Characterisation and Investigation of Alginate Dressings Containing Hydrogen Peroxide for the Promotion of Wound Healing

Submitted for the degree of Master of Science

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Submitted to the Higher Education and Training Awards Council (HETAC) in fulfilment of the requirements for Master of Science degree, September 2011

Declaration

I declare that the work herein, submitted for the degree of Master of Science, is the product of my own investigation, except where published literature is referenced. I also declare that that material submitted in this thesis has not been submitted for assessment for any other qualification.

Brendan Alexander

Acknowledgements

I would like to express my sincere gratitude to my supervisors Dr. Gertie Taggart, Dr. Joanne Gallagher, Dr. Garrett Farrell of Letterkenny Institute of Technology and Prof. Kathleen Murphy of Daemen College, New York State, USA for their instruction over the course of my studies.

I would also like to convey my deepest gratitude to Letterkenny Institute of Technology for providing the funding to make this project possible.

I would like to thank my laboratory colleagues Vicki Wallace and Grainne Quinn for their support during the project.

This project was also made possible by the generous use of equipment from the CAMBio research group, Daemen College and Michael Brooks of the Zwick/Roell Group.

I would also like to thank Ken McIntyre for his technical support over the course of this project and Dr. Joseph English for this guidance and help with the statistical analyses in this project. A sincere thank you to Dr. Anne Nelson for her support throughout this Master of Science Degree.

I also owe a deep debt of gratitude to my parents Mary and John who refused to give up on me during the very difficult days of my early education and who supported me in all my endeavours. Without their perseverance, patience and belief in me, I would not have achieved this degree or any other qualification.

Finally, I would like to reserve special thanks for my colleague and closest friend Vicki. Her support and guidance coupled with her vast patience and belief in me is the driving force behind all my personal and academic achievements.

Abstract

The care of chronic wounds carries a heavy financial burden on the healthcare industry, with billons being spent annually on their treatment. This, coupled with a decreased quality of life for sufferers, has led to an urgency in developing inexpensive wound dressings that promote wound healing. Alginate gels for application as wound dressings were formed by varying alginate (0-6 %w/v), calcium carbonate (0-1 %w/v), hydrogen peroxide (0-3.75 % v/v) and hyaluronic acid (0-1.25 mg/L) content. The aging effects on the physical properties of the gels over a 14 day period were also investigated. The results indicated that the concentration of calcium carbonate and hydrogen peroxide, as well as sample age, all had a significant effect on the rupture characteristics and gelation time of the gels. Increased calcium carbonate content caused an increase in rupture force values, whereas increased hydrogen peroxide content and sample age resulted in a decrease in rupture force measurements. Increased calcium carbonate and hydrogen peroxide content produced a decrease in the time required for gel formation. Statistical models were also produced to provide a means of estimating rupture characteristics and gelation times for gels containing other concentrations of these components. Chronic wounds endure a state of hypoxia that impairs the healing process. Thus, delivery of oxygen to the wound may benefit the healing of wounds. Gels containing hydrogen peroxide were shown to release oxygen when in contact with an artificial wound containing catalase. Gels not containing hydrogen peroxide did not release oxygen when in contact with the artificial wound. The incorporation of hyaluronic acid into the gel had no significant effect of the rate of oxygen release. These gels serve as an attractive vehicle for the delivery of oxygen to wounds, thus aiding in the healing process. Culturing synovial fibroblasts in the presence of the alginate gels had a detrimental effect on the cell migration and proliferation of these cells. Cell migration and proliferation are important phases in wound healing. These findings appear to suggest that the alginate gels are not beneficial in the wound healing. However, it is more likely that these results are due to the delicate nature of the *in vitro* system used for the assessment of the gels.

Abbreviations

AAD	Adipic Acid Dihydrazide
ATA	Atmospheres Absolute
ATR	Attenuated Total Reflectance
ANOVA	Analysis of Variance
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
FT-IR	Fourier Transform Infrared
G	α-L-guluronate
GDL	D-Glucono-δ-Lactone
GFP	Green Fluorescent Proteins
HA	Hyaluronic Acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
IL	Interleukin
kDa	KiloDalton
KGF	Keratinocyte Growth Factor
LT	Leukotriene
M	β-D-mannuronate
MMP	Matrix Metalloproteins
mrad	Millirad
mRNA	Messenger Ribonucleic Acid
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphphenyl)-2H-tetrazolium, inner salt
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
N	Newton
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
Nox	Nicotinamide Adenine Dinucleotide Phosphate Oxidase
PDGF	Platelet-Derived Growth Factor
PF	Platelet Factor

Abbreviations (continued)

PMS	Phenazine Methosulphate
RHAMM	Hyaluronic Acid-Mediated Motility
RNA	Ribonucleic Acid
RNAi	Ribonucleic Acid Interference
ROS	Reactive Oxygen Species
RPMI	Royal Park Memorial Institute
SEM	Standard Error of Mean
TGF	Transforming Growth Factor
TIMP	Tissue Inhibitors of Metalloproteinase
TNF	Tumour Necrosis Factor
VEGF	Vascular Endothelial Growth Factor

Units

%	Percent
>	Less Than
°	Degree
°C	Degree Celsius
µl	Microliter
µm	Micrometer
cm	Centimeter
cm^{-1}	Wavenumber
hr	Hour
mg	Milligram
mg/L	milligrames per Liter
min	Minute
ml	Milliliter
mm	Millimeter
mM	Millimolar
mmHg	Millimetre of Mercury
nm	Nanometer
U	Units
v/v	Volume per Volume
w/v	Weight per Volume

Tables:

Figures:

Table of Contents

Chapter 2:

Chapter 1:

Literature Review

1.0: Introduction

The term "wound" usually indicates a tissue lesion. It can also be an area of skin that has been destroyed by external factors or by the presence of an underlying pathological disorder (Thomas *et al.* 2000). Wounds can exist in two forms - acute or chronic. Acute wounds proceed to heal without complications. However, the normal process of healing does not extend to chronic wounds. In chronic wounds, the healing process is delayed or prevented by a persistent pro-inflammatory state (Stojadinovic *et al.* 2008). The care of chronic wounds carries a heavy financial burden on the healthcare industry, with billons being spent annually on their treatment. This, coupled with a decreased quality of life for sufferers, has led to an urgency in developing inexpensive wound dressings that promote wound healing.

This study documents the preliminary stages of developing a novel dressing for chronic wounds. This wound dressing is in the form of a calcium carbonate cross-linked alginate gel. Alginate has antibacterial properties and, because of this, it has been established as a wound dressing material in the healthcare industry. When combined with a slow cross-linker such as calcium carbonate, alginate can form gels that have good structural uniformity and can be easily shaped using moulds (Ma and Kuo 2001). It has been shown that low levels of hydrogen peroxide may be beneficial in wound healing (Roy *et al.* 2006). It may also be possible to use hydrogen peroxide to overcome problems in wound healing posed by. Hydrogen peroxide reacts with catalase present in wound tissue to produce oxygen. However, the excessive production of oxygen may be detrimental to wound healing due to the production of reactive oxygen species. Hyaluronic acid may act as an oxygen scavenger, thus eliminating some of the harmful effects caused by excessive oxygen in the wound environment, such as reactive oxygen species. Therefore, alginate gels incorporating calcium carbonate, hydrogen peroxide and hyaluronic acid were assessed in this study.

1.1: History of Wound Care

Since the earliest written records, mankind has been aware of the importance of wound care. This primarily consisted of simply covering wounds with a variety of natural materials. The past 25 years have witnessed a considerable increase in the types of materials utilized in wound dressings (Ovington 2007). In order to assess the suitability of any material for use as a wound dressing, the key aspects of wound healing and the ideal properties of wound dressings must be considered. The advent of sterile materials and techniques, spearheaded by Pasteur and Semmelweiss, followed by the development of the concept of moist wound healing, heralded a new era in wound care (Ovington 2007).

Moist wound healing replaced the practice of allowing or encouraging wound tissues to dehydrate, and ultimately dry out, by promoting and maintaining optimal hydration in the exposed tissues of the wound. The arrival of moist wound healing was marked by two seminal articles published in the journal, Nature, by Winter (1962) and Hinnman and Maibach (1963). Winter demonstrated that in swine, significant increases in re-epithelialization rates were displayed in acute, partial-thickness excisional wounds when the wound was maintained in a moist condition by semiocclusive polyethylene films when compared to uncovered wounds. Hinnman and Maibach (1963) confirmed this also to be true of human wounds. It is now widely accepted that wound dressings which maintain a moist condition for exposed tissue increase healing rates, lessen pain, reduce infection and lower overall healthcare costs (Nemeth *et al.* 1991; Hutchinson 1993; Ovilington 2001). However, it is not desirable to have a wound covered in fluid. The wound should not be dry or wet but physiologically moist (Ovington 2007). If wounds are overly moist in the early, inflammatory phases of healing, they may lose fluid through exudate production and evaporation.

Chronic wounds typically cease healing during the inflammatory stages of wound recovery. The excessive exudate production that follows can lead to maceration or "water-logging" of wound tissues (Ovington 2007). The potential soiling of patients' bandages with exudate also has to be managed at this stage. Wound etiologies such as

venous leg ulcers have been linked to very high levels of wound exudate. This can also be the case for wounds in the condition of lymphedema. In this case, the dressing must be capable of providing the optimal tissue moisture levels by managing the absorption of exudate. Provided the wound is suitably moist with low levels of exudate production, the dressing should be capable of maintaining an adequately moist environment while avoiding excessive exudate absorption leading to desiccation of the wound. However, if the wound's moisture levels are low, the dressing must be able to donate moisture to the wound to restore an optimally hydrated environment for wound recovery (Ovington 2007).

For the purpose of treating chronic or non-healing wounds, the dressing should be capable of absorbing excessive wound exudate, a property already well established for alginate wound dressings (Ovington 2007). It is known that wound dressings with good absorbent properties are more beneficial to chronic wounds as they require fewer dressing changes in a certain time period than less absorbent dressings such as gauze. This means the healing process is allowed to continue uninterrupted and also results in time and labour saving on behalf of the nurse or caregiver (Ovilington 2007).

Recent developments in wound care have led to the arrival of wound dressings that do more than simply manage exudate levels. These advanced dressings interact with the biochemical environment of the wound. The dressings quantitatively change some aspect of the wound and thus may be considered extremely beneficial to chronic non-healing wounds. Chronic wounds of multiple etiologies often exhibit similar biochemical dysfunction (Trengrove *et al.*1999). It has been shown that there is a difference in the variety and abundance of chemicals found locally in the tissues and fluids of slow healing or chronic wounds compared to normal wounds. Cell processes that are important to healing may be inhibited by biochemical aspects of the wound. These inhibitors include the presence of proteinases, intercellular communication chemicals such as cytokines and growth factors, reactive oxygen species (ROS), levels of bioburden (bacteria and bacteria by-products) and tissue hypoxia (where oxygen supply to tissue is below physiological

levels despite adequate perfusion of the tissue by blood) (Falanga *et al* 2008; Wysocki 1993; Cullen *et al.* 2002).

Chronic wounds contain a high proportion of cells that display altered physical shape and behave as if they are at the end of their lifecycle. These senescent cells exhibit decreased responses to growth factors and cytokines and also display reduced production of growth factors and extracellular matrix proteins. Cellular senescence can be affected by an underlying disease state (Mendez *et al.* 1998). However, it is now accepted that the biochemistry of the wound environment in chronic wounds causes senescent behaviour in cells (Loots *et. Al* 1999). A team of researchers lead by Raffetto (2001) found that exposing neonatal fibroblasts to wound fluid from venous leg ulcers greatly decreased the motility of these young, healthy cells. This and other findings have led to the development of advanced wound dressings focused on addressing the biochemical imbalance found in chronic wounds, as well as managing moisture levels in the wound environment (Ovington 2007).

Chronic wounds have been shown to have elevated levels of proteolytic enzymes compared to healing wounds. Matrix metalloproteases (MMP), a family of structurally related proteolytic enzymes, are found at persistently higher levels in chronic wounds, particularly those failing to progress towards closure. Matrix metalloproteases are involved in the inflammatory phase of wound healing in controlled proteolysis through stimulation by cytokines (Ovington 2002). They also play a part in the migration of cells through the extracellular matrix. In healing wounds, matrix metalloprotease levels peak during the inflammatory stage of healing. As the wound heals by filling in with granulation tissue and resurfacing with epithelial tissue, the levels of matrix metalloproteases then decrease (Ovington 2002).

In chronic wounds, levels of these proteases remain elevated, resulting in uncontrolled degradation of newly deposited extracellular matrix components. These include proteoglycans, glycosaminoglycans and collagen. Growth factors vital to coordinating healing are also degraded by persistent high levels of these proteases in chronic wounds.

This results in the failure of granulated tissue to accumulate, giving rise to the characteristic stalling of the healing process in the inflammatory phase. The development of elevated levels of matrix metalloproteases in chronic wounds is considered to have multiple causes such as excessive levels of bacteria in the wound, the presence of non-viable tissues and repetitive mechanical trauma to the wound (Ovington 2007).

1.2: Economic Impact of Chronic Wounds

Menke *et al.* (2007) states there is an absence of large scale, population based studies that assess the occurrence and economic cost of chronic wounds in the USA, and indeed, the global healthcare industries. In the USA alone, there are between three and six million patients suffering from non-healing wounds (Brem *et al.* 2000). Of these patients, 85% are over the age of 65 (Nelzen *et al.* 1991). Estimations of the cost of chronic wounds are in the range of billions of dollars. These figures exclude the knock-on costs such as loss of work time, decreased productivity, disability payments or the cost of rehabilitation (Menke *et al.* 2007). A much less quantifiable but none-the-less significant cost of chronic wounds was also not accounted for in these studies – the psychological damage endured by patients themselves, as well as their families, friends and spouses (Menke *et al.* 2007). This psychological distress can stem from complications and conditions often associated with non-healing wounds such as infection, chronic pain and amputation (Menke *et al.* 2007).

1.3: Alginates

Alginates are polysaccharides primarily extracted from brown algae, most commonly *Laminaria hyperborean* and *Laminaria lessonia.* These algae inhabit coastal waters across the globe (Augst *et al.* 2006). Stanford (1883) was the first to carry out the characterization of alginic acid. Stanford extracted alginic acid using sodium carbonate. The alginate was then precipitated out of solution at low pH. D-Mannuronic acid was shown to be a major product in alginate hydrolysates following the realization that uronic acid was a constituent of alginic acid (Atsuki and Tomoda 1926; Nelson and Cretcher

1926). Later, guluronic acid was identified as another major constituent of alginate hydrolysates. This discovery was made possible with the arrival of paper chromatography. It was then discovered that the guluronic to mannuronic acid ratios vary between alginates of different origins. It was also established that alginates are block polymers. This was shown by fractional precipitation with calcium and manganese salts (Haug 1959; Haug and Smidsrød 1965).

It is now established that alginates are a family of linear copolymers that contain 1-4-linked β-D-mannuronate (M) and α-L-guluronate (G) (Kuo and Ma 2001) (Figure 1.1). Augst *et al.* (2006) states L-guluronic acid is $^{1}C_{4}$ with diaxial links and D-mannuronic acid is $^{4}C_{1}$ with diequatorial links. The make-up of the blocks is dependent on the alginate source and are either similar (MMMM, GGGG) or alternating (GMGMGMGM) (Smidsrød and Skjåk-Bræk 1990). Smidsrød and Skjåk-Bræk (1990) also stated that G blocks exhibit greater stiffness than alternating blocks due to its diaxial links. Similarly, alternating blocks are more stable at low pH.

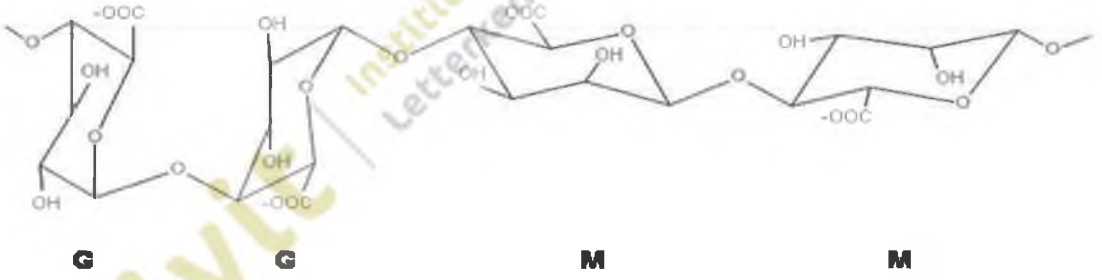

Figure 1.1: Alginate polysaccharide consisting of two guluronic acid and two mannuronic acid residues with (1,4)-linkages.

It is possible to prepare alginates with greatly varying molecular weights (50-100 kDa). The viscosity of aqueous solutions of alginates decreases with increasing shear rate (shear thinning). Therefore, they display non-Newtonian characteristics (Becker and Kipke

2002). The molecular weight distribution and polymer concentration also affects the viscosity of the alginate solutions (Kong *et al.* 2002). Divalent cations, such as Ca^{2+}, can interact ionically with alginate polymer chains to form a gel structure. These hydrogels are formed by each divalent ion interacting with two adjacent G-residues, as well as with two G-residues in an opposing chain. The resulting structure is often referred to as the "egg-box" model (Kristiansen *et al.* 2009) (Figure 1.2). The overall stiffness is dependent on the polymer molecular weight distribution and composition (M/G ratio) and the stoichiometry of the alginate with the chelating cation (Kong *et al.* 2002; Lee *et al.* 2000b).

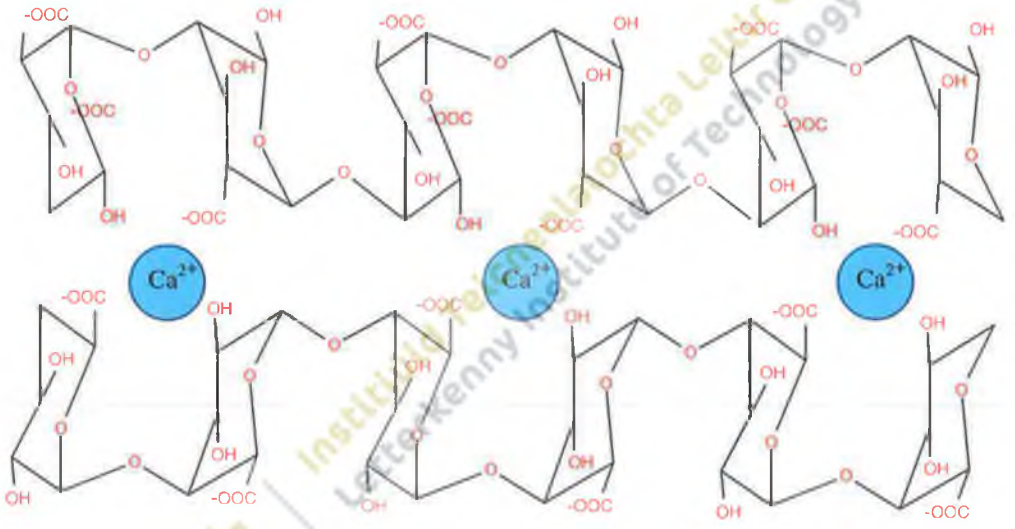

Figure 1.2: Alginate "egg-box" model formed by calcium ions interacting with two adjacent G-residues, as well as with two G-residues in an opposing chain.

1.3.1: Applications of Alginates

Historically, alginate has found the widest application in the food industry. It has been primarily utilized as an emulsifier and stabilizer in low-fat food due to its inherent properties and its interaction with proteins, fats and fibres (Augst *et al.* 2006). Alginate has been combined with pectin to form mixtures that gel independently of sugar content

and are low in calories. This has led to the widespread use of these alginate-pectin nixtures as gelling agents in various food products. However, it is alginate's application in the pharmaceutical industry that is of concern for this study. Its main application is as an excipient for drugs (Liew *et al.* 2006), dental impression material (Ashley *et al.* 2005) and wound dressings (Matthew *et al.* 1995). Alginate's biocompatibility and non-immunogenicity are extremely advantageous in the pharmaceutical industry. These properties are largely attributed to its hydrophilicity (Shapiro & Cohen 1997). Alginate also exhibits very gentle gelling behaviour, which is a major advantage for use in the pharmaceutical industry. This enables the encapsulation of numerous substances and reduces the risk of damage to the ingredient being encapsulated (Klöck *et al.* 1997).

Alginates receive widespread use as scaffolds in cell culture due to their excellent retention of initial size and shape regardless of the approach to cross-linking (Chang *et al.* 2001). Alginates have also been modified to allow its application as a clinical drug delivery vehicle for proteins that enhance regeneration of mineralized tissue (Amsden and Turner 1999). This polymer has also been used as a carrier of transplanted cells (Bent *et al.* 2001). Despite alginate's considerable advantages for use as a healthcare product, it does have its limitations. The past decade has witnessed significant research to overcome these problems to allow for its broader application. The main disadvantage is that the polymer does not naturally break down enzymatically in mammals. Another problem that researchers are working to overcome is alginate's inability to allow cells to adhere to it (Augst *et al.* 2006).

Alginate gels have long since found applications in the healthcare industry due to their suitability for use as wound dressings for exuding wounds. Alginate wound dressings may initiate or accelerate the recovery of chronic wounds, providing the underlying pathological condition is treated. This is due to two major actions of the alginate: firstly, the alginate fibres' moisture handling properties and secondly, the induced cytokine production by human monocytes that may result from bioactivity exerted from the alginate (Thomas *et al.* 2000).

1.3.2: Alginates use as Wound Dressings

Any biomaterial for use as a wound dressing requires specific and controllable properties. Degradation behavior and mechanical properties are two such important aspects that must be assessed. The mechanical properties of alginate gels can be influenced by modifying polymer chains and material composition. Increases in polymer concentration can bring about increases in gel stiffness (LeRoux *et al.* 1999). The resulting increase in viscosity of the pre-gel solution often poses problems. The more viscous solution is difficult to manage and the reduced time required for gelation increases the difficulty in forming a gel of desired shape and structure (Kong *et al.* 2003). A combination of high and low molecular weight alginates formulated to manipulate polymer molecular weight and molecular weight distribution has been used to overcome this problem (Kong *et al.* 2002). In these systems, the purpose of inclusion of low molecular weight alginates is to reduce the interaction between chains prior to gelation. These low molecular weight alginate chains also fully participate in gelation. High molecular weight alginate chains allow for long-range interactions with the gel. In gels formed exclusively with low molecular weight polymer chains, long-range interactions are absent and the gel is very brittle (Augst *et al.* 2006). The mechanical properties of alginate gels are also affected greatly by gelling conditions. Forming gels at low temperatures offers a number of advantages. The diffusion rate of Ca^{2+} ions is reduced at low temperatures. This results in slower gelation and enhanced mechanical properties through a more ordered network structure (Augst *et al.* 2006). The cross-linking agent also plays a major role in the mechanical properties of a gel. This is much more evident when the molecular weight between cross-links is lower than the molecular weight of the cross-linking molecule (Eiselt *et al.* 1999).

1.3.3: Alginate Gel Degradation and Kinetics

Another factor that has led to alginate's wide use as a biomaterial is the possibility of controlling its degradation (Meinel *et al.* 2005). Non-manipulated ionically cross-linked alginates degrade slowly and uncontrollably *in vivo*. This is as a result of the loss of divalent cross-linking cations at natural pH (Landsdown *et al.* 1994). Alginate gel

degradation has been controlled by physical and chemical methods. Manipulation of polymer composition and molecular weight can be used to control degradation.

Polymers of varying molecular weight and structures have been created through the gamma-irradiation of higher molecular weight alginates. Mannuronic-guluronic residues can be cleaved using irradiation doses below 8 Mrad (Alsberg *et al.* 2003). This means the G-block lengths and G content remain unchanged (Alsberg *et al.* 2003). Due to accelerated degradation, gels constructed from these polymers clear from the body quicker than gels formed from conventional polymers. This has considerable advantages for the formation of new bone from transplanted cells using these gels as scaffolds (Alsberg *et al.* 2003; Simmons *et al.* 2004). The method of combining alginates of varied lengths of G blocks to introduce a size mismatch between cross-linked blocks has been used to achieve a wide range of degradation kinetics (Kong *et al.* 2004a).

Alginates have also been produced that are susceptible to hydrolysis in order to manipulate their gel degradation kinetics. Partial oxidation of alginates by reacting them with sodium peroxate acts on the *cis*-diol group to cleave its carbon bond. Six membered hemi-acetal rings form as a result. The two unoxidized adjacent sugar residues play host to the closest hydroxyl group's urinate residues which revert to open chains creating an hydrolytically labile bond. The pH and temperature dictate the rate of hydrolysis (Bouhadir *et al.* 2001). The stiffness of the G block is affected by the degree of oxidation, thus partially oxidizing these polymers also has a profound effect on the mechanical properties of their molecules and gels (Lee *et al.* 2002). Chemical modification like this can be used in combination with other chemical or physical approaches to control gel degradation.

Gels can be more rapidly degraded if they have been formed using polymers with a bimodal molecular weight distribution. In this instance one chain is partially oxidized compared to gels formed from polymers with a single molecular weight distribution (Boontheekul *et al.* 2005). It has been shown that at low levels of oxidation degradation is enhanced, while cells in contact with the gel remain unharmed (Bouhadir *et al.* 2001).

Chain scission and the resulting separation of covalently cross-linked domains bring about the accelerated degradation of gels which are formulated from polymer mixtures of oxidation and bimodal molecular weight distribution (Kong *et al.* 2004b). The cross-linking of partially oxidized alginates (polyaldehyde guluronate) or polyguluronate can also be utilized to form gels (Bouhadir *et al.* 1999). The cross-linker density of these covalently cross-linked gels significantly effects the *in vivo* degradation of theses gels (Lee *et al.* 2000a). By simply doubling the cross-linker density, a virtually non-degrading gel can be produced from what was originally a readily degradable gel (Lee *et al.* 2001).

Increasing cross-linker density also acts as a means of increasing the gel's mechanical strength. Studies in mice have produced striking results. Lee *et al.* (2001) injected mice with polyaldehyde guluronate gels and osteoblasts with the goal of producing tissue. It was discovered that using excess adipic acid dihydrazide (AAD) to cross-link gels produced a high number of cross-linked molecules which only reacted on one end. This produced a delay in degradation as it was possible for re-cross-linking to take place after hydrolysis (Lee *et al.* 2001). Slower degradation rates can also be achieved by utilizing polyacrylamide-co-hydrazine (a multifunctional cross-linker) instead of bifunctional hydrazide to form polyaldehyde guluronate gels (Lee *et al.* 2004). These findings form a body of knowledge demonstrating that the fine tuning of alginate gel degradation kinetics is possible, thus broadening their range of applications in the healthcare industry (Augst *et al.* 2006).

1.4: Wounds and Wound Healing

Despite wound healing being commonly divided into phases (hemostasis, inflammation, proliferation and maturation), it is a complicated process which is coordinated by inflammatory cells and mediators. There is also a significant overlap of the wound healing phases (Stojadinovic *et al.* 2008). Hemostasis is the initial response following wounding of vascularized tissue. This is triggered by vasoconstriction and platelet-

mediated activation of the intrinsic clotting cascade. The clot then acts as support for the incoming inflammatory cells (Stojadinovic *et al.* 2008).

An array of inflammatory mediators such as cytokines, chemokines and growth factors are released from platelets embedded within the clot (Witte *et al.* 1997). The amount of inflammation at the surface will be determined by the condition of the wound bed. After a period of between 4-6 days, neutrophils, macrophages and fibroblasts arrive at the site of the wound. These are drawn by a variety of chemoattractants (CXC and CC), pro-inflammatory cytokines such as interleukin (IL)-1, tumour necrosis factor (TNF)-α, transforming growth factor (TGF)-β, platelet factor (PF)4, leukotriene (LT)B4 and mitogenic factors (Stojadinovic *et al.* 2008). These inflammatory cells then eradicate bacteria, clear the damaged extracellular matrix (ECM) molecules and inflammatory debris, and allow the migration of cells through the extracellular wound matrix. They achieve this by releasing nitric oxide and oxygen, free radicals, serum proteases and matrix metalloproteins (Broughton *et al.* 2006). Neutrophil numbers peak at 24-36 hr, after which circulating monocytes accumulate and mature into macrophages. These cells enhance the immune response and promote angiogenesis and the stimulation of fibroblasts. Fibroblasts move towards the centre of the wound 2-3 days after injury, synthesizing collagen and granulation tissue. After 1-2 weeks, fibroblasts are the primary cell type present. Granulation tissue replaces the fibrin clot until the wound is covered (Figure 1.3).

