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The Development of Novel Scaffolds for Tissue Engineering with a Range of Structural and Mechanical Properties

A thesis submitted to the University of Dublin in partial fulfilment of the requirements for the degree of

Doctor in Philosophy

Trinity College Dublin

February, 2009

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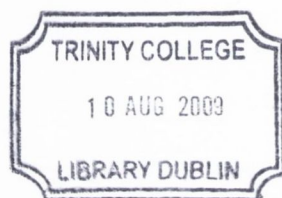
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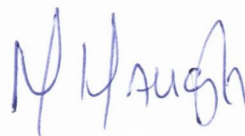


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February, 2009

Abstract

Tissue engineering (or regenerative medicine) is defined as the application of scientific principles to the synthesis of living tissues using bioreactors, cells, scaffolds, growth factors, or a combination (Rose and Oreffo, 2002). One of the principal methods in tissue engineering involves the use of a porous scaffold to support and guide synthesis of a 3D tissue or organ (Sachlos and Czernuszka, 2003). Collagen-Glycosaminoglycan scaffolds have found success in several clinical applications of tissue engineering (Yannas et al., 1989, Chamberlain et al., 1998).

However, it has been shown that the characteristics of these scaffolds must be tuned to their specific application if they are to be successful (Lee et al., 2001, Yannas, 2001, O'Brien et al., 2005, Byrne et al., 2008). This study sought to develop a series of novel scaffolds with a wide range of structural and mechanical properties, and to investigate the effects of these properties on cellular behaviour. Novel freeze-drying techniques were developed to vary the structure of the scaffolds. Using these techniques, I produced several scaffolds with individual mean pore sizes that could be varied within the range of 85-325 μm .

Studies were also carried out to determine the effect of several crosslinking techniques on the mechanical properties of the scaffolds and the activity of cells seeded within them. Using these crosslinking treatments, I produced scaffolds with compressive moduli that can be controlled between 0.44 and 1.8 kPa. Additionally, the improved mechanical properties of glutaraldehyde (GTA) and 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDAC) crosslinked scaffolds resulted in improved cell number, proliferation and cellular infiltration compared to the standard dehydrothermal crosslinked scaffolds. The enhanced cellular activity upon GTA and EDAC treated scaffolds, coupled with improved mechanical properties, increases their suitability for use both *in vitro* and *in vivo*.

In conclusion, it is proposed that the series of characterised scaffold variants produced in this study can be adapted for specific tissue engineering applications or used to further our knowledge of the effects of the structural and mechanical properties of scaffolds on the cellular activity and tissue synthesis within them.

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Acknowledgements

Firstly, I would like to thank my supervisor Prof. Fergal O'Brien, for taking me on as a student and guiding my research over the last four years. I'm very grateful to have worked on this project and believe I have become a better engineer due to Fergal's advice and support. Additionally, I'd like to thank Prof. Prendergast for advising me when I was considering postgraduate research and pointing me in Fergal's direction.

I have been lucky to carry out this work between two institutions, the Royal College of Surgeons in Ireland and the Trinity Centre for Bioengineering. I would like to thank the members of staff at both these institutions for making the last four years enjoyable. In the anatomy department at RCSI; Clive Lee, Tom, Alice, Sinead, Skantha, Claragh, Jane, Peter, Vinny, Terry, Katie, Amanda, Garry and Jacqueline. Likewise, at TCD; Joan Gillen, Sheena Brown, Peter O'Reilly, Gerard Byrne and Danny Kelly.

I have enjoyed my time as a postgraduate student and that is due to the people I've worked with in the office; Orlaith, Oran, Ruth, Mike J, Claire, Ciara, Laura, Grainne, Niamh, Mike K, Frank, Amir, Christian, Elaine, John, Tayna, Sonia, Peter, Johannes, Tim, Lise, Jakob, Jan, Cornelia, Hardeep, Ross, Eadaoin, Aidan, Danny and David. It's a long list, but everyone has helped me over the course of this project and made my time spent in the office/pub more enjoyable. Additionally, I would like to thank the other students from RCSI and TCD that I've spent time with in the pub or at conferences over the last few years; Aileen, Eric, Mohamed, Mark, Padraig, Cormac,

Kiva, Michael, Dave, Brianne, Jennifer, Stephen, Louise, Emma, Damien, Conor, Ciarán, John and Aidan.

Finally I would like to thank my family. My parents, George and Maureen who have guided me from day one. This would not be possible without their support and encouragement. My siblings Stephen, George, Suzanne and my niece Rosella. Paul Doyle also deserves special mention for helping me avoid library fines. I would also like to thank my close friends Tom, Dave, Johnny, Kev, Will, Ronan, Riona & John.

I gratefully acknowledge grant support from the Science Foundation Ireland, under the President of Ireland Young Researchers Award.

Nomenclature

3D	3-Dimensional
β -TCP	Tri-calcium Phosphate
ANOVA	Analysis of Variance
atm	Atmospheric Pressure
CG	Collagen-Glycosaminoglycan
CO ₂	Carbon Dioxide
CP	Calcium Phosphate
ΔG	Increase in Molar Free Energy
ΔW	Percentage Change in Weight
DHT	Dehydrothermal
DNA	Deoxyribonucleic Acid
DSC	Differential Scanning Calorimetry
DTA	Differential Thermal Analysis
ECM	Extra Cellular Matrix
EDAC	1-Ethyl-3-(3-Dimethyl Aminopropyl) Carbodiimide
FDA	Food and Drug Administration
FT-IR	Fourier Transform Infra-Red
GTA	Glutaraldehyde
GAG	Glycosaminoglycan
g	Gram
HA	Hydroxyapatite
Hyl	Hydroxylysine
H&E	Haematoxylin and Eosin
hr	Hour
IR	Infra-red
in	Inch
k	Re-crystallisation Rate
L	Liquid Phase
MC3T3	Mouse calvarial osteoblastic cell line
MGO	Methylglyoxal
MIT	Massachusetts Institute of Technology
MSC	Mesenchymal stem cell

min	Minute
mTgase	Microbial transglutaminase
N	Newton
P	Pressure
P	Passage
P_0	Surrounding Pressure
P_v	Vapour Pressure over a Curved Interface
P_v^0	Normal Vapour Pressure
PBS	Phosphate buffered saline
PGA	Polyglycolide acid
PLA	Poly lactide acid
PLGA	Poly-DL-lactic-co-glycolic acid
p	Probability
RCSI	Royal College of Surgeons in Ireland
R	Universal Gas Constant
r_1, r_2	Radii of Curvature
rpm	Revolutions per Minuet
S	Solid Phase
SS	Stainless Steel
T	Temperature
T_c	Critical Temperature
T_{eu}	Eutectic Melting Temperature
T_g	Glass Transition Temperature
T_f	Freezing Temperature
TM	Trade Mark
UV	Ultra-Violet
V	Vapour Phase
V	Specific Volume
v	Volume
WLF	Williams-Landell-Ferry Equation
W	Weight
w/v	Weight to Volume Solution

L	Litre
ml	Millilitre ($\times 10^{-3}$)
μ l	Microlitre ($\times 10^{-6}$)
M (or mol)	Molar
mmol	Milimolar ($\times 10^{-3}$)
m	Metre
cm	Centimetre ($\times 10^{-2}$)
mm	Millimetre ($\times 10^{-3}$)
μ m	Micrometre ($\times 10^{-6}$)
nm	Nanometre ($\times 10^{-9}$)
Pa	Pascal
kPa	Kilopascal ($\times 10^3$)
MPa	Megapascal ($\times 10^6$)
mTorr	Millitorr ($\times 10^{-3}$)
W	Watts
mW	Milliwatts ($\times 10^{-3}$)
°	Degrees
°C	Degrees Celsius
γ	Surface Free Energy
η	Re-crystallisation Rate

Publications and Presentations

Journal Articles:

Haugh M.G., Jaasma, M.J. and O'Brien F.J. (2008) The effect of dehydrothermal treatment on the mechanical and structural properties of collagen-GAG scaffolds. *J Biomed Mater Res A*. In Press.

Byrne E.M., Farrell E., McMahon L.A., Haugh M.G., O'Brien F. J., Campbell V.A., Prendergast P.J. & O'Connell B.C. (2008) Gene expression by marrow stromal cells in a porous collagen-glycosaminoglycan scaffold is affected by pore size and mechanical stimulation. *J Mater Sci Mater Med*, 19, 3455-63.

Tierney C.T., Haugh M.G., Liedl J., Mulcahy F., Hayes B., Jaasma M.J. & O'Brien F.J. (2008) The effect of collagen concentration and crosslink density on biological, structural and mechanical properties of collagen-GAG scaffolds for bone tissue engineering. *Journal of the Mechanical Behavior of Biomedical Materials*. Accepted for Publication.

The work contained in this thesis has been presented at the following international and national conferences:

Murphy C.M., Haugh M.G. and O'Brien F.J. (2008) The effect of pore size on osteoblast activity in collagen-glycosaminoglycan scaffolds. *In: Abstracts from the Tissue Engineering International and Regenerative Medicine Society- EU Chapter Meeting, Porto, Portugal*. Abstracts published in *Tissue Engineering* 14(5): 365.

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Altenbucher C., Haugh M.G., and O'Brien F.J. (2007) EDAC crosslinking of collagen-GAG scaffolds. In: *Proceedings of the 13th Annual Conference of the Section of Bioengineering of the Royal Academy of Medicine in Ireland* – F. Buchanan, N. Dunne and J. Orr, (eds) Queen's University Belfast and University College Dublin (ISBN 1 905254 18 0): 88.

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1.1 Overview

Repair is the standard mammalian response to significant organ injury. It is typically characterised by closure of the wound through contraction and scar formation. Generally this means that the organ in question loses most or all of its functionality. Regeneration on the other hand is characterised by restoration of the normal structure and function of the organ (Yannas, 2001). The particular response of an organ to injury is dependant on the location and extent of the damage as well as the organ in question. The consequences of healing by repair range from physical disfiguration to a complete loss of organ functionality. Due to these deficiencies, it is evident that the induction of regeneration is favourable to repair. There are several approaches to induce regeneration in cases of severe organ trauma.

Transplantation is the most widely used treatment to overcome the deficiencies associated with tissue repair. During transplantation, healthy tissue is removed from a donor site and subsequently grafted to the injured site in order to induce regeneration. Autografting is transplantation using healthy tissue harvested from the patient, whereas allografting utilises tissue taken from a separate donor. Despite the widespread use of transplantation, there are several shortcomings of this treatment. With autografting, a longer operation is needed to remove tissue from the donor site; this harvesting puts extra stress on the patient and can cause complications such as tissue morbidity at the donor site (Giannoudis et al., 2005). As many patients requiring grafts are elderly, the quality and quantity of tissue available for grafting can also be an issue. With allografting, there is the risk of immune response and the transmission of viral diseases and infections from the donor. In addition the number of suitable donors available is another limiting factor (Lanza et al., 1997). Processing of

allograft tissue reduces the risk of infection but can significantly weaken the biological and mechanical properties initially present in the tissue.

In some cases permanent prostheses may be used to restore partial function to the injured organ. Common examples of this are artificial joint replacements, heart valves and pacemakers (Nelissen, 2003, Pontefract et al., 2004, Chan and Cardall, 2006). As they are fabricated from materials that are biologically inert, the immune response associated with allografting is avoided. However, the unnatural or artificial nature of prostheses causes several problems. Artificial hip prostheses are commonly made from metallic alloys such as titanium, these materials are several times stiffer than the tissue they replace. This causes stress shielding and subsequent resorption of the surrounding bone leading to failure of the prosthesis (Lewis et al., 1984). In addition, even with biologically inert materials there may be an inflammatory response resulting in scar tissue encapsulation, preventing restitution of normal tissue structure (Mikos et al., 1998). These deficiencies and the lack of biological function classify permanent prostheses as an inferior treatment compared to regeneration of the injured organ.

Tissue engineering provides an alternative to transplantation and prosthesis, with the potential to overcome the limitations of these treatments (Langer and Vacanti, 1993). Tissue engineering (or regenerative medicine) is defined as the application of scientific principles to the synthesis of living tissues using bioreactors, cells, scaffolds, growth factors, or a combination (Rose and Oreffo, 2002). The scaffold may be implanted alone to induce host cell migration to the wound site and tissue regeneration, or it may be seeded with cells and/or growth factors and serve to control the release and targeting of these treatments. Bioreactors which apply biophysical

stimuli to the cell-seeded scaffold are also commonly used to encourage cells to form tissue on the scaffold *in vitro*. Tissue engineering has found initial clinical success in several areas including skin, nerve and bladder (Yannas et al., 1989, Chamberlain et al., 1998, Atala et al., 2006).

In our laboratory we use a composite scaffold that is fabricated from collagen and a glycosaminoglycan (GAG) as a basis for tissue engineering. This scaffold is currently being used in a number of tissue engineering applications. Collagen has received increasing attention over the past few years owing to its excellent biocompatibility, degradation into physiological end-products and suitable interaction with cells and other macromolecules (Geiger et al., 2003). GAGs are common constituents of cell surfaces and extracellular matrices. The first generation of collagen-GAG scaffolds was developed for skin regeneration in patients with massive skin loss by Ioannis Yannas at MIT (Yannas and Burke, 1980, Yannas et al., 1980, Dagalakos et al., 1980). These scaffolds have also been investigated for their potential use in induced regeneration of peripheral nerves and articular cartilage (Chamberlain et al., 1998, Lee et al., 2001).

Previous work has shown that internal architecture and the mechanical properties of scaffolds for tissue engineering can have a significant influence on the activity of cells within them (O'Brien et al., 2005, Lee et al., 2001, Byrne et al., 2008). The aim of this thesis is to develop a series of novel CG scaffolds with a range of structural and mechanical properties and to determine the effects of these properties on cellular behaviour. This will allow the production of a series of characterised scaffold variants

which can be adapted for specific tissue engineering applications or used to expand our understanding of the interactions between scaffold properties and cellular activity.

1.2 Scaffolds for Tissue Engineering

One of the principal methods in tissue engineering involves the use of a porous scaffold to support and guide synthesis of a 3D tissue or organ (Sachlos and Czernuszka, 2003). The scaffold itself is therefore a very important aspect of tissue engineering. It has been proposed that a scaffold must fulfil a number of key criteria (Hutmacher, 2001):

- Biocompatibility
- Biodegradability
- Mechanical properties
- 3-D Porous architecture

Biocompatibility is a concept that is simple in principle but complicated in practice. It is typically defined as “the ability of a material to perform with an appropriate host response in a specific application” (Williams, 1986). The broadness of this definition reduces its usefulness somewhat and in practice, the term is widely used to describe materials which are non-toxic when implanted into the body. In addition, a biocompatible scaffold should also sustain cell function and phenotype if it is to be successful as a tissue engineering scaffold. Biodegradability is another important aspect of scaffold performance. The scaffold must be designed to degrade into biocompatible products throughout the healing process, eventually leaving repaired or regenerated tissue (Yannas, 2001). This process should be controllable as the scaffold

must also last long enough to support the activity of the cells as they carry out the remodelling process.

The mechanical properties of the scaffold are important for several reasons. The scaffold must have suitable characteristics in order to facilitate *in vitro* handling and withstand the *in vivo* environment. It is often stated that scaffolds need to have mechanical properties resembling that of native tissue (Luo et al., 2007). However, standard treatments for load bearing tissues use supports such as fixation and sutures to bear mechanical loading during healing and rehabilitation (Koob, 2002, Finkemeier, 2002). Additionally, it is worth noting that a mixture of bone marrow and demineralised bone matrix with the consistency of sand has been successfully used to treat non-union bone fractures, while even mechanically durable treatments such as cortical bone grafts require the use of fixation (Tiedeman et al., 1995, Finkemeier, 2002).

In addition, the mechanical properties of the scaffold can direct cellular activity and further determine the functionality of a tissue-engineered construct (Engler et al., 2004a, Engler et al., 2006, Engler et al., 2004b, Yeung et al., 2005). In a study by Engler *et al.* (2006), the differentiation of mesenchymal stem cells (MSCs) was shown to be dependant on the mechanical properties of the substrate they were seeded upon. Neurogenic, myogenic and osteogenic markers were found on substrates with Young's moduli of 1, 11 and 34 kPa respectively. It has also been noted that mechanical properties can influence the interaction between cells and the scaffold (Torres et al., 2000, Lee et al., 2001). Chondrocytes and fibroblasts were shown to contract the scaffolds they were seeded upon; the level of contraction was shown to be

dependant on the mechanical properties of the scaffolds (Torres et al., 2000, Lee et al., 2001). Contraction causes several problems, the pore volume and permeability of the scaffold is reduced, limiting cellular proliferation. In addition, *in vivo* contraction would cause problems at the interface between the scaffold and the host tissue. It is therefore essential that a scaffold has appropriate properties to limit cell-mediated contraction.

The pore architecture of scaffolds for use in tissue engineering has been shown to have a significant effect on physical properties, cellular activity and vascularisation (Wake et al., 1994, Nehrer et al., 1997b, Tsuruga et al., 1997, Zeltinger et al., 2001, O'Brien et al., 2005, O'Brien et al., 2007). It has been hypothesised that the pore diameter must be large enough to allow infiltration of the cells towards the centre of the scaffold, whilst being small enough to present sufficient ligand density for cellular attachment (Yannas, 2001, O'Brien et al., 2005). The optimal pore size is dependant on both the cell type and scaffold material. Upon collagen scaffolds with a range of pore sizes (95-150 μm), O'Brien *et al.* (2005) found that osteoblast attachment increased with decreasing pore size, with optimal attachment upon scaffolds with a mean pore size of 95 μm . Zeltinger *et al.* (2001) report that upon PLA scaffolds endothelial cells prefer pores in a range of 30-80 μm . Furthermore, it is commonly reported that large pores (>100 μm) are required for bone formation and vascularisation (Tsuruga et al., 1997, Byrne et al., 2008, Cao et al., 2006). In addition, the pore size also determines permeability, which in turn influences the diffusion of nutrients and waste products within the scaffolds as well as the stimulus applied by flow perfusion bioreactors (Porter et al., 2005, O'Brien et al., 2007).

As the exact requirements are dependant on the specific application, many materials have been proposed as scaffolds suitable for tissue engineering. There are three major classifications of materials that have been used as scaffolds for tissue engineering: ceramics, synthetic polymers and natural polymers. These classifications are discussed in the following sections.

1.2.1 Ceramic Scaffolds

Ceramics are inorganic non-metallic compounds; they are crystalline in nature and are formed from a combination of metallic and non-metallic elements. They typically have a high stiffness but are very brittle. Because of their high stiffness, ceramic scaffolds are most commonly used in bone tissue engineering. The most common ceramics used are calcium phosphate (CP), tri-calcium phosphate (β -TCP) and hydroxyapatite (HA) (Barralet et al., 2002, Chu et al., 2002, Porter et al., 2004). HA is a natural constituent of bone and has been shown to be biocompatible and osteoconductive. Both β -TCP and HA are highly biocompatible. They differ however, in the biological response created at the host site. Porous β -TCP is removed from the implant site as bone grows into the scaffold while HA is more permanent (Giannoudis et al., 2005). In general, ceramic scaffolds have been found to be brittle and there is a lack of control of the degradation rate, which is generally very slow.

1.2.2 Synthetic Polymer Scaffolds

Many polymers have been investigated for use in tissue engineering applications. The most commonly used polymers are polylactide acid (PLA), polyglycolide acid (PGA) and a combination of the two, poly-DL-lactic-co-glycolic acid (PLGA) (Taboas et al., 2003, Lin et al., 2003). The popularity of these polymers as biomaterials is in no small part due to their FDA approval for use in joint replacements. The main advantage of

using synthetic polymers is the fact that they can be manufactured with a wide range of mechanical properties and architectures. The fundamental problem associated with the use of synthetic polymers is their degradation products, acidity and alcohol content. In particular, scaffold acidity has been implicated in accelerating the degradation of the scaffold and causing a pronounced inflammatory response in the surrounding tissue leading to an inhibition of tissue formation (Fischer and Reddi, 2003).

1.2.3 Natural Polymer Scaffolds

Natural polymers include collagen, chitosan, gelatin, silk fibrin, glycosaminoglycans and elastin (Park et al., 2002, Nazarov et al., 2004, Dawson et al., 2008). These materials are found naturally in the extra cellular matrix (ECM) of many tissues and as such contain many surface ligands and peptides appropriate for cellular attachment and cell-scaffold interactions (Harley and Gibson, 2008). As biological materials they have excellent biocompatibility and non-toxic degradation products. The shortcoming of natural polymers is their poor mechanical properties, which are not sufficient for high strength applications unless the polymer is chemically treated. In this study a composite of two natural polymers, collagen and chondroitin sulphate, is used as a scaffold.

1.3 Collagen-GAG Scaffolds

1.3.1 Collagen and Glycosaminoglycans

Collagen is the most abundant structural protein found in the body. There are more than twenty genetically distinct types of collagen molecules (Gelse et al., 2003), all of which are triple helical molecules based on polypeptide chains of amino acids.

Collagen type I is the most abundant collagen found in the human body. It is found in skin, ligaments, tendons and it is the main organic substance found in bone. The scaffolds used in this project are fabricated from collagen type I.

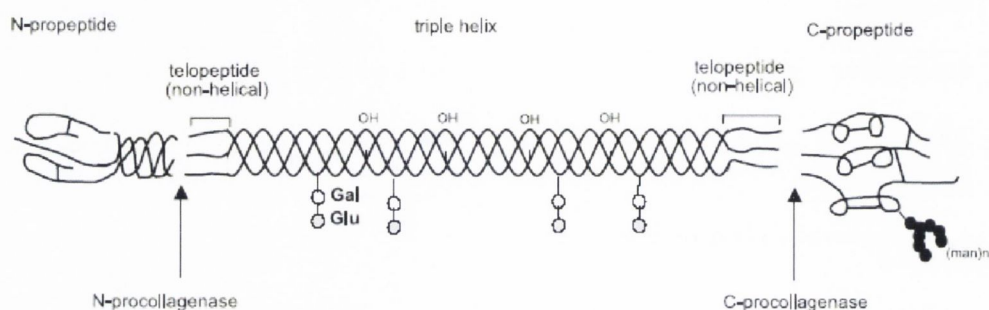


Figure 1.1 Collagen type I structure (Gelse et al., 2003).

Collagen type I is a heterotrimer (Figure 1.1), meaning it is a triple helix that consists of two $\alpha 1$ chains and an $\alpha 2$ chain. The $\alpha 2$ chain has a slightly different amino acid sequence compared to the $\alpha 1$ chains. Collagen type I triple helices are 300nm long and contain approximately 3000 amino acid residues. Each of the three α -chains within the molecule forms an extended left-handed helix with a pitch of 18 amino acids per turn (Hofmann et al., 1978). The three chains, staggered by one residue relative to each other, are supercoiled around a central axis in a right-handed manner to form the triple helix (Fraser et al., 1979). The polypeptide chains have a repeating unit (Gly-X-Y), with a glycine residue in every third position in the polypeptide chain. The plane of each peptide bond is positioned perpendicular to the axis of the helix so that the carbonyl groups are pointed in a direction where they form strong interchain hydrogen bonds with other chains in the molecule (Devlin, 2006). The triple helix is formed such that glycine, the smallest amino acid, is positioned at the centre of the helix and the more bulky side chains of other amino acids occupy the outer positions.

This allows a close packing along the centre of the molecule as shown in Figure 1.2 B (Gelse et al., 2003). The X and Y positions are most commonly occupied by the amino acids proline and hydroxyproline, respectively. A breakdown of the amino acid content of type I collagen is shown in Figure 1.2 C. The non-helical regions at the end of the collagen molecule are called telopeptides; these are involved in intermolecular crosslinking of collagen molecules. The propeptides are important in the formation of the collagen molecules. Once triple helices are formed, they group together to form collagen fibrils. These fibrils have a distinctive banded appearance illustrated in Figure 1.3, which is due the quarter-staggered alignment.

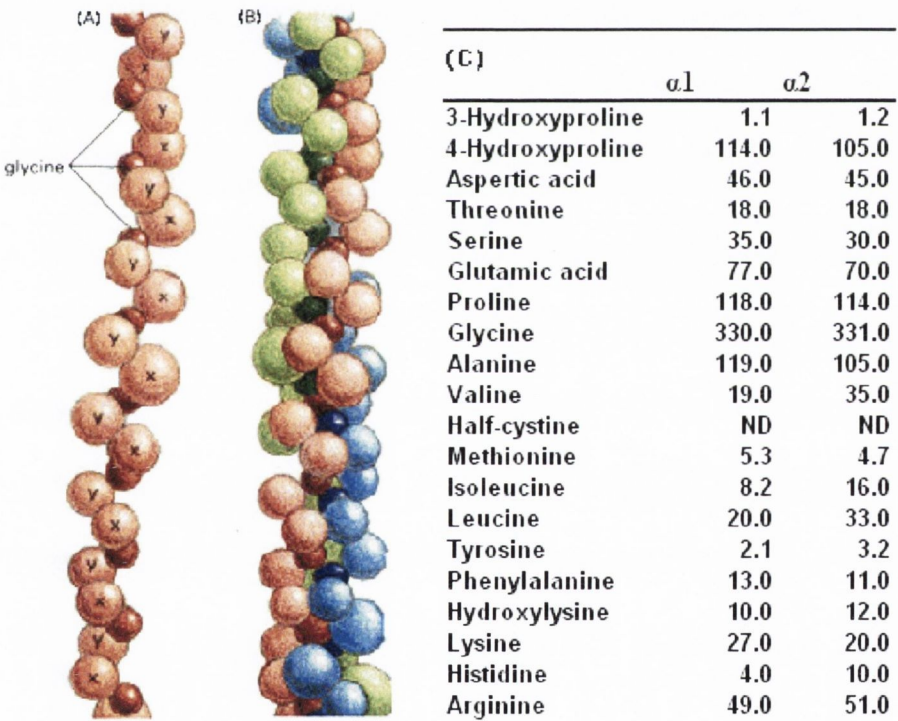


Figure 1.2 (A) Collagen chain (B) Collagen molecule (C) Amino acid composition of type I collagen (Burgeson et al., 1976, Alberts et al., 1998).

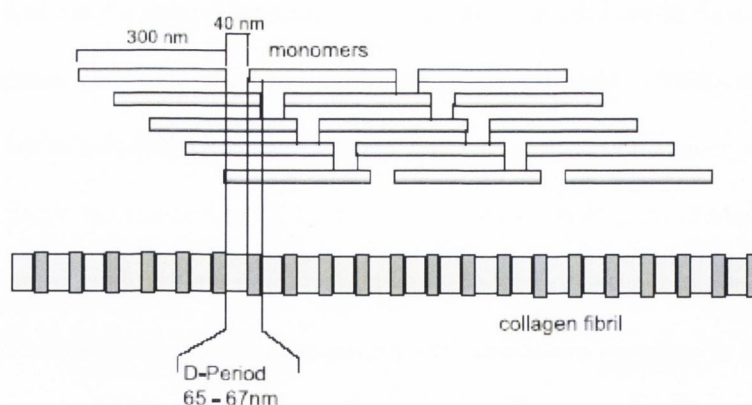


Figure 1.3 Collagen fibril structure, showing the banded structure (Gelse et al., 2003).

Glycosaminoglycans (GAGs) are added to the scaffold in order to further increase the biocompatibility. They are, with the exception of hyaluronic acid, found in the form of proteoglycans on cell surfaces and in the extracellular matrix. Chondroitin-6-sulphate is the GAG used to fabricate collagen-GAG scaffolds (Figure 1.4). It is a major constituent of cartilage and provides much of that tissue's compressive strength (Bayliss et al., 1999). GAGs are able to bind and modulate various proteins including growth factors, cytokines and extracellular matrix proteins. These interactions are essential for the adhesion, migration, proliferation and differentiation of cells. As a consequence, GAGs play a pivotal role in biological processes like morphogenesis and wound healing. Incorporation of GAGs into a tissue engineered construct may therefore enhance cellular activity (Pieper et al., 1999). Additionally, polymerisation of collagen with GAGs has been shown to improve the mechanical properties of collagen (Yannas et al., 1975).

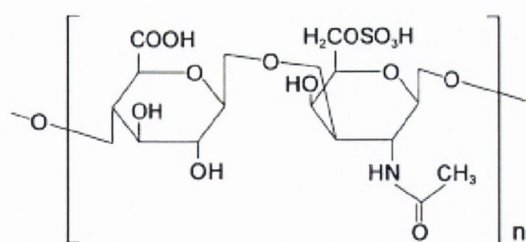


Figure 1.4 Schematic of chondroitin-6-sulphate (Huang and Yang, 2008).

1.3.2 Collagen-GAG Scaffolds

As a natural polymer, collagen is often utilised for its inherent biocompatibility. Collagen has been processed into a variety of physical forms, including mini-pellets, sponges and nanoparticles for the use as drug and gene delivery vehicles, as an injectable suspension for soft tissue augmentation, as matrices for use as wound dressings, and as constructs for engineered blood vessel, heart valve, tendon and skin substitutes (Dong et al., 2005). Collagen-GAG scaffolds were first developed by Ioannis Yannas at MIT for use in skin regeneration (Dagalakis et al., 1980, Yannas and Burke, 1980, Yannas et al., 1980). Since then, variants of these scaffolds have shown potential for use in nerve, cartilage and bone regeneration (Chamberlain et al., 1998, Lee et al., 2001, Farrell et al., 2006, Lynn et al., 2005). Chamberlain *et al.* (1998) found that peripheral nerve regeneration utilising CG scaffolds was of the same standard as autografting. Farrell *et al.* (2006) demonstrated that CG scaffolds support the differentiation of MSCs down chondrogenic and osteogenic lineages. The advantages of these scaffolds include their biocompatibility, porosity (99.5%) and non-toxic degradation products. Furthermore the natural availability of binding sites upon collagen, allows the scaffolds to be used a delivery vehicle for cytokines and growth factors.

