage whereupon the polymer traverses its gel transition temperature and solidifies. Agarose is an example of a polymer which has been printed in this manner where the polymer solution was held in the printer reservoir at 60°C-80°C and printed into a cool bath below the gel transition temperature (Figure 0-11a)\textsuperscript{204,205}. A limitation of this approach is that physical hydrogels tend to be very weak and may need to be reinforced using other polymers or with a post-print cross-linking step. A compromise must also be made with respect to the magnitude of the temperature drop; if the temperature drop is small, the polymer solution will have a high viscosity and require high pressure to expel, but if the temperature drop is large it will take a long time to cool down and gel. In some instances the initial and final temperature of the polymer solution/gel may also exclude it from being able to include cells during printing.

Photo-curable hydrogel-forming polymers can be printed onto an illuminated stage where they will form firm hydrogels upon the incidence of light\textsuperscript{206}. Some polymers can be directly photo-cured if the appropriate photoinitiator is incorporated. For example, it has been demonstrated that any proteinaceous biopolymer which contains
tyrosine residues (such as collagen, fibrin and gelatin) can be cross-linked with white light in the presence of Ru(II)bpy$_3^{2+}$ photoinitiator (Figure 0-11b)$^{207-209}$. Even polymers with are not ordinarily photo-curable (such as gellan gum or dextran) may be modified to become photo-curable, often through a straightforward reaction with an acrylate or methacrylate based agent$^{210,211}$. Photo-curable polymer printing has been reported using PEG-acrylate and PPO-acrylate functionalized polymers without cells, as well as with solutions of gelatin-methacrylate and hyaluronan-methacrylate functionalized polymers mixed with cells$^{206,212-214}$. The main advantage of photo-curable polymer printing is that this type of polymerization is usually very rapid (a few seconds to a few minutes) and is generally cell-friendly$^{210}$. Also, because the reactive stimulus is light in this case, so a bath is not needed and the time scale of gelation can be adjusted by changing the intensity of the light.

Reactive printing of ionotropic polymers is a very successful method used to date for extrusion printing of scaffolds. It involves printing a polymer solution into a bath of reactive substance that induces gelation. Usually, this is performed with ionotropic hydrogels and a bath containing a solution of the appropriate counter-ion$^{204,215}$. It has been reported in several instances that alginate has been printed into a calcium solution in this manner to produce microspheres as well as more complex structures$^{196,216,217}$(Figure 0-11c). The main advantage of reactive printing of ionotropic polymers is the very rapid gelation (approximately 1 second). The polymer solution and the bath can be held at cell culture temperature (37 °C) and the gel forming method itself is cell-friendly. In fact, it is possible to print gellan gum
solutions with cell culture media whereupon a gel is formed instantly (Figure 0-11d)

Figure 0-11: Examples of computer aided tissue engineering constructs made from hydrogels. A) a porous cube of agarose hydrogel printed using a thermal modulation approach; B) a porous mesh disc shaped scaffold printed from photo-curable gelatin-methacrylamide; C) an aortic valve conduit printed with a blend of alginate/gelatin hydrogel\textsuperscript{196} and differently scaled aortic valves printed from PEG-DA hydrogels (scale bar is 1 cm)\textsuperscript{218}; D) a cellular ink of gellan gum and mouse myoblasts is printed in spirals on a glass microscopy slide (scale bar is 500 µm)\textsuperscript{195}.

Although the pathway to the construction of engineered whole organs using computer aided approaches is obvious, the materials which are both tissue engineering and extrusion printing compatible are scarce. What is direly needed in this field are materials which meet the performance criteria of tissue engineering as well as being capable of being used in additive manufacturing, i.e., are capable of being reactive printed and gelling rapidly into firm gels.

1.4 Vascularisable tissue scaffolds
Arguably one of the greatest challenges faced in the field of tissue engineering today is mass transfer limitations in thick tissue components which limit the supply of
nutrients and oxygen to cells – the so called “thick tissue dilemma”\textsuperscript{3,219}. In natural human tissue, blood vessels act as service conduits and it has been observed that cells situated more than a couple hundred micrometres from a capillary blood vessel suffer from hypoxia and apoptosis because nutrients, waste, oxygen and carbon dioxide are unable to diffuse through more than several hundred micrometers of tissue/scaffold\textsuperscript{26}. As a consequence, the size of a non-vascularised tissue engineered component is limited to less than ~ 500 µm thicknesses or restricted to the few tissues types that do not require a vasculature such as heart valves, cartilage and bone.

People have sought to address the thick tissue dilemma using a variety of different approaches\textsuperscript{219}. Possibly the simplest strategy has been to increase the porosity and pore interconnectivity of tissue scaffolds so that oxygen and nutrients can diffuse a greater distance through the scaffold\textsuperscript{220}. However, this approach can mechanically weaken the scaffold and there is still a definite limit of diffusion, albeit an extended one\textsuperscript{220}. Researchers have also sought to circumvent the problem altogether by choosing cells that are resistant to low oxygen conditions, but these cell types are too constrictive for some engineered tissues\textsuperscript{3}. Some success has been made by accelerating the growth of vasculature in a scaffold by loading it with pro-angiogenic factors and/or cell types which stimulate the production of vasculature\textsuperscript{221}. This approach is limited somewhat by the slow growth rate of blood vessels (~5 µm.hour\textsuperscript{-1}) which must intrude into the scaffolds\textsuperscript{222}. Excessive loading of pro-angiogenic factors may also lead to the formation of tumours and other angiogenesis-related pathologies\textsuperscript{223}. A promising approach is pre-vascularisation of the scaffold before it is implanted into the patient\textsuperscript{9,14,224,225}. 
Generally speaking, blood vessels are formed either de novo by a process called vasculogenesis, or by angiogenesis where new vessels sprout from existing ones\textsuperscript{226,227}. In vasculogenesis, mesodermal cells first cluster and arrange themselves to form the basic framework of blood vessels\textsuperscript{228,229}. They then differentiate into angioblasts and hemangioblasts, followed by further differentiation into endothelial cells and mural cells\textsuperscript{230}. Angiogenesis begins when endothelial cells are stimulated by vascular endothelial growth factor (VEGF) to proliferate and produce matrix metalloproteases (MMP)\textsuperscript{231}. The MMP disrupt the basement membrane and extracellular matrix (ECM) to allow for endothelial cells to intrude into surrounding tissue and form tubular structures\textsuperscript{231}. Finally, mural cells are recruited from the surrounding tissue and other blood vessels to form a new basement membrane and stabilise the nascent capillaries\textsuperscript{231}.

With the in vitro tissue engineering approach, it is possible to develop a vasculature within the construct before it is implanted (in fact, it is the ability to do this that is a distinct advantage of in vitro strategies versus in vivo strategies). It is possible to completely guide the growth of the vasculature with topographical and chemical cues created during scaffold fabrication which provides an extra element of control over the design of the scaffold\textsuperscript{95}. It is also possible to use the vasculature of a pre-vascularised scaffold to nourish the bulk of the tissue during culture in a perfusion bioreactor\textsuperscript{232}.

Once a pre-vascularised tissue component is implanted it is not immediately integrated with the host vasculature so blood does not flow through the component straight away and apoptosis may subsequently occur\textsuperscript{233}. However, it has recently
been demonstrated that vascular inosculation can be stimulated to form functional junctions between scaffold and host vasculatures (Figure 0-12)\(^\text{222}\). Vascular inosculation is a process that involves the host microvasculature extending blood vessels into the scaffold where they abut other blood vessels belonging to the scaffold vasculature\(^\text{234}\). The vascular intrusion can be initiated and invigorated by pro-angiogenic growth factors\(^\text{222}\). Once blood vessels are touching they can knit together if the basement membrane of the vessels are destabilised which can be achieved through the action of certain angiopoeitins\(^\text{222,235}\).

Figure 0-12: Schematic representation of a) vascularisation of an un-vascularised scaffold by the process of infiltration, and b) vascular inosculation of a pre-vascularised tissue scaffold\(^\text{236}\). c) a typical mouse dorsal skinfold model for studying of vascularisation and d) a close up view of a pre-vascularised scaffold in the same model that has been inosculated over several days\(^\text{222}\).
Often, after successful connection of the blood supply, the vascular network of the scaffold will undergo remodelling which involves the regression of unnecessary blood vessels and creation of new blood vessels\textsuperscript{222}. The vascular tree is pruned where hyperoxic regions of tissue exist causing non-perfused blood vessels to regress, and extend into the anoxic regions of tissue where hypoxic endothelial cells up-regulate production of VEGF which in turn stimulates angiogenesis\textsuperscript{237}.

1.5 **Tough tissue scaffolds**
For some tissue engineering objectives (such as cartilage tissue scaffolds), tougher and more robust hydrogels which have the capacity to recover from mechanical deformation are required. Conventional hydrogels have poor mechanical properties because there are very few energy dissipation mechanisms to impede crack initiation and crack propagation. When under stress, forces cannot be evenly distributed over every polymer chain equally as the distance between the cross-linking points varies. When the shortest polymer chain fractures, all the load is immediately transferred to the next shortest chain which may subsequently break, and so on\textsuperscript{238}. Hydrogels can be made substantially tougher using a variety of modern hydrogel science strategies including topological hydrogels\textsuperscript{239}, tetra-PEG hydrogels\textsuperscript{240}, double network (DN) hydrogels\textsuperscript{241–244}, ionic covalent entanglement (ICE) network hydrogels\textsuperscript{68,69,245,246}, and microgel reinforced (MR) hydrogels\textsuperscript{247}. Figure 0-13 puts the mechanical performance of these various hydrogel types into perspective.
Figure 0-13: Charts depicting the different types of materials and hydrogels and their tensile mechanical properties a) work of extension and modulus, b) tensile strength and elongation at break$^{238}$. 
The DN approach has been proven to be one of the most successful approaches to preparation of tough hydrogels and has been explored extensively by J. P. Gong and co-workers using poly(2-acrylamido,2-methyl,1-propanesulfonic acid) and poly(acrylamide) (PAMPS/PAAm) systems. Here, gels are prepared in a two-step scheme where a PAMPS hydrogel network is swollen in acrylamide (monomer) which is subsequently photo cross-linked (Figure 0-14 a-c). They have determined that “the rigid and brittle PAMPS network serves as a sacrificial bond that fractures at a relatively low stress, while the soft, ductile PAAm network serves as hidden length that sustains stress by large extension afterwards” (Figure 0-14 d-e). Gong’s original DN hydrogels possessed an unprecedentedly high compressive fracture stress of 17.2 MPa, but as they indicate, the hydrogels are permanently deformed by the breaking of covalent bonds and do not recover like many tough biological materials. Gong has also hypothesised that “the introduction of any sacrificial bonds that yield and dissipate energy upon deformation will toughen the materials” and that “the same DN gel concepts can, in principle, be applied to other self-healing types of materials, if the covalent bonds are replaced by reversible bonds”. This hypothesis has been tested and found to be true by Gong and others by preparing gels with different types reversible sacrificial bonds including the hydrophobic interactions of molecules forming lamellar bilayers and ionic cross-links between ionotropic polymers.
Figure 0-14: a-c) Schematic representation of the formation of a double network hydrogel, where a highly cross-linked polyelectrolyte network (blue) is swollen in a solution of monomer (black dots) which is subsequently polymerised (c). Also depicted are schematic representations of the topological changes incurred in DN hydrogels during tensile strain (d) and a tearing fracture (e). Figure adapted from reference 238,242.
The incorporation of ionic sacrificial bonds into an interpenetrating network (IPN) of two polymers has led to a class of tough and self-healing hydrogels referred to as ionic-covalent entanglement (ICE) network hydrogels\textsuperscript{68,69,245}. ICE network hydrogels comprise a polyelectrolyte which is ionically cross-linked and a neutral polymer which is covalently cross-linked (Figure 0-15a)\textsuperscript{69}. Under stress, the sacrificial ionic cross-links are easily dissociated and when the stress is removed the ionic cross-links are able to reform and restore integrity to the hydrogel (Figure 0-15b). The ICE network gels reported so far have demonstrated enhanced strength and toughness compared to conventional hydrogels of the same polymers\textsuperscript{68,69,245,246}. The extra virtues of ICE network hydrogels compared to other tough hydrogels cannot be understated: i) ICE network hydrogels can recover their original structure after deformation, they are “self-healing”, ii) ICE network hydrogels are prepared in one-step syntheses and form firm gels upon cooling\textsuperscript{245}. This means that they can be printed or moulded into complex shapes, or even be formed \textit{in situ} – properties which are particularly attractive for the field of tissue engineering.
Hydrogels can also be made stronger and tougher by reinforcing them with fibres, plates and spheres much like conventional polymers can be. According to conventional theory of polymer reinforcement, successful reinforcing particles are ones which are stiffer and stronger than the matrix polymer; and have effective coupling to between the reinforcer and the matrix\textsuperscript{251}. Tough hydrogels have been prepared using nanocomposite reinforcing (NC gels) in the form of exfoliated clay platelets (~30 nm diameter) which act as multifunctional cross-linkers (Figure 0-16a)\textsuperscript{252}. These hydrogels possessed excellent mechanical performance (tensile...
strength of 109 kPa; tensile modulus of 9.9 kPa and elongation at failure of 857% when possessing a swelling ratio of 48). Microgels have also been used as multifunctional cross-linker reinforcing to improve the mechanical properties of hydrogels and are commonly referred to as microgel reinforced hydrogels (MR-hydrogels) (Figure 0-16b). MR-hydrogels exhibit improved mechanical properties because they too act as multi-functional cross-linking nodes that inhibit the formation of micro-cracks and voids in the hydrogel. Recently several double network hydrogel microspheres were reported to improve the mechanical properties of poly(acrylamide) hydrogels.
Figure 0-16: a) Formation of NC hydrogels, b) formation of MR-hydrogels. Both methods are similar in that the particles become a multi-functional cross-linking node. Figure adapted from reference 238.
The overall aim of this thesis was to explore the preparation of novel hydrogel materials and to characterise them with respect to their mechanical properties, swelling behaviour and degradability. In performing their research and developing these materials, it was intended that these materials might reveal the potential to be used as soft tissue engineering scaffolds so as to further advance the field of tissue engineering. For this reason, some tissue engineering specific properties were also investigated where appropriate such as the compatibility of the material with endothelial cells and exploration of gelation properties which might be amenable to extrusion printing in conjunction with cells.

Aim 1. Develop a gelatin based material that is thermally stable at 37°C, degradable, has stable swelling properties, is mechanically robust and is compatible with endothelial cells so that it might be vascularisable.

Aim 2. Develop a method for purification of LAGG and determine its resulting properties in terms of gel-transition temperature and mechanical performance so as to improve upon the original gelation properties of commercial LAGG.

Aim 3. Develop an ICE hydrogel material from gellan gum and gelatin that is stronger than conventional hydrogels and possess a self-recovering ability.

Aim 4. Explore the fabrication of ICE hydrogel microspheres and determine their ability to mechanically reinforce gelatin hydrogels.
1.6 Chapter summaries

1.6.1 Gelapin, a degradable genipin cross-linked gelatin hydrogel

Chapter 2 describes the preparation and characterisation of a hydrogel material prepared from gelatin which was cross-linked with genipin and could potentially be used as a vascularisable tissue engineering scaffold. The material was explored through compressive mechanical analysis, rheometric analysis, analysis of its swelling, leeching and degradation behaviour in a simulated physiological environment and its ability to be vascularised using a chick chorioallantoic membrane (CAM) assay. The concentration of gelatin and genipin used to prepare hydrogels was shown to affect the mechanical performance, swelling, leeching and degradation. Hydrogels prepared with 6% (w/v) gelatin and 20.3% (w/w) genipin were able to be vascularised in a chick CAM assay when loaded with pro-angiogenic substance phorbol-12-myristate-13-acetate.

1.6.2 Purification of gellan gum

Chapter 3 describes a method that can be used to purify commercial gellan gum from divalent cation contaminants and presents the properties of the purified gellan gum. Commercial gellan gum was shown to possess inorganic contaminants in the form of sodium, potassium, calcium and magnesium which increased the temperature and time needed to dissolve and hydrate the polymer and increased the temperature at which the polymer solutions gelled. The purification method described in this chapter could be used to prepare exclusively the sodium salt of gellan gum with 67% yield which had desirable hydration and gelation temperatures with some deleterious impact on mechanical performance.
1.6.3 Biopolymer based tough and self-recovering ionic-covalent entanglement hydrogels

Chapter 4 describes the development and characterisation of a hybrid hydrogel material which was substantially stronger than most conventional hydrogels as well as possessed the ability to recover its mechanical properties after compression. It was found that the combination of genipin cross-linked gelatin and calcium cross-linked gellan gum can be adjusted so that an ionic-covalent entanglement network hydrogel was formed which had significantly higher mechanical performance than either constituent polymer. Ionic cross-link sites in the gellan gum portion of the ICE network could be reconstructed after deformation which imparted recover-ability to the hydrogel. The gellan gum to gelatin ratio and genipin and calcium concentrations were observed to affect the mechanical performance with distinct optima occurring when the composition was 36% (w/w) gellan gum to 64% (w/w) gelatin, 2% (w/w) calcium and 20% (w/w) genipin. The toughest hydrogels prepared possessed 1.0 ± 0.2 MPa compressive failure stress, Young’s modulus of 890 ± 50 kPa, work of extension value of 126 ± 6 kJ.m⁻³ and could recover to 88% of their original work under compression when rested for as little as 10 minutes in simulated body fluid. The swelling and leaching behaviour of the hydrogels were also examined when immersed in simulated body fluid as well as the mechanical performance of fully swollen hydrogels.

1.6.4 Hydrogel microspheres

Chapter 5 describes the fabrication of hydrogel microspheres using emulsion based processing. It shows how hydrogel microspheres can be prepared using oil and water
emulsions and focuses on parameters including type of oil, type of surfactant and concentration of surfactant used to prepare the emulsions. The ability of the microspheres to mechanically reinforce a hydrogel was also demonstrated.
CHAPTER 2: GELAPIN, A DEGRADABLE GENIPIN CROSS-LINKED GELATIN HYDROGEL

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This chapter was submitted in its current form to RSC Advances.

Author statement
Damian M. Kirchmajer performed all research and wrote the manuscript. However, this work would not have been possible without the intellectual input and support from Marc in het Panhuis, Marie Ranson and Clare Watson. I would also like to thank Prof P. Calvert (USA) and Dr R. Clark (USA) for useful discussions.

Primary supervisor confirmation
I, Associate professor Marc in het Panhuis (primary supervisor), support and certify the above author statement.

Signature: 

Date: 

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Abstract
The synthesis of genipin cross-linked gelatin (Gelapin) hydrogel materials is presented. Gelapin hydrogels were comprehensively characterised through chemical, mechanical and physical analysis techniques. It was found that the hydrogels could be cross-linked to up to 90% using a genipin concentration of 24.4% (w/w). The hydrogels reach a stable swollen state and cease leaching of residual starting materials after 72 hours in phosphate buffered saline solution at 37°C. The proteolytic degradation of Gelapin by collagenase is tuneable through manipulation of the material composition with the rate of degradation ranging from 60 mg/day up to 500 mg/day. The mechanical characteristics (at 37°C) are controllable through adjustment of the gelatin and genipin concentrations resulting in compressive stress-at-failure values ranging from 26 kPa to 300 kPa. Gelapin gels were found to become more elastic and ductile during proteolytic degradation up to 70% mass loss. The ability for vascularisation of these hydrogels was demonstrated using a chick embryo chorioallantoic membrane assay method.