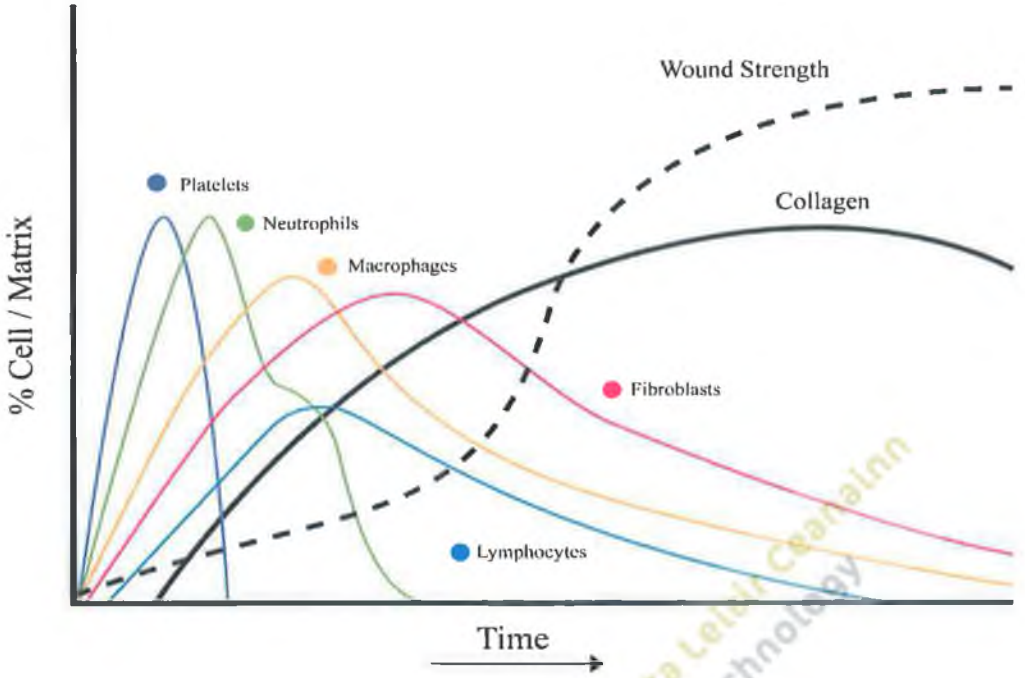

Figure 1.3: Schematic representation of cellular characteristics of the wound healing process. Platelets aggregate at the site of the wound due to the lattice formed by fibrin where they release growth factors which attract neutrophils, monocytes and fibroblasts to the site of injury.

Thermal exposure or mechanical forces can partially denature collagen fibrils and form collagen molecules. These need to be removed to allow newly synthesized collagen molecules to be aligned effectively into the non-denatured ECM (Stojadinovic *et al.* 2008). Epithelialization, angiogenesis and provisional matrix formation then follows. This proliferation phase of wound healing overlaps the initial phase of hemostasis and inflammation, between 4 and 14 days after the creation of the wound. TGF-α and epidermal growth factor (EGF) from activated platelets and macrophages stimulate epithelial proliferation (Grotendorst *et al.* 1989). Fibroblasts, stimulated by macrophage-released TNF-α and IL-1, secrete keratinocyte growth factor (KGF)-2 and IL-6, ultimately stimulating keratinocyte migration, proliferation and differentiation (Witte *et al.* 1997). Platelet-derived growth factor (PDGF) and TNF-α, synthesized and secreted by macrophages, initialize provisional matrix formation by recruiting and activating fibroblasts to synthesize matrix type III collagen, glycosaminoglycan, fibronectin and integrin (Grotendorst *et al.* 1989). Paracrine and autocrine stimulation by fibroblast-derived PDGF initiates early matrix production (Goldman 2004).

Fibroblasts stimulated by matrix-derived TGF-β produce type I collagen. The enhanced fibroblast synthesis of tissue inhibitors of metalloproteinase (TIMP) inhibits MMP (Goldman 2004). Macrophages (via IL-1 and TNF-α) and fibroblasts (via KGF-2 and TGF-β) stimulate keratinocytes to release vascular endothelial growth factor (VEGF) which is then upregulated by nitric oxide. VEGF then promotes endothelial proliferation and angiogenesis (Witte *et al.* 1997; Goldman 2004). Wound contracture and migration of epithelial cells from the wound edge act to eventually close the open wound bed. Wound contracture, mediated by myofibroblasts, draws the edges of the wound together. This reduces the area of the exposed wound and thus a smaller area is required to be repaired by scar formation. This system of wound closure is similar to the contractive forces in smooth muscles in that myofibroblasts are characterized by the action of myosin which also acts as the force generating system for the contraction of smooth muscle. Upon closure of the wound, apoptosis acts to dispose of myofibroblasts and a cellular scar results (Witte *et al.* 1997; Goldman 2004).

Delayed healing can arise from reduced wound contraction, while loss of function of tissue contracture and hypertrophic scarring can result from prolonged and/or excessive scar contracture (Stojadinovic *et al.* 2008). From eight days to one year after wounding, the maturation and remodeling phase of healing is in progress. This overlaps the proliferation phase. A matrix comprised of type I collagen replaces the provisional matrix made up of type II collagen, proteoglycan and fibronectin. The new matrix is strong and well organized. Wound maturation and remodeling is predominately mediated by TGF-β. This growth factor acts to inhibit MMP production and upregulate the expression of TIMP. It also plays a significant role in fibroblast-collagen matrix remodeling and ECM organization, wound contracture and remodeling (Grinell 2003; Lawrence and Diegelmann 1994). Collagen continues to be synthesized for over a month and the collagen matrix and remodeling continues for months. The new skin never achieves the collagen organization and wound breaking strength of uninjured skin. After three months, the new skin displays 80% of the strength of uninjured skin (Broughton *et al.* 2006).

1.4.1: Chronic Wounds

In chronic wounds, there is an absence of orderly, intricate and well-orchestrated processes that are present in normal wounds. Healing of chronic wounds is delayed and/or prevented by the existence of a pro-inflammatory state (Mast and Schultz 1996). The pro-inflammatory stimulus is multifactorial in nature. Factors such as local tissue hypoxia, repetitive tissue trauma, ischemia-reperfusion injury, tissue breakdown and necrosis and bioburden of the wound can all act as stimuli to the pro-inflammatory state (Mast and Schultz 1996). Chronic wounds do not exhibit the high levels of mitogenic activity which is normally present in acute wounds. In acute wounds, pro-inflammatory cytokines, chemokines, proteases and their natural inhibitors (MMP, TIMP) are delicately balanced (Schultz and Mast 1998). This is not the case in chronic wounds (Figure 1.4). During the formation of an acute wound, an insoluble fibrin clot is formed resulting in the accumulation and migration of platelets and inflammatory cells including neutrophils, macrophages and fibroblasts which fight infection and release growth factors (Figure 1.4 top panel). Under normal conditions, the wound healing proceeds with the growth of granulation tissue. Epidermis tissue then migrates over the wound. Tissue cells produce MMPs which aid their migration into the wound. Epidermal cells, fibroblasts, macrophages and endothelial cells release growth factors which promote healing (Figure 1.4 lower left panel). When the healing process is halted due to underlying disease or infection, a chronic wound results. Venous insufficiency can lead to fluid release or fibrin blockage of venules. Arterial occlusion can lead to tissue hypoxia and cell death. Bacteria may form a biofilm made up of polysaccharides which is resistant to antibiotics and phagocytosis. In reaction to the bacterial biofilm, phagocytes release proteases and toxic oxygen radicals, further damaging the wound area (Figure 1.4 lower right panel).

In chronic wounds, pro-inflammatory cytokines such as TGF-β and IL- β are released from resident neutrophils and activated macrophages. These increase MMP production and reduce TIMP synthesis, leading to ECM degradation, impaired cell migration and reduced fibroblast proliferation and collagen synthesis (Mast and Schultz 1996). Progression into the proliferation phase of healing is prevented by this sustained inflammation and proteolytic environment. The chronic non-healing state is perpetuated

when this is combined with impaired cellular and systemic host responses to stress (Mast and Schultz 1996; Mustoe 2004). Further complications may arise to inhibit wound healing such as malignancy. This can interfere with all stages of wound recovery through decreased tissue reperfusion and hypoxia, impaired inflammatory response rates and variation in fibroblast proliferation (Mast and Schultz 1996). Tumor progression involves many of the same components as wound healing, thus radiation and chemotherapy targets often include proteins involved in tissue repair and cellular proliferation. This leads to pre-operative radiation therapy having an increased risk of wound complications after incision (Falanga 1992). Further factors that may complicate the healing process are malnutrition and/or obesity. Diminished wound healing has shown correlations with hypoalbuminemia and anemia (Brem *et al.* 2007).

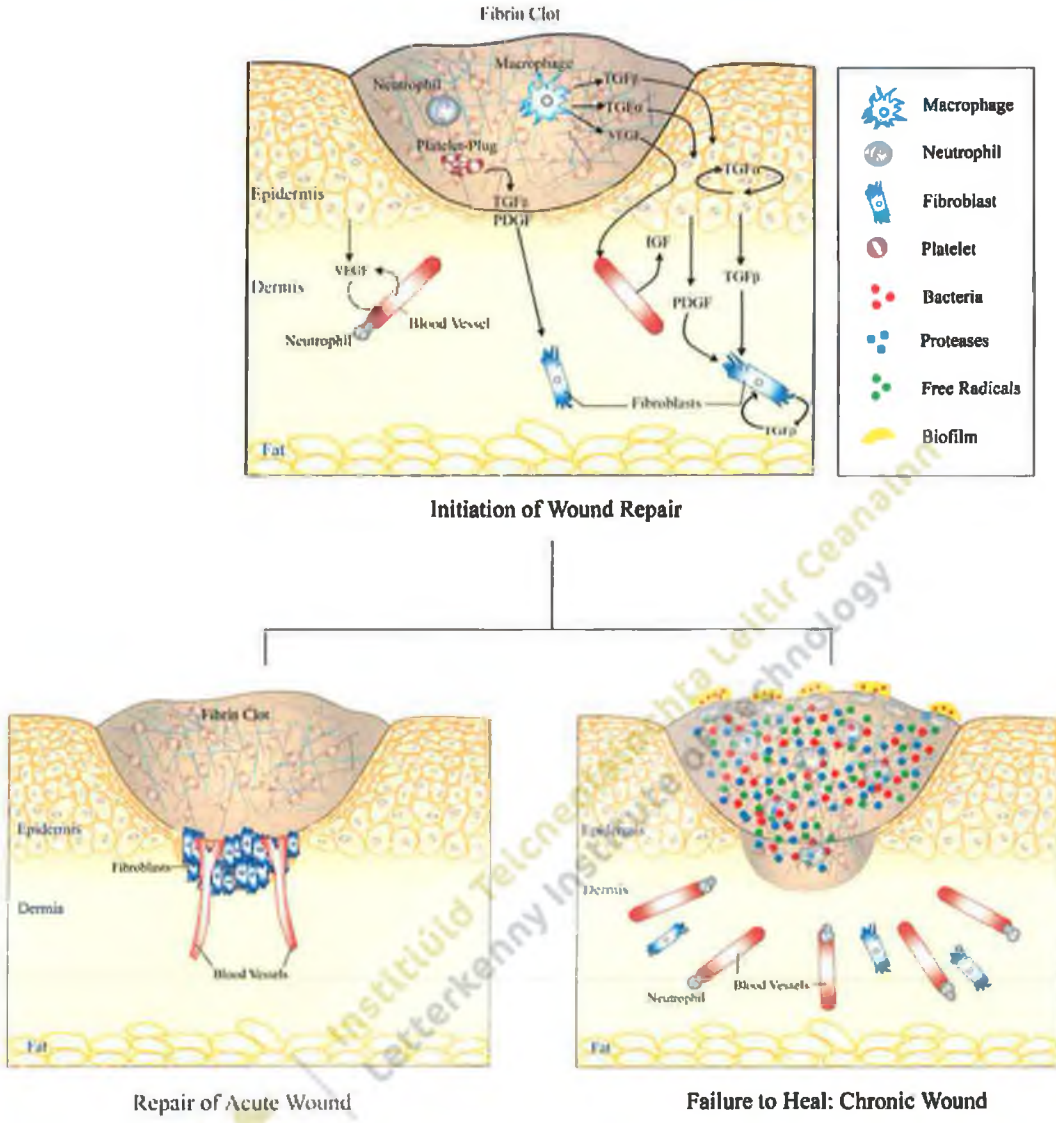

Figure 1.4: Schematic representation of the processes involved in acute versus chronic wounds. Following injury, an insoluble fibrin clot is formed resulting in the accumulation and migration of platelets and inflammatory cells including neutrophils, macrophages and fibroblasts which fight infection and release growth factors (top panel). Under normal conditions, the wound healing proceeds (lower left panel). When the healing process is halted due to underlying disease or infection, a chronic wound results (lower right panel) (Adapted from Clarke *et al.* 2007).

Careful evaluation of chronic wounds is important to ensure maximum treatment options and prevention of further wound degradation (Menke *et al.* 2008). Factors such as the extent of the wound, underlying etiology, odour, size, location, duration and appearance, as well as the appearance of the surrounding tissue, must all be documented by clinicians (Robson *et al.* 2000; Steed 2003). A chronic wound on a limb may be classified as non-limb-threatening, limb-threatening or life-threatening. Local (cellulitis or abscess) or systemic superinfection may be present in the wound. Signs of this range from erythema and lymphadenopathy to instability in vital signs and altered mental state (Menke *et al.* 2008). If the chronic wound contains a heavy bacterial load, healing will not proceed. The underlying organism must be identified. This may be achieved by taking a biopsy from the base of the wound, followed by identification by radiographic films. However, MRI or bone scans are required for evaluation by clinicians if bacterial infection is suspected in the bone. Venous and arterial dopplers or angiography may be required for additional evaluation of the vascular system on extremities (Grinnell and Zhu 1996).

1.4.2 Role of Oxygen in Wound healing

It is essential that oxygen is delivered to the wound site in order to ensure wound healing. However, wounded tissue will inevitably suffer from hypoxia. Impaired healing can result where normal oxygen levels and circulation are disrupted due to physiological disorders such as diabetes (Bishop 2008). Cell metabolism and oxygen consumption is increased following tissue injury, causing disruption to local blood vessels. This produces a hypoxic wound (Tandara *et al.* 2004; Whitney *et al.* 1989; Greif *et al.* 2000). Hypoxia then initiates wound repair by creating an oxygen gradient between the hypoxic tissue at the wound site and perfused uninjured tissue nearby (LaVan and Hunt 1990). It is thought that this oxygen gradient promotes the diffusion of oxygen to hypoxic tissue.

As earlier discussed, hemostasis is the first phase of the healing process. During this phase, vasoconstriction and coagulation take place. When hemostasis is established, the inflammatory phase is active. Increased capillary permeability from vasodilation then allows various cells, enzymes and oxygen to reach the wound site and sustain the healing

process (Whitney *et al.* 1989). Throughout the healing process, wounds generally display an increasing oxygen gradient towards the uninjured tissue and remain hypoxic at the wound centre (Gordillo and Sen 2003; Patel *et al.* 2005). The balance between oxygen perfusion and tissue consumption of oxygen (tissue oxygen tension) ranges from 60-70 mmHg at the wound edge to 0-20 mmHg at the wound centre (Ragheb and Buggy 2004). In blood or tissue, the partial oxygen pressure (the arterial oxygen tension) is approximately 100 mmHg (Gordillo and Sen 2003, Patel *et al.* 2005). When angiogenesis is complete and blood supply is restored at the end of the proliferation phase, this hypoxic state ceases (Bishop 2008). Niinikoski (1969) first proposed that higher cellular oxygen consumption at the wound site caused a steeper gradient, driving the diffusion of oxygen from oxygenated plasma to the wounded cells. This theory still finds support in the scientific community today (Gottrup 2004; Rodriguez *et al.* 2008).

The removal of debris and the control or elimination of invading bacteria are the main purposes of the inflammatory response (Worley 2004). Seminal research conducted by Sbarra and Karnorsky (1959) showed that there is a dramatic increase in leukocyte oxygen consumption during phagocytosis. This was supported by work carried out by Klebanoff (1980). Some of the oxygen which is not used for energy is converted into highly reactive ions and reactive oxygen species (ROS) including hydrogen peroxide and superoxide (LaVan and Hunt 1990). This process is referred to as the respiratory burst. ROS act to destroy bacteria through oxidative killing (Greif *et al.* 2000). Bacterial walls are broken down by superoxatives and leukocytes produce hydrogen peroxide during this process.

In a hypoxic environment, such as those found in acute and chronic wounds, there is evidence that superoxide production decreases. It has been suggested that oxygen tensions below 30-45 mmHg are most critical in decreasing the production of superoxides (LaVan and Hunt 1990; Allen *et al.* 1997). This means that superoxide production may be decreased within many parts of the wound (Bishop 2007). However, results of a study carried out by Gabig *et al.* (1979) contradicts this. Gabig and colleagues (1979) set up an *in vitro* experiment to investigate the effects of oxygen tension and pH on neutrophils

respiratory burst. It was shown that in hypoxic conditions, superoxide production displays minimal decline until the oxygen concentration fell below 1 % (7.6 mmHg). These results suggest that in an extremely hypoxic environment, neutrophils can continue to produce a respiratory burst and restrict bacterial activity. Despite this, it is now generally accepted that oxidative killing is dependant on all wounds being initially hypoxic and the maintenance of a local oxygen tension through ROS production by phagocytes (Tandara and Mustoe 2004; Gordillo and Sen 2003; Rodriguez *et al.* 2008). This means that unless there is sufficient oxygen perfusion, which is related to the oxygen gradient, oxidative killing cannot take place. The importance of sufficient oxygen supply in preventing infection has been acknowledged in numerous studies (Knighton *et al.* 1986; Hunt *et al.* 1975; Hohn *et al.* 1976).

Tissue injury can result from overproduction of ROS. Deoxyribonucleic acid (DNA) and cellular protein damage may result in cell death through necrosis or apoptosis (Pryor *et al.* 2004). It is thought that low concentrations of ROS play an important role in cell signaling and the re-establishment of homeostasis (Jurinek and Bezek 2005). This is supported by other researchers who theorize that these low concentrations of ROS may also stimulate growth factor release and angiogenesis (Gordillo and Sen 2003, Rodriguez *et al.* 2008; Sen 2003). Suggestions in the past decade have been made that initial ROS production is stimulated through hypoxia, although oxygen is required to sustain it and thus chronic hypoxia is unable to maintain the process (Rodriguez *et al.* 2008; Gordillo and Sen 2003; Jurinek and Bezek 2005).

As human cellular responses are notoriously challenging to measure *in vivo*, the majority of ROS research has been carried out on animal wound models or *in vitro* (Cho *et al.* 2001). As a result, clinical studies have been forced to focus on observing the effect of supplemental oxygen on the occurrence of wound infection (Bishop 2008). Most recent papers on the relationship between oxygen perfusion and the prevention of infection have focused on this. As discussed, primary growth factors such as TGF- β can stimulate wound healing through various actions. It has been shown that some of this growth factor production can be upregulated by either hypoxia or hypoxic conditions (Bishop

2008). Falanga *et al.* (1991) devised a study in which fibroblasts, removed from the dorsal forearm of healthy volunteers, were grown to almost confluent in normal oxygen conditions. These cultures were then subjected to normal oxygen conditions (15 % or 94 mmHg oxygen at cell surface) or hypoxic conditions (2 % or 14 mmHg oxygen at cell surface) for various time periods, not exceeding 72 hours. Falanga *et al.* (1991) observed that cells that were exposed to hypoxic conditions (2 % oxygen) secreted 9 times as much TGF-β as cells that were exposed to normal conditions (15 % oxygen). This finding was also reflected in the messenger ribonucleic acid (mRNA) levels of TGF-β which were 8 fold higher after 72 hours exposure to hypoxic conditions when compared with those exposed to normal conditions (Siddiqui *et al.* 1996). This demonstrated that TGF-β synthesis by human dermal fibroblasts was upregulated by an oxygen tension, increasing the secretion of this peptide. A few days after wounding, when the wound area is still hypoxic, fibroblasts migrate to the wound. The results of Falagan *et al.* (1991) show that collagen production may be initiated by this hypoxic environment.

Siddiqui *et al.* (1996) later examined the effects of chronic hypoxia on TGF-β secretion in a similar study. Dermal fibroblasts obtained from skin biopsies of healthy participants where grown in standard culture conditions (20 % or 140 mmHg oxygen) and in hypoxic conditions (1 % or 7 mmHg oxygen). Cells that where subcultured six times in the 1 % oxygen environment were defined as suffering from chronic hypoxia. Acute hypoxia was undefined. Chronic hypoxic cells (1 % oxygen) proliferated three times slower than cells exposed to standard oxygen conditions (20 % oxygen). This was once again reflected in the levels of TGF-β mRNA which displayed a 3.1-fold decrease in chronically hypoxiated cells compared to standard cells. This showed that TGF-β production from dermal fibroblasts was affected in a different manner, depending on whether cells were exposed to an acutely hypoxic environment or a chronically hypoxic environment. Acute hypoxia acts to stimulate the production of factors and other processes, such as VEGF and angiogenesis, whereas chronic hypoxia eliminates or inhibits these. Falagnga *et al.* (2002) later eluded that acute hypoxia may act as a stimulant for collagen synthesis, with a critical role being played by TGF-β1. The expression of VEGF by keratinocytes *in vitro* was examined by Patel and colleagues

(Patel *et al.* 2005). Cells were exposed to 20 % and 90 % normobaric oxygen, and hyperbaric oxygen (90 % oxygen at 3ATA (atmospheres absolute)). Limited cell growth resulted in cells that were exposed to 90 % normobaric oxygen compared to 20 % normobaric oxygen. Virtually no growth resulted when cells were subjected to hyperbaric oxygen. However, when mRNA analysis was carried out, the results suggested that oxygen therapy delivered at atmospheric pressure (normobaric) and above atmospheric pressure (hyperbaric) induced VEGF expression by keratinocytes. The investigators theorized that, although hypoxia can initiate angiogenesis, oxygen is required to allow the continued release of VEGF, thus sustaining the process (Patel *et al.* 2005).

It is well established that angiogenesis works to restore blood flow to wounded tissue (Whitney 1989). However, whether hypoxia or oxygen is needed to start this process requires clarification. The established theory is that angiogenesis is initiated by hypoxia. However, the oxygen gradient, arising from increased oxygen levels, acts to stimulate and maintain it (Gordillo and Sen 2003; Patel *et al.* 2005). Angiogenesis is enhanced by VEGF. Its relationship with oxygen has already been discussed earlier (Patel *et al.* 2005). Due to the challenges of controlling cofounding factors in humans and the difficulties in mimicking the complexity of *in vivo* interactions in *in vitro* studies, the majority of studies in this area were conducted in animal models (Bishop 2008). This means that results should be treated with caution as there is debate over the relevance of animal models to the healing process in humans.

Knighton *et al.* (1981) examined wounded rabbits' ears and measured the density and rate of capillary growth after inhalation of 12 %, 20 %, 40 % and 70 % oxygen concentrations. The authors failed to divulge the reason for using these concentrations or the length of time the rabbits were exposed to these oxygen levels. The investigators implanted a small flat circular object, referred to as an ear chamber, into the surface of the skin on the ventricle surface of each ear. Atmospheric oxygen was then prevented from reaching the wound by covering the ear chamber with an air-tight cover. The vascular pattern that developed over the chamber was traced and the number of

capillaries present was counted (Knighton *et al.* 1981). The rate of vessel growth was calculated by taking photographs and tracings of the chamber to calculating the percentage of the ear chamber covered by capillaries. Significantly slower vessel growth was observed after one day in the wounds exposed to 40 % oxygen. Days 2 – 4 saw a sharp increase in the rate of angiogenesis, meaning the difference ceased to be significant. At the end of the experiment, the rabbits who inhaled 40 % and 70 % oxygen displayed significantly higher capillary density than those which inhaled the lower concentrations of oxygen. When the chambers were removed and the hypoxic area was exposed to atmospheric oxygen, capillary growth was arrested. The investigators theorized that for angiogenesis to take place, an oxygen gradient is required. Removal of the oxygen gradient resulted in the arrest of capillary growth. The authors also suggested that the density and rate of capillary growth is affected by the concentration of oxygen, with more efficient angiogenesis resulting from the inhalation of higher oxygen concentrations. Use of an animal study allowed Knigton *et al.* (1981) to manipulate the oxygen concentrations and control the wound environment.

The effect of oxygen on angiogenesis was also investigated by Hopf *et al.* (2005). The team used a mouse subcutaneous wound model to assess the effect of oxygen on angiogenesis. Mice were exposed to oxygen concentrations of 13 % (hypoxic), 21 % (normoxic, the control group), 100 % (hyperoxic) and 100 % at 2.5 ATA and 3 ATA (all hyperoxic and all hyperbaric). Mice in the 13% and 21% oxygen groups were subjected to constant exposure to these oxygen levels. Mice in all the hyperoxic groups received 90 minute exposures, twice daily. This was in an attempt to re-enact hyperbaric oxygen therapy. Supplemented VEGF was given to half the mice in all groups. Assessment of angiogenesis was carried out by microscopic examination (Hopf *et al.* 2005). After seven days, all hypoxic groups displayed significant increases in angiogenesis, while hyperoxic groups showed inhibition of angiogenesis. The control group of non-VEGF supplemented mice displayed no significant increase in angiogenesis. While the quality of blood vessels was not assessed and sample sizes were not divulged, it can be interpreted with caution, that Hopf *et al.* (2005) proved their hypothesis that angiogenesis

is accelerated by hypoxia. However, prolonged hypoxia inhibits angiogenesis (Hopf *et al.* 2005).

Cho *et al.* (2001) discovered that animal wounds contain lower levels of oxidants than human wounds, which serves as a reminder that animal wounds are not a direct illustration of wounds in humans. Hopf *et al.* (2005) suggested that the results are relevant to the clinical setting, arguing that the study reflects the hypoxic environment and angiogenesis impairment that is commonly observed in chronic and acute wounds. However, a more thorough understanding of the underlying mechanisms is required through further *in vitro* studies.

Collagen fibres provide a scaffold for the healing process. They are synthesized by fibroblasts and collagen is considered an unusual protein in that it has a triple-stranded helix. The triple helix requires hydroxyproline to allow for its stable formation. Inadequate levels of hydroxyproline can lead to the unwinding of the helix. Hunt and Pai (1972) suggested that fibroblasts will only produce collagen if sufficient oxygen is present. The study discussed earlier by Siddiqui *et al.* (1996) on TGF-β1 supports this claim.

Niinikoski (1969) conducted pioneering work on the effects of oxygen in wound healing. It was shown that the inhalation of 30% - 70% oxygen significantly improved the tensile strength of wounds in rats. Hunt and Pai (1972) investigated the effects of varying oxygen levels in rabbits on the density and rate of collagen formation. Following the implantation of cylinders into six rabbits, the resulting wounds were allowed to heal in atmospheric conditions for 20 days. Two rabbits were then subjected to a hyperoxic environment (45 % oxygen), two were subjected to normoxic conditions (air) and two were exposed to hypoxic conditions (14 % oxygen) for a further five days after the initial 20 day period of healing. Observations suggested that subjecting the wounds to hyperoxic conditions led to an acceleration of collagen synthesis. As discussed earlier, arterial oxygen tension and possibly oxygen gradients were closely related to these results. However, the study by Hunt and Pai (1972) on the effect of hypoxia returned

inconclusive results. The reliability of the results was compromised by small sample numbers and experimental variability. Another factor that may have affected the results was the initial lack of moist wound healing.

Mehm *et al.* (1988) found that fibroblast proliferation and collagen synthesis were optimized at levels approximately double that of normal tissue. Cells were subjected to oxygen levels of 1788 mmHg, 722 mmHg, 160 mmHg, 80 mmHg, 38 mmHg (normal tissue level) and 15 mmHg for four days. Cells exposed to partial pressures under 80 mmHg showed the greatest cell growth, while cells exposed to 38 mmHg and 80 mmHg partial pressure displayed the highest collagen production. These results illustrated that hyperoxic levels of oxygen over 80 mmHg may be detrimental to fibroblast proliferation and collagen synthesis. The researchers suggested that the higher levels of oxygen (>89 mmHg) were toxic to the cells.