1.3.3 Fabrication of Collagen-GAG Scaffolds

Collagen-GAG scaffolds are manufactured using a freeze-drying (or lyophilisation) process (Dagalakis et al., 1980, O'Brien et al., 2004, O'Brien et al., 2005). Prior to freeze-drying, collagen and GAG are mixed together with acetic acid to produce a collagen-GAG suspension. This suspension is then placed into a freeze-dryer which cools it in a controlled manner. As the slurry freezes the collagen-GAG solute is localised between the growing ice crystals, forming a continuous, interpenetrating network of ice and solute. Sublimation of the ice crystals leads to the formation of a highly porous sponge. The pore size of the scaffold mirrors the size of the ice crystals formed during freezing and is therefore affected by the details of this process. O'Brien *et al.* (2004) developed a method for cooling the scaffolds at a constant rate to give a homogeneous scaffold with equiaxed pores. The effect of freeze-drying on the pore architecture of CG scaffolds is discussed in detail in Section 1.4 and Chapter 2. After freeze-drying, CG scaffolds are crosslinked in order to enhance their mechanical properties and reduce their degradation rate. The standard crosslinking method is dehydrothermal treatment (DHT), although several different treatments have been utilised in the literature (Lee et al., 2001, Harley et al., 2007, Pieper et al., 2000). This topic is discussed in further detail in Section 1.5 and Chapters 3 & 4.

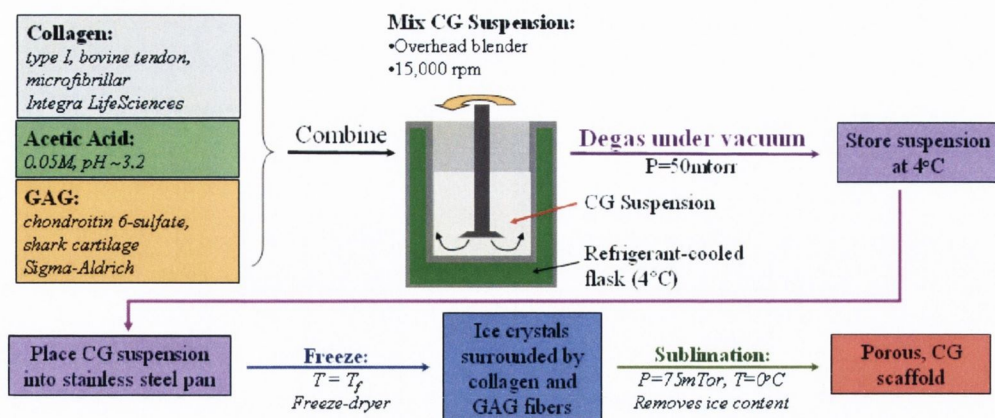


Figure 1.5 Scaffold fabrication (Harley et al., 2005).

1.4 Freeze-drying

Freeze-drying (or lyophilisation) is a process in which a solution, a mixture of a solute and a solvent, is frozen and then dried. It is primarily a means of increasing the stability of materials for long term storage and is most commonly used in the pharmaceutical and biological industries. The freeze-drying process has several advantages over other drying methods, the main one being that it can be applied to materials, such as proteins, which may be affected by the addition of heat. The major disadvantage is that it is an energy intensive process. In this study we use the freeze-drying process to fabricate porous collagen scaffolds; consequently an understanding of the process is needed to consistently produce homogeneous scaffolds.

The freeze-drying process can be split into two parts: freezing and drying. During freezing the temperature of the solution is lowered and a phase change occurs during which liquid water is converted into ice (Figure 1.6). The next step in the process is drying. During drying, heat is added to the material under a vacuum. Under these

conditions the ice crystals are sublimated from the solute. The sublimated water vapour is then trapped on a condenser, and the process is complete.

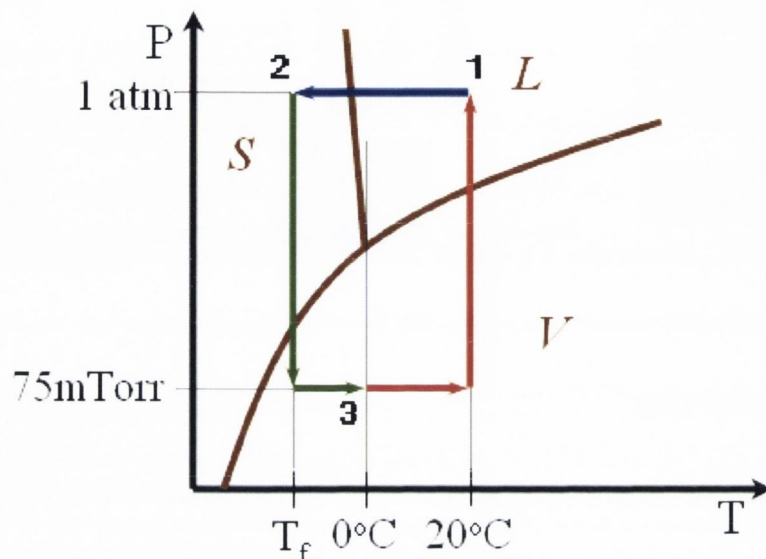


Figure 1.6 Phase diagram of the freeze-drying process (Harley et al., 2005). Where S indicates the solid phase, L the liquid phase, V the vapour phase and T_f the final freezing temperature. The process starts at point 1, the first step is freezing the solution to point 2. After freezing the frozen solution is brought to point 3 where drying takes place for 12 hours. When drying is complete, all the water has been removed from the solution and only the collagen scaffold remains on the shelf. The scaffold is then brought back to room to 20°C and 1 atmosphere before it is removed from the freeze-dryer.

1.4.1 Freezing

During freezing a two step process occurs in which ice crystals nucleate around particulate impurities within the solution and then grow in size. Conventionally, the structure of the ice crystals formed during freezing is only of significance because of its effect on drying; however, when producing scaffolds for tissue engineering it has further significance as the ice crystal structure will mirror the pore structure of the scaffold produced. The important structural characteristics are pore size, shape, distribution and interconnectivity.

Before describing the freezing process it is important that two words are defined: cooling and freezing. Cooling is a reduction in temperature, whereas freezing is an abrupt conversion of a solution into a mixture of ice and solute concentrate. Cooling below freezing temperature without freezing is called supercooling or subcooling (Biopharma, 2005). The cooling rate is an important aspect of the freezing cycle; traditionally these rates have been described in an unscientific manner, i.e. slow or fast. A better description is the rate at which the shelf or product is cooled per unit time; shelf temperature is most commonly used to control the process. Typically cooling rates of less than 1°C/min are considered slow and anything above (but usually in the region of 20-50°C/min) is considered fast (Biopharma, 2005).

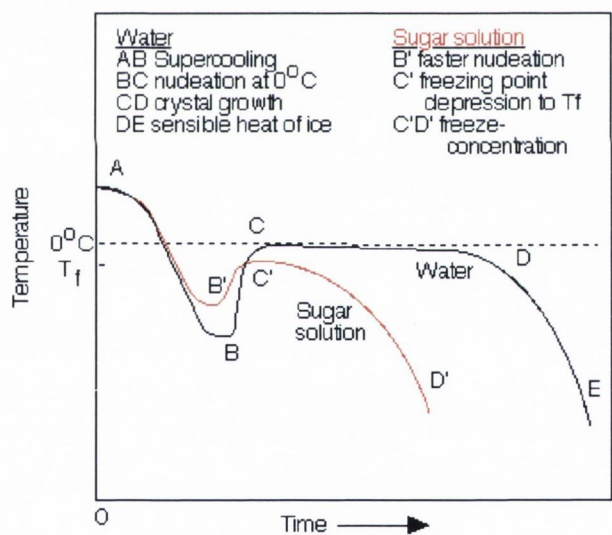


Figure 1.7 Product temperature during freezing (Sahagian and Goff, 1996).

In order to understand the freezing process, it is necessary to study the time-temperature relationships of pure water and aqueous solutions as they are frozen (Figure 1.7). There are four events in this process: supercooling, nucleation, crystal growth and freeze-concentration. From point A to B (and B') the solutions are supercooled below the freezing temperature of water before nucleation, the formation

of ice crystals throughout the liquid volume, takes place. The presence of the solute particles promotes nucleation; explaining why the sugar solution nucleates at a higher temperature than pure water. Supercooling is a metastable state which is analogous to an activation energy necessary for the nucleation process (Sahagian and Goff, 1996). Nucleation subsequently takes place from point B to C (B' to C'). However, only a fraction of freeze-able water crystallises during nucleation, as crystallisation is exothermic (Searles, 2004). Note that the T_f of the sugar solution is lower than the T_f of water, this freezing point depression is due to the presence of solutes.

Crystal growth and freeze concentration take place from C to D. The crystals grow as the remaining water in the nucleated solution is frozen. The partially frozen mixture will not cool until all of the freezeable water has crystallised. With the sugar solution, freeze concentration takes place from C' to D'. As water freezes in the solution, the concentration of the solute in the liquid phase increases. This phenomenon is termed freeze concentration. Freeze concentration lowers the freezing temperature and increases the viscosity of the solution. The increase in viscosity of the solution, caused by lower temperatures and freeze concentration, reduces the mobility of water molecules and hinders crystal growth.

The morphology of ice crystals in the frozen product is primarily dependent on nucleation. In particular, ice crystal size has been shown to be inversely proportional to the extent of supercooling at nucleation (Searles et al., 2001). Nucleation is, in turn, affected by the rate of cooling and the nature of particulate impurities within the solution. As nucleation is affected by environmental particulates, it is therefore not under direct control and leads to a degree of variability in the process. The rate of

cooling will also have an effect on the crystal formation during freezing. Fast rates of cooling have a tendency to produce many small ice crystals. These crystals may not be continuous, but they are generally homogeneously distributed throughout the frozen solution. A slow rate of freezing will result in a more connected ice crystal structure with larger crystals (Biopharma, 2005).

1.4.2 Drying

Drying is the second step in the freeze-drying process and it involves the removal of water from the frozen solution. Traditionally, drying can be split into primary and secondary drying, where primary refers to the removal of water by sublimation, the conversion of a solid directly into a vapour, and secondary drying is the removal of bound water by desorption. As the standard crosslinking treatment for the collagen-GAG scaffolds (DHT) removes bound water when it forms crosslinks, there is no need for secondary drying in our freeze-drying cycles.

Figure 1.6 shows how the phase of water is related to its temperature and pressure. From this chart it is clear that ice can be converted directly to vapour by adding heat at low pressures. This phase change requires a large amount of energy, approximately 2800 J to convert 1 g of ice to vapour (Biopharma, 2005). Having frozen the solution and created a solid, the pressure is reduced and heat is applied to sublimate the ice to a vapour. The vapour is then trapped on the condenser as ice. Because sublimation is endothermic, drying is sustained by applying heat to the solution. However, this heat must be applied with care as excessive heat input may melt or collapse the scaffold (described later). In contrast, applying insufficient heat will result in the solution cooling until its temperature approaches that of the condenser, which causes

sublimation to cease. The endpoint of the drying process is accepted to be the convergence of shelf and product temperature, i.e. when adding heat increases the temperature of the solution rather than fuelling sublimation (Biopharma, 2005).

In order to trap water vapour on the condenser, a pressure difference is needed between the ice interface in the product and the cold surface of the condenser. Additionally, a pressure gradient between the water molecules and the surrounding atmosphere is also necessary and this is provided by the vacuum. It is known that all materials exert a vapour pressure and this pressure is related to temperature; so we may define the driving force for sublimation as the difference in vapour pressure between the sample and the condenser. However, these concepts of sublimation do not take into account resistance to the vapour flow as it migrates from the drying solution to the condenser. Resistances to vapour flow include mechanical impedances by baffles, interconnections and valves in the system and impedances resulting from interactions between vapour molecules and the inner vial surface and stopper. These resistances have to be taken into account when choosing the condenser temperature of a cycle. Typically 15° below the product temperature is sufficient to overcome any resistance to vapour flow (Biopharma, 2005).

In drying a number of processing defects occur which need to be avoided. These include:

- Eutectic melting. This is defined as the melting of a eutectic solid (region of a frozen material outside of the 'pure ice' fraction). When under vacuum, this

can lead to severe eruption of the material. The temperature at which eutectic melting occurs is T_{eu} .

- Collapse. This is defined as the point at which a material softens to the extent of no longer being able to support its own structure as the sublimation interface passes through it. This will be above the glass transition temperature (T_g), but the extent above T_g depends on the solution constituents. The glass transition temperature is the temperature below which the viscosity of the frozen solution will increase and the material takes on a brittle glass like state, above this temperature the material becomes softer.

The critical temperature (T_c) is defined as the highest temperature that can be used in the drying phase without the occurrence of processing defects. Knowledge of the critical temperature of the solution is essential to avoid processing defects and use as high a drying temperature as possible, which speeds up the drying process. Various methods can be used to define T_{eu} and T_g . Collapse temperatures are determined by the glass transition temperature using Differential Scanning Calorimetry (DSC) or, alternatively, the temperature at which the drying solution is microscopically observed to distort and collapse, can be measured using freeze-drying microscopy. Freeze-drying microscopy provides the least ambiguous data, since it is used to assess the operational consequences of exceeding T_g or T_{eu} . Collagen-GAG scaffolds are dried above T_g , but as the associated collapse is not observed, this allows the use of high temperatures which allow fast drying.

1.4.3 Freeze-drying of Collagen-GAG Slurry

The standard freeze-drying cycle that is currently used to produce CG scaffolds is as follows (Table 1.1); the shelf is held at twenty degrees for 5 minutes and then cooled at a rate of 0.9°/min to the final freezing temperature. The final freezing temperature is then held for two hours. The drying phase is commenced; pressure is reduced to 200 mTorr and the temperature is ramped to 0°C in 160 minutes. This temperature is then held for 1020 minutes (17 hours). Finally the shelf is brought up to room temperature in order to prevent moisture from condensing upon the scaffolds on opening of the freeze-drying chamber. Altogether, the cycle takes approximately 24 hours.

Freezing Step	Temperature, °C	Time, min	R/H	Pressure, mTorr
1 (start)	20	10	H	Atm
2 (ramping)	-40	45	R	Atm
3 (annealing)	-40	120	H	Atm
4 (drying)	-40	5	H	0200
5 (drying)	00	160	R	0200
6 (drying)	00	1020	H	0200
7 (drying)	20	40	R	0200
8 (drying)	20	30	H	0200

Table 1.1 Freeze-drying cycle for collagen-GAG scaffolds.

An indication of the freeze-drying behaviour of CG slurry during this cycle can be obtained from the results of O'Brien *et al.* (O'Brien et al., 2005). Upon initial inspection of the cooling profiles of the solutions (Figure 1.7), it can be seen that they exhibit some of the behaviour of both pure water and solutions. The solute

concentration of the slurry is very low, 0.5%, explaining why the freezing profile of the solution is similar to pure water.

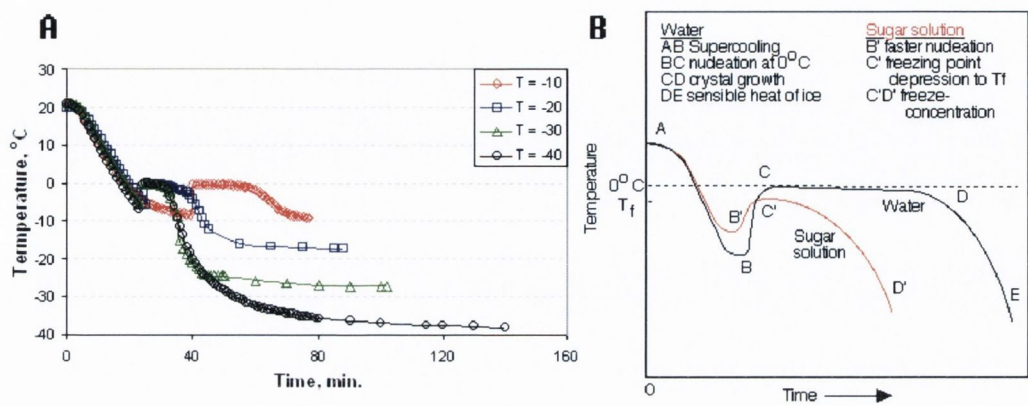


Figure 1.8 Product temperatures during freezing (A) Collagen-GAG (B) Water and sugar solution (Sahagian and Goff, 1996, O'Brien et al., 2005).

Scaffold pore size analysis (Table 1.2) demonstrated that crystal size decreased with final shelf temperature (O'Brien et al., 2005). From Figure 1.8 it can be seen that the crystal growth phase of the -10°C and -20°C cycles are shorter than the -30°C and -40°C cycles. This can only explain some of the differences in pore size however, as the crystal growth phases of the -30°C and -40°C cycles are identical, indicating that the crystals are still growing during freeze concentration (D to E). The difference in pore size between the -30°C and -40°C cycles can then be explained by a reduction in the rate of crystal growth due to increased viscosity caused by lower temperature and freeze concentration. The pores of the -20°C, -30°C and -40°C scaffolds were found to be equiaxed, while the -10°C scaffolds had pores which were larger in the longitudinal axis. This points to preferential cooling in the longitudinal direction during the -10°C cycle.

Freezing temperature (°C)	Pore size (µm)
-10	150.5
-20	121.0
-30	109.5
-40	95.5

Table 1.2 Effect of freeze-drying temperature on pore size for collagen-GAG scaffolds (O'Brien et al., 2005).

The work of O'Brien *et al.* (2005) will be expanded upon in this study, due to availability of a freeze-dryer with the ability to run cycles with a T_f of -70°C . This will allow investigation of the effects of T_f on pore size at temperatures below -40°C . Additionally, the effect of introducing an annealing step into the cycle will also be investigated in order to produce scaffolds with a larger range of mean pore sizes.

1.4.4 Annealing

The first study in this thesis investigates the potential of using an annealing step to increase the pore size of CG scaffolds. Heat annealing is an additional step in the freeze-drying cycle which involves raising the temperature of the frozen suspension after freezing in order to increase the rate of ice crystal growth by reducing viscosity (Searles, 2004). This step causes significant changes in the ice crystal structure in the frozen solution.

In general, ice crystals which existed prior to warming will become larger and solutes which are reluctant to crystallise may crystallise when the solution is cooled for the second time. Re-cooling after annealing should not result in new crystal growth as the volume is already nucleated. Rather upon re-cooling the remaining crystals will grow. Connectivity is also increased; and this coupled with the increase in ice crystal size

allows faster drying rates by decreasing resistance to vapour flow. Higher annealing temperatures and longer durations correlate with increased drying rates, but the effect of annealing on drying rates appears to be constrained by a maximum theoretical value. The cause of this limit was shown to be a shift from a mass transfer dependant process (water vapour transit through the dried layer of the product) to an energy transfer dependant process (energy from the shelf to the ice in the solution) due to decreased mass transfer resistance of annealed samples (Searles, 2004).

During annealing the increased water content and higher temperature increase the bulk mobility of the amorphous (unfrozen) phase and the diffusional mobility of all species in that phase. The increased bulk mobility of the amorphous phase during annealing allows it to relax into physical configurations of lower surface free energy (or surface tension). Surface free energy will provide a driving force for a contiguous volume to reduce its surface area so long as it is not already a perfect sphere. The structures within freeze-concentrated systems invariably possess junctions, edges, and other departures from the perfect sphere. Each of these three-dimensional features has two principal radii of curvature. The pressure within a structure with positive radii of curvature can be shown to be (Searles, 2004):

$$P = 2\gamma \left(\frac{1}{r_1} + \frac{1}{r_2} \right) + P_0$$

Where γ is the surface free energy (energy/area), r_1 and r_2 are the radii of curvature, and P_0 is the surrounding pressure. A practical example of how this works is a needle shaped structure, the tip will have very small radii of curvature and the body will have much larger radii of curvature. Using the above equation, the pressure in the tip will be much higher than the pressure in the body. If the viscosity is sufficiently low

(above T_g) the pressure will cause bulk flow of material from the tip into the body. Structures with negative internal radii, such as the junction between of two spheres in contact, will have a lower pressure than their surroundings, leading to flow of material into these areas. Sintering and accretion of high surface area particles into a single lower surface area structure are manifestations of this principle (Searles, 2004).

Another fundamental relationship of surface chemistry is the Kelvin equation, which states that smaller radii of curvature induce a higher vapour pressure:

$$\Delta G = RT \ln \frac{P_v}{P_v^0} = \gamma V \left(\frac{1}{r_1} + \frac{1}{r_2} \right)$$

Where ΔG is the increase in molar free energy, R is the universal gas constant, T is temperature, P_v^0 is the normal vapour pressure, P_v is the vapour pressure over a curved interface, V is the specific volume and r_1 and r_2 are the radii of curvature. Ice crystal regions with smaller radii will melt preferentially due to their higher free energy because smaller ice crystals have higher vapour pressures than larger ones. This may cause the smallest ice crystals to melt completely during annealing.

Ostwald ripening (re-crystallisation) can also occur during annealing. During Ostwald ripening dispersed crystals that are smaller than a critical size decrease in size while those larger than the critical size grow larger (Searles, 2004). In Ostwald ripening, the cubed average crystal diameter increases linearly with time, and the temperature dependence has been shown to follow the Williams-Landell-Ferry (WLF) equation:

$$\ln(k/k_0) = K(T - T_0)/(T - T_\infty)$$

Where k and k_0 are the re-crystallisation rates at T and T_0 , respectively. K is a constant and T_∞ is the temperature at which $k = 0$. Adherence to this equation suggests that the process is controlled by viscosity which Searles suggests is via translational diffusion of water through the non-ice phase, and this can be expressed in an alternate form as follows:

$$\ln(\eta/\eta_g) = C_1 (T - T_g) / (C_2 + (T - T_g))$$

Where η and η_g are the re-crystallisation rates at T and T_g , respectively, and C_1 and C_2 are constants.

1.5 Collagen Crosslinking

Crosslinks are chemical bonds between collagen molecules. They provide the mechanical strength of collagen and stabilise the collagen fibres by preventing the long rod-like collagen molecules from sliding past each other under stress (Bailey et al., 1980). Crosslinking is also an effective means of controlling the degradation rate of collagen scaffolds, as the crosslinks have to be broken down before the scaffold can be degraded. There are various methods of increasing the level of crosslinking in collagen scaffolds. The treatment used should depend on the application in which the scaffold is to be employed.

Crosslinking treatments are split into two groups: chemical crosslinking and biophysical crosslinking. Examples of chemical methods include the use of glutaraldehyde and various carbodiimides, whereas biophysical methods include the use of U.V. light and dehydrothermal crosslinking. The crosslinking efficiency of any treatment, physical or chemical, is a function of the number of amino acid residues

which can be affected by the treatment, the stability of the crosslink formed, and the bond energy associated with each crosslink (Weadock et al., 1983). Table 1.3 shows some of the reactive residues for various physical and chemical methods.

Procedure	Reactive Residues	Reactive Residues Per Alpha Chain		Sum of Reactive Residues
		$\alpha 1$	$\alpha 2$	
Short-wave U.V. Light	Tyrosine	4	3	51
	Phenyl-alanine	12	16	
Dehydro-thermal or Carbodiimide	Lysine	32	21	745
	Arginine	50	57	
	Aspartic acid	45	47	
	Glutamic acid	77	71	
	Serine	34	24	
	Threonine	16	17	
Gluter-aldehyde	Lysine	32	21	53

Table 1.3 Theoretical distribution of target amino acid residues for various collagen crosslinking treatments (Weadock et al., 1983).

When choosing a crosslinking treatment it must be kept in mind that there may be side effects. For example, many chemical treatments are unsuitable for tissue engineering because of toxicity or low water solubility; they may also leach out of the material, polymerise or form unstable crosslinks. Biophysical methods generally avoid introducing cytotoxic chemical residuals and therefore, retain the biocompatibility of the scaffold. The limitation of biophysical methods appears to be in trying to achieve a balance between attaining the desired amount of crosslinking whilst preventing degradation as a consequence of the long exposure times inevitable for physical processes (Khor, 1997).

This study will use several crosslinking methods to produce a collagen-GAG scaffold with a range of mechanical properties suitable for tissue engineering. The treatments used in this study are DHT, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDAC) and glutaraldehyde (GTA). These treatments are the most commonly used to

crosslink collagen in the literature. Microbial transglutaminase (mTgase) and ultraviolet light (UV) are also discussed as they are of interest. Additionally, chemicals such as formaldehyde, polyether oxide, hexamethylene diisocyanate and polyurethane have also been used to crosslink collagen. However they have been less successful due to their cytotoxicity and will not be examined in this study.

1.5.1 Glutaraldehyde

Glutaraldehyde (GTA) is one of the most popular crosslinking treatments for biological tissues. At low concentrations GTA produces intramolecular crosslinks in collagen, while at higher concentrations GTA forms long polymeric chains which produce intermolecular crosslinks (Weadock et al., 1983). Due to its potential to form long polymeric chains, glutaraldehyde can link residues that are spaced far apart and thereby enhance the extent of crosslink formation. Furthermore GTA crosslinking has been shown in some studies to induce calcification *in vivo*; this could be an advantage for bone graft substitutes (Kikuchi et al., 2004, Jorge-Herrero et al., 1999). Although it is widely used, GTA crosslinking is often cited as being undesirable as it introduces cytotoxic aldehyde molecules. The aldehyde may remain non-specifically bound to the matrix even after exhaustive rinsing and the aldehydes that have been incorporated into the matrix may be released from the matrix as it degrades (Lee et al., 2001). Heterogeneous crosslinking is another disadvantage associated with GTA (Weadock et al., 1983). Ruijgork *et al.* investigated the use of microwave irradiation to enhance the speed of GTA fixation (Ruijgrok et al., 1994). However this was shown to be only slightly more effective than conventional heating. The advantage of heating (to 50°C) is the formation of monomer GTA from polymer GTA. Monomer GTA is thought to crosslink collagen homogeneously as it is small enough to penetrate the matrix. The

diffusion rate will also be higher than at room temperatures and the reaction will be accelerated.

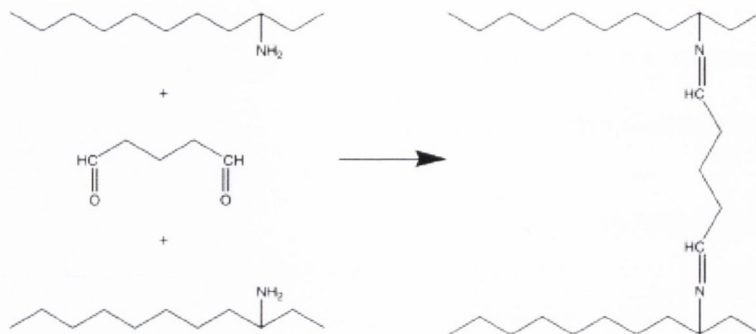


Figure 1.9 Glutaraldehyde crosslink formation (Lee et al., 2001).

1.5.2 Carbodiimides

Carbodiimides are used to crosslink many different types of functional groups. They can be used to form an isopeptide bond between the carboxyl and amino groups from different residues; the only by-product of this reaction is water-soluble urea which can easily be removed from the scaffold. The length of the particular carbodiimide chain used is a factor in its effectiveness as a crosslinking agent; shorter carbodiimides may be unable to crosslink residues with long separation distances.

In the literature two different types of carbodiimide have been used to crosslink collagen; cyanamide ($\text{H}-\text{N}=\text{C}=\text{N}-\text{H}$) and 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDAC) (Weadock et al., 1983, Pieper et al., 2000, Lee et al., 2001). Both EDAC and cyanamide provide no components to the final crosslinked product (Figure 1.10), because of this the two residues must be in direct contact *in situ* and the crosslinks formed are referred to as “zero length” crosslinks. Carbodiimide crosslinking has also been used to incorporate GAGs into the scaffold by introducing amide crosslinks (Pieper et al., 2000). Research by Ma *et al.* indicated that the

presence of lysine during EDAC crosslinking reduced the degradation rate of collagen scaffolds whilst degradation was accelerated by the addition glutamic acid (Ma et al., 2004). Unfortunately, no mechanical tests were carried out on the scaffolds in this study. Olde Damink *et al.* (1996) carried out an extensive study optimising the EDAC crosslinking of dermal collagen of sheep (non-collagenous constituents were removed from the dermal layer). The techniques employed in that study will serve as the basis of EDAC crosslinking during the course of this project.