2.1 Introduction
The engineering of tissue and organs in a laboratory is a comparatively cost effective and convenient alternative to human tissue and organ donations. The tissue scaffold, i.e. the material that reinforces the component’s structure, and holds the cells in place during maturation, is a critical component of all in vitro tissue engineering strategies. For engineering of soft tissues, hydrogel materials are the most appropriate scaffold materials because they have similar mechanical properties and chemical composition to natural tissues.
Hydrogels are a class of hydrated polymer materials whose polymer fraction generally lies between 0.1 – 10 % (w/v), with the remainder comprising water or an aqueous solution. Typical examples of commercial hydrogel products include toothpaste, foods, contact lenses, cosmetics, drug capsules and medical creams and ointments. Over the past 30 years an extensive array of both naturally derived and synthetic hydrogels have been utilised in research and development for all manner of tissue engineering objectives.

The study and use of gelatin hydrogels dates back centuries with the first documented use of gelatin materials for biomedical application as a haemostatic substance. Since then it has been also been utilised for surgical glues, sealants and wound dressings. Gelatin is denatured, hydrolysed collagen which itself is the primary constituent of all the connective tissues of the human body (within skin, muscle, bones, cartilage, fat). It is produced commercially via acid and alkaline hydrolysis and by proteolysis of collagen which can be derived from all manner of organisms including, but not limited to: pigs, cows, fish and rats. Depending on the type and extent of hydrolysis/proteolysis and the originating species, different gelatins can be produced with a variety of isoelectric points, molecular weights and Bloom numbers (indicative of gel strength). Gelatin hydrogels have the advantage of being biocompatible, non-toxic, non-immunogenic and biodegradable as well as being cheap and readily available, making them an ideal candidate materials for biomedical applications. However, unmodified gelatin hydrogels are mechanically weak and dissolve at temperatures above 29°C which are limitations that need to be addressed in order to develop them for practical applications.
It has been demonstrated that the mechanical and thermal properties of gelatin hydrogels can be improved by cross-linking\textsuperscript{116,160}. Examples of reagents and methods used for cross-linking gelatin include chemical cross-linking with gluteraldehyde, formaldehyde, diisocyanates, carbodiimides and acyl azides\textsuperscript{260}, as well as physical cross-linking using dehydrothermal treatment, ultraviolet irradiation and gamma irradiation\textsuperscript{162}. However, the physical treatments tend to achieve a very limited extent of cross-linking, and the chemical treatments have the potential to leave toxic residues in the gels which will release upon biodegradation \textit{in vivo}\textsuperscript{162}. Novel cross-linking reagents that are both effective and non-toxic are required to improve the mechanical and thermal properties of gelatin whilst retaining its inherent advantageous properties.

Genipin is a potent yet non-toxic cross-linker of proteins such as chitosan, collagen and gelatin that has been recently demonstrated to bestow anti-inflammatory properties to the materials it is incorporated into\textsuperscript{162,164,165,168}. It is produced both synthetically and naturally as the aglycon of geniposide, an iridoid glycoside that is the major component of the fruit of the gardenia plant, \textit{Gardenia jasminoides} Ellis\textsuperscript{46}. Genipin was first proposed and demonstrated for use as a biological tissue fixative and gluteraldehyde alternative in 1999 where it was demonstrated to be 10,000 times less toxic and almost equally efficacious\textsuperscript{46}. It has been established that genipin cross-linked gelatin via the epsilon amino group present in lysine and hydroxylysine residues (Supplementary Figure 2-1)\textsuperscript{167}. Genipin has been used in the preparation of cross-linked gelatin films and hydrogels\textsuperscript{161,162,261,262}, and employed in nerve guiding conduits, wound dressings, and cartilage scaffolds\textsuperscript{147,153,156}.
There is considerable interest in bioreactors for tissue engineering that can culture three-dimensional constructs in a dynamic fashion, and can also perfuse scaffold materials and stimulate their contents mechanically\textsuperscript{232,263–268}. In particular, mechanical stimulation has been shown to be crucial in directing the differentiation and phenotype of many cell types towards phenotypes which can produce functional extracellular matrix which will ultimately replace the scaffold and form the bulk of the new tissue component\textsuperscript{266,267,269–273}. In consideration of this aspect of tissue engineering, it is obviously of benefit to have a thorough understanding of the mechanical and rheological properties of candidate materials.

In this paper, the preparation and characterisation of genipin cross-linked gelatin (Gelapin) is presented. The ability of genipin to act as a gelatin cross-linker was investigated, including the stability and retention of both gelatin and genipin in phosphate buffer saline solution at 37°C. The degradation behaviour and mechanical characteristics of these hydrogels at 37°C are discussed in detail and their ability for vascularisation is demonstrated.

2.2 Materials and methods

2.2.1 Preparation of hydrogels

All reagents used were AR grade unless otherwise stated and deionised (DI) water (resistivity 18.2 MΩ cm) was prepared using a combined ion exchange and osmosis filtration system (Millipore, Australia). Gelapin hydrogels were prepared with type A, porcine gelatin (Bloom number of 300, molecular weight of 87,500 Da, Sigma-Aldrich, USA) with concentrations of gelatin ranging 2\% - 10\% (w/v) and genipin (Challenge Bioproducts, Taiwan) concentrations ranging 0 – 19.5\% (w/w).
Throughout this report Gelapin hydrogels will be referred to using nomenclature that describes their composition in the format Gelapin-[gelatin % (w/v)]/[genipin % (w/w)]. A summary of the preparation regime is as follows: Gelatin was initially dissolved with gentle stirring in DI water at 50°C on a magnetic stirrer/hot plate (Stuart CB162, UK). Subsequently, the appropriate amount of 20.3% (w/v) genipin in 60% (v/v) ethanol (Ajax Finechem, Australia) solution was added and stirred for a further 3 minutes at 50°C. The hot solution was then poured into glass Petri dishes (60 mm diameter x 15 mm height, Schott, Australia) and allowed to cure for 24 hours at 21°C. Silanisation of the Petri dishes with Coatasil (Thermofisher Scientific, Australia) prior to use facilitated the easy removal of the hydrogels without damaging them.

2.2.2 Extent of cross-linking

The extent of cross-linking in Gelapin hydrogels was determined by spectrophotometric comparison of the number of epsilon amino groups present in cross-linked and uncross-linked gelatin. The method used was based on the colorimetric assay developed by Offner and Bubnis\textsuperscript{274,275}: Gelapin samples were first lyophilised (LD Plus, Alpha, USA) and divided into 10 – 20 mg subsamples for individual analysis. Each subsample was combined with 4 mL of 4% (w/v) NaHCO\textsubscript{3} (Merck, Australia), 1 mL of 0.5% (w/v) 2,4,6-trinitrobenzenesulfonic acid (Sigma-Aldrich, USA) and heated in an oven (Binder-FD, USA) for four hours at 40°C. The subsamples were then acidified with 3 mL of 6 M HCl (Ajax Finechem, Australia) and hydrolysed in an autoclave (Tomy ES-315, 121°C, 186 kPa, 20 min). Three extractions using 20 mL diethyl ether (BDH, Belgium) were performed after addition
of 5 mL of DI water. A 5 mL aliquot of the aqueous extract was taken, heated on a steam bath for 15 minutes and allowed to cool to 20°C for 18 hours. The samples were then made up to 20 mL and measured using a dual beam spectrophotometer (Cary 500 Scan, Varian, Australia) in 1 cm path length glass cuvettes at 346 nm.

The extent of cross-linking (EC) was determined by comparing the cross-linked blank corrected sample absorbance (A) to the non-cross-linked blank corrected sample absorbance (A₀):

\[
EC = 1 - \frac{A}{A_0}. \quad (1)
\]

2.2.3 Swelling behaviour

Gelapin hydrogels were placed into round plastic containers (60 mm diameter x 40 mm, Chanrol, Australia), filled with 40 mL of phosphate buffered saline (PBS) solution (pH = 7.4, 3 mM NaN₃) and stored in a controlled temperature/humidity chamber (TRH-150-SD, Thermoline, Australia) at (37 ± 1)°C for up to 168 hours. The samples were removed from PBS solutions, padded dry with filter paper, and weighed after 3, 6, 12, 24, 48, 72 and 168 hours. The PBS solutions were replaced with fresh PBS solutions at 24, 48 and 72 hours. The swelling ratio (SW) was calculated:

\[
SW = \frac{W_t}{W_i} \times 100\%, \quad (2)
\]

where \( W_i \) and \( W_t \) are the initial mass and the mass at different swelling times, respectively.
2.2.4 Genipin and gelatin leaching

Gelapin hydrogels were immersed in 40 mL PBS solution and stored in sealed round container (60 mm diameter x 40 mm, Chanrol, Australia) at 40°C for up to 72 hours. At 24, 48 and 72 hours, the absorbance of the PBS solution \(A_{24h}, A_{48h}\) and \(A_{72h}\), respectively) was measured at 589 nm in the spectrophotometer in 1 cm glass cuvettes. After each 24 hour period, the hydrogels were re-immersed in fresh PBS solution. Although the specific quantity of genipin could not be determined in this way, the amount of genipin released relative to the total leachable genipin \((GR)\) was calculated as:

\[
GR = \frac{A_t}{A_{24h} + A_{48h} + A_{72h}} \times 100\%.
\] (3)

where \(A_t\) is the absorbance measured at either 24, 48 or 72 hours.

At 24, 48 and 72 hours, the gelatin concentration in the same PBS solution was measured using a Coomassie Blue protein assay kit (Coomassie Plus, Thermofisher Scientific, Australia) in 96 well microplate format using a plate reader (Polarstar, BMG Labtech, Germany) with an absorption spectrophotometer (595 nm). The gelatin concentration was calculated from interpolation of the linear regression of a standard curve. Calibration standards were prepared at gelatin concentrations of 0, 75, 150, 300, 450, 750, 1125 and 1500 μg/mL in duplicate and samples were prepared in quadruplicate with separate blanks.
2.2.5 Proteolytic and hydrolytic degradation

Gelapin hydrogels were preconditioned by soaking in PBS solution in the temperature/humidity chamber at (37 ± 1)°C for 14 days.

Hydrogel samples were placed into tissue cassettes (model M512, Simport, Canada) before being immersed in either collagenase solution for the proteolytic degradation study, or PBS for the hydrolytic degradation study. The collagenase solution was prepared from a lyophilized mixture of collagenases isolated from *Chlostridium histolyticum* (Sigma, USA) in PBS to a concentration of 10 2-furanacryloyl-L-leucylglycyl-L-prolyl-L-alanine (FALGPA) units/mL. One unit of FALGPA hydrolyses 1.0 mmol of FALGPA per min at 25 °C and pH 7.5 in the presence of calcium ions. The samples and solutions were kept at (37 ± 1)°C for the duration of the study and had the collagenase and PBS solutions changed regularly.

For each mass measurement, the samples in their cassettes were removed from the collagenase/PBS solution, dried and then weighted. The drying procedure involved drip-draining the majority of the solution from the cassettes, followed by blotting and wicking the solution away with tissue paper. The mass of the samples ($m_s$) were calculated by subtracting the mass of the cassettes ($m_c$) from the mass of the samples in their cassettes ($m_t$):

$$m_s = m_t - m_c. \quad (4)$$

The extent of degradation ($D$) was calculated every 24 hours as:

$$D = \left(1 - \frac{m_1}{m_i}\right) \times 100\% \quad (5)$$
where \( m_t \) is the mass of the sample at a particular point in time and \( m_i \) is the initial mass. The degradation rate and the induction period were determined from the slope and the intercept of the steady-state region in a plot of the extent of degradation versus time.

2.2.6 Rheological properties

The rheological properties of Gelapin hydrogels were examined using a rheometer (Physica MCR-301, Anton Paar, Australia) with Peltier temperature controlled bottom platen and 15.0 mm parallel plate measuring system. All measurements were conducted at \((37.0 \pm 0.1)\)°C with 15.0 mm diameter samples; a gap length of \((1.00 \pm 0.01)\) mm and \((1.0 \pm 0.1)\) N of normal force. The following experiments were conducted sequentially (with at least one minute rest between experiments): Strain-sweep experiment, where the oscillating frequency was held constant at 1 Hz and the oscillating strain was varied from 0.01% up to 10%; a frequency-sweep experiment, where the oscillating strain was held constant at 0.05% and the oscillating frequency was varied from 0.8 Hz –80 Hz; a creep recovery experiment, where samples were subjected to a non-oscillating shear stress of 10 Pa for a period of 5 seconds and then allowed to relax. The linear viscoelastic (LVE) region was defined in these experiments as the strain for which the storage modulus deviates by more than 5% of the initial (low strain) value.

2.2.7 Compressive mechanical properties

The mechanical properties of the full compositional range of Gelapin hydrogels was assessed using a universal mechanical analyser (EZ-S, Shimadzu, Japan). Prior to
analysis, the hydrogels were immersed in PBS solution within the temperature/humidity chamber (37 ± 1)°C for 72 hours. The instrument was adapted to incorporate a heated bath of PBS solution held at (37 ± 2)°C, so that samples were immersed during analysis. These compression tests were performed at a strain rate of 3 mm/min with samples that had been cut with a scalpel to rectangular prism geometry of ~ 10 mm x 10 mm x 8 mm. Stress-strain data was used to determine the compressive failure strain ($\varepsilon_{max}$), compressive tangent modulus over 20%-30% strain ($E_{tan}$), compressive failure stress ($\sigma_{max}$) and compressive strain energy to failure ($U$).

The mechanical properties of Gelapin hydrogels with composition of 4% (w/v) gelatin and 9.7% (w/w) genipin were assessed during degradation via collagenase proteolysis. Prior to testing, hydrogels (20 mm diameter, 5 mm height) were allowed to equilibrate in PBS solution followed by immersion in the collagenase solution. Partially degraded samples were removed at regular intervals during the proteolytic degradation process and subjected to compressive mechanical testing.

2.2.8 Angiogenesis

To examine the ability of blood vessels to intrude into Gelapin hydrogels and form nascent blood vessels the chick chorioallantoic membrane (CAM) on-plant assay was conducted which utilises the highly vascularised CAM as an experimental model of angiogenesis in situ$^{276}$. Grey rooster/white hen cross-bred fertile chicken eggs (Barter and Sons Hatchery, Australia) were incubated (HERAcell, Kendro Laboratory Products, Germany) for three days at 38°C and 60% relative humidity before separation of the shell from the embryo, yolk and albumin. The embryo was then incubated (37.8°C, 5% CO$_2$) for a further three days before Gelapin on-plants were
laid upon the surface of the vitelline. The embryos were then returned to incubate for a further four days, during which evidence of angiogenesis was monitored via optical microscopy (Z16, Leica, Germany).

Gelapin-6/9.7 on-plants were prepared in the absence (control) and presence of 0.5 μM phorbol-12-myristate-13-acetate (PMA) as a chemical stimulator of angiogenesis (Cayman Chemical, USA). Partially cured gels were then dispensed into the cavity of the on-plant device consisting of the space between two 5 mm squares of sterile mesh (Nylon-6,6, 180 μm grid size, 47% open area, Sefar Pty Ltd, Australia) and a spacer (Nylon-6,6, internal diameter 5.3 mm, external diameter 11.0 mm, 1.0 mm thickness, RS Components Pty Ltd, Australia). The gels were then allowed to cure and solidify over 4 more hours before being immersed in PBS solution and rested for an additional 18 hours to allow uncross-linked genipin to leach out and for the gel to shrink to its equilibrium size.

Macroscopic images were taken with a digital camera (Lumix DMC-FT1, Panasonic, Japan). Microscopic images were taken with the microscope with attached digital camera (DFC-290, Leica, Germany). Image analysis was performed with the Leica Application Suite (Leica, Germany). Assessment of the extent of angiogenesis, both in and around the on-plants were made on the basis of the number of blood vessels intersecting the on-plant boundary at 60°-120° in a spherical area with radius 10 mm from the centre of the on-plant. Other qualitative assessments were also made by inspecting above and below the on-plants and measuring the range and thickness of the surrounding blood vessels.
2.2.9 Statistical treatment of data

Dixon’s Q-test (95% confidence) was used to confirm and justify the removal of spurious data. Unless otherwise stated, the data presented in this manuscript are the mean ± one standard deviation (SD).

2.3 Results and discussion

2.3.1 Extent of cross-linking

Hydrogels were prepared by cross-linking gelatin with genipin. The extent of cross-linking (EC) is a measure of the effectiveness of genipin as the cross-linker for gelatin and was used to determine the amount of genipin required to obtain specific amounts of cross-linking in all subsequent experiments. The EC ratio presents the percentage of cross-linking sites in gelatin that form part of a cross-link versus the total amount of potential cross-linking sites and is calculated as described in Equation 1. The ratio was found to increase with increasing genipin concentration (Figure 2-1). The maximum extent of cross-linking attainable for Gelapin hydrogels was observed to be 84% – 90% for all concentrations of gelatin examined. This data can be used to predict the concentration of genipin required to attain any EC desired or vice-versa.

Our results are in agreement with those of Bigi and co-workers\(^{162}\), who suggested that genipin is unable to attain 100% EC due to a shielding effect of the gelation tertiary structure, which prevents some of the lysine residues from reacting with genipin. Smaller and more flexible cross-linking molecules, such as gluteraldehyde have been reported to attain a maximum EC of 100\(^{260}\). Notwithstanding, genipin should still be considered an effective cross-linker of gelatin, especially when one
considers that it has been demonstrated that genipin is not cytotoxic\textsuperscript{46,166} and bestows anti-inflammatory character\textsuperscript{164,165}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Extent of cross-linking in Gelapin hydrogels as a function of genipin concentration. Diamonds, squares and triangles represent gelatin concentrations of 2\%, 5\% and 10\% (w/v), respectively.}
\end{figure}

2.3.2 Swelling behaviour

The swelling ratio of Gelapin hydrogels was observed to be dependent on both the gelatin concentration and the cross-linker concentration (Supplementary Table 2-1). The swelling profiles of all hydrogels could be described as an initial rapid decrease in size which slowed to a steady state where no further significant change in mass occurred (an example profile for the Gelapin-10/X hydrogels is presented in Figure 2-2). The hydrogels all exuded water to varying extents, dependant on their composition (Supplementary Table 2-1). For 2\%, 3\% and 4\% (w/v) gelatin hydrogels it took 48 hours of soaking before the equilibrium swelling state was reached. For
5% and 10% (w/v) gelatin hydrogels, it took 72 hours to reach the equilibrium swelling state. The extent of de-swelling was found to be inversely proportional to the gelatin concentration and directly proportional to the genipin concentration. This behaviour is consistent with the Flory-Rehner theory where hydrogel volume is affected by the balance between osmotic pressure and elastic restorative forces\textsuperscript{278}. Hydrogels which contain more gelatin possess a greater osmotic pressure which acts to draw water into the gels and increases the equilibrium volume, while those with more extensive cross-linking possess a greater elastic restorative force which decreases the volume at equilibrium.