Work by Shandall *et al.* (1985) to predict leakages of colonic anastomses in rabbits using tissue oxygen tension paved the way for Sheridan *et al.* (1987) to investigate this in humans. Collagen plays a key role in the healing of anastomoses (Shandall *et al.* 1987). A Clarke electrode was used to measure the tissue oxygen of the colon. This was carried out on 50 patients undergoing colonic resection and anastomosis. Standardised 33.3 % oxygen was administered to patients. Anastomotic leakage was experienced in 10 % of patients with significantly lower oxygen tension ($p < 0.01$), despite 33.3 % oxygen being administered to them. Those experiencing leakage saw a decrease in tissue oxygen tension of up to 50 %. This suggests that prevention of separation in anastomotic healing requires good tissue oxygen tension levels. This is most likely due to the effects of oxygen on collagen synthesis (Sheridan *et al.* 1987). The majority of studies in this area have been conducted on animal models. However in recent times, studies like that by Falanga *et al.* (2002) have exploited advances in *in vitro* molecular techniques to investigate the effect of oxygen on collagen formation.

Successful wound healing requires oxygen, although hypoxia is a normal occurrence in all wounds. This state of hypoxia acts to stimulate important process such as growth

factor release, angiogenesis and the creation of an oxygen gradient (Bishop 2008). Sustained wound healing requires a significantly lower tissue oxygen tension at the wound centre than in the surrounding tissue. It has been suggested that excessive levels of oxygen may be detrimental to wound healing, specifically in relation to the synthesis of collagen (Bishop 2008). However, in clinical practice, complications such as dehiscence (release of material by splitting open of tissue) and infection are prevented by the use of supplemented oxygen. These apparent benefits of oxygen and the importance of an oxygen gradient mean that this area should be further studied (Bishop 2008). Animal and *in vitro* models have provided the context for most of the studies. In animal models, there is difficulty in measuring cellular responses and controlling confounding factors in humans is not always possible. Similarly, *in vitro* studies cannot allow for the cell-to-cell interactions that take place in *in vivo* models. Human factors such as nutrition and disease also play a role in complicating wound healing. Optimal oxygen levels for wound healing must be determined by further clinical trials (Bishop 2008). These would provide a basis for deciding the best forms of intervention for clinicians in promoting the healing of acute and chronic wounds.

1.4.3: Reactive Oxygen Species and Wound Healing

Although molecular oxygen is relatively unreactive, oxygen products which are more susceptible to participation in chemical processes are formed during the metabolism of oxygen. Both reducing and oxidizing agents are produced by superoxide, which is the main ROS produced *in vivo* (Bartosz 2009). The weak oxidant hydrogen peroxide attacks mainly thiols. Peroxynitrite, a strong oxidizing, nitrating and nitrosylating agent is produced through the reaction of superoxide with nitric oxide. Hydrogen peroxide reacts with the chloride ion, Cl^-, catalyzed by myeloperoxidase, to produce the oxidant and chlorinating compound, hypochlorite (Bartosz 2009). Hydrogen peroxide also reacts with transition metal ions to yield hydroxyl radicals ($^{\cdot}OH$). These are the most reactive species that occur *in vivo*. Rapid and indiscriminate reactions of $^{\cdot}OH$ with biomolecules of all classes such as carbohydrates, lipids, proteins, free nucleotides and nucleic acids,

results in the infliction of oxidative damage, possibly causing protein inactivation, DNA mutations and cell death (Bartosz 2009).

Biomedical interest in ROS initially stemmed from three events: Firstly, the suggestion that oxygen free radicals may be the driving force in hyperoxic injury (Gerschman *et al.* 1954), secondly, the emergence of the free radical theory of aging (Harman 1956) and finally, the discovery of superoxide dismutase (McCord and Fridovich 1969). They all postulated that ROS are an unavoidable consequence of the metabolism of oxygen. Ubiquitous ROS leads to the mounting of a complex antioxidant defense by the body from aerobes. This defense seems to be inadequate in ROS-mediated aging and in a number of diseases (Bartiosz 2009). These theories resulted in animal and human studies being carried out on the prevention of diseases linked to oxidative stress and the emergence of antioxidant supplements with the goal of prolonging life span. These studies, although promising in some cases, have failed to return desirable results in most instances. However, an alternative view has since formed. It is now suggested by some researchers that evolution has selected ROS production to perform useful roles in cellular metabolism. The abundance of data showing that ROS have important functions in cell signaling supports this view. Such important functions in cell signaling include ROS acting as participants and modifiers in signaling pathways which are essential for the complete development and proliferation of cells. ROS may also be able to mimic and amplify the actions of growth factors through mitogenic effects. This role in conveying information between cells shows that continuous formation and removal of ROS from the body is not altogether detrimental to cellular health. Since the discovery of the signaling role of nitric oxide, it has been generally accepted that small molecules have potential cell signaling capabilities (Bartosz 2009). Hydrogen peroxide and superoxide are considered to have possible signaling functionalities. Hydrogen peroxide is enzymatically produced, is involved in enzymatic removal, it has some selectivity of reactions through its low reactivity and it easily penetrates cell membranes. This points to hydrogen peroxide being a signaling molecule. The fact that hydrogen peroxide is also non-enzymatically produced is the only argument against it being a possible cell signaling molecule (Bartosz 2009). The arguments for superoxide being a possible

signaling molecule are that it is enzymatically produced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox), it is engaged in enzymatic removal and, due to its low reactivity, it has a degree of selectivity of reactions. The fact that it is also produced non-enzymatically, as well as its difficulty in penetrating membranes, form the main arguments against superoxide being a potential player in cell signaling. However, the precise identification of ROS involved in cell signaling is difficult. It is common place for one ROS to give rise to another species. Superoxide always gives rise to hydrogen peroxide by reduction through enzyme-catalyzed or spontaneous dismutation. Irreversible protein modifications may be executed by more aggressive ROS other than hydrogen peroxide and superoxide (Bartosz 2009).

Although exposure of cells to high levels of ROS has been shown to result in necrosis and apoptosis, it has also been demonstrated that exposure to low levels of ROS enhances cell division and proliferation (Toyokuni and Akatsuka 2007). Despite *in vitro* experiments failing to match the complexity of cell interactions at *in vivo* levels, experiments like these may shed light on some of the general principles of ROS actions. Several cell cycle proteins such as those containing cys residues and metal ci-factors have ROS-sensitive motifs in their active sites. This has led to suggestions that ROS signaling may be involved in the regulation of gap (G)0/G1 to synthesis (S) to G2 and mitosis (M) cell cycle progression (Bartosz 2009). Chronic granulation disease caused by Nox deficiency leads to compromised wound healing. Hydrogen peroxide is present at the wound site in micromolar concentrations. It supports wound healing by inducing expression of VEGF in human keratinocytes. While micromolar concentrations of hydrogen peroxide at the wound site can benefit the process, higher concentrations act to inhibit the healing process. Collagen synthesis is stimulated by ROS which also support angiogenesis. Monocyte and lymphocyte-induced angiogenesis is inhibited by antioxidants (Roy *et al.* 2006). Redox control affects numerous aspects of wound healing. Thus, it is critically important to develop a thorough understanding of how endogenous ROS, generated in wounds, influence the healing process. Understanding this could result in novel redox based strategies for wound healing. Hypoxia is the source of most problems in wounds so it is reasonable to assume that the correction of oxygen

levels in the wound may facilitate the generation of endogenous ROS by Nox in wound-related phagocytic and non phagocytic cells (Sen and Roy 2008).

1.4.4: The Role of Hyaluronic Acid in Wound Healing

One of the key factors in reconstituting supporting matrix at scar formation sites is the deposition of collagen by fibroblasts. The quality of the resulting scar is greatly determined by the nature of this collagen deposition (Price *et al.* 2007). Fibroblast proliferation appears to be stimulated by long chain hyaluronic acid (HA) in gingva (gum) and *in vitro* (Mesa *et al.* 2002; Greco *et al.* 1998; Mast *et al.* 1993). However, long chain HA does inhibit peripheral nerve adhesion, as well as decreasing total scar collagen production by adult dermal fibroblasts (Croce *et al.* 2001). There is evidence to suggest that enhanced ECM remodeling and more ordered collagen deposition results from HA application (Iocono *et al.* 1998; Kielty *et al.* 1992; Rooney & Kumar 1993). Increased scarring is caused by hyaluronidase, the enzyme responsible for the breakdown of HA, despite the fact that it was originally expected to increase tissue HA fragments. Also, persistent high levels of HA decreases fibroblast contraction (West *et al.* 1997; Huang-Lee & Nimni 1993). A number of growth factors may also affect fibroblast production of HA, while HA fragments of between 4 and 25 disaccharides in length act to promote angiogenesis (West *et al.* 1985). The search for scarless healing has seen fetal wound healing extensively studied (Price *et al.* 2007). After the initial proliferation phase in adult healing, the evaluated levels of HA decrease. However, throughout the maturation of fetal wounds, levels of HA remain elevated. This is a result of decreased HA turnover by fetal wounds rather than increased HA production (Longaker *et al.* 1991). In the fetal environment, it appears that HA fails to degrade, which may be due to rheological effects. When exogenous HA is applied to wounds induced on fetal forelimbs grown *in vitro*, reduced scarring results (Iocono *et al.* 1998). This may be partially explained by early fetal wounds displaying less expression of CD44 and receptors for hyaluronic acid-mediated motility (RHAMM) than in adult wounds (Lovvorn *et al.* 1998). Consistently elevated levels of macromolecular HA in fetal wounds may limit fibrosis, while dermal scare formation is associated with increased

breakdown products in adult wounds (Price *et al.* 2007). Keratinocyte production is enhanced by the application of exogenous HA both *in vitro* and *in vivo*. The addition of HA also enhances corneal cell migration *in vivo*. This effect is synergistic with the actions of endothelial growth factor (EGF) and fibronectin (Nishida *et al.* 1991). Increased keratinocyte proliferation and motility also results from the application of EGF to organotypic cultures (Pasonen-Seppänen *et al.* 2003). Laboratory and animal studies have produced strong evidence suggesting that hyaluronic acid may have a positive affect on many aspects of wound healing (Price *et al.* 2007). Collectively these studies suggest that HA has positive role in the wound healing process. However, further studies are required to elucidate the exact role and actions of HA in would healing.

1.5: Alginate Gel Structural Uniformity

Structural uniformity within the alginate gel is of paramount importance in the development of any alginate wound dressing. A homogeneous structure leads to more consistent structural properties throughout the gel (Kuo and Ma 2001). Alginate gel beads have been extensively used as encapsulation materials for bioactive compounds (Augst *et al.* 2006). Such systems have been used to treat type I diabetes by encapsulating insulin-producing cells in calcium-alginate capsules for delivery to the patient (Kristiansen *et al.* 2009). However, studies documenting this technique use fast gelling systems such as the drop-wise addition of sodium alginate slurry to a solution of calcium ions, often made by adding calcium chloride to water. The resulting rapid external gelling of the alginate produces non-homogeneous gels with varied crosslinking densities (Skjåk-Bræk *et al.* 1989). This non-homogeneity detracts from the functionality of a potential alginate wound dressing. Studies have found alternative methods of producing more homogeneous gels (Kuo and Ma 2001). The low solubility of calcium carbonate in water enables it to become uniformly distributed in the alginate solution. An internal, slow gelling system can then be created by releasing calcium ions through decreasing the pH, initiated by the addition of D-glucono-δ-lactone (GDL) (Zhang *et al.* 2005). The controlled release of Ca^{2+} permits a slower, more complete crosslinking of the gels. This allows the alginate-calcium mixture to be poured into moulds before

gelation is complete, enabling the formation of gels with complex structures that may be suitable for use as wound dressings. Although extensive research has been carried out demonstrating the effects of calcium ion concentration and alginate content on hydrogel properties (Draget *et al.* 1993), no such studies have been conducted that characterizes the effects of the addition of hydrogen peroxide or hyaluronic acid, or indeed aging effects during storage, on the properties of alginate gels.

1.6: *In Vitro* Scratch Wound Assay

The *in vitro* scratch wound assay involves inducing an artificial, so called "scratch" on a monolayer of confluent cells. It was observed that cells at either edge of this artificial gap moved towards each other until new cell-cell contacts were established once again, thus closing the gap (Liang *et al.* 2007). The basic procedure is initialized by inducing the scratch, followed by capturing images at the beginning and during regular intervals throughout the cell migration process, until eventually the scratch is closed. The rate of migration can then be determined by comparing these images (Liang *et al.* 2007). This method allows for the straightforward and economical study of *in vitro* cell migration (Todaro *et al.* 1965). This uncomplicated method mimics, to some degree, the migration of cells *in vivo*. When part of the endothelium is removed from blood vessels, migration of endothelial cells is induced into the stripped area to close to the wound (Haudenschild and Schwartz 1979). The behavior of cells *in vitro* also mimics the patterns of cell migration either in sheets (e.g. endothelial and epithelial cells) or as loosely connected populations (e.g. fibroblasts) (Liang *et al.* 2007). The *in vitro* scratch wound assay also has another major advantage in that it is particularly suited to the study of the regulation of cell migration by cell interactions with extracellular matrix and cell-cell interactions (Liang *et al.* 2007). The preparation of cells in suspension prior to the commencement of other popular methods, such as Boyden chamber assay, leads to disruption of cell-ECM and cell-cell interactions. The Boyden chamber assay was devised for the analysis of leukocyte chemotaxis. It consists of a chamber of two medium-filled compartments separated by a microporous membrane. Cells in the upper compartment are drawn across the membrane by chemotactic agents in the lower chamber. At the end of the assay, cells

on the membrane are fixed and stained prior to analysis (Chen 2005). The *in vitro* scratch wound assay's compatibility with microscopy allows for the live cell imaging which facilitates the analysis of intracellular signaling events during cell migration. This can be achieved with the use of proteins tagged with green fluorescent proteins (GFP) for subcellular localization or fluorescent energy transfer can be used for the detection of protein-protein interactions (Liang *et al.* 2007). These advantages, coupled with the assay's simplicity and the fact that it only requires common and inexpensive laboratory supplies, illustrate its suitability to this study for the examination of the effect of alginate-hydrogen peroxide wound dressings on healing. Despite the *in vitro* wound healing assay being developed for studying cell migration, it has been used in combination with other techniques. Examples of this include gene transfections or microinjections to evaluate the expression of exogenous genes on the migration of individual cells (Etienne-Manneville and Hall 2001; Fukata *et al.* 1999; Abbi *et al.* 2002). Time-lapse microscopy and image analysis software are utilized to track the migration path of cells in the leading edge of the scratch. Cells can be marked for the expression of exogenous genes or downregulated endogenous genes by ribonucleic acid interference (RNAi) through taking a fluorescent microscopy image at the beginning of the experiment. The role of a particular gene can then be ascertained by comparing the tracks of the marked cells with surrounding control cells under the same experimental conditions (Liang *et al.* 2007). The *in vitro* scratch wound assay also has limitations and disadvantages when compared to other well-established methods of chemotaxis such as the Boyden chamber assay. As no chemical gradient can be established in the *in vitro* scratch wound assay, it cannot replace the Boyden chamber method. This assay is also more time consuming than other techniques. Cell formulation requires 1-2 days and cell migration, to close the scratch, requires a further 8-18 hours. The majority of *in vitro* scratch wound assays take place in culture dishes rendering them unsuitable if availability of cells (e.g. specialized primary cells) and chemicals (e.g. expensive reagents) are a factor. Despite these limitations, the ease of use of the *in vitro* scratch wound assay means that it is still the technique of choice for the study of cell migration in many laboratories (Liang *et al.* 2007).

1.7: Objectives of Research

The antibacterial properties of alginate have established it as a wound dressing material in the healthcare industry. When combined with a slow cross-linker such as calcium carbonate, alginate can form gels that are easily shaped in moulds and have good structural uniformity (Ma and Kuo 2001). It has been shown that low levels of hydrogen peroxide may be beneficial in wound healing (Roy *et al.* 2006). It may also be possible to use hydrogen peroxide to overcome problems in wound healing posed by hypoxia. Hydrogen peroxide reacts with catalase present in wound tissue to produce oxygen. However, the excessive production of oxygen may be detrimental to wound healing due to the production of ROS. Hyaluronic acid may act as an oxygen scavenger, thus eliminating some of the harmful effects caused by excessive oxygen in the wound environment, such as ROS. In this study, alginate gels for use as wound dressings which incorporated varying concentrations of alginate, calcium carbonate, hydrogen peroxide and hyaluronic acid were assessed over time with regards to their gelation time, homogeneity, rupture force and rupture energy. Investigating the gelation time and homogeneity of these alginate gels allows for the assessment of their suitability for use as a wound dressing. A rapid gelation time has been shown to produce less structurally uniform gels (Kuo and Ma 2001). More homogeneous gels are more structurally sound than inconsistent gels (Draget *et al.* 1991; Van Susante *et al.* 1995). The assessment of rupture characteristics is also useful when evaluating the gel's suitability as a wound dressing. The more resistant the gel is to rupture, the more durable and workable it will be for patients and physicians alike. The profile of gelation and rupture characteristics and gel homogeneity provided a basis for the formulation of structurally robust gels which were then analysed using cellular models to determine the potential of these calcium-alginate gels which incorporated hydrogen peroxide in aiding wound healing. This was assessed by studying cell recovery and proliferation after the application of the aforementioned gels to scratch wounds induced in monolayers of synoviocytes (K4 IM) cells. The objectives of this research were to investigate the effects of important variables of gel formulation (Ca^{2+}, alginate, hydrogen peroxide, hyaluronic acid concentrations and storage time) on the gels' structural properties and to assess the effects of the resulting gels on cell proliferation and scratch wound recovery.

Chapter 2:

Materials and Methods

2.0: Materials and Methods

2.1: Spectral Analysis of Alginate

2.1.1: Materials

Sodium alginate (medium viscosity sodium alginate extracted from *Laminaria hyperborean*) was purchased from Sigma-Aldrich Ireland.

2.1.2: Determination of M/G Ratio by FT-IR

The M/G ratio was defined as proportion of mannuronic and guluronic acid blocks present in the alginate. Fourier transform infrared spectroscopy (FT-IR) was used to determine the M/G ratio of sodium alginate (medium viscosity sodium alginate extracted from *Laminaria hyperborean*). The instrument used was a Perkin Elmer Spectrum BX FT-IR System. A potassium bromide (KBr) disk of the alginate powder was prepared. An average of 32 scans at a resolution of 32 cm^{-1} were taken of the sodium alginate KBr disk to produce the final spectrum. The intensity of the bands at 1100cm^{-1} (mannuronic acid) and 1025cm^{-1} (guluronic acid) were then compared to derive the M/G radio of the alginate.

2.1.3: Spectral Analysis of Alginate Solutions Undergoing Gelation

The test alginate solution was thinly poured onto the attenuated total reflectance (ATR) crystal and the spectrum recorded. This spectrum recorded the structure of the alginate solution immediately after initialisation of gelation. Gelation was then initialised by placing filter paper soaked in a calcium nitrate solution on top of the alginate solution and spectra were recorded at intervals of 2, 4, 6 and 8 min after gelation was initialised.

2.2: Structural and Mechanical Properties of Alginate Gels

2.2.1: Materials

Sodium alginate (medium viscosity sodium alginate extracted from *Laminaria hyperborean*), calcium carbonate, hydrogen peroxide (30 %), hyaluronic acid sodium salt (from *Streptococcus equi sp.*) and D-glucono-δ-lactone (GDL) were purchased from Sigma-Aldrich Ireland.

2.2.2: Alginate Gel Preparation

Alginate gels were produced by varying concentrations of alginate (0-6 % w/v), calcium carbonate (0-1 %w/v), hydrogen peroxide (0.00-3.75 %v/v) and hyaluronic acid (0 - 1.25 mg/L). Three replicates of each sample were prepared.

Alginate and hyaluronic acid powders were dissolved in a suspension of calcium carbonate to which 1 ml of hydrogen peroxide was added. This mixture was vortexted for 60 seconds. To this mixture, 2 ml of GDL solution were then added, followed by vortexing for 15 seconds. The solution was then immediately poured into a petri dish (50mm diameter, 10mm height) and allowed to set for 48 hours in a cool, dry, dark location.

2.2.3: Gelation Time

Gelation time was assessed using a method adapted from Kuo and Ma (2001). Gelation time was defined as the time between the addition of GDL and the formation of the gel. The point at which gel formation was determined was when the sample no longer flowed when tilted at an angle of 45° for longer than 30 seconds (Kuo and Ma 2001).

2.2.4: Homogeneity

The method used by Kuo and Ma (2001) for determining homogeneity in alginate gels was adapted and used as follows: The dry to wet weight ratio of the gel was used to evaluate homogeneity. Each gel was cut horizontally to give four slices, which were numbered 1-4 (1 corresponding to the top slice and 4 to the bottom slice). These slices were weighed, dried to a constant weight and then reweighed. The dry to wet weight ratio of the slices provides an indication of the homogeneity of each gel. A homogeneous gel will have a consistent dry weight to wet weight ratio across its four constituent slices (Kuo and Ma 2001).

2.2.5: Rupture Characteristics

Adaptations were made to the method described by Roopa and Bhattacharya (2009) to carry out analysis of the rupture characteristics of the gels. Samples were subjected to penetration at two sites by a Zwick/Roell Universal Testing Machine using a 5 mm diameter probe. The 5 mm probe was attached to the testing machine's load cell via a custom designed adaptor. The adaptor which was constructed for use in this experiment is shown in Figure 2.1. Rupture characteristics were produced for each sample by taking the mean of six readings for the sample (three replicates, each measured twice). Rupture characteristics from the resulting force-deformation curve were obtained using Zwick's "test-X-pert" software.

Figure 2.1: Adaptor constructed for use in the rupture testing of alginate gels. The adaptor facilitates the attachment of a 5 mm probe to the load cell of the Zwick/Roell Universal Testing Machine.

Rupture force was defined as the maximum force immediately before rupture and was expressed in Newtons (N). Rupture energy was defined as the area under the force–deformation curve until the point of rupture (Roopa and Bhattacharya 2009).

2.2.6: Aging Effects During Storage

Rupture characteristics were assessed at two, seven and fourteen days after formulation to assess the effect of storage on the integrity of the gels.

2.2.7: Statistics

All graphs and statistical analyses were produced using Microsoft Excel 2007 and SPSS 17.0.

2.3: Alginate Gel Oxygen Release Analysis

2.3.1: Materials

Sodium alginate (medium viscosity sodium alginate extracted from *Laminaria hyperborean*), calcium carbonate, hydrogen peroxide (30 %), hyaluronic acid sodium salt (from *Streptococcus equi sp.*) and D-glucono-δ-lactone (GDL) were purchased from Sigma-Aldrich Ireland. Ground beef was purchased from a local supermarket and used as an artificial wound.

2.3.2: Alginate Gel Oxygen Release Analysis

The oxygen release of alginate gels when in contact with an artificial wound was analysed in Deamen College, Amherst, NY, USA. Ground beef, which acted as an artificial wound, was placed in a Vernier BioChamber. A 2 cm^2 piece of parafilm with a 1 cm^2 section removed from the centre of it was then placed on the artificial wound. This ensured a consistent contact area between the alginate gel and artificial wound. An alginate gel, prepared as described in section 2.2.2, was trimmed to 2 cm^2 and placed on top of the parafilm and the artificial wound. A Vernier oxygen gas sensor was then placed in the orifice provided for it in the BioChamber. This apparatus is illustrated in Chapter 4 (Figure 4.1). The BioChamber was then sealed and the oxygen levels within the BioChamber were recorded using Logger *Pro* software via a computer connected to the oxygen gas sensor. Catalase within the artificial wound reacted with the hydrogen peroxide in the alginate gel to produce oxygen gas and water.

2.4: Cell Culture and Wound Healing Assessment

All cell culture techniques were performed in a sterile environment in a FASTER UltraSafe laminar flow cabinet. Cells were incubated in a humid environment in a BINDER ATP Line C 150 CO_2 incubator set at 37 ^{o}C and 5 % CO_2. Cells were visualised using an Olympus CKX 41 inverted microscope.

2.4.1: Materials

Sodium alginate (medium viscosity sodium alginate extracted from *Laminaria hyperborean*), calcium carbonate, hydrogen peroxide (30 %), hyaluronic acid sodium salt (from *Streptococcus equi sp.*) and D-glucono-δ-lactone (GDL) were purchased from Sigma-Aldrich Ireland. Sterile filters (0.2 μm) were purchased from Sarstedt.

Royal Park Memorial Institute (RPMI) - 1640 medium (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) modification, with 25 mM HEPES, without L-glutamine, sterile-filtered, cell culture tested), 0.25 % trypsin- ethylenediaminetetraacetic acid (EDTA) solution, 200 mM L-glutamine, fetal bovine serum (FBS), penicillin-streptomycin (10,000 units penicillin and 10 mg streptomycin per ml in 0.9 % NaCl) and dimethyl sulfoxide (DMSO) were all purchased from Sigma-Aldrich Ireland. Phosphate-buffered saline tablets (Dulbecco A) were purchased from Oxoid. All reagents were sterile-filtered and cell culture tested.

2.4.2: Cell Line

The human synovial fibroblast cell line (K4IM) was established from a healthy donor and immortalized with SV40 T antigen by Haas *et al.* (1996). This cell line was used to assess cellular proliferation and migration in wound healing.

2.4.3: Supplementation of Media

Fetal bovine serum was decomplemented by allowing it to thaw from frozen at 4 °C. The serum was then placed in a waterbath at 56 °C for 30 minutes. The serum was then aliquotted into 50 ml lots and stored at -20°C.

RPMI-1640 medium was supplemented by adding the following:

Supplemented RPMI (500ml)

440 ml	RPMI-1640 media
50 ml	FBS
5 ml	Penicillin (10 000 U/ml) – Streptomycin (10 mg/ml)
5 ml	2 mM/ml L-glutamine solution

The supplemented medium was incubated at 37 °C and 5 % CO_2 for 24 hr and then examined for signs of contamination.

2.4.4: Seeding Cell Cultures

A 1 ml vial of cryopreserved cells was thawed at room temperature. The cells were transferred to a Sarstedt 75 cm^2 tissue culture flask. 20 ml of supplemented RPMI-1640 medium were added to the flask which was then placed in the humid atmosphere of the incubator at 37 °C and 5 % CO_2.

2.4.5: Feeding Cell Cultures

Supplemented medium was pre-heated in an incubator at 37 °C for 30 minutes. Cells were then examined under the microscope to examine their confluency, size, shape and for indications of contamination. The expended supplemented medium was removed and replaced immediately with fresh supplemented medium. The culture flasks were then returned to the incubator at 37 °C and 5 % CO_2. Upon initial feeding after seeding from cyropreservation, only half of the expended medium was removed and replaced with fresh supplemented medium. Subsequently, all expended medium was replaced.

2.4.6: Counting Cells

Cells were firstly trypsinized as follows: The expended medium was removed from the flask and cells were then washed with 2 ml of sterile phosphate buffered saline (PBS). Three milliliters of trypsin were added to the cells and the flask was transferred back to the incubator for 3 minutes. Following trypsinization (when cells were observed to be detaching from the surface of the flask), the cells were transferred back to laminar flow cabinet and 3 ml of supplemented medium were added to the flask. The cell suspension was pipetted up and down to disperse any clumps and transferred to a 15 ml sterile centrifuge tube. The cell suspension was centrifuged at 800 rpm for 5 minutes in a Heraeus Labofuge 400 R centrifuge. The supernatant was discarded and the cells were resuspended in 5 ml of supplemented medium. The cell suspension was mixed by inverting the container 2-3 times and 0.1 ml of the suspension was transferred to a 0.5 ml eppendorf. The surface of a coverslip and the hemocytometer slide (improved Neubauer) were cleaned with 70 % alcohol and lens tissue. The coverslip was then slightly wetted and placed on top of the hemocytometer. The cell suspension was mixed thoroughly by pipetting up and down and 20 µl were transferred to the edge of the hemocytometer chamber. Surplus fluid was blotted off without drawing out liquid from under the coverslip. The hemocytometer, containing the cell suspension, was viewed using a 10x objective. The cells contained in the 1 mm^2 (4 x 4 grid) were then counted for each of the four areas. The average number of cells per 1 mm^2 was then calculated. The hemocytometer slide (improved Neubauer) contains a volume of 1 x 10^4 ml per 1 mm^2. Therefore, the cell concentration was the average number of cells per 1 mm^2 per 1 x 10^4 ml. The hemocytometer slide and coverslip was then rinsed with disinfectant and cleaned using 70 % alcohol and lens tissue.