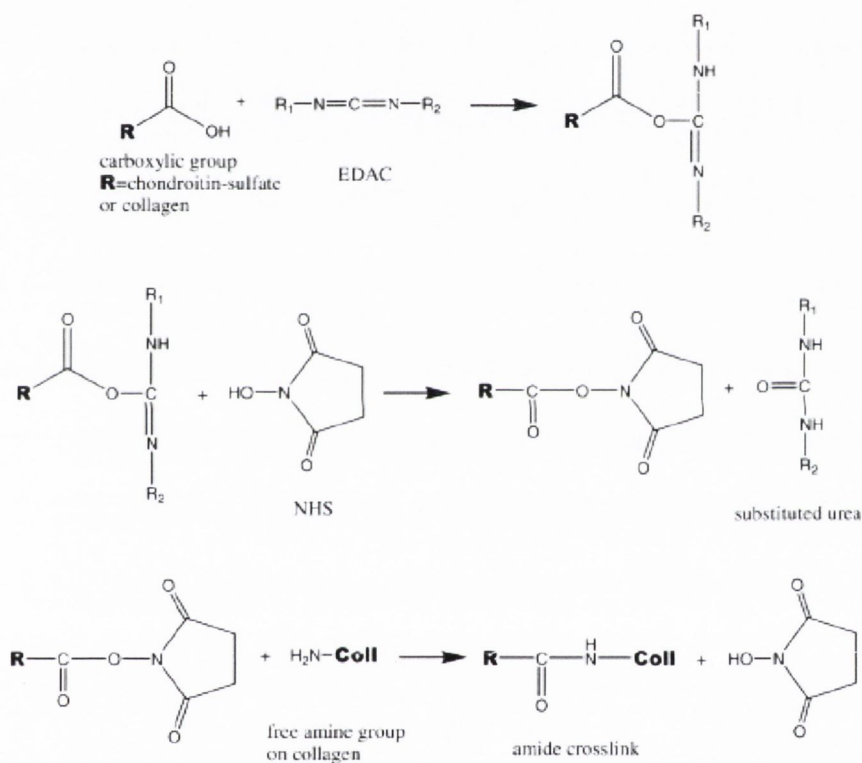


Figure 1.10 EDAC crosslinking (Lee et al., 2001).

1.5.3 Microbial Transglutaminase

Microbial transglutaminase (mTgase) is an enzyme which catalyses the formation of a crosslink between amino acid residues in adjacent collagen molecules. Ajinomoto Corporation Inc. Japan has recently made available a microbial form of transglutaminase which is suitable for industrial and laboratory use. As a crosslinker, transglutaminase is of particular interest as it has been shown to mimic *in vivo* crosslinking by creating crosslinks between collagen molecules without altering the natural characteristics of the material, such as the banding period or the triple helical structure of the molecule.

Transglutaminases catalyze an acyl-transfer reaction in which γ -carboxamide groups of peptide bound glutamine residues act as the acyl donor and, generally, the ϵ -amino groups of lysine residues or some naturally occurring primary amino groups that are the acyl acceptor. Thus, the polymerisation of proteins can be achieved as a result of the formation of intermolecular or intramolecular ϵ -(- γ -glutamyl) lysine bonds (Chen *et al.*, 2005). The treatment has been found to have no cytotoxic effects, when collagen scaffolds crosslinked in this manner were seeded with cells (Chau *et al.*, 2005). When compared to controls, Chen *et al.* (2005) found no increase in apoptosis or necrosis. Chau *et al.* (2005) also found an increase in cell attachment after mTgase crosslinking.

1.5.4 Ultraviolet Radiation

UV radiation produces radicals in the form of unpaired electrons located predominantly on the nuclei of the aromatic residues such as tyrosine and phenylalanine. Crosslinks are induced by bonding between these radicals. However,

long periods of irradiation, greater than five hours, have been shown to fragment and denature the collagen molecule. Additionally, the attenuation of UV light by collagen fibres also limits the achieved degree of crosslinking. Crosslinking occurs preferentially at the surface of the scaffold, producing a scaffold with heterogeneous mechanical properties. Photosensitisers are commonly used to encourage UV crosslinking of collagen. Dong *et al.* (2004) promoted UV crosslinking by introducing a photosensitive cinnamate to collagen (Figure 1.11). Riboflavin has also been used as a photosensitiser (Wollensak *et al.*, 2003, Wollensak and Spoerl, 2004).

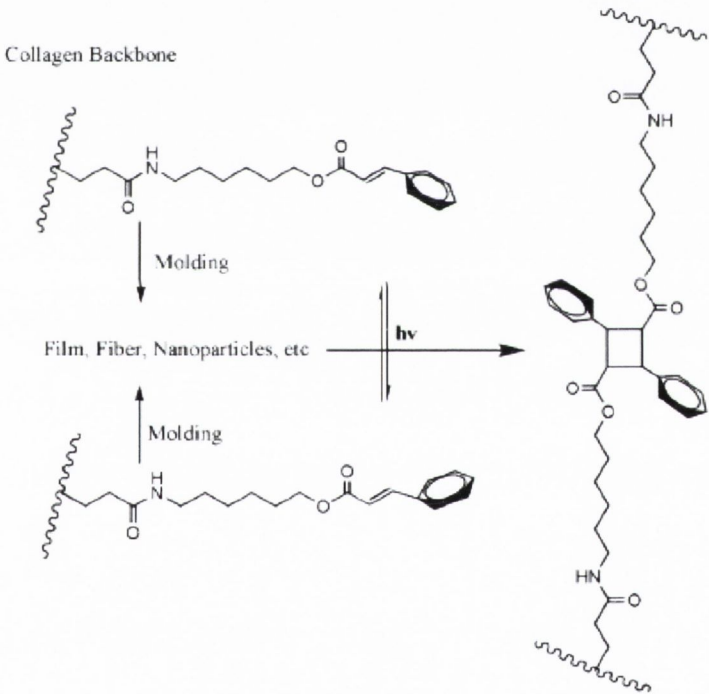


Figure 1.11 Photo-crosslinking of cinnamated collagen (Dong *et al.*, 2005).

1.5.5 Dehydrothermal Crosslinking

Dehydrothermal crosslinking has long been used to stabilise collagen and collagen composite materials (Yannas *et al.*, 1980, Gorham *et al.*, 1992, Weadock *et al.*, 1996). DHT crosslinking is a physical treatment that involves subjecting collagen to

increased temperature (>90°C) while under vacuum. This removes water from collagen, resulting in the formation of intermolecular crosslinks through condensation reactions either by esterification or amide formation (Weadock et al., 1983). The crosslinks formed by this reaction (Figure 1.12) are between the carboxyl groups of aspartate or glutamate residues and the amino groups of lysine or hydroxylysine residues (Gorham et al., 1992). It has also been postulated that a lysino-alanine crosslink would also be formed (Bohak, 1964, Patchornik and Sokolovsky, 1964). However, it was shown that very few of these crosslinks are formed by DHT (5 crosslinks per 1000 collagen molecules) and that their effect on the stability of collagen is negligible (Gorham et al., 1992). DHT crosslinking has been used to sterilise and improve the mechanical properties of collagen-GAG scaffolds originally developed at MIT (Yannas et al., 1980, O'Brien et al., 2004).

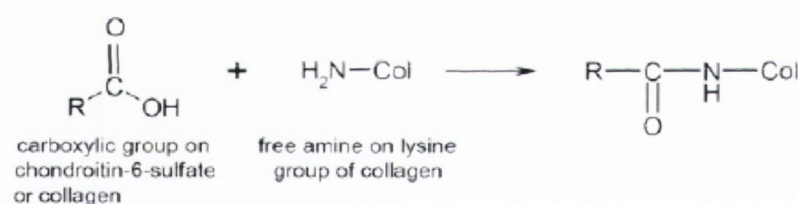


Figure 1.12 DHT crosslinking (Yannas and Tobolsky, 1967).

The drawback to the DHT process is that it is a time-consuming treatment which may not be complete even after three days. Studies have shown that crosslink density was still increasing after 5 days of DHT treatment (Weadock et al., 1995). In addition, the process has also been shown to denature the collagen and make it more susceptible to enzymatic degradation (Gorham et al., 1992, Weadock et al., 1995, Pieper et al., 1999). However, denaturation and crosslink formation are not mutually exclusive. The two processes have been shown to occur simultaneously and crosslink formation predominates at low temperatures (Gorham et al., 1992). DHT has also been shown to

make collagen less susceptible to degradation by some digestive enzymes but more susceptible to others. In a study by Gorham *et al.* (1992), DHT treated collagen was resistant to pepsin digestion but vulnerable to trypsin digestion. The crosslinks formed reduce the number of specific cleavage sites required for pepsin digestion (e.g. lysine residues). However, the denaturation caused by DHT makes the molecule more accessible to trypsin.

1.6 Thesis Objectives

This overall goal of this study was to develop a series of novel collagen-GAG (CG) scaffolds with a range of structural and mechanical properties and to determine how these properties influence cellular activity. The specific objectives of this thesis are:

1. To develop novel freeze-drying techniques in order to enlarge the range of mean pore sizes that scaffolds can be produced with.
2. To determine the effect of dehydrothermal (DHT) treatment parameters on the mechanical properties and denaturation of CG scaffolds.
3. To determine the effect of glutaraldehyde (GTA) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) crosslinking on the mechanical properties of CG scaffolds. Additionally, to explore the possibility of combining the two techniques to further improve properties.
4. To determine the effects of crosslinking and mechanical properties on cellular attachment, proliferation and infiltration within CG scaffolds.

Chapter 2. *Development of novel freeze-drying techniques in order to produce scaffolds with a range of pore sizes*

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2.1 Introduction

Porous scaffolds are used extensively in tissue engineering to provide a three dimensional structure on which tissue synthesis can occur. The pore architecture of these scaffolds has been shown to have a significant effect on both physical properties and cellular activity (Nehrer et al., 1997b, Tsuruga et al., 1997, Zeltinger et al., 2001, O'Brien et al., 2005, O'Brien et al., 2007). It has been hypothesised that the pore diameter must be large enough to allow infiltration of the cells towards the centre of the scaffold, whilst being small enough to present sufficient ligand density for cellular attachment (Yannas, 2001, O'Brien et al., 2005). In addition, the pore size also determines permeability which in turn influences the diffusion of nutrients and waste products within the scaffolds as well as the stimulus applied by flow perfusion bioreactors (Porter et al., 2005, O'Brien et al., 2007). The optimal pore size is consequently dependant on both the cell type and specifics of the tissue engineering application. Therefore, for a particular scaffold to be suitable for multiple applications, the ability to alter the pore size over a wide range is essential.

In this study we use a freeze-drying process to fabricate porous collagen-GAG (CG) scaffolds which have been used in a variety of studies (Chamberlain et al., 1998, Farrell et al., 2006, Byrne et al., 2008, Haugh et al., 2008). Using this technique, a suspension of collagen and chondroitin sulphate is frozen, trapping the collagen-GAG fibres between growing ice crystals. This produces a continuous network of ice crystals surrounded by CG fibres. Subsequent sublimation of the ice crystals during the drying phase leads to the formation of a highly porous CG scaffold (Freyman et al., 2001, O'Brien et al., 2004). As the pore structure of the scaffold mirrors the ice

crystal structure formed during freezing, pore structure can be effectively controlled by altering the freezing process used during freeze-drying (Schoof et al., 2001). Accordingly, modifications to this process are the key to creating a range of scaffolds with different pore structures.

Previous work has investigated the effects of the cooling rate and freezing temperature (T_f) used in the freezing process (O'Brien et al., 2004, O'Brien et al., 2005). Cooling rate was found to determine the homogeneity of the structure whereas freezing temperature influenced the pore size. The results showed that pore size decreased with T_f down to -40°C . However, that study was restricted to a T_f of -40°C due to technical limitations; the freeze-dryer used in this study has the ability to reach a T_f of -70°C , allowing the work of O'Brien *et al.* (2005) to be expanded upon. We hypothesise that reductions in T_f below -40°C will result in scaffolds with a smaller mean pore size. As described in Chapter 1, annealing is an additional step in the freeze-drying cycle which involves raising the temperature of the frozen suspension after freezing in order to increase the rate of ice crystal growth by reducing viscosity (Searles, 2004). While, annealing has been used to create larger ice crystals in order to facilitate shorter drying times for freeze-dried pharmaceuticals, the effects of annealing on the pore structure of freeze-dried scaffolds have yet to be investigated (Searles et al., 2001). We hypothesise that annealing can be used to increase the pore size of CG scaffolds. Therefore, the specific objectives of this study were 1) to determine effects of reducing T_f below -40°C and 2) to test our hypothesis on the effects of annealing on the pore size of CG scaffolds.

2.2 Materials and Methods

2.2.1 Slurry Fabrication

To prepare the slurry, type I bovine collagen (Integra Life Sciences, Plainsboro, NJ), chondroitin-6-sulphate (Sigma-Aldrich, Germany) and 0.05 M glacial acetic acid were blended together at 15,000 rpm using an overhead blender (Ultra Turrax T18, IKA Works Inc., Wilmington, NC). Blending was carried out in a reaction vessel, which was maintained at 4°C using a circulation cooling system (WKL 230, Lauda, Germany). The resulting collagen-GAG slurry contained 0.5% (w/v) collagen and 0.044% (w/v) chondroitin-6-sulfate. The slurry was degassed in a vacuum desiccator for 60 min to remove trapped air bubbles after blending.

2.2.2 Freeze-drying

The CG slurry was freeze-dried following the protocol developed by O'Brien *et al.* (2004). Briefly, 67.25 ml of the collagen-GAG slurry was pipetted into a stainless steel tray (5 x 5 in, grade 304 SS). The tray was placed onto the freeze-dryer shelf (Advantage EL, VirTis Co., Gardiner, NY) and the freezing cycle was started. Several variations of the freezing cycle were used in this study. In order to determine the effect of T_f on pore size, scaffolds were produced with a T_f of -10, -40, -50, -60 and -70°C (Figure 2.1). Producing scaffolds at a T_f of -10 and -40°C allowed us to determine any differences between scaffolds fabricated by O'Brien *et al.* (2005) at M.I.T. and those produced in our lab.

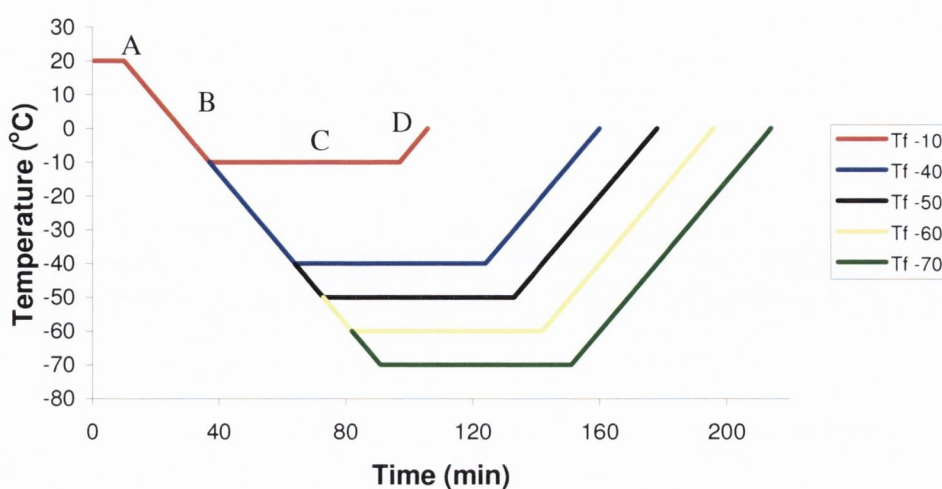


Figure 2.1 Time-temperature plot illustrating the standard freeze-drying cycle and alterations to the final freezing temperature. A) Temperature is held at 20°C for 20 min to allow the suspension to reach a uniform temperature. B) The shelf is cooled to the final freezing temperature at a rate of 0.9°C/min and C) held at this temperature for 60 min to allow the suspension to freeze. D) The sample is then heated to 0°C at a rate of 0.9°C/min before the onset of the drying cycle. The cycle outlined in blue (T_f -40) is the standard freeze-drying cycle developed by O'Brien *et al.* (2005).

Additionally, an annealing step was introduced into the freezing process (Figure 2.2). The samples were frozen using a T_f of -20°C after which the temperature was increased to -10°C in order to start annealing. Annealing times of 0.25, 6, 12, 18 and 24 hours were used to determine the effects of annealing on pore size (Figure 2.2, E). Once freezing was complete, the ice crystals were removed via sublimation for 17 h at 0°C and 200 mTorr. Samples were then crosslinked in preparation for histological analysis.

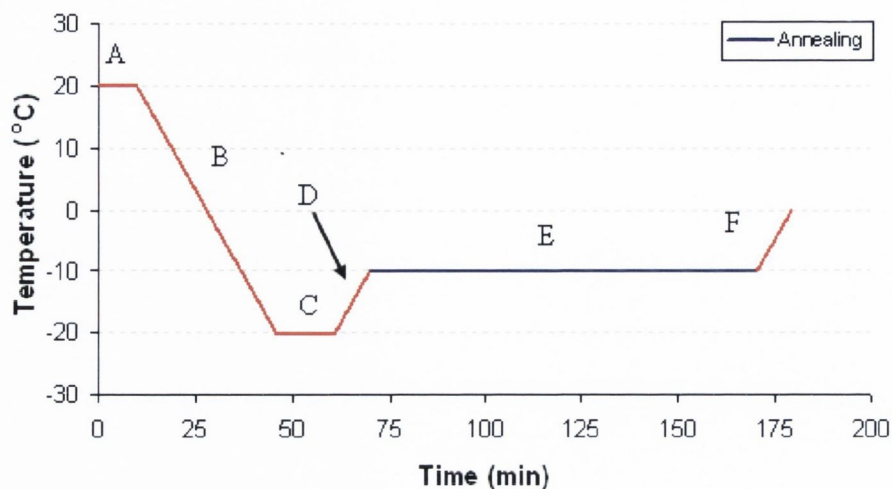


Figure 2.2 Time-temperature plot for a standard annealing cycle. A) Temperature is held at 20°C for 20 min to allow the suspension to reach a uniform temperature. B) The shelf is cooled to -20°C at a constant cooling rate of 0.9°C/min and C) held at this temperature for 20 min to allow the suspension to freeze. D) The frozen solution is then heated to -10°C at a rate of 0.9°C/min and E) held there for a set annealing time, which was varied from 0.25–48 hours in this study. F) The sample is then heated to 0°C at a rate of 0.9°C/min before the onset of the drying cycle.

2.2.3 Crosslinking

After freeze-drying, dehydrothermal (DHT) treatment was used to stabilise the CG scaffolds. DHT treatment was carried by placing the scaffolds in an aluminium foil packet inside a vacuum oven (Vacucell 22, MMM, Germany) under a vacuum of 0.05 bar at 105°C for 24 hours. This is the standard protocol used in several studies (Lee et al., 2001, O'Brien et al., 2004, O'Brien et al., 2005). Following DHT treatment, samples were prepared for pore size analysis.

2.2.4 Pore Size Analysis

Pore size was determined using a histological technique previously described by O'Brien *et al.* (2004). From each scaffold variant; four sets of cylindrical samples (8 mm diameter, 4 mm height) were cut using a punch. The samples were then embedded in JB4 glycomethacrylate (Polysciences, Germany), two in the longitudinal

plane and two in the transverse plane. The embedded samples were sectioned at 5 μm thickness using a microtome (Leica RM 2255, Leica, Germany), every 20th section was used to give a gap of 100 μm between sections. The sections were mounted on slides and stained using 2% aniline blue. Digital images were recorded using a microscope at 125x magnification (Optimphot2, Nikon, Japan). A total of 10 digital images were taken from each of the samples to give a total of 40 images from each scaffold variant.

Analysis of the digital images was carried out using a Matlab program developed in conjunction with Cian Maca Bhaird, Dr. Francois Petit and Prof. Anil Kokaram from the Electrical Engineering Department at Trinity College Dublin. Analysis was carried out in two stages. The program first thresholds the image, converting it into a binary image consisting of black and white pixels and an algorithm is run to remove blotches from the image. The second stage consists of pore analysis, which uses the following algorithm:

1. A random pixel within the image is selected. Reiterations of the algorithm select a random pixel that has yet to be selected.
2. The distance from this point to the nearest pore boundary is calculated through 360°. A distance vs. angle plot gives a polar representation of the pore. This is then post-processed to remove false measures due to blotches or low contrast edges.
3. The major and minor axes are calculated by selecting the largest and smallest orthogonal diameters of the pore's polar graph.
4. The pixels inside the pore are marked as selected and the process is repeated until at least 70% of the pixels in the image are marked as selected.

In this fashion the average pore size can be calculated for each section. The pore size referred to throughout this chapter, is the diameter of a circle with a cross-sectional area equivalent to that of the best fit ellipse that is generated by the software.

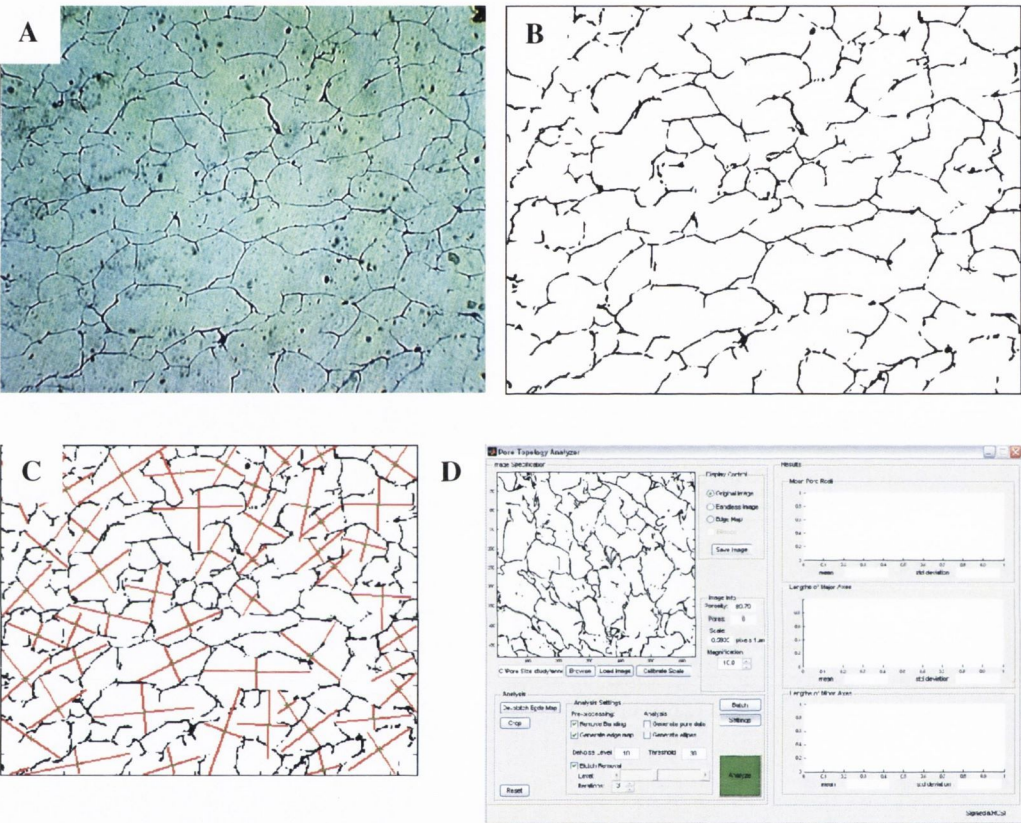


Figure 2.3 Matlab analysis of a stained section. A) Section prior to analysis, B) after threshold and cleanup, and C) after analysis using the Matlab code, displaying the max and min axes. The interface for the pore topography analysis program is shown in D.

2.2.5 Differential Thermal Analysis

Differential thermal analysis (DTA) was used in order to gather further information and explain the structural changes that take place as the T_f of the CG suspension is varied. DTA measures the difference in temperature between the sample and a reference, thus monitoring exothermic or endothermic changes observed as a solution melts. These exothermic and endothermic changes indicate subtle changes in the

viscosity of a frozen sample as it is melted. For example, if the viscosity of a sample reduces suddenly as it melts, the rate of increase in temperature will rise due to improved mobility within the frozen suspension. Using DTA, this will be observed as a reduction in the temperature difference (ΔT) measured between the sample and reference. DTA analysis was carried out using a Lyotherm 2 (Biopharama, UK). The temperature of the CG sample together and the reference material (AnalaR water) were measured at 5 second intervals as they were heated from -150°C . Raw data was exported, and interpretation of the cooling profile carried out to determine the temperatures of the significant events, which could be attributed to structural changes occurring in the CG suspension.

2.2.6 Statistical Analysis

Results are expressed as mean $\pm$ standard deviation. One-way analysis of variance (ANOVA) followed by pairwise multiple comparison procedures (SNK test) was used to evaluate the effects of T_f and annealing on the pore size of CG scaffolds. Statistical significance was declared at $p \leq 0.05$.

2.3 Results

2.3.1 Freezing Temperature

Pore size was found to decrease with T_f until -50°C ($p<0.05$), subsequent reductions in T_f did not result in a significant change in pore size (Figure 2.4, Table 2.1). The results show that alterations of the T_f can be used to produce scaffolds with a mean pore size ranging from 84.6 to 324.9 μm . A T_f of -10°C produced scaffolds with the largest pores at 324.91 μm , while scaffolds produced using a T_f of -60°C had the smallest pore size at 84.66 μm (Figure 2.5).

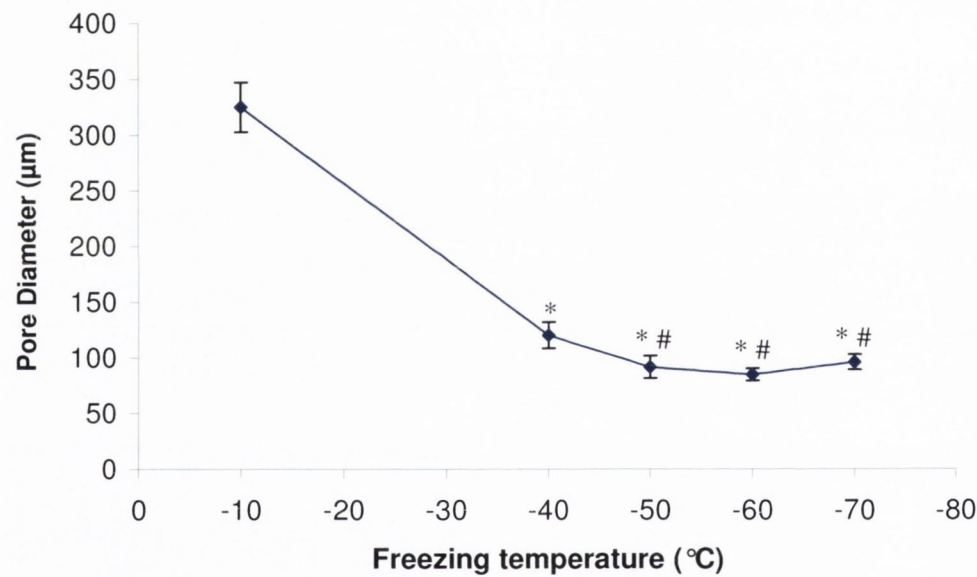


Figure 2.4 The effect of freezing temperature on pore size ($n=4$ per group, * $p<0.001$ compared to -10°C group, # $p<0.05$ compared to -40°C group, no differences were found between -50 , -60 and -70°C groups).

T_f ($^{\circ}\text{C}$)	-10	-40	-50	-60	-70
Pore size (μm)	324.9	120.0	91.3	84.6	95.8
Pore size SD (μm)	22.1	11.7	10.1	5.7	6.9

Table 2.1 The pore sizes produced at different values of T_f .

