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Assessing modified chitosan wound dressings to enhance wound healing in the porcine model

by

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Abstract

Dressings enhancing wound healing can improve the outcome of wounds where tissue replacement is required, like for burns and ulcers. Treatment of these wounds is complex due to their depth and excessive tissue loss. Replacement of the lost tissue and delivery of growth factors could enhance healing and reduce scarring. The natural biomaterial; chitosan is reported to bind growth factors, with reduced wound healing times when used in dressings. This study aimed to modify chitosan into a wound dressing filler that would optimise growth factor delivery to full-thickness wounds and overall reduce healing times with minimum scarring.

Lipophilic modified chitosan was chemically synthesised by addition of different percentages (10%, 20%, and 34%) of lauric acid residues into three lauroyl chitosan (LCs) derivatives (LCs_{10}, LCs_{20}, LCs_{34}). Lauric acid was the fatty acid of choice due to its superior antimicrobial properties among the saturated fatty acids.[1] The loading densities selected were based on commonly used concentration ranges as found in literature. The three derivatives were then characterised using Nuclear magnetic resonance (NMR) and Fourier-transform infrared (FT-IR) spectroscopy. Thereafter, swelling tests and water drop shape analysis followed to assess the physical characteristics of the derivatives. Cytotoxicity/proliferation assays using primary fibroblasts and sulphorhodamine-B for cell enumeration were performed followed by a preliminary skin sensitivity test. The acid phophatase assay was used to measure platelet adhesion while the enzyme-linked immunosorbent assay (ELISA) measured the release profile of platelet derived growth factor AB (PDGF-AB) over 24 hr. These assays assisted with determining which derivative had the optimum lauric acid loading density for wound healing.

After determining the derivative with the optimum loading density, porcine collagen was extracted from skin and added to the selected LCs derivative at the ratio 1:4 to make a wound filler paste that would increase cellular ingrowth. Wound healing studies using LCs_{10} enriched with collagen fibres (Co/LCs_{10}) alone and with platelet-rich plasma (Co/LCs_{10}/PRP) as dressing material were performed using the porcine full skin thickness wound healing protocol. Finally, histological analysis of the cellular events taking place in the wounds at different stages of healing were done using the Haematoxylin and Eosin and the Masson's Trichrome stains.

Evidently, the FT-IR and NMR, displayed successful modification of chitosan with the lauric acid side chains with a visible aliphatic group in both spectra. Comparison of the LCs derivatives to the underivatized chitosan using the drop shape analysis, showed increased contact angles with increased hydrophobicity. It appeared that as the molar concentration of lauric acid increased, the contact angle also increased. In the swelling tests, LCs_{34} had the highest swelling capacity.

Results from the *in vitro* assays showed that hydrophobic modification of chitosan reduced the adhesion capacity of platelets to chitosan as the lauric acid density on the underivatized chitosan increased. Cytotoxicity assays indicated that neither LCs nor chitosan were toxic to primary fibroblast cells, with the LCs_{34} significantly (43%) promoting fibroblast proliferation compared to the control. A preliminary skin sensitivity test comparing LCs_{34} to chitosan showed that LCs_{34} was compatible with human skin.

From the ELISA study the LCs_{10} sample exhibited a sustained release of growth factors over 24 hr compared to both chitosan and collagen.

Consequently, the LCs_{10} derivative was then selected for further analysis and for final analysis in the wound study.

Sixteen full-thickness skin wounds were thereafter made along the dorsum of each of four pigs with two treatments and a control (Jelonet®) randomly applied as dressing material: Co/LCs_{10}, Co/LCs_{10}/PRP and the Jelonet® treatment. The differences in wound healing were observed with biopsies taken at 3-day intervals over 21 days. By the 12^{th} day, all wounds had completely healed with little scarring. The Co/LCs_{10}/PRP dressing significantly induced haemostasis, wound contraction and accelerated wound closure and healing from the wound bed. Results from histological examinations demonstrated advanced granulation tissue formation, collagen deposition and epithelialisation in the wounds treated with Co/LCs_{10}/PRP.

This study therefore revealed that hydrophobically modified chitosan at 10% loading density provided a wound dressing material that allowed sustained growth factor release. The Co/LCs_{10}/PRP dressing also demonstrated that it was an improved wound dressing due to acceleration of wound healing, promotion of fibroblast proliferation with increased collagen deposition and minimal scarring. These materials may significantly reduce healing times of full-thickness wounds and should be studied further in *in vivo* models.

Declaration

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“And once the storm is over, you won’t remember how you made it through, how you managed to survive. You won’t even be sure, whether the storm is really over. But one thing is certain. When you come out of the storm, you won’t be the same person who walked in. That’s what this storm’s all about.”

— Haruki Murakami

Table of Contents

List of Figures

List of Tables

Abbreviations

°C	Degrees Celsius
%	Percent
AA	Acetic acid
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
bFGF	Basic fibroblast growth factor
BSA	Bovine Serum Albumin
CMC	Carboxymethylchitosan
CMCS	Carboxymethyl chitosan sulphate
Co	Collagen
CoLCs	Collagen/Lauroyl chitosan
Cs	Chitosan
DD	Deacetylation degree
DFUs	Diabetic foot ulcers
DMEM	Dulbecco's Modified Eagle Medium
DMF	Dimethylformamide
ECGF	Epithelial-cell growth factor
ECM	Extracellular matrix
EDC.HCl	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EtOH	Ethanol
FCS	Foetal calf serum
FDA	Food and Drug Administration of the United States of America
FGF	Fibroblast growth factor
GA	Gluteraldehyde
GAA	Glacial acetic acid
GP	General Practitioner
HDMS	Hexamethylsilazane
H & E	Haematoxylin and Eosin stain
HGB	Haemoglobin
HCT	Haematocrit
HGF	Hepatocyte growth factor
Hm-Cs	Hydrophobically modified chitosan

HS	Healthy skin
ICDRG	International Contact Dermatitis Research Group
IDF	International Diabetes Federation
IGF	Insulin-like growth factor
IL	Interleukin
IR	Infrared
KGF	Keratinocyte growth factor
LCs	Lauroyl chitosan
LD	Loading density
LSCS	Lauroyl sulphated chitosan
LMW	Low molecular weight
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
ml	Millilitre
mm	Millimetre
MIC	Minimum inhibitory concentration
min	Minutes
MMP	Matrix metalloproteinase
MT	Masson's Trichrome
MW	Molecular weight
MWT	Maggot wound therapy
NADH	1,4-dihydronicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
NHS	N-hydroxysuccinimide
nm	Nanometer
NMR	Nuclear magnetic resonance
OCNP	Oleoyl-chitosan nanoparticles
OH	Hydroxy
P. aeruginosa	*Pseudomonas aeruginosa*
PBS	Phosphate Buffered Saline
PDGF	Platelet derived growth factor
pg/ml	Picogram per millilitre
PMNs	Polymorphonuclear cells
PML	Polymorphonuclear leukocytes
p-NPP	para-nitrophenyl phosphate
PRP	Platelet-rich plasma

PPP	Platelet-poor plasma
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RBC	Red blood cell
S. aureus	*Staphylococcus aureus*
SD	Standard deviation
SEM	Standard error of the mean
SEM	Scanning Electron Microscopy
SRB	Sulphorhodamine-B
TCA	Trichloroacetic acid
TEM	Transmission Electron Microscopy
TGF-β	Transforming growth factor beta
TIMP	Tissue inhibitor of matrix metalloproteinase
TNF-α	Tumour necrosis factor alpha
Tris	Tris(hydroxymethyl)aminomethane
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
VLU	Venous leg ulcer
WA	Wound area
WBC	White blood cells
WHS	Wound Healing Society
w/v	Weight per volume
w/w	Weight per weight

Chapter 1

Literature review

1.1 Introduction

The high burden of wound management is challenging to healthcare systems as these wounds often become chronic, significantly affecting the quality of life and work productivity of the afflicted persons. The cost of managing chronic wounds in the United States of America is nearly US$32 billion.[2] In South Africa, however, prevalence and cost studies are lacking but it is estimated that R250 million was reportedly claimed from medical insurance providers in 2016 for wound care.[3] Furthermore, in Africa, treatment of chronic wounds poses a major challenge due to the lack of suitable resources. In addition to trauma related wounds, infectious tropical wounds such as leishmaniasis, buruli ulcer, phagedenic ulcer and leprosy are common occurrences.[4] These however do not receive the attention that they should, with the available treatments relying essentially on wound disinfection and drying out, subsequently delaying healing which in turn increases the cost of treatment.[4]

Regardless of the wound aetiology, the outcome of wound healing is restoration of the function and integrity of damaged tissues. It is an intricate process that involves the synchronised activity of various types of cells including endothelial cells, fibroblasts, keratinocytes, platelets and macrophages. Wound dressings are crucial in the management of serious wounds and these serve several purposes like protecting the wound area, maintaining an optimal moist environment which promotes healing while preventing microbial growth. To date, the wound dressing market has several polyurethane foam products and a number of biomaterials including those comprising of collagen, pectin, hydrocolloids, chitosan, alginates, and hyaluronic acid.[5-9]

Chitosan; which is the focus of this study, is a deacetylated derivative of the abundant natural, chitin. Chitin is readily available and economical as the molecule that forms the exoskeleton of invertebrates and the cell wall of fungi. It was first identified in 1811 by the French of natural history professor; Prof Henri Broconnot.[10] After isolation from ants, the name ‘chitin’ was then assumed in the 1830’s. This deacetylated product, chitosan, is widely used in medical and industrial applications and has been proven non-toxic, biodegradable,

biocompatible, hydrating and an antimicrobial agent showing positive properties on wound healing. Various materials derived from chitosan include: micro/nanoparticles, hydrogels, films, foam, fibres and powders have been successfully used.[11-14] The presence of the reactive primary amine and primary and secondary hydroxyl groups make chitosan of interest for chemical modification to improve certain functions including wound healing properties. The high proportion of protonated amino groups on the glucosamine units of chitosan explains its solubility in dilute aqueous acidic solvents.[15] Several investigations on the improvement of chitosan's wound healing properties through chemical modification have been reported.[16-20]

The specific characteristics of chitosan can be modified in several ways including modification with lauroyl chloride or combining with platelet-rich plasma (PRP) both of which have been touted as promoting wound healing. There are however no reports on the efficacy of this combination, which motivated the investigations in this study. This study thus aimed to prepare a chitosan based wound filling paste that would provide a matrix that enhances infiltration of fibroblasts while promoting growth factor release, angiogenesis and a reduction in overall wound healing times.

1.2 Wounds

According to the Merriam Webster dictionary, the Greeks use the word 'trauma' with reference to physical wounds.[21] The American Wound Healing Society (WHS) defines a wound as 'the disruption of cellular, anatomic and functional integrity of a living tissue'.[22] An expansive definition includes the disruption of the integrity of the skin as well as mucous membranes and organ tissues. A simple definition by Medline plus states that 'wounds are injuries that break the skin or other body tissues'.[23]

Traditionally there was a clear distinction between wounds and ulcers. Wounds were said to result from a break in the continuity of any of the bodily tissues due to violence while ulcers were from internal aetiology; characterised by inflammation and/or chronicity.[24] Wounds due to external injury were described as acute; healing in a timely and orderly manner.[25] However, the distinction between ulcers and wounds is seldom used. Regardless of aetiology (trauma or disease process), it is clear that any break in the integrity of the skin is regarded a wound.

Defined as the environment in direct contact with the wound surface but exterior to the wound, [26] the external wound microenvironment plays a key role in skin homeostasis. Its impact on the rate, duration and quality of healing (e.g. scar formation) is significant. The notion that wounds need fresh air to dry out and prevent infection was dispelled in 1962 when it was discovered that wounds re-epithelialise more rapidly under occlusion.[27] The interior of wound microenvironments are regulated by a variety of cells, cytokines and growth factors. During the inflammation phase; platelets, macrophages, polymorphonuclear cells (PMNs), and mast cells actively facilitate haemostasis and autolytic debridement while directly influencing the inflammation process. The proliferative phase through the mediation of growth factors such as fibroblast growth factor (FGF), platelet derived growth factor (PDGF), keratinocyte growth factor (KGF), epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) sees the formation of new cells by fibroblasts, keratinocytes, myofibroblasts and angioblasts.[28]

Invasion of pathogenic organisms in viable tissue around the acute or chronic wound is a common occurrence. However, it is factors such as wound location, type, quality, depth, the antimicrobial efficacy of the host immune response and the level of tissue perfusion that influence the microbial colonisation in any wound.[29] Both gram-negative and gram-positive bacteria are implicated in wound infection with the most commonly isolated pathogens being *Klebsiella spp, Escherichia coli (E. coli)*, *Staphylococcus spp,* and *Pseudomonas spp.*[30] Fungi such as *Candida albicans. Candida tropicalis*, *Candida parapsilosis*, *Trichosporon asahii*, and *Aspergillus* species [31] have also been reportedly isolated from wounds. Patients with highly glycosylated haemoglobin levels present with significantly higher fungal infections.[31] It is thus no surprise that healing of diabetic foot ulcers (DFUs) is complicated by the opportunistic fungi and by the immunocompromised status of these patients.

The normal pH of the skin which is between 4.2 and 5.6 becomes alkaline with wound infection [32], with most bacteria favouring a pH > 6.[33] For healing to occur, the pH has to progress to a slightly acidic state that favours healing. This implies that the pH in the wound influences infection control, oxygen release, antimicrobial activity, angiogenesis and protease activity.

Ranging from superficial cuts, limited to the epithelium, to deep wounds, extending into subcutaneous tissue and underlying organs; wounds can be of accidental or intentional aetiology or can result from a disease process (dermatological diseases, diabetes mellitus, and venous/arterial insufficiency). The numerous causes and nature of wounds result in many ways of classifying them. Characteristics such as inflammation, depth, duration, blood flow, repetitive trauma, nutrition, systemic factors and wound metabolism are used to describe wounds.[25] A further description of wounds is according to the type of wound healing; first intention, secondary intention or tertiary intention. Wounds are also classified according to whether they are open or internal. Open wounds have four classifications (abrasion, laceration, puncture, and avulsion) that are dependent on their cause.

Wounds may also be classified according to their appearance; sloughy, necrotic, granulating, malodorous/infected, epithelising. Classification according to depth gives; superficial, partial thickness and full-thickness wounds.[34] Superficial wounds only affect the epidermal layer of the skin, while partial thickness affect both the epidermis and dermis. Full-thickness wounds extend to the subcutaneous fat sometimes progressing to the bone. The time span and nature of the repair process leads to the classification of wounds into acute or chronic. The cause and type of wound influence the healing time. Acute wounds heal with minimum scarring within four weeks while chronic wounds heal over extended periods in a disorderly manner.

Chronic wounds have the greatest impact on the quality of life and work productivity of the afflicted persons. Prevalence of chronic wounds varies depending on diagnosis, year and country. Chronic wounds affect almost 8.2 million (15%) Medicare beneficiaries in the United States of America (USA)[2] per year, while statistics show that Canada has an estimated 4-7% cases[35]; Germany: 1.03 - 1.05%[36]; China: 1.7%[37] and the Indian population: 0.45%.[38] A global increase in chronic wounds was reported from 2012 – 2017 due to a rapidly aging population and a sharp rise in diabetes and obesity. In South Africa however, prevalence and cost studies are lacking, but it is known that the main cause of injury is trauma, with gunshot wounds costing the public healthcare system billions. Approximately 25% of emergencies in public hospitals in Kwazulu-Natal are due to trauma [39], while 23% of mortality in the Western Cape is a result of trauma. [40]

1.3 Socio-economic impact of wounds

Wound care is not a specialised field; dermatologists, podiatrists, vascular surgeons and geriatricians may be involved at some point of care of these wounds.[41] Successful wound care frequently involves a multidisciplinary team and regular visits to healthcare professionals. These basic wound related visits to health professionals contribute to the overall cost of wound care. The cost of wound care is also notably influenced by; the frequency of dressing change, duration of care and occurrence of complications.

The socio-economic impact of wound care is separated into direct costs and indirect costs. Direct costs are incurred directly by the healthcare provider from treating the wound e.g. wound dressings while indirect costs are those incurred by the patient, his/her family including the losses to society caused by the disease and its treatment e.g. inability to work. Indirect factors such as monthly income, number of dependents, cost of consultation, and cost of medication etc. contribute to the type of care sought and/or received by patients and the quality of life.[42] In a tertiary hospital in India, although the actual cost of managing the wound was not high, it was observed that patients with lower economic status did not come for follow up visits for regular wound management compared to the higher economic status patients. This was attributed to additional costs such as transport, attendant fees etc.[43] Thus, the socio-economic status of the patient had an indirect effect on the wound management.

The cost of managing wounds is enormous. The severity of the wound and the duration of treatment are also cost drivers associated with wound care. Uncomplicated wounds require basic resources and staff times, while chronic wounds that are complicated by several factors such as infection, low blood perfusion etc. sometimes require hospital stay or surgical intervention and longer term use of dressings/devices. Besides lowering the quality of life of the patients, chronic wounds often lead to serious life events such as limb amputations and/or premature death.[41] Up to 85% of all amputations in diabetics are preceded by DFUs. Needless to say, persons with an amputation may have difficulty being rehabilitated and may lose their employment further impacting their quality of life.

A few articles document the disproportionate cost of managing wounds. In the United Kingdom (UK) a relatively uncomplicated pressure ulcer will cost an estimated £1,200 to manage while a complicated one will have costs escalate to over £14,000.[44] In general, the

United Kingdom (UK) had an estimated 2.2 million wounds managed by the National Health Service (NHS) in 2012/2013. The annual NHS cost of managing these wounds and associated comorbidities was approximately £5.3 billion with these costs including 18.6 million practice nurse visits, 10.9 million community nurse visits, 7.7 million GP visits and 3.4 million hospital outpatient visits.[45] In the United States of America (USA), the cost of managing wounds for Medicare beneficiaries is just over US$32 billion. The highest cost drivers being surgical wounds and DFUs while hospital outpatients also accounted for a great proportion of the costs.[2] Hospital inpatient costs were found to be about half the hospital outpatient costs.[2] The total cost of wound care in Australia was estimated to be US$2.85 billion in 2014. Most of the costs were incurred in the hospital system while community care incurred lower costs.[46] In South Africa, an estimated R250 million was reportedly claimed from medical insurance providers in 2016 for wound care.[3]

Wounds do not only have medical and economic implications on the lives of those affected. While hand injuries have apparent consequences on the patient's ability to work, other types of wounds may result in mobility issues; moreover the physical effects of the wound such as pain, excessive exudate, mobility issues have a psychological impact and also have a bearing on the well-being of those affected[47] The consequences thereof is social isolation, stress, sleep disturbances, low self-esteem and negative mood.[47-49] Consistent with these findings, Jones *et al*, showed that excessive exudate and odour with leakages resulted in feelings of disgust, self-loathing and low self-esteem thus hampering their social lives, leading to social isolation and depression.[50] This vicious cycle of psychological stress leading to impaired healing is well researched.[47,51] Detillion *et al*, showed the effect a positive psychological state had on wound healing in rodents. They determined that social isolation impairs wound healing, while treatment with oxytocin blocked stress-induced surges in cortisol concentrations and expedited wound healing. [52]

Having a chronic wound can be a life-changing event that alters even the family of the affected individual. The obvious costs impose a financial hardship on the family by increasing expenses related to the cost of care and supplies.[48] Moreover, the family with the affected individual may have to adapt to meet the demands of the hardship of taking care of an ill individual. [48]

1.4 Pathophysiology of wounds

Despite various classification methods being used for wounds, the cellular and extracellular interactions that take place in wounds are similar. Restoration attempts of the damaged tissues begin very early after injury. Wounds go through four overlapping stages of healing that are not mutually exclusive. These four phases; haemostasis, inflammation, proliferation and maturation are coordinated and regulated by the action of several mediators including epidermal cells, dermal cells, inflammatory cells, growth factors, cytokines, platelets, (matrix metalloproteinases) MMPs and their inhibitors.[53] (**Table 1.1**)

Acute wounds heal timeously through an intricate, well-orchestrated sequence of the overlapping phases (**Figure 1.1**) that include cellular activities such as chemotaxis, phagocytosis, mitogenesis and synthesis of extracellular matrix (ECM) constituents. While the healing process is continuous, the division into different phases assists with understanding the physiology of the wound and surrounding tissue.[54]

Table 1.1 Wound healing associated cells and their function [55]

Cell type	Function related to wound healing
Platelets	• Thrombus formation • Inflammatory mediators including cytokines (e.g. TGF-β, PDGF, β-thromboglobulin, platelet factor-4) released by α granules • Key early stimulus for inflammation
Neutrophils	• First cells to infiltrate site of injury • Phagocytosis and intracellular killing of invading bacteria
Monocytes (macrophages)	• Phagocytise and destruction of invading bacteria • Clear debris and necrotic tissue • Rich source of inflammatory mediators including cytokines • Stimulate fibroblast division, collagen synthesis and angiogenesis
Lymphocytes	• Not clearly defined • May produce cytokines in certain types of wound
Fibroblasts	• Produce various components of the ECM, including collagen, fibronectin, hyaluronic acid, proteoglycans • Synthesise granulation tissue • Help to reorganise the 'provisional' ECM

1.4.1 Haemostasis

By and large, platelets circulate in close proximity but do not interact with the vascular walls.[56] However, when tissue injury disrupts blood vessels resulting in blood components being exposed to the ECM, potent vasoconstriction and formation of a fibrin plug is stimulated.

The first signal for tissue injury is given by release of interleukin (IL)-1α, by keratinocytes, from a preformed epidermal pool followed by synthesis of additional IL-1α.[57-58] Thereafter, blood components encounter exposed components of the ECM including collagen, thrombin, hydroxyproline and proline thus stimulating platelet activation through both the intrinsic and the extrinsic pathways of the clotting cascade. Once activated, the platelets release chemotactic mediators, cytokines and growth factors, which facilitate vasoconstriction, coagulation and the conversion of soluble fibrinogen to a network of insoluble fibrin fibres. The result is a stable platelet plug that consists of fibrin bound to the exposed integrin receptor ($\alpha_{IIb}\beta_3$) on activated platelets.[59] The clot offers a temporary matrix for cell migration in the ensuing phases of wound healing.

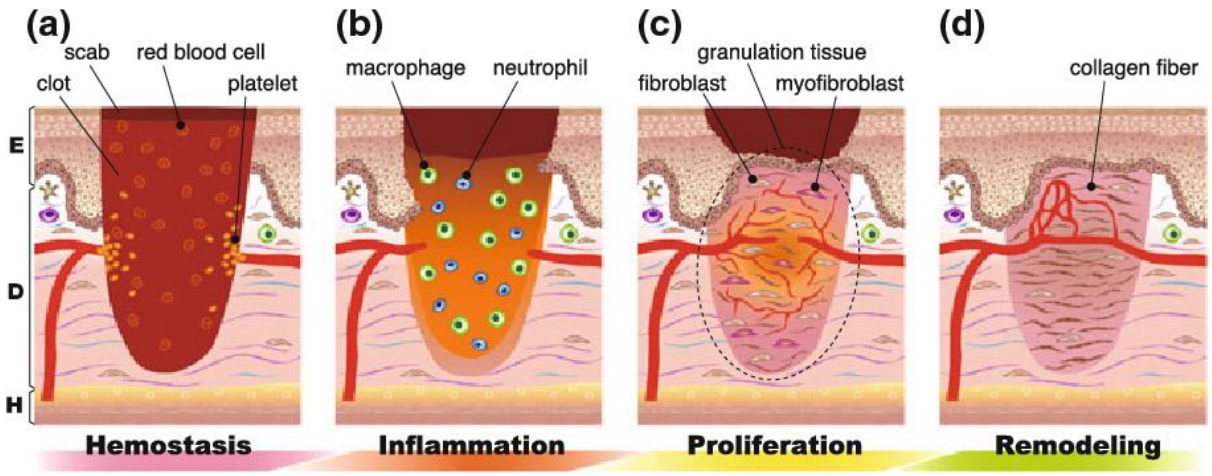


Figure 1.1 Phases of wound healing.[60]

1.4.2 Inflammation phase

The inflammation phase typically lasts between 24 to 48 hours. It begins with the activation of the compliment cascade and initiation of molecular events. Alpha granules in the cytoplasm of platelets release proinflammatory cytokines and growth factors such as transforming growth factor (TGF)-β, FGF, PDGF-β and EGF. Vascular endothelial cells, keratinocytes and fibroblasts in the lesion, also release cytokines such as tumour necrosis factor (TNF)-α. These

cytokines and growth factors promote chemotaxis of leukocytes to the site of injury facilitating the elimination of debris, bacteria and damaged tissue.[61] Ritsu *et al*, studied the effect of suppressing TNF-α activity by using neutralising monoclonal antibodies (mAb). It was evident that TNF-α is critical early in wound healing while neutralisation of TNF-α delayed wound closure by interfering with fibroblast proliferation and the formation of new ECM.[62]

Neutrophils, which rid the wound area of invading microbes and cellular debris, are active during the early stages of inflammation. Thereafter, monocytes infiltrate the wound site and mature into macrophages in a process mediated by IL-8. Macrophages play various crucial roles that include release of proinflammatory cytokines (IL-1, TNF, IL-6 etc.), resolution of inflammation in the later stages of inflammation by removing apoptotic cells and sustaining the proliferation of cells and restoration of tissue (**Figure 1.2**).[61] Macrophages also release prostaglandins, chemokines, leukotrienes, and complement which collectively induce increased vascular permeability and recruitment of inflammatory cells.[63] A study by Leivobich and Ross investigating the role of macrophages in wound healing showed that macrophage depleted wounds had defective repair with signs such as a severe lack of debridement and a delay in fibrosis.[64]

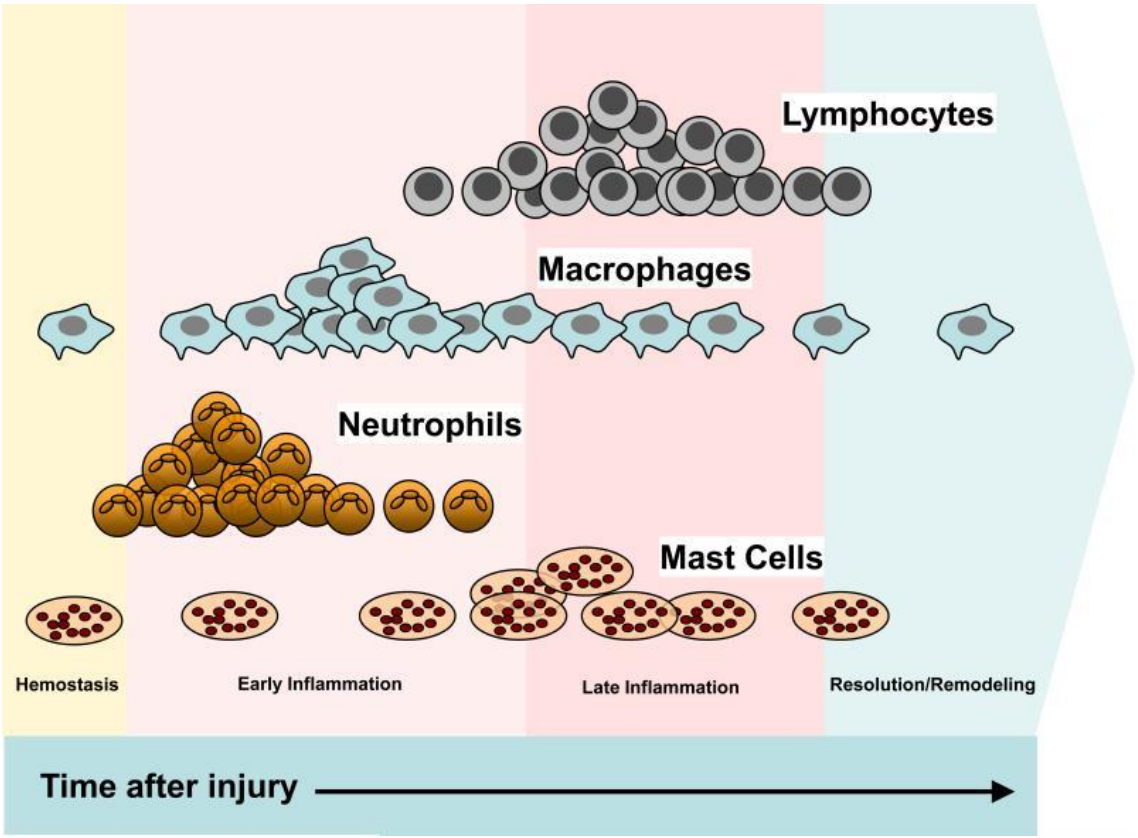


Figure 1.2 The pattern of leukocyte infiltration into wounds.
Inflammatory cells are present at all stages of wound healing.[65] The four most prominent types of leukocytes in wounds (mast cells, neutrophils, macrophages and lymphocytes) are depicted with their relative densities in each phase.

1.4.3 Proliferative phase

Once haemostasis and the immune system are effectively set, the proliferation phase, mediated mainly by fibroblasts, keratinocytes and endothelial cells begins 3 - 5 days after injury. The activity of these cells progressively modify the wound microenvironment from one that is inflammatory to a synthesis-driven phase.[66] Its onset is marked by the degranulation of macrophages resulting in the release of soluble mediators (e.g. FGF, TGF-β, EGF, PDGF) that activate and recruit endothelial cells for angiogenesis and fibrillogenesis.[67] The essential growth factor TGF-β stimulates matrix contraction[68] by fibroblasts and conversion of monocytes to macrophages. Fibroblasts and macrophages locally produce VEGF which stimulates new tissue and blood vessel formation, with the blood vessels formed sustained by VEGF.[69] Fibroblasts and myofibroblasts also produce the major extracellular matrix (ECM) components; collagen, glycosaminoglycans, fibronectin and proteoglycans which support the formation of granulation tissue. Fibronectin, a large major glycoprotein is found throughout all phases of wound healing; from the fibrin clot, the papillary dermis, to the newly

synthesised collagen in the granulation tissue.[70-71] Being the primary constituent of the provisional matrix, fibronectin provides migratory cells with a provisional matrix for collagen deposition and myofibroblast driven wound contraction.[71] This provisional matrix is eventually replaced by a more mature matrix. Thus, prolonged expression of fibronectin in chronic wounds might be a contributing factor to delayed healing. Re-epithelialisation begins within hours with the migration of keratinocytes (stimulated by growth factors such as EGF and TGF- β) and epidermal cells from the wound edge to the temporary matrix. This process may last up to 3 months.

1.4.4 Tissue remodelling phase

The final phase of wound healing; the remodelling phase initiates within the third week after injury and may continue for years. Fibroblasts play a key role in this phase with the production of fibronectin, proteoglycans, hyaluronic acid and collagen all of which are essential for cellular migration.