2.4.7: Cryopreservation of Cells from Culture

The cells were viewed using an inverted microscope. Following confirmation that the cells were contamination-free and displayed healthy cell morphology, they were trypsinized as described above. Zero point five milliliters of DMSO, 1 ml of FBS and 3.5 ml of supplemented medium were added to a 15 ml centrifuge tube. The cell suspension

was centrifuged at 800 rpm for 5 minutes. The supernatant was removed and then discarded. The cell pellet was transferred to the tube containing DMSO, FBS and supplemented medium and resuspended by pipetting up a down several times. The resulting cell mixture was then aliquotted out into 1 ml sterile labelled cryotubes and stored at -80 °C in a New Brunswick Scientific ultra low temperature freezer.

2.4.8: Formulation of Sterile Alginate Gels

Separate aqueous suspensions of alginate and calcium carbonate were prepared and autoclaved using a Tomy SX-700E autoclave. GDL was then weighed out into a sterile centrifuge tube and hydrogen peroxide (30 %) was transferred into a sterile centrifuge tube. All components of the gel were then placed in the laminar flow cabinet. Sterile water was added to the GDL. The aqueous GDL solution and hydrogen peroxide where then filtered through sterile 0.22 μm Sarstedt filters. Alginate gels were then produced, in the laminar flow cabinet, by varying concentrations of alginate (0-6 % w/v), calcium carbonate (0-1 % w/v) and hydrogen peroxide (0.0-0.5 % v/v).

2.4.9: Scratch Wound Assay

Alginate gels with varying compositions of alginate (0-6 % w/v), calcium carbonate (0-1 % w/v) and hydrogen peroxide (0.0-0.5 % v/v) were formed by transferring 2 ml of the pre-gelled solution to each well of a 6-well plate. These gels were allowed to set in the laminar flow cabinet for 24 hours. The scratch wound assay was performed by following the protocol outlined by Liang *et al.* (2007). Cells were seeded into COSTAR 6 well plates and grown to 80 % confluence. A straight line scratch was introduced to the monolayer by drawing a p200 pipette tip across the cells. Cell debris was then removed by washing the cells with 1 ml of growth medium. 5 ml of supplemented medium were then added to each well. Alginate gels were then transferred to the test wells and placed on top of the scratch-wounded cells. The scratch wound was then imaged at 0, 5, 24 and 48 hours after introduction of the wound using an Olympus IX51 inverted microscope and an Olympus DP70 camera. The images were analyzed at 100X magnification using Image J software to calculate the recovery of the wound in terms of the percentage of

original wound area populated by migrating cells after 5, 24 and 48 hr (Abramoff *et al.* 2004).

2.4.10: Proliferation Assay

Cells were seeded in COSTAR 12-well plates and grown to 60 % confluence. Growth medium was then removed and replaced with 800 µl of 0.1 % serum growth medium and incubated for a further 48 hours. This synchronized the cell cycle. Alginate gels with varying compositions of alginate (0-6 % w/v), calcium carbonate (0-1 % w/v) and hydrogen peroxide (0.00-0.25 % v/v) were formed by transferring 2 ml of pre-gelled solution to each well of a 12-well plate. These gels were allowed to set in the laminar flow cabinet for 24 hours. Following 48 hours incubation in 0.1 % serum growth medium, the cells were transferred from the incubator to the laminar flow cabinet. The 0.1 % serum growth medium was replaced with 800 µl of supplemented medium. Alginate gels were then transferred to the test wells and placed on top of the scratch-wounded cells. The proliferation assay was then carried out on separate plates of cells after 5, 24 and 48 hours of incubation. Cells were transferred from the incubator to the laminar flow cabinet. Alginate gels were removed from the wells and 160 µl of CellTiter 96 AQueous One Solution Reagent were added to each well. This plate was then incubated for a further 3 hours and the absorbance of the resulting wells was read at 490 nm on a BMG LABtech SPECTROstar Omega plate reader.

Chapter 3:

Structural and Mechanical Properties of Alginate Gels

3.1: Spectral Characterization of Alginate Gels

3.1.1: Introduction

This chapter documents the preliminary stages of characterising a novel dressing for wounds. Alginates are a family of linear copolymers that contain 1-4-linked β-D-mannuronate (M) and α-L-guluronate (G) (Jayakumar *et al.* 2009). Divalent cations, such as Ca^{2+}, can interact ionically with alginate polymer chains to form a gel structure. These hydrogels are formed by each divalent ion interacting with two adjacent G-residues, as well as with two G-residues in an opposing chain. The resulting structure is often referred to as the "egg-box" model (Kristiansen *et al.* 2009).

The most defining attribute of any alginate is its ratio of mannuronic acid (M) to guluronic acid (G), or its M/G ratio. Alginates with a higher proportion of guluronic residues, that is, a lower M/G ratio, will produce stronger, more rigid gels when cross-linked. Alginates which consist of a higher mannuronic content will yield softer, more malleable gels when cross-linked. These gels will also be more susceptible to degradation over time due to their inferior strength when compared to gels formulated from alginates with high guluronic content (Kuo and Ma 2001). Assessment of the alginates M/G ratio by FT-IR was carried out. This data was used to estimate the properties of gels produced from this alginate, thus determining the M/G ratio provides a means of characterising and consequently optimising the type of gel formulated.

3.1.2: Results

FT-IR spectral analysis of commercial sodium alginate (*Laminaria hyperborean*) used throughout this study was carried out using a FT-IR (Perkin Elmer Spectrum BX FT-IR System). The resulting spectrum is shown in Figure 3.1.

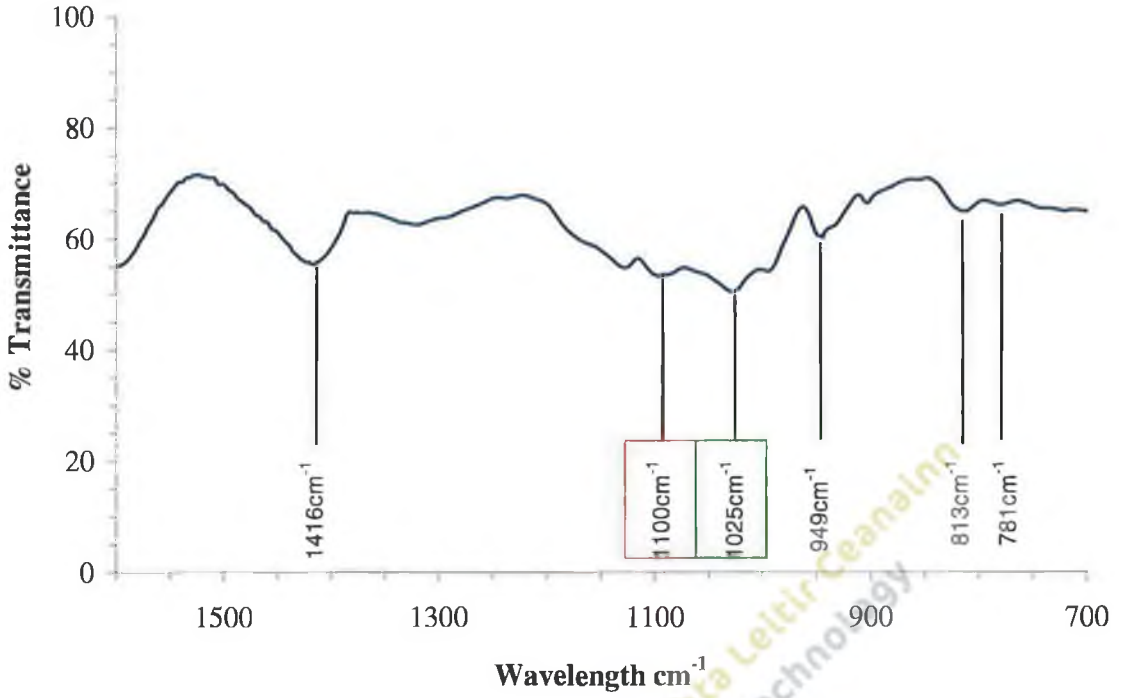

Figure 3.1: FT-IR spectrum of commercial sodium alginate (*Laminaria hyperborean*). A potassium bromide (KBr) disk of the alginate powder was prepared. An average of 32 scans were taken of the sodium alginate KBr disk at a resolution of 32 cm^{-1} to produce the final spectrum. The intensity of the absorbances at 1100cm^{-1} (mannuronic acid) and 1025cm^{-1} (guluronic acid) were then compared to give the M/G ratio of the alginate.

The bands at 1025 cm^{-1} and 1100 cm^{-1} are due to guluronic and mannuronic acid residues, respectively. This is due to the stretching of C-O-C and C-OH modes within both guluronic and mannuronic acid. Comparison of the relative intensity of these modes provides the M/G ratio of the alginate which was subsequently determined to be 0.9327. The bands at 813 cm^{-1} and 781 cm^{-1} also result from guluronic acid. The OH deformation mode gives rise to the absorbance at 949 cm^{-1}. The intense band at 1406 cm^{-1} is due to vibration of CH_2 groups (Pereira *et al.* 2003; Leal *et al.* 2008).

Spectral analysis of alginate solutions undergoing gelation was also carried out using the apparatus described below (Figure 3.2). This provided an understanding of the structural changes that the alginate solution undergoes during gelation. The results also gave an indication of the gelation time of the alginate gels studied.

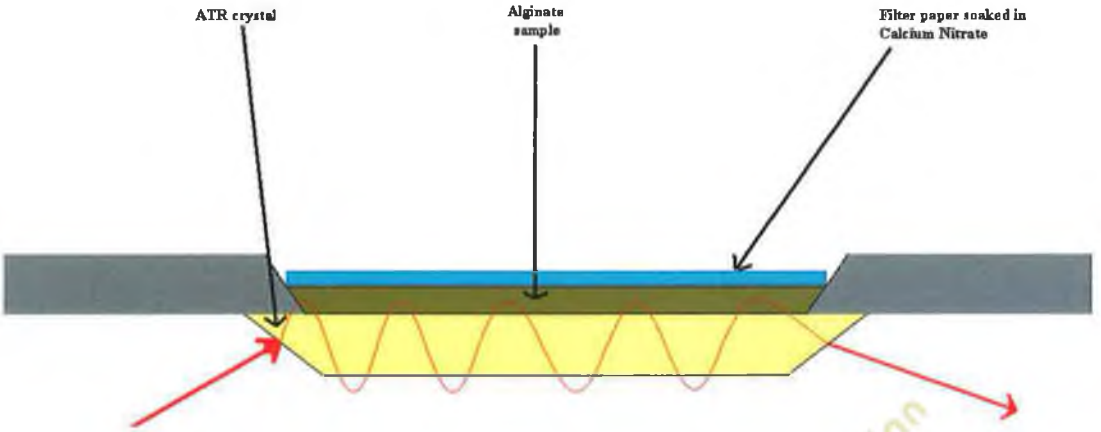

Figure 3.2: Apparatus used to measure FT-IR spectra of alginate solutions during gelation. The FT-IR beam is represented by the red line.

The test alginate solution was thinly poured onto the ATR crystal and the spectrum was recorded. This spectrum recorded the structure of the original alginate solution. Gelation was then initiated by placing filter paper which had been soaked in a calcium nitrate solution on top of the alginate solution and spectra were recorded 2, 4, 6 and 8 min after gelation was initiated.

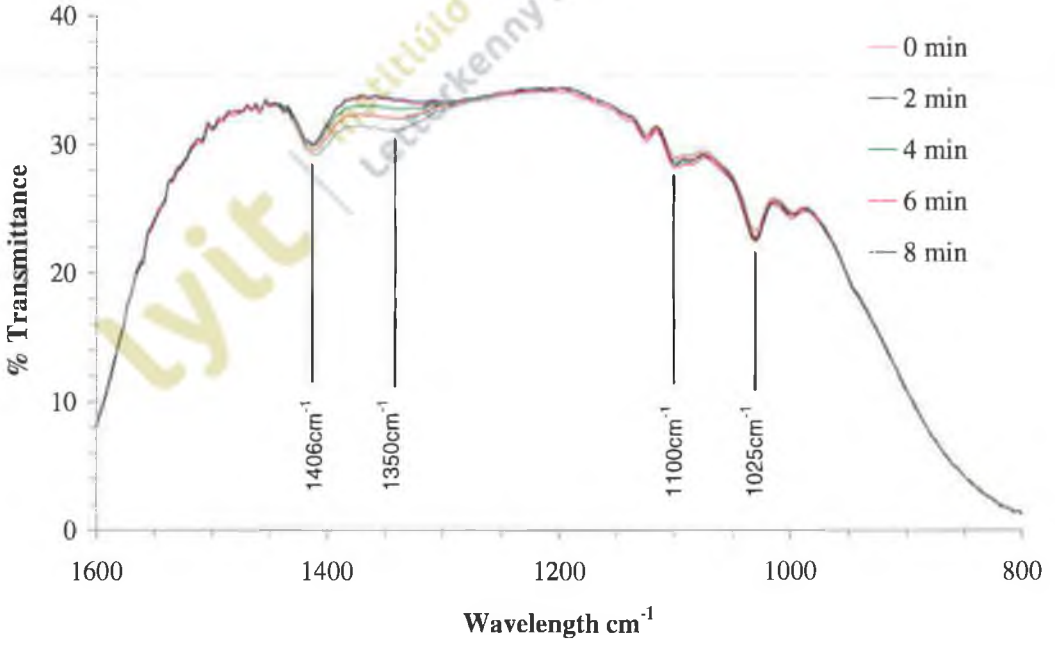

Figure 3.3a: FT-IR spectra of 4 % (w/v) sodium alginate solution without hydrogen peroxide undergoing gelation. An average of 32 scans were taken at a resolution of 32 cm^{-1} for each spectrum.

The incorporation of the alginate into an water based gel resulted in an apparent decrease in transmission at the extremes of the spectra in Figures 3.3a and 3.3b. Guluronic and mannuronic acid residues again give rise to bands at 1025 cm^{-1} and 1100 cm^{-1} respectively. The band at 1350cm^{-1} is attributed to NO_2 (resulting from the addition of calcium nitrate to initiate gelation) while the intense band at 1406cm^{-1} is accounted for by the vibrations of CH_2 groups (Figure 3.3a and 3.3b).

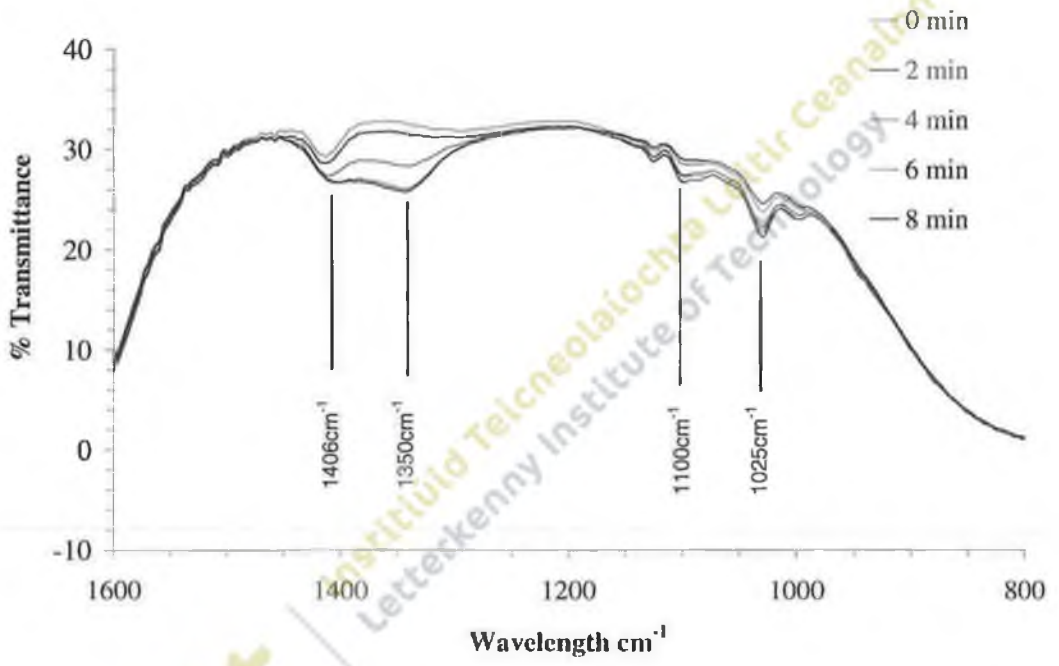

Figure 3.3b: FT-IR spectra of 4 % (w/v) sodium alginate solution containing 1 % (v/v) hydrogen peroxide undergoing gelation. An average of 32 scans were taken at a resolution of 32 cm^{-1} for each spectrum. Spectra were recorded at 0, 2, 4, 6, and 8 min after the addition of calcium nitrate solution.

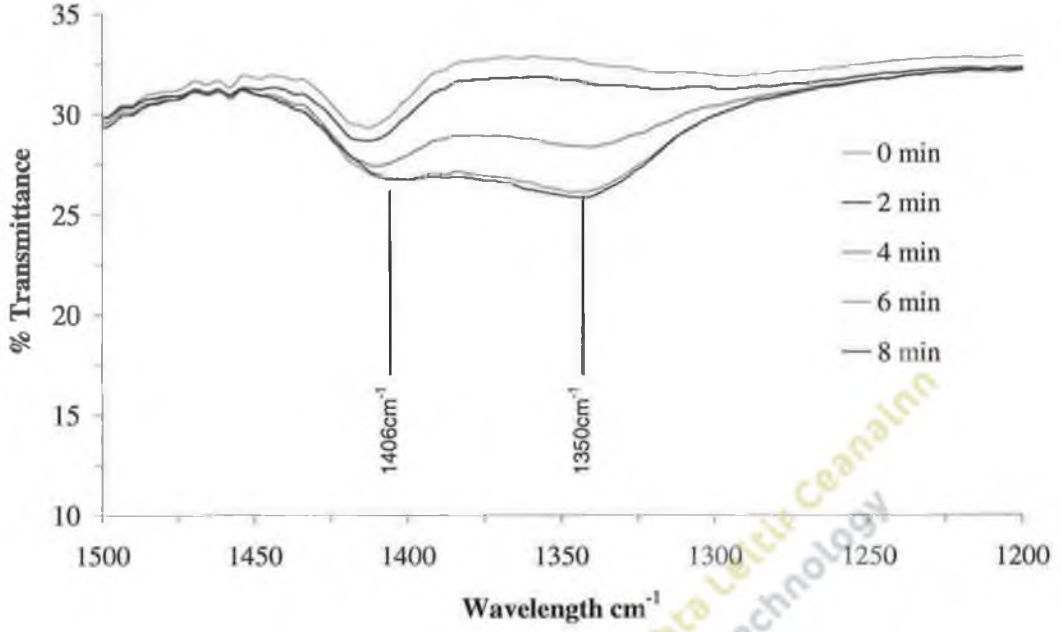

Figure 3.3c: Expanded FT-IR spectra of 4 % (w/v) sodium alginate solution containing 1 % (v/v) hydrogen peroxide undergoing gelation.

The intensity of the absorbances at 1350 and 1406 cm^{-1} resulting from the vibrations of NO_2 and CH_2 groups respectively were plotted with respect to time. As the period of gelation increased the absorbance of the bands for NO_2 and CH_2, at 1350 cm^{-1} and 1406 cm^{-1} respectively, intensified (Figure 3.3d).

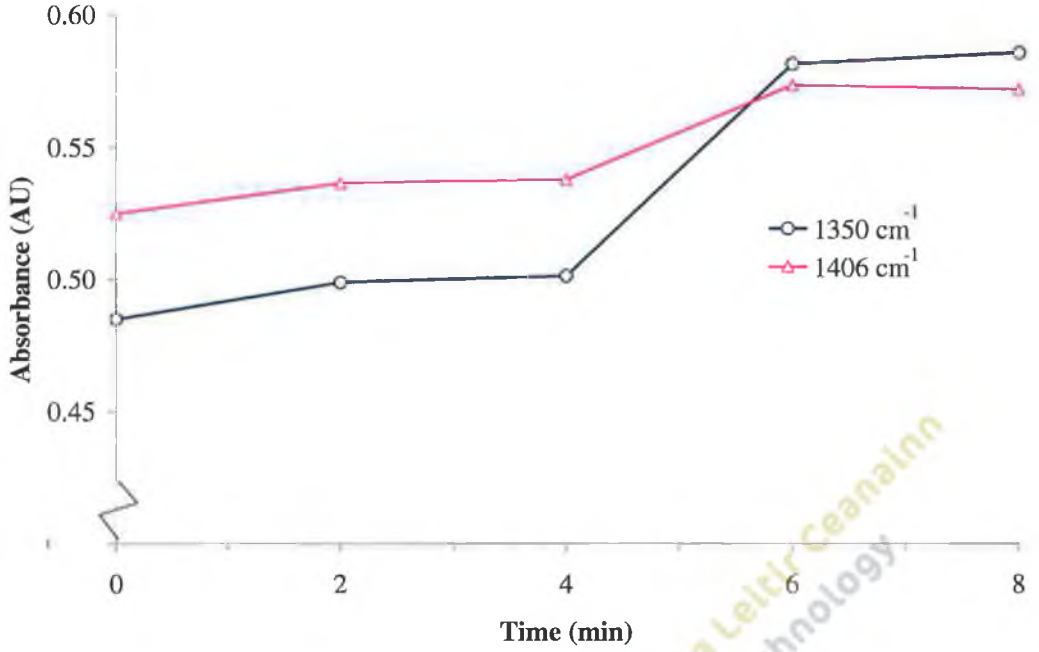

Figure 3.3d: FT-IR absorbance intensities for vibrations at 1350cm^{-1} and 1406cm^{-1} obtained for 4 % (w/v) sodium alginate solution containing 1 % (v/v) hydrogen peroxide undergoing gelation.

3.2: Gelation and Rupture Characteristics of Alginate Gels

3.2.1: Introduction

Structural uniformity within the alginate gel is of paramount importance in the development of any alginate wound dressing. A homogeneous structure leads to more consistent structural properties throughout the gel (Kuo and Ma 2001). Alginate gel beads have been extensively used as encapsulation materials for bioactive compounds (Augst *et al.* 2006). Such systems have been used to treat type I diabetes by encapsulating insulin-producing cells in calcium-alginate capsules for delivery to the patient (Kristiansen *et al.* 2009). However, studies documenting this technique use fast gelling systems such as the drop-wise addition of sodium alginate slurry to a solution of calcium ions, often initiated by adding calcium chloride to water.

The resulting rapid external gelling of the alginate produces non-homogeneous gels with varied cross-linking densities (Skjåk-Bræk *et al.* 1989). This non-homogeneity detracts

from the overall functionality of a potential alginate wound dressing. Studies have found alternative methods of producing more homogeneous gels (Kuo and Ma 2001). Gels formulated by varying the concentrations of alginate, hydrogen peroxide and calcium carbonate were tested with respect to gelation time, homogeneity and resistance to rupture. The rupture characteristics were derived from the force-deformation curve generated from the rupture testing of the gels (Figure 3.4). Rupture force was defined as the force in Newtons (N) at the point of rupture. Rupture energy is expressed as the area under the force-deformation curve before rupture and rupture strain was calculated as the rupture distance divided by the sample height (Figure 3.4). The systematic varying of the gel's composition followed by precise characterisation of the materials' functionality with regard to the aforementioned properties provide a means of tailoring the gels to the required needs.

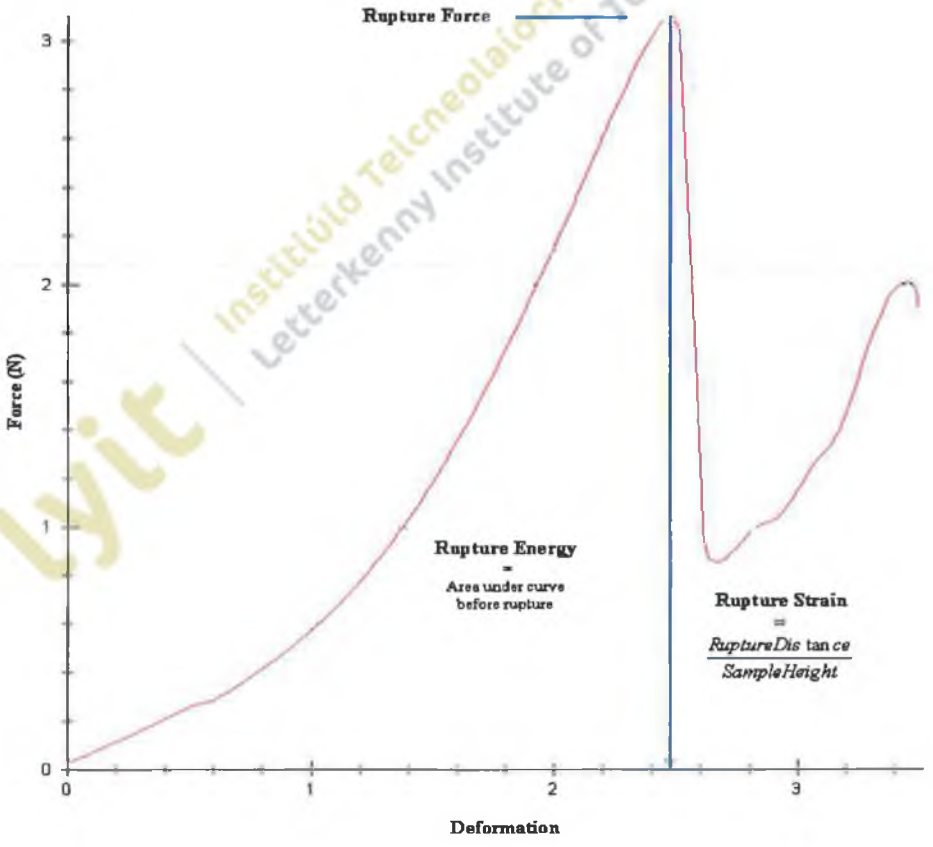

Figure 3.4: Force-deformation curve generated by Zwick-Roll test-X-pert software to determine gel rupture characteristics.

3.2.2: Results

A rapid gelation time is not desirable as it results in difficulties when moulding the gel and can lead to less homogeneous alginate gels (Kuo and Ma 2001). Figure 3.4a depicts the effects of calcium carbonate and hydrogen peroxide content on the gelation time of these hydrogels. It can be observed that gelation time decreases dramatically with increasing calcium carbonate content.

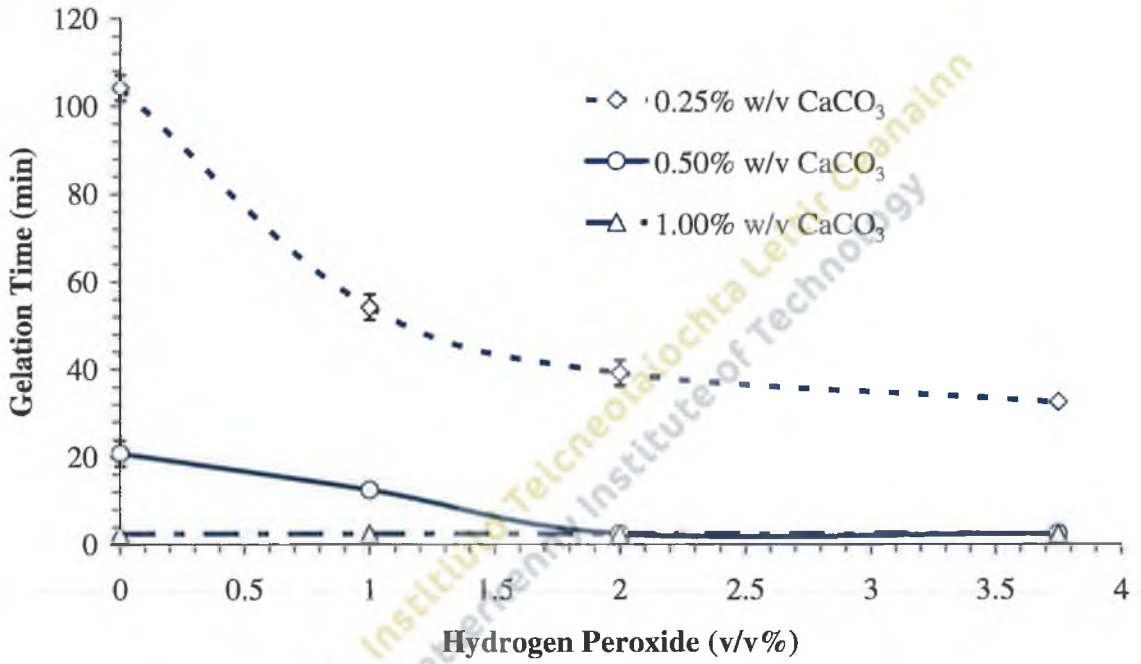

Figure 3.4a: The effects of hydrogen peroxide and calcium carbonate content on gelation time of alginate gels (4 % w/v alginate)

Figure 3.4b illustrates that as the concentration of alginate and hydrogen peroxide increases then the rate of gelation also increases. Figure 3.4a and Figure 3.4b display the time required for alginate gelation after the addition of calcium carbonate and GDL. The alginate solution was considered to be gelled when it no longer flowed when tilted at a 45° angle.