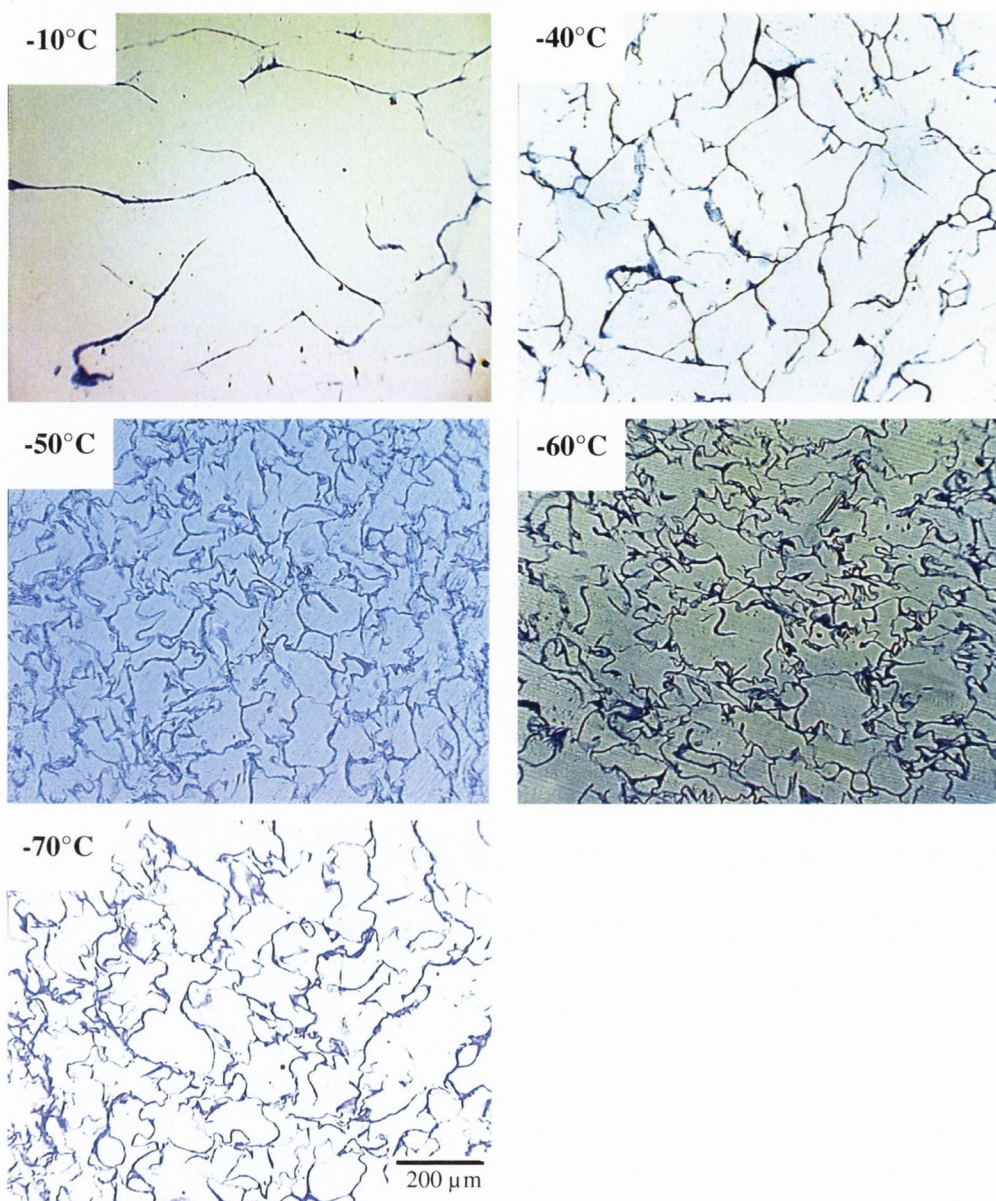


Figure 2.5 Digital images of the stained scaffold sections captured at 125x magnification. The mean pore size can be seen to decrease with T_f until -50°C .

2.3.2 Annealing

Results show that as annealing time increased an initial decrease in pore size can be seen from 0.25 to 6 h, after which the pore size increases to a peak at 18 h (Figure 2.6, Table 2.2). Annealing times beyond 18 h had no additional effect on pore size. Annealing for 18 h produced a 40% increase in the size of the pores initially produced (0.25 h, $p<0.001$). An annealing time of 18 h produced scaffolds with the largest pores at 191.4 μm , while scaffolds produced using an annealing time of 6 h had the smallest pore size at 112.5 μm (Figure 2.7).

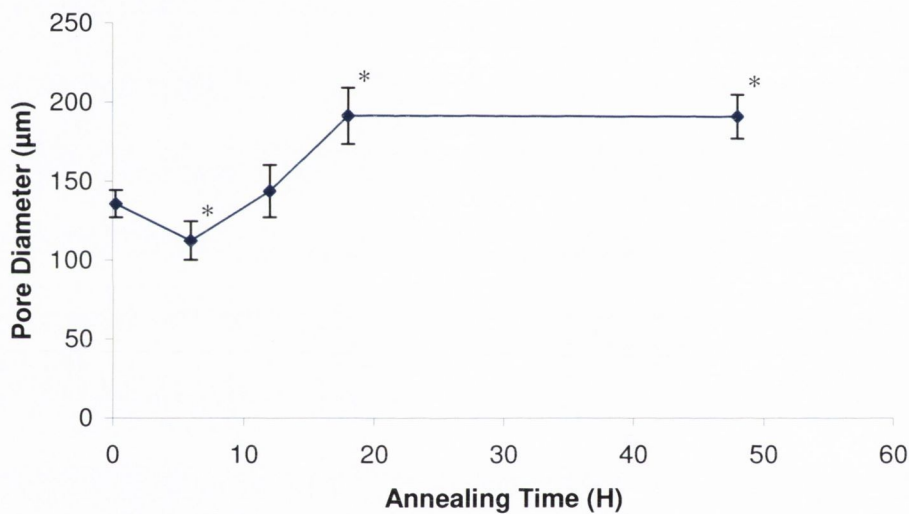


Figure 2.6 The effect of heat annealing on pore size (n=4 per group, * $p<0.05$ compared to 0.25 h group, no differences were found between 0.25 and 12 h groups).

Annealing time (h)	0.25	6	12	18	48
Pore size (μm)	135.6	112.5	143.8	191.4	190.6
Pore size SD (μm)	8.6	12.3	16.6	17.8	13.8

Table 2.2 The pore sizes produced at different annealing times.

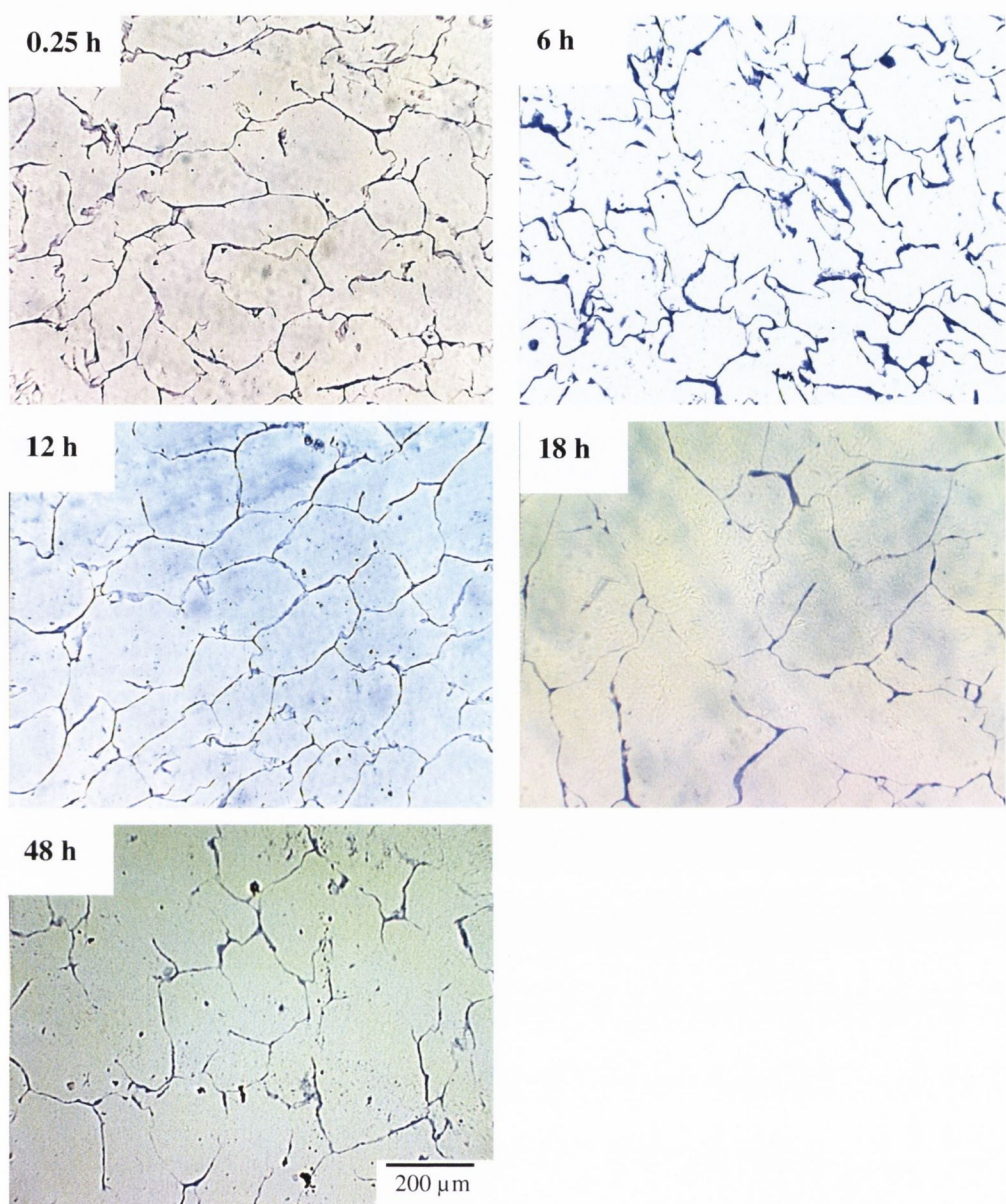


Figure 2.7 Digital images of the stained scaffold sections captured at 125x magnification. The mean pore size can be seen to increase after an annealing time of 18 hours.

2.3.3 Differential Thermal Analysis

DTA was used to determine the temperatures at which structural changes occur within the frozen CG suspension (Figure 2.8). Delta T represents the difference in temperature between the CG sample and pure water as they are heated from -100°C . An increase in delta T indicates an increase in the viscosity of the sample, while a reduction in delta T indicates the opposite. DTA analysis shows three distinct phenomena as the sample was heated from -100 to 0°C . An increase in viscosity (or hardening) can be seen up to -66°C (Figure 2.8, A), followed by a reduction in viscosity (softening) at -42°C (Figure 2.8, B). Finally, the onset of melting occurs at -6°C (Figure 2.8, A).

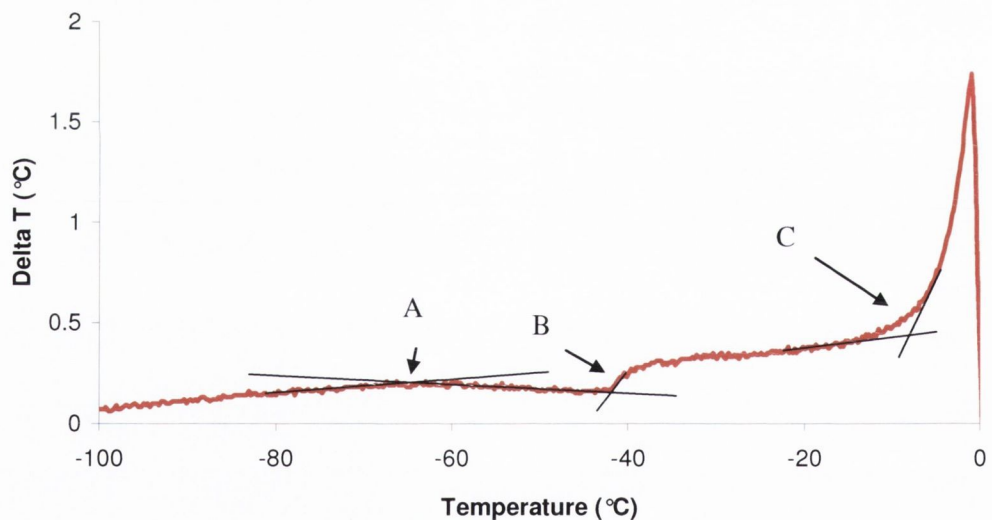


Figure 2.8 DTA analysis of the CG suspension. Delta T, represents the difference in temperature between the CG suspension and the reference. The following phenomena were observed: A) hardening of the structure at -66°C , B) softening of the frozen structure at -42°C and C) onset of ice melting at -6°C .

While this data relates to changes in the structure as it melts, it should be noted that the behaviour during freezing will be the opposite. For example, a reduction in the viscosity of a frozen suspension during melting points to an increase in viscosity at

the same temperature during freezing. Therefore, DTA indicates ice crystal nucleation at -6°C, followed by an increase in viscosity of the frozen suspension at -42°C and finally, a reduction in viscosity at -66°C. The increase in viscosity at -42°C may indicate the glass transition temperature of the suspension and explain why reductions in T_f have a limited effect below -40°C (Figure 2.4).

2.4 Discussion

The pore structure of 3-D scaffolds used in tissue engineering has been previously shown to influence cellular activity (Tsuruga et al., 1997, Zeltinger et al., 2001, O'Brien et al., 2005). With this in mind, the aim of this study was to develop methodologies to produce a range of scaffolds with different mean pore sizes. Scaffolds were fabricated using several alterations to the freeze-drying protocol developed by O'Brien *et al.* (2004). The freezing temperature (T_f) was varied from -10 to -70°C and an annealing step was introduced to the cycle. We found that the pore size of the scaffolds decreased with decreasing T_f until -50°C, after which no significant changes in pore size were observed. Additionally, annealing was found to result in a significant increase (40%) in pore size after 18 h. Taken together, these results demonstrate that methodologies developed in this study can be used to produce a range of CG scaffolds with mean pore sizes from 85 to 325 μm . This facilitates the investigation of the effects of pore size on both *in vitro* and *in vivo* performance, and the determination of the optimal pore structure for specific tissue engineering applications.

In this study we have investigated the effects of freezing temperature and annealing on the pore structure of CG scaffolds. The effects of freezing temperature have been

investigated in previous studies (Park et al., 2002, Zmora et al., 2002, Chen et al., 2007, Faraj et al., 2007). However, in these studies the cooling rate was also varied in conjunction with the freezing temperature; making it difficult to determine the specific mechanisms behind changes in pore structure. In addition, freeze-drying in this study was carried out using a freeze-dryer equipped with a temperature controlled shelf. This allowed control of the cooling rate and eliminated the possibility of sample thawing during transport between the freezing and drying apparatus, which were separate devices in many studies (Park et al., 2002, Zmora et al., 2002, Faraj et al., 2007). The use of annealing to alter the pore size of a freeze-dried scaffold is a novel aspect of this study. Annealing has been used previously to encourage crystallisation and reduce the drying cycle times of pharmaceuticals (Gatlin and Deluca, 1980, Korey and Schwartz, 1989, Searles et al., 2001), but has yet to be investigated for its effects on the structure of freeze-dried scaffolds.

Several studies have investigated the effects of cooling rate on the pore structure of freeze-dried scaffolds. It has been shown that slow cooling rates ($<1^{\circ}\text{C}/\text{min}$) produce scaffolds with large pores, while fast cooling rates ($>1^{\circ}\text{C}/\text{min}$) produce scaffolds with smaller pores (Park et al., 2002, Faraj et al., 2007). However, a study by O'Brien *et al.* (2004) has shown that cooling rate also affects the uniformity of the pore structure. It was found that a cooling rate of $0.9^{\circ}\text{C}/\text{min}$ produced scaffolds with homogeneous pore structure, while quicker or slower cooling rates reduced the uniformity of the structure formed (O'Brien et al., 2004). This has also been shown to be the case with freeze-dried alginate scaffolds (Zmora et al., 2002). On this basis, it was decided to keep a constant cooling rate of $0.9^{\circ}\text{C}/\text{min}$ and modify alternative aspects of the freezing cycle in this study.

O'Brien *et al.* (2005) reported a pore size of 150.5 and 95.9 μm for scaffolds fabricated at a freezing temperature of -10 and -40°C respectively. Our results however, show a pore size of 324.9 and 120.1 μm for scaffolds fabricated at the same temperatures. The discrepancy between these results can be accounted for by differences in the trays used to contain the CG suspension and the methods used to measure pore size. To prevent warping at low temperatures, the thickness of the stainless steel trays used in this study was twice that of the trays used by O'Brien *et al.* (2005). This change may decrease the cooling rate of the solution, which has been shown to increase pore size, therefore explaining the larger pore size found in our study. The tray thickness may have had less of an effect at a T_f of -40°C, because ice crystal growth rate is slower at this temperature due to increased viscosity. Additionally, differences in the methods used to measure pore size may also account for the discrepancy between the studies. In order to investigate this further, an experiment was carried out to quantify the difference between pore size analysis using the Matlab code employed in this study and the Scion Image™ macro used by O'Brien *et al.* (2005). The Matlab code was found to report a larger pore size than that reported by the Scion Image™ macro (Figure 2.9). However, significant differences in pore size were still found when slides from the two studies were analysed with the same software, demonstrating that differences in cooling rate are the most likely cause for the discrepancy between the studies. Additionally, it should be noted that the methods used are qualitative as they use 2D sections to study 3D structures.

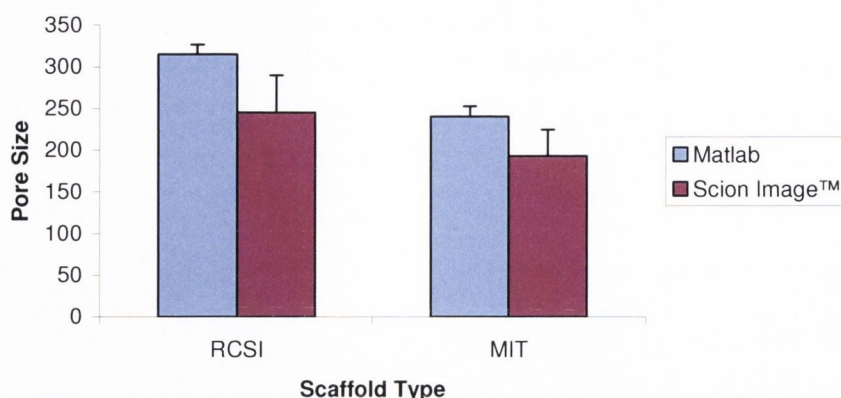


Figure 2.9 The effects of measurement technique and fabrication on pore size (n=10). Sample images from scaffolds fabricated using a T_f of -10°C at RCSI and at MIT (by O'Brien *et al.*) were analysed using both the Matlab code and the Scion Image™ macro. The pore size measured by the Matlab code was found to be on average 26% larger than that measured using the Scion Image™ macro.

It was found that while pore size decreased with T_f until -50°C , reductions in T_f below this did not result in a significant change in pore size. The size of ice crystals formed during freezing is dependant on the nucleation rate of the crystals and the rate of heat and protein diffusion within the frozen suspension. As the temperature is reduced, the rate of heat and protein diffusion also decreases, reducing the size of the ice crystals formed. However, when the temperature drops below the glass transition temperature (T_g), the viscosity of the frozen suspension increases to the point where ice crystal growth is negligible (Akyurt *et al.*, 2002, Searles, 2004). DTA analysis indicates an increase in viscosity at -42°C (Figure 2.8, B), which correlates with the reduction in ice crystal growth between -40 and -50°C indicated by the pore size data (Figure 2.4). These results suggest that the glass transition temperature is in the vicinity of -42 to -50°C , and that subsequent reductions in T_f have no effect on pore size due to the increased viscosity.

Annealing was found to cause an initial decrease in pore size followed by an increase after 18 hours annealing duration. During annealing a phenomenon known as Ostwald ripening occurs, which is the preferential melting of dispersed ice crystals that are smaller than a critical size, due to their higher vapour energy (Searles, 2004). The melting of these crystals coupled with increased temperatures reduces the viscosity of the unfrozen suspension allowing the remaining ice crystals to grow. This ripening can be seen as the pore size of the scaffolds initially decreases up to an annealing time of 6 h and then increases afterwards as the remaining ice crystals grow due to the reduction in the viscosity of the suspension.

2.5 Conclusion

Pore size has been previously found to be an important aspect of scaffold design (Nehrer et al., 1997b, Tsuruga et al., 1997, Zeltinger et al., 2001, O'Brien et al., 2005). The results of this study show that modifications to the freeze-drying cycle can be used to produce a variety of CG scaffolds with a wide range of mean pore sizes (85 to 325 μm). Altering the T_f was found to be the most effective method of varying pore size, although annealing may prove useful when combined with high T_f . In addition, DTA combined with pore size analysis indicate that the glass transition temperature of the CG suspension is between -42 and -50°C. The scaffolds produced in this study provide a basis for the investigation of the effects of pore size on cellular activity.

Chapter 3. *The effect of dehydrothermal treatment on the mechanical and structural properties of collagen-GAG scaffolds*

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3.1 Introduction

Dehydrothermal (DHT) treatment is a common technique for stabilising collagen and collagen composite materials (Yannas et al., 1980, Gorham et al., 1992, Weadock et al., 1996). It is a physical treatment that involves subjecting collagen to increased temperature ($>90^{\circ}\text{C}$) while under vacuum. These conditions remove bound water from collagen molecules, resulting in the formation of intermolecular crosslinks through condensation reactions either by esterification or amide formation (Yannas and Tobolsky, 1967, Yannas, 1972). DHT treatment is favourable to other crosslinking methods as it does not involve the use of cytotoxic reagents. A further advantage of DHT treatment is the sterilisation provided by the high temperatures and exposure periods used. Previous studies on DHT treatment have shown that increasing DHT temperature and exposure duration improves the mechanical properties of collagen fibres (Weadock et al., 1995, Wang et al., 1994).

In addition to forming crosslinks, the high temperatures used during DHT crosslinking have been shown to denature collagen (Yunoki et al., 2003). Denaturation is defined as rearrangement of the triple helix into a random chain configuration (Miles and Bailey, 2001). While generally considered undesirable as it disrupts the native collagen conformation, denaturation may reduce the inflammatory response and increase cellular infiltration *in vivo* (Koide et al., 1993). A greater knowledge of the level of denaturation after DHT treatment will enable further investigation of the relationship between cellular activity and denaturation.

Despite the widespread use of DHT treatment on collagen films, fibres and scaffolds, a detailed analysis has not been carried out to determine how the process parameters affect collagen-GAG (CG) scaffold parameters. Therefore, the specific objectives of this study were to 1) determine the range of compressive and tensile properties achievable using DHT by varying temperature and exposure period, 2) to evaluate the level of crosslinking after DHT treatment, 3) to establish the level of denaturation after crosslinking, and 4) to correlate changes in mechanical properties with crosslinking and denaturation.

3.2 Materials and Methods

3.2.1 Scaffold Fabrication

Scaffolds were produced by freeze-drying a collagen-GAG slurry as described in chapter 2 (Sections 2.2.1 & 2.2.2.). Briefly, a slurry containing 0.5% (w/v) collagen and 0.044% (w/v) chondroitin-6-sulfate was prepared. This slurry was then freeze-dried using a T_f of -40 (Section 2.2.2, Figure 2.1). This process produces a highly porous sheet of CG scaffold, with an average pore size of 120 μm (Section 2.3.1, Table 2.1).

3.2.2 Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) was used in order to determine how the temperatures used during DHT affect the physical and chemical properties of collagen. DSC is a method widely used in polymer science to study the thermal behaviour of materials as they undergo changes upon heating. DSC measures the heat flow necessary to maintain a constant heating rate ($^{\circ}\text{C}/\text{min}$) while heating a sample. When collagen is heated, the crystalline triple helix of collagen is transformed into

amorphous random coils, resulting in shrinkage of the collagen molecule. This shows up as a peak in the heat flow of a DSC curve (Mentink et al., 2002). Figure 3.1 shows a DSC curve of a collagen sample and the peak corresponding to the onset of denaturation (denaturation temperature).

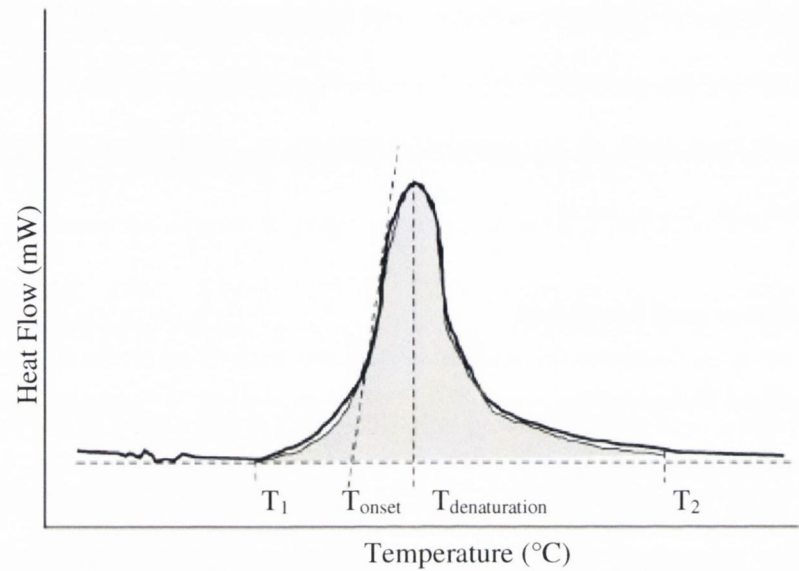


Figure 3.1 DSC scan of a collagen sample (Adapted from Mentink *et al.*, 2002).

Two different cycles were run on 8 mm diameter collagen samples. During the first cycle a sample was heated from -50°C to 200°C. This allowed measurement of the denaturation temperature as illustrated in Figure 3.1. During the second cycle the percentage weight of the collagen was measured as it was heated from 30°C to 700°C at 10°C per minute. This allowed me to examine the behaviour of collagen at the temperatures used during DHT treatment and determine the onset of thermal decomposition/degradation.

3.2.3 DHT Treatment

DHT treatment was carried out by placing the scaffolds in an aluminium foil packet inside a vacuum oven (Vacucell 22, MMM, Germany) under a vacuum of 0.05 bar. To determine the effect of DHT parameters on CG scaffold properties, exposure period and crosslinking temperature were varied. Exposure period was varied from 24 h to 120 h, at 24 h intervals, and four crosslinking temperatures were used: 105°C, 120°C, 150°C and 180°C.

3.2.3 Mechanical Testing

Compressive and tensile testing were used to determine the effect of DHT parameters on the mechanical properties of the scaffolds. Mechanical testing of scaffold samples was carried out using a mechanical testing machine (Z050, Zwick/Roell, Germany) fitted with a 5-N load cell. Samples were pre-hydrated in phosphate buffered saline (PBS) (Sigma-Aldrich, Germany) for one hour prior to testing and all testing was carried out in a bath of PBS. For unconfined compression testing with impermeable, un-lubricated platens, samples of 8 mm diameter were cut from the scaffolds using a punch. For tensile testing, rectangular strips (10 x 40 mm) were cut from the scaffolds using a razor blade. The gauge length used for tensile testing was 25 mm. Both tensile and compressive testing were conducted at a strain rate of 10%/min. The modulus was defined as the slope of a linear fit to the stress-strain curve over 2-5% strain, avoiding the less stiff toe region of the stress-strain curve.

3.2.4 Fourier Transform Infra-red Microscopy

Fourier transform infra-red (FT-IR) spectra were used to investigate the level of crosslinking and collagen denaturation after DHT treatment (Gordon et al., 1974, Yunoki et al., 2003, Sung, 1972). FT-IR microscopy was carried out using a Spectrum One FT-IR (Perkin Elmer, UK). Cylindrical samples (8 mm diameter) were used for the analysis. Spectra were analysed using a free-ware program, Spekwin (Daltrozso Group, University of Konstanz, Germany). Formation of crosslinks through DHT treatment can be monitored by analysing the amide II band absorbance peak at 1553 cm^{-1} (Wang et al., 2003, Muyonga et al., 2004). The band at 1553 cm^{-1} is proportional to the amount of NH_2 , which is converted to NH during the formation of crosslinks through condensation reactions (Yannas and Tobolsky, 1968). Therefore, a decrease in the absorbance at 1553 cm^{-1} corresponds to an increase in the number of crosslinks. The absorbance at 1553 cm^{-1} was normalised to the absorbance at 1450 cm^{-1} ($1553\text{ cm}^{-1}/1450\text{ cm}^{-1}$), which is unaffected by the formation of crosslinks or denaturation (Sung, 1972, Muyonga et al., 2004). The inverse of this ratio was defined as the effective crosslink density.

The absorbance peak at 1235 cm^{-1} (waves per cm) is affected by changes in the triple helical structure of collagen and can therefore be used to quantify the denaturation of a collagen sample. The absorbance at 1235 cm^{-1} was normalised to the absorbance at 1450 cm^{-1} (Yunoki et al., 2003, Sung, 1972, Gordon et al., 1974). By measuring the absorbance of an untreated sample (0% denatured) and gelatine (collagen that has lost its triple helical structure and is therefore 100% denatured), linear interpolation was used to measure the percentage denaturation of a test sample (Yunoki et al., 2003, Gordon et al., 1974).

3.2.5 Statistical Analysis

Results are expressed as mean $\pm$ standard deviation. Two-way analysis of variance (ANOVA) followed by pairwise multiple comparison procedures (Tukey test) was used to evaluate the effects of DHT exposure period and temperature on compressive modulus, tensile modulus, denaturation and crosslinking density. Multiple linear regression analysis was used to examine correlations between mechanical properties, crosslinking density and denaturation. Separate regressions for compressive and tensile moduli were carried out, with percentage denaturation and crosslink density set as the independent variables. Statistical significance was declared at $p \leq 0.05$. A correlation between tensile modulus and denaturation was found upon analysis. In order to further investigate this relationship a subset of the data was tested to failure in tension and the ultimate tensile strength and strain to failure were calculated.

3.3 Results

3.3.1 Differential Scanning Calorimetry

Figure 3.2 shows the percentage weight of the collagen as it is heated from 30°C to 700°C at 10°C per minute. This curve is characterised by three events: 1) removal of water (30-200°C), 2) thermal degradation of the collagen (200-450°C) and 3) combustion of degraded sample (450-700). Degradation of the collagen at 200°C is important as it indicates the highest possible temperature for thermal treatment of the collagen. DHT at or above this temperature would degrade the collagen and negate any benefit gained from crosslinking.