The fibrin clot from the haemostasis phase forms the premature composition of the regenerated matrix. This is followed by a significant event in connective tissue healing which is the differentiation of fibroblasts into myofibroblasts. Myofibroblasts then synthesise glycosaminoglycans, proteoglycans, and other proteins which form a temporary framework for the new matrix.[72-73] Eventually, a stronger and more organised matrix made of collagen replaces this temporary matrix.[73]

Collagen type III is remodelled to type I while the ECM is remodelled to bear a resemblance to normal tissue (scar tissue). Apoptosis rids the healing wound of the cells that were necessary in the repair process but are no longer needed. Collagen fibres realign and form crosslinks resulting in greater tensile strength and less scarring. However, scar tissue only attain up to only 80% tensile strength compared normal skin[55], while excessive collagen synthesis produces a hypertrophic scar or keloid.

1.5 Types of wound healing

1.5.1 Primary healing (healing by first intention)

Wounds that cause loss of a moderate amount of connective tissue cells and underlying epithelial cells heal by first intention. These types of wound which are a result of e.g. a surgical incision only cause focal damage to the skin and thus the wound edges are easily approximated with staples, sutures or adhesive and within 12-24 hours of its creation, the wound is closed. These wounds heal through re-epithelisation with minimum scar formation.

1.5.2 Secondary healing (healing by second intention)

Wounds that occur as result of major trauma causing extensive tissue loss (e.g. burns) undergo secondary healing. Due to the great loss in tissue resulting in full-thickness wounds, new granulation tissue ingrowth is necessary for restoration of the integrity of the skin. Healing is first by contraction and then re-epithelisation.

1.5.3 Healing by tertiary intention

Dehisced or infected wounds are cleaned, debrided and observed for a prescribed period (typically 4 - 5 days) to clear up any infection and allow new tissue growth before approximating the edges.

1.6 Chronic wounds

Disruption of the complex healing process results in chronic wounds, with some wounds recurring frequently and others progressing by involving new surrounding tissue. Mediators such as inflammatory cells, growth factors and proteases influence the healing process while exogenous factors such as disease, immunocompromised state, malnutrition, smoking and radiation exposure also have a significant impact on wound healing.[74] In contrast to acute wounds, chronic wounds are not only unsuccessful in advancing through an orderly and timely reparative process but also advance without creating a sustained anatomic and functional result.[22]

Different aetiologies and the various and complicated pathophysiology of these wounds yield poor or inappropriate management. However, it is commonly known that chronic wounds get trapped in the inflammation phase that becomes a continuous cycle with mutual features

such as high levels of reactive oxygen species (ROS), repeated tissue injury, platelet derived factors e.g. TGF-β, proinflammatory cytokines e.g. IL-1β and TNF, ECM fragments, proteases, senescent cells, and in some cases a persistent infection. [75-78] These factors, although impairing chronic wound healing; serve an important role in acute wound healing. (**Figure 1.3**)

Oxygen is essential for cellular respiration and subsequently production of adenosine triphosphate (ATP) by cells; providing the high energy required for angiogenesis and regeneration of damaged wound tissue. The role oxygen plays in wound healing depends on whether the wound is in a hypoxic, normoxic or hyperoxic state. In general, the wound oxygenation state influences collagen deposition, fibroplasia, angiogenesis, resistance to infection, and epithelialisation. [79] The oxygenation state of wounds frequently guides some treatment planning such as the decision to amputate a limb.[80] It is important to note that both high oxygen levels and too little may delay healing. Chronic and nonhealing wounds are often hypoxic as a result of poor blood perfusion from sympathetically induced vasoconstriction. The hypoxic environment created by the ischaemia and consumption of oxygen by facultative bacteria is ideal for the proliferation of anaerobes.[81] Adequate vascularisation is vital for proper oxygenation and nutrient supply to the healing tissue in the wound bed. Reduced blood supply to the wound aggravates it. PMNs, when in contact with specific stimuli; produce ROS that are derived from oxygen e.g. superoxide anion ($O_2^{-\bullet}$); that are essential for defence against bacteria and other pathogens. [82-83] In fact, all wound healing stages may be regulated by ROS. However, in chronic wounds there are numerous sources of ROS, which may lead to elevated levels. Inflammatory cells accumulated inside the chronic wound produce high levels of ROS.

These enhanced levels of ROS may result in oxidative stress, which further destroys the ECM and prolongs the inflammatory phase gives rise to the nonhealing wound. High MMP levels suppress angiogenesis and cell proliferation while aggravating the proteolytic process and thus uncontrolled tissue degradation.

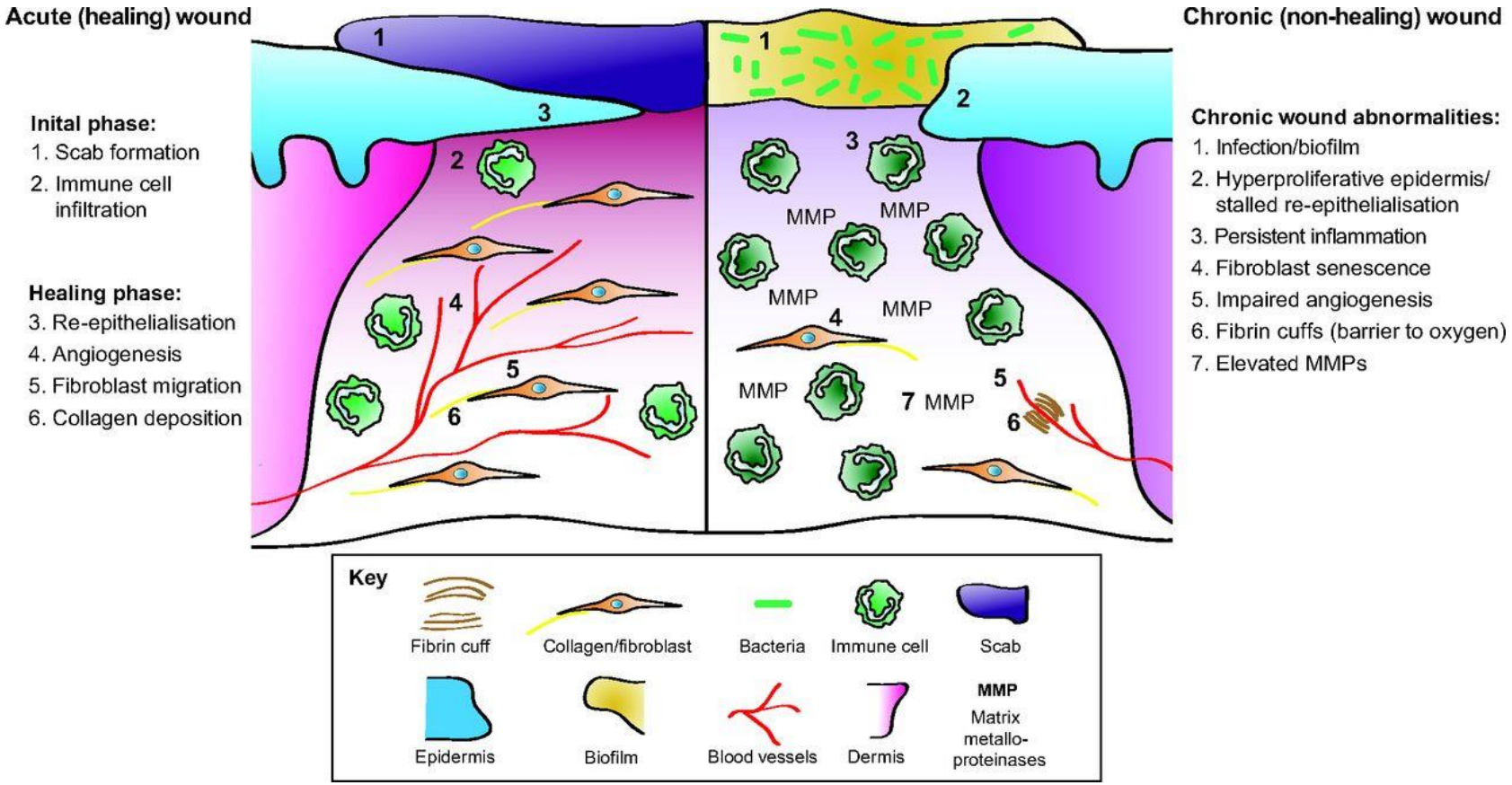


Figure 1.3 Cellular and molecular differences between acute and chronic wounds[84]

Acute wounds have a short-lived inflammatory response where granulation tissue is formed. Chronic wounds however, frequently present with stalled re-epithelialisation because of infection and persistent inflammation. Elevated matrix metalloproteinases (MMPs) together with poor infiltration of blood vessels and fibroblasts are a common occurrence. Open access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/3.0).

Activated PMNs and macrophages are the leading sources of proinflammatory cytokines (e.g. IL-1β) in wounds. The cytokines (TNF-α and IL-1β) are powerful mediators of various inflammatory processes at all phases of wound healing.[65] Wound macrophages also facilitate the phagocytosis of neutrophils during the proliferative and remodelling phase. In the early phases of healing, neutrophils facilitate effective decontamination and also produce a variety of growth factors that could promote revascularisation and repair of injured tissue. However, once decontamination is set, if neutrophils are not removed, they negatively influence repair, as they are capable of destroying healthy ECM components such as clotting factors, complement, immunoglobulins, and cytokines. Furthermore, neutrophils release collagenases and their inhibitors which prevent the accumulation of a collagen-rich matrix.[85] Delay in collagen deposition is also a common occurrence in DFUs where although collagen gene expression is elevated, there is decreased collagen deposition in the wounds because of the glycosylated collagen. Comparison of the changes in the ECM in DFUs and venous leg ulcers (VLUs) shows that these chronic wounds have in common the loss of collagen and elastin.[86]

Proteases hydrolyse the peptide bonds between amino acid residues in a polypeptide chain. During proteolysis, excess ECM components are edited, the ECM structure remodelled, and ECM assembly is regulated creating an equilibrium between ECM degradation and deposition.[87] Tissue metalloproteinase inhibitors (TMPI) inhibit and regulate protease activity in acute wounds; however, in chronic wounds there is overexpression of proteases and down regulation of TMPI[85] resulting in ECM and growth factor degradation, suppression of angiogenesis and cell proliferation while aggravating the proteolytic process and thus uncontrolled tissue degradation.

The elderly are also known to have higher incidences of chronic wounds. With advancing age, normal skin undergoes distinctive changes that have an effect on wound healing.[88] There are age-related alterations (**Table 1.2**) that influence wound healing during the human life span as well as susceptibility to conditions such as diabetes and vascular disease which adversely affect wound healing.[88] In contrast with neonates who after an injury are able to regenerate the lost tissue with minimum scarring, adult human wounds most often result in prolonged healing and scar formation.[89]

Table 1.2 Cutaneous ECM changes that affect wound healing over the human life span.[89-90]

Age	Properties
Foetal	Highly regenerative skin Large amount of cell mobility in a fragile ECM ECM rich in collagen III and hyaluronic acid Little inflammation in response to injury Scarless healing
Juvenile	Massive production of type I collagen Moderate and transient inflammation Cellular response in compliant ECM
Early adult	Scarring properties at maximum High production of type I collagen Fibrotic response in stiff ECM
Aged adult	Prolonged inflammation High matrix metalloproteinase and elastase expression Low expression of transforming growth factor beta Weakened cellular response in an atrophic ECM

Aged skin is more prone to cellular senescence than younger skin. *In vitro*, most cells have a finite life span before replication is arrested.[91] These cells are characterised by enlargement and spreading of the cells, an accumulation of lipofuscine, expression of senescence associated *β*-galactosidase (SA-*β*-gal), and an increase in polynucleation.[92] The above factors amplify the severity of wounds and may result in inadequate vascularisation, a lengthy inflammatory response, and a failure of re-epithelialisation.

The high significance of the ECM in wound healing is attributed to it being a major component of the dermal skin layer particularly in wounds that have a significant loss of tissue and cannot heal by primary intention. Scientific evidence has dismissed the notion that the ECM is only a scaffold that offers passive structural support for cells.[93] Some ECM interactions with cells are critical for cell regulation, adhesion, motility, growth, differentiation and even ECM synthesis. The mediation of ECM activities is regulated by the glycoprotein transmembrane receptors; integrins. Integrins are cell surface glycoproteins that have α and β subunit types that facilitate cell - ECM adhesion. In chronic wounds, there is defective ECM composition and remodelling. The provisional fibronectin matrix formed during proliferation is replaced by a

more mature matrix in normal wound healing. Consequently, prolonged expression of fibronectin in wounds might be a cause of delayed healing.

Prevalence of chronic wounds varies depending on diagnosis, year and country. As with other diseases, it is worth noting that in developing countries, due to lack of proper nutrition and medical care, chronic wounds have a higher prevalence.[94] Statistics show that Canada has an estimated 4-7% cases[35]; Germany: 1.03 - 1.05%[36]; China: 1.7%[37] and 0.45% in the Indian population.[38]

1.6.1 Types of chronic wounds

The American Wound healing Society (WHS) identifies four types of chronic wounds; DFUs, VLUs, arterial insufficiency ulcers and pressure ulcers.[89] Evidently, chronic wounds frequently result from an underlying pathologic condition.[95]

1.6.1.1 Diabetic foot ulcers

The International Consensus on the Diabetic Foot defined the DFU as a 'full-thickness wound below the ankle, in a diabetic, regardless of the duration'.[96] A purulent discharge, fever, vasculopathy, neuropathy, foul smell, osteomyelitis, cellulitis, gangrene and crepitus are all common in DFUs.[97]

Hyperglycaemia, a biochemical abnormality may accelerate neuropathy and vascular disease, thus inducing vascular damage through any of four pathways: (1) enhanced polyol activity leading to accumulation of fructose and sorbitol; (2) augmented glycation end product formation; (3) nuclear factor KB and protein kinase C activation; and (4) increased hexosamine pathway flux.[98] The process of high production of superoxide by the mitochondrial electron-transport chain activates all these harmful metabolic events. Thus, oxidative stress as a result of hyperglycemia is a contributing factor to the pathogenesis of diabetic complications,[99-100] including DFUs. Inflammatory cytokines and susceptibility to infection and other factors also contribute to the pathophysiology of DFUs. An estimated 15% of diabetic patients form DFUs.

DFUs can be classified into three groups: neuropathic, ischaemic or a combination of the two which is neuroischaemic.[101] Ischaemia leads to a lack of oxygen and nutrients[74], ultimately tissue necrosis therefore a common risk factor for amputation.

Due to their complicated pathophysiology and slow healing, DFUs are associated with long term disability and premature mortality.[102]

Neuropathy plays a significant role in the pathophysiology of most DFUs, with more than 60% of DFUs estimated to occur as a result of underlying neuropathy.[103] Neuropathy causes muscle weakness and numbness which allow the foot to be subjected to abnormal loading which the skin is unable to withstand because of diabetic ischaemia.[104] The resultant callus leads to further abnormal loading and oftentimes subcutaneous haemorrhage, which may eventually become infected. The infection complicates both neuropathy and ischaemia, thus increasing the risk of amputation of the limb.[105]

In order to control the diabetes epidemic and as a result effectively treat DFUs and reduce the number of amputations, prevention and early intervention is critical. Once a foot ulcer develops, the patient should be assessed for neuropathy and arterial blood supply to facilitate healing and if infection is present, it should be treated appropriately. Management of diabetic ulcers, includes debridement, restoration of arterial circulation to the limb to ensure adequate oxygen and nutrient delivery to the ulcerated area, application of medication and dressings, taking pressure off the area and administration of systemic antibiotics (if there is an infection) and improving plasma glucose control.

1.6.1.2 Venous leg ulcers (VLU)

Although the pathophysiology of venous ulcers remains ambiguously defined, they are characterised by venous incompetence and consequently venous hypertension, dysregulation of various cytokines, excessive deposition of fibrin around capillary beds.[106] With a 1% global prevalence, VLUs are the cause of up to 80% of the known leg ulcer cases.[107] The most common risk factors include female sex, increase in age, trauma, obesity, immobility, deep vein thrombosis and phlebitis.

Physical examination of the ulcers presents a shallow and irregular wound with the presence of granulation tissue and fibrin at the base of the ulcer. The study of the cellular infiltrate and ECM of chronic VLU and DFU compared to acute wounds showed that the chronic wounds had lower numbers of $CD4^+$ T cells, significantly higher B cells, plasma cells and macrophages compared to acute wounds.[108]

Venous ulcers are managed by elevation of the affected limb, compression therapy to correct impaired venous return, antibiotic treatment if necessary and most importantly constant monitoring of the ulcer by wound specialists. As already mentioned, wounds that fail to heal within four weeks are considered chronic. In the case of venous ulcers, it is recommended to reassess their pathophysiology and treat them further with topical or systemic agents.

1.6.1.3 Arterial insufficiency ulcers

Arterial insufficiency ulcers, frequently known as ischaemic ulcers are commonly caused by microangiopathy and macroangiopathy resulting in poor perfusion to the lower extremities. Insufficient oxygen and nutrient supply if left untreated ultimately lead to tissue necrosis. Characterised by deep wounds extending into the underlying tendons, these wounds are commonly deficient of new tissue growth. They present as black, yellow, brown or grey wounds that do not haemorrhage when debrided.

Like DFUs, arterial insufficiency ulcers of the lower limb increase the risk of limb loss. Like other chronic ulcers arterial insufficiency ulcers can be treated by debridement, management of infection, revascularisation and management of a moist wound environment.[109]

1.6.1.4 Pressure ulcers

The National Pressure Ulcer Advisory Panel defines chronic pressure ulcers (now known as pressure injury) as localised injury to soft tissues usually occurring over a bony prominence as a result of pressure and/or shear or friction.[110] The common predisposing factors are impaired mobility and exposure to undisturbed pressure for long periods. Patients, such as those with spinal cord injuries or bed-ridden patients who are unable to change positions on their own are at highest risk.

As with other chronic ulcers, management of these ulcers involves offloading, debridement to remove necrotic tissue, infection management and maintenance of a moist wound environment.

1.6.1.5 Tropical wounds

Tropical ulcers, also called phagedenic ulcer or malabar ulcer are unique to tropical and subtropical areas. They are large, infected (polymicrobial infection) ulcers that develop on skin predisposed to trauma (e.g. a leg with pre-existing sores, scratches or abrasions).

Communities with poor hygiene, malnutrition, underlying chronic diseases, low socio-economic conditions, poverty and lack of protection from insect bites are more susceptible to tropical wounds. [111]

The wound presents usually as a solitary ulcer with raised edges, foul-smelling discharge and oedema in the surrounding tissues. [112] It also features extensive necrosis of the skin, subcutaneous tissue, gangrene of muscles, and even osteomyelitis. Spontaneous healing is possible but in some cases the wounds are aggravated by superinfection with *Fusobacterium*, spirochetes and/or anaerobic microorganisms [113] resulting in deep wounds that penetrate into muscles, tendons, and bone. Infection control with systemic antibiotics is frequently indicated for these ulcers.

1.7 Full-thickness wounds

Full-thickness wounds are a consequence of great damage to the skin resulting in deep wounds that heal by simultaneous contraction and re-epithelialisation. They present with loss of tissue extending below the dermis into the subcutaneous tissues (**Figure 1.4**).

The pathophysiology of full-thickness wounds is diverse, but the extent of tissue damage is similar in these wounds. These wounds are deep and differ significantly from other wounds in appearance, yet the mechanism of wound healing proceeds through the same phases of healing viz haemostasis, inflammation, proliferation and tissue remodelling. [60]

If not complicated by underlying disease, full-thickness wounds will heal naturally by secondary intention.[114] However, it is imperative to take into consideration the location of the wound and the functionality of the tissue after healing. In areas where scarring could reduce the functionality, repair of the damaged tissue is a necessary intervention to minimise scarring and unwanted effects.

Deeper wounds re-epithelialise slowly and frequently require autologous skin grafting to prevent extensive scarring that results in reduced cosmetic and functional outcomes. The first line of treatment of these deep wounds includes the use of epidermal sheets or split-skin mesh grafts. However, suitable autologous skin grafts may not always be available, thus laboratory engineered skin substitutes have become a viable alternative.

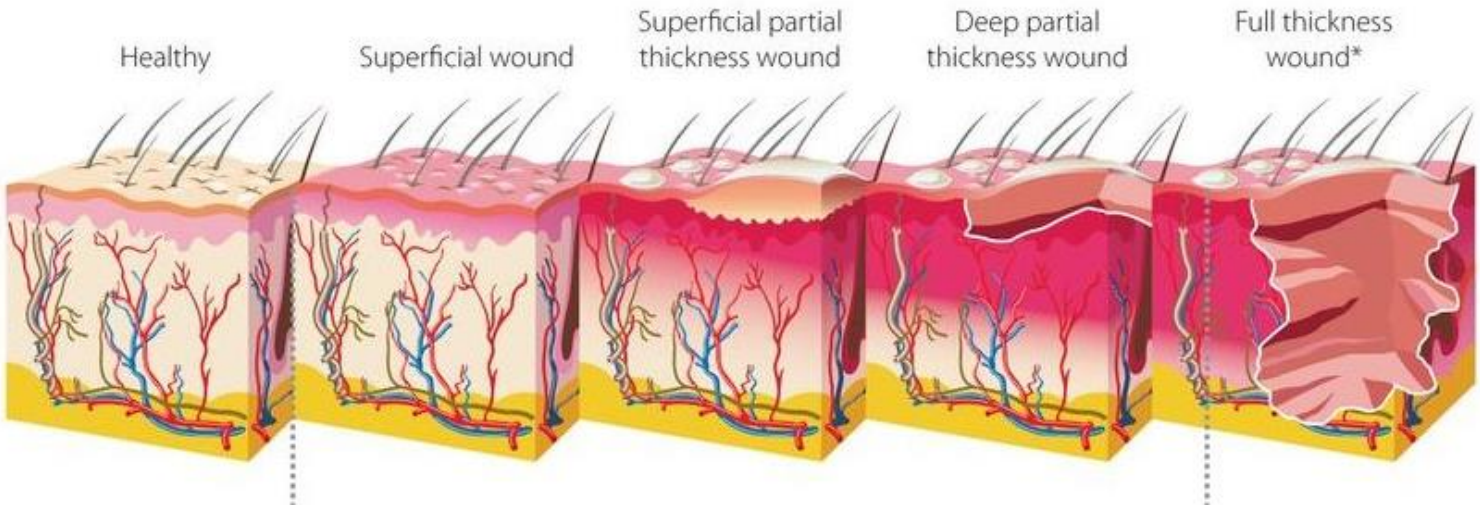


Figure 1.4 Wound classification according to the depth of the wound in relation to skin layers.[115]
Full-thickness wounds extend deeper than the epidermis and dermis right into the subcutaneous fat and in some cases up to the bone. They present as deep wounds with excessive tissue loss.

1.8 Keloids and hypertrophic scars

Scars are anticipated consequences of wound healing. Conversely, some wounds undergo excessive/dysfunctional scarring which results in the formation of keloids or hypertrophic scars. This is a consequence of excessive synthesis of connective tissue and excessive angiogenesis during the wound healing process. In mammals, the most important outcome of wound healing is restoration of the skin's protective and functional integrity, as rapidly as possible, rather than restoration of complete biological function[116] often resulting in scar tissue that lacks sweat and sebaceous glands, hair and melanocytes.[116]

Histopathologically, the differentiation between hypertrophic scars and keloids is difficult. While normal skin contains distinct collagen bundles that run parallel to the epidermis, collagen bundles in hypertrophic scars are flatter, less demarcated, and are arranged in a wavy pattern, although, still orientated to the epithelial surface. In keloids, collagen bundles are virtually non-existent, and the fibres lie in loose, haphazardly orientated sheets.

The management of wounds takes into account the outcome that comes from the use of the treatment option selected in relation to the specific wound treated. An example, as mentioned in **Section 1.7**, is the requirement of autologous skin grafting for extensive full-thickness wounds to prevent scarring that results from contraction over a large surface. The outcome-based approach of wound healing could decrease the potential for pathological scarring that could require further interventions.

1.9 Wound dressings

1.9.1 The history of wound healing

It is said that the history of wound healing is as old as history itself.[117] Beginning with the caveman who tended to his wounds acquired in battle; to the ancient Egyptians, Greeks, Romans and other ancient civilizations who used magical incantations, ointments and potions; wound healing evolved to a methodical script of wound care and surgery from Hippocrates and Celsus.[118] It is notable that some of the principles of wound healing applied prehistorically are still useful today.

The earliest recorded manuscripts related to wounds were papyri attributed to the ancient Egyptians from around 3000 BC; where honey was recorded as a wound healing agent. Other

records are the clay tablets discovered in Mesopotamia; dating back to about 2500 BC. The Edwin Smith papyrus, dated about 1700 BC, unquestionably is one of the most significant known 'surviving scientific treatise'.[119] Forty-eight wounds or trauma cases and their treatment were described in descending anatomical order. The Egyptians also used bandages made of linen with texture varying from the finest silk-like gauze to a canvas-like coarseness to cover and keep in place any medications applied.[120] In addition to being masters in applying and arranging bandages, the ancient Egyptians recognised the basic signs of infection and inflammation. The Ebers papyrus, also known as the most famous plant medicine "encyclopaedia" of ancient Egypt described and recommended several herbal remedies like *Aloe vera* and acacia which were used to treat skin diseases.[121]

The Greeks stressed the importance of handling wounds hygienically. They used boiled clean water, wine or vinegar (acetic acid) for cleaning the wounds. It was however only in the 19th century that the surgeon, Joseph Lister stressed the importance of antiseptic treatment and prevention of infection.[122] This was after he observed that pyaemia, erysipelas and gangrene were prevalent in open wounds of patients in some parts of the hospital building, especially those on the ground floor. At some point, the mortality in the male accident ward on the ground floor was so excessive that they shut down the ward and investigations led to the finding of a foul drain. In contrast to this, Lister showed that where the 'antiseptic system' was used, no cases of erysipelas, pysemia or hospital gangrene reported. [122]

The Romans were the first to describe the four cardinal signs of inflammation—*rubor, tumour, calor, et dolor* (redness, swelling, heat, and pain). [123] It is notable that some of the principles of wound healing applied prehistorically are still useful today.

1.9.2 Wound dressings; current trends

With over 6000 wound dressings currently available, the wound dressing market has shown considerable progress. However, only a few dressings have all the characteristics of an ideal dressing. It is important to use the wound environment as a guide to selecting a suitable wound dressing.[124]

As cited in **Section 1.9.1**, before the 1960s, wound dressings were considered to play a passive role in wound healing[125], creating a dry scab which functioned as a protective barrier against

bacterial contamination and also aided exudate absorption. Studies by Winter *et al* in the domestic pig showed that a moist wound environment significantly augmented re-epithelialisation rates when compared to wounds exposed to the air.[27,126] These findings were substantiated by Hinman and Maibach[127] who later did a parallel study comparing air exposed experimental split thickness wounds to wounds occluded with a polyethylene film in humans. As expected, occluded wounds re-epithelialised significantly faster than air exposed wounds.[127]

A moist wound environment ensures: improved interaction of growth factors and their target cells, accelerated angiogenesis, inhibits tissue dehydration and cell death, improves breakdown of dead tissue, and provides a significant reduction in pain.[128] The belief that the moist wound environment provided a medium for bacterial growth, was dismissed by a review of over 100 studies comparing wound infection in occlusive with non-occlusive dressings. [129] The results showed the overall infection rate under conventional dressings was 7.1% compared to 2.6% in the wounds under occlusive dressings. [129] These findings heralded the development of modern wound dressings.

Progression of research has brought an in-depth understanding of the underlying cellular and molecular mechanisms of wound healing, thus wound dressings have advanced from being passive to active material that manipulates the wound environment into an optimum milieu conducive for healing.[130] However, 50% of the wound dressing market is still accounted for by traditional dressings such as bandages, woven and non-woven sponges, etc.[131] with some of them modified and/or medicated. Various strategies are currently employed for enhancing the healing of wounds, ranging from skin grafts, to the inclusion of growth factors and gene delivery as well as cell therapy. Modern wound healing devices are continually progressing.

1.9.2.1 Gauze

Gauze is a timeless wound dressing material that encompasses woven or non-woven material. It was used for several wound types; from the dry wound to packing bleeding and exuding wounds. The common belief was that a dry wound environment was conducive for healing as it supposedly stalled the growth of bacteria.[132] Notwithstanding published evidence of the benefits of a moist wound environment, gauze is still commonly used worldwide. Several studies have even shown that modern dressings such as foam, hydrocolloids, etc. affording

superior healing, have lower infection rates, greater patient satisfaction and are overall more cost effective compared to gauze, as they require less frequent changes.[133] As cited above, modern wound management takes the wound environment into consideration to determine the suitability of the wound material selected. Traditional plain gauze dressings were known to adhere to the wound bed and thus result in trauma and bleeding upon dressing removal.[132,134] Consequently, impregnated gauzes were then developed to provide low adherence, antiseptic properties and moisture retention for the wound. The first modern low adherence dressing to be made was 'tulle gras'; developed in 1915 during World War I by the inventor Lumiere. This was gauze fabric impregnated with paraffin and balsam of Peru.[135] To date, gauze has also been impregnated with iodine, zinc, or petrolatum to avoid desiccation and afford nonadherence to the wound bed.[136]

Sood *et al* proposed three comprehensive wound dressing categories, each with a specific purpose.[134] The first interacting with the tissue itself, thus facilitating autolytic debridement. Wound dressings that regulate moisture level of the wound bed are in the second category, while the third category consists of dressings that control bacterial load.[134] Although the classes are sometimes not mutually exclusive, this classification is useful.

1.9.3 Wound dressings facilitating debridement

Foreign material in wounds promote an environment conducive for infection, extending the inflammatory period, and subsequently delaying contraction and epithelialisation of the wound.[137] Key to wound management is the use of debridement to prepare the wound bed for re-epithelialisation. Initially, wound care is aimed at reducing the incidence of foreign material, bacterial load, and damaged or necrotic tissue.

For ages, many methods of debridement were used. Mechanical debridement using sharp equipment has the disadvantage of lacking specificity for devitalised tissue. Surgical excision has the disadvantage of the trauma it causes, and the risk associated with the use of anaesthesia. Both surgical and mechanical debridement have increased risks of bleeding. A few modern wound dressings were designed to promote autolytic debridement by creating an environment conducive for proteases within the wound to liquefy necrotic tissue. Typically, a process that promotes natural wound debridement by endogenous phagocytic

cells and proteolytic enzymes. The highly selective method of autolytic debridement is indicated for necrotic, non-infected wounds.

Other wound dressings promote enzymatic debridement while biologic debridement using maggots is used with success in some refractory chronic wounds. Conversely, one debridement method may not be suitable/adequate for some wounds, e.g. infected wounds may need antibiotics while some wounds may require surgical debridement. The clinician who examines the wound determines the debridement method to use. Characteristics such as patient commodities, time, resources and available skills all impact the debridement method selected.[138] In many cases, a suitably qualified clinician is not always available to perform mechanical debridement, making autolytic/enzymatic debridement an important alternative. [138]

1.9.3.1 Hydrogels

A common debriding agent, hydrogel is a network of semisolid, hydrophilic polymers that are chemically or physically cross-linked to give a soft contour following three-dimensional structure.[139] Available as either amorphous or sheet hydrogels; they keep the wound environment moist[140] while their hydrophilic properties allow them to absorb large volumes of exudate without dissolving because of the crosslinks between their network chains.[141]

Research data comparing hydrogel dressings with other dressings shows that debridement followed by use of hydrogel is more effective and less complicated compared to standard wound care. Dumville and colleagues showed that hydrogel dressings heal lower grade DFUs more effectively than basic wound contact dressings.[142] However, comparison of debridement efficacy of collagenase vs. hydrogels shows that enzymatic debridement is superior to hydrogels in the debriding efficacy[138] Hydrogel dressings are important for promoting a moist wound environment in dry wounds and helps remove excess moisture in weeping wounds.