Some Gelapin hydrogels were not physically stable in simulated physiological conditions, PBS solution at 37 °C and pH 7.4. Hydrogels compositions comprising of 2% (w/v) gelatin and less than 7.3% (w/w) genipin; 3% (w/v) gelatin and less that 4.9% (w/w) genipin; and 4% (w/v) gelatin with 2.4% (w/w) genipin, dissolved completely over 24 hours of soaking. These hydrogels possess relatively low concentrations of gelatin and genipin and therefore possess only a small amount of polymer entanglement and covalent cross-links. It is suggested that this causes them to dissolve when the temperature of the hydrogels is raised from the curing temperature (~20°C) to the temperature (37°C) at which all testing is carried out.

It is well known that proteinaceous hydrogels, such as Gelapin are particularly sensitive to changes in pH which affect the balance of charges on the polymeric backbone\textsuperscript{278}. This was demonstrated by investigating the swelling behaviour in PBS soaking solutions (at 37°C) of pH 8.2. Under these conditions it was observed that the SW was higher compared to the corresponding gels swollen in PBS at pH 7.4.
Figure 2-2: Swelling ratio as a function of time for typical Gelapin hydrogels with gelatin concentration of 10% (w/v). Diamonds, squares, triangles and crosses indicate genipin concentrations of 4.9, 7.3, 12.2 and 19.5% (w/w), respectively.

2.3.3 Genipin and gelatin leaching

Gelapin hydrogels were immersed in PBS solution at 37 °C and pH 7.4 for several days during which the leaching of genipin and gelatin were monitored regularly. A noticeable amount of genipin in the form of partially reacted (not cross-linked) blue genipin pigments was observed to leach from the Gelapin hydrogels during 72 hours of immersion in PBS solution. The blue pigments are commonly known as the edible blue food dye “Gardenia Blue” and are a result of interactions between genipin and gelatin. The percentage of genipin released (GR) was calculated according to
Equation 3 at 24 h, 48 h and 72 h (data not shown). It was observed that the majority (>89%) of the genipin was released during the first 24 hours for all of the Gelapin hydrogels.

Insignificant amounts of gelatin were released from Gelapin hydrogels whilst being immersed in PBS (Supplementary Table 2-2). The majority of the gelatin was released within the first 24 hours of soaking with no detectable amount being released on subsequent days. The amount released was inversely proportionate to the extent of cross-linking. This is likely to be a result of cross-links tethering gelatin molecules together as well as contributing to confinement and entanglement of non-covalently cross-linked gelatin molecules. This initial release may represent the superficial, unbound gelatin. These results demonstrate that by increasing the amount of cross-linking, the gelatin release can be slowed or extinguished altogether.

2.3.4 Proteolytic and hydrolytic degradation

The extent of proteolytic and hydrolytic degradation (D) of Gelapin hydrogels were obtained using mass measurements of samples immersed in PBS solution with and without collagenase, respectively, over 93 days. Samples of hydrogel which had been immersed with collagenase were observed to lose mass at three different rates (Figure 2-3a, Table 2-1), referred to as the initial, steady state and tailing periods. In the initial (induction) period the infiltration of the hydrogel by collagenase commences and it begins to make scissions in the gelatin. During this period the hydrogel was being degraded but there was no significant change in mass loss until there were enough scissions made in the matrix to dislodge fragments from the bulk material. For samples immersed in collagenase, the induction period was found to be
proportionate to the amount of cross-linking in the Gelapin hydrogels, i.e. it was instantaneous for the least cross-linked hydrogels and up to 2 days for the hydrogels with the highest degree of crosslinking.

In the second (steady state) period, the degradation rate for the hydrogels immersed in collagenase solution ranged from 60 mg/day up to 500 mg/day and was observed to be dependent on both the gelatin concentration and the concentration of the genipin (Figure 2-3b). Increasing the gelatin concentration increased the density of the hydrogel producing a less penetrable matrix for collagenase and partially degraded matrix to diffuse through. As a result, the degradation rate observed for high gelatin content hydrogels was lower than that of the low gelatin content hydrogels. Gels with higher concentrations of the genipin cross-linker exhibited slower degradation rates. It is suggested that the covalent cross-links formed with genipin molecules are resistant to degradation by collagenase and may be tying partially degraded gelatin molecules together and contributing to their entanglement for longer periods of time than less densely cross-linked Gelapin hydrogels.

In the third (tailing) period, of the degradation rate decreased until the entire material was degraded. During this period, degradation is limited by the available surface area of the sample which diminishes in proportion to the radius of the sample squared. Consequently, as the sample is degraded to a smaller size, the sample area and degradation rate decrease quickly. The total time it took samples to degrade via proteolysis ranged from 3.5 ± 0.5 to 49 ± 3 days and was proportionate to the gelatin and genipin concentrations (Figure 2-3c). Slower degradation rates were observed for higher gelatin content and more extensively cross-linked Gelapin hydrogels which
had a direct effect on the overall time taken to degrade the component. In contrast, Gelapin hydrogel which were not exposed to collagenase did not show significant signs of degradation over 93 days, or a time period which is twice as long as it took for the most robust Gelapin hydrogels to degrade completely in collagenase. In fact, most samples increased in mass by a small amount which may be a result of swelling (Table 2-1). This suggests that in the absence of matrix metalloproteases such as collagenase, Gelapin hydrogels will not significantly degrade as a result of indiscriminate hydrolysis.
Table 2-1: The inductions times ($t_i$), degradation rates ($D$) and degradation times ($t_D$) for proteolytic degradation and the hydrolytic mass change ($ΔM$) for Gelapin hydrogels after a 93 day hydrolytic degradation period. The nomenclature of Gelapin hydrogels describes their composition in the format Gelapin-[gelatin %(w/v)]/[igenipin %(w/w)].

<table>
<thead>
<tr>
<th>Sample</th>
<th>$t_i$ (days)</th>
<th>$D$ (mg.day$^{-1}$)</th>
<th>$t_D$ (days)</th>
<th>$ΔM$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelapin-2/14.6</td>
<td>1.01 ± 0.06</td>
<td>400 ± 26</td>
<td>3.5 ± 0.5</td>
<td>-2 ± 2</td>
</tr>
<tr>
<td>Gelapin-2/19.5</td>
<td>1.0 ± 0.2</td>
<td>320 ± 17</td>
<td>4 ± 0</td>
<td>-2 ± 2</td>
</tr>
<tr>
<td>Gelapin-4/4.9</td>
<td>0.07 ± 0.06</td>
<td>520 ± 83</td>
<td>3.5 ± 0.5</td>
<td>+2.5 ± 0.6</td>
</tr>
<tr>
<td>Gelapin-4/9.7</td>
<td>0.83 ± 0.09</td>
<td>260 ± 33</td>
<td>5 ± 0</td>
<td>+2.7 ± 0.2</td>
</tr>
<tr>
<td>Gelapin-4/14.6</td>
<td>1.3 ± 0.1</td>
<td>180 ± 22</td>
<td>8.5 ± 0.5</td>
<td>+2.3 ± 0.2</td>
</tr>
<tr>
<td>Gelapin-4/19.5</td>
<td>2.1 ± 0.3</td>
<td>150 ± 22</td>
<td>10.5 ± 0.5</td>
<td>+2.9 ± 0.3</td>
</tr>
<tr>
<td>Gelapin-6/4.9</td>
<td>0.09 ± 0.03</td>
<td>360 ± 25</td>
<td>6 ± 0</td>
<td>+3.9 ± 0.3</td>
</tr>
<tr>
<td>Gelapin-6/9.7</td>
<td>0.84 ± 0.07</td>
<td>181 ± 9</td>
<td>11 ± 1</td>
<td>+3.44 ± 0.06</td>
</tr>
<tr>
<td>Gelapin-6/14.6</td>
<td>1.4 ± 0.3</td>
<td>120 ± 29</td>
<td>15.5 ± 1.5</td>
<td>+3.3 ± 0.2</td>
</tr>
<tr>
<td>Gelapin-6/19.5</td>
<td>1.5 ± 0.3</td>
<td>110 ± 12</td>
<td>17.5 ± 1.5</td>
<td>+5.6 ± 0.5</td>
</tr>
<tr>
<td>Gelapin-8/4.9</td>
<td>0.0 ± 0.1</td>
<td>240 ± 28</td>
<td>9.5 ± 0.5</td>
<td>+4.8 ± 0.4</td>
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<td>17.5 ± 1.5</td>
<td>+5.9 ± 0.2</td>
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<tr>
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<td>94 ± 1</td>
<td>22.5 ± 3.5</td>
<td>+6.5 ± 0.4</td>
</tr>
<tr>
<td>Gelapin-8/19.5</td>
<td>1.5 ± 0.3</td>
<td>90 ± 12</td>
<td>28.5 ± 3.5</td>
<td>+6.8 ± 0.5</td>
</tr>
<tr>
<td>Gelapin-10/4.9</td>
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<td>210 ± 34</td>
<td>13.5 ± 0.5</td>
<td>+5.9 ± 0.1</td>
</tr>
<tr>
<td>Gelapin-10/9.7</td>
<td>0.9 ± 0.2</td>
<td>100 ± 15</td>
<td>29.5 ± 1.5</td>
<td>+7.41 ± 0.07</td>
</tr>
<tr>
<td>Gelapin-10/14.6</td>
<td>2.0 ± 0.3</td>
<td>74 ± 9</td>
<td>44 ± 7</td>
<td>+7.9 ± 0.2</td>
</tr>
<tr>
<td>Gelapin-10/19.5</td>
<td>2.14 ± 0.05</td>
<td>60 ± 3</td>
<td>49 ± 3</td>
<td>+9.0 ± 0.3</td>
</tr>
</tbody>
</table>
Figure 2-3: (a) Typical proteolytic degradation profile for Gelapin-8/14.6. (i-iii) indicate the three period of the degradation, i.e. induction, steady state and tailing, respectively. (b) Proteolytic degradation rates of Gelapin hydrogels (± SD). (c) Degradation time for Gelapin hydrogels (± ½ range).
2.3.5 Rheological properties

Strain-sweep experiments were conducted to identify the linear viscoelastic (LVE) region which was observed to be up to 5% shear strain (data not shown). Hydrogels which possess a higher cross-linker concentration had shorter LVE regions as compared with hydrogels with smaller cross-linker concentration, which can be explained by the stiffening effect of cross-linking. For example, the shear strain values for the limit LVE region were 5% and 0.1% for Gelapin-4/4.9 and Gelapin-8/14.6 hydrogels, respectively. As expected, the values for the storage and loss moduli were larger for gels with a higher degree of cross-linking, consistent with a previous rheological study of cross-linked hydrogels using pollock gelatin. For example, Gelapin-4/4.9 and Gelapin-8/14.6 hydrogels exhibited storage modulus values in the LVE region of 9 kPa and 90 kPa, respectively. The data from the frequency-sweep and creep recovery experiments indicated the storage and loss moduli of Gelapin hydrogels were independent of frequency up to 80 Hz (data not shown).

2.3.6 Compressive mechanical properties

The mechanical properties of Gelapin hydrogels were examined in compression whilst immersed in PBS solution at 37°C (Figure 2-4). Compressive strain at failure ($\varepsilon_{\text{max}}$) values varied between 50 ± 3% and 74 ± 5% and were inversely proportional with increasing concentration of genipin (Table 2-2). This can be explained by the increased density of covalent cross-linking in the hydrogels induced by a higher genipin concentration – with more covalent cross-links, comes a greater amount of elastic stiffness reducing the extent of deformation before the hydrogel fails. The
gelatin concentration was also observed to have a similar effect on $\varepsilon_{max}$ values. The increased gelatin concentration is accompanied by more polymer entanglement which acts as a comparatively weak cross-link.

Tangent modulus ($E_{tan}$) values were observed to increase significantly with increasing concentration of genipin.
Table 2-2). $E_{tan}$ was also observed to be proportionate to the gelatin concentration. It is suggested that these observations are a result of increased polymer entanglement and physical cross-link density. In other words, the gels are becoming stiffer but less ductile with increasing genipin and gelation concentrations.

Figure 2-4: Typical stress-strain curves for, Gelapin-8/14.6 (line 1), Gelapin-6/9.7 (line 2) and Gelapin-4/4.9 (line 3).
Table 2-2: Summary of mechanical properties for Gelapin hydrogels. Compressive strain at failure, compressive tangent modulus (20%-30% strain), compressive stress at failure and compressive strain energy to failure are indicated by $\varepsilon_{\text{max}}$, $E_{\text{tan}}$, $\sigma_{\text{max}}$ and $U$, respectively. The nomenclature of Gelapin hydrogels describes their composition in the format Gelapin-[gelatin %/(w/v)])/[genipin %/(w/w)].

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\varepsilon_{\text{max}}$</th>
<th>$E_{\text{tan}}$</th>
<th>$\sigma_{\text{max}}$</th>
<th>$U$</th>
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<tr>
<td>Gelapin-2/9.7</td>
<td>67 ± 7</td>
<td>13 ± 6</td>
<td>26 ± 6</td>
<td>3 ± 1</td>
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<tr>
<td>Gelapin-2/14.6</td>
<td>57 ± 5</td>
<td>22 ± 4</td>
<td>26 ± 8</td>
<td>3 ± 1</td>
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<tr>
<td>Gelapin-2/19.5</td>
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<td>40 ± 10</td>
<td>27 ± 9</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>Gelapin-4/4.9</td>
<td>74 ± 5</td>
<td>18 ± 3</td>
<td>80 ± 40</td>
<td>11 ± 5</td>
</tr>
<tr>
<td>Gelapin-4/9.7</td>
<td>59 ± 5</td>
<td>90 ± 20</td>
<td>100 ± 20</td>
<td>16 ± 3</td>
</tr>
<tr>
<td>Gelapin-4/14.6</td>
<td>54 ± 2</td>
<td>130 ± 20</td>
<td>110 ± 30</td>
<td>18 ± 4</td>
</tr>
<tr>
<td>Gelapin-4/19.5</td>
<td>52 ± 5</td>
<td>160 ± 30</td>
<td>120 ± 30</td>
<td>18 ± 4</td>
</tr>
<tr>
<td>Gelapin-6/4.9</td>
<td>65 ± 6</td>
<td>50 ± 10</td>
<td>90 ± 30</td>
<td>14 ± 4</td>
</tr>
<tr>
<td>Gelapin-6/9.7</td>
<td>58 ± 5</td>
<td>150 ± 30</td>
<td>180 ± 50</td>
<td>26 ± 7</td>
</tr>
<tr>
<td>Gelapin-6/14.6</td>
<td>53 ± 5</td>
<td>200 ± 40</td>
<td>170 ± 70</td>
<td>30 ± 10</td>
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<tr>
<td>Gelapin-6/19.5</td>
<td>51 ± 5</td>
<td>270 ± 40</td>
<td>200 ± 60</td>
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<tr>
<td>Gelapin-8/4.9</td>
<td>62 ± 5</td>
<td>170 ± 20</td>
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<td>Gelapin-8/9.7</td>
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<td>Gelapin-8/14.6</td>
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<td>240 ± 60</td>
<td>38 ± 9</td>
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<td>Gelapin-8/19.5</td>
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<td>350 ± 40</td>
<td>270 ± 73</td>
<td>40 ± 10</td>
</tr>
<tr>
<td>Gelapin-10/4.9</td>
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<td>250 ± 90</td>
<td>40 ± 10</td>
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<tr>
<td>Gelapin-10/9.7</td>
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<td>240 ± 40</td>
<td>300 ± 100</td>
<td>50 ± 20</td>
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<tr>
<td>Gelapin-10/14.6</td>
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<td>380 ± 40</td>
<td>270 ± 60</td>
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<tr>
<td>Gelapin-10/19.5</td>
<td>51 ± 5</td>
<td>400 ± 50</td>
<td>300 ± 60</td>
<td>50 ± 10</td>
</tr>
</tbody>
</table>
Compressive stress at failure ($\sigma_{\text{max}}$) values ranged between 26 ± 6 kPa and 300 ± 60 kPa proportional with the gelatin concentration. It is likely that this can be explained by realising that gels with higher amounts of gelatin have a higher overall density. Hence, they require more stress to deformation to the point of failure (Table 2-2). The cross-linker concentration (genipin) did not have a significant effect on $\sigma_{\text{max}}$. Compressive strain energy at failure ($U$) values ranged from 3 ± 1 kJ/m$^3$ to 50 ± 10 kJ/m$^3$ and increased with increasing gelatin and genipin concentration. The compressive mechanical characteristics of our gels are in the same order of magnitude as those reported for a gelatin scaffold (100-140 kPa, cross-linked by immersion in a genipin solution) developed for cartilage tissue engineering$^{153}$. Our results demonstrate that Gelapin hydrogels are a versatile material which can have tuned mechanical properties through careful control over the gelatin and genipin concentration. Increasing the gelatin concentration increased the overall strength and toughness as indicated by the compressive stress and strain energy to failure, while increasing the genipin concentration increases the stiffness and toughness and limited the maximum strain attainable which is reflected in the $E_{\tan}$, $U$ and $\varepsilon_{\text{max}}$ values. The effect of degradation on the mechanical characteristics was examined using a typical Gelapin hydrogel (Gelapin-4/9.7) which was exposed to collagenase for up to 10 days. Figure 2-5 shows strain-at-break values increase, while tangent modulus values increase during proteolytic degradation of up to 70% (by mass). In other words, the gels become more ductile and elastic as a result of the mass loss. Once the
hydrogels had degraded by 70%, they became too fragile to handle and could not be subjected to mechanical testing.

Figure 2-5: (a) Tangent modulus (20%-30% strain) and (b) compressive stress at failure for typical Gelapin-4/14.6 hydrogels as a function of mass loss during proteolytic degradation. Arrows indicate trend with mass loss.
2.3.7 Angiogenesis

Materials used as a tissue scaffold have traditionally relied upon diffusion of nutrients and oxygen to provide nourishment and address the respiratory requirements of the cells therein. However, diffusion is limited to only several hundred microns in most cases and this presents a challenge for tissue homeostasis.14 This can be overcome by promoting blood vessel invasion into the scaffold.27 Vascularisation of Gelapin hydrogels from existing vasculature was investigated using the chick embryo CAM as an experimental model of angiogenesis in situ. Gelapin-6/9.7 hydrogels materials were selected for these studies as gels with this gelatin concentration (6% w/v) exhibit mechanical properties which are in the middle of the range of values of the investigated sample compositions (see Table 2-2). On-plants prepared using Gelapin-6/9.7 hydrogel materials in the absence or presence of the angiogenic stimulator PMA27 were placed on developing chick embryos and observed for up to 4 days. The presence of the on-plants did not compromise the viability of the embryos and their ability to grow blood vessels and evidence of blood vessel intrusion and formation within Gelapin on-plants is shown in Figure 2-6 a-b. Examination of the area surrounding the on-plants after 4 days of testing revealed that the number of blood vessels near both the control and PMA containing hydrogels was 19 ± 1. However, the blood vessels were generally very fine around the control on-plant and did not appear to extend into the hydrogel (Figure 2-6c). In contrast, there were also large blood vessels evident in the PMA-containing on-plants which intruded into the hydrogel (Figure 2-6 d-f) suggesting that the
Gelapin hydrogel acts as a vascularisable soft tissue scaffold for the pro-angiogenic effect of PMA.