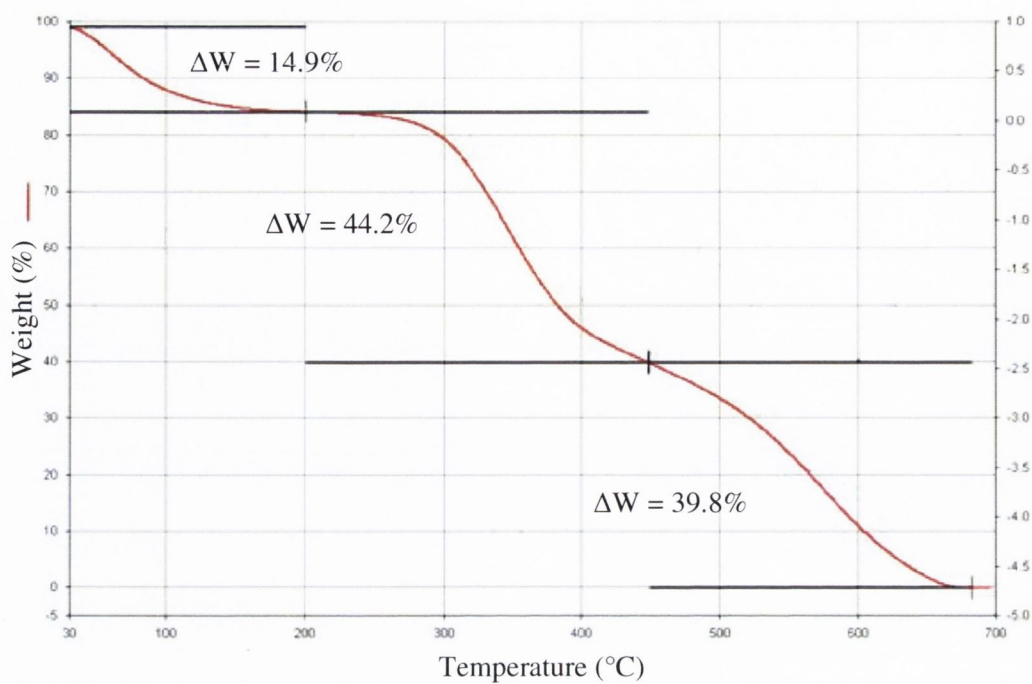


Figure 3.2 DSC scan 1: Percentage weight of collagen as it is heated.

Figure 3.3 shows a cycle in which the sample is heated from -40 to 200°C. The peak in the curve at 94.94°C indicates the denaturation temperature of the collagen sample. This suggests that DHT crosslinking denatures the collagen scaffolds, as the treatment takes place at temperatures above 94.94°C.

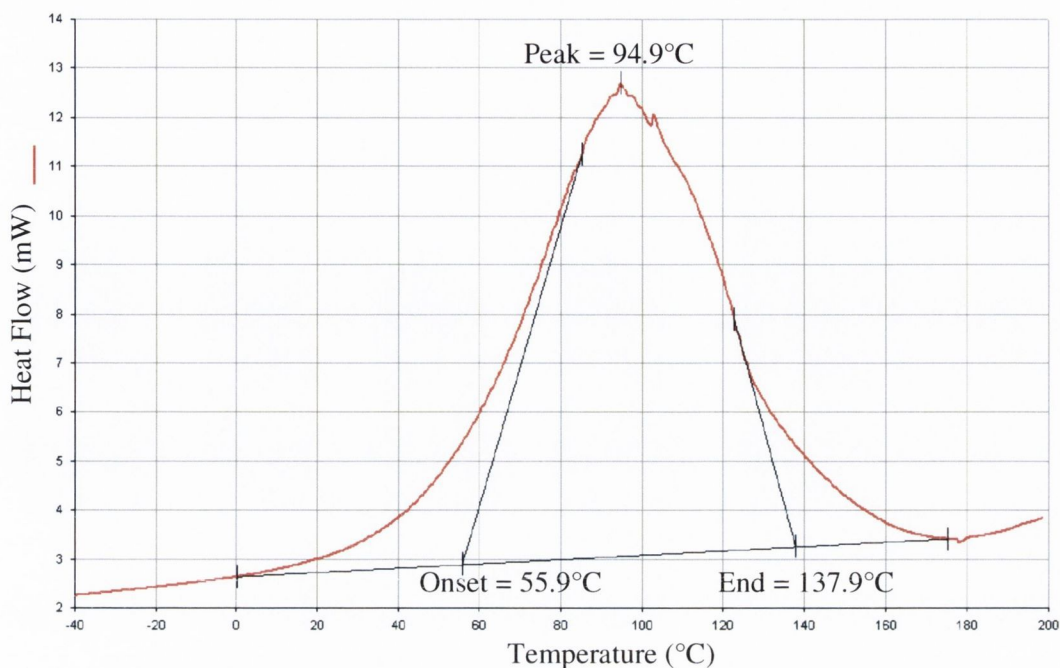


Figure 3.3 DSC Scan 2: Denaturation profile of a collagen sample.

3.3.2 Mechanical Properties

Analysis of the mechanical properties following DHT treatment revealed that compressive modulus increased with increasing temperature but not with increasing exposure period (Figure 3.4). At 24 h, compressive modulus increased 2-fold for scaffolds crosslinked at 180°C compared to 105°C ($p<0.001$). Exposure period had no effect on compressive modulus at 105, 120 and 150°C ($p>0.05$). However, at 180°C the modulus decreased by 20% when the exposure period was increased beyond 24 h ($p<0.001$).

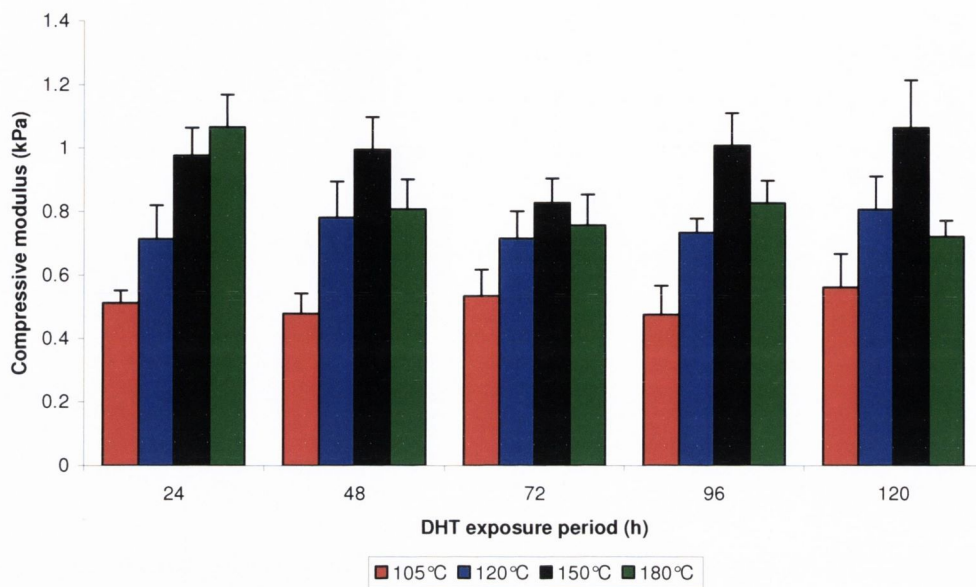


Figure 3.4 Compressive modulus versus DHT exposure period at four different crosslinking temperatures (n = 10 samples per temperature/duration combination).

Tensile modulus increased with both increasing temperature and increasing exposure period (Figure 3.5). Tensile modulus increased 3.8-fold when temperature was increased from 105°C to 180°C ($p < 0.001$). Increasing exposure duration had no effect on tensile modulus at 105°C ($p > 0.05$), whereas at 120 and 150°C there was an increase in tensile modulus with increased exposure duration ($p < 0.05$). At 180°C, the modulus decreased by up to 26% after 72 h ($p < 0.001$).

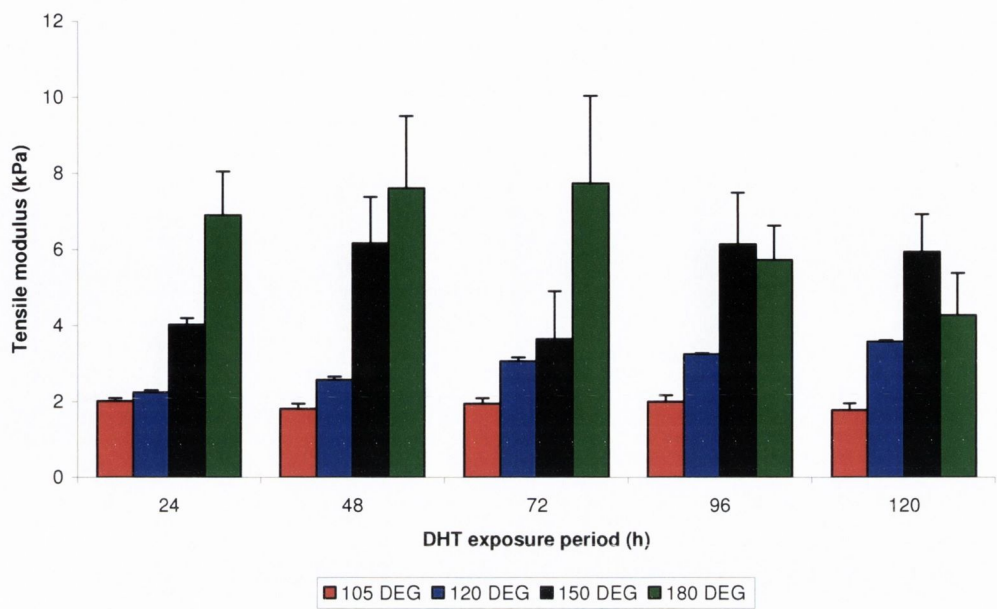


Figure 3.5 Tensile modulus versus DHT exposure period at four different temperatures (n = 6 samples per temperature/duration combination).

3.3.2 Crosslink Density

FT-IR analysis of DHT treated samples show that the level of NH_2 decreased with increasing temperature but not exposure period ($p < 0.05$). Since NH_2 levels are inversely proportional to crosslink density (Wang et al., 2003), this demonstrated an increase in the crosslink density with increasing temperature but not exposure period (Figure 3.6). This correlation is analysed in more detail in section 3.3.4.

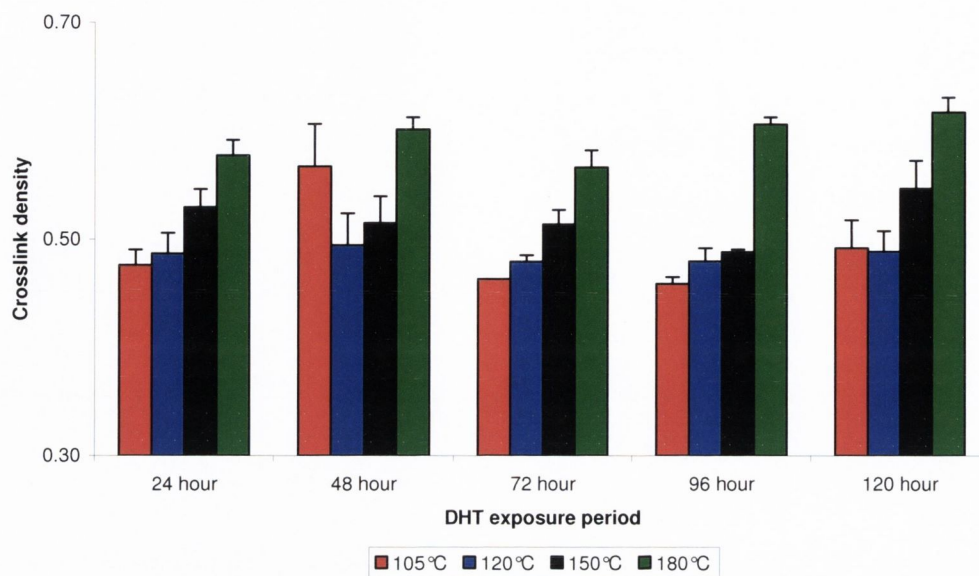


Figure 3.6 Crosslink density versus DHT exposure period at four different temperatures (n = 3 samples per temperature/duration combination).

3.3.3 Denaturation

FT-IR analysis showed that denaturation increased with DHT temperature (Figure 3.7, $p < 0.05$). Increasing exposure period had no effect on denaturation at 105 and 180°C ($p > 0.05$). However, at 120 and 150°C denaturation increased with exposure period ($p < 0.05$). Scaffolds contained 25% denatured collagen after treatment at 105°C for 24 h and 60% denatured collagen after extensive treatment at 180°C for 120 h.

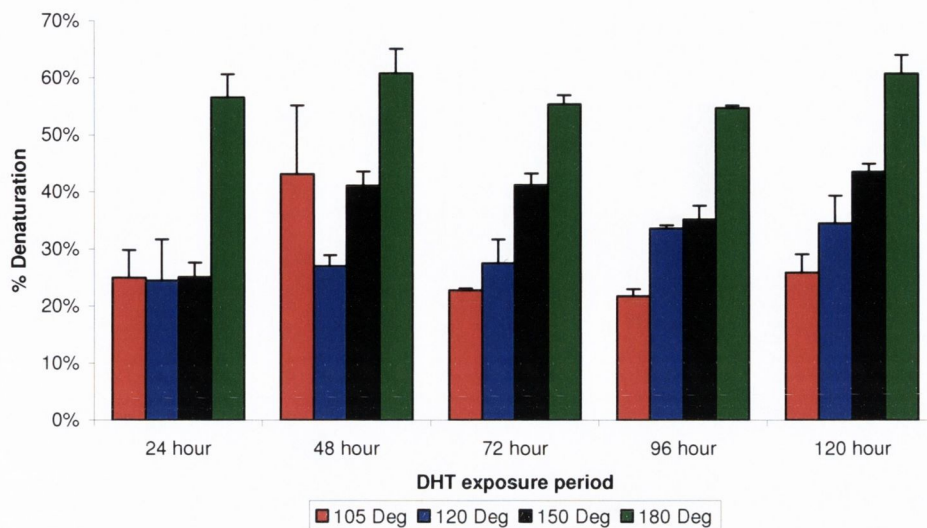


Figure 3.7 Denaturation versus DHT exposure period at four different temperatures (n = 3 samples per temperature/duration combination).

3.3.4 Multiple Linear Regression

Multiple linear regression analysis was carried out to investigate correlations between elastic moduli, denaturation and crosslink density. Analysis showed the effect of denaturation on compressive modulus was not significant, nor was the effect of crosslink density on tensile modulus. Therefore, these variables were removed in the relevant regressions and the regressions were subsequently simplified. The compressive modulus was found to be highly correlated with crosslink density (Figure 3.8 A, $p < 0.001$, $R^2 = 0.70$). The tensile modulus was highly correlated with percentage denaturation (Figure 3.8 B, $p < 0.001$, $R^2 = 0.85$).

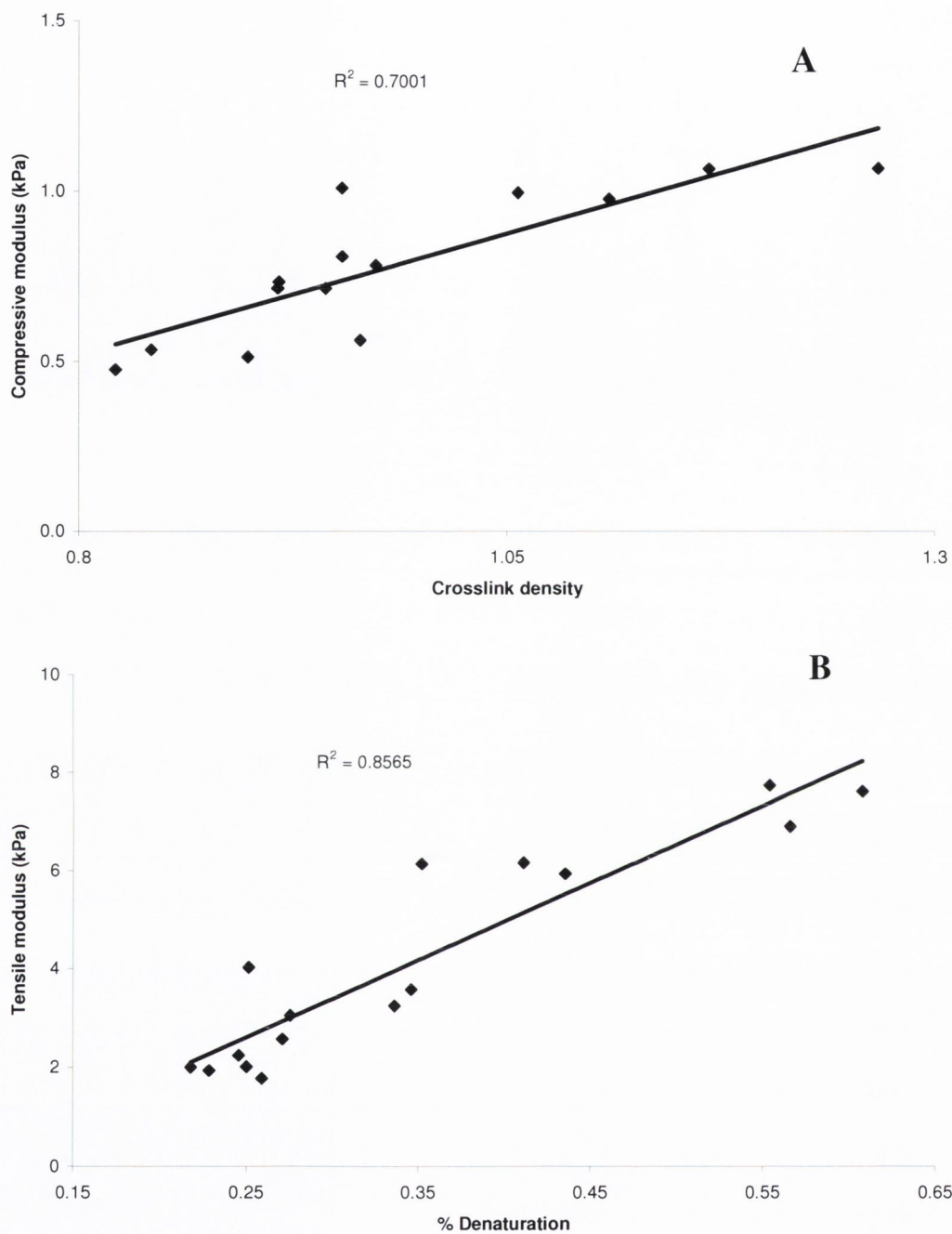


Figure 3.8 Linear regressions of moduli against crosslink density and denaturation. A: Compressive modulus correlated with crosslink density. B: Tensile modulus correlated with denaturation.

As tensile modulus was correlated with denaturation, samples at three levels of denaturation, (25, 40 and 60% corresponding to treatments of 105°C for 24 h, 150°C

for 48 h and 180°C for 48 h respectively) were tested to failure in tension. There was a significant decrease in UTS with increasing denaturation (Figure 3.9 A, $p<0.05$). Strain to failure also decreased significantly with increasing denaturation (Figure 3.9 B, $p<0.001$).

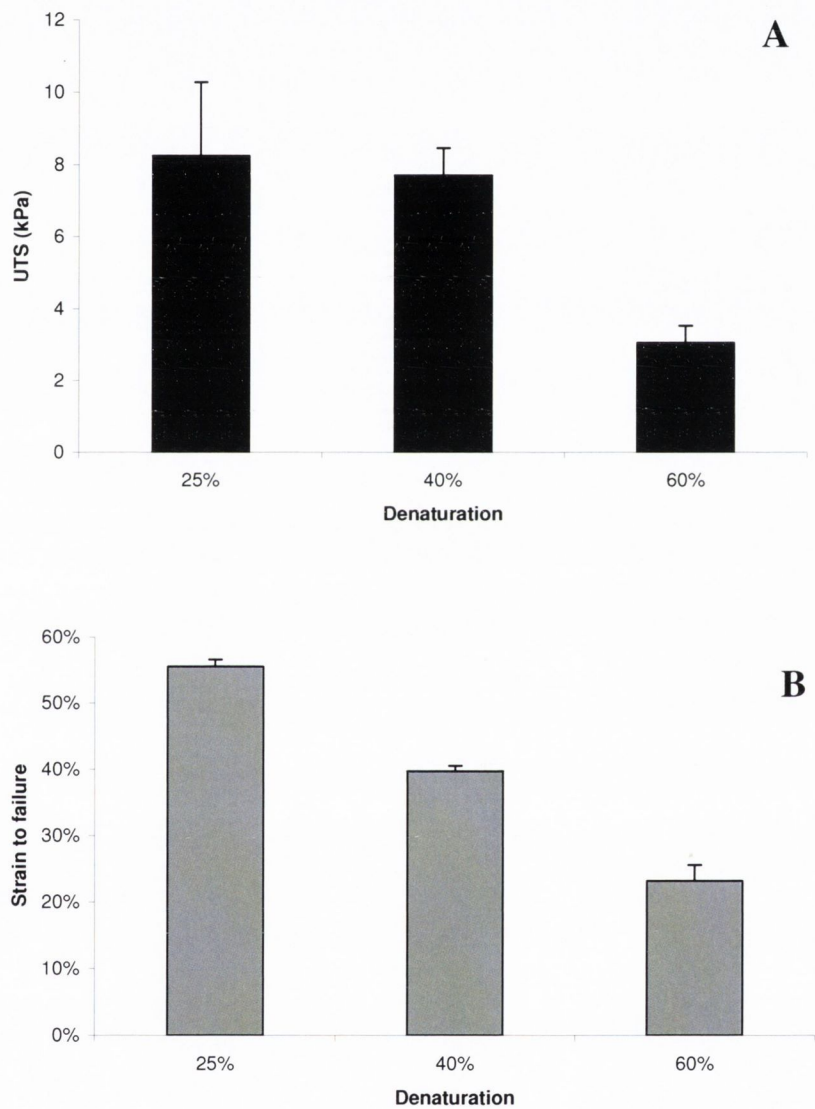


Figure 3.9 The relationship between tensile properties and denaturation. A: Ultimate tensile strength versus percent of denaturation (n = 3 per group). B: Strain to failure versus percent of denaturation (n = 3 per group).

Additionally, as denaturation increased the mode of tensile failure was also found to shift from ductile to brittle (Figure 3.10). Samples with moderate levels of denaturation (25-40%) can be seen to weaken before failure, while more extensively denatured samples fail abruptly.

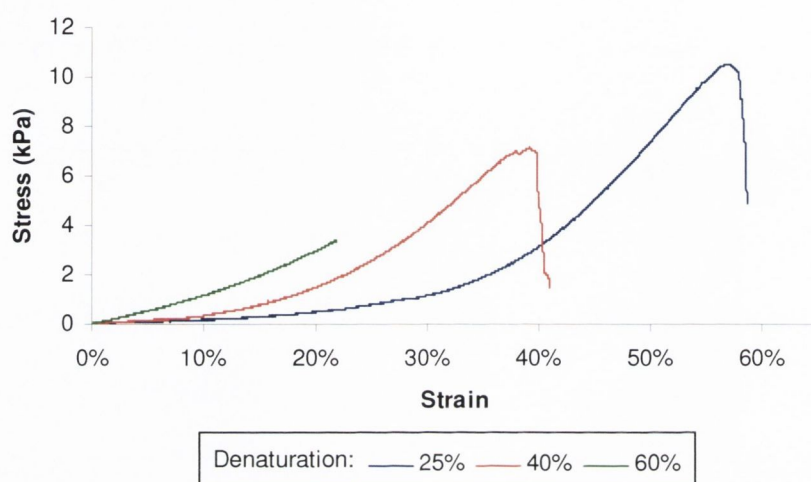


Figure 3.10 Stress-strain curves showing the effect of denaturation on the mode of tensile failure.

3.4 Discussion

The mechanical properties of scaffolds for tissue engineering are critical for preserving structural integrity and functionality during both *in vivo* implantation and long-term performance. In addition, the mechanical and structural properties of the scaffold can direct cellular activity and further determine the functionality of a tissue-engineered construct (Engler et al., 2004a, Engler et al., 2004b, Engler et al., 2006, Yeung et al., 2005). In this context, the aim of this study was to investigate the effects of dehydrothermal crosslinking treatment on the mechanical and structural properties of a CG scaffold used for tissue engineering applications. Temperature (105–180°C) and exposure period (24–120 h) of DHT treatment were varied to determine the effects of these parameters on the mechanical properties, crosslinking density and

denaturation of CG scaffolds. As expected, increasing the temperature and duration of DHT treatment resulted in an increase in Young's moduli. Compressive moduli increased up to 2-fold, while tensile moduli increased almost 4-fold. Crosslink density increased with DHT temperature but not exposure period. Denaturation increased with DHT temperature and exposure period, ranging from 20% to 60% denaturation. Taken together, these results indicate that DHT treatment is a viable technique for altering the mechanical properties of CG scaffolds. Accordingly, DHT treatment can be used to produce CG scaffolds with a range of mechanical properties and denaturation levels. This provides both a means to tailor CG scaffolds for specific tissue engineering applications and a 3D *in vitro* model system to study how mechanical properties and denaturation affect cell function.

In this study we investigated the effects of varying DHT parameters on both compressive and tensile properties of CG scaffolds. The effects of DHT parameters on crosslink density and denaturation were also investigated and correlated with the mechanical properties. The detailed analysis of DHT treatment and the resulting effects is a key strength of this study. In contrast, previous studies have predominantly investigated the effects of DHT treatment on collagen solubility or denaturation temperature (Yunoki et al., 2003, Weadock et al., 1996). Further to this, studies are often carried out on collagen fibres or films (Weadock et al., 1995, Weadock et al., 1996, Wang et al., 1994, Yunoki et al., 2003), which may not be comparable to the crosslinking of a CG co-polymer. Studies that have examined the mechanical properties of DHT-treated collagen have limited their investigation to tensile properties due to the difficulties of testing collagen fibres in compression (Wang et al., 1994, Weadock et al., 1995). Studies investigating crosslinking treatments

commonly evaluate the *in vitro* cytotoxicity of the method used (Pieper et al., 1999, Lee et al., 2001). Whilst DHT treatment does not introduce any potential cytotoxic reagents into the scaffold (Khor, 1997) and the viability of cells seeded on CG scaffolds crosslinked in this manner has been demonstrated extensively in the literature (Torres et al., 2000, Lee et al., 2001, Ueda et al., 2003, O'Brien et al., 2005, Farrell et al., 2006), the effects of DHT treatment of cellular activity are examined in chapter 5.

Through DHT treatment, we produced CG scaffolds with moduli ranging from 0.5–1 kPa in compression and 1.9–7.6 kPa in tension (Figure 3.4 & 3.5). These results compare well with previous studies (Weadock et al., 1995, Charulatha and Rajaram, 2003, Harley et al., 2007). After crosslinking CG scaffolds at 105°C for 24 h, Harley *et al.* (2007) reported a compressive modulus of 0.2 kPa and a tensile modulus of 2.0 kPa, which compare well with our results of 0.5 kPa in compression and 1.9 kPa in tension. In the study by Harley *et al.*, the compressive modulus was calculated from a linear fit to the stress-strain curve over 0–10% strain, while we limited evaluation to 2–5% strain. The 0–2% strain regime encompasses the less stiff ‘toe’ region of the curve, explaining the lower compressive modulus reported by Harley *et al.*

FT-IR analysis supported the theory that DHT treatment forms crosslinks through a condensation reaction between carboxyl and amino groups. During this reaction NH_2 is converted to NH (Yannas and Tobolsky, 1968), which was monitored using FT-IR. Results show that the crosslink density increased with the temperature of DHT treatment but was not affected by exposure period (Figure 3.6). However, it may be possible that additional crosslinks are formed by extended DHT treatment. For

example, it has also been postulated that a lysino-alanine crosslink may also be formed during DHT treatment (Bohak, 1964, Gorham et al., 1992). This type of crosslink would not be detected using FT-IR analysis.

Results confirm that the extended exposure to high temperatures during DHT treatment alters the structure of the collagen in CG scaffolds. FT-IR analysis demonstrated that the level of denaturation caused by DHT treatment increased with exposure period and temperature (Figure 3.7). Scaffolds were 25% denatured after 24 h at 105°C, which compares well with a previous report of 27% denaturation for a collagen film after treatment at 110°C (Yunoki et al., 2003). Denaturation increased to 60% after DHT treatment at 180°C for 120 h. Extended treatment at 180°C also produced a decrease in tensile and compressive moduli, which may be indicative of changes in scaffold integrity caused by thermal degradation at this temperature. The temperatures used during DHT treatment break the hydrogen bonds that maintain the triple helical structure of collagen, altering it to a random coiled structure (Miles et al., 1995, Wright and Humphrey, 2002). The denaturation temperature of collagen has been shown to be related to the level of moisture content (Yannas and Tobolsky, 1968, Yannas, 1972, Sung, 1972). Therefore, the introduction of a secondary drying step in the freeze-drying process would reduce the moisture content of the scaffolds and as a result lessen the level of denaturation caused by DHT treatment. Previous work suggests that denatured collagen matrices show better tissue regeneration than native collagen matrices (Koide et al., 1993, Taubenberger et al., 2008). Therefore, increased denaturation coupled with improved mechanical properties may enhance the performance of CG scaffolds for use in tissue engineering. However, denatured collagen has also been shown to degrade faster than native collagen (Pieper et al.,

1999). As degradation rate has been shown to be an important aspect of scaffold design (Yannas and Burke, 1980), further investigation is required to determine the effect of increased denaturation on cellular activity and scaffold degradation rate both *in vitro* and *in vivo*.