1.9.3.2 Hydrocolloid dressings

A combination of pectin, carboxymethylcellulose and gelatine[143] (e.g. Granuflex®), hydrocolloid dressings are widely used to absorb relatively large volumes of exudate and facilitate autolytic debridement [144] in sloughy wounds. However, there have been higher incidents of infection with their use, with Foster and colleagues even concluding that

hydrocolloid dressings should never be used on deep, infected, exudating diabetic wounds.[145] A podiatry study at the University of Huddersfield, concludes that the use of hydrocolloid dressings is controversial[146] while a review by Dumville and colleagues presented no research evidence proving that hydrocolloid wound dressings are superior to other types of dressing in healing DFUs.[147] Besides autolytic debridement, other advantages of hydrocolloid dressings include soothing of pain; autolytic debridement encouraged by the gel, the dressing can be retained on the wound for up to a week and easy removal without causing wound trauma.

1.9.3.3 Enzymatic dressings

Collagenase and proteolytic enzyme preparations are significant instruments in debridement of wounds as they break down non-viable tissue. Collagenase is specific for collagen and elastin and thus will not digest fibrin. It has been used for decades for enzymatic debridement.[148] These enzymes ultimately result in collagen fragments that are chemotactic for fibroblasts and macrophages. In addition to faster debridement, Palmieri *et al's* study showed that enzymatic debridement using Iruxol mono® (a collagenase wound ointment) gave a quicker reduction in inflammation, enhanced granulation tissue formation and improved epithelisation compared to the placebo dressing that comprised of liquid paraffin and white soft paraffin.[149] Even when compared to hydrogel, collagenase's significant debridement efficacy was apparent.[138]

1.9.3.4 Maggot wound therapy

Biological debridement using live, sterile larvae of the green fly (*Lucilia sericata)* is effectively employed in large wounds where painless debridement is required. Currently regulated and approved by the US Food and Drug Administration (FDA) since 2004[150], the use of maggots in wound healing is effective for necrotic wounds such as diabetic ulcers[151], pressure ulcers[152], venous ulcers[153] and postoperative wounds. Maggot wound therapy (MWT) significantly reduces treatment costs, healing times[154] and increases granulation tissue proliferation, while eradicating methicillin-resistant *Staphylococcus aureus* in infected wounds.[155]

The mechanism of action includes direct ingestion of necrotic tissue by maggots, leaving healthy tissue that promotes proliferation of granulation tissue. Maggots seem to indirectly stimulate healing by secreting protease inhibitor resistant digestive enzymes

(chymotrypsin 1) which contain serine proteases, collagenase, leucine aminopeptidase and carboxypeptidase enzymes.[156]

Maggot therapy is a highly effective debridement technique; however, it does not replace surgical treatment of large wounds, those that require amputation and those with large amounts of necrotic tissue.[156]

1.9.4 Wound dressings regulating moisture levels

Winter's studies showed that a moist environment is pivotal to wound healing as it increases the rate of epithelialisation.[27,126] Most modern wound dressings were developed to promote a moist wound environment in addition to other factors of wound healing, e.g. hydrogels and enzymatic dressings provide moisture while promoting wound debridement.

Wound exudate is a liquid containing high amounts of protein, nutrients, electrolytes and inflammatory cells. Increased permeability and blood vessel dilatation as a result of the inflammatory response leads to high levels of exudate. In acute wounds, wound exudate is a crucial constituent of the reparative process.[157] However, wound exudate is often believed to negatively impact wound healing. Deeper understanding of the constituents of exudate, shows that an exuding wound at optimum levels provides the wound with moisture, proteinases for autolytic debridement, tissue inhibitors of matrix metalloproteinases (TIMP) to regulate the autolytic debridement and cytokines.[157] High amounts of wound exudate however lead to maceration of surrounding tissues further impact the wound negatively. Thus, regulating the amount of exudate in a wound is key to effective wound management.

1.9.4.1 Foam dressings

Foam dressings are best suited for moderate to highly exuding wounds to facilitate faster healing.[135] They induce a moist wound environment, provide thermal insulation, have increased absorbency, promote gaseous exchange and increase hyaluronan synthesis in the epidermis which also promotes healing.[158] Commonly made from polyurethane or silicone, foam dressings can be used alone or in combination with an outer layer that acts as an antibacterial layer.

A review on foam dressings, showed that foam dressings do not have an advantage over other dressings in the healing of DFUs.[142] However, after consulting with several wound care

specialists, this study found that foam dressings were a dressing of choice for most full-thickness wounds because of their ability to absorb high quantities of exudate and to conform to the different wound shapes and aid in offloading pressure. They are suitable during the inflammatory phase especially after debridement. Foam dressings however, if left on the wound too long, can cause maceration of the wound due to absorbing large amounts of exudate, thus they need to be changed frequently.

1.9.4.2 Alginates

Alginates are gel-forming polysaccharides. Their mechanism of action involves absorbing exudate leading to the formation of a moist gel on the surface of the wound that facilitates healing of the wound. They are highly absorbent and can be packed into the wound easily; therefore, they are normally used as a primary layer on the surface of the wound, under a more absorbent dressing. Although these dressings are very effective for heavily exuding wounds,[159] there is no research evidence suggesting that they are more effective in healing chronic ulcers than other types of dressing.

1.9.5 Wound dressings controlling bacterial load

Wound dressings loaded with antimicrobial agents such as silver, ciprofloxacin,[160] zinc oxide[161] and mupirocin are viable alternatives to diminish wound bacterial colonisation and infection. Other therapies such as honey and silver sulphadiazine are already extensively used.

1.9.5.1 Honey

Since the beginning of time, honey has been known to have antimicrobial properties. Substantial evidence, both *in vitro* and *in vivo* has supported the positive wound healing effects of honey. With potent antibacterial activity, honey provides a moist wound environment favouring autolytic debridement. Furthermore, its viscosity protects the wound from additional bacterial colonisation. [162] Its antibacterial activity is even effective against multi-drug resistant bacterial such as *S. aureus*, *Acinetobacter baumanni, S. typhi, E. coli* and *P. aeruginosa*.[163]

Initially the limitation to the use of honey was licencing; however, there are many licenced sterile honey formulations now available for use.[162] Melladerm®, a modern honey gel, was found to heal wounds 26% faster than other high tech dressings.[164]

1.9.5.2 Silver

Silver is widely used for its broad antibacterial properties. Most commonly incorporated in creams e.g. Flamazine (10mg/ml silver sulphadiazine), these dressings are favoured for burn wounds. *In vitro* assessments of the activity of silver sulphadiazine against 409 bacterial strains showed activity against multi-resistant bacterial species such as methicillin-resistant *S. aureus* and *Acinetobacter spp*.[165] Slow release silver has recently been incorporated into wound dressings like actisorb™ and acticoat™.[166]

1.9.5.3 Mupirocin

Mupirocin (pseudomonic acid A), first isolated in 1971 from *Pseudomonas fluorescens,* is a potent topical bactericidal agent with *in vitro* activity against gram-positive bacteria that cause skin infections. Gram-negative bacterial are generally resistant to mupirocin. [167] Commonly marketed as Bactroban®, its mechanism of action involves inhibition of isoleucyl-transfer RNA synthetase, thus inhibition of RNA synthesis and bactericidal protein synthesis.[167]

Initially, mupirocin had activity against methicillin-resistant *S. aureus;* however, recent evidence has shown increasing bacterial resistance especially with repeated use.[168]

1.10 Skin grafts

Skin grafts are indicated for deep wounds with extensive tissue loss. The first skin graft was performed by Jaques-Louis Reverdin in 1869.[169] Since then, this technique has undergone significant development including the use of fibrin glue/tissue adhesives to approximate the edges.

The skin grafting procedure generally involves obtaining autologous skin from a donor site with minimum damage. After harvesting and transferring to the desired location, grafts do not have their own nutrient and blood supply and as a result, depend fully on the wound bed's nutrient and blood supply via absorption and diffusion respectively. Within the few subsequent days after placement, an intact vascular supply growing from inside the bed is

normally established.[114] Although grafts are frequently used to prevent the contracture observed in secondary intention healing, they do however demonstrate contracture although to a lesser extent. [114]

Skin grafting causes considerable pain for both the injury site and the donor site. Biomaterials are thus a desirable alternative for wound management in wounds with excessive skin loss.

1.11 Characteristics of the ideal wound dressing

No single dressing addresses all the ideal properties for optimal wound healing. Physical protection from external factors and conditions are a common feature of wound dressing materials. The ideal dressing should maintain some moisture, remove excess exudate, be impermeable to bacteria, should not delay healing, be free of particles or any contaminants, be non-allergenic and non-toxic, protect the wound from further trauma, thermally insulate the wound, allow gaseous exchange, be comfortable and also conformable, allow changing without causing further trauma and be able to release pharmacological agents to the wound.[145]

Not one single dressing has all these characteristics. Oftentimes a combination of wound dressings is used at the different stages of the wound healing cascade.

1.12 pH and wound healing

The pH of wounds which is a value expressing the negative logarithmic scale for the concentration of H^+-ions in the interstitial environment of wounds, has both a direct and indirect influence on all biochemical reactions taking place. The pH value is therefore an important factor for metabolism during wound healing and thus a significant parameter for therapeutic interventions in wound-care.[170] Healthy skin under normal conditions has a pH ranging between 4 and 6 which plays a critical role in the skin's barrier function. Different pH values are recorded for different stages of healing. In chronic wounds, the pH changes from alkaline to acidic as the wound heals.

For wound dressings, an acidic pH has been shown to have antibacterial and antifungal properties. A study by Basavraj *et al* showed that an acidic wound milieu resulting from the use of acidic wound dressings such as acetic acid and citric acid helps in wound healing by

increasing antimicrobial activity, releasing oxygen, controlling wound infection, altering protease activity, reducing toxicity of bacterial end products, and enhancing epithelialization and angiogenesis.[171] It follows that wounds with a high pH heal slower than wounds with a slight acidic environment and that the pH of chronic wounds decreases as the wound heals. These and other findings with regards to pH of wounds, should guide treatment selection.

1.13 Biomaterials as wound dressing material

The first ever biomaterial recorded in history were sutures used by the ancient Greeks, Indians, Romans and the Egyptians. The material used ranged from silk, hair, wool threads, tendons to plant fibres and later the discovery of the absorbable catgut in the 1960s.

Over the past three decades, a number of investigations have been done on wound healing material and technology with biomaterials emerging as excellent material for wound healing. Biomaterials are biocompatible, biodegradable, non-toxic and bioactive. There are several commercially available wound dressing biomaterials made from natural or synthetic polymers or a mixture of both.[172] These materials include tissue-engineered products such as collagen[173], chitosan[174], sericin[173], starch, alginates[8], hyaluronic acid and many others. Chitosan is the most extensively used due to its biocompatibility and biodegradability and almost unlimited supply of medicinal quality starting material.

Chitosan is structurally similar to hyaluronic acid (**Figure 1.5**) which is a common ECM component.[175] Hyaluronic acid, a glycosaminoglycan plays a vital role in the structure and organisation of the ECM.[176] Prominence of this polysaccharide is seen whenever prompt proliferation, regeneration, and repair of tissues is needed. [176]

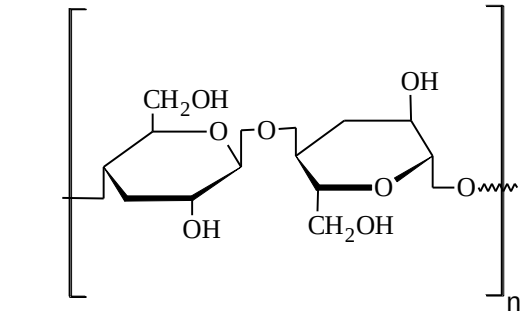
1.13.1 Chitin and chitosan

In 1811, Henri Braconnot, a Frenchman who was director of the national botanical garden at that time, discovered a material in mushroom that would not dissolve in sulphuric acid. [177] He named this material 'fungine'. A few years later the term 'chitin' was coined after similar material was isolated from beetle cuticles by another French scientist.

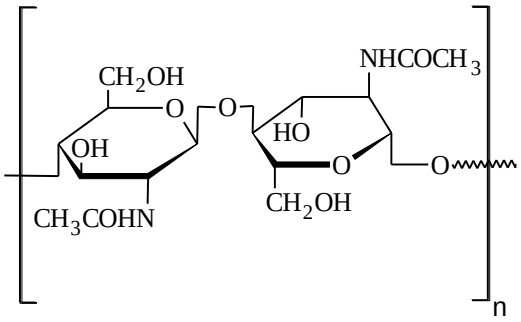
Chitin forms structural components of the exoskeletons of fungi and arthropods and naturally presents itself as an organised crystalline. Chitin essentially means 'the skeletal material of invertebrates'. An insoluble, inelastic, hard, white linear polysaccharide occurring naturally in

the crystalline state, chitin is composed of *β*-(1→4)-linked *N*-acetyl-D-glucosamine (d-GlcNAc) residues. The chitin chains are organised in sheets which are tightly bonded by a number of intra-sheet and inter-sheet hydrogen bonds[178], giving a highly hydrophobic semi-crystalline material that is insoluble in aqueous acetic acid and most organic solvents.[179]

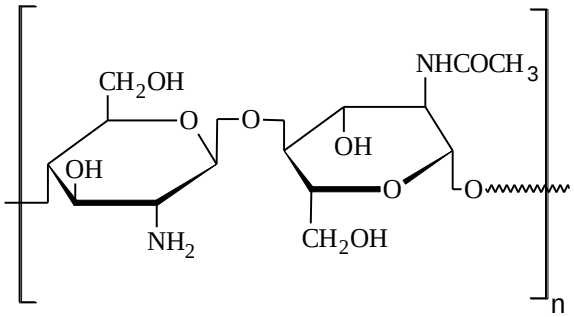
The chitin structure closely resembles cellulose (**Figure 1.5**), with chitin having the acetamide group (-$NHCOCH_3$) in place of the hydroxyl group (-OH) at the C2 position. Like cellulose, chitin has low chemical reactivity and is not freely soluble in water or organic solvents. It is the deacetylated derivative; chitosan that is used abundantly for biomedical applications.



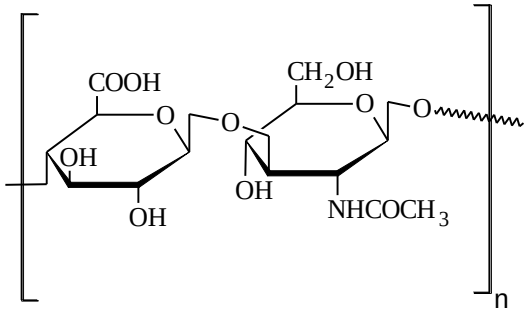
Cellulose



Chitin



Chitosan



Hyaluronic acid

Figure 1.5 Structures of cellulose, chitin, chitosan and hyaluronic acid

Despite its wide abundance, chitin is mostly collected from the shrimp and crab canning industry and processing involves demineralisation followed by deproteinization, decolourisation and deacetylation[180] (**Figure 1.6**). The natural source and biological function of chitin determines its structural form; which is either an *α*, *β* or *γ* allomorph that can be characterised by infrared and solid-state NMR spectroscopy, and X-ray diffraction.[181-182] The most stable and abundant form is *α*-chitin which is found in nematode and rotifer egg shells, arthropod cuticles and hydrozoan calyces. *β*-chitin occurs in cuttlefish bone, the squid pen, mollusc and brachiopod shells and the pogonophora tubes.

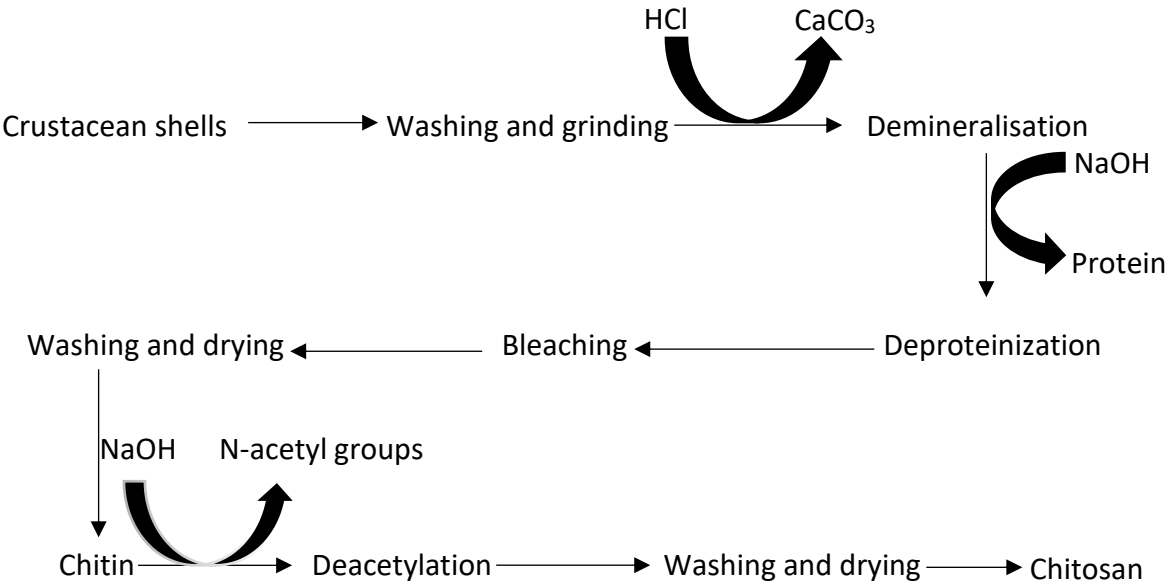


Figure 1.6 Chemical production of chitosan from chitin.[180]

Chitosan exists naturally in some microorganisms and in the cell wall of some fungi *(Mucoraceae).* However, commercial chitosan is produced from the partial deacetylation of chitin to chitosan through alkali *N*-deacetylation[183-184], enzymatic hydrolysis in the presence of a chitin deacetylase or by steam explosion. Chitosan is a copolymer of *N*-acetylglucosamine and D-glucosamine coupled through β-(1→4) glycosidic bonds, which when depolymerised gives a compound with lower molecular weight (MW)[185] and lower viscosity. It is also defined as a group of deacetylated chitins with an acetylation value of < 40% and nitrogen content > 7%.[186] The deacetylation of chitin into chitosan occurs in a highly concentrated base (NaOH) at high temperatures. Despite these harsh conditions, chitosan remains a semi-crystalline polymer.

Chitosan's NH_2 and OH groups result in a compound with high chemical reactivity; thus, the versatility of chitosan depends on its degree of deacetylation. Chitosan contains amino groups which strongly complex various species, such as metal ions, and are often used for waste water treatment, to remove heavy metal contaminants.[178] Several reports have mentioned chitosan's mucoadhesive[187], haemostatic[188], antifungal and antibacterial properties. Its excellent mucoadhesive properties result in chitosan being widely used in developing drug delivery systems. The mechanism for chitosan mucoadhesion as explained by Sogias *et al* appears to be through electrostatic attraction, hydrogen bonding and hydrophobic effects.[187] Other scholars reported that chitosan's positively charged amino groups and the negatively charged moieties of the mucus gel layer interact via hydrogen bonds and ionic interactions.[189]

1.13.1.1 Deacetylation degree of chitosan

The deacetylation degree (DD) is defined as the ratio between acetylated (GluNAc) and non-acetylated (GluN) glucosamine units. It is a process that involves nucleophilic substitution at the primary acetamido group of chitin, resulting in chitosan that has free amino groups ($-NH_2$) with deacetylation ranges from 40 to 98%.[190]

The DD is an important factor that determines various biological and physicochemical properties of chitosan, e.g. toxicity[191], degradation, crystallinity, solubility and hydrophilicity. Chitosans with higher DD have more positively charged amine groups when dissolved in acid solution compared to chitosans with a lower DD[192] and this has a direct effect on the biological activity of the chitosan. Schipper and colleagues compared eight chitosans with varying DD and MWs. Their results showed that chitosans with low DD displayed a clear dose-dependent toxicity, while those with high DD showed low toxicity.[191] Other studies indicated that the radical scavenging activities of chitosans,[193] and the effect on fibroblast proliferation and collagenase activity[194] depend on the degree of deacetylation and concentration.[193] Prasitsilp and colleagues' study showed that their high DD chitosans regardless of the source (shrimp or cuttlefish) supported adhesion and proliferation of two different cell-lines (L929 and BHK21) under investigation; while the lower DD chitosans did not favour this attachment of these cells.[195] It is thus important to accurately determine the DD of each chitosan sample used.

Various methods[196] are used to measure the DD of chitosan including: IR spectroscopy, UV spectrophotometry, X-ray diffraction, colloidal titration, potentiometric titration, circular dichroism (CD), ninhydrin assay, NMR spectroscopy, elemental analysis, gel permeation chromatography, conductimetry and *N*-acetyl group hydrolysis.[196] Most of the methods mentioned above are too costly for routine analysis, time consuming or destructive to the sample. However, the ^{1}H NMR spectroscopy has proven to give more accurate results with the use of less resources.[197]

1.13.1.2 Wound healing properties of chitosan

Chitosan derivatives in the form of gels, sponges, powders, scaffolds, nanoparticles or films have been used successfully to accelerate wound healing.[198] Ranging from hydrogels to scaffolds/membranes, there are various kinds of chitosan wound dressings available commercially.[199-200] Chitosan promotes wound healing using different mechanisms (**Figure 1.7**) at different stages of the wound healing process.

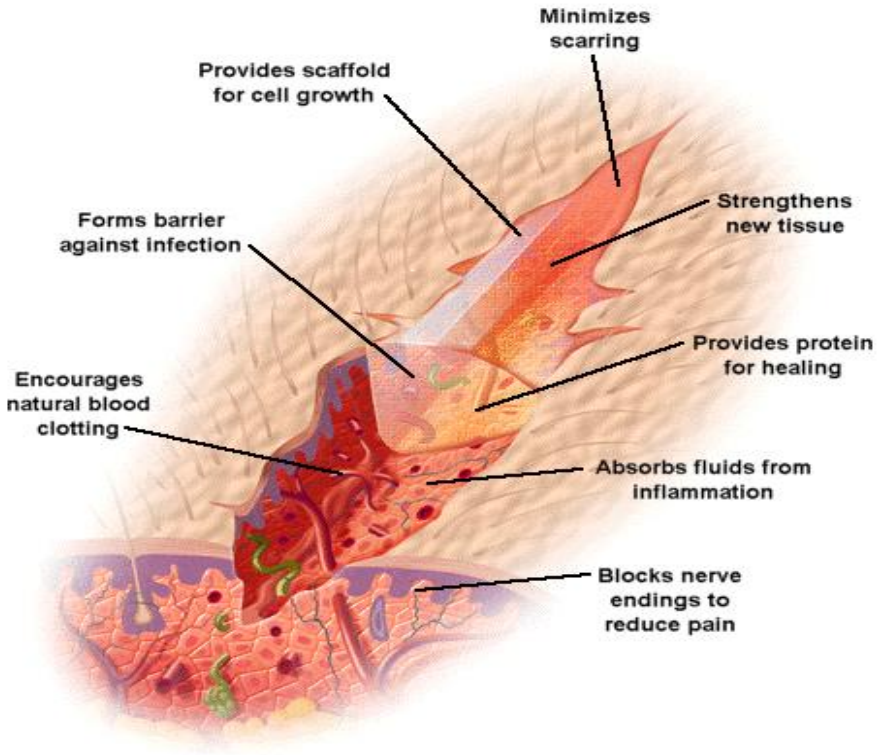


Figure 1.7 Schematic representation of the properties of a chitosan wound dressing material.[201]

Previous studies have shown that chitosan based dressings can expedite contraction of wounds, accelerate healing of different tissues,[202] accelerate the infiltration of the PMNs, activate migration and proliferation of fibroblasts[203], promote granulation tissue formation, stimulate production of cytokines[204] and attenuate production of proinflammatory cytokines such as IL-1β and TNF-α formation but increases the concentration of the anti-inflammatory cytokine; IL-10.[205]

Haemostasis

Considerable research data shows the haemostatic effect of chitosan in a normal environment[206] and in the absence of platelets or coagulation factors.[207] While some scholars have proven that chitin and chitosan both enhance blood coagulation[188], He and his colleagues' study showed that chitosan's positive charge retarded blood coagulation on chitosan films.[208] They further proved that chitosan's positive charge resulted in inhibited activation of the contact system.[208] However, the exact mechanism by which chitosan causes haemostasis is not fully elucidated. The haemostatic efficacy is highly influenced by physicochemical properties like DD, MW, polymeric structure, cationic properties and intrinsic viscosity. Both high viscosity and a high degree of deacetylation increase chitosan's haemostatic efficiency.[209] It is suggested that the haemostatic effect of chitosan is mainly due to the agglutination of the red blood cell component rather than interaction with the normal clotting cascade.[210] The cationic chitosan interacts with the negatively charged molecules at the cell surface in its agglutination.[210]

Janvikul *et al* showed a significant reduction in the whole blood clotting time (WHBCT) with the use of either chitin and chitosan, while only chitin significantly shortened platelet recalcification time.[211] Rao and Sharma also showed that chitosan brought down the WHBCT by up to 40 % of the normal. They proceeded to study the effectiveness of the interaction between red blood cells and chitosan and concluded that even small amounts of chitosan can cause agglutination of erythrocytes from any species.[210] In another study, chitin aggregated platelets more than chitosan. The conclusion was therefore made that platelet aggregation did not necessarily reflect blood coagulation.[188]

Thus, there is sufficient evidence from literature supporting the use of chitosan as a haemostat.[209,212] The FDA approved HemCon™ chitosan dressing when used in pre-hospital

combat patients; successfully arrested bleeding in > 90% of cases.[213] Other haemostatic chitosan wound dressings already approved by the FDA for external use include TraumaStat and Celox dressings.[214] TraumaStat, a silica and chitosan based dressing is proven to have superior haemostatic properties than HemCon™ .[215]

Inflammation

A study investigating the effects of chitosan on wound healing in dogs showed accelerated migration of macrophages and severe infiltration of PMNs on the 3rd day.[203] Inan and Saraydin also showed that during the early phases of wound healing, chitosan stimulates inflammatory cells and the immune-localisation of the growth factor; VEGF and the receptor; FGFR3.[216]

In another study however, evaluation with the enzyme-linked immunosorbent assay (ELISA) showed that water soluble chitosan oligosaccharides with MW < 5 kDa had the ability to reduce inflammation by inhibiting the secretion of proinflammatory cytokines; IL-6 and TNF-α.[217]

Proliferation

Ueno and colleagues showed that from the 9th to the 15th day after treatment of wounds with a chitosan based dressing; there was a great amount of collagen deposition indicating that chitosan promotes granulation tissue formation. [203] In another study, chitosan stimulated fibroblast proliferation *in vitro* at a rate dependent on the DD. [218] However, in the same study, the higher DD (89%) chitosan inhibited keratinocyte proliferation compared to the lower DD (37%) which had no effect on them .[218] This proliferation of fibroblasts induced angiogenesis and collagen formation in the wound area.[218] These findings were consistent with Prasitsilp *et al* whose study exhibited high DD chitosans regardless of the source (shrimp or cuttlefish) as supportive of cell adhesion and hence proliferation of two different cell-lines (L929 and BHK21) under investigation; while the lower DD chitosans did not favour this attachment of these cells.[195] Another study showed an increased synthesis of type III collagen with the use of chitosan.[203] In yet another study, Zhou *et al* prepared biocompatible carboxyethyl chitosan/poly (vinyl alcohol) nanofiber wound dressings through electrospinning of aqueous carboxyethyl chitosan/poly(vinyl alcohol) solution. Cell culture results showed that these fibrous mats successfully promoted attachment and proliferation of L929 mouse fibroblasts without any cytotoxicity towards these cells.[219]

Tissue remodelling

Scar formation is a clinical feature of the remodelling phase of wound healing. A balance between collagen deposition and degradation or collagen remodelling from type III to type I is of paramount importance as it has a bearing on the tensile strength of the scar. As mentioned in **Section 1.8**, excessive collagen deposition results in hypertrophic scars or the formation of keloids.[220] While chitosan is known to induce collagen synthesis during the early phases of wound healing, further investigations on chitosan's role in the remodelling phase are ongoing.

A study by Wang and colleagues showed superior inhibition of scar formation in wounds treated with chitosan than those treated with hyaluronate while those treated with saline solution and free fat did have some scar formation.[221] Consistent with these findings, research data from Hilmi *et al* demonstrated that use of a skin substitute derived from chitosan in irradiated wounds yields significant tissue-engineered skin with significantly smaller scar sizes when compared to the biomaterial Duoderm® CGF™.[222]

Stone's study did not show a difference between wound size reduction of wounds treated with chitosan compared to their gold standard wound dressing (Kaltostat®). However, another important dimension of wound healing was shown; the colour of the scar site treated with chitosan showed an earlier return to normal skin colour compared to the site treated with the Kaltostat® control.[223]

In an attempt to explain the mechanism of action of inhibition or reduction in scar formation Lv *et al* cultured hypertrophic scar fibroblasts (HSF), keloid fibroblasts (KF) and normal dermal fibroblasts (NDF) in the presence or absence of different doses of chitosan.[224] They found that to a small extent chitosan exhibited proliferation-inhibiting effect on NDF while inhibiting proliferation of HSF and KF in a dose-dependent manner. Their findings further demonstrated that chitosan could significantly ($p < 0.01$) suppress the expression of type I and III procollagen in both HSF and KF.[224]

Chitosan is also said to play a role in decreasing wound fibrosis[225] thereby, decreasing the chances of hypertrophic or dysfunctional scarring.

1.13.2 Modified chitosan-based materials in wound healing applications

1.13.2.1 Modification to enhance solubility of chitosan

The solubility of chitosan is influenced by its DD, the distribution of free amino and N-acetyl groups along the polymer chain, MW, pH and temperature of the solvent.[178] Chitosan being a weak base is completely soluble in aqueous acidic media which come with several complications such as toxicity, chain hydrolysis and the removal of residual solvents.[226]

Various studies to modify chitosan to improve its solubility in organic solvents[179] and water[179,186,227] are reported. Crustacean chitosan, carboxylated in equimolar ratio glyoxylic acid: amino groups, produced the water soluble anionic derivative; carboxymethylchitosan (CMC).[228] Sometimes CMC is formed from chitosan's reaction with monocholoroacetic acid in alkaline medium.[16] A porous, physically stable, self-sustaining CMC membrane was prepared using the natural crosslinker, genipin. CMC with high MW permitted the preparation of a lightly cross-linked membrane which showed improved swelling capacity and good mechanical properties.[17] Recent data has shown possible solubility of chitosan at neutral pH. From a histological perspective, wounds treated with water soluble chitosan have fine matured collagen fibres, arranged in a similar fashion to those in normal skin and an overall higher tensile strength than those treated by chitin or normal chitosan.[226]

1.13.2.2 Modification to improve physical properties of chitosan

The hygroscopic chitosan is susceptible to degradation when exposed to extreme temperatures during processing, e.g. high temperatures. Furthermore, the diverse sources of chitosan mean that the chitosan material differ significantly in terms of MW and stability. These and several other factors affect chitosan's stability (**Figure 1.8)**, thus application of chitosan might be limited.