Figure 2-6: Optical images of (a) typical chick embryo after 6 days prior to placement of the Gelapin-6/9.7 on-plants, (b) typical chick embryo after 4 days with on-plants prepared in the absence (1) or presence (2 and 3) of PMA in place, (c) enlargement of control on-plant, (d) enlargement of on-plant 2 prior to excision, and (e-f) enlargements of on-plant 2 after excision showing red blood cells within the confines of the on-plant.
2.4 Conclusions
We successfully prepared genipin cross-linked gelatin hydrogel materials which were characterised in terms of extent of cross-linking, swelling behaviour, degradability and mechanical characteristics as well as ability for vascularization. It was shown that soaking Gelapin in phosphate buffered saline solution (37 °C, pH 7.4) for 72 hours resulted in the removal of 99% of the leachable gelatin and genipin. The swelling behaviour, mechanical properties and degradability of these hydrogels can be controlled through adjustment of the genipin and the gelatin concentrations. The degradation rate could be tuned between 50 mg/day and 500 mg/day. Compressive stress at strain value could be tuned over one order of magnitude (26 ± 6 kPa to 300 ± 60 kPa). Proteolytic degradation studies showed that typical Gelapin hydrogels became more ductile and elastic until they degraded by more than 70% (by mass). It was shown that typical Gelapin hydrogels have the ability to become vascularised with incorporation of pro-angiogenic factors. This paper contributes to the development of Gelapin hydrogels as a potential material for use in future tissue engineering applications.
2.5 Supplementary Information

Supplementary Figure 2-1: Schematic of the reaction scheme for the cross-linking of gelatin by genipin. The abbreviation, LYS, is short for lysine which is the main cross-linkable residue in gelatin.
Supplementary Table 2-1: The swelling ratio for Gelapin hydrogels after various soaking periods at pH 7.4 and 8.2. The sample naming convention is in the format Gelapin-[gelatin %(w/v)]/[genipin %(w/w)]. The dash (-) indicates where a sample has dissolved.

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<th>Swelling ratio (%), pH = 8.2</th>
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<td></td>
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<td>3 h</td>
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</tr>
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Supplementary Table 2-2: Gelatin released from Gelapin hydrogels of various compositions after 24 hours of soaking in PBS.

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</tr>
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</tr>
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<tr>
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</tr>
<tr>
<td>GELAPIN-4/7.3</td>
<td>0.1 ± 0.001</td>
</tr>
<tr>
<td>GELAPIN-4/12.4</td>
<td>-</td>
</tr>
<tr>
<td>GELAPIN-4/19.5</td>
<td>-</td>
</tr>
<tr>
<td>GELAPIN-5/2.4</td>
<td>0.6 ± 0.01</td>
</tr>
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<tr>
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</table>
CHAPTER 3: ENHANCED GELATION PROPERTIES OF PURIFIED GELLAN GUM

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This chapter was submitted in its current form to Carbohydrate Polymers.

Author statement
Damian M. Kirchmajer performed the majority of the research and wrote the entire manuscript. Benedikt Steinhoff also performed some experimental research which contributed to this manuscript. This work would not have been possible without the intellectual input and support from Holly Warren, Ross Clarke and Marc in het Panhuis. I would also like to thank Cathy Lancaster for technical assistance.

Primary supervisor confirmation
I, Associate professor Marc in het Panhuis (primary supervisor), support and certify the above author statement.

Signature: __________________________

Date: __________________________
Abstract
Gellan gum is a hydrogel-forming polysaccharide when combined with monovalent or divalent cations such as sodium, magnesium, potassium or calcium. Commercially, gellan gums are sold with trace amounts of these cations which have been proven to affect the gelation properties and mechanical properties of the resultant hydrogels. Our research describes a method for purifying gellan gum of these contaminants. The sodium salt form of gellan gum is shown to have lower dissolution and gel transition temperatures (the improved gelation behaviour could have significant benefit in its ability to be utilised as a cell culture or tissue engineering material as it can be both dissolved and gelled at approximately 37°C).

3.1 Introduction
Gellan gum is an anionic polysaccharide hydrogel-forming polymer produced from the bacteria Sphingomonas elodea176. Structurally, it comprises a tetrasaccharide repeat unit of two β-D-glucoses, one β-D-glucuronate, and one α-L-rhamnose120 (Figure 3-1). Gellan gum is available commercially under the trade names Gelrite™ and Kelcogel™ in “high acyl” and “low acyl” forms with the high acyl form being the native state96. The low acyl gellan gum is prepared via alkali treatment of the native gellan gum and is distinctively different in its gelation behaviour and mechanical properties279 - high acyl gellan gum will form a gel upon cooling from 65°C creating a flexible, soft hydrogel while low acyl gellan gum will form a gel upon cooling below 40°C creating a rigid and brittle hydrogel178.

In recent years, low acyl gellan gum has become an attractive biopolymer for applications in tissue engineering as a cellular scaffold because it resembles the
natural extracellular matrix (ECM) and is bio-inert\textsuperscript{51}. In particular, gellan gum hydrogels have been used to encapsulate chondrocytes and were shown to have a high cellular entrapment efficiency where cell viability and ECM production was stimulated over a two week period\textsuperscript{119,188}. Gellan gum has also been used as an injectable matrix for cellular therapies as well where it was remarked that gelation behaviour permitted cells to be mixed homogenously through the gel solution, then injected into the body where it formed firm gels within a short time period (~20 s)\textsuperscript{184}.

Gellan gum has also recently been demonstrated to be a material suitable for rapid prototyping techniques such as 3D gel printing\textsuperscript{280}. Gellan gum microgel suspensions have been combined with several cell types to create a cellular ink for drop-on-demand cell placement using a microvalve printing approach\textsuperscript{197}. There is therefore potential for gellan gum based materials to be used for computer aided tissue engineering, however, this potential may be realised more fully if the gelation behaviour of gellan gum were improved\textsuperscript{200}. In particular, if the gel transition temperature were closer to the temperature required to keep cells active and viable (37°C).

![Figure 3-1](image-url): The tetrasaccharide repeating unit of acid-form gellan gum. When deacylated, the indicated acetyl and glyceryl units are removed.
Gellan gum, like many anionic polysaccharides forms a physical gel by undergoing a random coil to double helix transition upon cooling. Stronger gels are formed if cations are present during the sol-gel transition. In this case, divalent cations form particularly strong gels through the aggregation of helixes and monovalent cations form intermediate strength hydrogels through electrostatic interactions with carboxylate groups. The presence of divalent cations also inhibits the ability of the un-hydrated gellan gum to become hydrated. In the food industry, it is common practice to add calcium sequestrants (citrates and phosphates) to water to improve the ability of low acyl gellan gum to be hydrated. The presence of cations in commercially provided gellan gum is ordinarily minimal and may not impede their use in food and pharmaceutical applications. However, very small amounts of calcium present in commercial gellan gums may still affect the more sophisticated chemistries used to modify gellan gum for tissue engineering applications. Calcium impurities may also affect the gel transition temperature so significantly that it precludes it from being utilised in rapid prototyping technology.

A method for the rapid purification of gellan gum was established two decades ago which employed an ion-exchange resin to capture cationic contaminants present in commercial gellan gum. They reported that after purifying the gellan gum of divalent cation contaminants, the acid form gellan gum could be converted to a monovalent salt using a corresponding hydroxide salt. Sodium or potassium gellanate salts were able to be hydrated at much lower temperatures and formed gels of comparable strengths to un-purified gellan gum hydrogels.
The research reported herein elaborates on Doner’s purification method, and provides quantitative information regarding the concentration of sodium, magnesium, potassium and calcium ions before and after purification; the temperature of hydration and gel transition temperature; and the mechanical properties of hydrogels prepared from purified and un-purified gellan gum solutions.

In this paper, gels are purified following Doner’s method. We provided for the first time quantitative information regarding the concentrations of the cation before and after purification. Furthermore, we present a new method based on impedance analysis for determining the gel transition temperature of purified and un-purified gellan gum. Rheological and mechanical compression results are also presented.

3.2 Experiment

All reagents used in these experiments were AR Grade or better with inorganic contaminants present at 0.01% or lower. Deionised water (DI water) was prepared using a combination reverse osmosis and ion exchange filter (Millipore, Australia) to a resistivity of 18.2 MΩ.cm. All glassware was soaked in 10% (v/v) hydrochloric acid (Ajax Finechem, Australia) for 24 hours prior to use to minimise contamination of the purified products and also to minimise contamination during inorganic elemental analysis. Purified gellan gum was prepared from low acyl gellan gum (GELZAN-CM, Lot #1/1443A, CP Kelco, Singapore) which was generously gifted from CP Kelco.

3.2.1 Moisture content

The moisture content of un-purified gellan gum was determined using an infrared moisture determination balance (AD-4712, A&D Company Ltd., Australia). A 5 g
sample of gellan gum was heated on the balance to 80°C and weighed periodically until a steady mass was attained.

3.2.2 *FTIR spectroscopy*

FTIR spectroscopy of dried samples was performed using a diamond attenuated total reflectance spectrometer (IRAffinity-1, Shimadzu, Japan) with 2 cm⁻¹ resolution and Happ-Genzel apodisation.

3.2.3 *Purification of gellan gum*

Gellan gum (3 g) was dissolved in 300 mL of DI water at 80°C whilst being stirred by an overhead mixer (RW 20, IKA, Australia) for 10-15 minutes at 300 rpm. Once dissolved, the gellan gum solution was cooled to 60°C before adding 8 g of cation exchange resin (50WX8 DOWEX, Sigma Aldrich, USA). The mixture was stirred for 30 minutes at 60°C before stirring was stopped and the resin was allowed to settle. The supernatant was then decanted and filtered (grade 165, Filtech, Australia) into a chilled reservoir containing 300 mL of 2-propanol (Ajax Finechem, Australia) with rapid stirring whereupon fibrous agglomerates of acid-form gellan gum (A-GG) precipitated. The A-GG was recovered from the 2-propanol solution using vacuum assisted filtration and freeze-dried (Alpha 1-2LDplus, Christ, Germany) for 48 hours to remove residual water and 2-propanol.

The purified gellan gum sodium salt (Na-GG) was prepared in the same manner described above excepting that the supernatant, after being decanted, was neutralised with ~10 mL 0.4 M standardised NaOH solution until the pH of the solution reached 7.4 (826 pH-Mobile pH meter, Metrohm, Australia). After being neutralised, the
supernatant was precipitated into 2-propanol and freeze dried as previously described.

3.2.4 Atomic absorption spectroscopy

Atomic absorption spectrometry of gellan gum samples was performed using a flame atomisation atomic absorption spectrometer (flame-AAS, Spectra AA 220FS, Varian, Australia) with hollow cathode lamp light sources for sodium (589.6 nm), potassium (769.9 nm), calcium (422.7 nm) and magnesium (285.2 nm), and an air/acetylene oxidant/fuel mixture.

Samples, blanks and calibration standards were prepared with a 5% (v/v) sulfuric acid matrix with CsCl (Sigma Aldrich, USA) added as ionisation suppressant. Calibration standards were prepared from certified multi-element standard solution (Lot #A2-MEB2366019, Inorganic Ventures, Australia). Samples were digested prior to analysis as follows: Approximately 1.00 g of sample was weighed and transferred quantitatively into a 150 mL Erlenmeyer flask. 5.0 mL of concentrated sulfuric acid (Ajax Finechem, Australia) was then added, followed by heating until the solids dissolved and the solution turned dark brown and started to fume. Hydrogen peroxide (30% v/v, Ajax Finechem, Australia) was then added drop-wise until the solution turned clear. The solution was then allowed to cool to room temperature before having 16 mL of 12.6 mg/mL CsCl solution was added. The solution was then diluted to 100 mL with DI water, mixed thoroughly, and measured within the day.
3.2.5 Gel transition temperature

The gel transition temperature for solutions of GG and Na-GG was assessed using a custom-designed electrical impedance instrument as well as a rheometer (Physica MCR-301, Anton Paar, Australia) in rotation mode. For electrical impedance analysis, hot gel solutions were poured into rectangular plastic troughs (1 cm x 1 cm x 4.5 cm) with reticulated vitreous carbon electrodes (ERG Aerospace, USA) placed at either end such that the distance between the electrodes was 2.5 cm. The impedance analysis was performed by applying 1 V peak voltage and alternating current signals from a waveform generator (U2761A, Agilent, USA) to a circuit comprised of the gel sample cell and a 10 kΩ resistor in series while the temperature was measured simultaneously with a digital thermometer probe (Jaycar Electronics, Australia). Impedance across the gel sample cell for frequencies ranging from 100 Hz to 100 kHz were measured as the gel cooled using an oscilloscope (U2701A, Agilent, USA). The gel transition temperature is defined as the point where the slope of the impedance divided by the temperature of the gel changes from one constant to another.

For rheometry based measurements, hot gel solution was sandwiched between a temperature controlled stage (Jaluba, AWC 100) and 50 mm diameter and 1° cone (CP50-1, Anton Paar, Australia) with a gap length of 0.097 mm. The sample was then sheared in rotation at a shear rate of 100 s⁻¹, while the temperature controlled stage was cooled from 70°C to 20°C at a rate of 5°C.min⁻¹. The temperature of gelation was determined graphically, as the temperature at the point where viscosity started the increase dramatically.
3.2.6 Compressive mechanical analysis

Hydrogel samples for mechanical analysis were prepared by dissolving gellan gum in DI water at 80°C for 30 minutes with gentle stirring followed by addition of 1 M NaCl solution to a concentration of 100 mM or 0.5 M CaCl₂ solution to a concentration of 5 mM. The hot gel solutions were then poured into moulds (17.5 mm diameter x 10 mm high, PVC rings) and cooled to 21°C to form firm hydrogels. Samples were compressed at a rate of 1 mm.min⁻¹ at 21°C using a universal mechanical testing apparatus (EZ-S, Shimadzu, Japan). The resulting stress-strain data was used to determine the compressive failure strain (\(\varepsilon_c\)), compressive secant modulus over 20% - 30% strain (\(E_c\)), compressive failure stress (\(\sigma_c\)) and compressive strain energy to failure (\(U\)).

The swelling ratios (SW) of hydrogels were calculated as the mass of the swollen hydrogel (\(m_s\)) divided by the mass of the dried hydrogel (\(m_d\)).

\[
SW = \frac{m_s}{m_d} \quad (1)
\]

3.2.7 Statistical treatment of data

Unless otherwise stated, the errors presented herein are the standard deviation of at least three measurements.

3.3 Results

3.3.1 Purification of gellan gum

As supplied, the un-purified gellan gum (GG) was a beige coloured powder which dissolved slowly at a concentration of 1% (w/v) at 80°C in DI water to a produce
clear, pale yellow solution from which hydrogels of gellan gum could be prepared by cooling the solution to room temperature (21°C). The moisture content of un-purified GG was found to be 1.1 ± 0.2%.

A procedure based on the method described by Doner was used to purify the gellan gum to produce the acid-form gellan gum (A-GG) with an average yield of 58 ± 2%. After drying, the A-GG was in the form of a dry, paper-like fibrous mat which was beige in colour. The solubility of the A-GG in DI water was especially poor and would not dissolve at a concentration of 1% (w/v) at 80°C like GG did. We suggest that this may be because the neutral acid form of gellan gum is less polar than the carboxylate salt form which is present to some extent in commercial gellan gum. Review of the FTIR spectra of GG and A-GG indicates that the peak at 1740 cm\(^{-1}\) is only apparent in the A-GG spectrum which is typical of the carbonyl stretching mode of carboxylic acids. This peak is replaced by one at 1600 cm\(^{-1}\) in the GG and Na-GG spectra which is typical of the carbonyl stretching mode of a deprotonated carboxylic acid. This suggests that during the purification process, GG became acidified and the cations were stripped away from the carboxylate groups (Figure 3-2). This hypothesis is also supported by the observation that addition of a small amount of sodium hydroxide (which changes the acid form gellan gum to the sodium carboxylate salt of gellan gum) drastically improved the solubility and clarity of A-GG solutions.

Acid-base titration of A-GG using standardised potassium hydroxide solution (0.01 M, Sigma Aldrich, USA) were conducted to determine the number of cation binding sites available to interact with cations such as Na\(^+\), Mg\(^{2+}\), K\(^+\) and Ca\(^{2+}\). The number
of cation binding sites was determined to be $1.27 \pm 0.08 \text{ mmol.g}^{-1}$ which is marginally less than the theoretical number cation binding sites ($1.55 \text{ mmol.g}^{-1}$).

The purified sodium salt form gellan gum (Na-GG) was prepared with an average yield of $64 \pm 6\%$. In contrast to A-GG, the dried product was bright white and dissolved very quickly and easily at a concentration of 1% (w/v) at 80°C.

![Figure 3-2: ATR-FTIR spectra of GG, Na-GG and A-GG.](image)

3.3.2 *Elemental analysis of impurities*

Elemental analysis of A-GG indicates that the purification method effectively removed Na, Mg, K and Ca from GG. The concentration of Na, Mg, K and Ca in GG, A-GG and Na-GG were measured using Flame-AAS (Table 3-1). The analysis revealed that an appreciable amount of Na, K and Ca was present in the commercial
GG which accounts for approximately 6.4% of the dry weight of GG and would be expected to occupy a large proportion of the available cation binding sites. Of particular significance is the concentration of Ca in commercial GG (0.34 ± 0.06 mmol.g⁻¹) which is known to form strong associations with the cation binding sites in gellan gum¹²⁰.

The concentration of Na, Mg, K and Ca present in the A-GG and Na-GG samples were significantly lower than those found in GG (Table 3-1) which attests to the efficacy of the purification methods. The concentration of Mg and Ca in A-GG and Na-GG were both reduced to below the limit of detection for the method (0.013 mmol(Mg).g⁻¹ and 0.01 mmol(Ca).g⁻¹, respectively). With respect to Na-GG, Mg²⁺ and Ca²⁺ were replaced mainly by Na⁺ and to a smaller extent K⁺; the aggregated concentration of Na and K (1.35 ± 0.05 mmol.g⁻¹) approximately equals the previously measured number of cation binding sites (1.27 ± 0.08 mmol.g⁻¹).