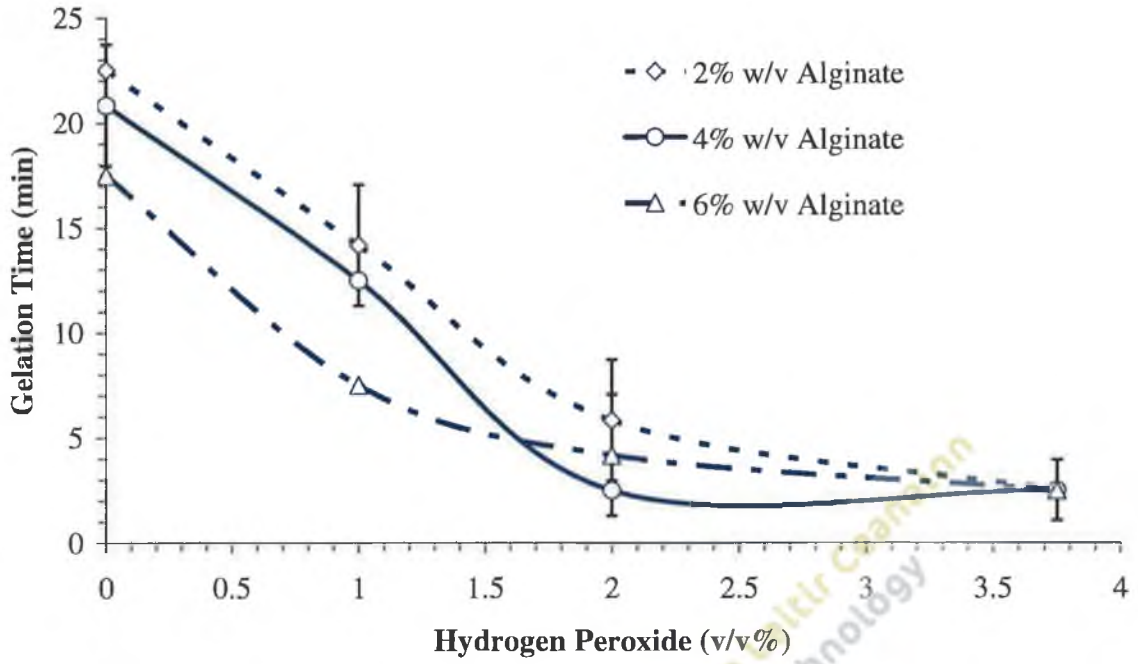

Figure 3.4b: The effects of hydrogen peroxide and alginate content on gelation time of alginate gels (0.5 % w/v calcium carbonate)

Table 3.1 confirms that only calcium carbonate concentration has a significant effect on the gelation times of these alginate gels ($p<0.001$). Gels formulated using different calcium carbonate concentrations were tested for statistical difference in their mean values of gelation time, homogeneity, rupture force and rupture energy. The same tests were carried out for gels of varying hydrogen peroxide and alginate content. It is clear that calcium carbonate content has a significant effect on all four parameters tested. Hydrogen peroxide content has a significant effect on the rupture force and rupture energy of gels ($p<0.001$). Alginate content, for the concentrations tested, does not significantly affect any of the four parameters tested.

Table 3.1: ANOVA test for significant difference between groups for varying compositions of alginate gels for potential use as wound dressings.

Parameter Measured	ANOVA p-value between groups		
	Calcium Carbonate	Hydrogen Peroxide	Alginate
Gelation Time	0.000	0.177	0.502
Homogeneity	0.000	0.797	0.174
Rupture Force	0.000	0.000	0.375
Rupture Energy	0.000	0.000	0.422

Figures 3.5a, 3.5b and 3.5c display gel homogeneity. Alginate gel homogeneity was assessed by allowing samples to gel for 48 hours and then slicing the gels horizontally into four slices of equal size. These slices were labelled "Slice 1" (top slice) to "Slice 4" (bottom slice) and the dry weight to wet weight ratios was calculated for each slice and used as an indication of gel homogeneity. A homogeneous gel displays equal values of dry weight to wet weight ratios for all four of its constituent slices. Figure 3.5a suggests that gel homogeneity improves with increasing alginate content. However, this is not confirmed by the ANOVA analysis in Table 3.1. The p-value of 0.174 illustrates that there is no significant difference in the homogeneity values of gels containing varying amounts of alginate.

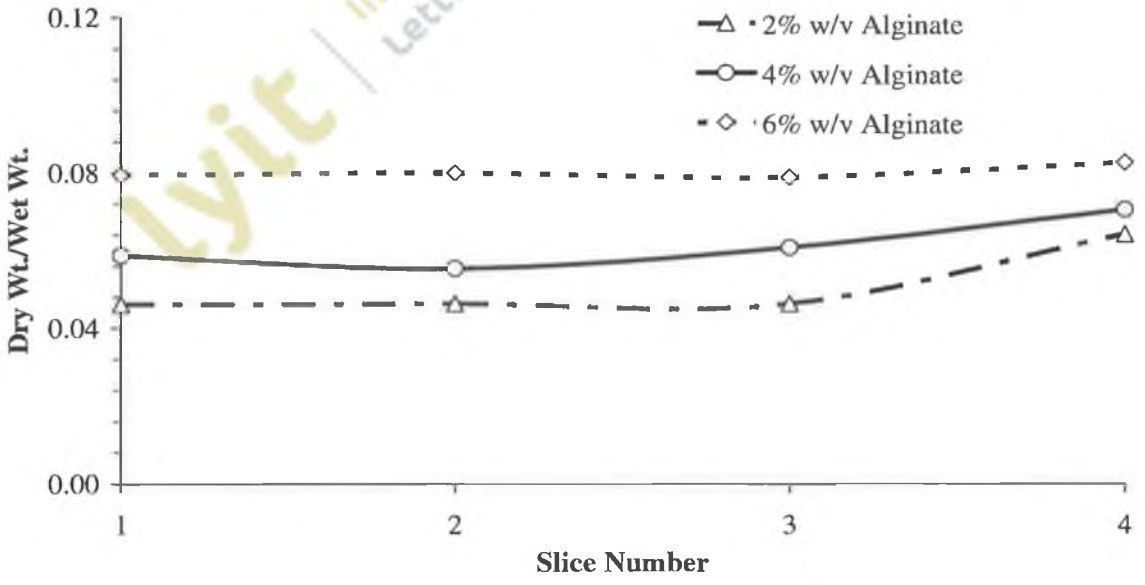

Figure 3.5a: The effect of alginate content on gel homogeneity

Calcium carbonate content appears to have the most significant effect on gel homogeneity, as shown in Figure 3.5b. Gel homogeneity decreases with increasing calcium content. This is confirmed by the ANOVA analysis documented in Table 3.1, which shows that there is a significant difference between the homogeneity values of gels with varying calcium carbonate concentrations ($p<0.001$).

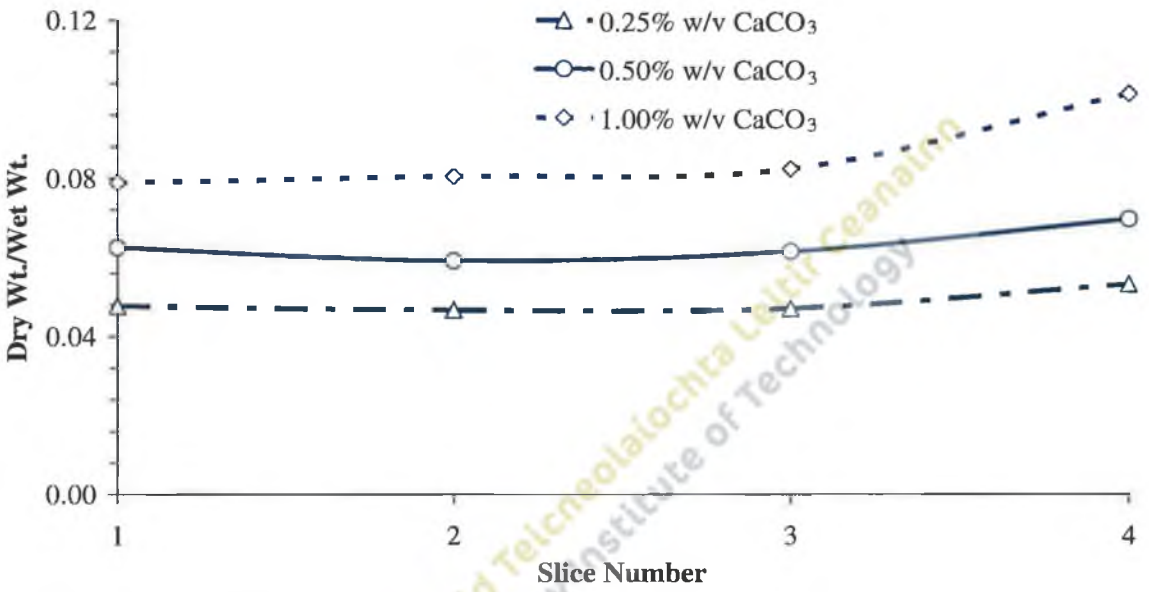

Figure 3.5b: The effect of calcium carbonate content on gel homogeneity

Hydrogen peroxide concentration has no obvious effect on the homogeneity of the gels, as shown in Figure 3.5c. This is confirmed in Table 3.1 which returned a p-value of 0.797.

Figure 3.6 indicates that increasing calcium carbonate content leads to an increase in the force required to rupture the gel. Increased hydrogen peroxide content produces gels with a decreasing rupture force. Table 3.1 supports these observations, with both calcium carbonate and hydrogen peroxide groups returning significant p-values ($p<0.001$). Table 3.1 also shows that alginate content does not have a significant effect on rupture force for the concentrations tested. Similar effects are observed for the action of calcium carbonate, hydrogen peroxide and alginate content on the rupture energy of the gels.

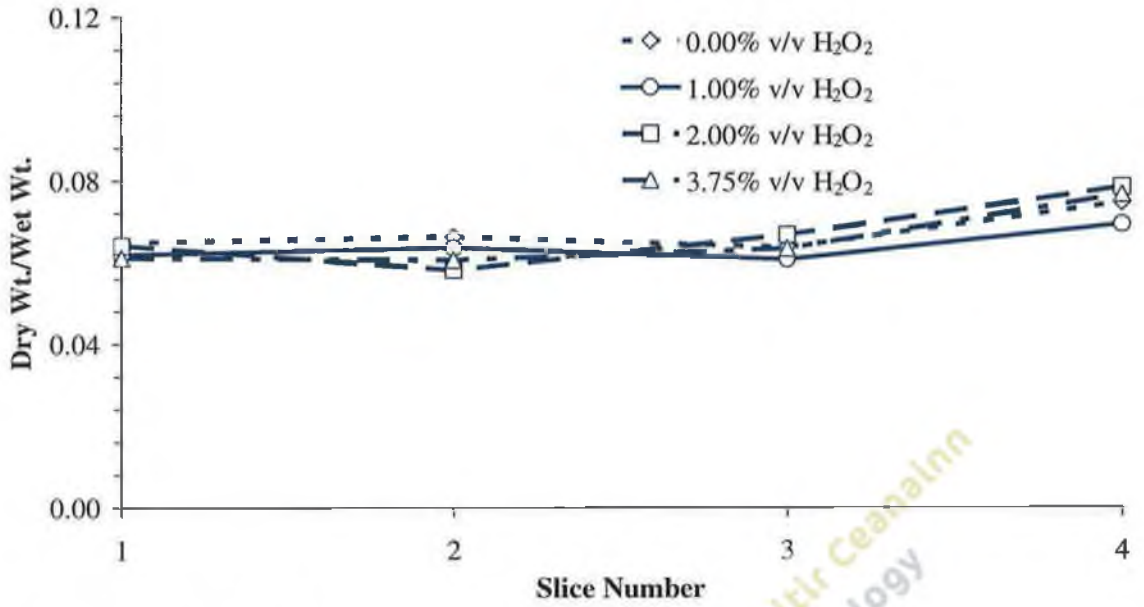

Figure 3.5c: The effect of hydrogen peroxide content on gel homogeneity

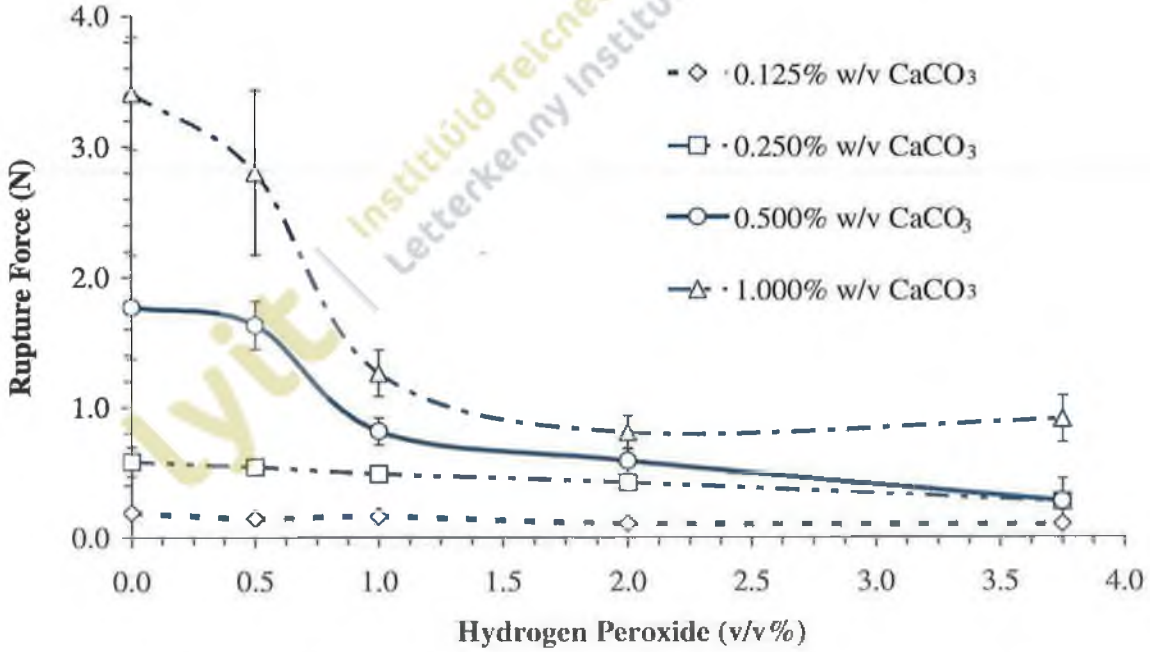

Figure 3.6: The effects of hydrogen peroxide and calcium carbonate content on rupture force of alginate gels (4 % w/v alginate)

Figures 3.7a, 3.7b and 3.7c indicate that the rupture force of alginate gels containing no hydrogen peroxide remain unchanged over time. However, samples containing hydrogen peroxide degraded over time, producing lower rupture force values. This effect is more pronounced with increasing hydrogen peroxide content and with increasing time.

Figures 3.7a, 3.7b and 3.7c also depict the effect of hyaluronic acid on the rupture force of the gels over time. Table 3.7a shows that hyaluronic acid delays the degradation of gels containing higher concentrations of hydrogen peroxide after two days of aging.

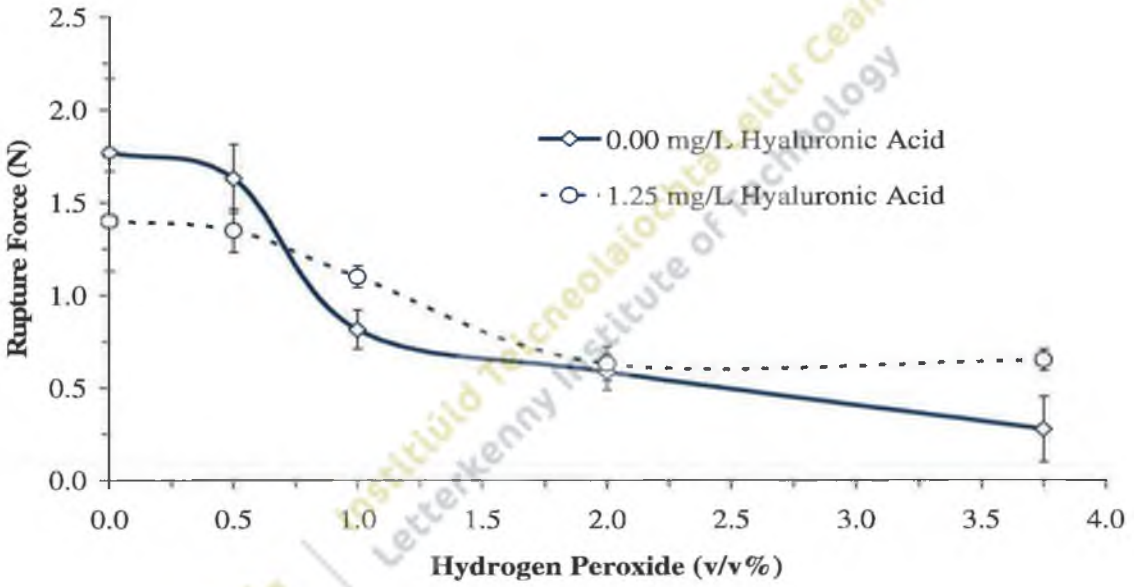

Figure 3.7a: The effect of hyaluronic acid (H.A.) content on rupture force of alginate gels after 2 days of storage (4 % w/v alginate, 0.5 % calcium carbonate)

The effect of the hyaluronic acid is more pronounced after seven days of aging, as shown by the higher rupture force values for gels containing hyaluronic acid compared to those not containing hyaluronic acid (Figure 3.7b).

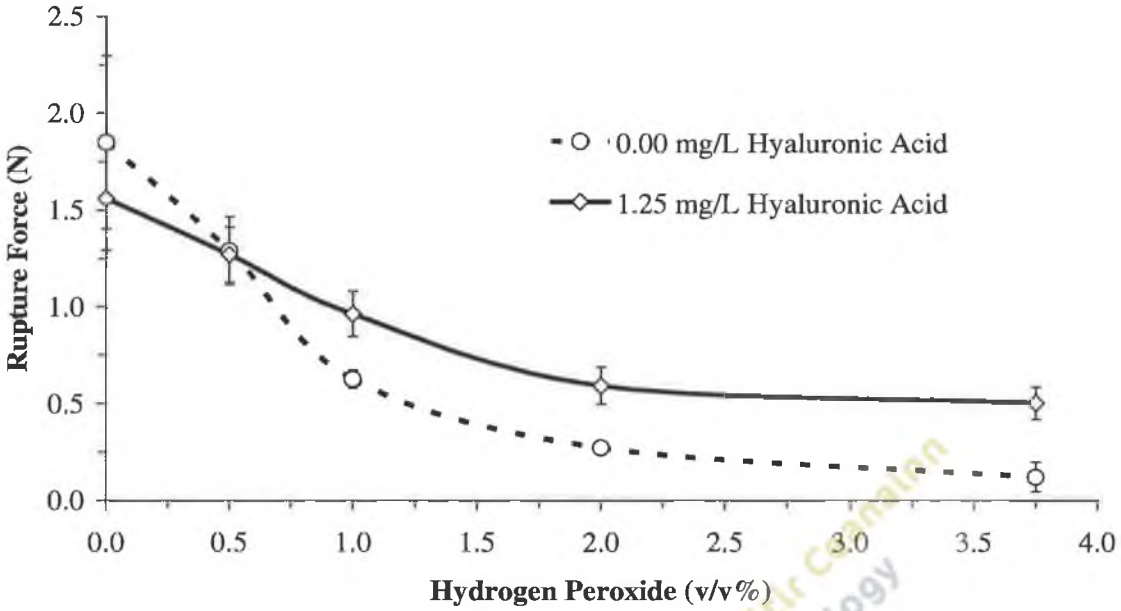

Figure 3.7b: The effect of hyaluronic acid (H.A.) content on rupture force of alginate gels after 7 days of storage (4 % w/v alginate, 0.5 % w/v calcium carbonate)

However, after 14 days of aging, rupture force values for samples containing hyaluronic acid are very similar to those without hyaluronic acid (Figure 3.7c). These values are considerably lower than those observed after two and seven days of aging (Figure 3.7b).

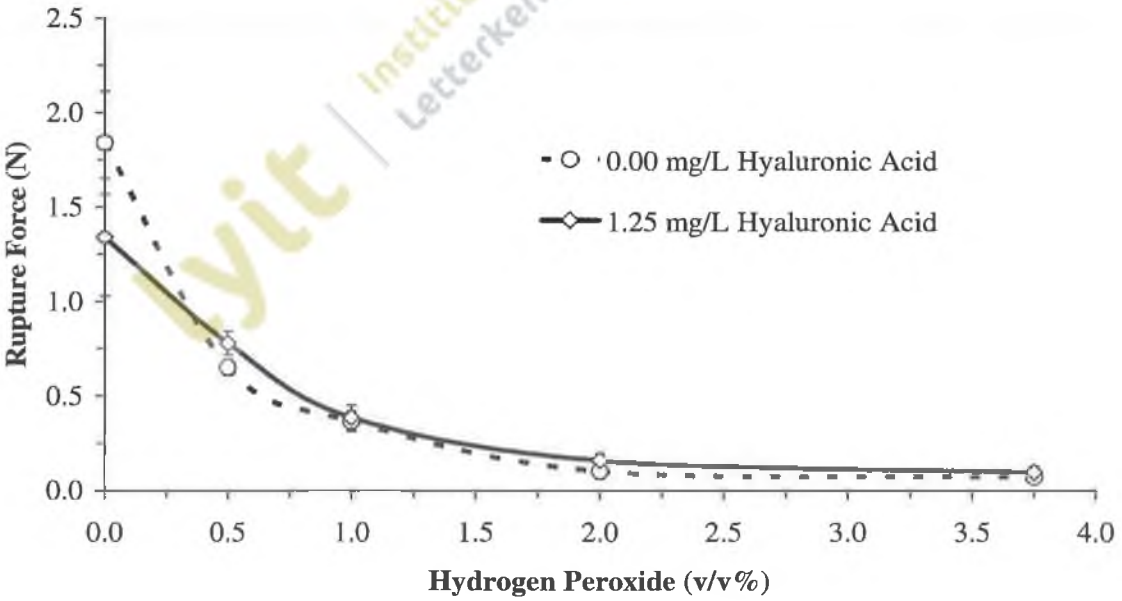

Figure 3.7c: The effect of hyaluronic acid (H.A.) content on rupture force of alginate gels after 14 days of storage (4 % w/v alginate, 0.5 % w/v calcium carbonate)

This suggests that gels containing hydrogen peroxide have a finite lifetime before degradation renders them unsuitable for use wound dressings. However, hyaluronic acid clearly offers some protection against gel degradation. Table 3.2 shows models produced through multiple linear regression for the prediction of gelation time, homogeneity and rupture force for varying compositions of alginate gels for use as wound dressings. Interpretation of these models allow for the estimation of a gel's characteristics within the experimental range. This can then be used to determine the gel composition most suited for use as a wound dressing.

Table 3.2: Multiple linear regression for the prediction of gelation time, homogeneity and rupture force values for varying compositions of alginate gels for potential use as wound dressings. The models constructed from this analysis will allow for the estimation of the gelation time, homogeneity and rupture force of gels of various compositions within the limits of the analysis.

Parameter	**Component**	**B**	**Std. Error**	**Sig. (p-value)**	**R^2**
Gelation Time (Min)	Constant	120.33	25.25	0.000	0.373
	$CaCO_3$	-82.90	23.40	0.001	
	H_2O_2	-11.35	5.27	0.039	
	Alginate	-6.076	4.47	0.183	
Homogeneity (standard deviation of the four constituent slices)	Constant	0.003	0.002	0.276	0.644
	$CaCO_3$	0.016	0.002	0.000	
	H_2O_2	0.001	0.001	0.277	
	Alginate	-0.001	0.000	0.005	
Rupture force (N)	Constant	0.515	0.071	0.000	0.578
	$CaCO_3$	1.509	0.083	0.000	
	H_2O_2	-0.243	0.021	0.000	
	Alginate	-0.250	0.006	0.000	

3.3: Discussion

The M/G ratio of alginate governs a number of its structural attributes. The M/G ratio of the alginate used for this study was determined to be 0.9327. This measurement was achieved by recording a FT-IR spectrum of the alginate and comparing the intensity of the bands due to mannuronic acid (1100 cm^{-1}) and guluronic acid (1025 cm^{-1}) (Figure 3.1). Gels formed from alginate with a high proportion of guluronic acid are more porous, thus more structurally robust, compared to those with a high proportion of mannuronic acid. G blocks exhibit greater stiffness than alternating blocks due to its diaxial links (Smidsrød and Skjåk-Bræk 1990). Similarly, alternating blocks are more stable at low pH. As the M/G ratio of this alginate is close to 1, it can be assumed that gels formed from it should be more stable at low pH. This makes gels formed from this alginate more suited to the incorporation of hydrogen peroxide than those with either high M block or G block content. With gels produced from alginate with a high G block content, the overall stiffness of the gel is also dependent on the polymer molecular weight distribution and the stoichiometry of the alginate with the chelating cation. This means that by selecting a strong and complete cross-linking agent gels that are both stable lower pH and structurally strong can be formed (Kong *et al.* 2002; Lee *et al.* 2000b).

Gelation time is an important variable in gel formulation, as previous studies have shown that a faster gelation time can lead to a less homogeneous gel. It can also have a major effect on the gel's application to cellular models. A gel formulated for testing with cells must be uniform, both in shape and composition. If a gel displays a rapid gelation time, then it becomes almost impossible to produce a morphologically uniform gel (Kuo and Ma 2001).

Figures 3.3a and 3.3b outline the results of the FT-IR analysis of an alginate solution undergoing gelation using calcium nitrate as a calcium ion source. A reduced intensity of bands between 1200cm^{-1} and 900cm^{-1} was observed after the addition of calcium in the sample in both the presence and absence of hydrogen peroxide (Figures 3.3a and 3.3b respectively). This dampening is more pronounced, however, in the sample containing hydrogen peroxide. There is also a marked increase in the intensity of bands at 1350 and

1406 cm^{-1} attributed to NO_2 (resulting from the addition of calcium nitrate to initiate gelation) and CH_2 groups (Figure 3.3b, 3.3c and 3.3d). The fact that the NO_2 absorbance is more intense in samples containing hydrogen peroxide may indicate that these samples have a faster gelation rate as Ca^{2+} is used up faster, giving more free NO_2. This may account for the higher degree of dampening in samples containing hydrogen peroxide, as a more complete gel is formed faster. However, the formation of a gel structure within 10 minutes of the addition of calcium ions is not necessarily desirable, as a faster gelation time can lead to a less homogeneous gel. Additionally, the use of an external gelling system by the diffusion of readily available calcium ions from a calcium nitrate solution leads to problems in producing a gel for application as a wound dressing. As the gel networks forms from the outside of the alginate towards the centre of the alginate solution, a gelation gradient is created. Thus, an alginate capsule which is gelled on the outside but not gelled in the centre results. A slow, internal gelling system is much more suited for use as an alginate wound dressing. The system that was employed from this point on in the study was the alginate-GDL-calcium carbonate system. Calcium ions within a calcium carbonate solution are not readily available for cross-linking. This allows the calcium to become evenly distributed throughout an alginate solution. The addition of GDL to this system then slowly releases the calcium ions from calcium carbonate, leaving them available for cross-linking (Kuo and Ma 2001).