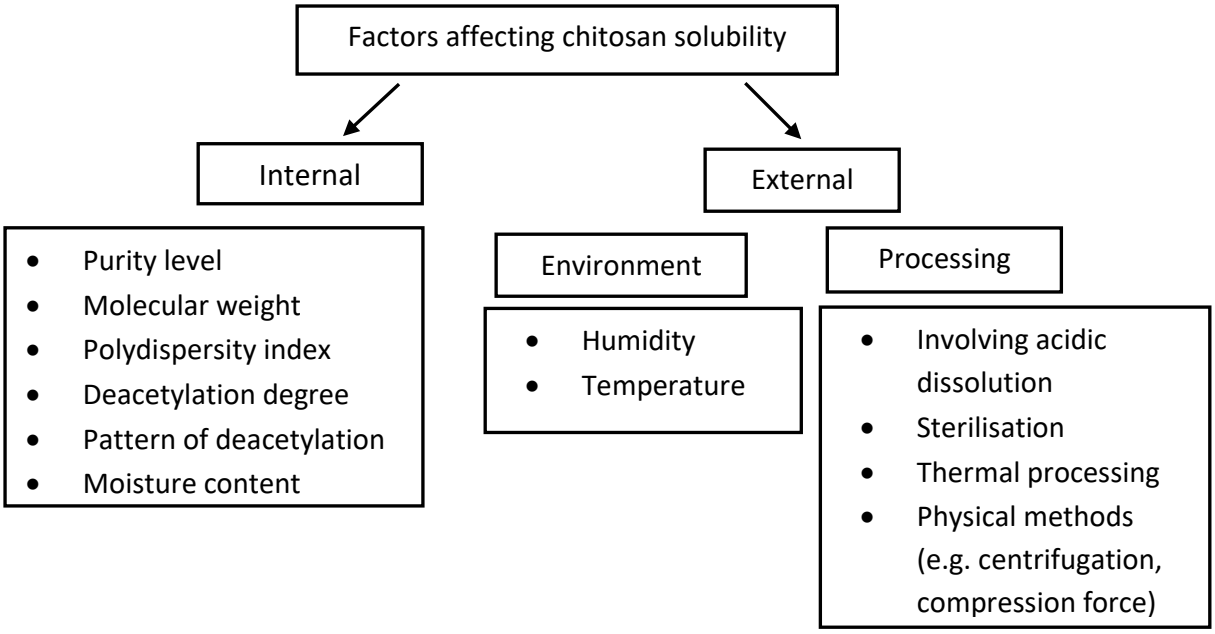


Figure 1.8 Factors affecting stability of chitosan based products[229]

1.13.2.3 Modified antibacterial chitosan

In recent years, extensive attention has been drawn on the antimicrobial activity of chitosan and its derivatives against different groups of microorganisms, such as bacteria, yeast, and fungi.[230] The physical characteristics of chitosan such as DD and MW have a great influence on the antimicrobial activity of chitosan. Liu *et al* showed low MW chitosans to have higher antibacterial activity than that of high MW samples; however, the middle MW chitosans promoted the growth of bacteria.[231]

Chitosan's antimicrobial activity occurs through three proposed mechanisms. One means is the interaction of charged groups in the polymer backbone with bacteria wall constituents,[18] leading to hydrolysis of the peptidoglycans in the microorganism wall, causing the escape of intracellular electrolytes, resulting in the death of the microorganism. The other mechanism, is the charge on the bacterial surface which was shown to influence the activity of chitosan in *in vitro* experiments.[232] Some authors reported that chitosan had less effect on gram-negative bacteria (*Salmonella typhymurium, E. coli, Vibrio parahaemolyticus, etc.)* compared to gram-positive strains (*S. aureus, Lactobacillus plantarum, Listeria monocytogenes, etc)*. However,

several *in vitro* experiments proved that gram-negative bacteria were more sensitive to chitosan due to their hydrophilicity compared to the gram-positive strains.

In 2009, Xing and his colleagues prepared oleoyl-chitosan nanoparticles (OCNP) whose antimicrobial properties they investigated against *E. coli* and *S. aureus***.** They found that *OCNP* bound to intracellular targets (like DNA and RNA or extracellular targets such as phosphate groups) and damaged the cell membrane.[19] In another study; Huang *et al* prepared chemically modified chitosan sulphate derivatives and also investigated their antibacterial activity against *E. coli* and *S. aureus* using the optical density method. The results showed that the acylated chitosan sulphates not only increased the *S. aureus* inhibition activities but also marginally inhibited the growth of *E. coli*.[233] Tao *et al*[20] prepared a carboxymethyl chitosan sulphate (CMCS) derivative and that they evaluated for its effects on the proliferation of fibroblasts using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. The results showed that the CMCS promoted the proliferation of fibroblasts. Vo and Lee[234] prepared a very stable hydrophobically modified chitosan sponge by reacting chitosan with dodecyl aldehyde. This hydrophobic sponge had an improved ability to capture *E. coli* cells (~4.0×10^8 cells/mg sponge) by intercalating into the outer membrane of *E. coli* cells compared to a sponge made with chitosan alone. Although this was a water treatment study, the evidence substantiated the notion that hydrophobically modified chitosan has antibacterial activity.[234]

1.13.2.4 Modified haemostatic chitosan

Various *in vivo* experiments have reported enhanced haemostasis using chitosan.[235-237] However, chitosan has been modified to further enhance its haemostatic potential through chemical modification. Several chitosan derivatives with different structures have been discovered. Ong and colleagues fabricated a dressing incorporating polyphosphate which is a pro-coagulant and the antimicrobial silver.[238] The haemostatic efficacy was evaluated on full-thickness wounds on a mouse model. At the optimal chitosan-polyphosphate concentration, there was increased platelet adhesion, an acceleration in blood clotting, faster thrombin generation and more blood absorption compared to the plain chitosan.[238]

In a more recent study, the use of chitosan modified to produce an amphiphilic biopolymer as a “reversible” haemostatic agent was explored. A few hydrophobic tails were attached to

the backbone of chitosan, thus producing a hydrophobically modified chitosan (hm-chitosan) (**Figure 1.9**). Upon addition of hm-chitosan to liquid blood a liquid gel was instantly observed. When the same experiment was repeated using unmodified chitosan, the blood remained a flowing liquid, with only an increase in viscosity. (**Figure 1.10**)[239]

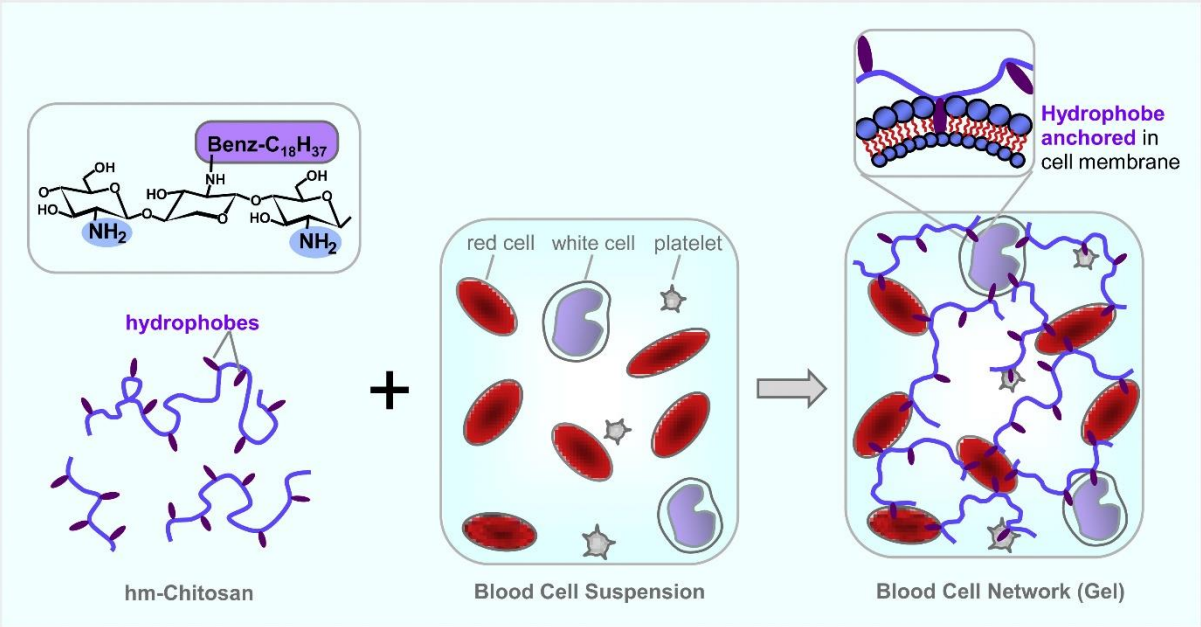


Figure 1.9 Schematic representation of the gelation of blood by hm-chitosan.
The left showing the polymer with its hydrophilic backbone in blue and the attached benzyloctadecyl hydrophobes in purple. Insertion of hydrophobes into blood cell membranes (as depicted in the top inset); resulted in the polymer chains bridging the cells into a self-supporting network. Upon addition to liquid blood, the components assembled into a three-dimensional network (gel), as shown on the right.[239] (with permission)

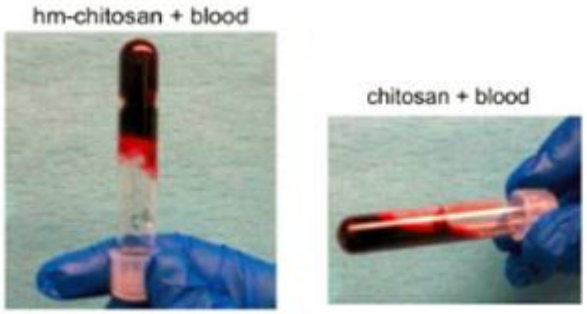


Figure 1.10 Effect of hm-chitosan and chitosan on heparinised human blood.
The hm-chitosan/blood mixture formed a gel that held its weight in the inverted tube while the chitosan/blood mixture remained a freely flowing mixture. (with permission)

Shi and colleagues[240] developed a haemostatic powder consisting of microspheres made from CMC, collagen and sodium alginate. Collagen was added because of its ability to combine with platelets, activate the intrinsic pathway and accelerate wound healing at different stages of

the wound healing phases. The result was a powder, which when tested for swelling rate, platelet adhesion strength and platelet aggregation, and toxicity illustrated respectively; that this material exhibited very high water absorption ability (> 50 self-weight) and very active haemostatic function, in addition to good biodegradability and non-cytotoxicity.[240]

In another study[241], azide and lactose moieties were introduced to the chitosan and cross-linked to an insoluble hydrogel using UV irradiation. Addition of the lactose moieties increased the water solubility of the chitosan. Overall, a hydrogel with the effectiveness that compares to fibrin glue was produced. To assess the sealing ability of chitosan, a rabbit model was used. The chitosan hydrogel when used to seal punctures made on the carotid artery and lungs, was able to stop the bleeding and air leakage respectively.[241]

An exceptional polymer solution containing chitosan and glycerol phosphate when mixed with whole blood forms a solid polymer-blood implant.[242] Research by Marchand *et al* investigated the mechanism of solidification of the chitosan–GP/blood implant and found that it solidifies through coagulation mechanisms involving activation of thrombin, platelets, FXIII and through fibrin polymerisation, resulting in a dual fibrin–polysaccharide clot scaffold that has more tensile strength than normal blood clots and resists lysis.[243]

Increasing amino groups on chitosan may improve blood-clotting properties of chitosan. Ogino and colleagues focused on introducing amino groups to the surface of chitosan using O_2, Ar, NH_3 and NH_3–He mixed gas plasmas.[244] The results showed a significant reduction in blood clotting time in the sample pre-treated with O_2 and then treated with NH_3 and He plasma compared to the untreated sample.

1.14 Hydrophobically modified wound dressings

In the last decade, wound dressings with a hydrophobic contact layer have been used to reduce infection by adsorbing bacteria onto the dressing surface.[245] A study by Bowler and his colleagues, showed that the chemical nature and the surface properties of bacteria influenced the interactions with the wound dressings. They proved that particularly the gram-negative *P. aeruginosa* bound more to the wound dressing compared to the gram-positive bacteria.[245]

An invention by Tomas Fabo emphasised on a hydrophobic contact layer preventing the wound dressing from sticking to the wound bed.[246] Hydrophobic dressings are ideal for wounds with exudate as hydrophobic material has poor solubility in aqueous wound exudate.[246-247]

1.15 Lauric acid

Lauric acid, a saturated 12 carbon medium chain fatty acid is found in coconut, palm kernel and several other vegetable oils. It presents as a white, soapy odour powder, that is widely used in the production of cosmetics and soap.

Lauric acid's strong bactericidal properties are reported in various studies including acne treatment[248], burn wound healing[249] and as a general wound treatment.[250] Lauric acid has also been shown to have 5α-reductase enzyme activity making it a suitable treatment for testosterone induced hyperplasia.[251] Lauric acid's bactericidal properties are consequently beneficial in wound healing.

1.16 Collagen

Collagen, a fibrous material is the most abundant protein in the body; being the major component in skin and bones. It is a material that is extensively studied and applied in tissue engineering due to its biocompatibility and biodegradability. Its use alone, is however limited due to the rapid biodegradability and low mechanical strength. Collagen is produced mostly by fibroblasts in most connective tissues. Fibroblasts in turn also exhibit a chemotactic response to type I, II, and III human collagens.[252] There are several types of collagen identified in the human body with the most common types found in the dermal and epidermal layers being type I-IV.

It is biodegradable and biocompatible thus making it a very good material for wound healing. Within normal tissue the collagen in the ECM has been shown to provide binding sites for cells, cytokines and growth factors in addition to acting as a scaffold in the intracellular space. Collagen plays a role in all the phases of wound healing. It can protect and deliver to the wound, healing promoting factors such as growth factors[253] while providing physical support and allowing infiltration of cells into the injury site.

There is renewed interest in the use of collagen material for tissue engineering and in wound dressings. Promogran®, a novel, spongy, collagen dressing containing oxidised cellulose is patented for treating exuding ulcers in the USA and Europe.[254] Micronized collagen is beneficial in remodelling of the ECM and accelerating wound closure thus stabilising the environment in a chronic wound, resulting in healing,[255] thus collagen is a widely used material for wound dressings.

1.17 Platelet-rich plasma and growth factors

Presently, platelet derived growth factors, including platelet derived growth factor-BB (PDGF-BB), basic fibroblast growth factor (bFGF), and granulocyte macrophage-colony stimulating factor (GM-CSF) are used for wound healing in the USA. Of these growth factors, only PDGF-BB has successfully completed randomised clinical trials for treatment of chronic DFUs.[256]

Platelet-rich plasma is a concentrated source of platelets with up to 8-fold increase in platelet concentration compared to that of normal plasma.[257] Platelets are small anucleate cells ranging from 1-3 µm in diameter; produced by large polyploid megakaryocytes (MKs) in the bone marrow (**Figure 1.11)**. This process involves endomitosis of the MKs and a maturation phase in which the greater part of the cytoplasm is packed into 10-20 blunt tube-like protrusions called proplatelets, and the nucleus is extruded.[258] The blunt proplatelets elongate over time and eventually platelets form at their tips.[259]

Preplatelet fragments are released into the blood where maturation occurs. All the platelet constituents are derived from the megakaryocytes. Their outer membrane encompasses the cytoplasm and its constituents which include the mitochondria, lysosomes, storage granules, microfilaments, endoplasmic reticulum or Golgi apparatus.

Platelets play a significant role in innate immunity, haemostasis, thrombosis and inflammation and are an important source of growth factors. Initially it was thought that platelets are only involved in thrombosis and haemostasis but there is overwhelming evidence that they have other important functions. Published data demonstrates that growth factors and their receptors are major mediators of tissue repair[260]; however, it is the synchronised effects of a blend of growth factors that produces the maximum tissue response.[261]

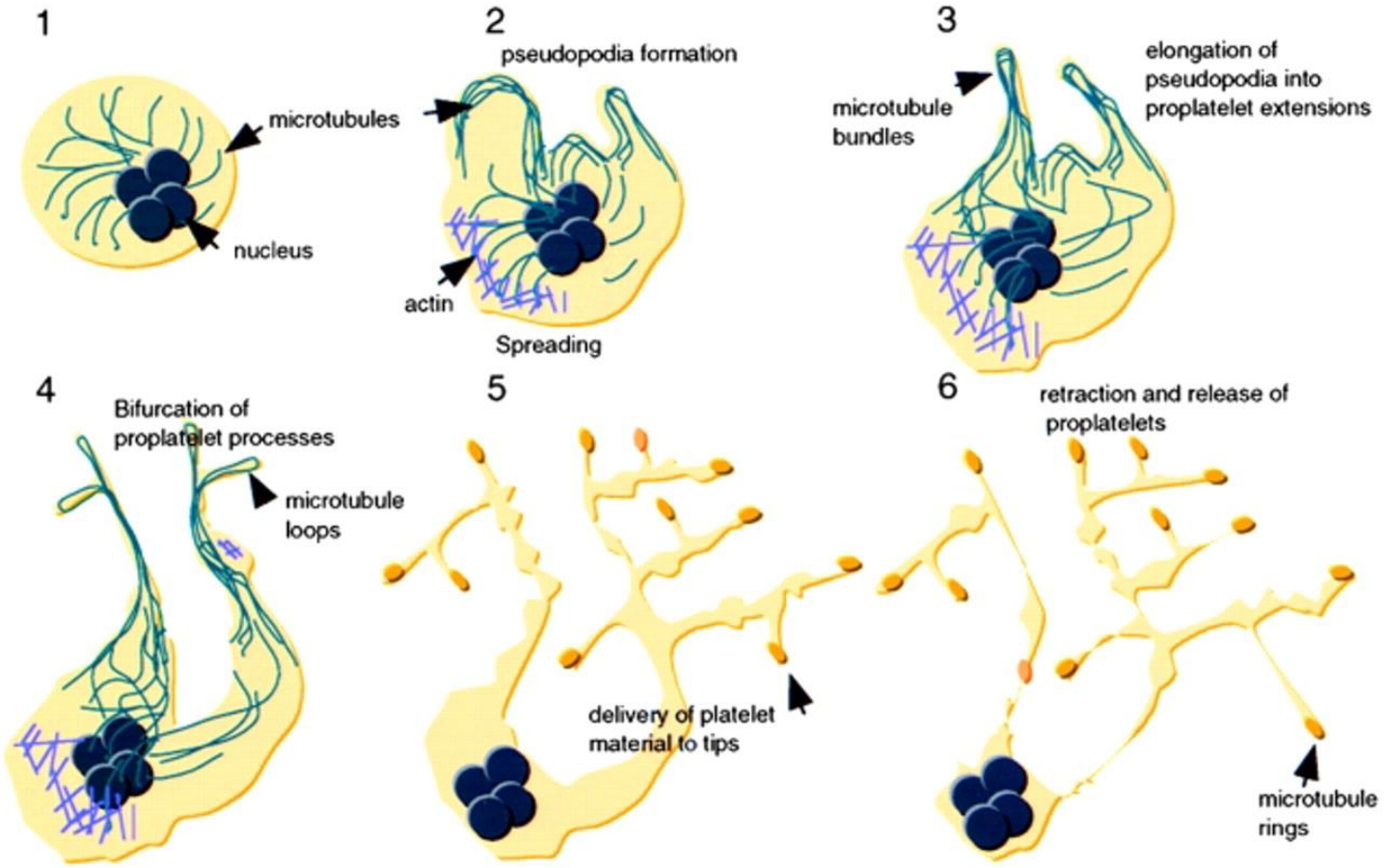


Figure 1.11 Model of platelet production from megakaryocytes.[259]

(Stage 1) Polyploidisation and cytoplasmic maturation of MKs. (Stage 2) Remodelling of megakaryocyte cytoplasm into thick pseudopodia containing bundles of microtubules (Stage 3) Blunt pseudopodia stretch into proplatelet processes, with thick bundles of microtubules in their core. (Stage 4) Bending of proplatelets to form a branched structure from which new processes extend. (Stage 5) Constrictions on length of proplatelets give the beaded appearance to proplatelets. (Stage 6) Retraction to release proplatelets from the megakaryocyte body and may undergo further fragmentation to yield preplatelets.

Table 1.3 gives a summary of the function of some cytokines and growth factors in wound healing. The cytokine IL-1 is the first cytokine to be released from keratinocytes after tissue injury, signalling barrier damage.[260,262] Immediately after injury, platelets infiltrating the wound site are activated and trigger the clotting cascade.[263] The platelet plug formed provides a matrix for the influx of inflammatory cells. Thereafter, the platelets degranulate releasing alpha granules which secrete growth factors such as: PDGF, TGF-β and EGF including the proinflammatory cytokines IL-1 which are chemotactic for neutrophils.

TGF-β promotes the conversion of monocytes to macrophages which augment inflammation and tissue debridement. Macrophages in turn secrete IL-1, IL-6, FGF, EGF, TGF-β and PDGF. Growth factors FGF, TGF-β and PDGF promote the infiltration of fibroblasts. Phenotypic changes of fibroblasts to myofibroblasts which align themselves at wound edges to facilitate wound closure. [53,264]

Table 1.3 **Growth factors and cytokines and their effects on wound repair[53]**

Process	Growth factors/cytokines involved
Neutrophil infiltration	TGF-β, MCP-1, MIP2/GRO-α, IL-8, IL-6, IL-10 (-)
Macrophage infiltration	TGF-β, MCP-1, MIP-1α, IL-10 (-)
Angiogenesis	VEGF-A, PLGF, FGF2, Angiopoietins, HGF, Cyr61, MCP-1, IL-8, GRO-α, GM-CSF, IP-10 (-)
Fibroplasia	PDGF, TGF-β, CTGF, GM-CS F, IGFs
Matrix deposition	FGF2, IGF-1, NGF, TGF-β, Activin, MCP-1, CTGF, Cyr61
Scarring	IGF-I, TGF-β, Activin, CTGF, IL-6, IL-10 (-)
Re-epithelialisation	FGF2, FGF7, FGF10, EGF, TGF-α, HB-EGF, NDF, IGFs, NGF, Activin, MCP-1, IL-6, GM-CSF, Leptin, TGF-β, BMP-6 (-), IP-10 (-)

1.18 Problem statement

Although wound healing strategies have evolved significantly over the ages, the basic principles have remained the same. Wounds go through similar phases of healing; however, the duration of healing is influenced by several factors including aetiology and management of the wounds. Evidently, not all wounds are the same, therefore the treatment plan for each wound is specific for the patient.

Deep chronic wounds still remain a challenge for clinicians. Skin grafts and other autologous technologies are currently used for these wounds; nevertheless, biopolymers offer a feasible substitute. Biopolymers are important alternatives as wound healing material as their availability, biocompatibility and efficacy is proven. Chitosan is a naturally abundant polymer that is already used in some wound healing materials. The hydrophobic material, lauric acid is known to have antibacterial properties but its wound healing potential for full-thickness wounds has not been reported.

As full-thickness wounds present with excessive tissue loss, minimum growth factor activity and impaired angiogenesis, dysfunctional scarring emanating from these factors is inexorable, unless some skin substitutes are used. A wound filler that promotes tissue growth, angiogenesis and combats infection is seen as a viable option for treating these wounds.

1.19 Hypotheses, aims and objectives

1.19.1 Hypothesis

Wound management using hydrophobically modified chitosan-based dressings combined with collagen and platelet-rich plasma will enhance the wound healing rate of full-thickness wounds measured using the porcine wound model.

1.19.2 Aim

The aim of this study was to determine which of three lauric acid conjugated chitosan derivatives (LCs_{10}, LCs_{20}, LCs_{34}) showed the best physicochemical properties for use in a wound dressing and to test the treatment efficacy of a wound filling dressing material based on the selected conjugate in a full-thickness wound model.

To prepare a chitosan based wound filling paste that would provide a matrix that enhances infiltration of fibroblasts while promoting growth factor release, angiogenesis and a reduction in overall wound healing times.

1.19.3 Research objectives

The properties of a chitosan based wound dressings with and without added platelet-rich plasma (Co/LCs_{10} and Co/LCs_{10}/PRP) were compared to the standard of care wound dressings where healing rates and tissue characteristics were compared.

To achieve the aim of this study, the following objectives were set:

- Synthesis of three different hydrophobic modified chitosan products with different percentages of lauric acid loading densities.
- Chemical characterisation of the lauroyl chitosan derivative (LCs).
- *In vitro* assays to determine optimum loading density of lauric acid on chitosan based on platelet binding and growth factor loading.
- Assessing different lauroyl chitosan conjugates to determine desirable physical properties and compare these as new improved wound dressings.
- Preparation of a space filling wound dressing using the most suitable modified chitosan product combined with collagen and platelet-rich plasma.
- Testing the modified dressing in synergism with autologous PRP on a porcine full skin thickness wound model.
- Identify significant histological differences in the wound bed and the wound edges of chitosan dressings compared to the control.

Chapter 2

Lauroyl chitosan synthesis and characterisation

2.1 Introduction

Chitosan is a significantly versatile polymer existing naturally in some microorganisms and in the cell wall of some fungi *(Mucoraceae).* However, to supply chitosan's enormous market, commercial chitosan is produced from the partial deacetylation of its natural form, chitin through alkali *N*-deacetylation.[183-184] Chitin forms structural components of the exoskeletons of fungi and arthropods and naturally presents itself as an organised crystalline. Its physical characteristics include; a white, hard, inelastic, insoluble linear polysaccharide. It has low chemical reactivity and is not readily soluble in water or organic solvents, thus compared to its deacetylated form, chitosan is the preferred form. While chitin is composed of *β*-(1→4)-linked *N*-acetyl-D-glucosamine (d-GlcNAc) residues, deacetylation yields chitosan, which is composed of *N*-acetyl-D-glucosamine and D-glucosamine residues. Chitosan is therefore defined as 'a group of deacetylated chitins with an acetylation value of < 40% and nitrogen content > 7%'.[186]

Chitosan has been used in a vast number of applications in different fields ranging from water treatment, cosmetic, weight loss, drug delivery and wound healing.[265] Several modification strategies of chitosan have been applied to improve the physical characteristics, processability and in some cases suitability for a specific biological application. Hydrophobic modification of chitosan increases its processability, drug delivery properties and interaction with biological tissues.

This chapter reports on the chemical modification of chitosan with lauric acid to produce the hydrophobic derivative lauroyl chitosan. The synthesis of lauroyl chitosan involved the formation of amide linkages between the primary amine groups of chitosan and the carboxylic acid functional group of lauric acid. Three loading densities (10%, 20% and 34%) of lauric acid on chitosan were synthesised to give LCs_{10}, LCs_{20} and LCs_{34}, respectively. These loading densities were selected according to the commonly used concentrations in literature.

The lauroyl chitosan was extensively characterised with the Drop Shape Analyser (DSA), Moisture Analyser, Fourier-Transform Infrared (FT-IR) Spectroscopy and Proton Nuclear

Magnetic Resonance (^{1}H NMR) Spectroscopy. DSA provided information on the contact angle of a drop of water deposited on the surface of a thin compressed disc of lauroyl chitosan as a measure of the hydrophobicity of the material. It is a simple method for analysing the relationship between the wetting of a liquid and the solid properties. The swelling index, measured using the Moisture Analyser, is an important parameter used in the production of wound dressing materials. It was used to evaluate the capacity of the material to absorb moisture and in wounds this will be the exudate. FT-IR spectroscopy offered a molecular 'fingerprint' of the material by exposing a sample to infrared radiation. Each different functional group in the compound produces specific spectral signal. NMR spectroscopy is used to determine a compound's unique structure by defining, often, the hydrogen and carbon composition of the compound.

2.2 Materials

Acetic acid was purchased from SAARchem` Modderfontein, South Africa. Chitosan (Mw: 50 - 190 kDa and degree of deacetylation: 75% – 85%), dimethylformamide (DMF), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloric acid (EDC.HCl), lauric acid, lauroyl chloride (d = 0.946 g/ml), N-hydroxysuccinimide (NHS) and ninhydrin solution (90%, d = 0.788 g/ml) were purchased from Sigma-Aldrich, South Africa. Methanol (99.9%), sodium hydroxide (NaOH), ethanol (99.5%) and acetone (99.5%) were purchased from Merck (Pty) (Ltd) Modderfontein, South Africa.

The BBA^{TM} FTA Hemagglutination buffer consisting of sodium chloride 7.65 g/l, disodium phosphate 1268.8 mg/l, monosodium phosphate 0.1 g/l and monopotassium phosphate 211.3 mg/l at pH 7.2 purchased was from Becton, Dickson and Company, Sparks, USA. It was prepared to a volume of 1 l and stored.

Deuterium chloride (DCl) 35 wt% was obtained from Sigma-Aldrich, South Africa and was diluted to a 2% solution using D_2O. The 2 wt% DCl/D_2O was made up by diluting 0.057 ml 35 wt% DCl with 1 ml D_2O).

Distilled water (ddH_2O) was used in the experiments.

2.3 Methods

2.3.1 Chemical synthesis of lauroyl chitosan

Method A

Conjugation of lauric acid to chitosan using carbodiimide chemistry

Chitosan, 1 g, was dissolved in a binary mixture of aqueous acetic acid (100 ml 1% v/v) and DMF (40 ml), and left to stir for 24 hr. In a separate reaction flask, EDC.HCl and NHS were added to a solution of lauric acid (0.422 g, which was equal to 34% loading density) dissolved in DMF (10 ml) and left to react for 2 hr. The activated solution of lauric acid was added drop wise to the chitosan solution under constant stirring to prevent precipitation and left to react for 72 hr at ~23 °C. At the end of the experiment, the reaction was quenched by precipitating the chitosan with 100 ml of an ethanol/NaOH (0.5 M) solution (60 ml/40 ml). The precipitate was then washed copiously with acetone and water. The gelatinous product obtained was rid of residual solvent using a Buchi Interface R-300 Pro Rotary Vaporizer (790 mbar at 49 °C), followed by freeze-drying for 72 hr at -55 °C.

Method B

Conjugation of lauric acid to chitosan using acyl chloride chemistry

Chitosan, 1 g, was dissolved in a binary mixture (100 ml) of aqueous acetic acid (1% v/v) (57 ml) and methanol (43 ml) and left to stir for 24 hr. Thereafter, lauroyl chloride at 143 µl, 286 µl and 486 µl (which equated to 10%, 20% and 34% loading densities, respectively) was added drop wise to the chitosan solution under constant stirring to prevent precipitation and left to react for 24 hr at ~23 °C. A precipitate of the conjugate was extracted using acetone (100 ml) and washed copiously with acetone and water. The material was then centrifuged at 6000 x g for 10 min. The gelatinous product obtained was rid of residual solvent using the Buchi Rotary evaporator (790 mbar at 49 °C), followed by freeze-drying for 72 hr at -55 °C using the Telstar LyoAlfa 10 (2013) freeze-dryer instrument.