Table 3-1: Concentration of metal cations in GG, A-GG and Na-GG (±SD). *These concentrations were below the limit of detection for these elements.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>GG</th>
<th>A-GG</th>
<th>Na-GG</th>
</tr>
</thead>
<tbody>
<tr>
<td>mmol (Na).g⁻¹</td>
<td>0.24 ± 0.06</td>
<td>0.0044 ± 0.0004</td>
<td>1.09 ± 0.04</td>
</tr>
<tr>
<td>mmol (Mg).g⁻¹</td>
<td>0.048 ± 0.003</td>
<td>&lt; 0.013*</td>
<td>&lt; 0.013*</td>
</tr>
<tr>
<td>mmol (K).g⁻¹</td>
<td>1.14 ± 0.01</td>
<td>0.0240 ± 0.0006</td>
<td>0.26 ± 0.03</td>
</tr>
<tr>
<td>mmol (Ca).g⁻¹</td>
<td>0.34 ± 0.6</td>
<td>&lt; 0.01*</td>
<td>&lt; 0.01*</td>
</tr>
</tbody>
</table>
3.3.3 Dissolution and gel transition temperatures

Purified Na-GG was observed to dissolve at lower temperature than GG or A-GG. The temperature at which 1% (w/v) mixtures of GG, A-GG and Na-GG would dissolve was examined by slowly warming the mixtures from 5°C to the temperature where solutions ceased to be turbid. According to technical data from CP Kelco\textsuperscript{178}, GG requires heating to approximately 80°C for full dissolution\textsuperscript{4}, however we observed that the 1% (w/v) solution of GG went from turbid to clear at a temperature of 57 ± 3°C. A-GG was observed to be completely insoluble even at temperatures up to 90°C; this observation was also noted in Doner’s previous experiments\textsuperscript{279,281}. It was possible to prepare a clear solution of A-GG by boiling at ~ 96°C, however it is likely this occurred because the gellan gum was depolymerising to a lower molecular weight. Na-GG was observed to dissolve at a temperature of 39 ± 4°C which, incidentally, is very close to the optimal temperature for mammalian cell culture (37°C).

The gel transition temperature of Na-GG was observed to be lower than that of GG, regardless of the cross-linking cation used. Samples which were cross-linked with 100 mM sodium possessed the highest gel transition temperature of 46 ± 2°C and 45 ± 2°C for GG and Na-GG, respectively. Gellan gums which were cross-linked with 5 mM calcium possessed an intermediate gel transition temperature of 37 ± 2°C and 36 ± 2°C for GG and Na-GG, respectively. Gellan gums which were not cross-linked with cations also exhibited a gel transition at 30 ± 2°C and 29 ± 2°C (GG and Na-GG, respectively).
A new method using a custom-designed electrical impedance apparatus was used for determining the gel transition temperature of 1% (w/v) GG and Na-GG with no added cross-linker, 100 mM Na\(^+\), and 5 mM Ca\(^{2+}\) (these concentrations are typical cross-linker concentrations for the formation of firm gellan gum hydrogels\(^{120,177}\)). In this method, a gel solution was placed in between two electrodes and slowly cooled while the electrical impedance and temperature were measured simultaneously. As the solution cools, the ion mobility decreased and the electrical impedance increased at a linear rate. The gelation event is apparent by a distinct change in the rate of change of electrical impedance (Figure 3-3a). Using this method, the temperature of gelation for 1% (w/v) Na\(^+\) and Ca\(^{2+}\) cross-linked GG and Na-GG were determined (Table 3-2). For comparison, the gel transition temperature was also measured using a rheometry based method. In the rheometric analysis, samples of gel solution were sandwiched between a temperature controlled stage and a rotating cone. While the temperature dropped, the viscosity of the gel solution was measured and the gelation event was apparent when the viscosity increased dramatically (Figure 3-3b). There was excellent agreement between the two methods (Table 3-2). This indicates the validity of the impedance method.

We thus suggest that hypothetically, a solution containing of Na-GG and cells (possibly in conjunction with other materials) could be used to create a “cellular ink” that could be extruded to create complex 3D structures with potential application in tissue engineering/organ printing.

Another advantage of Na-GG is that it makes its carboxylic acid groups more accessible, which could be used to functionalise the gellan, for example, with the
integrin binding RGD sequence using EDC chemistry. This strategy has been shown previously to enhance cellular adhesion to gellan gum matrixes but has a low functionalization efficiency due to the poor access to carboxylic acid groups\textsuperscript{96,283}.

Figure 3-3: Gel transition temperature determinations of a typical Na-GG solution cross-linked with 5 mM Ca\textsuperscript{2+}. a) Average electrical impedance between 100 Hz and 100 kHz as a function of temperature b) viscosity at 100 s\textsuperscript{-1} as a function of temperature. Straight lines are linear fits of the data.
Table 3-2: Gel transition temperatures of GG and Na-GG with either Na⁺, Ca²⁺ or no cross-linking ions. T_{impedance} and T_{rheometric} indicate the gel transition temperatures determined using the electrical impedance method and rheometric method, respectively. *T_{impedance} could not be determined for those samples as the gel transition temperature was below the minimum measurable temperature.

<table>
<thead>
<tr>
<th>Cross-linker</th>
<th>T_{impedance}</th>
<th>T_{rheometric}</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG None</td>
<td>*</td>
<td>29.5 ± 2</td>
</tr>
<tr>
<td>GG Na⁺</td>
<td>44 ± 1</td>
<td>46 ± 2</td>
</tr>
<tr>
<td>GG Ca²⁺</td>
<td>37 ± 1</td>
<td>37 ± 2</td>
</tr>
<tr>
<td>Na-GG None</td>
<td>*</td>
<td>28.5 ± 2</td>
</tr>
<tr>
<td>Na-GG Na⁺</td>
<td>44 ± 3</td>
<td>45 ± 2</td>
</tr>
<tr>
<td>Na-GG Ca²⁺</td>
<td>36 ± 1</td>
<td>35.5 ± 2</td>
</tr>
</tbody>
</table>

3.3.4 Mechanical analysis

Hydrogels prepared from Na-GG were observed to be weaker and less stiff than those prepared from GG. The mechanical properties of hydrogels prepared from 1% (w/v) solutions GG and Na-GG which were cross-linked with either 100 mM Na⁺ or 5 mM Ca²⁺ were examined using compressive mechanical analyses (swelling ratio of all hydrogels was 101). Calcium cross-linked hydrogels were observed to have higher compressive stress to failure and secant moduli than sodium cross-linked hydrogels for both purified and un-purified gellan gums (Table 3-3). In Doner’s previous report, it was remarked that hydrogels prepared from the purified Na-GG were of comparable strength to the un-purified gellan gum. However, Doner used different brands/grades of gellan gum (Phytigel from Sigma Chemical Company and GelGro from ICN Biochemicals) which may have had different degrees of
acetylation and molecular weights which would be expected to effect the gelation behaviour and gel strength. Also, the cross-linker concentrations stated are the nominal concentrations resulting from direct addition of Na⁺ or Ca²⁺ solution; the concentration of Na⁺ and Ca²⁺ which were already present in GG were not taken into consideration for these measurements.

Table 3-3: Compressive mechanical properties of hydrogels prepared from GG and Na-GG (100 mM Na⁺ and 5 mM Ca²⁺ nominal cross-linker concentrations were used). SW – swelling ratio, εc - compressive failure strain, Ec - compressive secant modulus over 20%-30% strain, σc - compressive failure stress and U - compressive strain energy to failure, *where samples fractured for less than 30% strain, the average modulus over 0-25% strain is presented.

<table>
<thead>
<tr>
<th></th>
<th>SW</th>
<th>σc (kPa)</th>
<th>εc (%)</th>
<th>Ec (kPa)</th>
<th>U (kJ.m⁻³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG (Na⁺ cross-linked)</td>
<td>101</td>
<td>30 ± 5</td>
<td>34 ± 3</td>
<td>114 ± 5</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>GG (Ca²⁺ cross-linked)</td>
<td>101</td>
<td>130 ± 20</td>
<td>61 ± 3</td>
<td>108 ± 2</td>
<td>28 ± 4</td>
</tr>
<tr>
<td>Na-GG (Na⁺ cross-linked)</td>
<td>101</td>
<td>10 ± 1</td>
<td>35 ± 2</td>
<td>41 ± 2</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>Na-GG (Ca²⁺ cross-linked)</td>
<td>101</td>
<td>19 ± 1</td>
<td>25 ± 1</td>
<td>78 ± 3</td>
<td>2.3 ± 0.2</td>
</tr>
</tbody>
</table>

3.4 Conclusions

Commercial gellan gum (GG) was shown to possess inorganic contaminants which affected its dissolution and gelation behaviour as well as the mechanical properties of resultant hydrogels. Purified gellan gum (Na-GG) was dissolved fully at approximately 39 ± 4°C and could form a firm hydrogel at approximately the same temperature (36 ± 2°C) in the presence of Ca²⁺ ions. These temperatures coincide with the temperature required for cell culture (37°C). A new impedance method for
determination of gel transition temperature was validated in this research. It should be noted that a deeper investigation of the imaginary component of the impedance spectrum might provide further insight into the electrical properties of this material and others and should be considered in future work. This paper contributes to the development of gellan gum as a material for tissue engineering applications.
CHAPTER 4: ROBUST BIOPOLYMER BASED IONIC-COVALENT ENTANGLEMENT HYDROGELS

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This chapter was submitted in its current form to Macromolecules.

Author statement

Damian M. Kirchmajer performed all research and wrote the manuscript. However, this work would not have been possible without the intellectual input and support from Marc in het Panhuis. I would also like to thank Dr P. Whitten for useful discussions.

Primary supervisor confirmation

I, Associate professor Marc in het Panhuis (primary supervisor), support and certify the above author statement.

Signature: _________________________

Date: ___________________________
Abstract

We report the development of a robust biopolymer based ionic-covalent entanglement network hydrogel made from calcium cross-linked gellan gum and genipin cross-linked gelatin. The ratio of the two polymers and the cross-linker concentrations significantly affected the mechanical characteristics of the hydrogels. Hydrogels with optimized composition exhibited compressive fracture stress and work of extension values of up to 1.1 ± 0.2 MPa and 230 ± 40 kJ.m\(^{-3}\) for swelling ratios of 37.4 ± 0.6 and 19 ± 1, respectively. The compressive and tensile mechanical properties, swelling behaviour (including leachage), pH sensitivity and homogeneity are discussed in detail. Fully swollen hydrogels (swelling ratio of 37.4 ± 0.6) were able to recover 95 ± 2% and 82 ± 7% of their energy dissipation (hysteresis) at 37 °C after reloading to either constant stress (150 kPa) or constant strain (50%), respectively.

4.1 Introduction

Hydrogels are highly swollen, materials prepared from hydrophilic polymers that can absorb up to a thousand times their dry weight in water\(^{40}\). As a result of their high water content, most hydrogels are soft and weak materials compared to other polymeric materials such as rubbers. For this reason, hydrogels are typically utilised for applications which do not require them to be particularly strong or resilient (for example, in foods, ointments and creams)\(^{40,139,284}\). As soft and wet materials, hydrogels are a substance that is reminiscent of soft biological tissue and have been investigated over the past 30 years as candidate materials for soft tissue engineering scaffolds\(^{2,34,40,139}\). However, new applications of hydrogels such as soft robotics\(^{285–287}\)
and cartilage tissue scaffolds require hydrogels with enhanced mechanical performance which has stimulated an investigation into how hydrogels may be made tougher and more enduring.

Tough hydrogels have been prepared using methods such as slip-ring hydrogel synthesis, nano-composite hydrogels, and double network hydrogels. Of all the tough hydrogel synthetic strategies, the double network approach affords the highest versatility in terms of composition and resulting properties. Double network (DN) hydrogels are interpenetrating polymer networks (IPN) which are formed in a two-step synthesis where a highly cross-linked, rigid and brittle polyelectrolyte is swollen in a monomer solution of a ductile, neutral polymer that it is subsequently polymerised. This approach has been recently adapted for the formation of tough, IPN hydrogels in a “one-pot” synthetic approach. These new hydrogels referred to as ionic-covalent entanglement (ICE) network hydrogels consist of a tough and self-recovering, interpenetrating network of an ionotropic polymer and a chemically cross-linkable polymer. ICE network hydrogels prepared from gellan gum and PAAm possessed compressive strain energy to failure of 44 kJ.m\(^{-3}\) and were able to recover 53% of their hysteresis (within 1 hour) when compressed to a constant stress (25 kPa) at 21 °C. ICE network hydrogels made from alginate and PAAm were able to be stretched a phenomenal 23 times their original length and resulting in fracture energies of 9000 J.m\(^{-2}\). They were able to recover up to 74% of their hysteresis (upon stretching to a constant strain) when rested for 1 day at 80 °C. Recently, we demonstrated that the ICE approach can be adapted for printing these alginate-PAAm tough gels.
Gellan gum is an anionic polysaccharide biopolymer derived from the bacteria \textit{Pseudomonas elodea}\textsuperscript{176}. It comprises a tetrasaccharide repeating unit structure of β-D-glucose, β-D-glucuronic acid and α-L-rhamnose with varying degrees of acetylation. Low-acyl gellan gum is a polymer which gels ionotropically in the presence of cations when its temperature is reduced below the coil-helix transition temperature\textsuperscript{120}. Gellan gum has been used as a food additive for a long time and recently, as a material for biomedical applications such as cartilage tissue engineering and as an injectable, \textit{in situ} forming hydrogel polymer for cellular delivery\textsuperscript{119,120,184}.

Gelatin is a denatured and partially hydrolysed protein produced from collagen – a biopolymer present in almost all connective tissue in the human body\textsuperscript{258}. Gelatin is a highly versatile biopolymer which can be obtained from a variety of animal sources, is cheap, and can be produced at a range of isoelectric points, molecular weights and gel strengths\textsuperscript{258}. It has been used in biomedical devices, pharmaceuticals and tissue engineering for over fifty years, is biocompatible, non-cytotoxic, non-immunogenic and presents no risk of pathogen transfer from originating organism unlike its parent protein collagen\textsuperscript{104,117,144,146,147,152,172,257,259,261,295–297}. Without cross-linking, gelatin hydrogels are very weak and readily dissolve at temperatures above 29°C which prohibit their use in tissue engineering\textsuperscript{54,158}. However, covalent cross-linking significantly improves the mechanical performance and stability of gelatin hydrogels\textsuperscript{162,260}.

Genipin is the algycone of geniposide, an iridoid glycoside that is the major component of the fruit of the gardenia plant, \textit{Gardenia jasminoides Ellis} and is
emerging as a popular chemical cross-linker of choice for proteinaceous biopolymers such as gelatin, collagen and chitosan. Genipin forms chemical cross-links between primary amino groups present in either the ε-amino groups of lysine and hydroxylysine residues, or the guanidinium group of arginine residues in gelatin. Genipin has been reported to be a non-cytotoxic cross-linker and anti-inflammatory agent and has been used to prepared scaffolds for tissue engineering and materials for biomedical engineering applications over the past 68 years.

In this paper, we report the preparation of a new type of ICE network hydrogel based on the biopolymers gellan gum (calcium cross-linked) and gelatin (genipin cross-linked). We investigated the mechanical properties in compression and tension as well as in “as prepared” state and equilibrium swollen state. The behaviour of the hydrogels when immersed in simulated body fluid was also investigated with respect to polymer leaching, cation migration, pH and dimensional changes. Finally, the ability of these hydrogels to dissipate energy after repeated compressions with different length resting periods in ambient conditions and simulated body fluid at 37 °C was examined.

4.2 Experimental section

4.2.1 Materials

All reagents used were AR grade unless otherwise stated and deionised (DI) water was prepared using a combined ion exchange and osmosis filtration system (Millipore, Australia) to a resistivity 18.2 MΩ cm. Low acyl gellan gum (Lot #1/1443A, Gelzan-CM, CP Kelco, Singapore) and type A, porcine gelatin (Bloom
number 300, molecular weight 87.5 kDa, Sigma Aldrich, USA) were used to prepared hydrogels. A 20.3% (w/v) genipin (Challenge Bioproducts, Taiwan) solution in 60% (v/v) ethanol (Ajax Finechem, Australia) was used for cross-linking gelatin and a 1 M CaCl$_2$ (Sigma Aldrich, Australia) solution was used for cross-linking gellan gum.

Simulated body fluid (SBF) was prepared with DI water and contained 0.035% (w/v) NaHCO$_3$; 0.0548% (w/v) MgCl$_2$·6H$_2$O; 2% (v/v) HCl; 0.02% (w/v) NaN$_3$; 0.80% (w/v) NaCl; 0.0224% (w/v) KCl; 0.0174% (w/v) KH$_2$PO$_4$; 0.0368% (w/v) CaCl$_2$·2H$_2$O; 0.0071% (w/v) Na$_2$SO$_4$; and 0.606% (w/v) tris(hydroxymethyl)aminomethane (Chem-Supply, Ajax Finechem, and Sigma Aldrich, Australia). The pH of SBF was adjusted to 7.4 ± 0.2 at 37˚C using 1 M NaOH solution and pH meter (826 pH Mobile, Metrohm, Australia).

4.2.2 Hydrogel preparation

Hydrogels were prepared as follows: gellan gum was added to 80˚C DI water with rapid stirring until dissolved on a hot plate/stirrer (CB162, Stuart, UK). Gelatin was then added and stirred until dissolved. Subsequently, 1 M CaCl$_2$ solution and 20.3% (w/v) genipin solution was added to reach the desired cross-linker concentrations. The solutions were then stirred for 3 more minutes before being poured into glass petri dish moulds (60 mm diameter x 15 mm height, Schott, Australia) and left to cure for 24 hours, covered at 21 ± 5˚C.
4.2.3 Mechanical analysis

Mechanical analyses were performed using a universal mechanical testing apparatus (EZ-S, Shimadzu, Japan). For compressive mechanical analysis, samples were cut from slabs of hydrogel into rectangular prisms 10 mm x 10 mm x 7 mm, and subsequently compressed at a rate of 1 mm.min\(^{-1}\) at 21°C. The resulting stress-strain data was used to determine the compressive failure strain (\(\varepsilon_c\)), compressive secant modulus over 20%-30% strain (\(E_c\)), compressive failure stress (\(\sigma_c\)) and compressive strain energy to failure (\(U\)).

For tensile mechanical analysis, samples were cut with a “dog-bone” shaped cutter (conforming with JIS – K6250\(^{300}\)) with a thickness of 1.7 mm, neck width of 4 mm and gauge length of 50 mm, and subsequently pulled at a rate of 4 mm.min\(^{-1}\) at 21°C. The resulting stress-strain data was used to determine the elongation to failure (\(\varepsilon_t\)), Young’s modulus (\(E_t\)), tensile fracture stress (\(\sigma_t\)) and work of extension (\(W\)).

Trouser tear tests based on the Japanese Industrial Standard method were used for fracture analysis\(^{301}\). Trouser shaped samples were cut with a steel cutter 1.7 mm thick (\(T\)), 50 mm long and 4 mm wide with a 25 mm split length. The legs of the trousers were pulled in tension perpendicular to the direction of crack propagation (mode III tearing) at a rate of 4 mm.min\(^{-1}\) and at 21°C. The critical fracture energy (\(G_c\)) was calculated as follows:

\[
G_c = \frac{2F}{T}, \quad (1)
\]

where \(F\) was the force require to propagate the crack in a hydrogel.
Recovery of hysteresis behaviour of swollen hydrogels was examined in compression to either a specific stress, or to a specific strain value. Samples of the hydrogels were prepared in disc moulds (17.5 mm diameter, 5 mm height) and then immersed in SBF for 3 days at 37°C to allow them to reach their equilibrium swollen state. The hydrogels were then loaded in compression at a rate of 1 mm.min\(^{-1}\) until they reached either a stress of 150 kPa, or strain of 50% and then unloaded at a rate of 1mm.min\(^{-1}\) to the original height. Samples were then subjected to up to 4 subsequent loading and unloading cycles after a period of resting in SBF at 37°C (wet recovery) or wrapped in plastic wrap (Gladwrap, Clorox Australia Pty Limited, Australia) at 37°C (dry recovery). The energy dissipated during a cycle (hysteresis, \(U\)) was calculated using

\[
U_i = \int_{\text{loading}} \sigma \, d\varepsilon - \int_{\text{unloading}} \sigma \, d\varepsilon , \quad (2)
\]

where \(\sigma\) and \(\varepsilon\) are the compressive stress and strain during cycle \(i\).