Previous studies on collagen films have reported a decrease in tensile modulus and UTS with increasing denaturation (Bigi et al., 1998, Bigi et al., 2004). While it was found that the UTS and strain to failure were both negatively correlated with denaturation (Figure 3.9), the tensile modulus of CG scaffolds was positively correlated with denaturation (Figure 3.8 B). Therefore, DHT treatment, and possibly denaturation itself, causes the CG scaffold to become more brittle. However, the unwinding of the collagen triple helix caused by denaturation may allow the formation of additional crosslinks by bringing side chains into alignment. This would increase modulus while increasing brittleness. In contrast to tensile modulus, compressive modulus was only correlated with crosslink density. This suggests that structural changes that occur during DHT treatment, either through denaturation or the formation of crosslinks not detected using FT-IR, restrict fibre sliding but do not affect fibre buckling or bending stiffness.

Scaffolds fabricated using DHT treatment at 105°C for 24 hr and 150°C for 48 hr will be used in Chapter 5 to study the effects of crosslinking on cellular activity. 105°C for 24 hr will be used as it is the standard crosslinking treatment used in a range of studies and therefore provides a basis for comparison (Torres et al., 2000, Lee et al., 2001, O'Brien et al., 2005, Farrell et al., 2006). DHT treatment at 150°C for 48 hr provides a combination of improved mechanical properties (2-fold) without excessive

denaturation (40%) and will accordingly be used to determine the effects of extended DHT treatment on cellular activity.

3.5 Conclusion

Previous work has shown that mechanical properties must be considered when designing a scaffold for a specific tissue engineering application (Torres et al., 2000, Yeung et al., 2005, Powell and Boyce, 2006, Engler et al., 2006). This study demonstrates that tensile and compressive moduli of CG scaffolds can be significantly increased through DHT treatment. Both the temperature and duration of DHT treatment were found to have a significant effect on scaffold moduli and denaturation. Results show a correlation between increases in tensile modulus and the level of denaturation. The enhanced mechanical properties of DHT-treated CG scaffolds improves their suitability for use both *in vitro* and *in vivo*. In addition, this work facilitates investigation into the effects of mechanical properties and denaturation on cellular activity in a 3D environment.

Chapter 4. *The effect of chemical crosslinking on the mechanical properties of collagen-GAG scaffolds*

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4.1 Introduction

Chemical crosslinking has been used to enhance the stability of collagen based biomaterials for several years (Weadock et al., 1983, Park et al., 2002, Powell and Boyce, 2006, Olde Damink et al., 1996, Ruijgrok et al., 1994). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) and glutaraldehyde (GTA) are two of the most commonly used chemical crosslinking agents. These two chemicals work in distinctly different fashions. EDAC forms “zero length” crosslinks whereas GTA crosslinks take the form of long polymer chains (Powell and Boyce, 2006, Khor, 1997). This means that EDAC is limited to crosslinking collagen molecules which are directly adjacent to each other (1 nm) whereas GTA can crosslink molecules that are separated by a distance (Zeeman et al., 1999). However, the incorporation of GTA into CG scaffolds for tissue engineering leads to some disadvantages, as GTA has the potential to leach out of the scaffold as it degrades, with potentially toxic repercussions for cells in the immediate area (Wiebe et al., 1988, Gendler et al., 1984). EDAC on the other hand, produces substituted urea as a by-product of the crosslinking reaction. Urea is cytotoxic and needs to be washed out of the scaffold prior to use (Powell and Boyce, 2006).

Previous studies have concentrated on optimizing the effectiveness of one reagent, a study directly comparing the effectiveness of several grades of the two treatments has yet to be carried out (Powell and Boyce, 2006, Kikuchi et al., 2004, Lee et al., 2001, Ruijgrok et al., 1994). Additionally many studies have investigated crosslinking of collagen fibres or films which may not be directly comparable to the crosslinking of a collagen-GAG scaffold (Weadock et al., 1983, Olde Damink et al., 1996, Ber et al.,

2005, Ruijgrok et al., 1994). Furthermore, as each treatment affects specific residues, there is the potential for combining the two crosslinking methods (Weadock et al., 1983). Table 4.1 shows the specific amino acid residues that are affected by various crosslinking treatments. It can be seen that glutaraldehyde reacts solely with lysine residues whereas EDAC has the capacity to react with several different residues. In order to combine EDAC and GTA in the most effective manner, we must also take into account the strength of the bonds formed by each treatment. GTA affects a relatively small number of residues, yet GTA treated collagen has a comparable mechanical properties to EDAC-treated collagen (Weadock et al., 1983). This suggests that the crosslinks formed by GTA are stronger than other crosslinks. Therefore, to effectively use GTA in combination with EDAC (which affects many residues, including the same residue as GTA), Table 4.1 suggests that GTA crosslinking followed by EDAC crosslinking would yield the best results. Using EDAC first may consume many of the lysine residues that GTA needs to create stronger crosslinks.

Procedure	Reactive Residues	Reactive Residues Per Alpha Chain		Sum of Reactive Residues
		$\alpha 1$	$\alpha 2$	
Short-wave U.V. Light	Tyrosine	4	3	51
	Phenyl-alanine	12	16	
Dehydro-thermal or Carbodiimide	Lysine	32	21	745
	Arginine	50	57	
	Aspartic acid	45	47	
	Glutamic acid	77	71	
	Serine	34	24	
Gluter-aldehyde	Threonine	16	17	53
	Lysine	32	21	

Table 4.1 Theoretical distribution of reactive amino acid residues for various crosslinking treatments (Weadock et al., 1983).

The overall goal of this study is to determine the effects of chemical crosslinking on the mechanical properties of CG scaffolds. Despite the widespread use of chemical treatments on collagen films, fibres and scaffolds, a detailed analysis has not been carried out to determine how the process variables affect CG scaffold properties. Therefore, the specific objectives of this study were to determine the effects of 1) EDAC concentration, 2) GTA concentration and 3) the effects of combined EDAC and GTA crosslinking on the mechanical properties of CG scaffolds.

4.2 Materials and Methods

4.2.1 Scaffold Fabrication

Scaffolds were produced by freeze-drying a collagen-GAG slurry as described in chapter 2 (Sections 2.2.1 & 2.2.2.). Briefly, a slurry containing 0.5% (w/v) collagen and 0.044% (w/v) chondroitin-6-sulfate was prepared. This slurry was then freeze-dried using a T_f of -40 (Section 2.2.2, Figure 2.1). This process produces a highly porous sheet of CG scaffold, with an average pore size of 120 μm (Section 2.3.1, Table 2.1).

4.2.2 Crosslinking

Cylindrical samples (8 mm diameter, 4 mm height) were cut from the scaffolds prior to crosslinking. Samples were then prehydrated in phosphate buffered saline (PBS, Sigma-Aldrich, Germany) for 24 h prior to crosslinking. Five different concentrations of EDAC (Sigma-Aldrich, Germany) were used: 0.6, 3, 6, 48 and 96 mmol EDAC per g collagen. These concentrations were based on the results of a study by Olde Damink *et al.* (1996), in which the optimum concentration was found to be 6 mmol. These concentrations were prepared in 2 ml distilled water and the samples were crosslinked

in 24 well plates for 24 h. A molar ratio of 2.5 mol NHS/mol EDAC was used for all EDAC crosslinking (Olde Damink et al., 1996). GTA (Sigma-Aldrich, Germany) crosslinking was also carried out using five concentrations: 0.025, 0.05, 0.125, 0.25 and 0.375 g GTA per g of collagen. These concentrations were based on the results of a study by Ruijgrok *et al.* (1994). GTA crosslinking solutions were prepared in PBS and the samples were crosslinked in 24 well plates for 24 h. In order to determine the effects of combined EDAC and GTA crosslinking, samples were crosslinked using 0.125 g GTA followed by 96 mmol EDAC or 96 mmol EDAC followed by 0.125 g GTA.

4.2.3 Mechanical Testing

Compressive testing was used to determine the effect of DHT parameters on the mechanical properties of the scaffolds. Mechanical testing of scaffold samples was carried out using a mechanical testing machine (Z050, Zwick/Roell, Germany) fitted with a 5-N load cell. Samples were pre-hydrated in phosphate buffered saline (PBS) for one hour prior to testing and all testing was carried out in a bath of PBS. For unconfined compression testing with impermeable, un-lubricated platens, samples of 8 mm diameter were cut from the scaffolds using a punch. Testing was conducted at a strain rate of 10%/min. The modulus was defined as the slope of a linear fit to the stress-strain curve over 2-5% strain.

4.2.4 Statistical Analysis

Results are expressed as mean $\pm$ standard deviation. One-way analysis of variance (ANOVA) followed by pairwise multiple comparison procedures (Tukey test) was

used to evaluate the effects of EDAC and GTA concentrations on the compressive modulus of CG scaffolds. Statistical significance was declared at $p \leq 0.05$.

4.3 Results

4.3.1 EDAC Crosslinking

As expected, analysis of the mechanical properties following EDAC treatment revealed that compressive modulus increased with increasing EDAC concentration (Figure 4.1, $p < 0.05$). A concentration of 96 mmol EDAC per g collagen produced the stiffest scaffolds, with a compressive modulus of 1.8 kPa. This was a 4-fold increase over the untreated scaffolds ($p < 0.05$). However, it is worth noting that a concentration of 6 mmol EDAC per g collagen produced a significant increase in modulus whilst using one 16th the amount of EDAC, this can be seen clearly when compressive modulus is plotted against EDAC concentration using a linear scale (Figure 4.2).

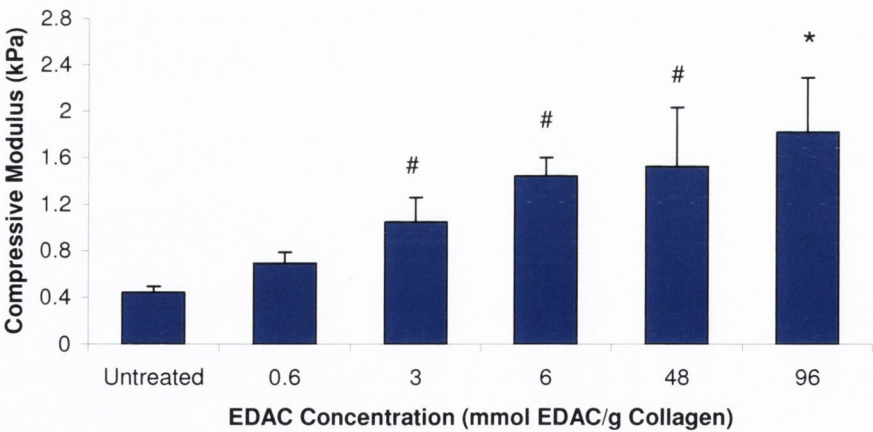


Figure 4.1 Compressive modulus vs. EDAC concentration (n = 10 samples, * $p < 0.05$ compared to all other groups, # $p < 0.05$ compared to the untreated group).

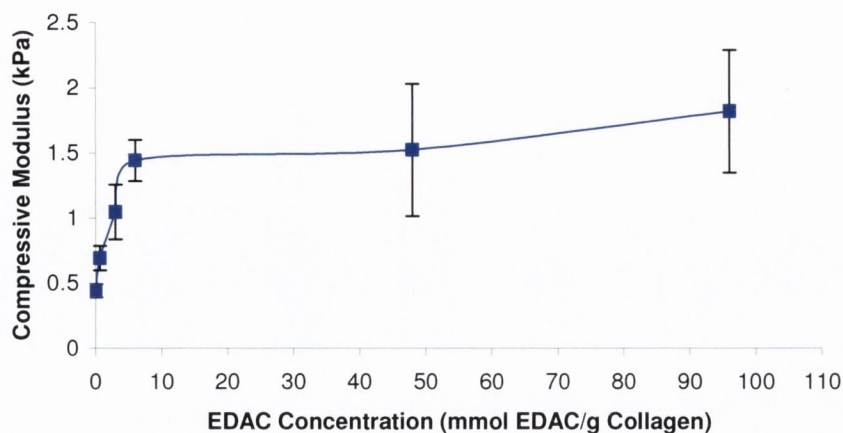


Figure 4.2 Compressive modulus vs. EDAC concentration. EDAC concentration is shown on a linear scale ($n = 10$ samples per group).

4.3.2 GTA Crosslinking

Analysis of the mechanical properties following GTA treatment revealed that compressive modulus increased with concentration up to 0.125 g GTA per g collagen (Figure 4.3). Further increases gave insignificant changes in modulus, with a reduction in modulus comparing 0.375 g GTA to 0.125 g GTA ($p > 0.05$). GTA crosslinking using 0.125 g produced the stiffest scaffolds, with a compressive modulus of 1.45 kPa. This was a 3-fold increase over the untreated scaffolds ($p < 0.05$).

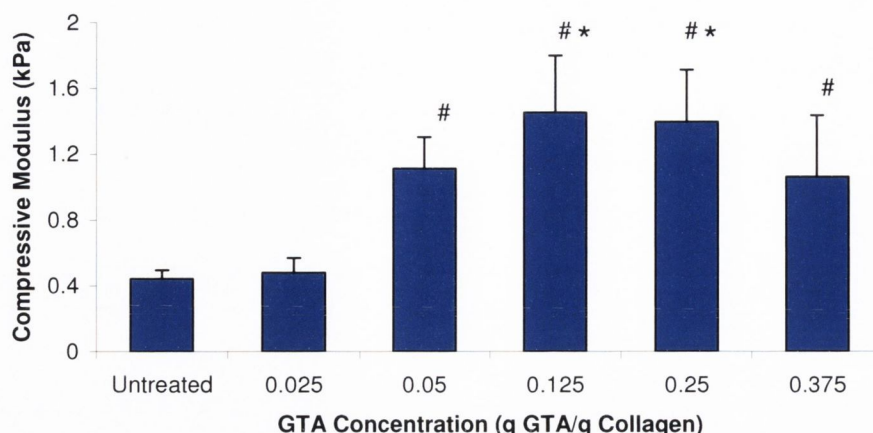


Figure 4.3 Compressive modulus versus GTA concentration (n = 10 samples, * p<0.05 compared to 0.05 g GTA group, # p<0.05 compared to the untreated group).

4.3.3 Combined Crosslinking

In order to establish the effects of combining EDAC and GTA crosslinking, the concentrations which yielded the stiffest scaffolds were chosen from the two techniques, EDAC 96 mmol and GTA 0.125 g. Scaffolds were crosslinked using EDAC first followed by GTA or GTA first and then EDAC so as to determine if order had an effect on crosslinking. The results from mechanical testing are compared to the stiffest EDAC and GTA treatments in figure 4.4. No significant differences were found between the crosslinking groups, suggesting that there was no benefit of combining the two crosslinking treatments (p>0.05).

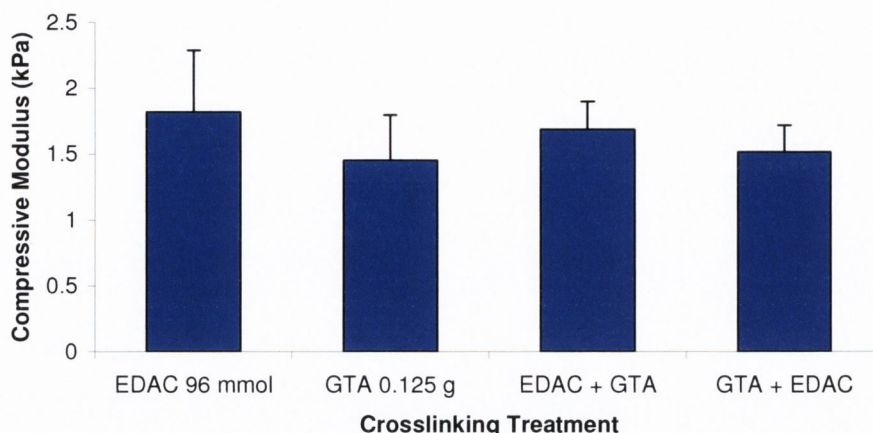


Figure 4.4 Comparison of the optimal treatments and the combined crosslinking treatments (n = 10 samples per group).

4.4 Discussion

Chemical crosslinking has been used previously to provide stabilisation and improve the mechanical properties of collagen films, sheets and dermal layers (Weadock et al., 1983, Ruijgrok et al., 1994, Olde Damink et al., 1996). With this in mind, we sought to determine the effects of EDAC and GTA crosslinking on CG scaffolds. Our experiments have demonstrated that a wide range of compressive moduli can be attained by varying the concentration of the two chemical crosslinking agents (Figure 4.1 & 4.3). The stiffest scaffolds were produced using 96 mmol of EDAC and 0.125 g GTA per a gram of collagen. We also sought to explore the possibility of using EDAC and GTA in combination to achieve further increases in mechanical properties. Results show that the use of EDAC and GTA in combination had no effect on mechanical properties when compared to the use of the treatments on their own. Taken together, these results indicate that GTA and EDAC crosslinking can be used effectively to produce CG scaffolds with a range of mechanical properties.

This study presents a comparison of the effects of EDAC and GTA crosslinking at range of different concentrations which can be directly related to the collagen content of the scaffolds. The majority of the work previously published has neglected to give the molar ratios of the crosslinking agents to the weight of collagen treated (Lee et al., 2001, Powell and Boyce, 2006, Jorge-Herrero et al., 1999, Torres et al., 2000). This prevents the results from being used in an effective manner, as the exact crosslinking conditions can not be reproduced. Many studies use tensile testing to determine the effects of crosslinking on the mechanical properties of collagen (Torres et al., 2000, Weadock et al., 1983). However, previous work has shown that the compressive properties are of greatest interest when studying the effects of substrate properties on cellular activity such as cell-mediated contraction and differentiation (Harley et al., 2007, Engler et al., 2006). Taking into account these studies and the material demands of tensile testing, it was decided that compressive testing would provide sufficient data for mechanical characterisation of the crosslinked scaffolds. Additionally, the potential cytotoxicity of chemically treated scaffolds is of interest; this topic is addressed in Chapter 5.

Harley *et al.* (2007) also examined the effectiveness of EDAC crosslinking of CG scaffolds and reported a compressive modulus of 1.497 kPa when treating scaffolds with 6 mmol EDAC per gram of collagen. This compares well with our result of 1.442 kPa with scaffolds crosslinked at the same molar ratio. Olde Damink *et al.* (1996) reported that the optimal increase in shrinkage temperature and crosslinks formed was achieved using molar ratio of EDAC to collagen was 6 mmol, our results show the highest Young's modulus at 96 mmol. However, the differences can be explained by

the fact that we tested a collagen glycosaminoglycan composite scaffold whereas they tested collagen fibres. Additionally, Pieper *et al.* (1999) have shown that EDAC forms crosslinks between chondroitin sulphate (GAG) and collagen, therefore increasing the amount of possible crosslinks that can be formed. Furthermore, 6 mmol of EDAC per g of collagen was the largest molar ratio used in the study by Olde Damink.

Surprisingly, the results show that there is no benefit of combining GTA and EDAC crosslinking. Figure 4.1 shows the particular amino acids that each crosslinking treatment reacts with. Specifically, GTA forms crosslinks by bonding the amine groups found upon lysine residues. Whereas, EDAC crosslinks form bonds between an amine group, again from lysine, and a carboxylic group, which can be found in several amino acids. The mechanisms for the crosslinking reactions are shown in figure 4.5. It was thought that EDAC crosslinking would be unable to react with all the available amine groups as it can only form short “zero length” crosslinks. GTA would therefore be able to react with the remaining amine groups as it constructs crosslinks in the form of long polymer chains. A possible explanation for this may be that the positioning of the un-reacted amine groups after EDAC crosslinking is unfavourable for additional GTA crosslinking or that the number of GTA crosslinks formed in such circumstances has negligible effects on the mechanical properties of the scaffolds. Likewise, when GTA crosslinking is carried out first, the positioning of un-reacted amine groups or the small number of additional crosslinks formed makes secondary EDAC crosslinking ineffective. Additionally, when GTA is used as the primary crosslinking treatment there is the possibility that it may form incomplete crosslinks, therefore using up all the available amine groups. EDAC does not create

this problem as it catalyses a reaction between a carboxylic group and an amine group only when both groups are present.

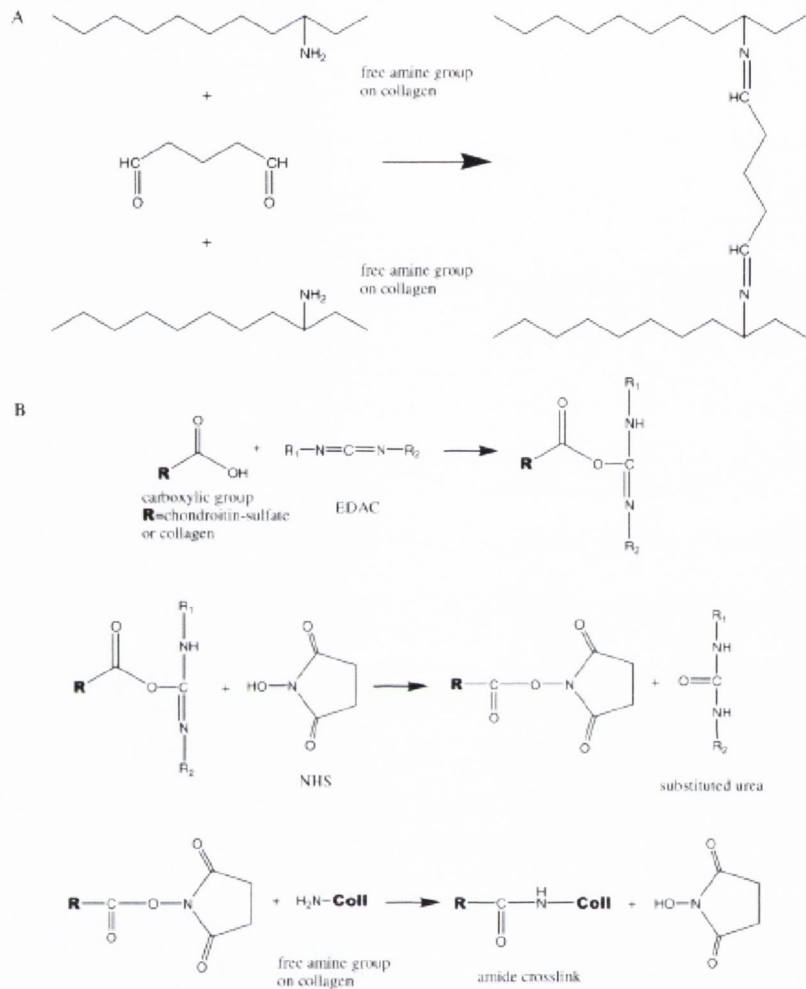


Figure 4.5 (A) GTA and (B) EDAC crosslink formation (Adapted from Lee *et al.*, 2001).

The most favourable mechanical properties were attained using 96 mmol of EDAC and 0.125 g GTA per a gram of collagen. Crosslinking using these treatments resulted in a 3 to 4-fold increase in compressive modulus. In addition, it is worth noting that 6 mmol EDAC crosslinking imparted mechanical properties comparable to the EDAC 96 mmol group whilst making efficient use of the crosslinking reagent (80% modulus using 6% the amount of EDAC), this may be of importance when the cytotoxicity of

the treatment is evaluated. The results from this study and Chapter 3 will be used in the cellular experiments described in Chapter 5 (Figure 4.6).

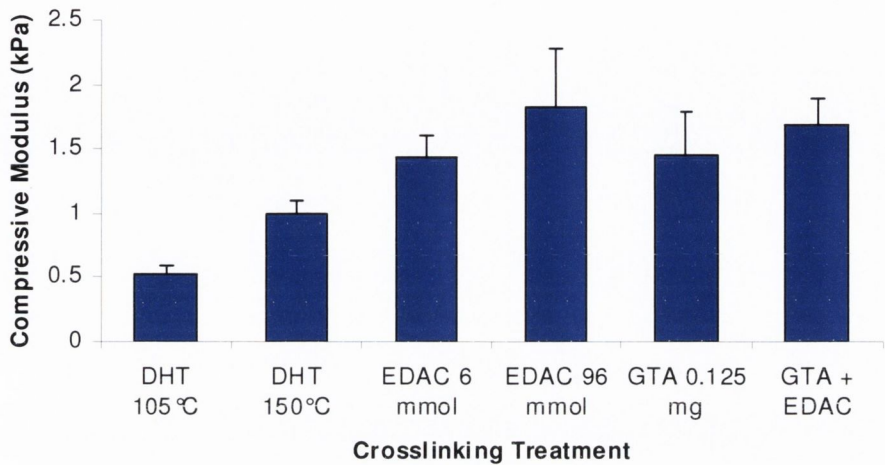


Figure 4.6 Compressive modulus of scaffolds crosslinked using various chemical and physical treatments (n = 10 samples per group).

4.5 Conclusion

The results of this study show that EDAC and GTA crosslinking can be used to produce substantial increases in the mechanical properties of CG scaffolds. The optimal properties were attained using 96 mmol of EDAC and 0.125 g GTA per a gram of collagen. Using these techniques a 4-fold increase in the compressive properties of CG scaffolds was attained ($p<0.05$). Results show that the mechanisms of EDAC and GTA crosslinking prevent their use in combination.

Chapter 5. *The effect of crosslinking on the activity of osteoblasts within collagen-GAG scaffolds*

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5.1 Introduction

Crosslinking is a technique that can be used to enhance the mechanical properties of collagen-based biomaterials (Weadock et al., 1983, Khor, 1997, Lee et al., 2001). In the previous two chapters we evaluated the effects of several crosslinking techniques on the mechanical properties of collagen-GAG (CG) scaffolds (Figure 5.1). While the effects of crosslinking on the mechanical properties of CG scaffolds are important, it is also of interest to determine the effects of crosslinking, and the subsequent changes in mechanical properties, on cellular activity within the scaffolds.

Primarily, the improvements required in the mechanical properties of CG scaffolds, via crosslinking, are needed in order to facilitate *in vitro* use and implantation. Additionally, significant cell-mediated contraction of collagen-based scaffolds during culture has been reported (Nehrer et al., 1997a, Lee et al., 2000). Contraction causes a reduction in the pore size and permeability of the scaffolds, which subsequently reduces the diffusion of nutrients and waste products. The degree of contraction also creates problems when designing a tissue graft to repair a particular size defect. The use of crosslinking to improve the mechanical properties of CG scaffolds would significantly reduce contraction and the problems associated with it. In addition, previous work has shown that the mechanical properties of scaffolds can influence cellular differentiation, adhesion and proliferation (Engler et al., 2004a, Engler et al., 2004b, Engler et al., 2006, Yeung et al., 2005). Therefore, the use of crosslinking to alter the mechanical properties of CG scaffolds may offer further insight into the mechanisms of these effects.

The nature of chemical crosslinking involves the use of reagents which have the potential to cause cell death if they remain within the scaffold. It is therefore necessary to carry out experiments to determine if these chemicals have any cytotoxic side effects. EDAC crosslinking does not attach any harmful molecules to the scaffold, but it does need to be thoroughly washed out of the scaffolds prior to use as the chemical itself is toxic, as is urea, which is the by-product of the crosslinking reaction. The potential cytotoxicity of GTA has been discussed previously in Chapter 4, although there is some debate as to whether it is cytotoxic on a clinically relevant level (Lee et al., 2001, Kikuchi et al., 2004, Chen et al., 2007).

In order to determine the effects of crosslinking on cellular activity, the most mechanically promising and efficient groups were chosen from the previous studies (Chapter 3 & 4) of crosslinking techniques (Figure 5.1). DHT treatment at 105°C for 24h was chosen as it is the standard crosslinking treatment used in many studies (Yannas et al., 1980, O'Brien et al., 2005, Tierney et al., 2008). DHT treatment at 150°C for 48h was chosen as the optimal DHT treatment, as it gave a good balance between improved mechanical properties and low denaturation. EDAC 96 mmol/g collagen and GTA 0.125 g/g collagen were chosen as the optimal treatments from the previous studies. EDAC 6 mmol/g collagen was also used as it combined good mechanical properties with efficient use of the crosslinking reagent. Finally EDAC 96 mmol/g collagen and GTA 0.125 g/g collagen were used in combination. The MC3T3 osteoblastic cell line was used as it is used currently in our lab, thus allowing comparison to previous studies (Menard et al., 2000, O'Brien et al., 2005, Tierney et al., 2008). The specific goals of this study were to investigate the effects of crosslinking techniques and thus scaffold stiffness, on 1) the metabolic activity, 2)

seeding efficiency, 3) proliferation and 4) spatial distribution of MC3T3 osteoblasts seeded onto collagen-GAG scaffolds.

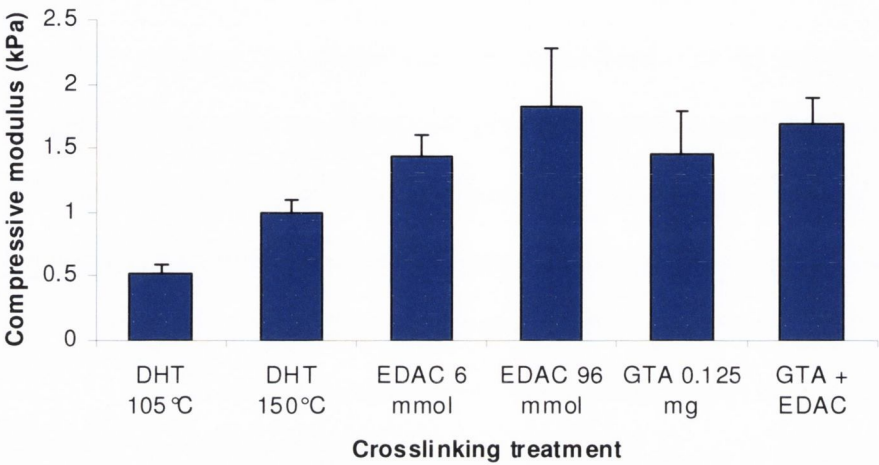


Figure 5.1 Compressive modulus of scaffolds crosslinked using various chemical and physical treatments.