2.3.2 Characterisation of lauroyl chitosan

FT-IR analysis

The Perkin Elmer Spectrum 100 Fourier-Transform Infrared (FT-IR) Spectrometer was used to obtain the infrared absorption spectra. Briefly, a dry sample of lauroyl chitosan (2 mg) was

scanned at wave numbers between 4000 cm^{-1} and 650 cm^{-1}. A standard 32 scans procedure at a resolution of 4 cm^{-1} was ran at room temperature.

^{1}H NMR analysis

Proton (^{1}H) nuclear magnetic resonance (^{1}H NMR) experiments were conducted at 60 °C on a Bruker Ascend^{TM} 500 MHz NMR Spectrometer with Prodigy Probe. Dry lauroyl chitosan (8 mg) was dissolved in 1 ml of 2 wt % deuterated HCl (DCl) in deuterated water (D_2O) and then added to a 5 mm NMR tube. The tube was kept in a water bath at 60 °C prior to being analysed. Tetramethylsilane (TMS) was used as an internal reference and chemical shifts (δ) were reported in ppm. Hirai *et al* method[266] was used for structural analysis.

2.3.3 Scaffold preparation

The lauroyl chitosan samples obtained after freeze-drying were dissolved in distilled water to give a 2% solution. This was seeded onto 48-wells plates at 500 µl/well and left to stand for +/- 30 min until the air bubbles disappeared. Thereafter, the plates were frozen at -80 °C for 2 hr after which they were freeze-dried for 24 hr. Each scaffold, obtained as a soft foam, was then soaked in 0.5 M NaOH for 10 min and thereafter soaked twice in distilled water for 20 min each time. This was followed by freezing at -80 °C for 2 hr followed by freeze-drying for 24 hr to give the scaffolds.

2.3.4 Drop shape analysis

The wettability of lauroyl chitosan was evaluated using the Kruss contact angle video-based goniometer system (DSA100), which utilises a dispensing needle for dosing and an on-board camera for image acquisition. All measurements were made at room temperature (~23 °C) using deionised distilled water as the probe liquid. A mass of ~7.5 mg of each sample was pressed into a disc using a manually-operated hydraulic press. A 10 µl droplet of ddH_2O was deposited onto the test sample material using a luerlock syringe. Within 10 s of droplet deposition the image of the water droplet was captured. The contact angle between the drop and the material's surface was used to determine their relative hydrophilicities. Measurements were made by the sessile drop method on three randomly selected surface positions for each sample. For each specimen of modified chitosan, three different samples were used. Hydrophilicities of chitosan and lauroyl chitosan were compared.

2.3.5 Swelling index

Approximately 100 mg sample of the scaffold of each loading density was completely immersed in 5 ml of phosphate buffered saline solution (PBS) pH 7.4 at 37 °C (**Figure 2.1**) and weighed at predetermined time intervals, i.e. 1 hr, 2 hr, 3 hr, 6 hr, 12 hr for up to 24 hr. The swelled samples were removed, and the excess liquid was carefully wiped off with paper towels and placed on the aluminium weighing pan of a Radwag® IRDW MA 50.R Moisture Analyser (Poland) balance for mass determination. The average of three measurements for the samples of chitosan and lauroyl chitosan scaffolds was used to determine the swelling index.

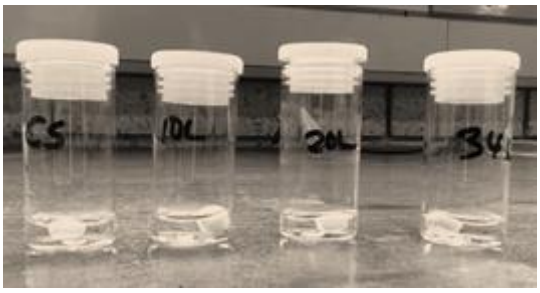


Figure 2.1 Scaffold samples soaked in PBS pH 7.4

The following equation was used to calculate the swelling index of the scaffolds. Where W_i is the initial weight of a dry sample and W_f is the final weight of a soaked sample.[267]

$$S_i\,(\%) = \frac{W_f - W_i}{W_i} \text{ X } 100\%$$

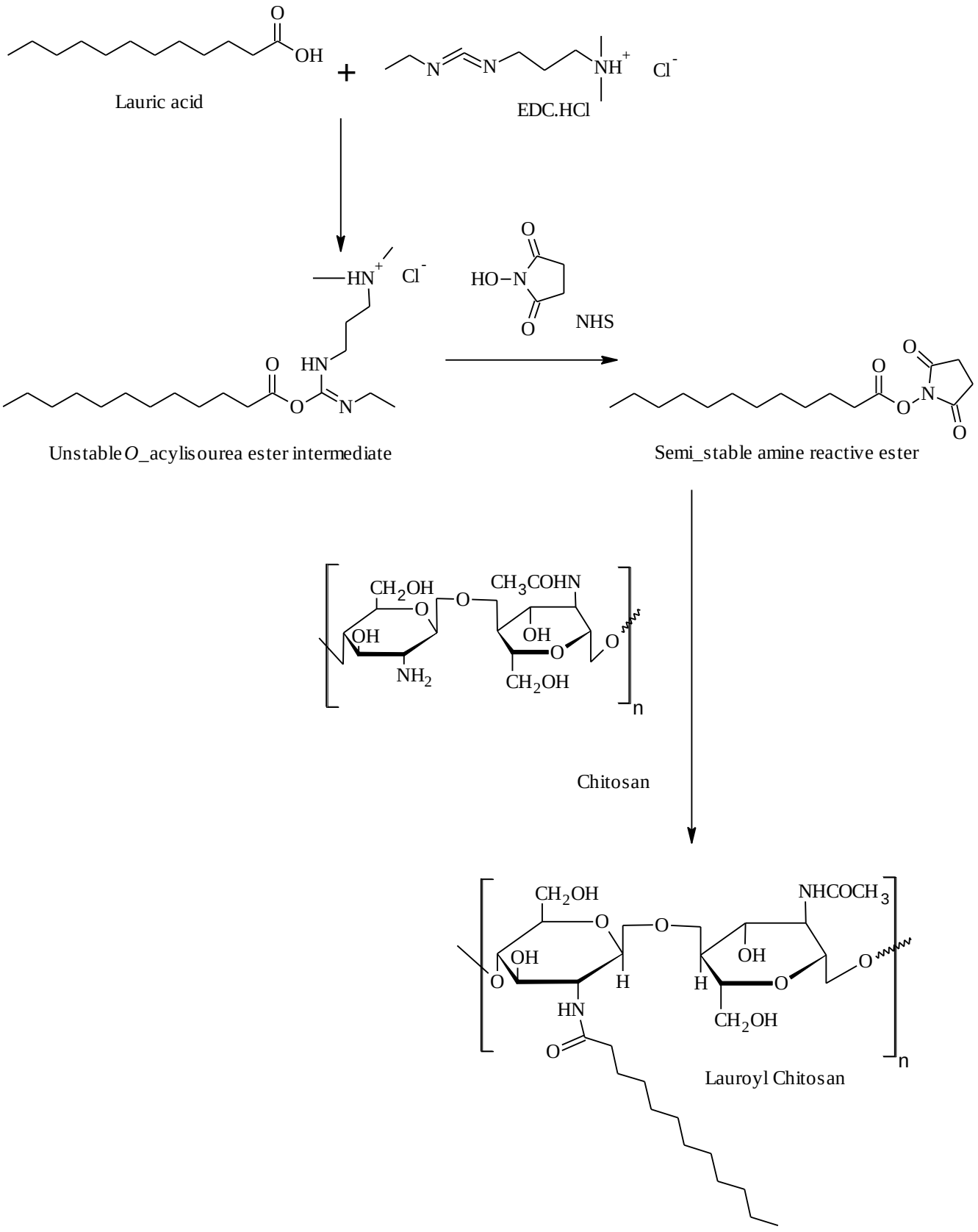
2.4 Results and discussion

Chemical synthesis of lauroyl chitosan

Both **Methods A** and **B** for conjugating lauric acid to chitosan yielded an off white coloured, fluffy foam which was soluble in dilute aqueous acetic acid (1% v/v). The samples for each of the lauric acid loading densities; 10%, 20% and 34%, were each named LCs_{10}, LCs_{20} and LCs_{34}. It was observed that as the loading density of lauric acid increased, the material became more plasticised with a hard textured material that was almost yellow in colour. This was observed predominantly for the LCs_{34} sample. This sample did not easily go into solution in 1% v/v acetic acid compared to the LCs_{10} sample due to its increased lipophilicity. It was therefore immersed in a water bath at ~55 °C and further vortexed to finally get it into solution.

The hydroxyl and primary amino functional groups of chitosan offer a multi-nucleophilic property to the polymer which enables several chemical and physical modification strategies to improve its application. In this study, the chemical modification strategy involved coupling lauric acid to the primary amine of chitosan to yield an amide. Discrimination between the nucleophilic amino and hydroxyl groups was achieved because the acidic medium (1% acetic acid) used readily protonates the more nucleophilic amino groups than the –OH which makes the former the preferred site for substitution.[268] Furthermore, the reaction was carried out at 23 °C to prevent *O*-acylation. Initially, **Method A** adopted from Chen *et al*[269], linked lauric acid to chitosan using carbodiimide chemistry. The chloride salt of EDC was used to activate the carboxylic group of lauric acid. The EDC salt is one of the most commonly used agents for the activation of the carboxyl or phosphate groups of organic acids for conjugation to primary amines or alcohols. It is particularly attractive for biopolymer functionalisation because of its water solubility. The mechanism of action involves a nucleophile, in most cases a primary amine functional group, reacting with the electrophilic EDC-activated carboxyl functional group to form an ***O***-acyl urea ester intermediate. Although commonly used in aqueous-based coupling reactions, the reagent (EDC) rapidly degrades in water while the complex formed with the acid functional group is relatively unstable and hydrolyses to an inactive water soluble by-product iso-urea. ***N***HS is included in the reaction to rapidly react with the EDC-activated complex to form a stable NHS-ester.[270] The rate of hydrolysis of this ***N***HS-ester is slower than that of the ***O***-urea derivative of EDC making it possible to even isolate and purify the ***N***HS-ester.[271] ***N***HS-ester formation thus allows for increased coupling efficiency. In the presence of chitosan, the ***N***HS ester of lauric acid reacts further with the nucleophilic primary amine to form an amide of lauric acid and chitosan (Scheme 2.1).

The final product was washed twice using methanol and NaOH (5:2) and further washed with acetone to remove the iso-urea by-products.



Scheme 2.1. Conjugation of lauric acid to chitosan using carbodiimide chemistry.

While the carbodiimide coupling chemistry involving EDC is a widely used method of conjugating carboxylic acids for biopolymers, acid chlorides are significantly more reactive than the ***N***HS esters. The chloride is an excellent leaving group. A second method using commercially available lauroyl chloride (**Method B**). The highly reactive lauroyl chloride significantly reduced the reaction time from 72 hr to just 24 hr.

The products were characterised using FT-IR to confirm the conjugation of the acyl groups onto the chitosan. Consistent with literature reports, some of chitosan's specific peaks included the amide I (1656 cm^{-1}), amide II (1548 cm^{-1}), and the amide III (1320 cm^{-1}).[272]

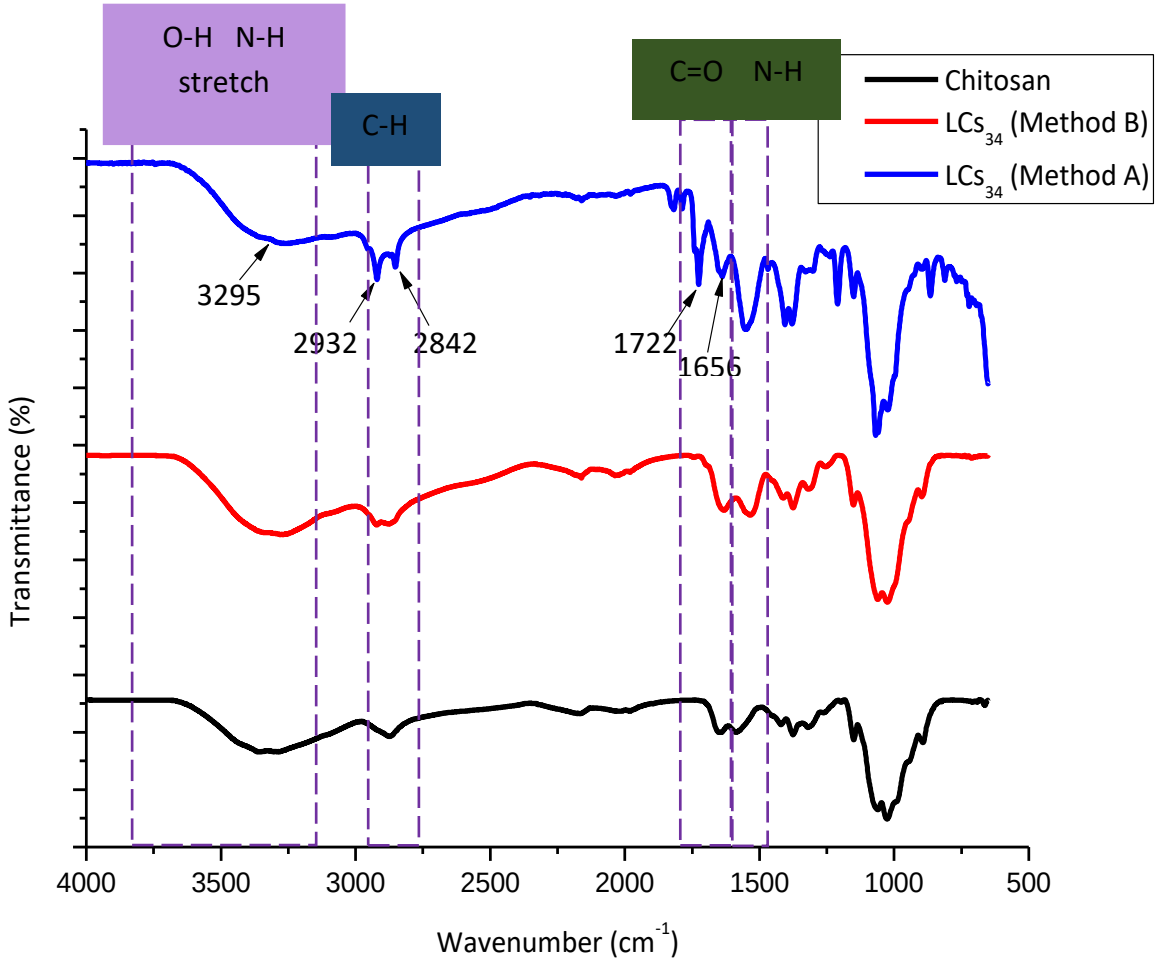


Figure 2.2 FT-IR of chitosan and LCs_{34} from both Method A and Method B

The FT-IR spectra for both **Method A** and **B** (**Figure 2.2**) showed that lauroyl chitosan when compared to the underivatised chitosan had increased intensity of absorption peaks at 2842 cm^{-1} and 2932 cm^{-1} which were attributed to symmetric and asymmetric C-H stretching, respectively. These stretching vibrations accounted for the methyl, methylene and methine

groups in $NHCOCH_3$, CH_2OH and CH in the pyranose ring, respectively.[273] An increase in the intensity of these peaks in the lauroyl chitosan compared to the chitosan spectra in both **Methods A** and **B** confirmed successful coupling of the saturated aliphatic fatty acid chain to chitosan. Both **methods A** and **B** showed an increase in the carbonyl band at 1656 cm^{-1} of lauroyl chitosan confirming the formation of an amide linkage between amino groups of chitosan and carboxyl groups of lauroyl chloride.

The FT-IR also displayed very slight ***O***-acylation in the LCs_{34} from **Method A** (1722 cm^{-1}) while this phenomena did not occur using **Method B**, as the typical ester band, above 1700 cm^{-1} was absent in the spectra of all the **Method B** samples.[274] It was rather the amide carbonyl at ~1656 cm^{-1} that was present in all samples. It was suggested that N-acylation rather than O-acylation of chitosan occurred. The FT-IR peaks showed clear differences between chitosan and the lauroyl modified chitosan.

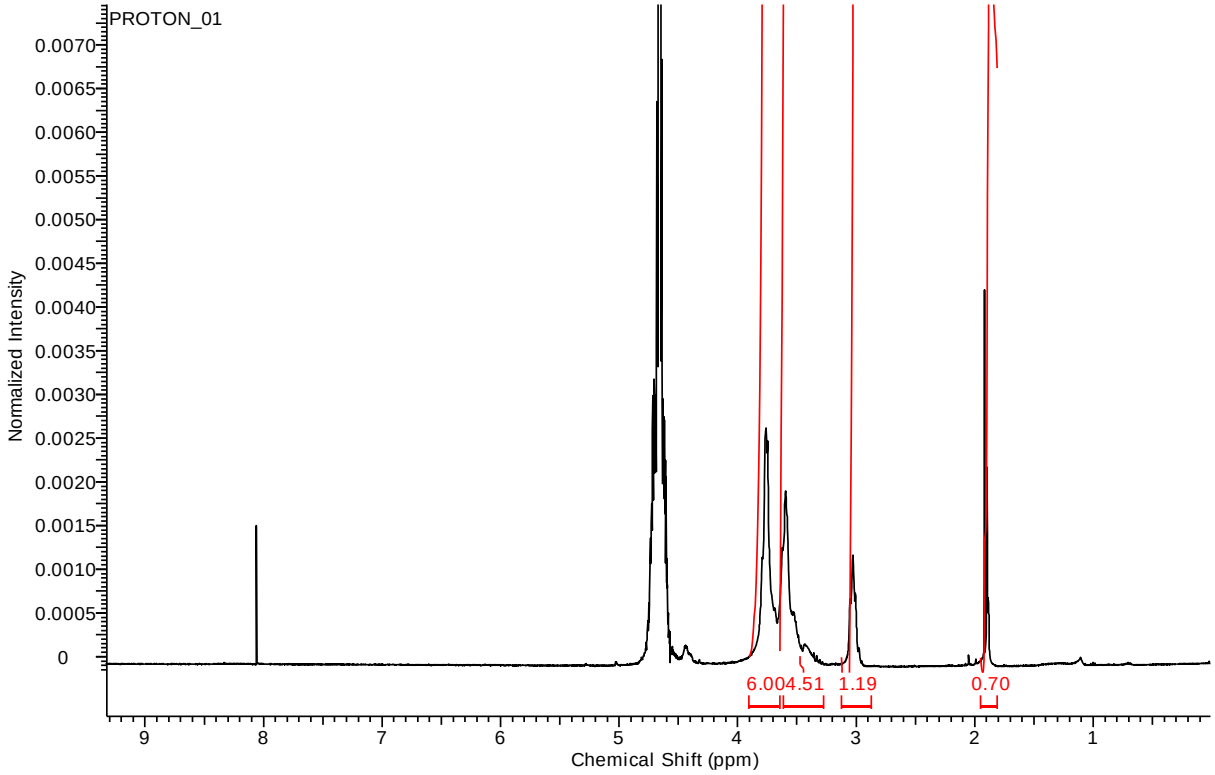
This broadening around 3319/3327 cm^{-1} was assigned to the OH stretching and this overlapped with the NH_2 stretching for both chitosan and lauroyl chitosan. The OH were more than the NH in both chitosan and lauroyl chitosan; hence, the OH peak overshadowed the NH. In this case, coupling of lauric acid to chitosan resulted in a decrease in NH_2 groups which presented as a decrease in the intensity of the absorption band at 3000 – 3600 cm^{-1}. The N-H bending vibrations for the deacetylated primary amines in chitosan were seen at ~1521 cm^{-1}. Furthermore, the intensity of the band at 1548 cm^{-1}, did not change for chitosan before and after modification to lauroyl chitosan. Greater intensity of the band than that of the carbonyl peak at the 1649/1656 cm^{-1} region typically suggested a highly deacetylated chitosan.[273]

The FT-IR spectra obtained for lauroyl chitosan was highly consistent with the results of Chiandotti *et al*. [268]

NMR analysis

The ^1H NMR spectra of chitosan and lauroyl chitosan from **Method B** were given in **Figure 2.3.** The Cs ^1H NMR showed: δ=4.7, δ=3.1, δ= 3.4-3.9 δ= 1.8, δ= 0.9. The ^1H NMR spectra of lauroyl chitosan showed: δ=4.6, δ=3.0, δ= 3.4-3.8 δ= 1.9.

(A) Chitosan



(B) Lauroyl chitosan

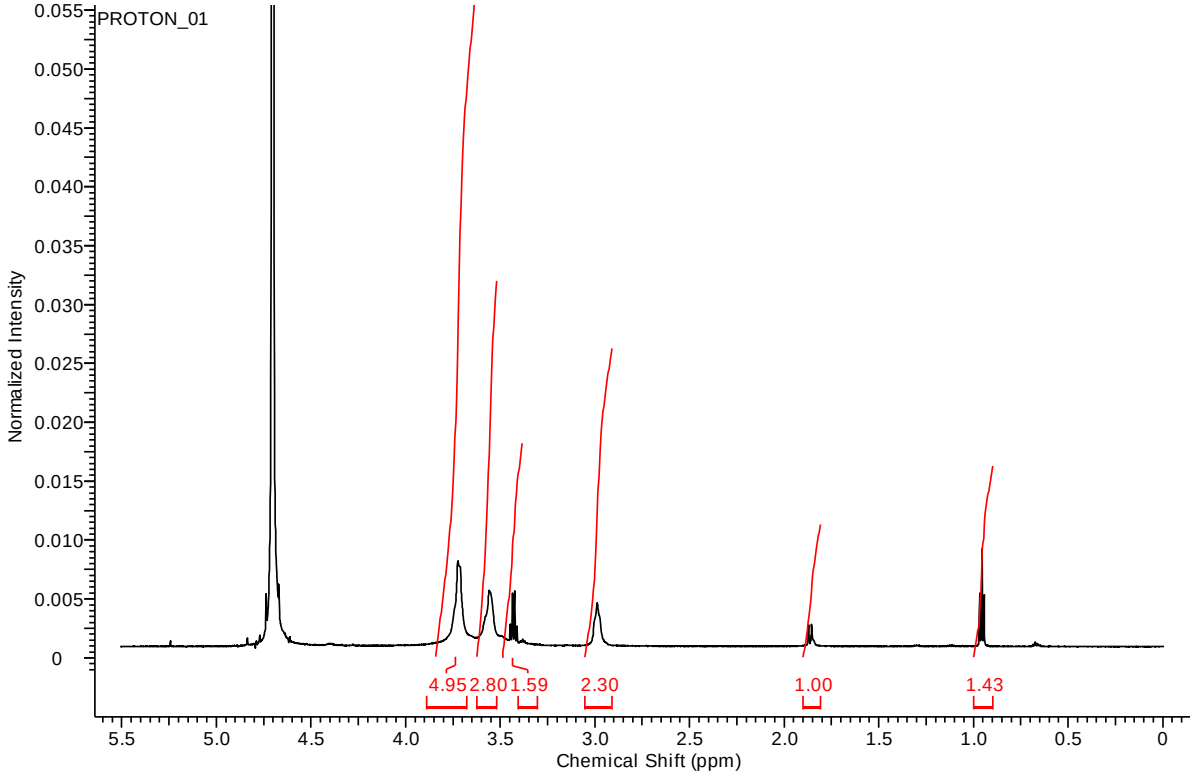


Figure 2.3 NMR peaks of (A) chitosan and (B) lauroyl chitosan

Consistent with the spectrum of Hirai *et al*[266] and Shelma & Sharma[275], ^{1}H NMR (**Figure 2.3**) analysis showed a peak at 4.95 ppm which was assigned to the solvent (D_2O. DCl). The peaks resonating at 3.6 ppm and 3.75 ppm were considered to be from the ring protons. The singlet peak at 1.80 was a result of the methyl groups of the *N*-acetylglucosamine residue of chitosan. Consistent with the FT-IR spectrum, the ^{1}H NMR spectrum of lauroyl chitosan displayed a more intense peak than that of chitosan at 0.9 ppm that was attributed to the aliphatic protons of lauric acid.[266]

Drop shape analysis

The degree of water repellency confered on chitosan by hydrophobical modification with lauric acid was assessed by determination of the contact angle of a drop of water deposited onto the material using a DSA. The basic principle of this being that the shape or angle 'θ' at the base of a sessile drop of liquid on a solid surface gives the surface energy of the solid surface. A large contact angle indicates a more hydrophobic surface, while a smaller contact angle indicates a greater surface hydrophilicity (**Figure 2.4**).

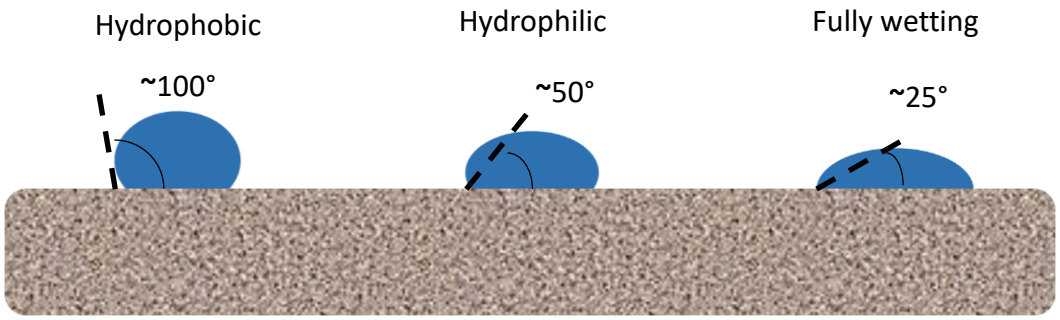
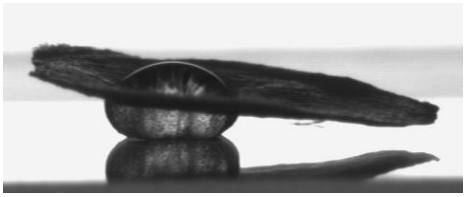


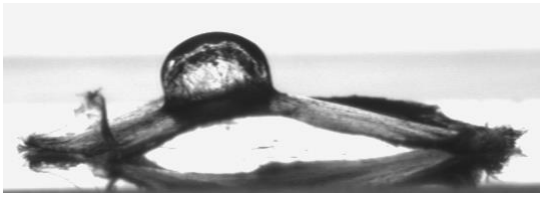
Figure 2.4 Schematic representation of a sessile drop contact angle

Contact angle also gives valuable information with regards to surface roughness, energetics and chemical heterogeneity.[276] Contact angle measurements of the samples before and after pressing with the hydraulic press gave different measurements.

(A)



(B)



(C)

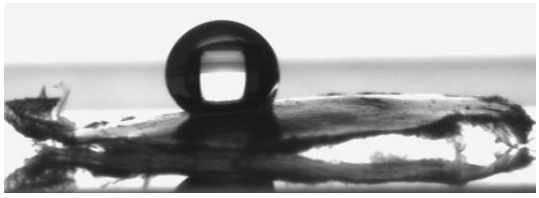


Figure 2.5 Contact angle images of water
(A) non-compacted (non-pressed) chitosan. (B) Chitosan pressed with a hydraulic press gave a 106.38° angle. (C) compacted (pressed) LCs_{34} showed a 149° angle.

Table 2.1 Contact angles of chitosan and lauroyl chitosan films

		Lauroyl chitosan		
	Chitosan	**LCs_{10}**	**LCs_{20}**	**LCs_{34}**
Contact angle	106.38°	94°	104°	149°

The LCs_{34} material was found to have increased water repellence as seen in the high contact angles compared to chitosan. The chitosan surface prior to modification had a contact angle of 106.38° and increased considerably to 149° for the LCs_{34} sample (**Table 2.1**).

The elucidation of surface characteristics like the wettability studies are important in determining the potential biological properties materials may have.[277] The surface wettability of biomaterial substances which can be characterised using water contact angle measurements, has a great influence on cell adhesion and spreading. Proteins are reported to have a greater affinity for hydrophobic surfaces compared to hydrophilic surfaces.[278] The

sessile drop method was used to give information on the contact angle of a small drop of deionised water placed on the sample.

An increase in water repellence with increasing concentration of the lauroyl groups on chitosan led to an increased contact angle as a proportion to the loading density. Chitosan's contact angle before modification was 106.38 ° which is an indication of significant water repellence. This high contact angle might be partly due to the physical processing of the material into a disc before analysis and relatively high degree of acetylation (as much as 25%) of the chitosan used. Compacting with a hydraulic press was necessary because loosed lyophilised chitosan was not suitable for a sessile drop experiment as the water percolated through the test sample (**Figure 2.5**). Significantly high contact angle for chitosan has also been reported in the literature.[279-281] These reports gave a minimum of 88.4°.

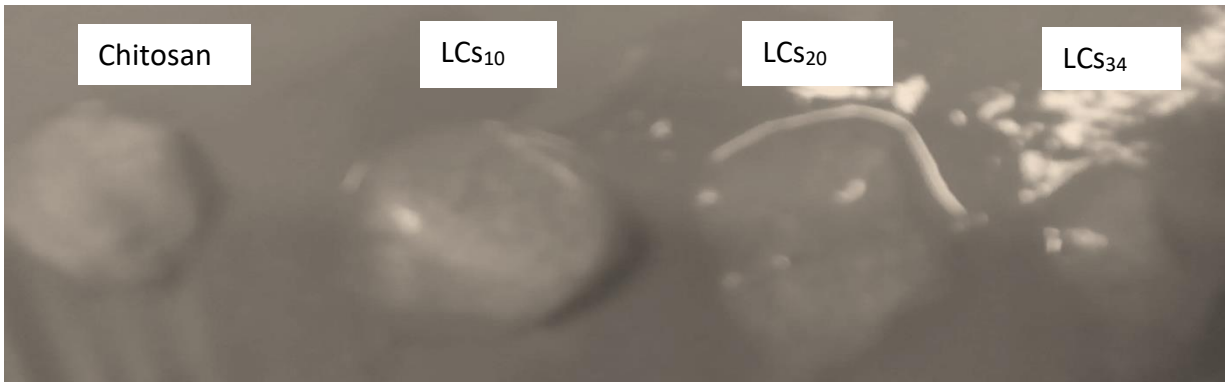
The highest contact angle of 149° for the sessile drop of water on the modified chitosan samples was observed, expectedly, for LCs_{34}. The lower loading concentrations of LCs_{10} and LCs_{20} actually demonstrated contact angles lower than unmodified chitosan, 94° and 104°, respectively.

Research findings showed that as the concentration of lauroyl groups on chitosan increase, so does the hydrophobicity, while the positive charge disappears[282], consequently, the degree of substitution of chitosan with the hydrophobic aliphatic chain affects its swelling properties[283] and subsequently, the preservation of the material's shape[284], therefore the swelling ratio was used to measure the performance of the modified polymer in physiological media solution.[267]

Figure 2.6 Scaffolds prepared in 48-well plates after lyophilisation.
The cylindrical scaffolds measured 7 mm in diameter, with a thickness of 4 mm.