4.2.4 Optical microscopy

The homogeneity of the hydrogels was examined using light microscopy (Z16, Leica, Germany) of hydrogels which had been paraffin embedded, sectioned and stained using periodic acid-Schiff (PAS) staining\(^{302}\). The hydrogels were first embedded using automated tissue processing and embedding stations (ASP200S, EG1150C and EG1150H, Leica, Germany) which dehydrated the hydrogels using a solvent gradient of 70% (v/v) ethanol, 90% (v/v) ethanol, absolute ethanol and xylene before embedding in paraffin. The gels were then sectioned using a microtome (RM2255, Leica, Germany) to a thickness of 10 μm and collected on
glass microscopy slides (Knittel, Germany). The hydrogel sections were then rehydrated using the reverse of the abovementioned solvent gradient; oxidised with 5\% (w/v) periodic acid (BDH, England) for 5 minutes; and stained with Schiff’s Reagent (Merck, USA) for 15 minutes before being dehydrated using an automated staining station (ST4020, Leica, Germany).

4.2.5 FTIR spectroscopy

The connectivity between gellan gum and gelatin polymer networks in the hydrogels was examined using Fourier transform infrared spectrometry (IRAffinity-1, Shimadzu, Australia). Hydrogel samples were oven dried (FD, Binder, USA) at 80 ± 5°C for 4 hours prior to analysis with a diamond ATR accessory. The spectra were processed using Happ-Genzel apodisation, ATR correction, smoothing and baseline correction algorithms.

4.2.6 Immersion studies

Gels were immersed in SBF at 37 ± 1°C in a temperature controlled chamber (Thermoline, Australia) for 148 hours. The swelling ratio of the hydrogels as well as the pH and gellan gum, gelatin, sodium, magnesium, potassium and calcium concentrations in the immersion solutions were measured at 0, 3, 6, 12, 24, 48, 72, 96, 120 and 144 hours.

Gelatin and gellan gum concentrations were measured with a Coomassie Plus assay (Thermo Scientific, Australia) and a Total Carbohydrates assay (Biovision Incorporated, USA), respectively, in microplate format using a plate reading spectrophotometer (Polarstar, BMG Labtech, Germany).
The sodium, magnesium, potassium and calcium concentration of the immersion solutions was measured using inductively coupled plasma mass spectrometry (7500CE, Agilent Technologies, Japan). The element concentrations were determined using standard curves based on $^{23}\text{Na}$, $^{25}\text{Mg}$, $^{39}\text{K}$ and $^{48}\text{Ca}$ isotopes. Samples were prepared for analysis by diluting to the working ranges of the standard curves with high purity DI water containing 2% (v/v) HNO$_3$ (Suprapur, Merck Millipore, Australia). Calibration standards were prepared from a certified multi-element standard (Lot# A2-MEB236019, Inorganic Ventures, Australia) in 2% (v/v) HNO$_3$. High purity argon was used as the plasma/crrier gas and helium was used as the collision/reaction gas.

The swelling ratio (SW) was calculated as the mass of the swollen hydrogels ($m_s$) divided by the dry mass ($m_d$). Swollen hydrogel mass measurements were taken on a top-loading balance (PB3002/-S/FACT, Metler-Toledo, Australia) after the gels were extricated from the immersion solutions and blotted dry with filter paper (165 hardened and ashless papers, Filtech, Australia).

The effect of pH on the SW of hydrogel was studies by immersing the hydrogels in 0.1 M phosphate buffered saline (PBS) solution for 24 hours. The pH of all immersion solutions was measured with an electrode based pH meter (826 pH Mobile, Metrohm, Australia).
4.2.7 **Statistical treatment of data**

Dixon’s Q-test (95% confidence) was used to confirm and justify the removal of spurious data. Unless otherwise stated, the data presented in this manuscript are the mean ± one standard deviation (SD).

4.3 **Results**

4.3.1 *Optimisation of hydrogel composition*

Ionic-covalent entanglement network hydrogels (Figure 4-1) were prepared from calcium cross-linked gellan gum and genipin cross-linked gelatin. The values of the mechanical properties exhibited by the ICE gels is better than the sum of its constituent gel materials, i.e. hydrogels comprising of only calcium cross-linked gellan gum, or only genipin cross-linked gelatin (Figure 4-2, Table 4-1).

![Figure 4-1: Photographs of typical a typical ICE network hydrogel (2.75% w/v polymer) subjected to tensile (a) and trouser tear (b) tests.](image-url)
Table 4.1. Mechanical properties of ICE network, gellan gum and gelatin hydrogels with varying polymer concentrations (± SD).

<table>
<thead>
<tr>
<th></th>
<th>ICE network (2.75% wt)</th>
<th>ICE network (4.12% wt)</th>
<th>ICE network (5.50% wt)</th>
<th>Gellan gum (1% wt)</th>
<th>Gelatin (1.75% wt)</th>
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<tbody>
<tr>
<td>$\sigma_c$ (kPa)</td>
<td>1100 ± 200</td>
<td>1000 ± 200</td>
<td>1000 ± 200</td>
<td>360 ± 80</td>
<td>20 ± 10</td>
</tr>
<tr>
<td>$\varepsilon_c$ (%)</td>
<td>85 ± 1</td>
<td>81 ± 5</td>
<td>72 ± 3</td>
<td>82 ± 2</td>
<td>74 ± 5</td>
</tr>
<tr>
<td>$E_c$ (kPa)</td>
<td>120 ± 20</td>
<td>260 ± 30</td>
<td>490 ± 30</td>
<td>70 ± 10</td>
<td>4.5 ± 0.6</td>
</tr>
<tr>
<td>$U$ (kJ.m$^{-3}$)</td>
<td>147 ± 9</td>
<td>200 ± 40</td>
<td>200 ± 40</td>
<td>57 ± 7</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>$\sigma_t$ (kPa)</td>
<td>270 ± 20</td>
<td>510 ± 30</td>
<td>620 ± 60</td>
<td>70 ± 10</td>
<td>*</td>
</tr>
<tr>
<td>$\varepsilon_t$ (%)</td>
<td>64 ± 4</td>
<td>66 ± 6</td>
<td>69 ± 3</td>
<td>16 ± 2</td>
<td>*</td>
</tr>
<tr>
<td>$E_t$ (kPa)</td>
<td>420 ± 20</td>
<td>780 ± 30</td>
<td>890 ± 50</td>
<td>460 ± 30</td>
<td>*</td>
</tr>
<tr>
<td>$W$ (kJ.m$^{-3}$)</td>
<td>80 ± 10</td>
<td>170 ± 30</td>
<td>230 ± 40</td>
<td>6 ± 2</td>
<td>*</td>
</tr>
<tr>
<td>$G_c$ (J.m$^{-2}$)</td>
<td>40 ± 10</td>
<td>98 ± 4</td>
<td>126 ± 6</td>
<td>*</td>
<td>*</td>
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<tr>
<td>SW</td>
<td>40 ± 5</td>
<td>25 ± 5</td>
<td>23 ± 5</td>
<td>101 ± 5</td>
<td>58 ± 3</td>
</tr>
</tbody>
</table>

$\varepsilon_c$, compressive failure strain; $E_c$, compressive secant modulus; $\sigma_c$, compressive failure stress; $U$, compressive strain energy to failure; $\varepsilon_t$, elongation to failure; $E_t$, Young’s modulus; $\sigma_t$, tensile fracture stress; $W$, work of extension; $G_c$, critical fracture energy; SW, swelling ratio. Properties marked with “*” were unable to be determined because the hydrogel samples were too fragile to undergo testing.
Figure 4-2: Compressive stress-strain of a typical ICE network hydrogel (2.75% w/v polymer) and its constituent gellan gum and gelatin hydrogel components.

Gels of various compositions were examined to determine the optimum polymer and cross-linker ratios in terms of mechanical characteristics. For example, the polymer ratio was changed by increasing the gelatin content while keeping the amount of gellan gum constant. The compressive stress at failure reached a maximum of 1.1 ± 0.2 MPa for ICE network consisting of 36% (w/w) gellan gum to 64% (w/w) gelatin (Figure 4-3a). A similar trend was observed for compressive strain energy to failure (Supplementary Figure 4-1, Supplementary Table 4-1), while secant modulus increased with gelatin content (Figure 4-3b). Similar trends were observed for the ratio between Ca$^{2+}$ cross-linker and gellan gum (Figure 4-3c-d, Supplementary Figure 4-2, Supplementary Table 4-2). In contrast, changing the ratio of the genipin to gelatin did not result in a maximum (Supplementary Figure 4-3, Supplementary
Table 4-3). Rather, compressive stress to failure and compressive strain energy increase, while the compressive secant modulus decreases with increasing genipin to gelatin ratio. It is not clear at present why some of the mechanical characteristics decrease with this increasing ratio. However, this result appears to suggest that gellan gum network may impede the formation of covalent cross-links in the gelatin network due to a shielding effect.

The distribution of gellan gum and gelatin throughout the hydrogels was confirmed to be homogenous based on microscopic investigations of stained hydrogel sections. Gelatin was stained blue from the genipin, and gellan gum was stained pink using periodic acid-Schiff (PAS) staining. There were no distinct blue (gelatin rich) regions or pink (gellan gum rich) regions in the micrographs (Figure 4-4a).

Figure 4-3: Compressive mechanical stress at failure and compressive secant modulus of gellan gum-gelatin ICE network hydrogels with: a, b) varying polymer ratios (changed by increasing gelatin content while keeping gellan gum content constant) with constant cross-linker concentrations; c-d) Varying Ca\(^{2+}\) concentration with constant polymer and genipin concentrations.
FTIR spectroscopy suggested that the gellan gum and gelatin polymer networks were not covalently cross-linked. FTIR spectra of the gellan gum-gelatin ICE network hydrogels were observed to be a simple combination of the spectra of gellan gum hydrogels and gelatin hydrogels spectra (Figure 4-4b). This indicates that no new covalent bonds were formed or existing covalent bonds were broken during the preparation of ICE network hydrogels. This strongly suggests that the two polymer networks are covalently independent of one another.

The mechanical properties were also investigated by testing the most robust hydrogels in tension (Table 4-1). It is well known that mechanical properties such as fracture energy ($G_c$) and Young’s modulus ($E$) values increase with decreasing swelling ratio. Figure 4-5a shows that the $G_c$ and $E$ values of our gels are smaller than those of DN gels and rubbers, but larger compared to conventional gels. It is likely that the difference in $G_c$ values between DN and our ICE gels can be (partially) attributed to the difference in swelling ratio. Our ICE gels have swelling ratios of 20-40, which is larger than the corresponding ratios for DN gels ($<10$).
However, comparing gels at similar swelling ratios (20-40) reveals that the work of extension values ($W$) of our gels is better than those of conventional gels, but not as good as NC hydrogels (Figure 4-5b).

Figure 4-5: a) Fracture energy ($G_c$) versus Young’s modulus ($E$) and b) work of extension ($W$) versus swelling ratio ($SW$) comparison charts of ICE network hydrogels reported in this work (crosses) and conventional hydrogels (diamonds), DN hydrogels (triangles), NC hydrogels (circles), rubbers (squares) adapted from reference 238.
4.3.2 Immersion studies

ICE network hydrogels were immersed in simulated body fluid (SBF) for up to 144 hours. The gellan gum, gelatin, sodium, magnesium, potassium and calcium concentrations and the pH of the SBF was measured at regular intervals in addition to the swelling ratio of the hydrogel samples.

The concentrations of gellan gum and gelatin in the SBF reached a plateau value after 48 hours of immersion (Supplementary Table 4-4). It is suggested that this is release of free polymer chains (those unassociated with the gel network)\textsuperscript{180,303}. The relative amount of gellan gum leached was observed to increase in proportion to the total polymer concentration to a maximum of 0.042 ± 0.009\% (w/w) for 5.500\% (w/v) ICE network hydrogels (Figure 4-6a). The amount of gelatin leached as a percentage of the gelatin used to prepare the hydrogels was observed to fluctuate around an average value of 2 ± 2\% (w/w), irrespective of the total polymer concentration in the hydrogels (Figure 4-6b). This suggests that gellan gum and gelatin release is small and not likely to have a large impact on gel properties.

The concentration of calcium in the SBF surrounding 2.75\% (w/w) ICE network hydrogels increased from 0.20 ± 0.05 μg.L\textsuperscript{-1} to 0.5 ± 0.3 μg.L\textsuperscript{-1} over the first 48 hours and did not change significantly afterwards (Figure 4-6c). The concentrations of sodium, magnesium and potassium did not change significantly during the immersion period (Error! Reference source not found.). Calcium ions are directly involved in the cross-linking of gellan gum and it is possible that they were exchanged with other ions such as sodium in the SBF resulting in weaker networks\textsuperscript{120}. 

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The pH of SBF was observed to decrease by ~0.4 over the 144 hour study period with the most dramatic change occurring within the first 24 hours for most polymer concentrations (Figure 4-6d). The gellan gum possesses carboxylic acid functional groups and the type of gelatin used to prepare the hydrogels is an acid hydrolysed porcine gelatin so it is unsurprising that leaching of these materials lowered the pH of solutions in which they were immersed.

All of the hydrogels de-swelled to some extent during the immersion study with the majority of the de-swelling occurring within the first 24 hours. The hydrogels with 1.375% (w/v) polymer de-swelled from a swelling ratio of 75 ± 3 to 64 ± 4 over 144 hours, while all of the other hydrogels only changed marginally (Figure 4-6e). The swelling ratio of ICE network hydrogels did not change significantly when immersed in 0.1 M phosphate buffered saline (PBS) solutions at pHs between 4.5 and 8.6 (Figure 4-6f). Ordinarily, proteinaceous polymers such as gelatin change their volume in response to being immersed in solution of different pH. For example, a genipin cross-linked gelatin hydrogel (without gellan gum) de-swelled to 23% of its original volume in a previously reported experiment\textsuperscript{117}. This phenomenon occurs as a result of ionisation of functional groups in these proteins at pHs above, below and at the isoelectric point of the protein.
Figure 4-6: a) The percentage of gellan gum leached from hydrogels of different polymer concentrations after 144 hours immersion in SBF, b) the percentage of gelatin leached from hydrogels of different polymer concentrations after 144 hours immersion in SBF, c) the concentration of calcium in SBF containing a 2.75% (w/w) polymer hydrogel at different immersion intervals, d) The pH of SBF containing a 2.75% (w/w) polymer hydrogel at different immersion intervals, e) the change in swelling ratio of hydrogels of different polymer concentrations after 144 hours immersion in SBF, f) the swelling ratio of 2.75% (w/w) polymer hydrogels after being immersed in 0.1 M PBS solutions for 24 h of different pHs (±SD).

4.3.3 Characteristics of swollen hydrogels

In the previous section, the composition was optimised and it was determined that 48 hours immersion in SBF was sufficient time for the hydrogels to reach an equilibrium swelling state. ICE network hydrogels comprising of 36% (w/w) gellan gum, 64% (w/w) gelatin, 2% (w/w) Ca$^{2+}$ and 20% (w/w) genipin were prepared with polymer concentrations between 1.375% (w/v) and 5.500% (w/v), and immersed in SBF. The compressive mechanical properties of ICE network hydrogels immersed in SBF were compared to analogous as-prepared hydrogels, i.e. gels which had not been immersed in SBF. The compressive failure stress and strain energy to failure
increased with increasing polymer concentration between 1.375 and 2.750\% (w/v) but plateaued for higher polymer concentrations (Figure 4-7a-b). As-prepared hydrogels possessed higher failure stresses and strain energies than fully swollen hydrogels. The compressive secant moduli of both the as-prepared and fully swollen hydrogels increased with increasing polymer concentration. However, the moduli of immersed hydrogels were slightly lower than the non-immersed hydrogels (Figure 4-7c). The compressive strain to failure decreased with increasing polymer concentration. Fully swollen hydrogels fractured at a lower strain than the corresponding as-prepared hydrogels (Figure 4-7d).

Overall (and as expected), the magnitude of the mechanical properties of hydrogels decreased as a result of swelling (Figure 4-7 and Supplementary Table 4-4). It is suggested that the diminution of mechanical properties of immersed hydrogels may be caused by loss of calcium ions from the hydrogels. This suggestion is supported by a previous study where calcium cross-linked gellan gum hydrogels were shown to be degraded via sodium-calcium ion exchange \textit{in vivo}^{304}. 

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4.3.4 Recovery of hysteresis

Gellan gum-gelatin ICE network hydrogels comprising 2.75% (w/w) polymer were compressed to either a constant stress of 150 kPa or a constant strain of 50% to determine their ability to recover after a resting period in air (dry recovery) or immersed in SBF (wet recovery). The energy dissipated (hysteresis) during the first loading/unloading cycle to constant stress (150 kPa) or constant strain (50%) was 24 ± 2 kJ.m$^{-3}$ (Figure 4-8). The hysteresis reduced to 6 ± 2 kJ.m$^{-3}$ when the gels were immediately subjected to a second loading/unloading cycle (Figure 4-9). This
reduction could be partially attributed to observed expelling of water during the initial loading cycle. This could be indicative of a de-swelling effect and contributing to the observed stiffening of the gels. Regardless of the resting time, the gels do not improve the value of their hysteresis in the experiments to either constant strain (Figure 4-9a), or constant stress (Figure 4-9b).

Figure 4-8: a) and b) Stress-strain curves for loading/unloading cycles 1 and 2 of typical ICE network hydrogel (2.75% w/w) samples compressed to 50% after resting in air or immersed in SBF for 10 min between cycles, respectively. c) and d) Stress-strain curves for loading/unloading cycles 1 and 2 of typical ICE network hydrogel (2.75% w/w) samples compressed to 150 kPa after resting in air or immersed in SBF for 10 min between cycles, respectively.
However, placing the gels in SBF results in a significant improvement in the hysteresis (Figure 4-8). Allowing the gels to rest between cycles 1 and 2 for 5 hours in SBF resulted in an increased hysteresis value (to constant strain) during the second cycle of $U_2 = 20 \pm 4$ kJ.m$^{-3}$, which is $82 \pm 7\%$ of the hysteresis of the first cycle (Figure 4-9a). It is likely that the re-swelling of the gels is responsible for the observed improved recovery in hysteresis. Similar results were obtained for gels subjected to loading/unloading cycles to constant stress (Figure 4-9b). Resting the gels between cycles 1 and 2 for 4 hours in SBF resulted in $U_2 = 23.4 \pm 0.8$ kJ.m$^{-3}$, which is equivalent to $95 \pm 2\%$ recovery of the hysteresis value of the first cycle.