5.2 Materials and Methods

5.2.1 Scaffold Fabrication

Scaffolds were produced by freeze-drying a collagen-GAG slurry as described in chapter 2 (Sections 2.2.1 & 2.2.2.). Briefly, a slurry containing 0.5% (w/v) collagen and 0.044% (w/v) chondroitin-6-sulfate was prepared. This slurry was then freeze-dried using a T_f of -40 (Section 2.2.2, Figure 2.1). This process produces a highly porous sheet of CG scaffold, with an average pore size of 120 μm (Section 2.3.1, Table 2.1).

5.2.2 Crosslinking

Cylindrical samples (12.7 mm diameter, 4 mm height) were cut from the scaffolds prior to crosslinking. DHT treatment was carried by placing the samples in an aluminium foil packet inside a vacuum oven (Vacucell 22, MMM, Germany) under a

vacuum of 0.05 bar. To determine the effect of DHT parameters on osteoblast activity, exposure period and crosslinking temperature were varied. Two settings were used 1) exposure at 105°C for a period of 24 h and 2) exposure at 150°C for a period of 48 h. EDAC (Sigma-Aldrich, Germany) crosslinking was carried out using two concentrations, 6 and 96 mmol of EDAC per gram of collagen. GTA (Sigma-Aldrich, Germany) crosslinking was carried out at a concentration of 0.125 g GTA per gram of collagen. Additionally, a group of scaffolds were crosslinked using 0.125 g GTA followed by 96 mmol of EDAC. All chemical crosslinking was carried out over 24 h, after which the scaffolds were washed twice using sterile PBS (Sigma-Aldrich, Germany).

5.2.3 Cell Culture & Seeding

MC3T3-E1 osteoblasts (ATCC, Manassas, VA) were cultured in T175 culture flasks (Sarstedt, Nuembrecht, Germany) using alpha minimum essential media (Sigma-Aldrich, Germany) supplemented with 10% fetal bovine serum (BioSera, East Sussex, UK), 1% L-glutamine (Sigma-Aldrich, Germany) and 2% penicillin/streptomycin (Sigma-Aldrich, Germany). Prior to seeding, cells (Passage 26) were detached using trypsin-ethylenediaminetetraacetic acid (Sigma-Aldrich, Germany) and suspended in supplemented medium at 10^7 cells/mL. Crosslinked scaffolds were placed into 6-well tissue culture plates (Sarstedt, Nuembrecht, Germany). The top surface of each of the scaffolds was then seeded with 100 μ L of the cell suspension (1×10^6 cells). The 6-well plates were then placed in an incubator for 15 min to allow initial cellular attachment. The scaffolds were then turned over and the opposite surface was seeded with 100 μ L of the cell suspension and incubated for a further 15 min. After the second incubation period, 5 ml of supplemented medium was added to each well and

the plates were returned to the incubator. Both culture flasks and seeded scaffolds were cultured under standard conditions (37°C, 5% CO₂).

5.2.4 AlamarBlue™ Analysis

Reduction of AlamarBlue™ (BioSource, Belgium) was used as a measure of metabolic activity (O'Brien et al., 2000). After 1, 2 and 7 days of incubation, seeded scaffolds were transferred to 6-well plates containing 10% AlamarBlue™ in supplemented media. The plates were placed on an orbital shaker (Biosan, Riga, Latvia) and incubated for a further 2 hours. 100 µl of the supernatant was then plated, in triplicate, into a 96-well plate and absorbance of the samples were read on a spectrometer (Titertrek Multiscan, MSC, Ireland) at 540 nm and 620 nm. Percentage reduction of the AlamarBlue™ solution was then determined using a standard curve.

5.2.5 Hoechst DNA Assay

Cell number was evaluated using Hoechst 33258 assay, which fluorescently labels double-stranded DNA (Sigma-Aldrich, Germany), according to a previously published protocol (Kim et al., 1988). After 1, 2 and 7 days of incubation, seeded scaffolds were flash frozen in liquid nitrogen and stored at -80°C. Samples were then thawed and digested in Papain (Sigma-Aldrich, Germany) for 1.5 hours at 60°C. Hoechst dye solution was then added to the digested samples and 210 µl was then plated, in triplicate, into a 96-well plate. Fluorescence was then measured (excitation: 355 nm, emission: 460 nm) using a fluorescence spectrophotometer (Wallac Victor2, PerkinElmer Life Sciences). Readings were converted to cell number using a standard curve, with unseeded scaffolds used as blanks.

5.2.6 Histology

Histological analysis was used to evaluate the spatial distribution of cells within the scaffolds. After 1 and 7 days of incubation, scaffold samples were placed into a solution of 10% formalin for 30 minutes and then embedded in paraffin wax using an automatic tissue processor (ASP300, Leica, Germany). All samples were sectioned longitudinally at a thickness of 10 μm throughout the depth of the sample using a rotary microtome (Leica microtome, Leica). Sections were mounted on slides and placed in an oven at 70°C overnight to remove wax from the sample and adhere the sections to the slides. The slides were then stained using haematoxylin and eosin (H&E) to evaluate spatial distribution of cells within each of the scaffolds. Digital images were recorded using a microscope at 125x magnification (Optimphot2, Nikon, Japan).

5.2.7 Statistical Analysis

Results are expressed as mean $\pm$ standard deviation. Two-way analysis of variance (ANOVA) followed by pairwise multiple comparison procedures (Tukey test) was used to evaluate the effects of crosslinking on metabolic activity and cell number. Statistical significance was declared at $p \leq 0.05$.

5.3 Results

5.3.1 Metabolic Activity

The metabolic activity within the scaffolds was found to be significantly higher upon the GTA crosslinked scaffolds when compared to other crosslinking groups (Figure 5.2, $p < 0.001$). GTA crosslinked scaffolds showed a 70% increase in metabolic

activity when compared to the standard DHT 105°C crosslinked scaffolds ($p<0.001$). Comparing 1 and 7 day time points, metabolic activity was found to increase over incubation period in the GTA and GTA+EDAC crosslinking groups ($p<0.05$).

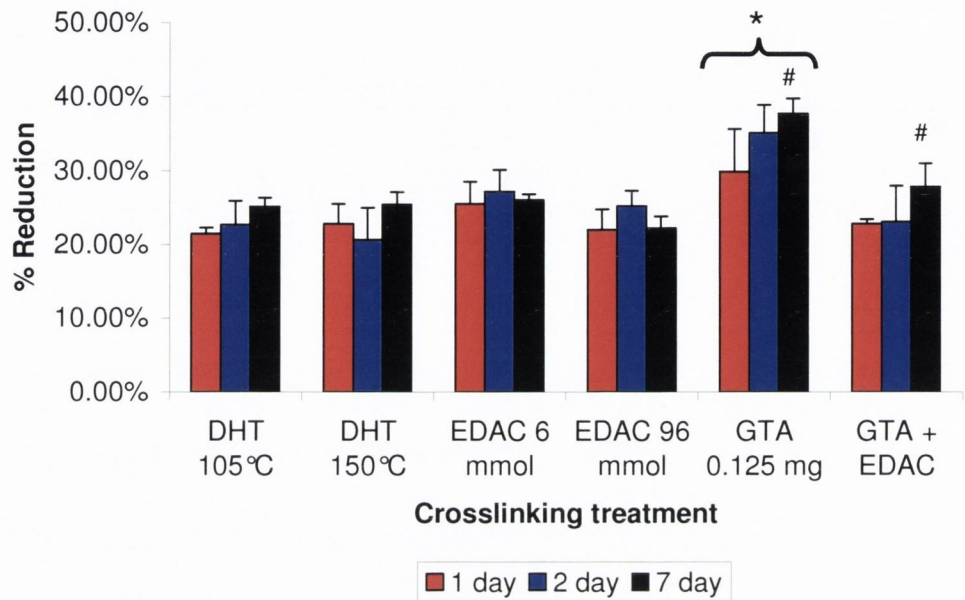


Figure 5.2 Metabolic activity of MC3T3 osteoblasts within the scaffolds at 1, 2 and 7 days as measured using the AlamarBlue™ assay (n=4 per group, * $p<0.001$ compared to all other groups, # $p<0.05$ compared to 1 day time point in the same group).

5.3.2 Cell Number

The number of cells within the scaffolds was found to vary with the method of crosslinking used (Figure 5.3). EDAC 6 mmol, GTA and GTA+EDAC groups showed a significant increase in cell number over the standard, DHT 105°C treated scaffolds ($p<0.05$). In particular, GTA crosslinked scaffolds had a significantly higher cell number when compared to all other treatment groups, showing a 5-fold increase in cell number when compared to the standard DHT 105°C crosslinked scaffolds ($p<0.001$).

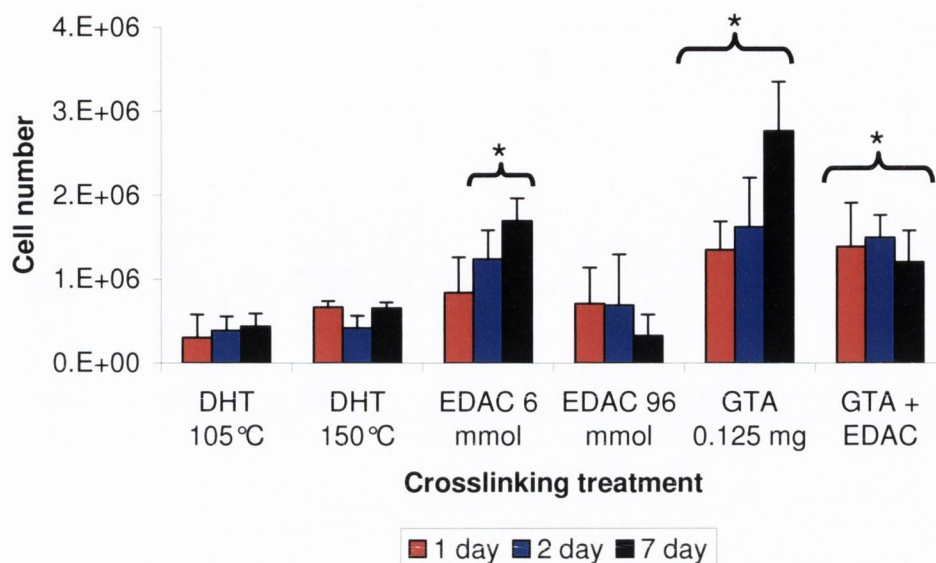


Figure 5.3 MC3T3 cell number within the scaffold at 1, 2 and 7 days measured using Hoechst DNA assay (n=4 per group, * p<0.05 compared to DHT 105°C group).

The effect of crosslinking on seeding efficiency was measured by expressing the number of cells attached after 1 day as a percentage of the number of cells initially seeded (2×10^6 cells, Figure 5.4). The GTA and GTA+EDAC groups had the highest seeding efficiency, with 70% of seeded cells attached after 1 day. This represents a 4.5-fold increase over the standard crosslinking treatment ($p < 0.001$).

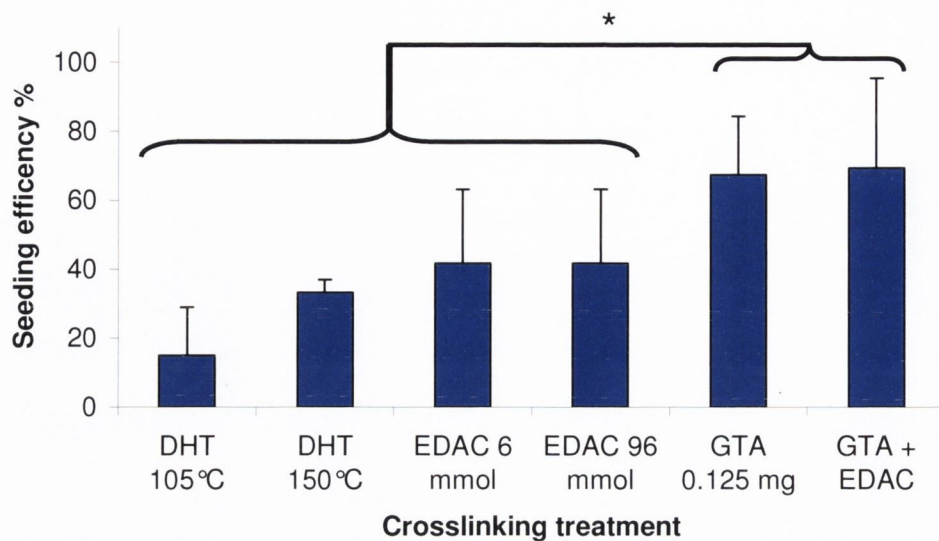


Figure 5.4 An assessment of seeding efficiency on the various scaffold types, by comparison of the number of cell seeded against the number attached after 1 day of incubation (n=4 per group, * p<0.05).

Cellular proliferation upon the scaffolds was measured using the difference between the cell number at 1 and 7 days (Figure 5.5). Significant levels of proliferation were found in the EDAC 6 mmol and GTA crosslinking groups (p<0.05). In particular, a 2-fold increase in cell number was found in the GTA crosslinked group (p<0.001). Insignificant reductions in cell number were found in the EDAC 96 mmol and GTA+EDAC crosslinking groups.

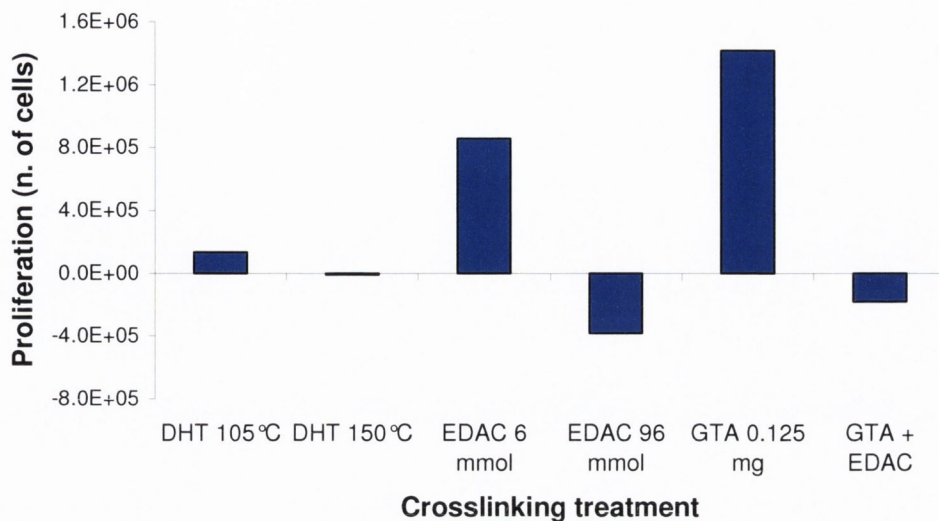


Figure 5.5 Proliferation of MC3T3 osteoblasts upon the various scaffold types, by comparison of the cell number at 1 day against the cell number after 7 days of incubation (n=4 per group, * p<0.05).

5.3.3 Cell Distribution

Histology was used to determine the effect of crosslinking on the spatial distribution of cells within the collagen-GAG scaffolds at 1 and 7 days (Figure 5.6-5.11). All treatment groups show cellular attachment at the edge of the scaffolds at 1 and 7 days (Figure 5.6-5.11, A & C). There was no evidence of cellular infiltration into the centre of the scaffolds after 7 days in the DHT, EDAC 96 mmol and GTA+EDAC groups (Figure 5.6 D, 5.7 D, 5.9 D and 5.11 D). However, EDAC 6 mmol and GTA crosslinking groups show notable levels of cellular infiltration at the centre of the scaffolds after 7 days of culture (Figure 5.9 D & 5.10 D).

DHT 105°C

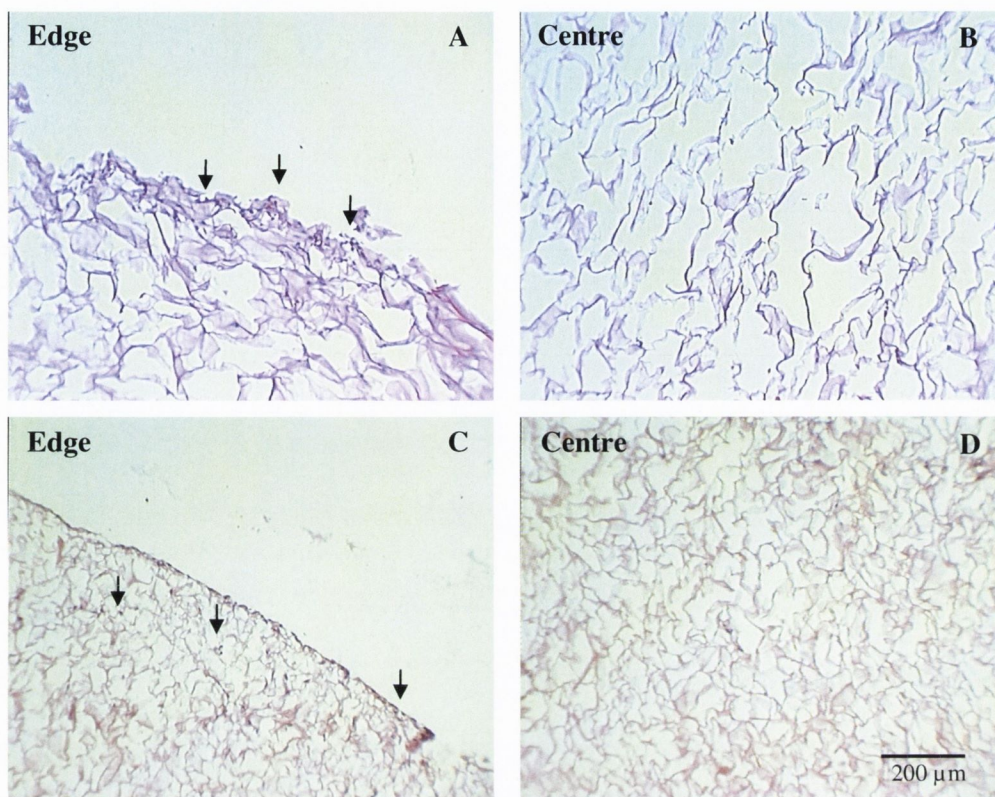


Figure 5.6 Spatial distribution of MC3T3 osteoblasts at the edge and centre of DHT crosslinked scaffolds (105°C, 24 h) after 1 (A&B) and 7 days (C&D). At 1 day cells can be seen attached to the periphery of the scaffolds (A), with no cells present at the centre (B). At 7 days cells can be found at the periphery of the scaffolds (C), but not at the centre (D).

DHT 150°C

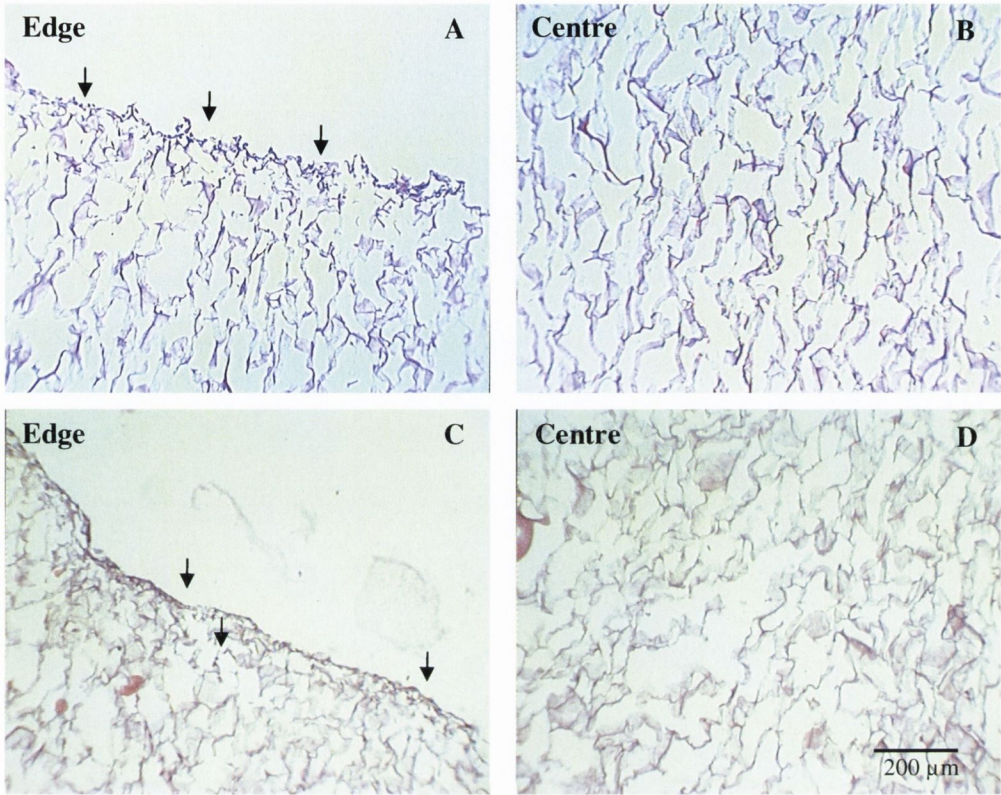


Figure 5.7 Spatial distribution of MC3T3 osteoblasts at the edge and centre of DHT crosslinked scaffolds (150°C, 48 h) after 1 (A&B) and 7 days (C&D). At 1 day cells can be seen attached to the periphery of the scaffolds (A) with no cells present in the centre (B). At 7 days cells can be found at the periphery of the scaffolds (C) but not at the centre (D).

EDAC 6 mmol

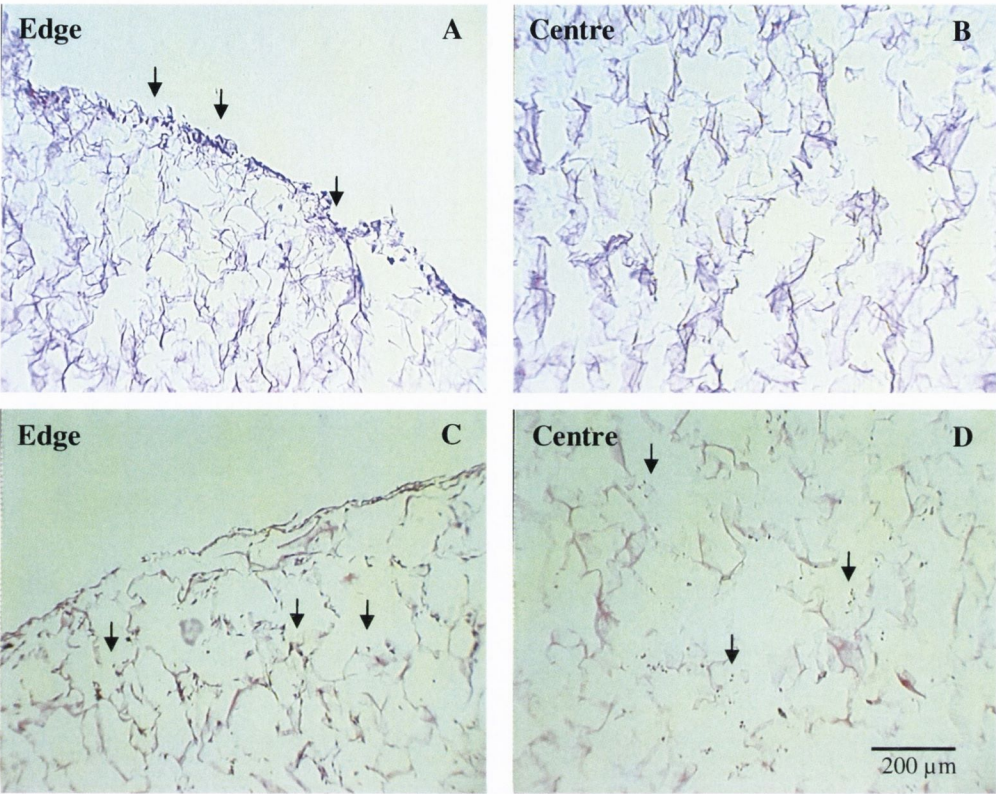


Figure 5.8 Spatial distribution of MC3T3 osteoblasts at the edge and centre of EDAC crosslinked scaffolds (6 mmol) after 1 (A&B) and 7 days (C&D). At 1 day cells can be seen attached to the periphery of the scaffolds (A), with no cells present at the centre (B). At 7 days cells can be found evenly distributed at edge of the scaffolds (C), with some cells reaching the centre (D).

EDAC 96 mmol

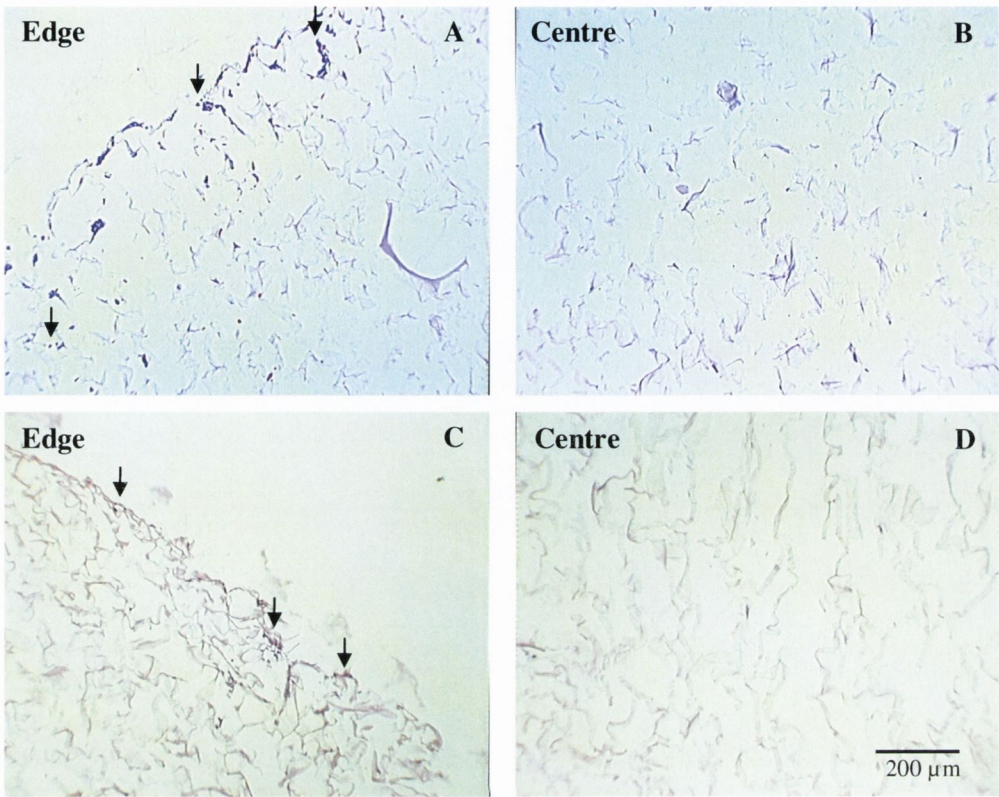


Figure 5.9 Spatial distribution of MC3T3 osteoblasts at the edge and centre of EDAC crosslinked scaffolds (96 mmol) after 1 (A&B) and 7 days (C&D). At 1 day cells can be seen attached to the periphery of the scaffolds (A), with no cells present in the centre (B). At 7 days cells can be found at the periphery of the scaffolds (C), but not at the centre (D).