Swelling index

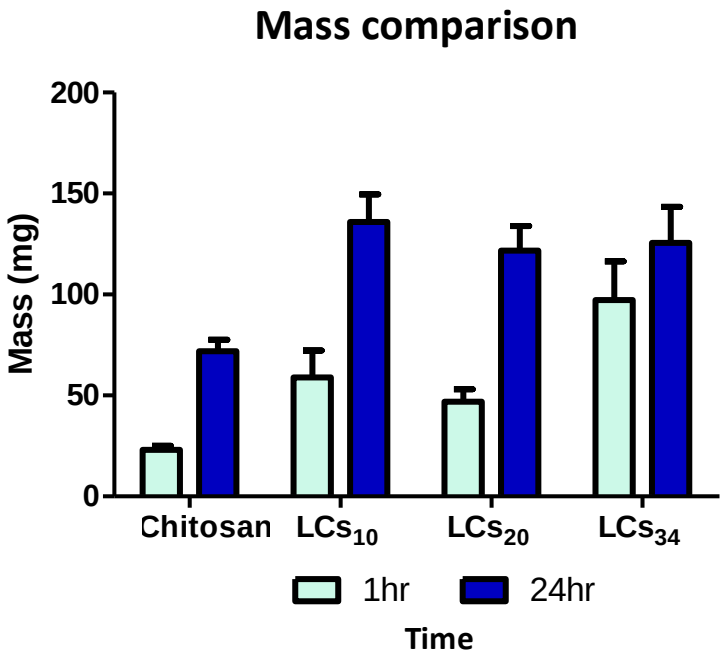
(A)



(B)

Figure 2.7 Non-neutralised and neutralised scaffolds swollen in PBS pH 7.4.
(A) Non-neutralised scaffolds in PBS. From the left showing: chitosan, lauroyl chitosan (LCs_{10}, LCs_{20} and LCs_{34}), respectively. Scaffolds before neutralisation were unstable in PBS and fully disintegrated within 4 hr (B) Neutralised chitosan before (left) and 24 hr after (right) saturation in PBS, pH 7.4.

(A)



(B)

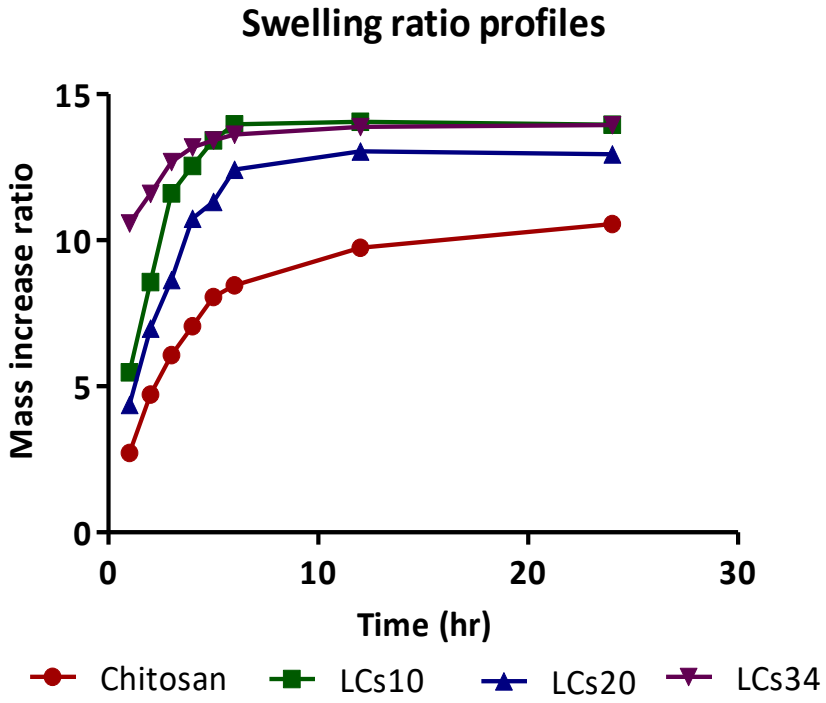


Figure 2.8 Swelling behaviour of chitosan and lauroyl chitosan in PBS

*(A) Mass comparison of chitosan and lauroyl chitosan after 1 hr and 24 hr saturation in PBS, pH 7.4 at 37 °C. (B) Swelling profile of chitosan compared to lauroyl chitosan in PBS at pH 7.4 (37 °C). Triplicates (n = 3) of each type of scaffold were measured and swelling percentage was calculated ± SD. *, ** and *** represent significant difference $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.*

Swelling of chitosan is dependent on the temperature and pH of the media.[285-286] An acidic medium would result in dissolution or degradation of chitosan through protonation of the amino groups (NH_2) or hydrolysis.[286] Swelling experiments were therefore conducted in PBS (pH 7.4) at 37 °C. A 2% w/v chitosan solution was lyophilised into a scaffold as control material. Since deformations in the material structure have an effect on cell proliferation and migration[284], the scaffolds were stabilised in 0.5 M NaOH to enable them to maintain their morphologies when soaked in PBS. Not doing so resulted in the lauric acid-modified materials dissolving into gelatinous forms. The scaffolds were then lyophilised once again before use. Chitosan scaffolds were able to maintain their shapes although from physical assessment they appeared unstable. The LCs_{20} scaffold almost completely dissolved into a gel within 2 hr and no further readings could be taken beyond this time point (**Figure 2.7**) **(A)**.

By the fourth hour of the experiment, the scaffolds had absorbed moisture to a maximum, ranging between 271% and 1400% mass increase. The swelling of all modified chitosan scaffolds had stabilised and further exposure to PBS did not cause any significant change in swelling of the scaffolds **(Figure 2.7) (B))**.

In **Figure 2.8 (A)** comparison between the swelling ratio of chitosan and lauroyl chitosan after 1 hr and 24 hr showed that chitosan almost doubled its initial weight after 24 hr while LCs_{34} swelled almost to its maximum potential within an hour followed by slow constant swelling thereafter. After 24 hr saturation, both chitosan and lauroyl chitosan had absorbed PBS, with lauroyl chitosan showing a greater swelling degree than chitosan. This was contrary to what is observed in literature. It was expected that as the concentration of the aliphatic chains on chitosan increased, the swelling of the scaffold would decrease as a result of the increase in their hydrophobic character.

The LCs_{10} sample swelled more than LCs_{20} which made the trend not clear cut. Overall, hydrophobic modification of chitosan increased its swelling capacity up to a certain extent. At higher lauric acid concentrations, the swelling capacity decreased. This was accounted for by the rigid nature of the chitosan as the hydrophobic loading increased. However, the hydrophobically modified scaffolds did not show obvious differences regardless of the concentration.

2.5 Conclusion

Hydrophobic modification of chitosan with lauric acid was achieved successfully as shown by the presence of lauric acid's aliphatic chain in both the FT-IR and ^{1}H-NMR spectra of LCs compared to the underivatized chitosan. The conjugation was completed successfully using the lauroyl chloride addition method. This method significantly shortened the reaction time from 72 hr for the carbodiimide chemistry method to 24 hr. The reaction conditions favoured *N*-acylation rather than *O*-acylation as shown by the FT-IR results.

Further characterisation using drop shape analysis and swelling ratio were performed. Results from the water drop shape analysis showed that the LCs_{34} sample made a larger contact angle with water compared to chitosan while the swelling ratio showed that the hydrophobicity of the lauroyl conjugates affected the degree of swelling to a great extent especially for the conjugate with the highest lauric acid loading density (LCs_{34}). The swelling ratio also showed that prior neutralisation of the scaffolds with 0.5 M NaOH as the unneutralised forms disintegrated in water.

Contact angle measurements were useful for predicting the adhesive properties of platelets to LCs. The derivatives with the lower loading densities did not show significant differences in contact angles to the underivatized chitosan. It was only the LCs_{34} derivative that showed a significantly higher contact angle compared to chitosan indicating its increased hydrophobicity. It was estimated that LCs_{10} with a contact angle of 94° would give the best adhesion, however this would be determined with *in vitro* studies.

Chapter 3

Bioassays of chitosan and lauroyl chitosan samples

3.1 Introduction

Platelet-rich plasma (PRP) is an autologous whole blood product which has a high concentration of platelets suspended in plasma and may contain additional leukocytes. Platelets play an important role in the blood clotting and initiation of damaged tissue repair and thus have significance for promoting healing of internal and external injuries.

Immediately after an injury, the first reaction is binding of platelet glycoprotein receptors to ECM collagen followed by platelet adhesion, which is mediated by von Willebrand factor (vWf). This is followed by platelet activation and degranulation as a consequence of a combination of factors including the platelets releasing activating agents such as adenosine diphosphate (ADP), localised thrombin activation and contact with exposed ECM proteins, e.g. collagen.[287] Platelet activation subsequently results in the release of peptide type growth factors, which together with other factors; mediate all phases of wound healing (haemostasis, inflammation, proliferation and maturation).

Throughout the entire healing process, it is important to maintain an optimum concentration of growth factors at the wound site. To this end several biomaterials have been used as platelet carriers to aid in the delivery of these PDGFs. The adhesion and activation properties of these biomaterials for platelets are a very important measure of the ability of the material to increase bioavailability of growth factors and important cytokines that regulate the immune response during the phases of wound healing. Protein–surface interactions of biomaterials play a major role in the biocompatibility or lack thereof on medical devices and have been a major focus area for several decades.[288] A number of these biomaterials including alginates, sericin, collagen, chitosan and starch have mostly been applied in enhancing skin related wound healing. Among these biomaterials, chitosan, which is the deacetylated form of chitin, has emerged as a popular biomaterial owing to its biocompatibility, ease of use, relatively low cost and availability.

The modification of chitosan to form a more hydrophobic material alters its interaction with blood[239] and other biological tissues. Chitosan was modified by conjugation of lauric acid to increase the hydrophobic properties. Lauric acid is a saturated 14-carbon fatty acid demonstrating strong bactericidal properties.[248,289]

Platelet adhesion to polymer surfaces occurs after initial adsorption of plasma proteins such as fibrinogen and thrombin on the foreign surface[290], however, the most abundant plasma protein, albumin does not favour platelet adhesion as much as fibrinogen.[291] Thus, the adsorption of specific proteins onto the biomaterial can greatly influences platelet adhesion.

There are conflicting reports regarding the benefits of using PRP; however, growth factor delivery for promoting tissue regeneration has increased in popularity. Platelets are reported to be more mitogenic for endothelial cells than whole blood.[292] While platelet adhesion plays a defined role in haemostasis and thrombosis, it also provides significant improvement in wound healing times.

Chitosan has haemostatic activity that is independent of the normal clotting cascade[210] but its degree of deacetylation influences its affinity for different plasma proteins with the highly deacetylated chitosan preferentially binding fibrinogen that leads to platelet adhesion and activation.[293] As chitosan has natural haemostatic properties[235] and has been reported to expedite contraction of wounds, this study set out to investigate the effect of various loading densities (LCs_{10}, LCs_{20} and LCs_{34}) of lauric acid during conjugation of chitosan by assessing which sample had the best activity in appropriate bioassays. It was hypothesised that hydrophobically modified chitosan biomaterial would increase interaction with platelets and therefore enhance delivery of growth factors to a treated wound while stimulating fibroblast infiltration and an overall increase in collagen deposition at the wound site. Since not much data with respect to the effects that hydrophobicity or hydrophilicity of chitosan-based materials have on platelet activation and growth factor release has been reported, it was thus one of our objectives to distinguish and quantify the interaction of the platelets with the modified hydrophobic material. *In vitro* assays were performed to determine the feasibility of utilisation of the prepared LCs samples (LCs_{10}, LCs_{20} and LCs_{34}) as wound dressing material and which lauric acid conjugation densities of chitosan provided an optimum material for wound healing.

3.1.1 Platelet-rich plasma

Platelet-rich plasma (PRP) is a small volume of plasma where most blood cells have been removed but when compared to whole blood have up to an 8-fold increase in platelet concentration.[257] It was first used for its wound healing properties in the 1980s.[294] However, it was only in 1999 that a method was described that allowed the quick and relatively easy preparation of PRP for outpatient use.[295] Of the numerous methods of separating PRP and platelet-poor plasma (PPP) from red blood cells (RBCs) described in subsequent years,[296-298] the buffy coat and density gradient centrifugation separation PRP methods are most commonly used.

In the PRP method, PRP is extracted by concentrating platelets using two gradient density centrifugation steps to ensure complete separation.[299] The first centrifugation step separates the platelets in plasma from the erythrocytes. The second centrifugation of the plasma layer (including the buffy coat) then separates the PRP from the PPP. The buffy coat method utilises one centrifugation step. Whole blood is centrifuged at relatively high centrifugal force and the thin layer of buffy coat is subsequently collected.

Thrombin or calcium addition to the PRP activates platelets to degranulate; releasing the growth factors essential for tissue repair. Collagen also has platelet-activating properties upon surface contact with the key receptor; glycoprotein (GP) Ia/IIa, integrin [alpha]$_2$[beta] taking part in the adhesion.[300] In one study, soluble or micronized type 1 collagen was used to activate platelets.[301] However, Scherer *et al* showed that non-activated PRP could be more effective in wound healing than activated PRP.[302] Based on this report, non-activated PRP was used in all the assays in this study.

3.1.2 Fibroblasts

When observed under a microscope, fibroblasts appear as flattened and elongated cells with cytoplasm filled with spherical lipid droplets that compress the nucleus.[303] They take an oblong or triangular shape when attached to a flat surface, while detachment gives rise to a change in shape to a spherical shaped cell. Actively modulated by a number of growth factors and cytokines like EGF, fibroblasts play an active role in promoting angiogenesis during wound healing.[303] Fibroblasts also play an important role in collagen architecture. In fact, fibroblasts

are responsible for synthesising glycosaminoglycans, collagen fibres, elastic and reticular fibres.

Fibroblasts are important in wound healing as they are the predominant cell type of the ECM and provides a scaffold that maintains the structure of the ECM in healthy tissue. The critical role they play in wound healing and their presence in healthy wounds from the inflammation phase right up until the wound is fully epithelialized[304] confirms their significant role. Fibroblast infiltration is an assay that measures the extent of infiltration by fibroblasts into the different LCs scaffolds being tested.

Chitosans with high degree of deacetylation stimulate fibroblast proliferation while samples with lower degree of deacetylation show less activity[218], the effect of the hydrophobic modification on fibroblast proliferation was investigated.

3.1.3 Objectives of the bioassays

Three samples of the modified lipophilic chitosan with different loading densities of lauric acid were tested *in vitro,* while a skin sensitivity patch assay was performed to determine if any of the three LCs elicited an immune response and which biomaterial showed the best properties to evaluate further *in vivo*.

The following were the aims and objectives set for the *in vitro* assays performed.

1. Platelet adhesion assay

   To measure the effect that hydrophobicity had on the adhesion of platelets to the sample surface.

2. Growth factor release assay

   To investigate the release kinetics of PDGF-AB from the hydrophobic derivatives compared to the starting chitosan and collagen using the ELISA.

3. Fibroblast infiltration

   To evaluate the extent to which fibroblasts infiltrate the different hydrophobically modified chitosan.

4. SRB assay

   To assess potential cytotoxicity or proliferation enhancement of primary fibroblasts using the Sulphorhodamine-B for cell enumeration.

5. Skin sensitivity test

To assess the sensitivity of human skin to LCs.

3.2 Materials and reagents

Dulbecco's Modified Eagle's Medium (DMEM) (with low glucose concentration) powder, was obtained from Sigma-Aldrich (St. Louis, Mo, USA). A 1.04% solution was prepared in autoclaved, pyrogen-free, ultra-pure de-ionised water and adjusted to pH 7.4 using sodium hydrogen carbonate was obtained from Merck Chemicals (Darmstadt, Germany). The solution was filtered three times using *in vacuo* filtration (Sartorius, 0.22 µm). It was enhanced with addition of 1% streptomycin/penicillin, L-glutamine and 10% foetal calf serum and stored at 4°C until use.

Foetal calf serum (FCS) was obtained from The Scientific Group (South Africa), and heat inactivated by heating at 56 °C for 45 min. It was then stored at – 20°C until use. Human PDGF-AB ELISA kit was purchased from Reliable Diagnostic Supplies (Pty) Ltd, Midrand, South Africa. The 96-well antibody coated plate, standard, detection Reagent A and detection Reagent B were stored at -20 °C while all the other reagents were stored at 4 °C until use.

Researchers from the Pharmacology Department cell culture laboratory kindly donated primary human fibroblasts isolated from human skin biopsies. Third or fourth passage cells were used for all the assays in this study.

PBS (BBA^{TM} FTA Hemagglutination buffer) consisted of sodium chloride 7.65 g/l, disodium phosphate 1.27 g/l, monosodium phosphate 0.1 g/l, monopotassium phosphate 0.21 g/l at pH 7.2. An amount of 9.32 g buffer salt was reconstituted to 1 l, autoclaved at 121°C for 25 min and stored between 2°C to 8°C. Glacial acetic acid was sourced from Merck (Darmstadt, Germany) and stored at room temperature. For the purposes of our research 1% AA was made by adding 1 ml GAA to 100 ml of distilled water and stored at room temperature in a glass container until use. Human PRP was prepared in the laboratory using freshly drawn whole venous blood obtained from healthy donors (see **Section 3.3.1**).

Lauroyl substituted chitosan with the three lauric acid loading densities (10%, 20%, 34%) were prepared as described in **Chapter 2** and stored as the freeze-dried sponges at 4°C until use. Para-nitrophenyl phosphate (p-NPP, P4744) powder was obtained from Sigma-Aldrich, South Africa and stored at -20 °C until use.

Penicillin/streptomycin (10 000 U/10 000 µg/ml respectively) solution was acquired from BioWhittaker (Walkersville, USA) and was added to the DMEM to a final concentration of 1%.

BD Vacutainer® Sodium Citrate 8 ml Glass CPT™ tubes with blue /black conventional closure, 16 x 125 mm were obtained from The Scientific Group (PTY) LTD, Randburg, South Africa. Saponin was purchased from Sigma-Aldrich (St. Louis, USA) and a final concentration of 1% w/v was prepared using deionised water and stored between 2 and 8 °C. Sodium hydroxide was obtained as pellets from Merck, (Darmstadt, Germany). The 2 M solution was made up of 8 g NaOH in 100 ml deionised water. Sodium chloride crystals were obtained from Merck (Darmstadt, Germany).

Sulphorhodamine-B (SRB) powder was purchased from Sigma-Aldrich (St. Louis, USA) and 285 mg dissolved in 500 ml 1% aqueous acetic acid and stored in a dark plastic container until use. Trypan blue powder was from Sigma-Aldrich (St. Louis, USA). A 0.1% was prepared with distilled water and stored at room temperature in a dark plastic tube. Triton-X 100 was obtained from Fluka Biochemica (Switzerland).

Acid phosphatase assay reagents[305]

Acid phosphatase buffer (APH) was made up using 0.1 M sodium acetate and 45 ml of 0.1% Triton-X. This was made up to 40 ml and could be stored at 2-8 °C for up to 4 weeks. A 1.5 ml volume of 3 M sodium acetate buffer was added to 45 µl Triton-X and this was made up to 45 ml with sterile deionised water. APH assay solution was prepared immediately before the assay by dissolving 30 mg of 4-nitrophenyl phosphate in 15 ml APH buffer. The prepared solution was protected from light and used immediately.

Sodium acetate 3 M solution was made up by dissolving 20.41 g sodium acetate trihydrate in 40 ml deionised water. Thereafter, the pH was adjusted to 5.2 with glacial acetic acid followed by making up the volume to 50 ml and finally filter sterilising using a 0.22 µm filter.

Reagents required for SEM and TEM: (provided by the microscopy unit)

The following reagents were supplied by the University of Pretoria's Microscopy Unit:
2.5 % Glutaldehyde/formaldehyde fixative, 0.075M sodium phosphate wash buffer, several solutions ranging from 30 - 100% ethanol in H_2O at 10% intervals for sample dehydration.
1% Osmium tetroxide, Hexamethyldisilazane (HMDS) and resin.

3.3 Methods

3.3.1 PRP preparation

This study was structured in accordance with the Declaration of Helsinki (last update: October 2013), and was approved by the University of Pretoria's Ethics committee (173/2018) (**Annexure 4**). From each of 10 healthy volunteers who gave informed consent (**Annexure 2**), 16 ml of blood was collected from a forearm vein by venepuncture using a 21-gauge BD Vacutainer® Safety-Lok™ blood collection set (SLBCS). The blood was drawn directly into two 8 ml BD Vacutainer® CPT™ tubes containing sodium citrate as the anticoagulant and a wax separating layer, then mixed by gentle inversion several times to ensure that the blood and anticoagulant were completely mixed. The whole blood was centrifuged at 1800 x g in an Allegra™ X-12R centrifuge for 20 min at 22 °C. The platelet-poor plasma (upper half of the plasma layer) was removed using a Pasteur® pipette and care was taken not to remove any PRP layer. Carefully, PRP (buffy coat) was collected with another Pasteur® pipette and transferred to a 1.5 ml Eppendorf® tube leaving the RBCs in the bottom layer. Aliquots of the PRP was sent to Ampath (Centurion, RSA) for counting using a Sysmex XS-1000i haematology analyser. A haemocytometer was also used to confirm the platelets counts. It was not necessary to activate the PRP as chitosan has platelet-activating properties[306], and exposure of platelets to collagen; a constituent of the modified dressing, also activates platelets.[307] Platelets were stored at room temperature with gentle agitation using a VRN-200 rocker (Taiwan) for up to 48 hr before use as Moore *et al* validated that platelets are stable at room temperature for up to five days.[307]

3.3.2 Platelet counting

Before counting the platelets, extracted PRP was rocked on a VRN-200 shaker (Taiwan) to ensure even mixing and prevent clumping. Manual platelet counts were performed using an improved Neubauer haemocytometer (Beight-line, USA) and were viewed on a Reichert-Jung Microstar 110 phase contrast microscope at 40 x magnification. These counts were validated by comparing the platelet counts with those made at Ampath using a Sysmex XS-1000i (Kobe, Japan) Automated Haematology Analyser device.

For the haemocytometer method, platelets were diluted in PBS to give a 1/10 dilution and thereafter four of the 16-set squares in the Neubauer chamber were counted and averages calculated. The following equation was used to give the platelet count per ml:

$$\boldsymbol{Cell\ count = x \text{ x } 10^5 \text{ x } 4}$$

Where x is the platelet count for four of the 16 squares. Platelet counts obtained were compared using the student t-test in Microsoft Excel 2010 and the minimum statistical significance value was set at $p < 0.05$.

3.3.3 Collagen extraction

Collagen type 1 was prepared using modified methods of Martinez-Ortiz and Sheu *et al*.[308-309] Freshly harvested porcine skin was scraped with a scalpel to remove hair and fat and cut into squares of about 0.5 cm^2 in size. A 2:1 chloroform: methanol solution was used to extract the remaining fat at 23 °C for 18 hr (the chloroform: methanol solution was changed every 6 hr). Thereafter, the skin was rinsed with methanol to remove residual chloroform. The treated skin was suspended in 0.5 M acetic acid at a ratio of 1:10 (w/v) then homogenised with an Ultra-turrax homogeniser at 15 000 rpm. This was then digested at pH 2 for 20 hr at 23 °C using pepsin (1 g/L) mixed with the NaN_3 (1 g/L) preservative. Finally, to precipitate the collagen out, the pH was adjusted to 10 using 0.5 M NaOH after which it was made to stand for 24 hr at ambient temperature. The pH was then readjusted to pH 7 with hydrochloric acid (HCl). The precipitate was filtered out and then centrifuged for 10 min at 6000 x g in an Allegra^{TM} X-12R centrifuge. The precipitated collagen was then dissolved in a 0.5 M acetic acid solution and freeze-dried.

3.3.4 Cell line preparation

Jacques Snyman of the University of Pretoria (Pharmacology Department) who isolated fibroblasts from human skin tissue biopsies kindly donated the cells used in this study. The fibroblasts were incubated in DMEM supplemented with 10% FCS, 1% Glutamax and 1% pen/strep at 37°C, 5% CO_2, 95% humidity incubator with medium changes every three days. Cells were passaged at approximately 80% confluence and third or 4th passage fibroblasts were used in this study.

To harvest the cells from the flasks, media was poured off and the cells quickly washed with DMEM without any additives before adding commercial trypsin/EDTA solution and incubated at 37°C for 4–5 min and the detached cells diluted in supplemented DMEM and harvested by centrifugation at 120 x *g* for 5 min.

3.3.5 Acid phosphatase assay for platelet adhesion

The platelets were prepared as described in **Section 3.3.1.** A method described by Chou *et al*[310] was modified to evaluate the platelet adhesion. Furthermore, the Bellavite and colleagues'[311] colorimetric acid phosphatase method was suitably modified to confirm platelet numbers. The activity of the platelet enzyme; acid phosphatase, is independent of platelet stimulation thus rendering it stable. Acid phosphatase enzyme in platelets reacts with the p-nitrophenyl phosphate (p-NPP) substrate. The platelet enzyme; acid phosphatase,[311] catalyses the breakdown of the phosphate into p-nitrophenol and phosphoric acid (H_3PO_4) as illustrated in the equation below. The yellow p-nitrophenol obtained is then measured at 405 nm using a spectrophotometer.

p-Nitrophenyl phosphate + H_2O + Phosphatase → p-Nitrophenol + H_3PO_4 + Phosphatase

To quantitatively measure the amounts of platelets adhered, a standard curve was prepared using a platelet suspension with a known platelet count. This calibration curve was used to estimate actual platelet counts of the adhered platelets. The assay was performed in 96-well culture plates that were coated with a volume of 100 µl of 1% acetic acid (negative control), 2% collagen in 1% acetic acid as positive control or LCs (LCs_{10}, LCs_{20} and LCs_{34}) at 2% for 1 hr at 37°C. This was followed by washing twice in 0.9% NaCl by plate inversion. Thereafter, 50 µl of the platelet suspension was added at 3.175 x 10^7 platelets/well followed by incubation for 60 min at room temperature without agitation. The non-adherent platelets were then removed by aspiration and the wells once again washed three times with 0.9% NaCl by plate inversion. This was followed by addition of 100 µl of 1 mg/ml p-nitrophenyl phosphate (Sigma-Aldrich) dissolved in 0.1 mol/l sodium citrate/0.1 mol/l citric acid and 0.1% (w/v) Triton-X-100 (pH 5.4). The blank value consisted of 50 µl 0.5% aqueous acetic acid solution. To stop the reaction and develop colour, 100 µl of 2 M NaOH was added to all wells followed by measurement of optical densities (ODs) at 405 nm of the yellow p-nitrophenol product using

a microplate reader. Background absorbance was measured at 605 nm and these values were subtracted from the readings obtained.

3.3.6 Growth factor release assay

The release of PDGF AB was measured following the protocol described by Shimojo *et al*.[312] To prepare PRP/LCs samples, PRP immediately after isolation was dripped onto LCs at a concentration of 200 µl of PRP/10 mg of LCs in 48-well plates. Chitosan samples were used as control and each sample was assayed in duplicate. This was allowed to interact with the scaffolds for one hour before adding 1.5 ml PBS. Thereafter, the plates were placed in an incubator at 37 °C with 5% CO_2 and after 3 hr, 6 hr, 12 hr and 72 hr, 1.5 ml samples of the culture medium were taken out of the well and stored at -80 C until further use. Fresh culture medium was added to each well. The ELISA was performed according to the Cloud Nine Protocol as seen in Section 3.3.5.

3.3.7 Enzyme-linked immunosorbent assay Protocol (As supplied by Cloud-Clone kit)

Before use, all kit components and samples were brought to room temperature (18 - 23°C). Standards were prepared 15 min before the assay. Thereafter, the standards were reconstituted with 0.5 ml of standard diluent and kept at room temperature while shaking gently. The stock solution concentration was 5.000 pg/ml. Half dilution series in 0.25 ml standard diluent were prepared in Eppendorf (EP) tubes. The 7-point diluted standards set had concentrations of: 0 pg/ml, 78 pg/ml, 156 pg/ml, 312 pg/ml, 625 pg/ml, 1 250 pg/ml, 2 500 pg/ml and finally 5 000 pg/ml. After clarifying detection, Reagent A and detection Reagent B by brief centrifugation at 12 000 x g, they were diluted 100 times for working concentration with assay diluent A and B.

Previously frozen supernatant samples were brought to room temperature and thereafter 100 µL of each added to appropriate wells in duplicate, including blank and the standard series were added to the wells of the ELISA kit plate. This was incubated at 37°C for 1 hr while covered with the plate sealer. Liquid from each well was aspirated and 100 µL of Detection Reagent A working solution was added followed by incubation at 37° C for another hour while covered. The solution was aspirated and thereafter wells were washed with 350 µL wash solution for 2 min before removing by decanting. This wash was repeated two times, removing final wash buffer residue using absorbent paper by inverting plate and blotted

against absorbent paper. Working solution of detection Reagent B was then added to each well and covered with a plate sealer and incubated again for 30 min at 37°C. The aspiration and wash procedures were repeated five times as conducted above. A volume of 90 µL substrate solution was added to each well and covered with a new plate sealer. This was incubated for 20 min while protected from light. The solution turned blue as a result of adding substrate solution. Stop solution (50 µL) was then added to each well with the solution turning yellow. To ensure thorough mixing, the side of the plate was tapped gently. Readings were conducted immediately on a microplate reader at 450 nm. The PDGF-AB ELISA kit has high sensitivity. The minimum PDGF-AB quantification detection dose of the kit as specified in the manufacturer's manual is typically less than 31.2 pg/ml.

3.3.8 Scaffold sample sterilisation

Prior to seeding the well plates, LCs scaffolds were sterilised using 70% ethanol for 12 hr then rinsed 3 times using sterile PBS via solvent exchange for 15 min each time. This was followed by overnight incubation in fortified DMEM.

3.3.9 Sulphorhodamine (SRB) assay

The SRB assay, developed in 1990 by Skehan *et al*,[313] is a rapid, sensitive, simple and cost effective in vitro colorimetric assay for cell enumeration used for cytotoxicity testing.[21] Basically, SRB, a negatively charged, bright pink aminoxanthene dye binds to basic amino acids in cells[22] that have been fixed on culture plates with trichloroacetic acid (TCA). The stoichiometric binding of SRB to cells under mild acidic conditions allows the quantity of bound dye to be used as a measure of relative cell number and overall, as a measure of cell proliferation or cytotoxicity.[23]

A slightly modified method of Orellana and Kansiki[314] was used to describe the proliferation of fibroblasts when exposed to LCs. A volume of 50 µL of human fibroblasts at a concentration of 2×10^4 cells/well were seeded into a 96 well plate and allowed to attach overnight. The blank wells contained 100 µL 10% FCS-supplemented DMEM while the cells were exposed to 50 µL non-supplemented DMEM (negative control), 1% acetic acid (vehicle control), 1% saponin (positive cytotoxicity control) or the LCs samples (LCs_{10}, LCs_{20} and LCs_{34}) in supplemented DMEM for 72 hr. The cells were then fixed with 50 µL cold 50% (w/v) TCA and incubated for 1 hr at 4 °C. This was followed by washing the plate 4 times in slow running

water. The plate was left to air-dry at room temperature. A volume of 50 µL of 0.04% (w/v) SRB was then added to each well. This was left at room temperature for 1 hr and thereafter rinsed 4 times with 200 µL 1% (v/v) aqueous acetic acid to remove unbound SRB dye. A 10 mM tris(hydroxymethyl)aminomethane (Tri base) solution (pH 10.5) (100 µL) was added to each well and plates rocked for 10 min to dissolve the dye. Absorbance was measured at 510 nm using a microplate reader. Percentage proliferation or cytotoxicity of the cells was expressed relative to the negative control.