Furthermore, our hydrogels were subjected to 5 cycles in succession each with 10 min resting in SBF between cycles. The hysteresis value decreased between cycles 1 and 2, but remained constant for subsequent cycles 3-5 (≈ 80\% of the hysteresis value of the first cycle). This behaviour combined with the observation that the re-swollen gels regain their initial volume (but not swell beyond that volume) appears to suggest that our gels might not be permanently damaged by the first loading/unloading cycle as reported for some microgel reinforced gels$^{253}$. 
4.4 Conclusions

This paper describes the preparation and characterization of a robust biopolymer based ionic-covalent entanglement network hydrogels from gellan gum and gelatin. The optimal concentrations of gellan gum, Ca\(^{2+}\), gelatin and genipin were identified and the resulting hydrogels were demonstrated to be homogenous. The compressive fracture stress and work of extension values of the optimized hydrogels were 1.1 ± 0.2 MPa (swelling ratio 37.4 ± 0.6) and 230 ± 40 kJ.m\(^{-3}\) (swelling ratio 19 ± 1), respectively. The behaviour of the hydrogels when immersed in simulated body fluid was investigated and it was observed that calcium was leached from the hydrogels over time. It was suggested that the observed reduction in the magnitude of the mechanical properties for swollen hydrogel could be attributed to the observed loss of the calcium cross-linker.
The ICE network hydrogels were able to recover to a significant proportion of their hysteresis when rested in simulated body fluid (37 °C) for more than 10 minutes between compression cycles 1 and 2. We showed that the hysteresis recovery was 95 ± 2% under cyclic compression to a constant stress and 82 ± 7 under cyclic compression to a constant strain. This paper contributes to the development and understanding of interpenetrating polymer network hydrogels with (reversible and sacrificial) ionic cross-linkers.
4.5 Supporting information

4.5.1 Mechanical Characterisation

Supplementary Table 4-1: The compressive mechanical properties of gellan gum-gelatin hydrogels possessing various molar network ratios (± standard deviation). Hydrogels were prepared with the same concentrations of calcium and genipin cross-linkers but varying ratios of gellan gum and gelatin.

<table>
<thead>
<tr>
<th>Gelatin / (gelatin + gellan gum) (%)</th>
<th>$\sigma_c$ (MPa)</th>
<th>$\varepsilon_c$ (%)</th>
<th>$E_c$ (kPa)</th>
<th>$U$ (kJ.m$^{-3}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>33</td>
<td>0.27 ± 0.06</td>
<td>76 ± 2</td>
<td>56 ± 6</td>
<td>40 ± 5</td>
</tr>
<tr>
<td>50</td>
<td>0.6 ± 0.2</td>
<td>81 ± 3</td>
<td>56 ± 5</td>
<td>70 ± 20</td>
</tr>
<tr>
<td>56</td>
<td>0.8 ± 0.2</td>
<td>85 ± 2</td>
<td>60 ± 20</td>
<td>100 ± 10</td>
</tr>
<tr>
<td>60</td>
<td>0.9 ± 0.2</td>
<td>85 ± 2</td>
<td>79 ± 9</td>
<td>110 ± 20</td>
</tr>
<tr>
<td>64</td>
<td>1.1 ± 0.2</td>
<td>85 ± 1</td>
<td>120 ± 20</td>
<td>150 ± 20</td>
</tr>
<tr>
<td>69</td>
<td>0.8 ± 0.3</td>
<td>81 ± 4</td>
<td>180 ± 20</td>
<td>130 ± 40</td>
</tr>
<tr>
<td>71</td>
<td>0.5 ± 0.1</td>
<td>74 ± 4</td>
<td>180 ± 20</td>
<td>90 ± 20</td>
</tr>
<tr>
<td>80</td>
<td>0.25 ± 0.04</td>
<td>64 ± 2</td>
<td>260 ± 40</td>
<td>50 ± 20</td>
</tr>
</tbody>
</table>
Supplementary Figure 4-1: The polymer network ratio affects the a) maximum compressive stress, b) secant modulus, c) maximum compressive strain, and d) strain energy to failure (± SD).
Supplementary Table 4-2: The compressive mechanical properties of gellan gum-gelatin hydrogels possessing various calcium concentrations (± SD). Hydrogels were prepared with the same concentrations of gellan gum and gelatin polymers and genipin cross-linker but concentrations of calcium.

<table>
<thead>
<tr>
<th>[Ca^{2+}]% (w/w)</th>
<th>$\sigma_c$ (MPa)</th>
<th>$\varepsilon_c$ (%)</th>
<th>$E_c$ (kPa)</th>
<th>$U$ (kJ.m$^{-3}$)</th>
</tr>
</thead>
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<tr>
<td>0.0</td>
<td>0.30 ± 0.08</td>
<td>72 ± 4</td>
<td>120 ± 20</td>
<td>50 ± 10</td>
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<td>77 ± 3</td>
<td>160 ± 10</td>
<td>80 ± 10</td>
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<tr>
<td>0.8</td>
<td>0.6 ± 0.2</td>
<td>82 ± 2</td>
<td>130 ± 10</td>
<td>100 ± 20</td>
</tr>
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<td>150 ± 20</td>
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<td>4</td>
<td>0.9 ± 0.2</td>
<td>82 ± 2</td>
<td>140 ± 10</td>
<td>130 ± 20</td>
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<tr>
<td>8</td>
<td>0.5 ± 0.1</td>
<td>69 ± 4</td>
<td>290 ± 30</td>
<td>100 ± 20</td>
</tr>
<tr>
<td>20</td>
<td>0.29 ± 0.01</td>
<td>64 ± 1</td>
<td>350 ± 20</td>
<td>72 ± 4</td>
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</tbody>
</table>
Supplementary Figure 4-2: The concentration of calcium cross-linker affects the a) maximum compressive stress, b) secant modulus, c) maximum compressive strain, and d) strain energy to failure (± SD).
Supplementary Table 4-3: The compressive mechanical properties of gellan gum-gelatin hydrogels with genipin concentrations (± SD). Hydrogels were prepared with the same concentrations of gellan gum and gelatin polymers and calcium cross-linker but concentrations of genipin.

<table>
<thead>
<tr>
<th>[Genipin] (%(w/w))</th>
<th>$\sigma_c$ (MPa)</th>
<th>$\varepsilon_c$ (%)</th>
<th>$E_c$ (kPa)</th>
<th>U (kJ.m$^{-3}$)</th>
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<td>74 ± 4</td>
<td>150 ± 10</td>
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<td>8</td>
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<td>100 ± 20</td>
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<td>1.1 ± 0.2</td>
<td>85 ± 1</td>
<td>120 ± 20</td>
<td>150 ± 20</td>
</tr>
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<td>28</td>
<td>1.0 ± 0.1</td>
<td>86 ± 2</td>
<td>100 ± 10</td>
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Supplementary Figure 4-3: The concentration of genipin cross-linker affects the a) maximum compressive stress, b) secant modulus, c) maximum compressive strain, and d) strain energy to failure (± SD).
4.5.2 *Immersion studies*

Supplementary Table 4-4: Gellan gum and gelatin concentration and pH of simulated body fluid and the corresponding swelling ratio of hydrogels with different polymer concentrations with respect to immersion time.

<table>
<thead>
<tr>
<th>Polymer % (w/v)</th>
<th>Immersion time (h)</th>
<th>Gellan gum leached (%)</th>
<th>Gelatin leached (%)</th>
<th>pH</th>
<th>Swelling ratio</th>
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<td>SD</td>
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<td>SD</td>
<td>AVG</td>
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Supplementary Figure 4-4: Change in concentration of sodium, magnesium, potassium and calcium ions in SBF with a 2.75% (w/w) polymer hydrogel immersed in them for different periods of time.
CHAPTER 5: REINFORCING BIOPOLYMER HYDROGELS WITH IONIC-COVALENT ENTANGLEMENT HYDROGEL MICROSPHERES

Damian M. Kirchmajer,\textsuperscript{a} and Marc in het Panhuis\textsuperscript{a}

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This chapter was submitted in its current form to Soft Matter.

Author statement
Damian M. Kirchmajer performed all research and wrote the manuscript. However, this work would not have been possible without the intellectual input and support from Marc in het Panhuis.

Primary supervisor confirmation
I, Associate professor Marc in het Panhuis (primary supervisor), support and certify the above author statement.

Signature: ______________________

Date: ______________________
Abstract
Microscopic hydrogel spheres can be used to improve the mechanical properties of conventional hydrogels. We prepared ionic-covalent entanglement (ICE) hydrogel microspheres of calcium cross-linked gellan gum and genipin cross-linked gelatin using a water-in-oil emulsion based processing technique. The method was optimised to produce microspheres with number average diameter 4 ± 1 µm. These ICE microspheres were used to reinforce gelatin hydrogels and improve their compressive mechanical properties. The strongest microsphere reinforced hydrogels possessed a compressive mechanical stress at failure of 0.50 ± 0.1 MPa and a compressive secant modulus of 0.18 ± 0.02 MPa.

5.1 Introduction
Tissue engineering technologies may one day provide a plentiful supply of highly compatible laboratory grown tissue and organs for implantation. The tissue scaffold, i.e. the material that reinforces the component’s structure and holds the cells in place during maturation, is a critical component of all in vitro tissue engineering strategies. For engineering of soft tissues, hydrogel materials are the most appropriate scaffold materials because they have similar mechanical properties and chemical composition to natural tissues.

Hydrogels are a class of polymeric materials whose composition comprises a small fraction of hydrophilic polymer network with water or aqueous solution as the major fraction. Hydrogels make excellent materials for engineered tissue scaffolds because they resemble the composition of natural extracellular matrix and possess mechanical properties similar to most soft tissue. Hydrogels are also excellent
materials to use for drug delivery because they can easily absorb, retain and release bioactive molecules\textsuperscript{284}.

Many hydrogels have very weak mechanical properties despite having excellent biocompatibility and utility as tissue scaffolds. Certain tissue types, such as cartilage, require scaffolds which are strong, highly durable and resistant to fracture. Recently, topological hydrogels\textsuperscript{239}, tetra-PEG hydrogels\textsuperscript{240}, double network (DN) hydrogels\textsuperscript{241–244,290,291}, ionic covalent entanglement (ICE) network hydrogels\textsuperscript{68,69,245,246,293} have been reported which are significantly stronger and tougher than conventional hydrogels. However, some of these strategies only work with very specific polymers and reagents and/or have very constricting methods for their preparation which greatly limits the options for polymer choice and fabrication approach. For example, DN hydrogels must be prepared in a two-step method where one network is swollen in a monomer solution of the secondary network, a preparation method which is not amenable to printing or extrusion\textsuperscript{242}. Reinforcement of regular hydrogels with microgel particles is an alternative method for improving hydrogel properties which is in many ways more versatile than the abovementioned methods\textsuperscript{247}.

Hydrogel microspheres can be used to reinforce hydrogels where they act as multifunctional cross-linking nodes\textsuperscript{254,255}. In a microgel reinforced hydrogel (MR-hydrogel), the microspheres inhibit the formation of micro-cracks and voids in the bulk hydrogel which are responsible for the rapid and catastrophic fracturing observed in unreinforced hydrogels\textsuperscript{254}. MR-hydrogels have also demonstrated effectiveness at presenting biological cues in a controlled fashion when used as a cartilage scaffold\textsuperscript{256}. Previously it was shown that double network hydrogel
microspheres improved the mechanical properties of poly(acrylamide) hydrogels and we propose to use ICE hydrogel microspheres to strengthen gelatin hydrogels in a similar way\textsuperscript{247,249,253}. ICE hydrogels are an interpenetrating network of an ionotropic polymer and a chemically cross-linkable polymer which possess above average strength and toughness\textsuperscript{68,69,245,246}.

A variety of methods have been reported for making hydrogel microspheres of gelatin and other polymers which have typically been used for drug delivery and controlled drug release applications\textsuperscript{171,259,297,305}. Of the many available options, emulsion based strategies offer a fast and scalable method for the production of nano- and micro- sized droplets which can be set as spheres upon cooling below the gel transition temperature\textsuperscript{306}. Basic water in oil emulsions are very easy to prepare and require only simple shearing that can, and often is, achieved with bench-top stirrers. However, these basic emulsions will prepare droplets/spheres between 10 µm and 1000 µm with a large size distribution\textsuperscript{307}. The finest emulsions are prepared under high shear and highly controlled shear in the presence of an appropriate emulgent where almost monodisperse droplets form as a result of Rayleigh instability\textsuperscript{307}. Many factors affect the ability to emulsify a solution (for example, of gellan gum and gelatin), including the choice of emulgents, emulgent concentration, and the choice of oil\textsuperscript{307}.

The microspheres presented in this manuscript are comprised of an ICE hydrogel prepared from calcium cross-linked gellan gum and genipin cross-linked gelatin. Gellan gum is a polysaccharide biopolymer produced from the bacteria \textit{Pseudomonas elodea} that forms a firm gel upon cooling to below its gel transition
temperature (~45°C). The strength of gellan gum hydrogels is significantly affected by the presence of cross-linking cations and in particular, calcium cations produce strong thermally irreversible hydrogels that enable microspheres to be produced from emulsions by thermal modulation. Gelatin is a proteinaceous biopolymer produced from the controlled hydrolysis of collagen. Ordinarily, gelatin hydrogels swell extensively; rapidly dissolve above the gel transition temperature (~29°C), and lose their strength and rigidity rapidly. However, covalently cross-linking the gelatin with genipin (the algycone of geniposide, an iridoid glycoside that is the major component of the fruit of the gardenia plant, Gardenia jasminoides Ellis) remedies these limitations. Genipin forms chemical cross-links mainly between the primary amino groups of either the ε-amino groups of the lysine residues in gelatin and it is this reaction that is responsible for both the formation of the ICE network but also the coupling of the microsphere phases to the bulk hydrogel phase in MR-hydrogels.

In this paper, we present a simple method for the preparation of gellan gum-gelatin ICE hydrogel microspheres. The optimal emulgent hydrophile-lipophile balance (HLB) was identified, and the effect of emulgent concentration was explored. A variety of different oils were examined for their effectiveness at producing the microsphere-forming emulsions. The size of the resulting microspheres was characterised and their ability to reinforce gelatin hydrogels was evaluated.
5.2 Experiment

5.2.1 Materials and equipment

Hydrogels were prepared from acid hydrolysed porcine gelatin (Bloom number of 300, molecular weight of 87,500 Da, Sigma Aldrich, USA) and low acyl gellan gum (CP Kelco, Singapore) using deionised water (DI) which had been purified using a combination ion-exchange/reverse osmosis filtration system (Millipore, USA) to a resistivity of 18 MΩcm. The hydrogels were cross-linked using solutions of genipin (Challenge Bioproducts, Taiwan) and CaCl$_2$ (Sigma Aldrich, USA). Water-in-oil (w/o) emulsions were formed using canola oil (Crisco, USA), hydraulic oil (Ultramax 46, Valvoline), machine oil (X68, Caltex), 3-in-1 oil (3-in-1, USA) and paraffin oil (Recochem, Australia) with non-ionic surfactants Tween 80 (Sigma Aldrich, USA) and Span 80 (Merck, Australia). Solvents used for washing residual oils and surfactants from hydrogel microspheres included toluene (BDH, Australia), petroleum spirit (BDH, Australia), dichloromethane (Ajax Finechem, Australia), acetone (Ajax Finechem, Australia) and ethyl acetate (BDH, Australia).

5.2.2 Microsphere fabrication

ICE hydrogel solutions comprising 1% (w/v) gellan gum, 1.75% (w/v) gelatin and 2% (w/w) Ca$^{2+}$ were prepared by dissolving the gellan gum and gelatin in DI water at 80°C with gentle stirring on a combined magnetic stirrer/hot plate (CB162, Stuart, UK) for 30 minutes before 1 M CaCl$_2$ solution was added. These ICE hydrogels possessed compressive failure stress of 1.1 ± 0.2 MPa, strain to failure of 85 ± 1% and secant modulus of 120 ± 20 KPa. Oil/emulgent mixtures were prepared
separately at 60°C. Unless otherwise stated, the oil/emulgent mixtures were 0.1% (w/w) Tween 80 and 0.9% (w/w) Span 80 in paraffin oil.

Emulsions, unless otherwise stated, were prepared using a tissue homogeniser (HG-15D Wisemix, Daihan Scientific, Korea) with dispersing tool (HT1018, Wisemix, Daihan Scientific, Korea) which was pre-warmed to 60°C by immersing the tool in hot water. 200 mL of hydrogel solution was added slowly to 300 mL of oil-emulgent mixture whilst stirring at 2,000 rpm in a 600 mL low-form glass beaker (Crown, Australia). After the solutions were combined, the speed was increased to 7,000 rpm and maintained for 5 minutes (Figure 5-1 i). The emulsion was kept warm throughout the homogenising using a hotplate (CB162, Stuart, UK).

Figure 5-1: Procedure for the fabrication of ICE hydrogel microspheres using emulsification to create micro-sized droplets of hydrogel solution and thermal modulation to set the droplets into firm hydrogel microspheres.
The emulsion was gelled into a microsphere suspension immediately after stirring by pouring it into an ice-cold, 1L glass bottle (Schott, Germany), and then plunging the bottle into a bucket of ice for approximately 15 minutes (Figure 5-1 ii). The oil and emulgents were removed from the microspheres by washing with two 500 mL portions of petroleum spirits, and one 450 mL portion of petroleum spirits with 50 mL ethyl acetate added (Figure 5-1 iii). The microspheres were then freeze dried completely (Alpha LD plus, Christ, Germany) (Figure 5-1 iv).

5.2.3 MR-hydrogel preparation

MR-hydrogels were prepared by combining freeze-dried microspheres with a solution of gelatin at 50°C and stirring gently for 10 minutes to allow the microspheres to swell and rehydrate (Figure 5-2 i). Subsequently, the solution was cross-linked by addition of genipin solution (20.3% w/w in 60% ethanol) to a concentration of 200 mg genipin per gram gelatin and stirring for a further three minutes (Figure 5-2 ii). The gel solution was then poured into glass Petri dish moulds (60 mm diameter x 15 mm height, Schott, Australia) and left to cure, covered, for 24 hours at 21°C. Silanisation of the Petri dishes using Coatasil (Ajax Finechem, Australia) glass treatment solution prior to casting facilitated easy removal of the cured hydrogels from the dishes. The swelling ratio ($SW$) of hydrogels were calculated as the mass of the swollen hydrogel ($m_s$) divided by the mass of the dried hydrogel ($m_d$).

$$SW = \frac{m_s}{m_d} \quad (1)$$
5.2.4 Rheology

The viscosities of the gel solution and various oils were analysed using a rheometer (Physica MCR-301, Anton Paar, Australia) with temperature controlled stage (AWC100, Julabo, Germany), a 50 mm diameter and 1° cone and plate tool (CP50-1, Anton Paar, Australia), with a gap length of 0.097 mm. The viscosity was measured over a range of temperatures spanning from 80°C down to 4°C by applying a rotational shear of 100.s⁻¹ while gradually lowering the temperature at a rate of -5°C.min⁻¹.