GTA 0.125 mg

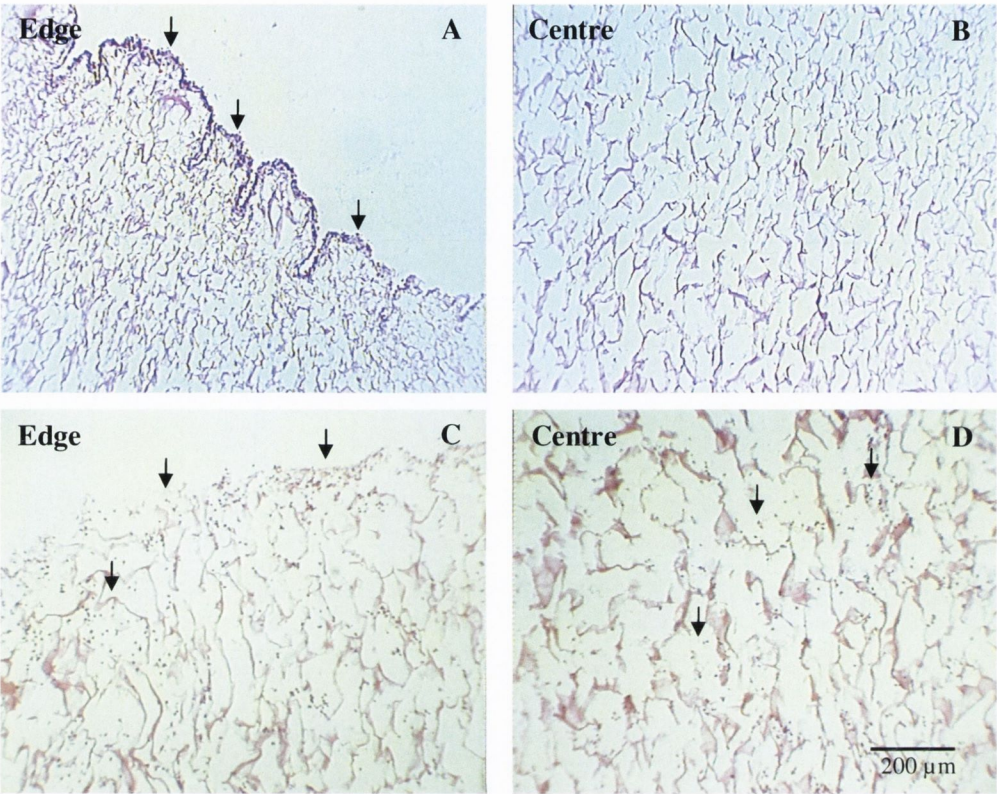


Figure 5.10 Spatial distribution of MC3T3 osteoblasts at the edge and centre of GTA crosslinked scaffolds (0.125 mg) after 1 (A&B) and 7 days (C&D). At 1 day cells can be seen attached to the periphery of the scaffolds (A), with no cells present at the centre (B). At 7 days cells can be found evenly distributed throughout the entirety of the scaffold, with numerous cells at both the edge (C) and centre (D).

GTA + EDAC

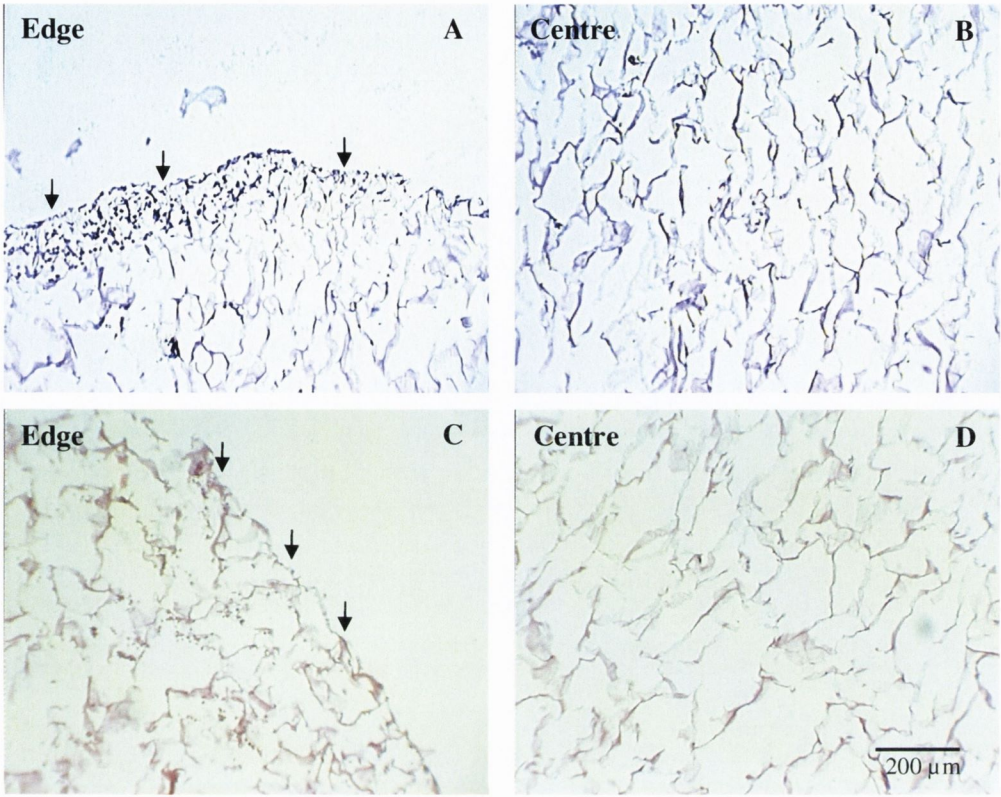


Figure 5.11 Spatial distribution of MC3T3 osteoblasts at the edge and centre of GTA+EDAC crosslinked scaffolds (96 mmol EDAC and 0.125 mg GTA) after 1 (A&B) and 7 days (C&D). At 1 day cells can be seen attached to the periphery of the scaffolds (A), with no cells present in the centre (B). At 7 days cells can be found at the periphery of the scaffolds (C), but not at the centre (D).

5.4 Discussion

Crosslinking, and the resultant changes in mechanical properties, have previously been shown to influence cellular activity. With this in mind, the aim of this study was to determine the effects of crosslinking on osteoblast activity within CG scaffolds. Scaffolds were crosslinked using six different treatments (DHT 105°C, DHT 150°C, EDAC 6 mmol, EDAC 96 mmol, GTA and GTA + EDAC), seeded with cells and incubated for 1, 2 and 7 days in order to evaluate the effects of crosslinking on metabolic activity, cell number and cellular distribution. The GTA crosslinked scaffolds showed increased metabolic activity, cell number and cellular distribution when compared to the other crosslinking treatments. The EDAC 6 mmol treated group also showed increased cell number and infiltration. However, we found that increasing the EDAC concentration to 96 mmol showed some evidence of cytotoxicity. Increasing the degree of DHT treatment (from 105°C to 150°C) was found to have no significant effects on cellular activity. Taken together, these results indicate that the enhanced cellular activity upon GTA and EDAC treated scaffolds demonstrates their suitability for use both *in vitro* and *in vivo*.

This study presents a direct comparison of the effects of several crosslinking treatments on cellular activity within CG scaffolds. Many studies present limited information with regard to the crosslinking procedure used (Lee et al., 2001, Powell and Boyce, 2006). For example percentage crosslinking solutions are provided without the volume of the solution used to treat the samples. This is insufficient information for calculation of the molar ratio of the crosslinking agent to collagen, limiting the constructive use of the data, particularly when the relationship between

cytotoxicity and the concentration of a crosslinking agent is of interest. Several studies examine the effect of crosslinking on cell mediated contraction (Lee et al., 2001, Powell and Boyce, 2006). Although we make observations on contraction, no measurements were taken. However, measurement of contraction was not believed to be useful due to the short term nature of the cell culture (up to 7 days) used in this study and the limited contraction observed within the majority of the groups.

We report a 15% seeding efficiency at 24 hr upon the standard scaffolds compared to 40% reported by O'Brien *et al.* (2005). However, the scaffolds in the O'Brien study were seeded dry, which would aid infiltration as the cell suspension would not have to displace any fluid already within the scaffolds. The DHT treated scaffolds in this study were seeded in a hydrated state in order to enable a direct comparison to chemical treatments which required the scaffolds to be hydrated at the seeding stage. This limitation could have been overcome through freeze-drying the chemically crosslinked scaffolds, though it is likely that this would have caused some damage to the structure of the scaffolds. In particular, GTA crosslinked scaffolds showed significantly enhanced seeding efficiency when compared the standard DHT group (70% compared to 15%, Figure 5.4). EDAC crosslinked scaffolds also showed increased seeding efficiency, however the difference was not statistically significant when compared to DHT groups (40% compared to 15%). The increased mechanical properties of GTA and EDAC crosslinked scaffolds allows them to retain their porous structure upon hydration while DHT treated samples contract upon hydration reducing the surface area available for seeding. However, GTA and EDAC crosslinked scaffolds have comparable mechanical properties, yet GTA crosslinked scaffolds

show superior seeding efficiency. This suggests that the presence of GTA crosslinks in some way affects seeding and cellular attachment.

GTA 0.125 g and EDAC 6 mmol treatment groups show enhanced proliferation and infiltration (Figure 5.5-5.11). In particular GTA crosslinked scaffolds showed a 6.4-fold increase in cell number at 7 days when compared to the standard DHT 105°C group. This compares well with previous work which has shown no proliferation upon the standard scaffolds after 7 days (Tierney et al., 2008). Additionally, the proliferation data correlates well with infiltration as the GTA 0.125 g and EDAC 6 mmol groups are the only groups to show infiltration into the centre of the scaffolds after seven days. Previous work has postulated that cell mediated contraction may reduce proliferation of chondrocytes (Lee et al., 2001). The only groups observed to show any cell mediated contraction were the groups treated using DHT treatment. Therefore the lack of proliferation in these groups may be caused by contraction of the scaffold structure, which among other things causes a collapse of the scaffold pore structure reducing the diffusion of nutrients and waste products within the scaffold. As the EDAC and GTA treated groups did not contract, the porous structure was retained allowing proliferation and infiltration within these groups. The GTA 0.125g group showed enhanced proliferation compared to EDAC 6 mmol, which may be a result of higher cell number at day 1 due to superior seeding efficiency.

The EDAC 96 mmol and GTA+EDAC groups did not perform as well as the other chemical crosslinking groups, showing a reduction in cell number after 7 days. This may be evidence of the cytotoxic effects of EDAC at high concentrations. Although EDAC is not incorporated into the scaffold when it forms crosslinks the byproduct of

this reaction is urea which is cytotoxic if left within the scaffolds. Previous work has found a reduction cellular activity when high concentrations of EDAC are used to crosslink collagen scaffolds (Powell and Boyce, 2006). Powell and Boyce postulated that it becomes more difficult to wash out the resultant urea and unused EDAC when high concentrations of EDAC are used. We found no evidence of cytotoxicity caused by GTA crosslinking. GTA is integrated into the crosslinks that are formed and unreacted GTA may also remain within the scaffold after crosslinking. Previous studies have found some evidence of cytotoxicity, causing reduced proliferation, with cells cultured on GTA crosslinked CG scaffolds (Lee et al., 2001). Contrary to this we found an increase in cellular activity with GTA crosslinked scaffolds. However there is still the potential for damage to the cells to occur as GTA is released as the scaffold degrades, although some *in vivo* studies show no evidence of adverse effects (Kikuchi et al., 2004).

In the context of bone tissue engineering it is also important to note that there are differing reports in the literature on the relationship between GTA crosslinking and mineralisation. In some studies GTA crosslinking has been shown in some studies to induce calcification *in vivo* (Jorge-Herrero et al., 1999, Kim et al., 1999). However there are reports that GTA may also compromise mineralisation in CG scaffolds (Chen et al., 2007). Long term cell culture experiments in osteogenic media are needed to clarify these findings.

5.5 Conclusion

When considering a crosslinking method for scaffolds used in a particular tissue engineering application, the effects of the various treatments on cellular activity is just

as important as the effect on mechanical properties. GTA and EDAC crosslinked scaffolds exhibit enhanced cell number, proliferation and cellular infiltration when compared to the standard DHT treatment. However, increased levels of EDAC crosslinking show some evidence of cytotoxicity, which may be due to the increased difficulty of removing reaction by-products. GTA crosslinked scaffolds show the greatest potential for tissue engineering, having the ability to support 6.4 times as many cells as the standard scaffolds. The enhanced cellular activity upon GTA and EDAC treated scaffolds, coupled with improved mechanical properties, increases their suitability for use both *in vitro* and *in vivo*.

Chapter 6. Discussion

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6.1 Collagen-Glycosaminoglycan Scaffolds and Tissue Engineering

Collagen-based scaffolds have found success in several clinical applications of tissue engineering (Yannas et al., 1989, Chamberlain et al., 1998, Atala et al., 2006). The success of these scaffolds can in part be attributed to their compositional similarity with the extra cellular matrix found in many tissues. However, it has been shown that the characteristics of these scaffolds must be tuned to their specific application if they are to be successful (Lee et al., 2001, Yannas, 2001, O'Brien et al., 2005, Byrne et al., 2008). This thesis describes a project which sought to develop a series of novel scaffolds with a range of structural and mechanical properties and to determine the effects of these properties on cellular behaviour.

The structural and mechanical characteristics are of particular importance as they have been shown to strongly influence cellular differentiation, attachment, gene expression, matrix deposition and *in vitro* degradation rate (Lee et al., 2001, Yannas, 2001, Engler et al., 2006, O'Brien et al., 2005, Byrne et al., 2008). Several studies have been published describing various methods for altering the properties of collagen scaffolds. However, the work presented in this study contains significant improvements of the methods used. For example, freeze-drying is often used to control the pore architecture of collagen scaffolds and several studies have investigated the effect of the freezing temperature on the mean pore size of the scaffolds produced (Park et al., 2002, Zmora et al., 2002, Chen et al., 2007, Faraj et al., 2007). However, these studies fail to control the rate of freezing and use crude methods such as immersion in oil baths or liquid nitrogen. In comparison, this study uses a temperature-controlled shelf to determine the effect of freezing temperature on pore size. It is proposed that the

series of characterised scaffold variants produced in this study can be adapted for specific tissue engineering applications or used to further our knowledge of the effects of the structural and mechanical properties of scaffolds on the cellular activity and tissue formation within them. The methods used to control the properties of CG scaffolds and their effects on cellular activity are described in the following sections.

6.2 Pore Architecture

The work described in Chapter 2 has led to the development of two distinct techniques which allow the pore structure of CG scaffolds to be modified in a controlled fashion. The first technique investigated the effect of altering the freezing temperature (T_f) used in the freeze-drying cycle. Additionally the introduction of an annealing step was also investigated; annealing is defined as a holding step at increased temperature prior to drying. This technique was based on the hypothesis that increasing the temperature of the frozen suspension would reduce the viscosity and hence increase the rate of ice crystal growth. Using these techniques, we produced several scaffolds with individual mean pore sizes that could be varied within the range of 85-325 μm . This is a significant improvement compared to our previous ability to control pore size (96-150 μm , O'Brien et al., 2005).

In order to determine the effect of T_f on pore size, scaffolds were produced with T_f ranging from -10 to -70°C. It was found that pore size reduced with T_f until -50°C, after which reductions in T_f failed to produce a significant change in pore size. Using this data and differential thermal analysis (DTA) we determined that the glass transition temperature of the CG suspension upon freezing is located between -42 and -50°C. To our knowledge, the glass transition temperature of a collagen suspension

has yet to be described in the literature. When the glass transition temperature is reached, a large increase in the viscosity of the suspension is observed, impeding any further changes in ice crystal structure, consequently explaining why pore size is not affected by T_f below -50°C . In order to determine the effect of annealing on pore size, the duration of the annealing step was varied. We found that annealing had a significant effect on pore size up to 18 h annealing time, which resulted in a 40% increase in pore size.

Previous studies have shown that pore size has a significant effect on cellular attachment, activity, gene expression and vascularisation (Wake et al., 1994, Nehrer et al., 1997b, Tsuruga et al., 1997, Zeltinger et al., 2001, O'Brien et al., 2005, Byrne et al., 2008). The optimal pore size has been shown to be dependant on both cell type and scaffold material. For a particular scaffold to be suitable for a wide range of applications, it is therefore necessary to be able to control the pore sizes over a wide range. In addition to allowing the elucidation of the optimal pore size for specific application, the ability to alter pore size also allows the influence of ECM pore size on cellular activity to be determined. The work contained in Chapter 2 has been used by another researcher in our lab, Ciara Murphy, to investigate the effect of pore size on the cell number within CG scaffolds 2 days post seeding (Figure 6.1).

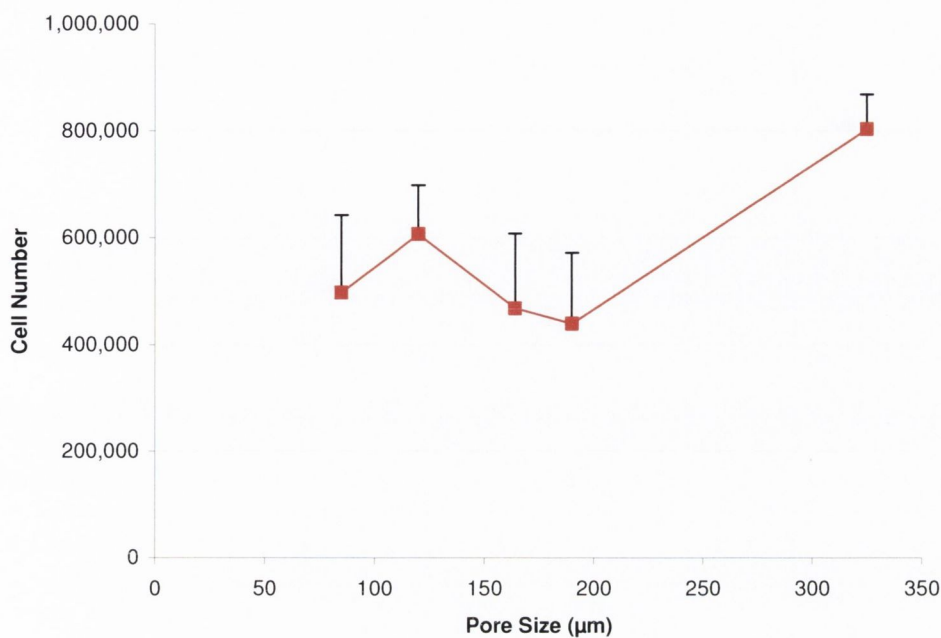


Figure 6.1 The effect of pore size on cell number, 2 days post seeding. MC3T3 osteoblasts were seeded onto CG scaffolds to determine the effect of pore size on cellular attachment and proliferation (n = 6, Murphy et al., 2008).

This follows on from previous work by O'Brien *et al* (2005), which determined the effect of pore size in the range of 95-150 μm on the attachment of osteoblasts within CG scaffolds. They determined that within this range cellular attachment increased with reducing pore size. However, when this work was repeated over a larger range of pore sizes it was found that while attachment decreased with increasing pore size from 120-200 μm, it began to drop off below 120 μm and peak levels of cellular attachment were found when the pore size was increased above 200 μm (Figure 6.1). It is hypothesised that the peak at 100 μm is due to the benefits of increased surface area with smaller pore sizes, while the second peak at 325 μm is due to the increase in permeability observed as pore size increases. This compares well to previous work which has shown that a pore size above 300 μm is required for bone tissue

engineering (Chu et al., 2001). The additional findings of the second study (Murphy et al., 2008) reveal the complexity of the interactions between pore structure and cellular activity, thus demonstrating the need for the ability to manufacture scaffolds with a wide range of mean pore size.

6.3 Mechanical Properties

In Chapters 3 & 4 I describe the effects of dehydrothermal (DHT), 1-ethyl-3-(3-dimethyl aminopropyl carbodiimide (EDAC) and glutaraldehyde (GTA) crosslinking on the mechanical properties of CG scaffolds. Crosslinking was used to strengthen the scaffolds as it provides an effective method of improving the mechanical properties without altering the porous nature of the scaffolds. DHT treatment is the standard method used to stabilise CG scaffolds in a number of studies, though it has yet to be investigated in a thorough fashion. EDAC and GTA, whilst considered alone for their effects on collagen, have not been studied in a manner that allows their relative merits to be directly compared for use in tissue engineering. In addition, it was hypothesised that it may be possible to combine EDAC and GTA treatments in order to further increase scaffold properties. Using these crosslinking treatments, we produced scaffolds with a compressive modulus that can be controlled between 0.44 and 1.8 kPa.

Temperature and exposure period of DHT treatment and the concentration of EDAC and GTA used were varied to determine the effects of these parameters on scaffold properties. Increasing the temperature and duration of DHT treatment resulted in a 2-fold increase in compressive modulus, while tensile moduli increased almost 4-fold. In addition, denaturation increased with DHT temperature and exposure period,

ranging from 20% to 60% denaturation. Denaturation is generally considered undesirable as it disrupts the native collagen structure and increases the degradation rate (Pieper et al., 1999, Miles and Bailey, 2001). However, denaturation has also been shown to reduce the inflammatory response and increase cellular activity *in vivo* (Koide et al., 1993, Taubenberger et al., 2008). Further work is required to clarify its impact on the performance of the scaffolds. As expected, the effectiveness of EDAC and GTA increased with concentration. The optimal mechanical properties were attained using 96 mmol of EDAC and 0.125 g GTA per a gram of collagen. Using these techniques a 4-fold increase in the compressive properties of CG scaffolds was attained ($p < 0.05$). However, results indicated that the mechanisms of EDAC and GTA crosslinking prevent their use in combination. Taken together, these results indicate that crosslinking is a viable technique for altering the mechanical properties of CG scaffolds.

Despite these improvements, the mechanical properties of these scaffolds are in a range that is not directly comparable to native tissue in many applications. Unquestionably, the mechanical properties of scaffolds are critical for preserving structural integrity and functionality during both *in vivo* implantation and long-term performance. In addition, a great deal of work has been published demonstrating the influence of mechanical properties on cellular activity, thus mechanical properties further determine the functionality of a tissue-engineered construct (Engler et al., 2004a, Engler et al., 2004b, Engler et al., 2006, Yeung et al., 2005, Lee et al., 2001). However, while it is often stated that scaffolds need to have mechanical properties resembling that of native tissue (Hollinger and Chaudhari, 1992, Hutmacher, 2000, Luo et al., 2007), it is the author's opinion that this is not required for a scaffold to be

successful in many tissue engineering applications. It is worth noting that the standard treatments for repair of load bearing tissues use supports such as fixation and sutures to bear mechanical loading during healing and rehabilitation (Koob, 2002, Finkemeier, 2002). Furthermore, approaches using materials with compliant mechanical properties have been successfully used to induce regeneration of bone (Tiedeman et al., 1995). It also has to be considered that the scaffold is a starting point for engineering a tissue; many approaches envisage *in vitro* conditioning, using bioreactors and growth factors to grow a functional tissue prior to implantation. Therefore, the influence of scaffold mechanical properties on ECM production and tissue regeneration is of greater importance than a desire for the scaffolds to be load bearing when implanted, provided the properties are sufficient to survive surgical implantation and the *in vivo* environment.

6.4 Crosslinking and Cellular Activity

Chapters 3 and 4 describe the effects of DHT, EDAC and GTA crosslinking techniques on the mechanical properties of CG scaffolds. In Chapter 5 we sought to explore the effects of crosslinking, and the subsequent changes in mechanical properties, on cellular activity within the scaffolds. In addition, the nature of crosslinking involves the use of reagents which may have the potential to cause cell death if they remain within the scaffolds. It was therefore necessary to carry out experiments to expose any cytotoxic side effects. To this aim, treatment groups that showed significantly enhanced mechanical properties were chosen from the previous studies of crosslinking techniques and used to treat scaffolds in order to investigate the effects of crosslinking and mechanical properties on the metabolic activity,

seeding efficiency, proliferation and spatial distribution of MC3T3 osteoblasts seeded onto collagen-GAG scaffolds crosslinked.

The results presented in Chapter 5 show that cellular activity can be improved through crosslinking of CG scaffolds. GTA and EDAC 6mmol treatment groups show enhanced cell attachment, proliferation and infiltration. In particular GTA crosslinked scaffolds showed a 6.4-fold increase in cell number at 7 days when compared to the standard DHT 105°C group. This compares favorably with previous work in our laboratory which has shown no proliferation upon the standard scaffolds after 7 days (Tierney et al., 2008). Additionally, there was evidence of cytotoxicity when using high molarity EDAC crosslinking. Cellular proliferation was found to decrease when the molarity of EDAC crosslinking was increased from 6 to 96 mmol. Taken together, these results indicate that the enhanced cellular activity upon GTA and EDAC treated scaffolds demonstrates their suitability for use both *in vitro* and *in vivo*.

Previous work has postulated that cell mediated contraction may reduce the proliferation of chondrocytes (Lee et al., 2001). In the study contained in Chapter 5, the only groups observed to show any cell mediated contraction were the groups crosslinked using DHT treatment. Therefore the lack of proliferation in these groups may be due to contraction of the scaffold, which, among other things, results in collapse of the pore structure reducing the diffusion of nutrients and waste products within the scaffold. The increased mechanical properties of GTA and EDAC crosslinked scaffolds allows them to retain their porous structure upon hydration while DHT treated samples contract upon hydration reducing the surface area available for seeding. This demonstrates the importance of mechanical properties in

providing an appropriate environment for cellular activity. Additionally, previous work has shown that the mechanical properties are capable of influencing cellular differentiation, adhesion and proliferation (Lee et al., 2001, Yeung et al., 2005, Engler et al., 2006). Therefore the control of mechanical properties via crosslinking may have further benefits. However, longer term culture experiments are needed to shed further light on this and the influence of mechanical properties on tissue formation. Increasing the degree of DHT treatment (from 105°C to 150°C) was found to have no significant effects on cellular activity. It appears that the increase in mechanical properties obtained through extended DHT were not sufficient to withstand cell-mediated contraction.

There is some evidence of the cytotoxicity due to GTA treatment in previous work (Wiebe et al., 1988, Gendler et al., 1984). In contrast, GTA-treated scaffolds in this study show enhanced cellular viability when compared to EDAC treated scaffolds with similar mechanical properties. However, GTA treatment has also been shown to induce calcification *in vivo* (Jorge-Herrero et al., 1999, Kim et al., 1999). This may enhance the suitability of GTA-treated CG scaffolds for use in bone regeneration, while limiting their use for regeneration of non-calcified tissues. Long term cell culture experiments using osteoblasts and osteogenic factors are needed to clarify these findings.

6.5 Future Work

- Though annealing produced an increase in pore size, it did not expand upon the range of pore sizes that could be produced by altering T_f . Therefore, the possibility of producing scaffolds with larger pore sizes by combining high freezing temperature, which produced the largest pores, and annealing, which causes ice crystal growth, is of interest.
- Differences in freeze-drying equipment were deemed to cause a discrepancy between the pore size of scaffolds produced at MIT (O'Brien et al., 2004, O'Brien et al., 2005) compared to RCSI. This demonstrated the influence of cooling rate on pore size. Detailed investigation of the influence of cooling rate on pore size may yield interesting results.
- DHT treatment resulted in significant denaturation of the collagen within the scaffolds. Denaturation is generally considered undesirable as it disrupts the native collagen structure and increases the degradation rate, yet it may also enhance cellular activity and reduce the inflammatory response. DHT treated scaffolds may be of use in determining the effects of denaturation on scaffold performance.
- Investigation of the effects of EDAC and GTA crosslinking on the tensile properties of CG scaffolds would be worthwhile. This information is important if the scaffolds are to be used in tensile loading bioreactors or to regenerate

tissues which are loaded in a tensile fashion (i.e. tendon or ligament regeneration).

- The effect of mechanical properties on cellular activity (independent of the influence of crosslinking technique) can be investigated using EDAC crosslinking to produce scaffolds with a range of mechanical properties. In particular, advancing the experiments of Engler *et al.* (2006) concerning the influence of substrate stiffness on stem cell differentiation, by moving from a 2D substrate to a 3D construct is of interest.
- The induction of calcification caused by GTA treatment is of interest in the context of bone tissue engineering. Long term culture of osteoblasts within GTA treated CG scaffolds is needed to provide further information.

6.6 Conclusions

1. A process has been developed that allows the mean pore size of collagen-GAG (CG) scaffolds to be varied in a controlled manner from 85-325 μm . The freezing temperature used during fabrication of CG scaffolds was varied from -10 to -70°C in order to determine its effect on pore size. The pore size was found to reduce with temperature until -50°C, after which there are no further changes in pore size. It was hypothesised that this was due to a large increase in the viscosity of the frozen suspension caused by glass transition between -42 and -50°C.
2. An annealing step was introduced into the fabrication process and optimised in order to increase the mean pore size of the scaffolds. The duration of annealing was varied between 0.25 and 48 h to determine its effect on pore size. The pore size was found to increase with annealing time until 18 h, producing a 40% increase in pore size (from 135 to 190 μm).
3. Dehydrothermal (DHT) treatment was found to increase the mechanical properties and denaturation levels of CG scaffolds. The effects of the temperature and exposure period of DHT treatment were varied to determine their effects on the mechanical and structural properties of CG scaffolds. Statistical correlations demonstrated that compressive modulus increased with temperature (2-fold), while the tensile modulus (3.8-fold) and levels of denaturation (15-60%) were found to increase with both temperature and exposure.

4. Chemical crosslinking techniques produced a significant increase in the compressive modulus of CG scaffolds. The concentration used during 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDAC) and glutaraldehyde (GTA) crosslinking was varied to determine its effects on the mechanical properties of CG scaffolds. Both EDAC and GTA crosslinking significantly enhanced the compressive modulus of CG scaffolds. Using these techniques a 4-fold increase in the compressive properties was attained.
5. The possibility of combining EDAC and GTA crosslinking was investigated by using the treatments in succession. However, no increase in compressive modulus was found when the combined treatments were compared to the individual EDAC and GTA treatments. It was hypothesised that the mechanisms of the crosslinking treatments prevented them from being combined effectively.
6. Crosslinking and the mechanical properties were demonstrated to have significant effects on cellular behaviour. Scaffolds were treated using DHT, EDAC and GTA crosslinking. The improved mechanical properties of EDAC and GTA crosslinked scaffolds were shown to enhance cellular attachment, proliferation and infiltration when compared to the standard DHT crosslinked scaffolds. In particular, GTA crosslinked scaffolds show the greatest potential for tissue engineering, having the ability to support 6.4 times as many cells as the standard scaffolds. In addition, EDAC crosslinking showed evidence of cytotoxicity at high concentrations.

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