The decreased gelation time for gels containing higher concentrations of calcium carbonate is due to the increased availability of calcium ions for cross-linking (Figure 3.4a). Gelation time also decreases in gels with higher alginate content (Figure 3.4a and 3.4b). This is an expected effect of additional alginate chains being available for cross-linking with calcium ions, due to the increased alginate content. The gelation rate is faster for gels containing higher concentrations of hydrogen peroxide (Figure 3.4b). This was also observed in Figure 3.3b. One possible explanation for this is that the presence of hydrogen peroxide may lead to the depolymerisation of alginate chains, producing more numerous chains that are shorter in length and more readily available for cross-linking with calcium ions, thus yielding faster gelation rates.

Samples with higher amounts of calcium carbonate display more unequal values for the dry weight to wet weight ratios of its constituent slices, given in Table 3.5b. This is probably due to more powder (calcium carbonate) falling to the bottom of the liquid samples, prior to gelation. More viscous samples (containing higher alginate content) appear to be more homogenous (Table 3.5a), since the calcium carbonate stays in suspension more effectively than in less viscous samples and is, therefore, more evenly distributed within the gels. This sedimentation of sample constituents leading to less homogeneous gels is supported by the observation that in all cases, slice 4 (the bottom slice) has the highest dry weight to wet weight ratio of that gel's constituent slices (Figure 3.5a, 3.5b and 3.5c).

The rupture characteristics of a gel indicate its structural integrity. It is desirable to produce a gel that will not rupture easily, since such a gel would be impractical for use as a wound dressing. It is observed from Figure 3.6 that calcium carbonate content has a strong positive effect on the rupture characteristics of alginate gels. This may be explained by the more complete cross-linking of the gels which occur from a greater amount of calcium ions being available for cross-linking. This more complete cross-linking is further supported by the strong positive effects that calcium concentration has on gelation time. Hydrogen peroxide content acts negatively on rupture force and rupture energy. This could be due to hydrogen peroxide degrading the gels by depolymerisation or due to the production of CO_2 from the CO_3^{-2} + GDL reaction. While it may be desirable to produce a gel that will yield the highest resistance to rupture, which could be achieved by increasing the calcium content of the gel, it is important to remember that in doing so, the gel would become more brittle (higher rupture strain value) and more difficult to mould into a desirable uniform structure (faster gelation time). For these reasons, it is important to consider all response values of the gel in order to produce the most structurally suitable gel for use as a wound dressing.

Figures 3.7a, 3.7b and 3.7c depict increasing hydrogen peroxide content leading to decreasing rupture force values for the gels over time. This degradation of the gels is much less pronounced at lower concentrations of hydrogen peroxide over the 14 day

storage period and can be minimised by increasing the calcium content of the gels. It may be possible to produce structurally stable gels using lower concentrations of hydrogen peroxide. It is highly likely that these lower concentrations of hydrogen peroxide could still aid in wound healing.

Inspection of Figures 3.7a, 3.7b and 3.7c shows that the presence of hyaluronic acid may slow the rate of gel degradation. It is evident that the rupture force of gels without hydrogen peroxide is lower when the gel contains hyaluronic acid. This may arise from the hyaluronic acid acting in a similar manner to the hydrogen peroxide and initialising depolymerisation of the alginate chains within the gel, resulting in weaker gels. However, in gels also containing hydrogen peroxide, the oxygen scavenging capabilities of hyaluronic acid can be observed. This slows the degradation of the gels containing hydrogen peroxide. By comparing Figure 3.7a with Figure 3.7b, it is observed that samples containing hyaluronic acid have a similar rupture force value after two and seven days, whereas samples not containing hyaluronic acid returned notably lower rupture force values after seven days than after two days aging. This indicates that the use of hyaluronic acid in the gels can help slow the effect of aging. However, inspection of Figure 3.7c shows that after 14 days of storage in a dark place at room temperature, gels have considerably lower rupture force values, whether they contain hyaluronic acid or not. This suggests that gels have a finite lifetime, after which they are unsuitable for use as wound dressings. But the use of hyaluronic acid in the gel may prolong this lifetime.

The data collected was used to produce a model for predicting the rupture characteristics of gels containing other concentrations of alginate, calcium carbonate, hydrogen peroxide and hyaluronic acid and for samples of varying age.

Table 3.2 shows the multiple linear regression models for the prediction of gelation time, homogeneity and rupture force of a gel. The model for homogeneity displays a reliable R^2 value of 0.64, while the gelation time model has an R^2 value of 0.37. The model for the prediction of rupture force shows a reliable R^2 value in excess of 0.57. Using these models, the estimation of gel characteristics within the experimental range is possible.

The rupture force in Newtons (N) of a gel can be calculated using the following equation derived from the statistical analysis in Table 3.2:

$$Rf = 0.515 + 1.509_C - 0.243_H - 0.250_A$$

Where *Rf* is the Rupture Force in Newtons (N), C is Calcium Carbonate (% w/v), H is the percentage hydrogen peroxide (% v/v) and A is the sample age (days). The rupture force of a one day old gel containing 0.75 % w/v $CaCO_3$ and 1.25 % v/v H_2O_2 can be estimated as follows:

$$\boldsymbol{Rf} = 0.515 + (1.509 \text{ x } 0.75) + (-0.243 \text{ x } 1.25) + (-0.250 \text{ x } 1.0)$$
$$= 1.09 \text{ N}$$

Gelation time and homogeneity of gels with various compositions within the experimental range can be estimated in a similar fashion using the models given in Table 3.2. This will provide important information on the structural attributes of gels which can be used in future studies.

3.4: Conclusion

Important variables of alginate gel formation were tested to assess their effects on gel homogeneity, gelation time and rupture characteristics. Increased calcium carbonate concentration led to increased rupture values of the gels, while it decreased gelation time and gel homogeneity. Increased hydrogen peroxide content also decreased gelation time and gel homogeneity, as well as the rupture values of the gels. Alginate content did not have a significant effect on the rupture values of the gel. Gels containing hyaluronic acid had lower mean rupture values than those formulated in the absence of hyaluronic acid ($p<0.05$). The rupture characteristics of gels not containing hydrogen peroxide remained unchanged over the two week storage period, while gels containing hydrogen peroxide displayed decreasing rupture values with increasing hydrogen peroxide concentration and storage time. However, gels containing hyaluronic acid displayed a marked decrease in

gel aging over a seven day period. The multiple linear regression models produced in this study allow for the estimation of rupture characteristics of gels with varying compositions. If the dressing is required to be intact after removal from the wound, a gel with a greater resistance to rupture is required. Knowing a gel's rupture characteristics will be useful in assessing its suitability as an effective wound dressing.

Chapter 4:

Oxygen Release of Alginate Gels Containing Hydrogen Peroxide and Hyaluronic Acid

4.1: Introduction

It is essential that oxygen is delivered to the wound site in order to ensure wound healing (Bishop 2008). However, wounded tissue will inevitably suffer from hypoxia. Impaired healing can result where normal oxygen levels and circulation are disrupted due to physiological disorders such as diabetes. Cell metabolism and oxygen consumption is increased following tissue injury, causing disruption to local blood vessels. This produces a hypoxic wound (Tandara *et al.* 2004; Whitney *et al.* 1989; Greif *et al.* 2000). While this hypoxic state initiates wound repair by creating an oxygen gradient between the hypoxic tissue at the wound site and perfused uninjured tissue nearby, in chronic wounds local tissue hypoxia can promote their characteristic pro-inflammatory state (LaVan and Hunt 1990). Therefore, it may be possible to promote the healing of chronic wounds by delivering oxygen to the site of wound.

In vivo trials are outside the scope of this study. For this reason, it was necessary to devise an artificial wound. As the goal of this experiment was to measure the rate of oxygen release from alginate gels containing hydrogen peroxide, it was essential that the artificial wound contained the enzyme catalase that is present at the site of any wound. Ground beef fitted the required profile of an artificial wound in that it contained catalase, which would then be able to react with hydrogen peroxide contained in the gels to produce oxygen.

In almost all living organisms exposed to oxygen, catalase is present to convert hydrogen peroxide to water and oxygen (Chelikani *et al.* 2004). Catalase has four subunits (tetrametic), thus it is referred to as a tetramer. Each of its four polypeptide chains contain over 500 amino acids. The enzyme's reaction with hydrogen peroxide is facilitated through its four iron (prophyrin heme) groups (Maehly and Chance 1954). The reaction below illustrates the decomposition of hydrogen peroxide by catalase to produce water and oxygen.

$$2\,H_2O_{2\,(l)} \rightarrow 2\,H_2O_{(l)} + O_{2\,(g)}$$

When alginate gels containing hydrogen peroxide are placed in contact with wounded tissue, catalase within the tissue reacts with hydrogen peroxide to release oxygen. This oxygen may then aid in the healing process. However, excessive production of oxygen may be detrimental to wound healing due to the production of ROS. Hyaluronic acid may act as an oxygen scavenger, thus eliminating some of the harmful effects caused by excessive oxygen in the wound environment, such as ROS.

The apparatus for measurement of oxygen release from alginate gels containing hydrogen peroxide in contact with an artificial wound is shown in Figure 4.1.

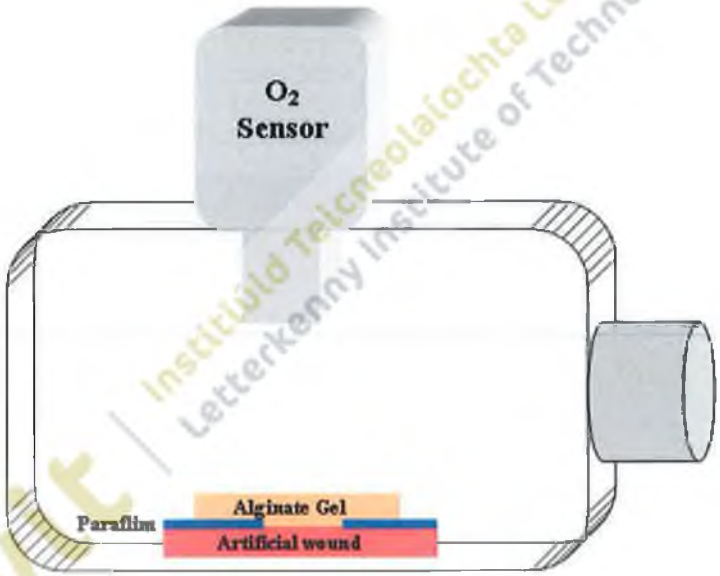

Figure 4.1: Apparatus for measurement of oxygen release from alginate gels containing hydrogen peroxide in contact with an artificial wound. The experiment took place inside a sealed 250 ml Vernier Biochamber. A layer of parafilm with a 1 cm^2 section removed from its center was placed between the artificial wound and alginate gel to ensure a constant area of contact.

4.2: Results

The rate of oxygen release was calculated by taking the total oxygen present in the chamber after ten minutes and subtracting the initial amount of oxygen present in the Biochamber. This number was then divided by ten to give the rate of oxygen release in ml^3 / min. This was carried out for ten minute intervals over the course of an hour.

Figures 4.2, 4.3 and 4.4 display the rate of oxygen release per minute for alginate gels containing varying concentrations of hydrogen peroxide and hyaluronic acid when in contact with an artificial wound. The results are the mean of three separate one hour experiments. The error bars represent the standard error of the mean (SEM) for the samples. At the start of each set of analysis, the artificial wound (ground beef) was placed in the Biochamber and the oxygen levels were recorded over a one hour period. The control samples show the oxygen levels in the Biochamber containing only an alginate gel formulated with 2 % hydrogen peroxide. For the test samples, the artificial wound was placed inside the Biochamber and the test alginate gel was then placed on top of the artificial wound. The Biochamber was then sealed and the oxygen levels were recorded for one hour.

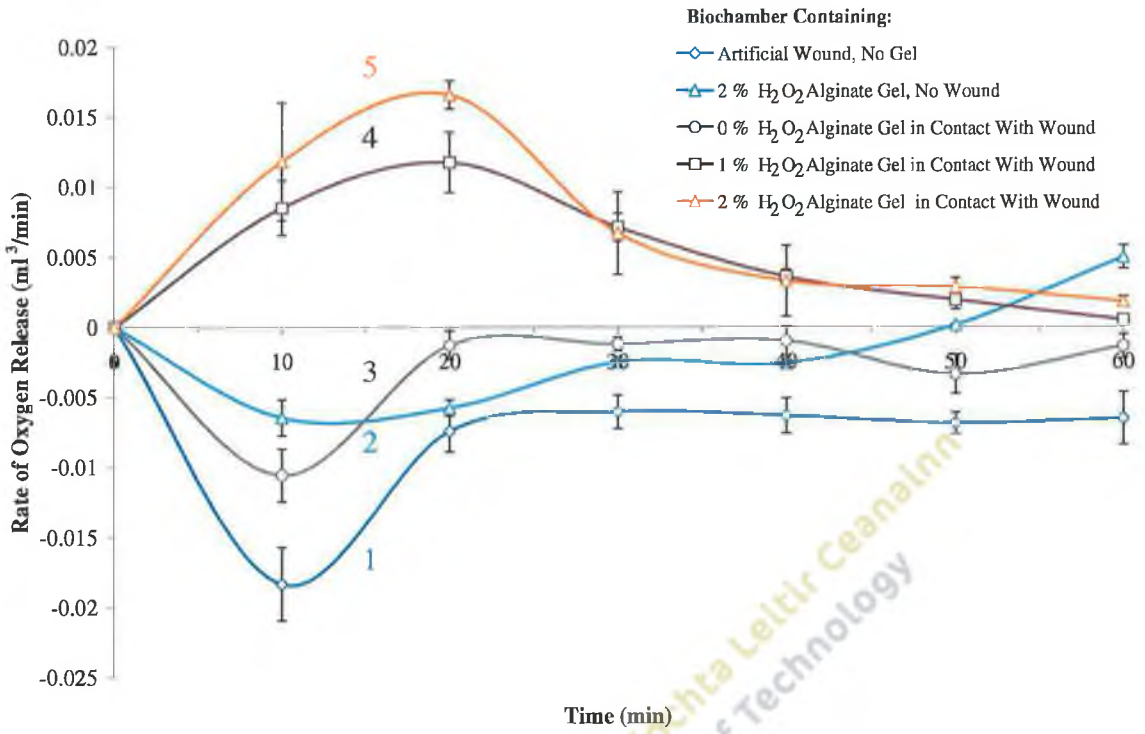

Figure 4.2: Rate of oxygen release of alginate gels in the absence of hyaluronic acid containing varying concentrations of hydrogen peroxide.

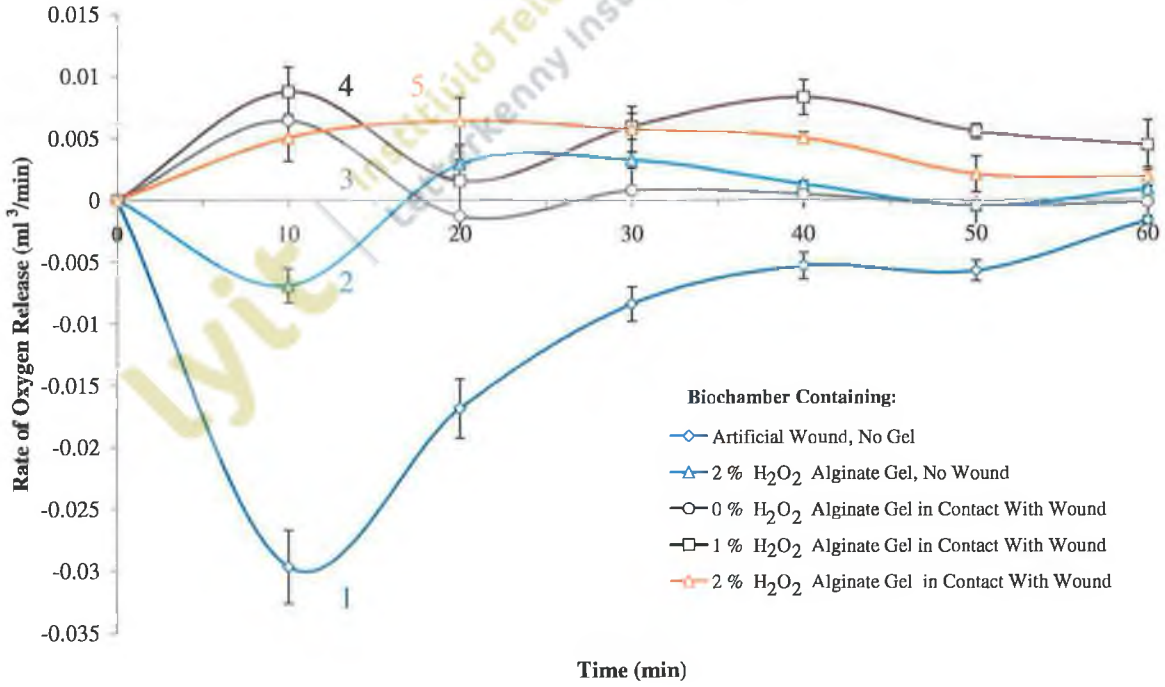

Figure 4.3: Rate of oxygen release of alginate gels containing varying concentrations of hydrogen peroxide and 1.25 mg/L hyaluronic acid.

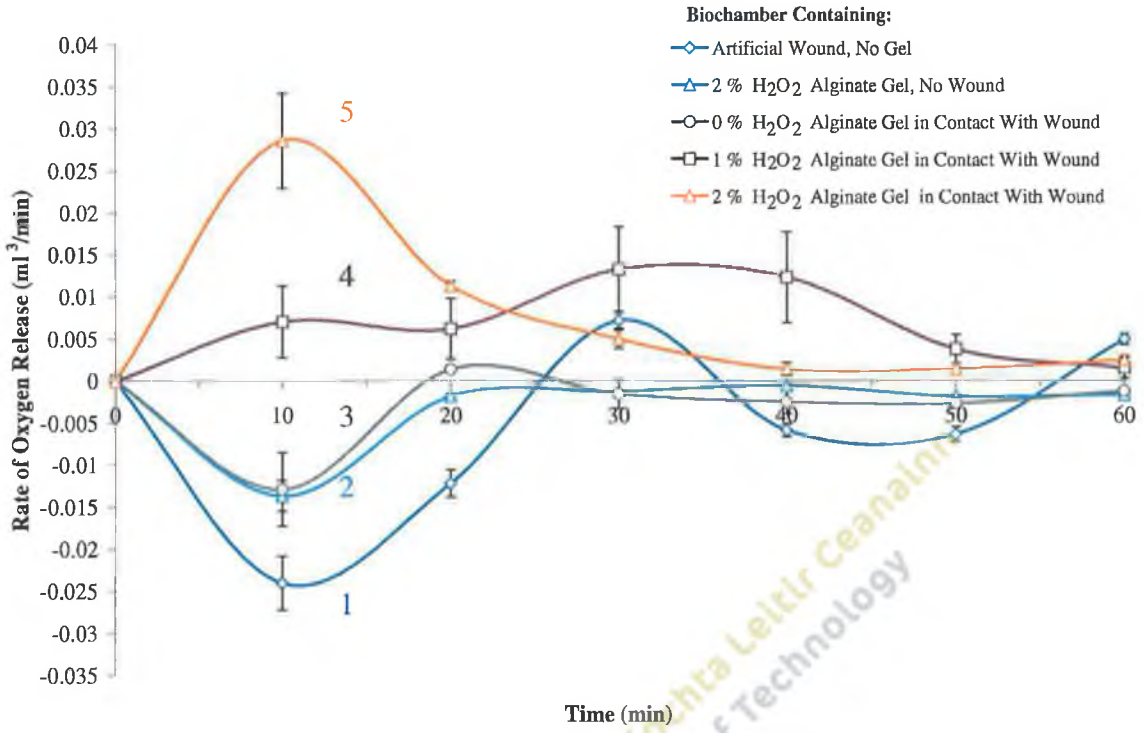

Figure 4.4: Rate of oxygen release of alginate gels containing varying concentrations of hydrogen peroxide and 2.5 mg/L hyaluronic acid.

Line 1, in Figures 4.2, 4.3 and 4.4, represents a control and suggests that oxygen is being absorbed when only the artificial wound is present in the Biochamber. Oxygen is also absorbed when only an alginate gel is placed in the Biochamber (line 2 in Figures 4.2, 4.3 and 4.4). This indicates that the presence of the gel in the Biochamber is producing this absorption.

Lines 4 and 5 in Figures 4.2, 4.3 and 4.4, where gels containing 1 and 2 % hydrogen peroxide were placed in contact with the artificial wound, display a dramatic oxygen release, particularly during the first 20 minutes. This indicates that the presence of hydrogen peroxide in the gel which is in contact with the wound must engage in a process that releases oxygen.

The overall results from Figures 4.2, 4.3 and 4.4 indicate that gels without hydrogen peroxide (line 3 in each Figure) do not display a marked release of oxygen. This would

suggest that the reduced level of oxygen released is as a result of the absence of hydrogen peroxide.

Statistical analysis confirms that for the first time period of 0 -10 min, there is a possible significant difference in the rate of oxygen release for gels containing differing amounts of hydrogen peroxide ($p < 0.1$). The rate of oxygen release of these gels is significantly different for the other five time periods ($p < 0.05$) (Table 4.1).

Hyaluronic acid content appears to have no effect on the rate of oxygen release for the concentrations tested. This is confirmed from the statistics displayed in Table 4.1. There is no significant difference in the rate of oxygen release for alginate gels containing varying concentrations of hyaluronic acid ($p < 0.05$ for all six time periods). While a lower rate of oxygen release may be expected from gels containing hyaluronic acid due to its oxygen scavenging properties, this is not the case. Samples containing 2 % hydrogen peroxide and the highest concentration of hyaluronic acid (2.5 mg/L) returned the highest rate of oxygen release for the first ten minutes after contact with the artificial wound (Figure 4.4).

Table 4.1: ANOVA test for significant difference between groups for varying compositions of alginate gels for potential use as wound dressings. Gels formulated using different hydrogen peroxide and hyaluronic acid concentrations were tested for statistical difference in their mean values of rate of oxygen release when in contact with an artificial wound.

Rate of Oxygen Release	ANOVA p-value between groups	
	Hydrogen Peroxide	Hyaluronic Acid
0 – 10 min	0.082	0.785
10 – 20 min	0.039	0.882
20 – 30 min	0.008	0.696
30 – 40 min	0.023	0.896
40 – 50 min	0.006	0.806
50 – 60 min	0.039	0.876

4.3: Discussion

It is clear from the analysis that neither the artificial wound nor the alginate gels released oxygen when placed in the Biochamber separately. In all cases, the rate of oxygen release returned negative values for the first ten minutes. This was due to oxygen absorption by the artificial wound and by the gel. The fact that alginate gels containing hydrogen peroxide do not release oxygen when not in contact with the artificial wound is favourable for its application as a wound dressing for the delivery of oxygen to hypoxic tissue. The gels are able to retain oxygen in the form of hydrogen peroxide until the gel is brought into contact with the wound. It is also clear that hydrogen peroxide is acting as the source of oxygen being released from the system. Gels without hydrogen peroxide do not release a marked amount of oxygen compared to those containing hydrogen peroxide (Figure 4.2, 4.3 and 4.4).

The amount of hydrogen peroxide in the gel was also shown to have a significant effect on the rate of oxygen release. There was a possibly significant difference in the rate of oxygen release for gels of differing hydrogen peroxide concentrations in the first time period ($p < 0.1$) and a significant difference in the rate of oxygen release for the remaining five time periods ($p < 0.05$) (Table 4.1). This is important for the application of the alginate gel as a wound dressing because if the source of oxygen is known, then its by-products are also known. In this case, the oxygen is released from the reaction between hydrogen peroxide and catalase. Water and oxygen are the only products of the reaction. Further confirmation of this is shown in gels containing 1 % and 2 % hydrogen peroxide. When these gels were placed in contact with the artificial wound (source of catalase), oxygen was released during all time periods for all three sets of experiments (Figure 4.2, 4.3 and 4.4). The greatest oxygen release was produced from 2 % hydrogen peroxide and 2.5 mg/L hyaluronic acid alginate gels. However, there does not appear to be any definite trend in relation to hyaluronic acid and hydrogen peroxide concentrations and oxygen release, other than the fact that oxygen is released when hydrogen peroxide is present in the gel. This is not the case for gels without hydrogen peroxide. In two of the three hyaluronic acid concentrations, the 0 – 10 min time period saw the greatest oxygen release from gels containing hydrogen peroxide (Figure 4.3 and 4.4). Alginate gels not

containing hydrogen peroxide showed the greatest oxygen release between 10 and 20 minutes (Figure 4.2). Thereafter, the rate of oxygen release from gels containing hydrogen peroxide generally decreased. This is in line with expectations drawn from enzyme kinetics (Hames and Hooper 2000).

Gels containing 1.25 mg/L hyaluronic acid displayed more stable oxygen release than the gels formulated from other concentrations of hyaluronic acid. It can be concluded from these experiments that hyaluronic acid has no definite effect on the rate of oxygen release. This is further supported by the results of the statistical analysis (Table 4.1). There was no significant difference in the rate of oxygen release for gels containing varying amounts of hyaluronic acid. This was the case for all six time periods of the experiment ($p > 0.05$) (Table 4.1). A possible conclusion is that the concentration range tested (0.0 – 2.5 mg/L) was too low to detect any of the oxygen scavenging expected. The concentrations selected were chosen as they encompass the concentration at which hyaluronic acid is found in human tissue (1.25 mg/L). It appears from these results that hyaluronic acid is not having any effect on the oxygen release of the alginate gels containing hydrogen peroxide. Further studies, outside the scope of this project, would need to be developed to further investigate oxygen release from alginate gels containing hydrogen peroxide and hyaluronic acid. However, it is conclusive that the incorporation of hydrogen peroxide into alginate gels results in the release of oxygen when in contact with the enzyme catalase.

4.4: Conclusion

It can be concluded from the results that the incorporation of hydrogen peroxide into alginate gels results in the release of oxygen when in contact with catalase. This would indicate that these gels serve as an attractive vehicle for the delivery of oxygen to wounds, thus aiding the healing process. It is also clear that the oxygen release is the result of the reaction between hydrogen peroxide and catalase. Therefore, the only by-product other than oxygen is water. This means the gels are non-toxic to the wound and

the oxygen released from these gels may be beneficial in overcoming wound hypoxia and its associated problems.

Chapter 5:

The Effects of Alginate gels on Cell Migration and Proliferation

5.1: Cell Migration

5.1.1: Introduction

Cellular migration and proliferation are required to replace damaged cells at the site of a wound. The following experiments document preliminary studies to determine the potential of alginate dressings to facilitate wound healing in *in vitro* cellular models of wounds. The effects of alginate gels containing hydrogen peroxide on cell migration and proliferation were assessed by the production of a controlled wound in a monolayer of synovial fibroblasts. Injury was introduced to the cells by scratching the monolayer culture using a sterile p200 pipette tip. These monolayer cultures (with scratch wounds) were then grown for 48 hours in the presence of alginate gels of varying composition. The alginates gels' effect on cell migration, a key process in wound healing, during this time was assessed photographically and the scratch wound area was measured using Image J software according to the *in vitro* scratch assay protocol (Liang *et al.* 2007).

5.1.2: Results

Results obtained from the scratch wound assays appear to indicate that wounds cultured without alginate gels recovered better than those cultured in the presence of alginate gels as indicated by cell migration. Cells grown without gels exhibited complete recovery after 48 hr (Figure 5.1.4). This can also be observed in Figure 5.1.1. Photographic analysis demonstrated cells migrating across the original scratch wounds 5, 24 and 48 hr after the induction of the scratch wound.