3.3.10 Skin sensitivity test

A 2% lauroyl chitosan solution was tested on 16 healthy participants lacking any skin irritation that might be confused with an allergic reaction. Each participant was provided the information and allowed to ask questions before signing a written consent (**Annexure 1**) Ethics approval (99/2016) (**Annexure 3**) was obtained from the University of Pretoria ethics committee prior to the commencement of the study.

The inclusion criteria below were followed:

- 18 to 70 years
- Volunteers not participating in another clinical trial already
- Volunteers not on any topical or oral corticosteroid or antihistamine medication up to 7 days before the test.

Exclusion criteria were as follows:

- Volunteers who have a history of allergies to any cosmetics could not take part in this study.

An aliquot of 300 mg lauroyl chitosan (LCs) was weighed and dissolved in 1% acetic acid. A total of 16 healthy volunteers aged 25 – 57; 6 males and 10 females participated in the study. The mean age of the participants was 34. Each participant signed an informed consent form (Annexure 1). The patch consisted of 1 cm^2 gauze soaked in 0.5 ml of 2% lauroyl chitosan dissolved in 1% acetic acid solution. The control was the same gauze soaked in 0.5 ml 1% acetic acid solution. The patches were applied directly to the inner forearm at about 2 cm apart and covered with Opsite® Flexifit film dressing and left on for 48 hr. After removal of

the patches the skin was observed for signs of irritation, swelling, discolouration and observed for a further 24 hr after removal.

Any changes to the skin, e.g. itching, erythema were recorded according to the interpretation adapted from the International Contact Dermatitis Research Group (ICDRG). (Error! Reference source not found.)

Table 3.1 Recording of patch test reactions according to the ICDRG

Notation	Description	Interpretation
-	No changes to the skin	Negative
+?	Faint erythema	Doubtful reaction
+	Palpable erythema - moderate oedema or infiltrate	Weak reaction
++	Numerous papules and vesicles present	Strong reaction
+++	Coalescing vesicles, bullae or ulceration	Extreme reaction

3.3.11 Fibroblast infiltration

TEM and SEM were used to assess the extent of infiltration of human fibroblasts seeded on the surface of chitosan, LCs_{10}, LCs_{20} and LCs_{34}. Briefly, sterilised scaffolds were incubated in supplemented DMEM for 24 hr. A volume of 200 µL of human fibroblast suspension at a concentration of 2×10^4 cells/well were seeded directly onto each of the different LCs samples. A volume of 300 µL media was added to the wells 4 hr later. The samples were maintained in supplemented DMEM for 7 days. The medium was changed every third day.

3.3.11.1 Sample preparation for SEM and TEM

The samples were washed with the sodium phosphate buffer (0.07 M) and after removing the buffer, fixed for 1 hr with 2.5% GA/FA solution. The fixative solution was removed and thereafter the sample was washed three times with sodium phosphate buffer for 15 min each time. After removal of the buffer, the samples were post fixed in the fume hood with 1% osmium tetroxide for one hour. The samples were once again washed three times for 15 min each time and finally dehydrated using a graded series of ethanol (30%, 50%, 70%, 90% and

3 x 100%) for 15 min each. The last ethanol was left for 30 min. The ethanol was removed and half the samples were then prepared for SEM while half were prepared for TEM.

3.3.11.2 SEM sample preparation

A 1:1 mixture of HDMS and 100% ethanol was added to the samples and left for one hour with the samples covered. After removal of this mixture, HDMS was added and left for another hour. This was removed, fresh HDMS was added, and the container left open for the samples to dry. The samples were then mounted on suitable grids and carbon coated using an automated coater.

3.3.12 TEM sample resin embedding

After removal of ethanol from the samples, a 1:2 mixture epoxy resin:ethanol was added to the samples and left for an hour. This was replaced with a 1:1 mixture of epoxy resin:ethanol and left for another hour. This was followed by a 2:1 mixture of epoxy resin:ethanol and left for another hour. Finally, the mixture was replaced by 100% epoxy resin and was left to stand for 4 hr. After removal of the resin, fresh resin was added and a sample number was assigned to the samples before embedding them in moulds. The samples were placed in the oven for 36 hr for polymerisation and thereafter block trimmed and sectioned.

3.4 Statistics

For all the *in vitro* experiments, means of triplicates were used for calculations while the Student t-test and one-way analysis of variance (ANOVA) in Microsoft Excel 2010 and GraphPad 5 software were used unless otherwise indicated. The p-values of 0.05 or less were considered statistically significant.

3.5 Results and discussion

Biological response to biomaterials is significantly influenced by surface properties such as topography, chemistry, elasticity, surface free energy and charge which may affect protein and cell interactions, and eventually the host response.[278,315] The current *in vitro* studies investigated interaction between hydrophobically modified chitosan with platelets and fibroblasts. The use of PRP for wound healing has been studied extensively and proven exceptional in wound healing. Initial adhesion of platelets to the surface has a great impact on their performance *in vivo/in vitro*. Fibroblasts, a basic component of a healthy healing

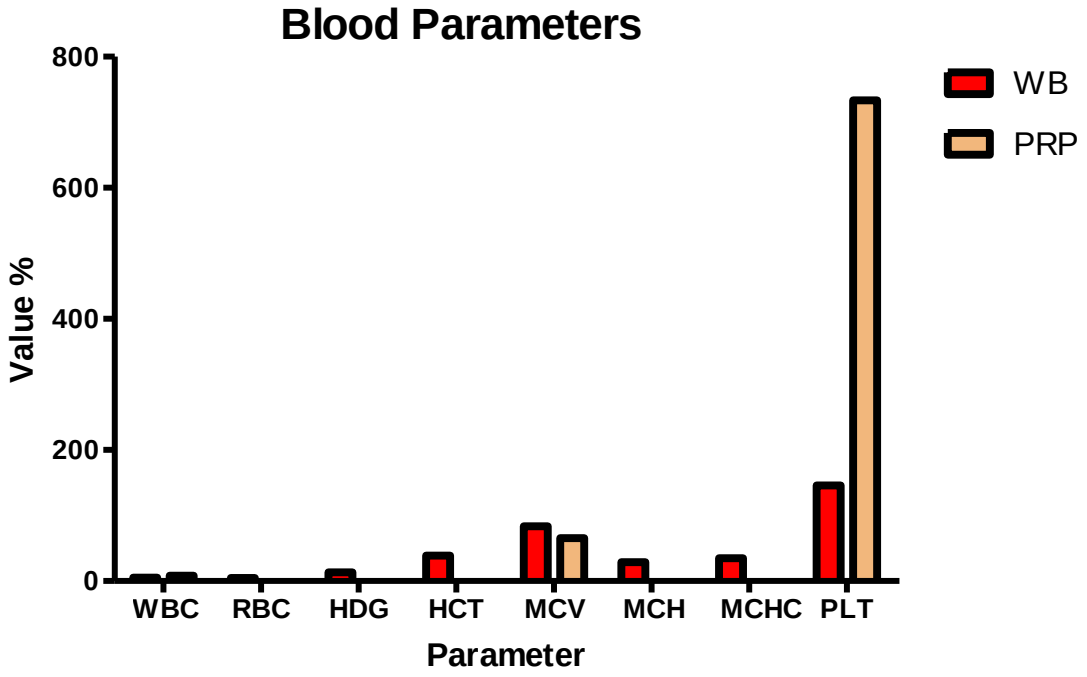
wound, are a significant factor in the wound healing process. Their role in fundamental processes such as breaking down the fibrin clot, synthesising collagen, generating a new ECM along with contracting the newly formed granulation tissue is well elucidated[68,304] and was assayed further.

Haematology analysis of platelet-rich plasma

Platelet counts from whole blood and platelet-rich plasma samples from each study participant gave on average 18 ± 2 percent PRP extract, with an average increased platelet count from 146 x 10^9 platelets/L in blood to 733 x 10^9 platelets/L in the PRP extract. The median platelet count in PRP was 765.5 x 10^9 platelets/L. Platelet counts from whole blood were all within the normal human range; ranging from 100 to 209 x 10^9 platelets/L. Contrary to literature reports, PRP preparation resulted in an average 5-fold increase in platelet concentration which was less than the 8-fold expected.[257] Only 16.27% participants gave an 8-fold increase in the platelet count before and after extraction.

Further factors measured of the complete blood count compared to the PRP demonstrated that RBC, HDG and HCT concentration decreased by 99% while WBC in the concentrated plasma had an increase of close to 60% of the initial value (**Figure 3.1**). The high WBC concentration is an added benefit of PRP. In the early phases of wound healing, platelets release mediators, which are chemoattractant to leukocytes, which assist in cleaning and removing damaged tissue debris and foreign particles.

(A)



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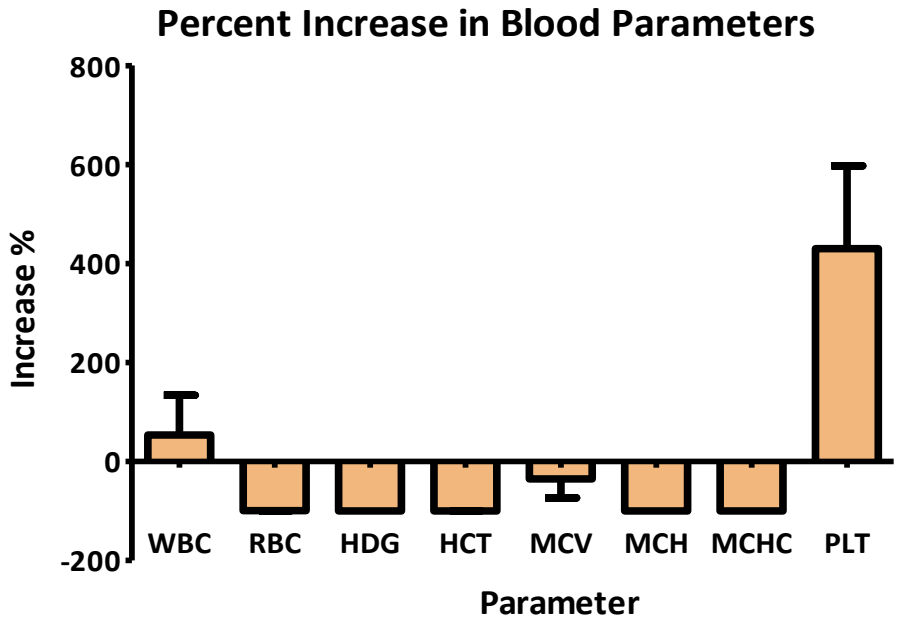

Figure 3.1 Comparison of average white blood cells, red blood cells and platelet counts between whole blood and PRP of 10 participants

Collagen extraction

Collagen, the main activator of platelet response after injury, was the positive control for all platelet assays performed. Types I, III, and VI promote platelet adhesion and aggregation[316] resulting in platelet activation and growth factor release.

Type 1 collagen extracted from porcine skin and was prepared and characterised using FT-IR. Infrared spectra of diverse collagens extracted differently showed strong peak similarities for the major amides (**Table 3.2**) with however, fine structural differences in the amides I and II. The FT-IR spectrum of the extracted collagen had several similarities with the FT-IR spectra of collagen in other animals (**Figure 3.2**)**(A)**.[317] CoP's Amide A peak was at 3307 signalling strong binding to a hydrogen as the typical N-H stretching vibration occurs around 3310-3270 cm^{-1}. Consistent with literature reports, the amide B peak was at 3084 cm^{-1}. Amide I band's frequency typically occurs at 1700-1600 cm^{-1} and exhibits a strong protein signal in this region.[318] Approximately 80% of Amide I is the stretching vibration of the C=O peptide bonds[319] with minor contribution from the C-N stretching vibration.[320] Amide I at 1640 cm^{-1} showed a weak band due to the lower frequency showed a weak band. The downward shift of the band was a result of many reasons including a possible higher concentration of imine groups compared to amino groups. Between the 1510 and 1580 cm^{-1} region the amide II frequency occurs as a consequence of the N-H bending vibration and the C-N stretching vibration. CoP's amide II which occurred at a higher frequency of 1543 cm^{-1} could have had an upward shift as a result of a partially hydrated collagen sample as suggested previously by Susi *et al.*[321] Amide III band at 1326 cm^{-1} was easier to determine compared to Amide I as water vibrations do not interfere in this region.[322]

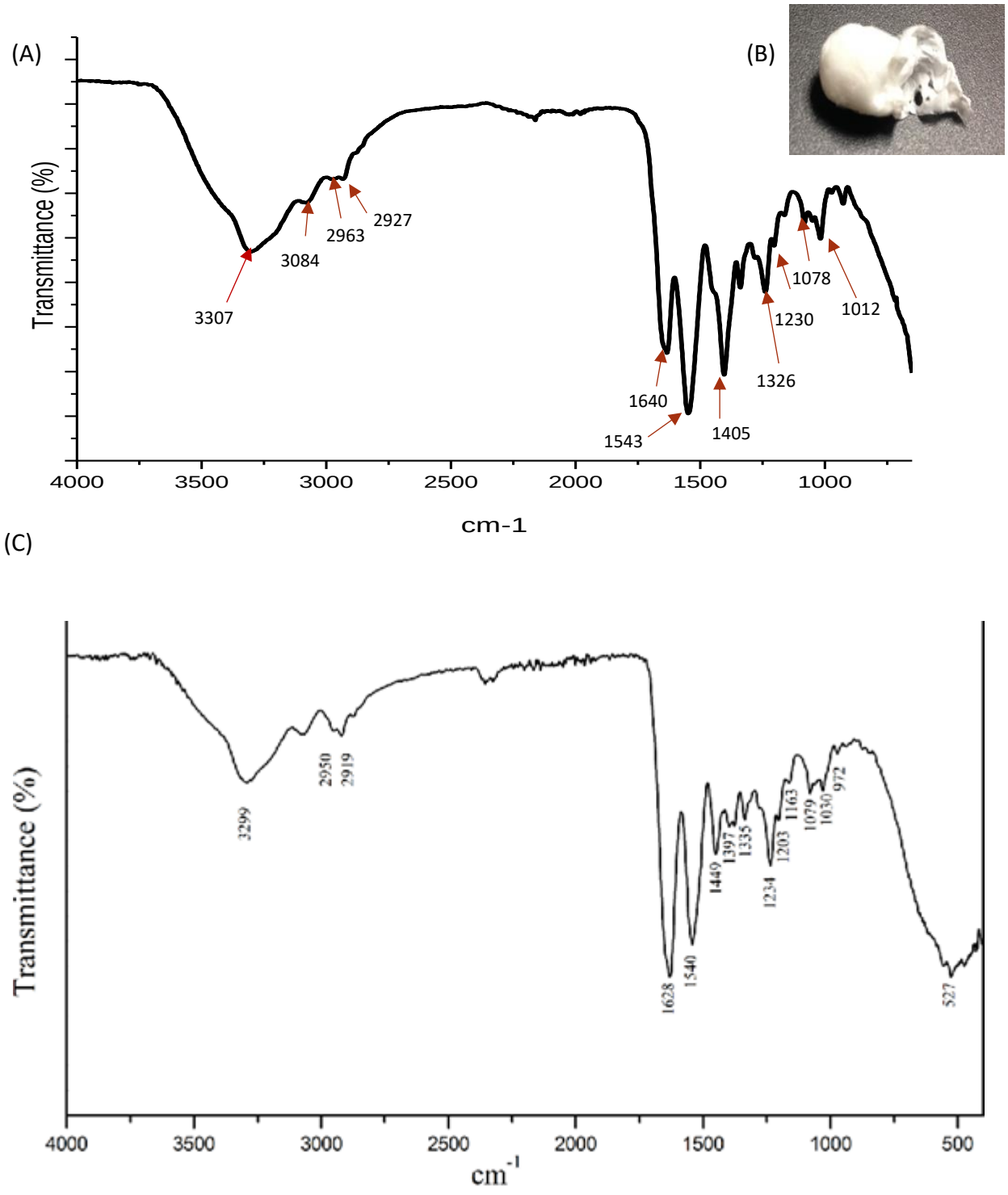

Figure 3.2 FT-IR of collagen extracted from porcine skin compared to Buffalo skin collagen
(A) FT-IR of extracted collagen (B) Extracted collagen after lyophilisation at -55°C (C) Rizk and Mustafa 2016. Extraction and Characterisation of Collagen from Buffalo Skin for Biomedical Applications

Table 3.2 Amide band frequencies (cm^{-1}) of native and denatured collagen dry films.[317]

Band	Native Collagen				
	RTT Section	*Earthworm cuticle*	*Cartilage ex H_2O*	*RTT ex 1% acetic acid*	*Denatured Collagen RTT ex hexafluoro-isopropanol*
Amide A	*3326 (perp)*	*3330*	*3321*	*3329*	*3307*
Amide B	*3076 (perp)*	*3079*	*3074*	*3072*	*3073*
Amide I	*1661 (perp)*	*1663*	*1659*	*1661*	*1656*
Amide II	*1529 (par)*	*1533*	*1533*	*1540*	*1533*

Consistent with peaks shown in **Table 3.2** and collagen from buffalo skin (**Figure 3.2**); CoA showed Amide A at 3307 cm^{-1}, Amide B was at 3084 cm^{-1}, Amide I at 1640 cm^{-1} and Amide II at 1543 cm^{-1}.

Acid phosphatase assay for platelet adhesion

Albeit the mechanism of chitosan's haemostatic activity remains abstract, its ability to interact with blood is well known and reported. Some research findings suggest that this activity is independent of platelets or coagulation factors[323] and also independent of the intrinsic pathway.[293] In fact, the positive charge on chitosan was found to inhibit contact system activation.[208] Electrostatic interactions between chitosan's positively charged amino groups and the negatively charged moieties of the mucus gel layer have however been shown to proceed via hydrogen bonds and ionic interactions.[189] Chitosan's influence on adsorption of certain proteins like fibronectin or perlecan results in augmented platelet adhesion and activation[324]; thus, increasing the interaction of growth factors with the wound.

Plasma protein adherence to foreign surfaces is a major drawback in the use of cardiovascular and other implants[290]; however, when it comes to wound healing material, it is a positive attribute. The next objective of this study was to investigate how derivatisation with different concentrations of the lipophilic lauric acid moiety affected platelet binding capacity of chitosan. The adhesion of platelets to the conjugated LCs surfaces compared to chitosan was examined. The expectation was that hydrophobically modified chitosan paste used

synergistically with PRP would have high adhesion properties for platelets and would thus be used as a platelet delivery vehicle for the wounds. In some studies, in line with the study's hypothesis, platelets adhered more strongly onto the hydrophobic surfaces than to the hydrophilic surfaces.[277] Wu *et al* found that surfaces with contact angles above 65 °C favoured higher fibrinogen adsorption and hence, platelet adhesion.[325] Fibrinogen and von Willebrand factor are essential proteins in platelet adhesion and aggregation. Adsorption of fibrinogen onto a surface creates a suitable environment for the adhesion of platelets.[326]

Assessments were made to determine if the adhesion was a result of the modified material or came about spontaneously and/or if hydrophobic modification resulted in superior adhesion of platelets.

Initially, the adhesion was measured by coating a 6-well plate with the different material and followed by the addition of PRP for an hour, finally washing out with PBS and thereafter quantifying the adhesion microscopically. The interference of the dried PBS crystals with the adhered platelets made viewing of the platelets microscopically a challenge. The dried refractive PBS crystals sometimes overlapped with the platelets obscuring the view. The protocol of Chou *et al*[310] was also followed; however, similar challenges with the PBS were experienced. The present study omitted the washing of platelets in order to decrease the occurrence of spontaneous activation of platelets[327] and the alteration of platelet function. Additionally, washed platelets are devoid of fibrinogen and other important clotting factors[328] that also influence platelet adhesion. Use of PRP also gave a better estimate of platelet function in the physiological environment.[284] PRP used eliminated the need to count platelets. However, platelet concentration was still determined for each blood sample using a haemocytometer and verified using an automated platelet counter at Ampath. Platelets were viewed as small, round refractile bodies at 40 x magnification. Manual platelet counts gave values of 59.4 x 10^6 platelets/ml. These platelet values were significantly low compared to what is commonly seen in PRP. Although Umshakar's study showed that unstained platelets counts give reliable results,[329] samples were still sent to Ampath to validate these findings. The average count was approximately 733 x 10^6 platelets/ml in PRP when counts were done at Ampath. Ampath platelet counts were consistent with the average platelet counts seen in PRP.

Spectrophotometric assay comparison of collagen, the positive control to Cs and the three conjugated LCs samples (LCs_{10}, LCs_{20} and LCs_{34}) gave more reliable results, using para-nitrophenyl phosphate (p-NPP) substrate in the acid phosphatase assay. In the said assay, phosphatase enzymes hydrolyse the p-NPP substrate releasing p-nitrophenol, which is then measured in a colorimetric assay. A standard curve (**Figure 3.3**) drawn using known platelet concentrations was used to calculate platelet concentration from each sample.

The chitosan material adhered more than 90% platelets than the collagen positive control (**Figure 3.4).** The acetic acid vehicle control adhered the least concentration of platelets while adhesion capacity decreased as the hydrophobicity increased. No significant differences in adhesion capacity was seen between LCs_{20} and LCs_{34}, while all the LCs surfaces adhered significantly less than the chitosan surface.

Platelets adhering to chitosan had pseudopods while those adhering to LCs_{10} were firmly adhered whereas maintaining their initial shape (**Figure 3.5**).

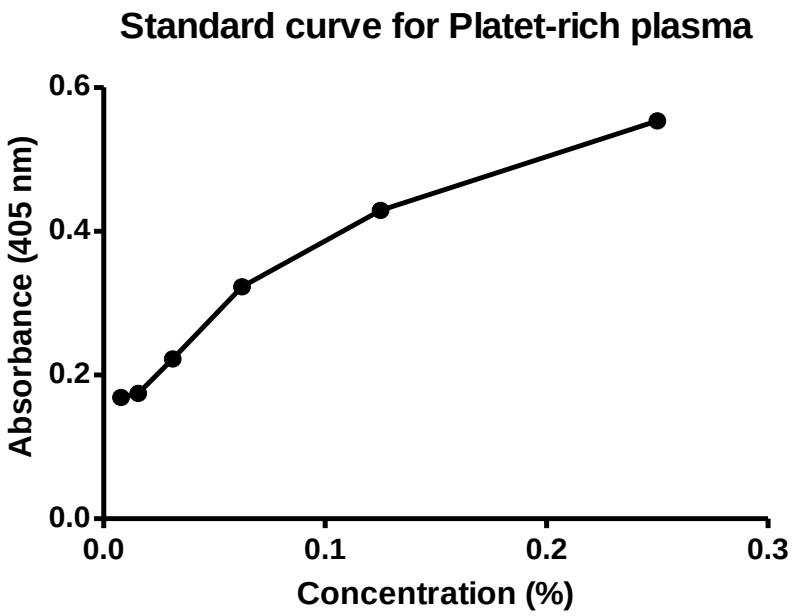

Figure 3.3 Standard curve of PRP in NaCl
Concentrations ranged from 0.78%, 1.56%, 3.13%, 6.25%, 12.50% and up to 25.00%. The curve displayed that p-NPP absorbance was directly proportional to platelet count from 159 × 10^6 to 5 × 10^6 platelets/ml. Total platelet concentration before dilution was 635 x 10^6 platelets/ml.

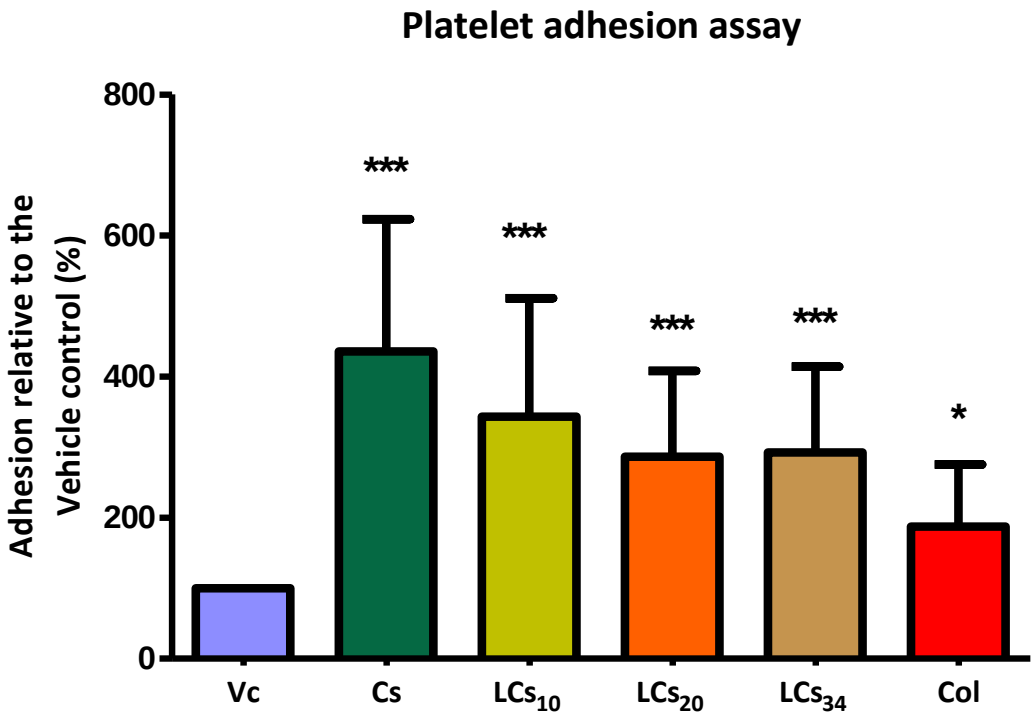

Figure 3.4 Platelet adhesion to the samples: collagen (Col), chitosan (Cs), lauroyl chitosan
*The three loading densities (10%, 20% and 34%) (LCs_{10}, LCs_{20} and LCs_{34}) were compared to the vehicle control (Vc). The Cs sample had the most pronounced concentration of adhered platelets. The average total platelet count used was 635 x 10^9 platelets/Litre. Results were expressed as mean +/- standard deviation of the mean *p < 0.05; **p < 0.01; and ***p < 0.001 vs. the vehicle control.*

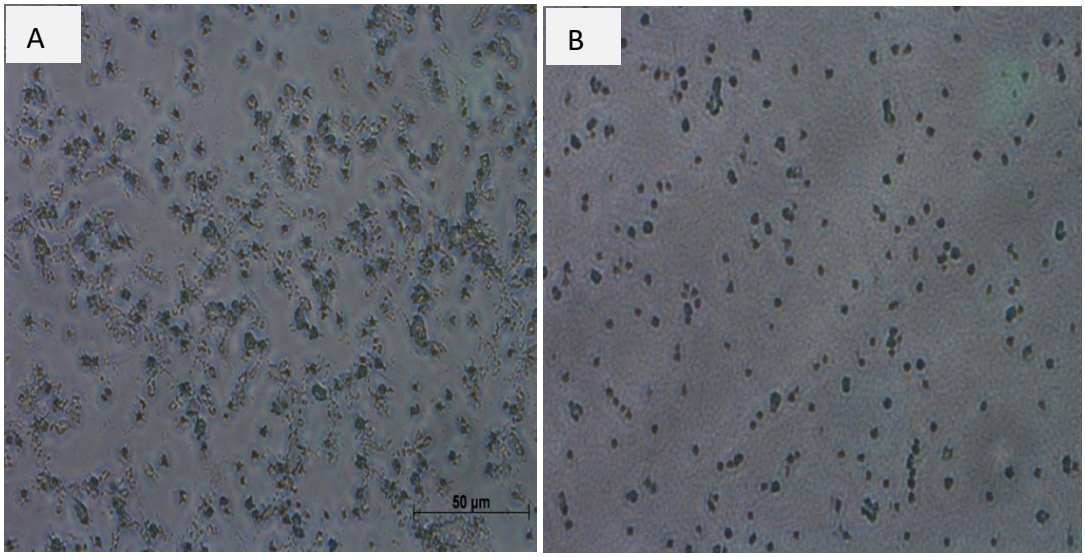

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Figure 3.5 Microscopic images of platelet adhesion to chitosan (A) and LCs_{10} (B).

Of the initial 3.175 x 10^7 platelets per well, only an average of 47.7% adhered. The expectation was that as sample hydrophobicity increased, so would platelet adhesion. Contrary to this, chitosan had the highest concentration of adherent platelets followed by LCs_{10}. Evidently;

increased hydrophobicity of the samples seemed to cause a decrease in platelet adhesion. A 90% increase in platelet adhesion was seen with chitosan compared to collagen, while LCs_{34} had a 38.35% increase in adhesion. These findings contradicted the study's hypothesis and were inconsistent with the commonly perceived observations. However, they were consistent with Neumann and colleagues' study on the adhesion of platelets to different polymer surfaces. Neumann found that with increasing hydrophobicity as well as water contact angle, the density of adhered platelets declined.[291] In line with these findings, Yeh and Lin also contradicted this study's hypothesis, with their hydrophilic sulphonated chitosan membranes attaching more platelets than the hydrophobic membranes. They attributed this attachment to the indigenous characters of sulphonate/sulphonic acid groups.[330]

In some studies, in line with the study's hypothesis, platelets adhered more strongly onto the hydrophobic surfaces than to the hydrophilic surfaces.[277] Wu *et al* found that surfaces with contact angles above 65 °C favoured higher fibrinogen adsorption and hence, platelet adhesion.[325] Fibrinogen and von Willebrand factor are essential proteins in platelet adhesion and aggregation. Adsorption of fibrinogen onto a surface creates a suitable environment for the adhesion of platelets.[326]

These results also contradicted the study by Xu *et al* [277] on the adhesive force of albumin, fibrinogen and FXII. Their findings presented a step increase in adhesion force of all the samples as the surface contact angles increased above $\vartheta \sim 60°$ ($\tau < 36.4$ dyn/cm). Xu *et al* thus proceeded to suggest that the data they obtained could possibly be used as a standard for distinguishing a surface as either 'protein adherent' or 'protein non-adherent'. In a study by Shi and colleagues, a hydrophilic scaffold with a water contact angle value of 34.81 ± 2.8° hardly adhered any platelets at all while however promoting the adhesion and proliferation of human umbilical vein endothelial cells.[331]

Research data suggested that chitosan induced platelet adhesion and activation proceeds via augmented expression of GPIIb/IIIa complex and increased $[Ca^{2+}]_i$ mobilisation.[310] Fibrinogen is binds to the GPIIb/IIIa receptor on platelets[332] while the same receptor increases $[Ca^{2+}]_I$; thus, leading to increased platelet adhesion. Chitosan's adhesive potential lies mainly on its positive charge that interacts with the negatively charged platelets and plasma proteins. It was therefore suggested that the lauroyl modified chitosan because of the hydrophobic

lauroyl groups resulted in a negatively charged polymer surface that repelled the negatively charged platelets. Increased hydrophobicity is also known to reduce the shear stress of a surface, decrease the surface area available for adhesion and overall reduce the concentration of adherent platelets.[333] Fatty acids induce platelet aggregation, however, when it comes to adhesion, fatty acids most likely form a smooth surface that repels platelets.