5.2.5 Size analysis

An optical microscope (DM6000, Leica, Germany) was used to take micrographs of the microspheres. Leica Application Suite (Leica, Germany) was used to measure the size and size distribution of the microspheres. Samples prepared for optical microscopy were prepared by suspending the microspheres in DI water and dropping onto glass microscopy slides. Freeze dried microspheres were rehydrated for 10 minutes in DI water prior to analysis.
5.2.6 Mechanical analysis

The mechanical properties of the hydrogels were examined in compression using a universal analyser (EZ-S, Shimadzu, Japan). Samples were cut from slabs of hydrogel into rectangular prisms 10 mm x 10 mm x 7 mm, and subsequently compressed at a rate of 2 mm.min\(^{-1}\) at 21˚C. The resulting stress-strain data was used to determine the compressive failure strain (\(\varepsilon_c\)), compressive secant modulus over 20%-30% strain (\(E_c\)), compressive failure stress (\(\sigma_c\)) and compressive strain energy to failure (\(U\)).

5.3 Results

5.3.1 Optimisation of microsphere production

For the first time, microspheres of ICE hydrogels were prepared using emulsion based processing and thermal modulation of the gel solution (Figure 5-1). It was found that ICE hydrogel microspheres could be prepared in approximately 1-2 hours and that it could be performed easily on a 200 mL scale which is much quicker and larger scale than all previously reported DN microspheres which took over 8 hours to prepare a significantly smaller quantity\(^{247,249,253}\). The optimal process for preparing hydrogel microspheres was determined by independently examining the effects of i) the type and viscosity of oil; ii) the emulgent HLB value; and iii) the emulgent concentration.

The hydrophile-lipophile balance (HLB) of the emulgent used to prepare emulsions was observed to have an effect on the type of emulsion formed, its stability, and the ultimate quality of the microspheres produced. Emulgents with HLB values between 4.3 and 15 were used to prepare emulsions of paraffin oil and gel solution. The
emulsions were assessed qualitatively by inspecting micrographs of the emulsions which had been set by dropping the temperature immediately after shearing ceased (Figure 5-3). The stability of the emulsions were also assessed qualitatively by comparing micrographs of the same emulsion taken immediately after shearing ceased and 10 minutes afterwards. Emulsion prepared with emulgent HLB values between 15.0 and 11.8 formed oil-in-water (o/w) type emulsions. These emulsions created porous hydrogel sponges with droplets of oil trapped in the pores after being cooling. Emulsions prepared with HLB values of 10.7 and 9.6 produced oil-in-water-in-oil (o/w/o) double emulsions initially, which would transform into single o/w emulsions gradually over 10 minutes. Emulsions prepared with emulgent HLB values of 8.6 and 7.5 produced unstable water-in-oil (w/o) emulsions that would coalesce and agglomerate noticeably over 10 minutes. The best quality hydrogel microspheres and most stable w/o emulsions were prepared using emulgents with HLB values between 4.5 and 6.4.

Figure 5-3: Micrographs of emulsions prepared with different HLB values. a) An o/w emulsion formed with emulgent HLB value of 13.9. b) An o/w/o emulsion formed with emulgent HLB value of 9.6. c) A w/o emulsion formed with emulgent HLB value of 6.4.
The concentration of emulgent affected the stability of the emulsion and the size of microspheres prepared from the emulsion. Emulsions were prepared using different concentrations of emulgent (HLB value = 5.4) ranging from 0.1% (w/w) to 8% (w/w). Emulsions formed with 0.1% emulgent were not stable and coalesced back into two separate layers before the gel solution set. When 0.5% or more emulgent was used, stable emulsions were formed for long enough to cool the gel solution to below the gel transition point and form microspheres. With increasing emulgent concentration, the average microsphere diameter decreased as did the size distribution of the microsphere (Figure 5-4). When more than 4% emulgent was used the microspheres produced were very small (<10 µm) and highly stabilised in paraffin oil by the presence of a large amount of surfactant; this made it difficult to separate the microspheres from the oil after setting. In general, the time it took for microspheres to settle out of suspension increased in proportion to the concentration of emulgent used and was between 5 minutes and 2 hours for all concentrations tested excepting those made with 8% (w/w) emulgent which took one week to settle. An optimal range of emulgent concentration was identified as 1 – 2% (w/w), where the average diameter and size distribution of microspheres are minimised and the ease of processing them was maximised.
Figure 5-4: a) The number average diameter of microspheres prepared from emulsions with different concentration of emulgent. Error bars indicate the standard distribution of microsphere diameters which also decrease with increasing emulgent concentration. b) Photograph of 10 mL of microspheres taken approximately 5 minutes after resuspending in 30 mL of petroleum spirit. Orange lines on the photo indicate the extent of settling at this point in time – the settling time increases with increasing emulgent used to prepare the microspheres. The concentration of emulgent used to prepare the microspheres increases from left to right in sequence: 0.5%, 1.0%, 2.0%, 4.0%, 8.0%

Rheological measurements of ICE hydrogel solution over the temperature range of 80 - 10°C indicated that the gel transition temperature of the ICE network was approximately 45°C (Figure 5-5a). The viscosities of five different oils were also examined over the same temperature range and found to range from 11 to 42 mPa.s at a shear rate of 100 s⁻¹ at 45°C (Figure 5-5b). These five different oils were then used to prepare ICE microspheres under identical conditions (1% emulgent with HLB value of 5.4) and it was observed that the type of oil affected the size of the microspheres produced which ranged from 14 – 60 µm and with varying size distributions (Table 5-1). A correlation between the size distribution (expressed as the relative standard deviation of the diameter measurements) of the microspheres and the viscosity of the oil used to prepare them was observed (Figure 5-5b) – in general, it was observed that higher viscosity oils, produced microspheres with
smaller size distributions. This observation is explicable as the effect caused Rayleigh instability which causes droplets in an emulsion to fragment towards a size distribution that is dependent on the relative viscosities of the dispersed and continuous phases\textsuperscript{307}. Despite the large size distribution attained through using paraffin oil (the lowest viscosity oil), it was selected for continued use throughout our experiments because it was the easiest oil to extricate fully from the microspheres after fabrication.

Table 5-1: Tabulated viscosity (@ 100s\textsuperscript{-1}, 45°C) different oils used to prepare ICE microspheres and their corresponding microsphere diameters.

<table>
<thead>
<tr>
<th>Viscosity @ 100s\textsuperscript{-1}, 45°C (mPa.s)</th>
<th>Number average diameter (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paraffin oil</td>
<td>60 ± 50</td>
</tr>
<tr>
<td>3-in-1 oil</td>
<td>14 ± 9</td>
</tr>
<tr>
<td>Canola oil</td>
<td>14 ± 8</td>
</tr>
<tr>
<td>Hydraulic oil</td>
<td>50 ± 20</td>
</tr>
<tr>
<td>Machine oil</td>
<td>25 ± 8</td>
</tr>
</tbody>
</table>
Figure 5.5: a) Viscosity at 100 s\(^{-1}\) versus temperature during cooling from 80 – 10°C of ICE hydrogel solution, b) viscosity at 100 s\(^{-1}\) versus temperature of different oils c) Microsphere size distribution expressed as relative standard deviation versus oil viscosity (at 100 s\(^{-1}\), 45 °C). Solid line is a guide to the reader’s eye.
5.3.2 Mechanical reinforcement of hydrogels

Gellan gum-gelatin ICE hydrogel microspheres were used to reinforce gelatin hydrogels (Figure 5-6). These hydrogels were prepared with a total polymer concentration of 6% (w/w) (swelling ratio of 17.7) made up of gelatin and freeze-dried ICE microspheres in different proportions. The freeze-dried microspheres were observed to hydrate and swell almost immediately upon mixing with the polymer solution to a final size of 4 ± 1 µm (Figure 5-6).

Figure 5-6: a) Micrograph of gelatin hydrogel which has been reinforced by replacing 40% of the polymer with freeze-dried microspheres; b) size distribution of microspheres in MR-hydrogels.
Hydrogels comprised entirely of genipin cross-linked gelatin (no added microspheres) were relatively soft and ductile (Figure 5-7) and possessed compressive failure stress of 0.41 ± 0.09 MPa, compressive strain to failure of 69 ± 2%, compressive secant modulus of 0.074 ± 0.008 MPa and compressive strain energy to failure of 39 ± 7 kJ.m⁻³. The MR-hydrogels exhibited a maximum in mechanical properties at a microsphere fraction of 40% (Figure 5-8). In comparison to gelatin only hydrogels, the MR-hydrogels were stiffened (increase in $E_c$ to 0.18 ± 0.02 MPa) without losing their ductility and had a resultant increase in compressive strain energy to failure to 70 ± 10 kJ.m⁻³.

In comparison to DN MR-hydrogels presented in recent literature our hydrogels appear weaker. Jian Ping Gong’s PAMPS/PAAm microgel reinforced PAAm hydrogels possessed tensile fracture stresses of 2.46 MPa and Young’s modulus of 0.22 MPa, however, these hydrogels had lower swelling ratio 6.7. The scale that our ICE MR-hydrogels were prepared was also significantly larger (~15 mL pieces of hydrogel) than the DN MR-hydrogels (~1 mL). Increasing the microsphere fraction above 40% led to a reduction in the values of the mechanical properties (Figure 5-8). For example, increasing the fraction from 40% to 60% resulted in a significant reduction in stress at failure (from 0.5 MPa down to 0.1 MPa). When gels were composed entirely of microspheres (100%), a self-supporting but weak gel which behaved like a semi-viscous fluid with an undetermined yield stress was apparent. This indicates that ratio of microspheres to bulk phase polymer is an important determinant of the mechanical performance of these hydrogels.
The mechanical behaviour of our gels is consistent with MR-hydrogels being a two-phase composite consisting of gelatin network and microgels\textsuperscript{253}. It has been previously proposed that the stiffening (increase in $E_c$) is a result from topologically constrained chain entanglements of the gelatin matrix and the ICE microgels\textsuperscript{253}.

Figure 5-7: Stress versus strain curves for gelatin hydrogel reinforced with 40% (w/w) ICE hydrogel microspheres (unbroken line), and 6% (w/v) gelatin hydrogel (dashed line).
Figure 5-8: Compressive mechanical properties of 6% gelatin hydrogels where 0% to 80% of the polymer is replaced by gellan gum-gelatin ICE hydrogel microspheres.

5.4 Conclusion
The preparation of gellan gum-gelatin, ICE hydrogel microspheres using the oil-in-water emulsion method was optimised. It was determined that the optimal mixture of emulgents was 1 part Tween 80 to 9 parts Span 80 (by weight) which produced an emulgent mixture with a HLB value of 5.4. It was observed that the emulgent concentration was inversely proportionate to microsphere number average diameter, however, the optimal concentration of emulgent was considered to be 1% (w/w) because it provided a good compromise between small microsphere diameter and ease of process-ability. A variety of different oils were also successfully used to
prepare the microspheres and a correlation between oil viscosity and size distribution was observed.

Microspheres were used to reinforce gelatin hydrogels where it was demonstrated that an optimal proportion of microspheres was 40% (w/w) of the total polymer in the hydrogel. These produced hydrogels which exhibited reinforcement compared to the gelatin matrix.
GENERAL CONCLUSIONS

Tissue engineering is a rapidly developing field that will one day address the supply shortage of replacement tissue and organs for the world’s growing population. Hydrogel materials have been studied extensively over the past three decades as candidate materials for soft tissue scaffolds which form the fundamental support structure for engineered soft tissues. One of the greatest challenges faced by tissue engineers to date is the identification of suitable hydrogel-forming polymers that meet the extensive list of performance criteria including, but not limited to; mechanical robustness and compatibility; non-toxicity and biocompatibility of the polymers and reagents required to process them; degradability or non-degradability as the situation requires; and process-ability of the materials to form complex scaffold shapes and structures. This thesis presents gelatin and gellan gum based hydrogel materials and demonstrates their mechanical, degradation and physical properties.

Chapter 2 reported the development of a hydrogel material from genipin cross-linked gelatin. It details the extent of cross-linking; swelling behaviour; degradation and leeching profiles; rheological behaviour; mechanical properties and ability of the hydrogels to be vascularised. It was revealed that hydrogels comprising between 2% and 10% (w/v) gelatin with up to 20% (w/w) genipin would form self-supporting hydrogels with highly tuneable mechanical, rheological and degradation properties. The strongest gels possessed compressive mechanical performance similar to other previously reported genipin and gluteraldehyde cross-linked gelatins\textsuperscript{160,161,260}. It was also demonstrated that the degradation rate could be adjusted most effectively.
through modulation of the genipin cross-linker concentration. This material was also shown to have the potential to be vascularised through a modified chick CAM experiment. In this experiment, it was observed that over several days, blood vessels emerging from a chick embryo were attracted to PMA loaded hydrogels and intruded into them. The research presented in Chapter 2 addresses Aim 1 of this thesis by describing the preparation of a material which was able to be vascularised and had tuneable mechanical, degradation and swelling properties. The presence of a vasculature in a cell scaffold, like in natural tissue, enables nutrients and oxygen to be delivered to cells throughout the component entirely thus overcoming limitations of mass transfer. The material described in this chapter might potentially be used in the future as a thick tissue scaffold for tissue engineering, however, it would be necessary to confirm the material’s biocompatibility and degradability in vivo first.

Chapter 3 reported the purification and properties of low acyl gellan gum. Commercially prepared low acyl gellan gum is an ionotropic hydrogel-forming biopolymer which has potential as a material for soft tissue engineering and in particular 3D extrusion printing of scaffolds. However, it is known that gellan gum contains divalent cation contaminants as supplied which affect the hydration and gel transition temperatures. A method was adopted from the one previously reported by Doner and Landis\textsuperscript{279,281} to purify commercial gellan gum to be free from divalent cation contaminants and prepare a sodium salt of the gellan gum. The purification method successfully removed Ca\textsuperscript{2+} and Mg\textsuperscript{2+} cations with a 64 ± 6% yield of the sodium salt gellan gum product. It was demonstrated that both the dissolution temperature and the gel transition temperature could be lowered by purifying the
commercial gellan gum to the sodium salt but that this process had a deleterious effect on the mechanical properties of hydrogels prepared from these polymers. Notwithstanding, the sodium salt gellan gum possess a dissolution temperature (at 1% w/v) of 39 ± 4°C and a gel transition temperature with calcium ions present of 37 ± 2°C. The research presented in this Chapter (3) addresses Aim 2 of this thesis by presenting a material that can be prepared and gelled at normal body temperature (37°C) providing it with the potential to be combined with cells safely and produce a cellular ink which could potentially be fabricated using extrusion or ink jet printing into complex 3D structures and tissue scaffolds.

Chapter 4 reports the preparation of a hybrid, ionic-covalent entanglement (ICE) hydrogel comprised of calcium cross-linked gellan gum and genipin cross-linked gelatin. This novel hydrogel type builds upon the toughening principals of double network hydrogels and adds to them a self-recovering aspect which was demonstrated in this work. It was shown that the proportions of the two network polymers and their respective cross-linkers have a significant effect on the compressive mechanical properties with the optimal hydrogels prepared possessing high swelling ratios. The mechanical properties of the ICE hydrogels were shown to be significantly better than hydrogels prepared of their constituent network polymers combined. The effect of immersion of these hydrogels in SBF was investigated on compressive mechanical properties; the leeching of calcium, genipin, gelatin and gellan gum; and the degradation and swelling of the hydrogels. It was shown that immersion of ICE hydrogels in SBF leached calcium from the hydrogels and that the mechanical performance of the hydrogels diminished but the hydrogels themselves
were dimensionally stable (non-swelling) and they did not degrade otherwise over a 144 hour period. The ICE hydrogels possessed the ability to almost completely recovery from a small compressive load if allowed to rest for 10 minutes in SBF. The research presented in Chapter 4 addresses Aim 3 of this thesis by demonstrating the preparation of a material which has a mechanical robustness greater than conventional hydrogels. The mechanical stiffness of the ICE hydrogel was similar to that of cartilage itself\textsuperscript{308,309}, so the material might be potentially used as a temporary cartilage tissue scaffold or for any other biomedical application where mechanical robustness is desirable.

Chapter 5 reports a method for the preparation of ICE hydrogel microspheres of gellan gum and gelatin. This method involved the setting of a water-in-oil emulsion prepared using the non-ionic surfactants Tween 80 and Span 80. The optimal mixture of emulgents was 1 part Tween 80 to 9 parts Span 80 (by weight) which produced an emulgent mixture with a HLB value of 5.4 and total concentration of 1\% (w/w) which were effective at producing microspheres 4 ± 1 \textmu m in diameter. The effect of other emulgent HLB values were examined where it was found that HLB values above 11.8 produce oil-in-water emulsions, and HLB values below 7.5 produced water-in-oil emulsions of varying stability. The total concentrations of emulgent and choice of oil was also examined and found to affect the microsphere diameter and size distribution, respectively. It was then shown that microspheres could be used to reinforce gelatin hydrogels with the optimal fraction of polymer present as microspheres was 40\% (w/w). However, it was shown that the ICE microspheres could not be used to reinforce bulk ICE hydrogels. The research presented in Chapter
5 addresses Aim 4 of this thesis as it demonstrates how hydrogel microspheres can be prepared and incorporated into a hydrogel to improve the mechanical performance. This material may also be a potential candidate material for cartilage tissue scaffolds provided the materials recoverability could be shown. The microsphere may also be a useful material for inclusion in ink formulation for 3D printing as they impart thixotropic properties to hydrogel solutions – however, a much more thorough study on this aspect would be required to confirm this.

This thesis has contributed to the field engineered soft tissue scaffolds by developing new hydrogel materials which are highly versatile in terms of mechanical properties, gelation behaviour and degradability. The new materials highlight the potential of genipin cross-linked gelatin based materials to be used as vascularisable scaffold materials, and for hybrid hydrogels of genipin cross-linked gelatin and calcium cross-linked gellan gum to prepare stronger than normal hydrogels which can recover from mechanical strains. Gellan gum was altered/purified to enhance its gelation properties and make it further amenable to computer aided tissue engineering of soft tissue scaffolds. It was also demonstrated that ICE hydrogel microspheres of gelatin and gellan gum can be fabricated and incorporated into gelatin hydrogels to improve the mechanical performance. In summation, genipin cross-linked gelatin and calcium cross-linked gellan gum were shown to be versatile hydrogel-forming polymers with tremendous potential for use as engineered soft tissue scaffold materials.

Now that the mechanical properties of these materials has been thoroughly explored, future work with these materials should involve in vivo studies that could confirm the biocompatibility of the materials with cells and animal models as well as gauge the
degradability in vivo. The printability of purified gellan gum based materials should be explored thoroughly as this may have significance to the combined extrusion printing of cells and hydrogels which is an important step towards computer aided tissue engineering and whole organ tissue engineering objectives. The ICE hydrogels materials showed excellent mechanical properties and recoverability which are important features with respect to their potential to be used for cartilage tissue scaffolds, however, it may be possible to further improve the mechanical performance of these hydrogels by making them non-independent IPN hydrogels. This could be accomplished through imbuing covalent cross-links between the gelatin and gellan gum networks which could be accomplished by creating the hydrogels with modified (methacrylated) polymer analogues, or perhaps using high energy radiation based cross-linking approaches. A more thorough study of the rheological properties of ICE hydrogel microsphere suspensions in hydrogel solutions would be beneficial to confirm their thixotropic properties. Thixotropic hydrogel solutions are useful for extrusion printing of hydrogels because they are an avenue to layer-upon-layer construction of 3D scaffolds.
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