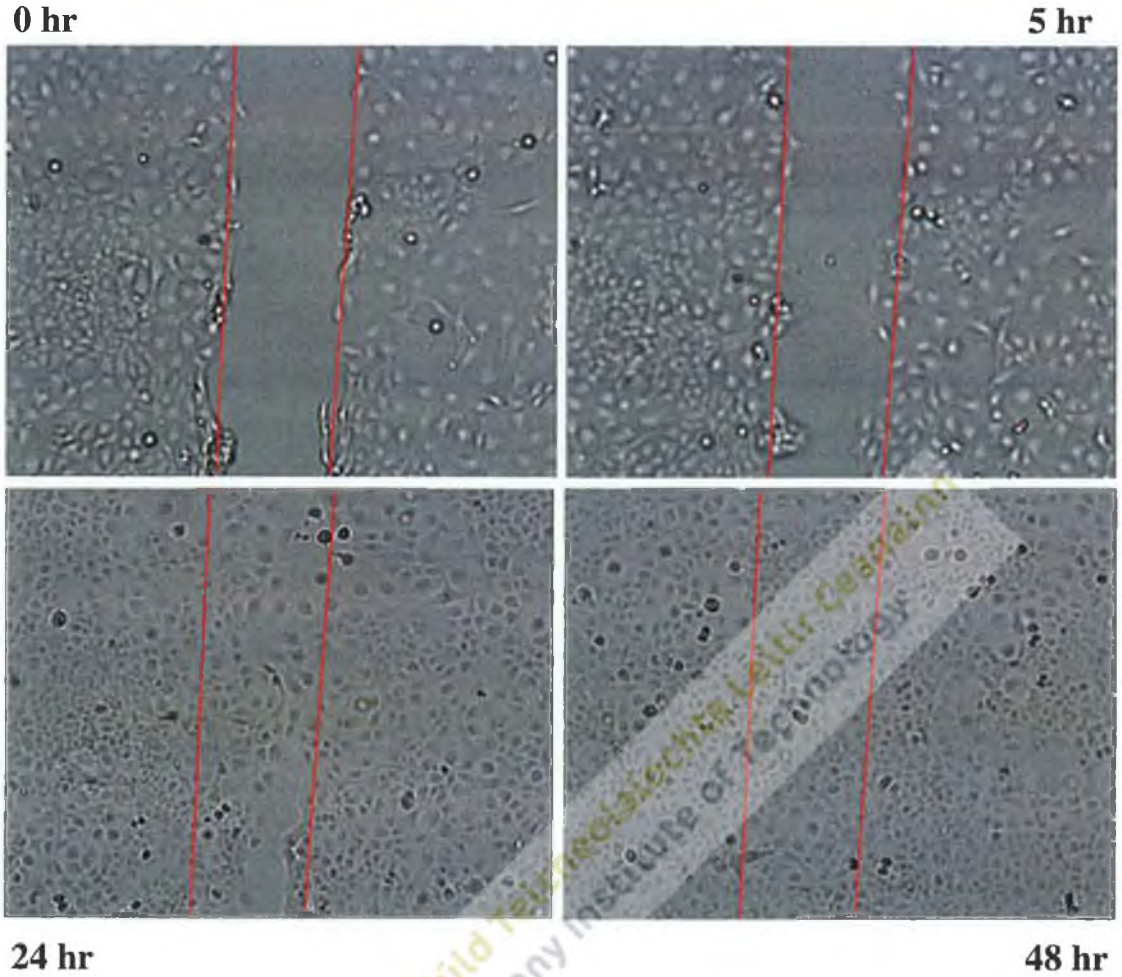

Figure 5.1.1: Analysis of K4IM synovial fibroblast cell migration in the *in vitro* scratch wound assay. Original scratch wound area is marked with red lines and cells were photographed at 100 X magnification using an Olympus IX51 inverted microscope and an Olympus DP70 camera at 0, 5, 24 and 48 hr after induction of the scratch wound.

Cells cultured in the presence of alginate gels without hydrogen peroxide exhibited some recovery, displaying 22.7 % recovery after 48 hr (Figure 5.1.4). Figure 5.1.2 displays one replicate of cells cultured with alginate gels not containing hydrogen peroxide.

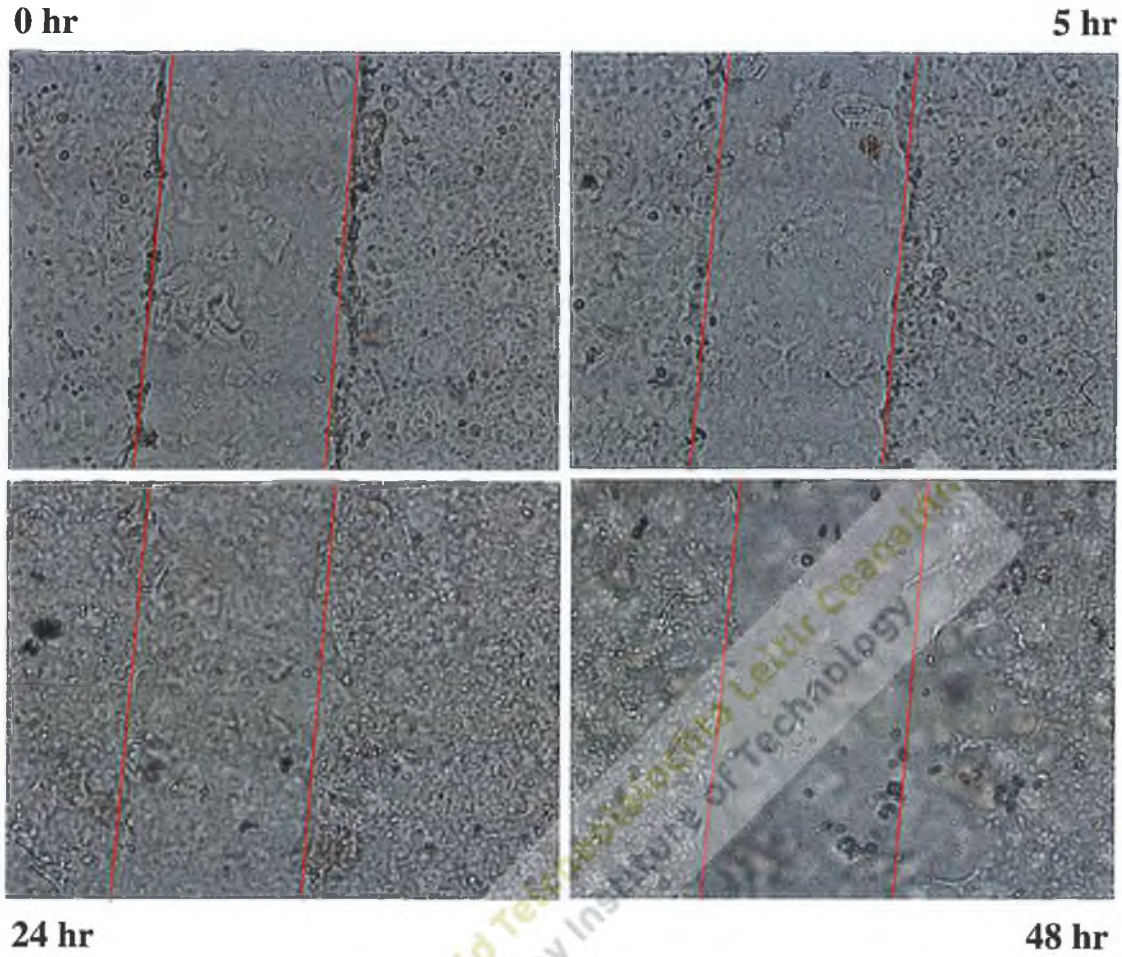

Figure 5.1.2: Analysis of K4IM synovial fibroblast cell migration during incubation with an alginate gel containing 2 % (w/v) alginate, 0.25 % (w/v) calcium carbonate and without hydrogen peroxide in the *in vitro* scratch wound assay. Original scratch wound area is marked with red lines and cells were photographed at 100 X magnification using an Olympus IX51 inverted microscope and an Olympus DP70 camera at 0, 5, 24 and 48 hr after induction of the scratch wound.

In all cells cultured in the presence of alginate gels containing hydrogen peroxide, there was a marked increase in scratch wound area, returning negative wound recovery values (Figure 5.1.4). Figure 5.1.3 illustrates this photographically. It is observed that the original area of the scratch expands after 24 and 48 hr indicating that the presence of the alginate gel is inhibiting cell migration.

Figure 5.1.3: Analysis of K4IM synovial fibroblast cell migration during incubation with an alginate gel containing 2 % (w/v) alginate, 0.125 % (v/v) hydrogen peroxide and 0.25 % (w/v) calcium carbonate in the *in vitro* scratch wound assay. Original scratch wound area is marked with red lines and cells were photographed at 100X magnification using an Olympus IX51 inverted microscope and an Olympus DP70 camera at 0, 5, 24 and 48 hr after induction of the scratch wound.

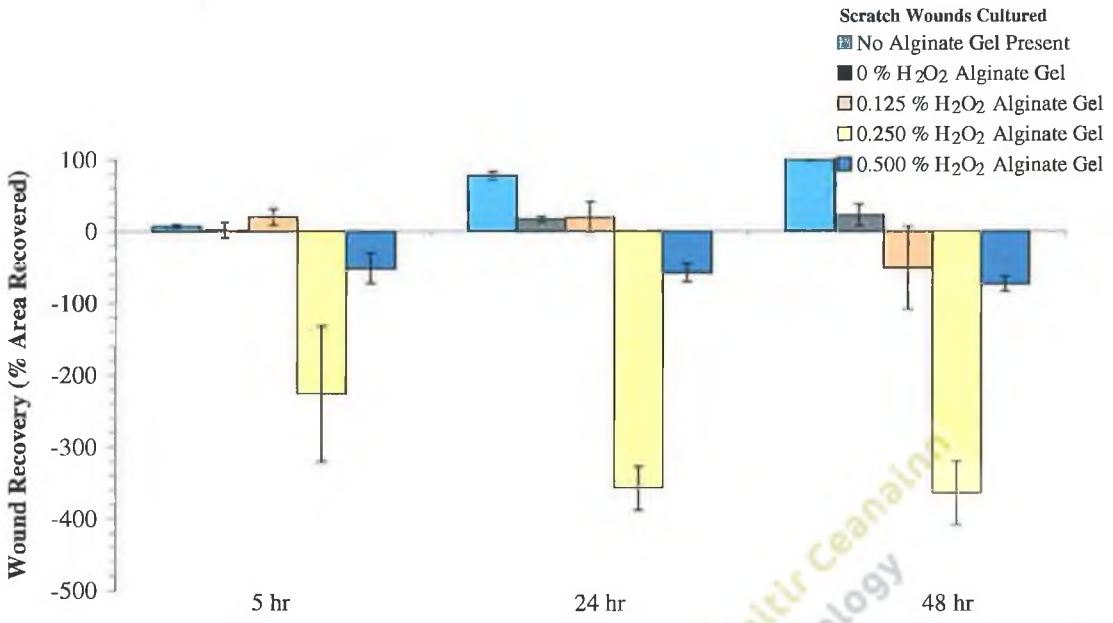

Figure 5.1.4: Analysis of the effects of alginate gels on synovial fibroblast cell migration by *in* vitro scratch wound assay. Scratch wound area was assessed at 0, 5, 24 and 48 hr after induction of the scratch wound. Wound recovery was calculated as a percentage of the original wound area populated with migrating cells measured after 5, 24 and 48 hr. A completely recovered wound displayed a wound recovery value of 100%. In cases were the wound area expanded due to clearing of cells caused by possible cell death, negative wound recovery values were observed.

5.1.3: Discussion

Cell migration is a key process in wound healing. The *in vitro* scratch wound assay was used to assess cell migration. When a confluent layer of cells is "scratched" to create an artificial gap in the layer of cells, the cells along the edge of the gap or "scratch" migrate towards each other to close the gap (Liang *et al.* 2007). The goal of this experiment was to assess the effects of alginate gels with and without hydrogen peroxide on the rate of cell migration, thereby potentially indicating the effects of these gels on wound healing. The scratch wound was assessed at 0, 5, 24 and 48 hr after introduction of the scratch. Results were expressed as the percentage of original wound area recovered (i.e. covered with migrating cells). This was recorded photographically and Image J Image analysis software was employed to measure the area of the wound before and after incubation with the alginate gels.

After 48 hr, full recovery of the scratch wound was observed in cells that were cultured without alginate gels. Cells that were cultured in the presence of alginate gels displayed lower rates of scratch wound recovery after 48hr. Scratch wounds that were cultured in the presence of alginate gels without hydrogen peroxide displayed 22.7 % recovery. However, when this was compared to the control samples, it was clear that the alginate gels were having a detrimental effect on cell migration. This can be observed in Figure 5.1.1 and 5.1.4. Complete scratch wound closure is evident in cells cultured without alginate gels after 48 hr (Figure 5.1.1 and Figure 5.1.4). Cell migration progressed more slowly in cells that were cultured in the presence of alginate gels without hydrogen peroxide when compared to cell culture without alginate gels (Figure 5.1.3 and Figure 5.1.4).

Inspection of cell recovery values for cells incubated in the presence of gels containing 0.125, 0.25 and 0.5 % hydrogen peroxide illustrated that the presence of hydrogen peroxide led to expansion of the wound area (Figure 5.1.4). Detached spherical cells suspended in the growth medium were observed and recorded photographically in these samples during the analysis (Figure 5.1.3). The death of cells in a monolayer culture causes them to detach from the surface and become suspended in the growth medium. This suggests that expansion of the wound area was caused by cell death. Cells that were cultured in the presence of alginate gels containing 0.25 % hydrogen peroxide displayed the highest rates of cell death, with the scratch wound expanding to over 400 % of its original area after 48 hr (Figure 5.1.4). If hydrogen peroxide in the gels causes cell death, it would be expected that the highest rate of cell death would result in samples cultured with alginate gels containing the highest concentration of hydrogen peroxide. Indeed, inspection of the photographic analysis in this experiment suggests that this is the case, as the greatest degree of cell clearing is observed in cells cultured with alginate gels containing 0.5 % hydrogen peroxide. The fact that alginate gels containing 0.5 % hydrogen peroxide appeared to have higher recovery rates compared to gels containing 0.25 % hydrogen peroxide may be due to the immediate effect, possible cell death, which the higher hydrogen peroxide concentration had on the scratch wound.

Figure 5.1.4 displays the scratch wound recovery rates of cells grown with an alginate gel containing 0.5 % hydrogen peroxide. At 0 hr, the scratch wound appears larger than the scratch wound in other samples. There is less definition in the edges of the scratch wound and detached, suspended (dead) cells are also present. This suggests that gels containing higher concentrations of hydrogen peroxide, such as 0.5 %, are causing cell death and expansion of the scratch wound immediately after contact with cells. In these samples, the scratch wound has already expanded after 0 hr. This means that subsequent measurement of the wound area after 5, 24 and 48 hr returns higher % recovery values than those obtained for gels containing less or no hydrogen peroxide. This may explain why the samples cultured in the presence of gels containing 0.25 % hydrogen peroxide displayed the highest rates of cell death after 48 hr rather than those grown in the presence of gels containing 0.5 % hydrogen peroxide (Figure 5.1.4).

5.2: Cell Proliferation

5.2.1: Introduction

In order to examine the effect of alginate gels containing hydrogen peroxide on cell proliferation, the 3-(4,5-dimethylthiazol-2-yl) -5- (3-carboxymethoxyphenyl) -2- (4-sulphphenyl) -2H-tetrazolium (MTS) cell proliferation assay was utilized. MTS is a tetrazolium compound. When MTS is in the presence of the cellular metabolite phenazine methosulphate (PMS), it is reduced by living cells to produce a formazan product. This formazan product can then be colorimetrically assayed. The bioreduced product is stable in culture medium (Buttke *et al.* 1993). The quantity of formazan product, which is directly proportional to the number of viable cells in the culture, was measured by absorbance at 490 nm.

5.2.2: Results

Cells were grown in 12 well plates to 80 % confluence. A scratch wound was introduced to the cell monolayer and the cells were incubated in the presence or absence of alginate gels containing varying concentrations of hydrogen peroxide. Cellular proliferation was

assessed at 5, 24 and 48 hr. The results obtained indicate that the presence of alginate gels has an inhibitory effect on cellular proliferation during wound recovery. Cells that were scratched and allowed to recover without an alginate gel displayed the greatest cell proliferation throughout the experiment. Cells that were not scratched and simply continually cultured without an alginate gel also displayed high rates of proliferation at 48 hr. Cells that were induced with a scratch and cultured in the presence of alginate gels without hydrogen peroxide displayed lower levels of proliferation after 5 and 24 hr than cells cultured in the absence of alginate gels. However, these low levels of proliferation ceased after 48 hr. All scratch wounds that were cultured in the presence of alginate gels with and without hydrogen peroxide displayed less proliferation than the controls throughout the experiment, suggesting that the presence of alginate gels has an inhibitory effect on cellular proliferation in scratch wound recovery (Figure 5.2). However, this may be as a result of the gels preventing cellular gaseous exchange for example, rather than the alginate gels themselves inhibiting cellular proliferation.

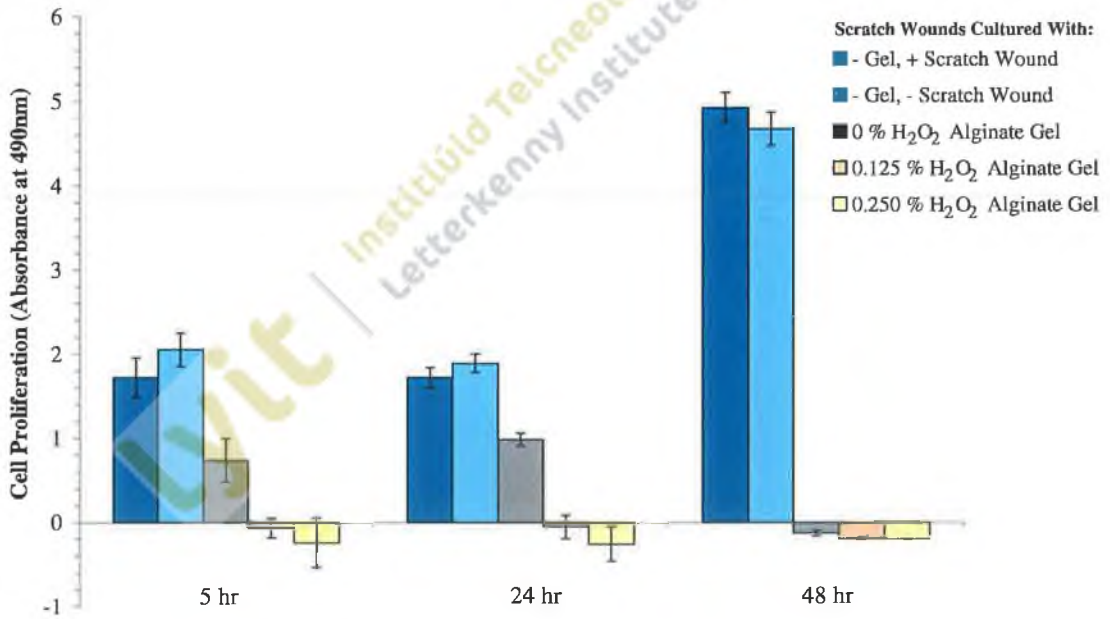

Figure 5.2: Cellular proliferation was assessed at 5, 24 and 48 hr. Cells were cultured in 12 well plates in the presence or absence of alginate gels containing varying concentrations of hydrogen peroxide. Cell proliferation is directly proportional to the sample absorbance at 490 nm. Proliferating cells display elevated absorbance readings. A negative absorbance reading indicates that those cells were not proliferating. The results indicate that the presence of alginate gels has an inhibitory effect on cellular proliferation in scratch wound recovery. The error bars represent the SEM of 3 independent experiments.

5.2.3: Discussion

The results of the proliferation assay suggest that the presence of alginate gels inhibits cellular proliferation during scratch wound recovery. Cells that were cultured without alginate gels after inducing the scratch wound displayed the greatest proliferation values after 48 hr, with similar values obtained for cells which were not scratched and cultured without alginate gels. As in the cell migration assay, cells grown in the presence of alginate gels without hydrogen peroxide returned the highest cellular proliferation rates of cells incubated with alginate gels. These cultures demonstrated proliferation up to 24 hr into the experiment. Cells grown in the presence of with gels containing 0.125, 0.25 and 0.5 % hydrogen peroxide did not display proliferation throughout the experiment, returning negative absorbance readings for all three time periods (Figure 5.2).

It is clear from the results of both the cell migration and proliferation assays that cells cultured without alginate gels were much healthier than those cultured in the presence of alginate gels. This would appear to indicate that these gels would have a detrimental effect on cells at the site of a wound if they were applied as a wound dressing. However, cells that were cultured in the presence of alginate gels without hydrogen peroxide returned elevated rates of migration and proliferation compared to those that were cultured with gels containing hydrogen peroxide, although both resulted in less proliferation than the cells grown without any alginate gels. This would indicate that in addition to the presence of the alginate gel, the presence of hydrogen peroxide is also having a detrimental effect. However, the gels containing hydrogen peroxide have already been shown in Chapter 4 to release oxygen when in contact with catalase, which is known to benefit in the healing of chronic wounds (Bishop 2008). It is possible that the delicate nature of cells grown in monolayer rendered them unable to withstand the weight of the gels. This would not be a problem in the *in vivo* environment. Although gels were produced to be as thin as practically possible (1.5 mm) they may still have altered cell morphology and function. The gels may also have blocked gaseous exchange in this *in vitro* environment which would not be a problem in *in vivo* conditions as surrounding tissue and blood would be able to provide cells with a means of gaseous

exchange. The cell monolayer may not promote oxygen release from the gels containing hydrogen peroxide at the same level as in an *in vivo* environment or in the artificial wound system used in Chapter 4 of this study. This would mean that oxygen may not have been released from gels containing hydrogen peroxide as it was in Chapter 4. Also, the simple nature of a single cell type grown in monolayer only allows for the estimation of the effects of the gel on that cell type and does not facilitate analysis of complicated cell-cell and cell-matrix interactions that take place at the site of the wound. For a fuller understanding of the effects of the alginate gels at cellular level, it wound be necessary to carry out trials using gels containing hydrogen peroxide as wound dressings in animal models. Doing so would give a clearer indication of the value of a hydrogen peroxide containing alginate gel in clinical practice.

5.3: Conclusion

Alginate gels containing varying compositions of hydrogen peroxide had a detrimental effect on the cell migration and proliferation of synovial fibroblasts. Migration and proliferation are important phases in wound healing, so this would appear to suggest that the alginate gels are not beneficial in the wound healing. However, it is possible that these results are due to the nature of the *in vitro* system employed in these experiments for the assessment of the gels. Theories in the literature and results from Chapter 4 suggest that these gels may play a beneficial role in wound healing. Therefore, a more complete understanding of the value of alginate gels containing hydrogen peroxide in the promotion of wound healing should be elucidated in further *in vitro* and *in* vivo experiments.

Chapter 6:

Summary

6.0: Summary

The care of chronic wounds requires heavy financial input from the healthcare industry, with billons being spent annually on their treatment. The decreased quality of life for patients suffering from chronic wounds is also a major concern for the healthcare sector. This has led to a requirement for inexpensive wound dressings that promote wound healing. In this study, alginate gels for use as wound dressings which incorporated varying concentrations of alginate, calcium carbonate, hydrogen peroxide and hyaluronic acid were assessed over time with regards to their gelation time, homogeneity and rupture force. Investigating the gelation time and homogeneity of these alginate gels allowed for the assessment of their suitability for use as a wound dressing.

Increased calcium carbonate concentration led to increased rupture values for the gels, while it decreased gelation time and gel homogeneity. Increased hydrogen peroxide content also decreased gelation time and gel homogeneity, as well as the rupture values of the gels. Alginate content did not have a significant effect on the rupture values of the gels. Gels containing hyaluronic acid had significantly lower mean rupture values than those formulated in the absence of hyaluronic acid ($p<0.05$). The rupture characteristics of gels not containing hydrogen peroxide remained unchanged over the two week storage period, while gels containing hydrogen peroxide displayed decreasing rupture values with increasing hydrogen peroxide concentration and storage time. This indicated that the presence of hydrogen peroxide accelerated decomposition of the gels. However, gels containing hyaluronic acid displayed a marked decrease in gel aging over a seven day period.

The multiple linear regression models produced in this study allow for the estimation of rupture characteristics of gels with varying compositions. If the dressing is required to be intact after removal from the wound, a gel with a greater resistance to rupture is required. Knowing the rupture characteristics of a gel would be useful in assessing its suitability as an effective wound dressing.

The incorporation of hydrogen peroxide into alginate gels resulted in the release of oxygen when in contact with the enzyme catalase. This indicated that these gels may serve as an attractive vehicle for the delivery of oxygen to wounds, thus aiding the healing process.

Alginate gels containing varying compositions of hydrogen peroxide had a detrimental effect on the cell migration and proliferation of synovial fibroblasts. Migration and proliferation are important phases in wound healing, so this would appear to suggest that the alginate gels were not beneficial in the wound healing. However, it is more likely that these results were due to the nature of this *in vitro* system for the assessment of the gels. Theories in the literature and results from Chapter 4 suggest that these gels may play a beneficial role in wound healing. Therefore, *in vivo* experiments may offer a more complete understanding of the value of alginate gels containing hydrogen peroxide as wound dressings.

Chapter 7:

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Appendices

Appendix 1.1:

ANOVA test for significant differences between alginate concentrations for rupture force, gelation time and homogeneity values of alginate gels for potential use as wound dressings.

ANOVA

		Sum of Squares	Df	Mean Square	F	Sig.
RuputreForce	Between Groups	1.281	2	.641	.983	.375
	Within Groups	232.707	357	.652		
	Total	233.989	359			
GelationTime	Between Groups	4002.736	2	2001.368	.704	.502
	Within Groups	93766.089	33	2841.397		
	Total	97768.825	35			
Homogeneity	Between Groups	.000	2	.000	1.845	.174
	Within Groups	.001	33	.000		
	Total	.002	35			

Appendix 1.2:

ANOVA test for significant differences between calcium carbonate concentrations for rupture force, gelation time and homogeneity values of alginate gels for potential use as wound dressings.

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
RuputreForce	Between Groups	95.240	3	31.747	81.456	.000
	Within Groups	138.748	356	.390		
	Total	233.989	359			
GelationTime	Between Groups	36252.726	2	18126.363	9.724	.000
	Within Groups	61516.099	33	1864.124		
	Total	97768.825	35			
Homogeneity	Between Groups	.001	2	.000	20.288	.000
	Within Groups	.001	33	.000		
	Total	.002	35			

Appendix 1.3:

ANOVA test for significant differences between hydrogen peroxide concentrations for rupture force, gelation time and homogeneity values of alginate gels for potential use as wound dressings.

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
RuputreForce	Between Groups	50.067	4	12.517	24.159	.000
	Within Groups	183.922	355	.518		
	Total	233.989	359			
GelationTime	Between Groups	13776.252	3	4592.084	1.750	.177
	Within Groups	83992.573	32	2624.768		
	Total	97768.825	35			
Homogeneity	Between Groups	.000	3	.000	.339	.797
	Within Groups	.002	32	.000		
	Total	.002	35			

Appendix 1.4:

Linear regression model for the prediction of alginate gel rupture force by calcium carbonate and hydrogen peroxide concentration and sample age.

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.760(a)	.578	.574	.5268168

a Predictors: (Constant), Time, HydrogenPeroxide, CalciumCarbonate

ANOVA(b)

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	135.186	3	45.062	162.364	.000(a)
	Residual	98.803	356	.278		
	Total	233.989	359			

a Predictors: (Constant), Time, HydrogenPeroxide, CalciumCarbonate

b Dependent Variable: RuputreForce

Coefficients(a)

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta	B	Std. Error
1	(Constant)	.515	.071		7.239	.000
	CalciumCarbonate	1.509	.083	.627	18.215	.000
	HydrogenPeroxide	-.243	.021	-.400	-11.624	.000
	Time	-.025	.006	-.155	-4.492	.000

a Dependent Variable: RuputreForce

Appendix 1.5:

Linear regression model for the prediction of the gelation time of alginate gels by alginate, calcium carbonate and hydrogen peroxide concentration.

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.580(a)	.337	.297	44.32150

a Predictors: (Constant), HydrogenPeroxide, CalciumCarbonate

ANOVA(b)

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	32943.780	2	16471.890	8.385	.001(a)
	Residual	64825.045	33	1964.395		
	Total	97768.825	35			

a Predictors: (Constant), HydrogenPeroxide, CalciumCarbonate

b Dependent Variable: GelationTime

Coefficients(a)

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta	B	Std. Error
1	(Constant)	96.025	18.071		5.314	.000
	CalciumCarbonate	-82.897	23.691	-.496	-3.499	.001
	HydrogenPeroxide	-11.348	5.334	-.302	-2.128	.041

a Dependent Variable: GelationTime

Appendix 1.5:

Linear regression model for the prediction of alginate gel homogeneity by alginate, calcium carbonate and hydrogen peroxide concentration.

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.802[a]	.644	.611	.0042375

a. Predictors: (Constant), Alginate, HydrogenPeroxide, CalciumCarbonate

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	.001	3	.000	19.288	.000[a]
	Residual	.001	32	.000		
	Total	.002	35			

a. Predictors: (Constant), Alginate, HydrogenPeroxide, CalciumCarbonate

b. Dependent Variable: FinalHomo

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	.003	.002		1.107	.276
	CalciumCarbonate	.016	.002	.728	6.902	.000
	HydrogenPeroxide	.001	.001	.117	1.107	.277
	Alginate	-.001	.000	-.317	-3.001	.005

a. Dependent Variable: FinalHomo

Appendix 2.1:

ANOVA test for significant differences between hydrogen peroxide concentrations for rate of oxygen release from alginate gels for potential use as wound dressings.

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
0 - 10 min	Between Groups	.001	2	.000	3.896	.082
	Within Groups	.001	6	.000		
	Total	.001	8			
10 - 20 min	Between Groups	.000	2	.000	5.818	.039
	Within Groups	.000	6	.000		
	Total	.000	8			
20 - 30 min	Between Groups	.000	2	.000	11.709	.008
	Within Groups	.000	6	.000		
	Total	.000	8			
30 - 40 min	Between Groups	.000	2	.000	7.577	.023
	Within Groups	.000	6	.000		
	Total	.000	8			
40 - 50 min	Between Groups	.000	2	.000	13.951	.006
	Within Groups	.000	6	.000		
	Total	.000	8			
50 - 60 min	Between Groups	.000	2	.000	5.828	.039
	Within Groups	.000	6	.000		
	Total	.000	8			

Appendix 2.2:

ANOVA test for significant differences between hyaluronic acid concentrations for rate of oxygen release from alginate gels for potential use as wound dressings.

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
0 - 10 min	Between Groups	.000	1	.000	.080	.785
	Within Groups	.001	7	.000		
	Total	.001	8			
10 - 20 min	Between Groups	.000	1	.000	.024	.882
	Within Groups	.000	7	.000		
	Total	.000	8			
20 - 30 min	Between Groups	.000	1	.000	.166	.696
	Within Groups	.000	7	.000		
	Total	.000	8			
30 - 40 min	Between Groups	.000	1	.000	.018	.896
	Within Groups	.000	7	.000		
	Total	.000	8			
40 - 50 min	Between Groups	.000	1	.000	.065	.806
	Within Groups	.000	7	.000		
	Total	.000	8			
50 - 60 min	Between Groups	.000	1	.000	.030	.867
	Within Groups	.000	7	.000		
	Total	.000	8			

Appendix 4.1:

Copy of article accepted for publication in the Journal of Biomedical Materials Research: Part B - Applied Biomaterials. The paper, entitled "Gelation Time, Homogeneity and Rupture Testing of Alginate-Calcium Carbonate-Hydrogen Peroxide Gels for Use as Wound Dressings" is based on data obtained as part of this masters degree and is documented in Chapter 3 of this thesis.

Gelation time, homogeneity, and rupture testing of alginate-calcium carbonate-hydrogen peroxide gels for use as wound dressings

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Received 19 May 2011; accepted 3 August 2011
Published online 00 Month 2011 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jbm.b.31965

Abstract: The care of chronic wounds carries a heavy financial burden on the healthcare industry, with billions being spent annually on their treatment. This, coupled with a decreased quality of life for sufferers, has led to a real urgency in developing inexpensive wound dressings that promote wound healing. Alginate gels for application as wound dressings were formed by varying alginate (0%–6% w/v), calcium carbonate (0%–1% w/v), hydrogen peroxide (0%–3.75% v/v), and hyaluronic acid (0–1.25 mg/L) content. The aging effects on the physical properties of the gels over a 14-day period were also investigated. The results indicated that the concentration of calcium carbonate and hydrogen peroxide, as well as sample age, all had a significant effect on the rupture characteristics and gelation time of the gels. Increased calcium carbonate content caused an increase in rupture force and rupture energy values, whereas increased hydrogen peroxide content and sample age resulted in a decrease in rupture force and rupture energy measurements. Increased calcium carbonate and hydrogen peroxide content produced a decrease in the time required for gel formation. Statistical models were also produced to provide a means of estimating rupture characteristics and gelation times for gels containing other concentrations of these components. © 2011 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 000B:000–000, 2011.

Key Words: wound dressing, alginate gel, hydrogen peroxide, hyaluronic acid, rupture characteristics

How to cite this article: Alexander BR, Murphy KE, Gallagher J, Farrell GF, Taggart G. 2011. Gelation time, homogeneity, and rupture testing of alginate-calcium carbonate-hydrogen peroxide gels for use as wound dressings. J Biomed Mater Res Part B 2011:00B:000–000.

INTRODUCTION

The term "wound" usually indicates a tissue lesion. It can also be an area of skin that has been destroyed by external factors or by the presence of an underlying pathological disorder.[1] Wounds can exist in two forms—acute or chronic. Acute wounds heal without complications. However, the normal process of healing does not extend to chronic wounds. In chronic wounds, the healing process is delayed or prevented by a persistent proinflammatory state.[2]

This study documents the preliminary stages of developing a novel dressing for such wounds. This wound dressing is in the form of an alginate gel. Alginates are a family of linear copolymers that contain 1-4-linked β-D-mannuronate (M) and α-L-guluronate (G) (Figure 1).[3] Divalent cations, such as Ca^{2+}, can interact ionically with alginate polymer chains to form a gel structure. These hydrogels are formed by each divalent ion interacting with two adjacent G-residues, as well as with two G-residues in an opposing chain. The resulting structure is often referred to as the "egg-box" model.[4] Such alginate gels have long as found applications in the healthcare industry due to their suitability for use as wound dressings for exuding wounds.[5] Alginate wound dressings may initiate or accelerate the recovery of chronic wounds, provided that the underlying pathological condition is treated. This is due to two major actions of the alginate; first, the alginate fibers' moisture handling properties and second, the induced cytokine production by human monocytes that may result from bioactivity exerted from the alginates.[3]

Studies have shown that low levels of hydrogen peroxide can also aid in wound healing by stimulating angiogenesis (the formation of new blood vessels).[6] It also stimulates macrophage vascular endothelial growth factor release, which also contributes to the healing process.[6] Another critical participant in delaying the healing process is the presence of excess reactive oxygen species (ROS).[7] Their presence can result in indiscriminate damage to cellular constituents, such as DNA, lipids, and proteins.[8] ROS can also result in the destruction of extracellular matrix (ECM) components, such as collagen and proteoglycans.[9] Hyaluronan is a component of the ECM. It is important during the healing process of chronic wounds.[10] Hyaluronan modulates the inflammatory response by acting as an antioxidant and

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Appendix 4.1:

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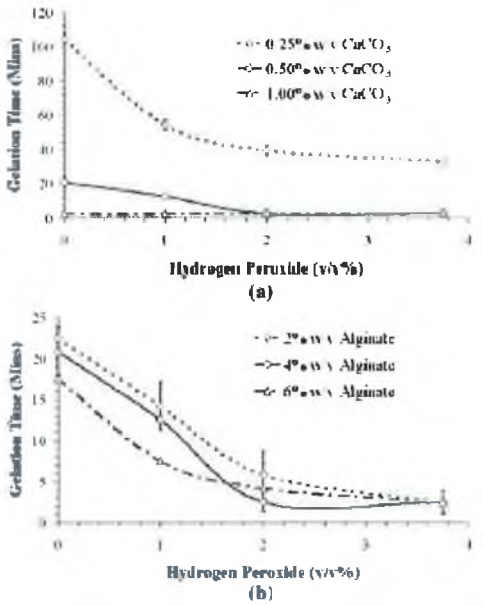

FIGURE 1. Alginate polysaccharide consisting of two guluronic acid and two manuronic acid residues with (1,4)-linkages. (a) The effects of hydrogen peroxide and calcium carbonate content on gelation time of alginate gels (4% w/v alginate). (b) The effects of hydrogen peroxide and alginate content on gelation time of alginate gels (0.5% w/v calcium carbonate) [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com].

scavenging ROS.[11] Studies have demonstrated the beneficial effects of the application of exogenous hyaluronic acid in the healing of chronic wounds.[12]

Structural uniformity within the alginate gel is of paramount importance in the development of any alginate wound dressing. A homogeneous structure leads to more consistent structural properties throughout the gel.[13] Alginate gel beads have been extensively used as encapsulation materials for bioactive compounds.[14] Such systems have been used to treat type 1 diabetes by encapsulating insulin-producing cells in calcium-alginate capsules for delivery to the patient.[4] However, studies documenting this technique use fast gelling systems such as the drop-wise addition of sodium alginate slurry to a solution of calcium ions, often made by adding calcium chloride to water. The resulting rapid external gelling of the alginate produces nonhomogeneous gels with varied crosslinking densities.[15] This nonhomogeneity detracts from the functionality of a potential alginate wound dressing. Studies have found alternative methods of producing more homogeneous gels.[13]

The low solubility of calcium carbonate in water enables it to become uniformly distributed in the alginate solution. An internal, slow gelling system can then be created by releasing calcium ions through decreasing the pH, initiated by the addition of D-glucono-δ-lactone (GDL).[16] The controlled release of Ca^{2+} permits a slower, more complete crosslinking of the gels. This allows the alginate-calcium mixture to be poured into molds before gelation is complete. This allows the formation of gels with complex structures that may be suitable for use as wound dressings.

Although extensive research has been performed demonstrating the effects of calcium ion concentration and alginate content on hydrogel properties,[17] no such studies have been conducted that characterizes the effects of the addition of hydrogen peroxide or hyaluronic acid, or indeed aging effects during storage, on the properties of alginate gels.

In this study, alginate gels for use as wound dressings which incorporate varying concentrations of alginate, calcium carbonate, hydrogen peroxide, and hyaluronic acid were assessed over time with regards to their gelation time, homogeneity, rupture force, and rupture energy. Investigating the gelation time and homogeneity of these alginate gels allows for assessment of their suitability for use as a wound dressing. A rapid gelation time has been shown to produce less structurally uniform gels.[13] More homogeneous gels are more structurally sound than inconsistent gels.[18,19] Assessment of rupture characteristics is also useful when evaluating the gel's suitability as a wound dressing. The more resistant the gel is to rupture, the more durable and workable it will be for patients and physicians alike. The profile of gelation and rupture characteristics and gel homogeneity will provide a basis for the formulation of structurally robust gels which will then be analyzed using cellular models to determine the potential of these calcium-alginate gels, incorporating hydrogen peroxide and hyaluronic acid, in aiding wound healing.

The objective of this research is to investigate the effects of important variables of gel formulation (Ca^{2+}, alginate, hydrogen peroxide, hyaluronic acid concentrations, and storage time) on the gels' structural properties.

EXPERIMENTAL

Materials

Sodium alginate (medium viscosity sodium alginate extracted from *Laminaria hyperborean*), calcium carbonate, hydrogen peroxide (30%), hyaluronic acid sodium salt (from *Streptococcus equi* sp.) and GDL were purchased from Sigma-Aldrich Ireland.

Alginate gel preparation

Alginate gels were produced by varying concentrations of alginate (0%–6%), calcium carbonate (0%–1% w/v), hydrogen peroxide (0.00%–3.75% v/v), and hyaluronic acid (0–1.25 mg/L). Three replicates of each sample were prepared.

Alginate and hyaluronic acid powders were dissolved in a suspension of calcium carbonate to which 1 mL of hydrogen peroxide was added. To this mixture, 2 mL of GDL solution was then added. The solution was immediately poured into a petri dish (50 mm diameter, 10 mm height) and allowed to set for 48 h in a cool, dry, dark location.

Gelation time

Gelation time was assessed using a method adapted from Kuo and Ma.[13] Gelation time was defined as the time between the addition of GDL and the formation of the gel.

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TABLE I. ANOVA Test for Significant Difference Between Groups for Varying Compositions of Alginate Gels for Potential Use as Wound Dressings

	ANOVA *p*-value between groups		
Parameter Measured	Calcium Carbonate	Hydrogen Peroxide	Alginate
Gelation time	0.000	0.177	0.502
Homogeneity	0.000	0.797	0.174
Rupture force	0.000	0.000	0.375
Rupture energy	0.000	0.000	0.422

Gels formulated using different calcium carbonate concentrations were tested for statistical difference in their mean values of gelation time, homogeneity, rupture force, and rupture energy. The same tests were performed for gels of varying hydrogen peroxide and alginate content. It is clear that calcium carbonate content has a significant effect on all four parameters tested. Hydrogen peroxide content has a significant effect on the rupture force and rupture energy of gels ($p < 0.001$). Alginate content, for the concentrations tested, does not significantly affect any of the four parameters tested.

The gel was said to be formed when the sample no longer flowed when tilted at an angle of 45° for longer than 30 s.[13]

Homogeneity

The method used by Kuo and Ma[13] for determining homogeneity in alginate gels was adapted and used as follows: the dry weight to wet weight ratio of the gel was used to evaluate homogeneity. Each gel was cut horizontally to give four slices, which were numbered 1–4 (1 corresponding to the top slice and 4 to the bottom slice). These slices were weighed, dried to a constant weight, and then reweighed. The dry weight to wet weight ratio of the slices provides an indication of the homogeneity of each gel. A homogeneous gel will have a consistent dry weight to wet weight ratio across its four constituent slices.[13]

Rupture characteristics

Adaptations were made to the method described by Roopa and Bhattacharya[20] to perform analysis of the rupture characteristics of the gels.

Samples were subjected to penetration at two sites by a Zwick/Roell Universal Testing Machine using a 5 mm diameter probe. The value of each rupture characteristic was produced for each sample by taking the mean of six readings for the sample (three replicates, each measured twice).

Rupture characteristics from the resulting force-deformation curve were obtained using Zwick's "test-Xpert" software.

Rupture force was defined as the maximum force immediately before rupture and was expressed in Newtons (N). Rupture energy was defined as the area under the force–deformation curve until the point of rupture (Roopa and Bhattacharya).[20]

Aging effects during storage

Rupture characteristics were assessed at 2, 7, and 14 days after formulation to assess the effect of storage on the integrity of the gels.

Statistics

All graphs and statistical analyses were produced using Microsoft Excel 2007 and SPSS 17.0.

RESULTS

Figure 1(a,b) display the time required for alginate gelation after the addition of calcium carbonate and GDL. The alginate solution was considered to be gelled when it no longer flowed when tilted at a 45° angle. A rapid gelation time is not desirable as it results in difficulties when molding the gel and can lead to less homogeneous alginate gels.[13] Figure 1(a) depicts the effects of calcium carbonate and hydrogen peroxide content on the gelation time of these hydrogels. It can be observed that gelation time decreases greatly with increasing calcium carbonate content, while Figure 1(b) illustrates that raising the concentration of alginate and hydrogen peroxide also leads to faster gelation of the gels. Table I confirms that only calcium carbonate concentration has a significant effect on the gelation times of these alginate gels ($p < 0.001$). Figure 2(a–c) display gel homogeneity.

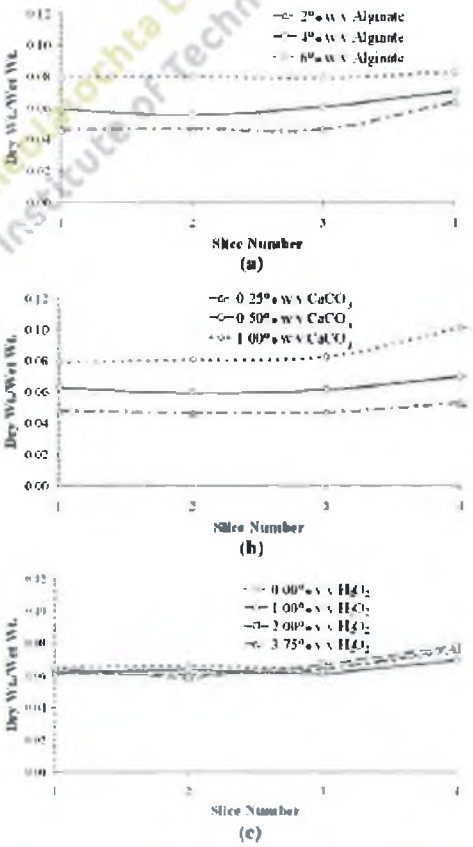

FIGURE 2. (a) The effect of alginate content on gel homogeneity. (b) The effect of calcium carbonate content on gel homogeneity. (c) The effect of hydrogen peroxide content on gel homogeneity. (Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.)

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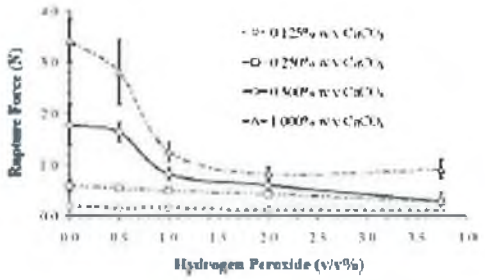

FIGURE 3. The effects of hydrogen peroxide and calcium carbonate content on rupture force of alginate gels (4% w/v alginate) [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com].

Alginate gel homogeneity was assessed by allowing samples to gel for 48 h and then slicing the gels horizontally into four slices of equal size. These slices were labeled with "slice 1" (top slice) to "slice 4" (bottom slice), and the dry weight to wet weight ratios was calculated for each slice and used as an indication of gel homogeneity. A homogeneous gel displays equal values of dry weight to wet weight ratios for all four of its constituent slices. Calcium carbonate content has the largest effect on gel homogeneity, as shown in Figure 2(b). Gel homogeneity decreases with increasing calcium content. This is confirmed by the ANOVA analysis documented in Table I, which shows that there is a significant difference between the homogeneity values of gels with varying calcium carbonate concentrations ($p < 0.001$). Figure 2(a) suggests that gel homogeneity improves with increasing alginate content. However, this is not confirmed by the ANOVA analysis in Table I. The p-value of 0.174 illustrates that there is no significant difference in the homogeneity values of gels containing varying amounts of alginate. In Figure 2(c), it can be observed that hydrogen peroxide concentration has no obvious effect on the homogeneity of the gels. This is confirmed in Table I which returned a p-value of 0.797.

It is observed in Figure 3 that increasing calcium carbonate content leads to an increase in the force required to rupture the gel. Increased hydrogen peroxide content produces gels with a decreasing rupture force. Table I supports these observations, with both calcium carbonate and hydrogen peroxide groups returning significant p-values ($p < 0.001$). Table I also shows that alginate content does not have a significant effect on rupture force for the concentrations tested. Similar effects are observed for the action of calcium carbonate, hydrogen peroxide, and alginate content on the rupture energy of the gels. Figure 4(a–c) shows that the rupture force of alginate gels containing no hydrogen peroxide remains unchanged over time, whereas samples containing hydrogen peroxide degraded over time, producing lower rupture force values. This effect is more pronounced with increasing hydrogen peroxide content and with increasing time.

Figure 4(a–c) also depict the effect of hyaluronic acid on the rupture force of the gels over time. Figure 4(a) shows that hyaluronic acid delays the degradation of gels containing higher concentrations of hydrogen peroxide after two days of aging. The effect of the hyaluronic acid is more pronounced after 7 days of aging, as shown by the higher rupture force values for gels containing hyaluronic acid than those not containing hyaluronic acid [Figure 4(b)]. However, after 14 days of aging, Figure 4(c) shows that the rupture force values for samples containing hyaluronic acid and those not containing any are very similar and considerably lower than the values observed after 2 and 7 days of aging. This suggests that gels containing hydrogen peroxide have a

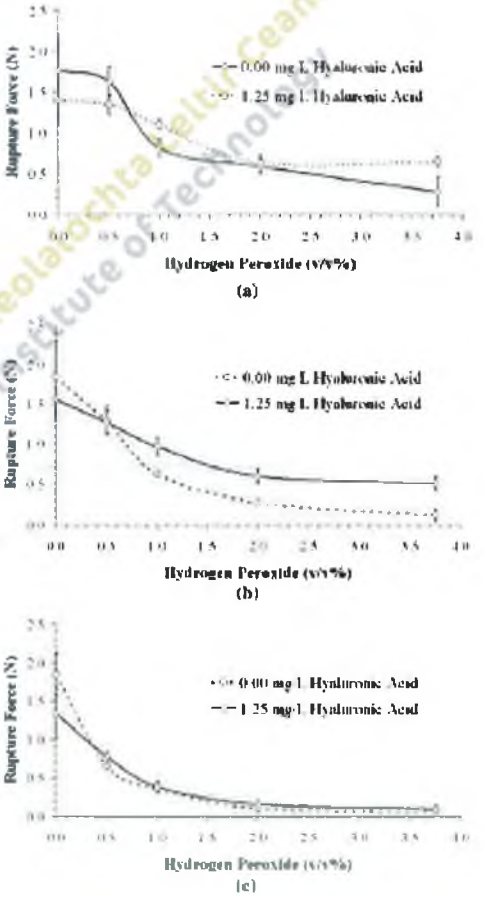

FIGURE 4. (a) The effect of hyaluronic acid (H.A.) content on rupture force of alginate gels after 2 days of storage (4% w/v alginate, 0.5% calcium carbonate). (b) The effect of hyaluronic acid (H.A.) content on rupture force of alginate gels after 7 days of storage (4% w/v alginate, 0.5% w/v calcium carbonate). (c) The effect of hyaluronic acid (H.A.) content on rupture force of alginate gels after 14 days of storage (4% w/v alginate, 0.5% w/v calcium carbonate) [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com].

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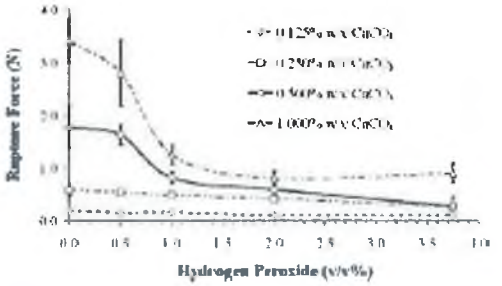

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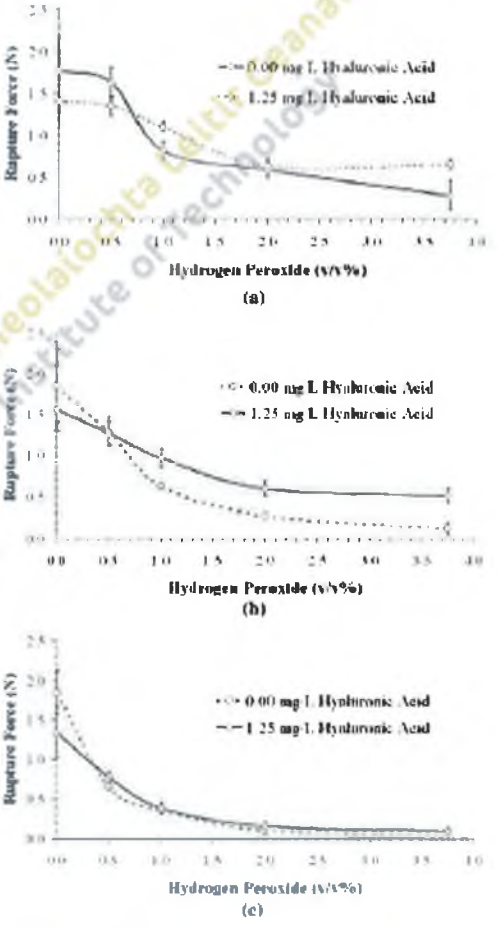

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samples. It can be observed that the rupture force of gels containing no hydrogen peroxide is lower when the gel also contains hyaluronic acid. This may be the hyaluronic acid acting in a similar manner to the hydrogen peroxide and depolymerizing the alginate chains within the gel, resulting in weaker gels. However, in gels also containing hydrogen peroxide, the oxygen scavenging capabilities of hyaluronic acid can be observed. This slows the degradation of the gels containing hydrogen peroxide. By comparing Figure 2(a,b), it is observed that samples containing hyaluronic acid have a similar rupture force value after 2 and 7 days, whereas samples not containing hyaluronic acid returned notably lower rupture force values after 7 days than after 2 days aging. This indicates that the use of hyaluronic acid in the gels can help slow the effect of aging. However, inspection of Figure 2(c) shows that after 14 days of storage in a dark place at room temperature, gels have considerably lower rupture force values, whether they contain hyaluronic acid or not. This suggests that gels have a finite lifetime, after which they are unsuitable for use as wound dressings. But, the use of hyaluronic acid in the gel may prolong this lifetime.

It may be useful to use the data collected to produce a model for predicting the rupture characteristics of gels containing other concentrations of alginate, calcium carbonate, hydrogen peroxide, and hyaluronic acid and for samples of varying age. The next stage of this study requires testing the alginate gels on cellular models of wound healing to assess their effectiveness. This will require trial and error to obtain the most effective gel composition to promote wound healing. When the composition of this gel is defined, these models can then be used to evaluate its rupture characteristics to assess if its structural integrity is suitable for practical use.

Table II gives the multiple linear regression models for the prediction of gelation time, homogeneity, and rupture force of a gel. The model for homogeneity displays a reliable R^2 value of 0.64, while the gelation time model has an R^2 value of 0.37. The model for the prediction of rupture force shows a reliable R^2 value in excess of 0.57. Using these models, the estimation of gel characteristics within the experimental range is possible. The rupture force (N) of a gel can be calculated using the following equation based on the analysis in Table II:

$$Rf = 0.515 + 1.509_C - 0.243_H - 0.250_A$$

where: Rf = Rupture Force (N)
C = calcium carbonate (% w/v)
H = hydrogen peroxide (% v/v)
A = sample age (days).

The rupture force of a 1-day-old gel containing 0.75% w/v $CaCO_3$ and 1.25% v/v H_2O_2 can be estimated as follows:

$$Rf = 0.515 + (1.509 \times 0.75) + (-0.243 \times 1.25) + (-0.250 \times 1.0) = 1.09\,N$$

Gelation time and homogeneity of gels of various compositions within the experimental range can be estimated in a similar fashion using the models given in Table II. This will provide important information on the structural attributes of gels which can be used in future studies.

CONCLUSION

Important variables of alginate gel formation were tested to assess their effects on gel homogeneity, gelation time, and rupture characteristics. Increased calcium carbonate concentration led to increased rupture values of the gels, while it decreased gelation time and gel homogeneity. Increased hydrogen peroxide content also decreased gelation time and gel homogeneity, as well as the rupture values of the gels. Alginate content did not have a significant effect on the rupture values of the gel. Gels containing hyaluronic acid had lower mean rupture values than those formulated in the absence of hyaluronic acid ($p < 0.05$). The rupture characteristics of gels not containing hydrogen peroxide remained unchanged over the 2-week storage period, while gels containing hydrogen peroxide displayed decreasing rupture values with increasing hydrogen peroxide concentration and storage time. However, gels containing hyaluronic acid display a marked decrease in gel aging over a 7-day period. The multiple linear regression models produced in this study allow for the estimation of rupture characteristics of gels with varying compositions. If the dressing is required to be intact after removal from the wound, a gel with a greater resistance to rupture is required. Knowing a gel's rupture characteristics will be useful in assessing its suitability as an effective wound dressing.

ACKNOWLEDGMENTS

The authors thank Michael Bronks of the Zwick/Roell Group for generously allowing the use of a Zwick/Roell Universal Testing Machine and Dr. Joe English for helpful discussions on statistics.

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Appendix 4.2: Letter confirming acceptance of the manuscript entitled "Gelation Time, Homogeneity and Rupture Testing of Alginate-Calcium Carbonate-Hydrogen Peroxide Gels for Use as Wound Dressings" for publication in the Journal of Biomedical Materials Research: Part B - Applied Biomaterials. This paper is based on data obtained as part of this masters degree and is documented in Chapter 3 of this thesis.

Preview

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Dear Mr. Alexander:

I am pleased to inform you that your manuscript entitled "Gelation Time, Homogeneity and Rupture Testing of Alginate-Calcium Carbonate-Hydrogen Peroxide Gels for Use as Wound Dressings" has been accepted for publication in the Journal of Biomedical Materials Research: Part B - Applied Biomaterials.

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