The platelets adhered to the Cs sample had pseudopodia indicating activation, while those adhered to LCs_{10} were firmly adhered but still maintained their discoid shape (**Figure 3.5**) **(b)**). The formation of dendritic pseudopodia marks one of the initial events in platelet adhesion onto foreign substances. Based on Hattori and Ishikara's results[306], chitosan activates platelets and releases growth factors but the current hypothesis was that the hydrophobic chitosan would enhance binding of platelets and thereafter activate them. Hattori and Ishikara's study of 13 types of chitosan to see how their varying MWs and DD would affect platelet activation showed that chitosan with a DD of > 75% increased the release of platelet factor 4 into the plasma.[306] The MW and DD, however, had an impact on platelet activation which in turn influenced the quantities of growth factors released into the plasma. Their results suggested that the chitosan with a similar DD and MW (DD of 75–85% and an MW of 50,000–190,000 Da) to the chitosan in the present study was the most effective for PRP therapy.[306]

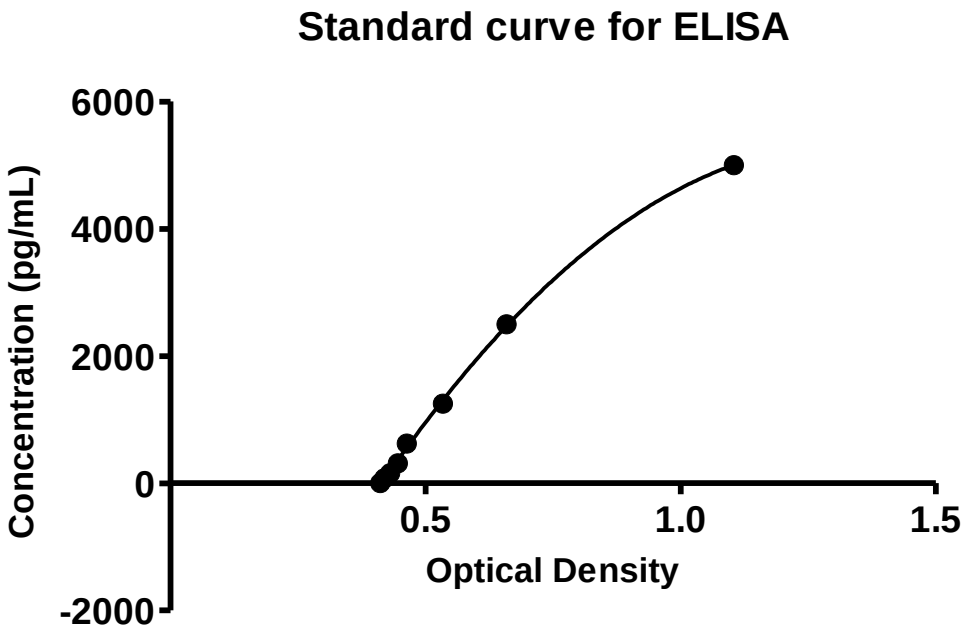
PDGF-AB release kinetics

Growth factors play an important role in the regulation of cellular events involved in granulation tissue formation and wound healing.[334] Their release into the wound is essential for healing to occur. PDGF and TGF-β are significant in the initial phase of wound healing chemo-attracting neutrophils and macrophages and also stimulating the proliferation of fibroblasts.[335] PDGF-AB plays a key role in the formation of blood vessels as it enhances cell growth and division.

After determining that the LCs_{10} exhibited overall superior *in vitro* properties, the study proceeded to measure its PDGF-AB release kinetics compared to Cs and collagen. As cited previously, chitosan's positive charge interacts with the positive charge on biological membranes; thus, activating or binding to them. Hattori and Ishikara investigated the feasibility of optimising PRP therapy by using chitosan in synergism with PRP to improve

platelet activation ability. [306] Their findings displayed an increased growth factor release and increased proliferation of fibroblasts and adipose tissue-derived stromal cells which depended on the MW and DD of chitosan.[306] Results from the ELISA assay showed that LCs_{10} exhibited a sustained release of PDGF-AB over 24 hr compared to both chitosan and collagen. In the first hour, chitosan and collagen had a burst of growth factors released while LCs_{10} only released 41% when compared to collagen. Within 3 hr, the released PDGF-AB from both chitosan and collagen had stabilised; however, the LCs_{10} sample released growth factors for a further 18 hr at a sustained rate. Collagen and chitosan both activate platelets immediately upon contact[301], thus the burst release of growth factors within the first hour was possibly a consequence of the activated platelets. The LCs_{10} sample because of the hydrophobic groups did not immediately activate the platelets; thus, the initial slow release of the growth factors. The use of PRP with a carrier provides a controlled release system for growth factors. Chitosan, when modified is able to control growth factor release when used as a carrier for platelet-rich plasma.[312]

(A)



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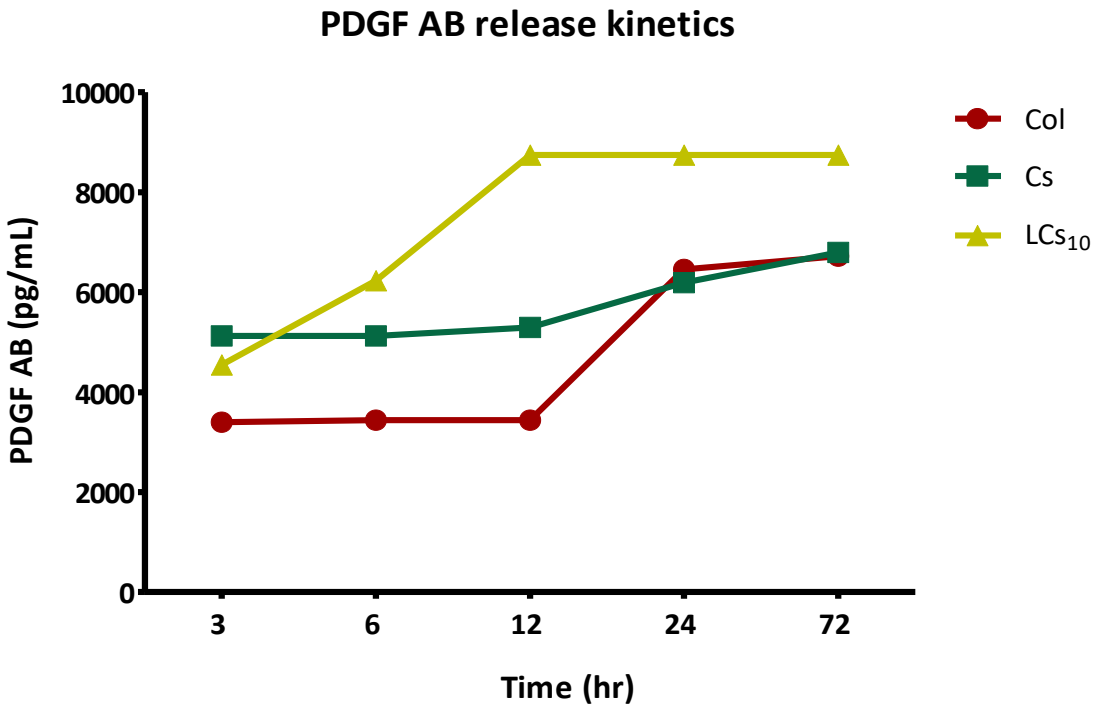

Figure 3.6 (A) Standard curve for ELISA (B) ELISA PDGF-AB release profiles for chitosan, LCs_{10} and collagen over 72 hr

Toxicity assays

One of the key benefits of the use of the polymer chitosan is its biocompatibility. Used extensively in several fields and even as a drug excipient[336], chitosan is generally accepted as a non-toxic biopolymer. Lauric acid on the other hand is non-toxic but rather induces cell proliferation and has strong bactericidal properties.[248,289] With a plethora of background knowledge on the biocompatibility of our material, the authors proceeded to conduct toxicity studies as the chemical modification of chitosan to LCs could possibly alter its pharmacodynamics. A skin sensitivity test and an *in vitro* toxicity assay were done as preliminary studies to evaluate the possible adverse reactions and skin sensitivity to the LCs_{34} material.

Skin sensitivity test

The solvent; 0.5% acetic acid (CH_3COOH), used for solubilising the samples, is the second simplest carboxylic acid and an important chemical reagent in industrial chemistry. In the food industry, it is the main ingredient in vinegar besides water, making up 3 - 9% acetic acid by volume. Glacial acetic acid is corrosive to skin, however, 1% acetic acid is safe and non-toxic in the treatment of wounds.[337-338] The acidic nature of acetic acid and other varied mechanisms that include protein denaturation, account for acetic acid's antibacterial properties. For the purposes of wound healing, the commonly used acetic acid concentrations range between 0.5 and 5.0%. Therefore, no skin toxicity was expected to result from the use of 0.5% acetic acid. However, one study participant; P13 had a rash showing sensitivity to acetic acid even in the 0.5% solution. The fact that P13 showed no reaction to the LCs_{34} patch suggested that the 2% LCs_{34} mixture formed a barrier that protected P13 from the harmful effects of the dilute acetic acid. Further investigations into this matter are important. Reduction in pH as a result of the exposure to 0.5% acetic acid for an extended period possibly caused dry skin experienced after removal of the patches.

The inclusion criteria included males and females between the ages 18 to 65. Although it is reported that the level of skin sensitivity between males and females varies[339], no significant differences between the male and the female responses were observed. The sex and age of the participants were not determinants of the reaction of the skin to LCs_{34}. Contrary to typical expectations relating to age and sex, the most severe response displayed in a 25-year old

male who was sensitive to both LCs_{34} and the control. Overall, 56.25% of the participants did not respond to the test material nor the control (**Figure 3.8**).

Table 3.3 **Skin sensitivity test results**

Participant	**Age**	**Sex**	**Results**		
			Control		**Test**
				Itching?	
P1	57	F	-		-
P2	34	F	-		-
P3	33	F	-		+?
P4	30	M	-		-
P5	51	M	-		-
P6	35	M	-		-
P7	35	F	-		-
P8	30	M	-		-
P9	25	F	-		-
P10	29	F	-		-
P11	41	F	-		-
P12	23	M	-		-
P13	25	M	+?		+?
P14	35	M	-		-
P15	26	F	-		-

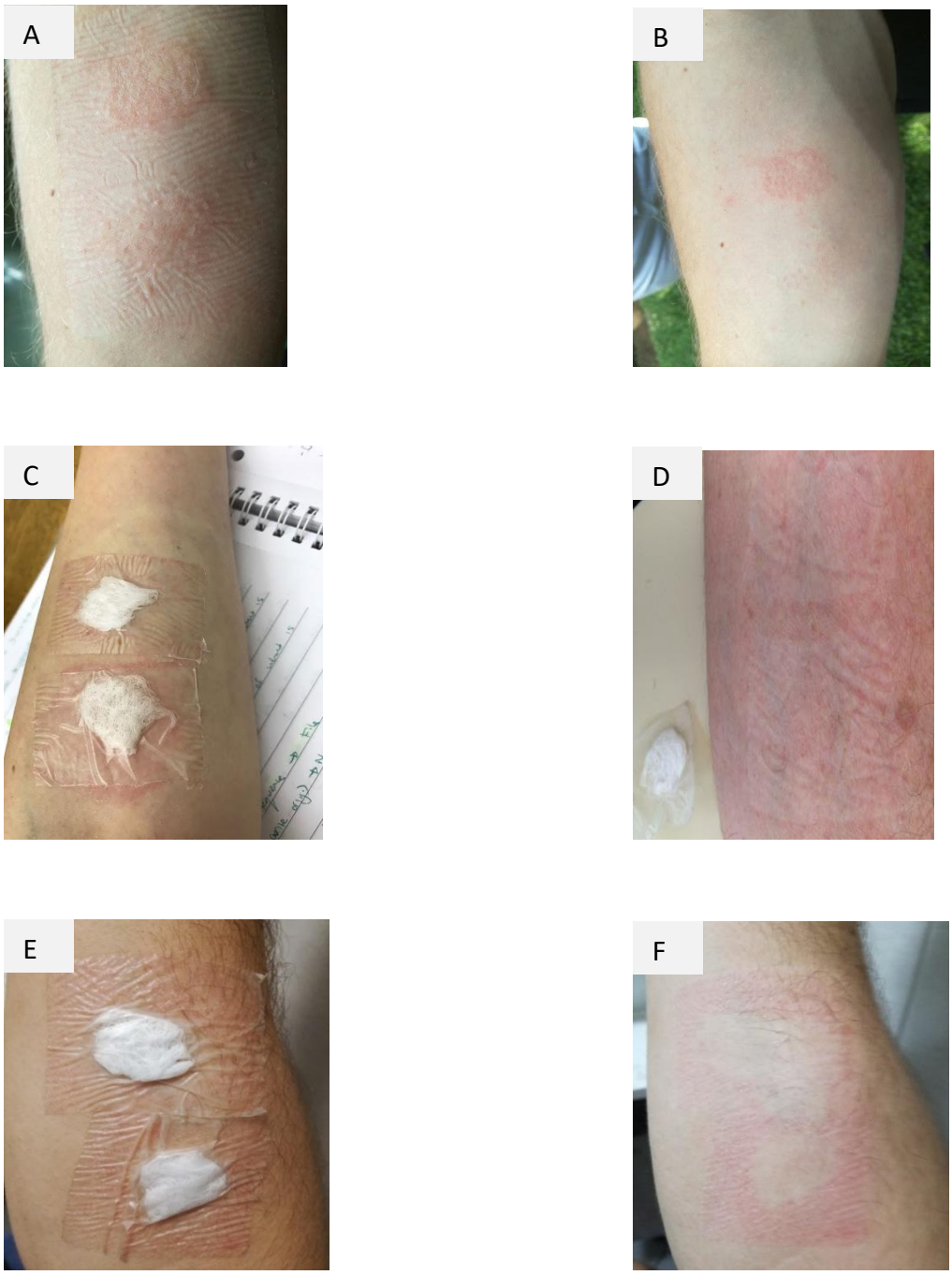

Figure 3.7 Patch test results from P13 after removing patch
(A) skin immediately after removing the patch (B) skin 24 hr after removal of patch (C) P3 within 30 min of applying patch (D) P5 after removal of patch (E) P12 24 h after treatment initiation (F) P12 after removal of patch

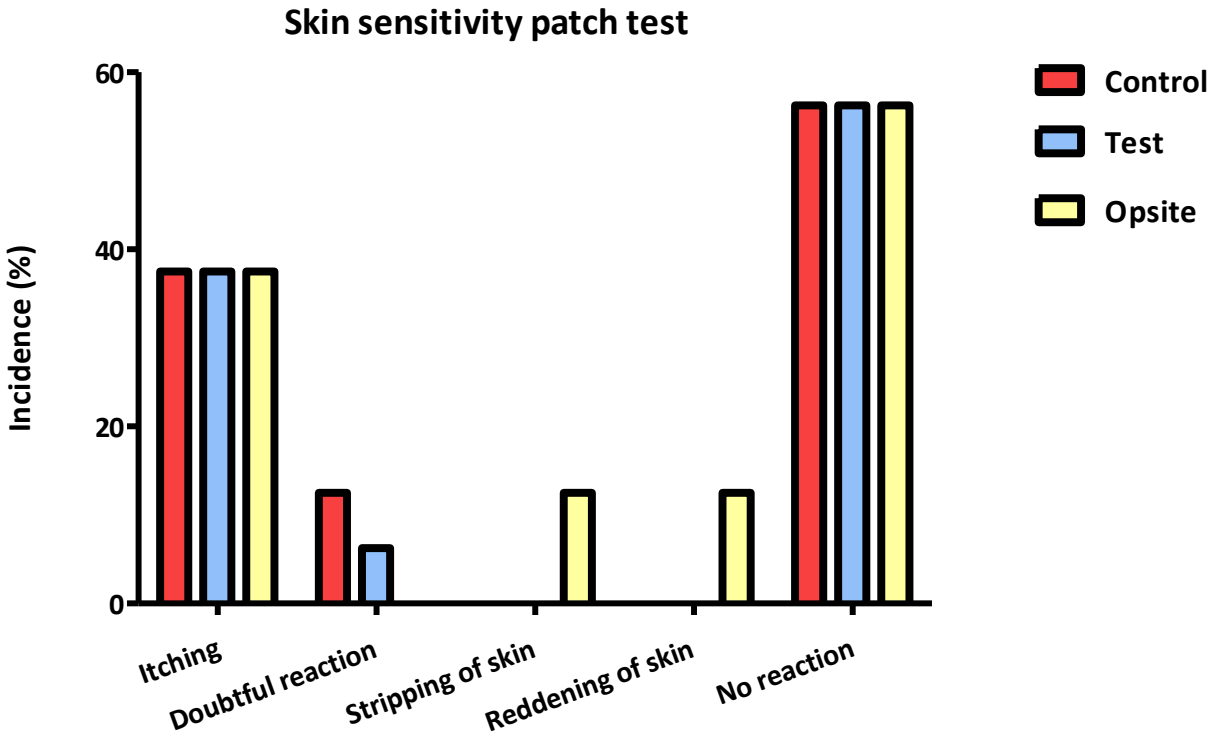

Figure 3.8 Patch test of sensitivity of skin to LCs_{34}, 1% acetic acid (control) and the OpSite® dressing

Two participants' skin, P3 and P13 reddened within an hour after applying the patches with P3's reaction resolving after a few minutes while P13 was slightly red until removal of the patches. P13 presented with erythema in the control after removal of the patch, even 24 hr after (**Figure 3.7**). After disappearance of the rash, dry scaly skin remained. Six participants reported itching after removing the patch. This itching disappeared within 24 hr. P5 reported smooth looking skin for a few hours after removal of the patch. Overall, the majority of the skin reactions seemed to be allergic reactions to the Opsite®Flexifit film dressing or the glue that the participants were exposed to for a prolonged period. Out of the 16 participants, 10 reported no reaction at all to any of the material that was applied on them.

The patch test results relied mainly on self-diagnosis as skin sensitivity is largely a subjective sensory skin discomfort.[339] Another drawback of the present study was the small group of participants, which cannot be used conclusively to give an indication of the safety of the material. However, further toxicity tests were performed *in vitro* using the sulphorhodamine-B (SRB) assay.

The SRB assay was used to measure cell viability as it quantifies cell density based on the uptake of the negatively charged pink aminoxanthine dye, sulphorhodamine-B (SRB) by amino

acids in the cells. This method is more accurate than the commonly used MTT assay which relies on activation of mitochondrial activity which may be inhibited by varied glucose levels in the cells, presence of NADH and other factors. The exposure of chitosan to fibroblast cells for 72 hr resulted in cell density reduction of not less than 50% in all the cell-lines. Consistent with the reports of Shelma and Sharma who studied the toxicity of their LCs using the MTT assay L929 mouse fibroblast cells[274], all the conjugated LCs samples (LCs_{10}, LCs_{20}, and LCs_{34}) including the underivatised chitosan were non-toxic. In fact, after 72 hr they had all stimulated fibroblast cell proliferation significantly with increasing lauric acid concentration compared to the chitosan sample. Our findings were also consistent with Shelma and colleagues' toxicity study results from the MTT assay on their amphiphilic lauroyl sulphated chitosan (LSCS) derivatives on L929 cells.[275] They found that chitosan and LSCS displayed 85 ± 1.5% and 100% cell viability after the experiment confirming its biocompatibility. [275]

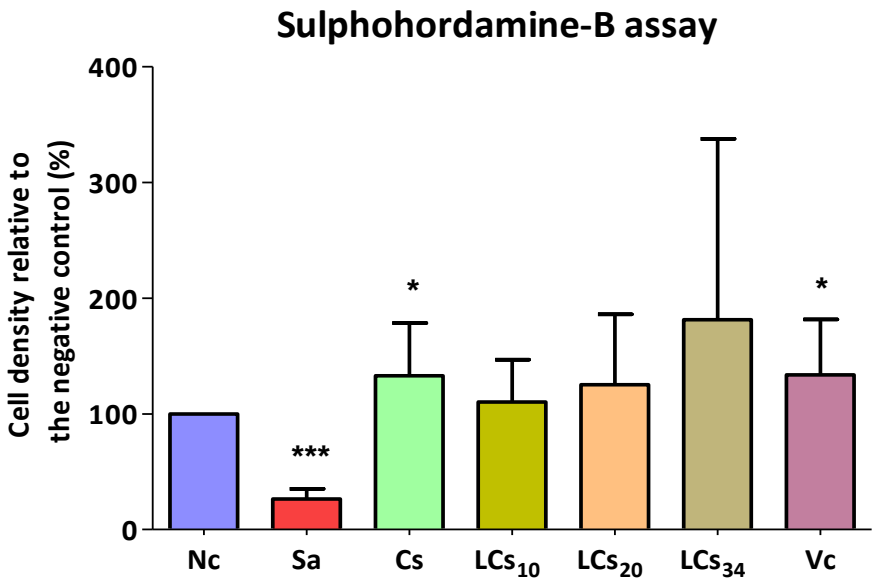

Figure 3.9 Effect of chitosan (Cs) and LCs on the cell density of fibroblast cells after exposure for 72 hr
*(NC – negative control, Sa – saponin, Cs – chitosan, Vc – vehicle control. ***p < 0.001. All samples showed increased protein density compared to the negative control. The LCs_{34} sample had a 43% increase in cellular protein compared to the control. Comparison of the proliferation of Cs, LCs_{10} and LCs_{34} exhibited non-significant differences in the proliferation of the fibroblasts.*

The next objective was to measure the effect the hydrophobicity had on the proliferation and infiltration of fibroblasts. In 1994, Frederick Grinnell suggested that fibroblast migration at the wound edges produced enough force to promote wound contraction[340] thus fibroblast migration and subsequently infiltration would be a good measure of wound contractibility; consequently, wound healing. Infiltration and proliferation of cells on the biomaterial are key

for tissue regeneration. Contrary to what was expected, the SEM and TEM images did not show any fibroblasts on the scaffolds. The infiltration of fibroblasts into the scaffolds was most likely influenced by the porosity of the scaffold rather than by the DD and MW. Hamilton *et al* found no clear relationship between chitosan material characteristics such as MW, DD and wettability on cell attachment or proliferation.[282] Groth and Altankov's study showed that surfaces with contact angles of 60 °C and above resulted in decreased adhesion of fibroblasts due to the considerable reduction in the deposition of endogenous fibronectin on the substratum as a consequence of surface hydrophobicity.[341] With this published data as reference, the expectation was that the fibroblast cells would bind less to the more hydrophobic scaffold compared to the Cs sample. As expected, chitosan showed the highest adhesion, even surpassing the positive control (collagen). This is consistent with research data that suggests that a low hydrophobicity results in a larger pore size which favours fibroblast adhesion; however, as the hydrophobicity increases, adhesion decreases.

Finally, infiltration into the scaffolds was measured by culturing human fibroblasts for 72 hr on the LCs scaffolds. From the SEM images (**Figure 3.10**), it was not easy to differentiate the concentration of fibroblasts on the scaffold as both the SEM and TEM images did not show any fibroblast attachment. Furthermore, the SEM images showed only the fibrous structure of the scaffolds. It was disappointing not to be able to view the fibroblasts tightly adhered on the walls of the scaffold. The TEM images likewise did not yield any good results.

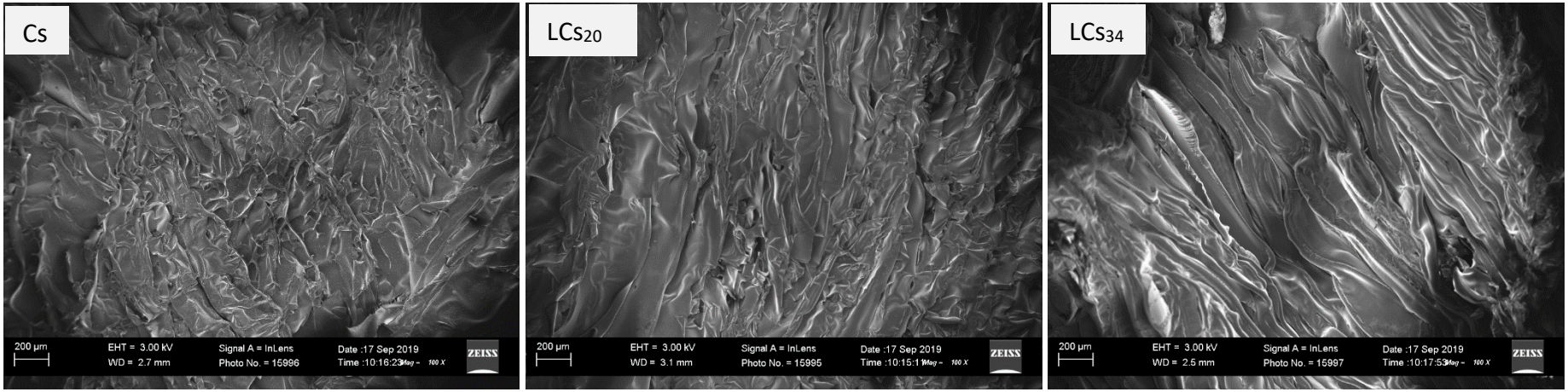

Figure 3.10 SEM images of chitosan and lauroyl chitosan at 34% lauroyl loading density (LCs_{34}) after culturing with fibroblast cells for 72 hr.
Cells were expected to attach, proliferate and infiltrate the scaffold material within 3 days of culture. Cells did not show significant proliferation into the scaffold and SEM images after culture did not indicate cell infiltration but showed only the morphology of the scaffolds.

3.6 Conclusion

An optimum platelet concentration should bind to the biomaterial to enable a positive healing effect of the platelets. However, a very high platelet adhesion does not necessarily yield positive results.[342] Platelets adhered firmly to chitosan compared even to the positive control, collagen. Hydrophobic modification of chitosan diminished its platelet adhesion capacity with increasing hydrophobicity. Proving that hydrophobic modification would not necessarily result in increased platelet binding. The FT-IR peaks of CoP strongly correlated with several collagen extracts seen in literature demonstrating successful isolation of collagen while the ELISA showed that the LCs_{10} sample exhibited a sustained release of growth factors over 24 hr compared to both chitosan and collagen.

Cytotoxicity assays showed that neither LCs nor chitosan was toxic to primary fibroblast cells, with the LCs_{34} significantly (43%) promoting fibroblast proliferation compared to the control. From the skin sensitivity assay, LCs_{34} did not cause significant irritation to the skin. However, from the results obtained from P13 1% acetic acid is a possible skin irritant. P13 results showed no positive reaction to the test patch, which can prove that lauroyl chitosan, could have protected the skin from irritation that the 1% acetic acid caused. Fibroblast infiltration tests did not show any visible growth into all the different scaffolds; thus, findings from this assay were inconclusive.

In summary, hydrophobic modification of chitosan reduced the adhesion capacity of chitosan to platelets as the lauric acid density on the underivatised chitosan increased. Although this was contrary to the hypothesis of the project, an optimum concentration of platelets would still be required to deliver a sufficient concentration of growth factors for healing. Platelet counts of the extracted PRP were reliably done using an automated system at Ampath as the haemocytometer gave very low counts. Lauroyl chitosan at 10% loading density (LCs_{10}) allowed sustained release of growth factors over 24 hr and from the skin sensitivity test and SRB assay showed that it is a safe material to use for a wound dressing thus this was selected for further analysis in a wound healing study.

Chapter 4

Wound healing study using porcine model

4.1 Introduction

An ideal skin substitute should have suitable mechanical properties, be safe to use, be biodegradable while retaining sufficient durability to allow cell infiltration and growth and should support vascularisation.[343] In short, the success of a biomaterial for wound healing depends on its ability to stimulate healing, assuage inflammation and infection and result in less scar formation or prevent dysfunctional scarring. A wound filler paste that would function as a skin substitute while the body regenerates its own skin was hypothesised to reduce healing times, while reducing wound contraction and scar formation. The lauric acid conjugated chitosan paste that was shown in the earlier characterisation studies to have both good chemical and cell growth characteristics was tested as an *in vivo* wound healing enhancer on full-thickness wounds in the porcine model.

It is not possible to create an *in vitro* model of a full-thickness wound that would closely mimic the characteristics of an *in vivo* wound environment and its complexities[344]; hence, animal models are frequently used for wound healing studies. To date, a number of wound healing models have been used in biomedical research and regenerative medicine. The porcine model was selected for this study because it had the best chance of answering the hypothesis. It is widely used because it closely resembles human skin[345] both anatomically and physiologically.[126] As in humans, porcine skin has sparse body hair and very similar structural and functional characteristics. Anatomically, the epidermis of the pig closely matches the thickness of human skin with the thickness varying according to the body area. The epidermis of the pig ranges from 30 to 140 µm while that of humans from 50 to 120 µm.[346] Porcine wounds like in humans, heal through re-epithelisation as compared to contraction in small animal wound models.

Debeer *et al's* study highlighted the main immunohistochemical differences and similarities between porcine and human skin. They found that keratinocytes as well as Langerhans cells are similarly organised in human and porcine epidermis.[347] The ECM of both humans and pigs has about 95% collagen and 2% elastic fibres, which affects the elasticity of the skin,

which is vital in wound contraction. Porcine dermal collagen is similar to human collagen.[348] The main differences between the porcine and human skin as shown by Debeer *et al* were less melanin and melanocytes in the basal cell layer, smaller and sparsely distributed sebaceous glands, a thinner granular cell layer and wider distribution of apocrine (vs eccrine) sweat glands in the porcine skin.[347]

Collagen fillers and wound healing accelerators have been used extensively as implants in cosmetics and reconstructive surgery and in complicated bone fractures; thus, verifying that collagen is non-immunogenic and non-toxic *in vivo*.[349-350] Washed and soluble porcine collagen type 1 was extracted from abdominal porcine skin (**Section** Error! Reference source not found.) and then incorporated into the modified chitosan at relatively high content (25%) to form a thick paste that could be spread and formed into the wound. The collagen-lauroyl chitosan ($CoLCs_{10}$) paste was made to provide a template that would contain collagen, which is produced by fibroblasts and a major protein of the ECM, and is a significant component of a healing wound.

The animal study was carried out at the Central Animal Services (CAS) at the University of Witwatersrand under the supervision of Profs Kennedy and AD Cromarty. The Animal Ethics Committees of both the Universities of Pretoria (H009-17) (**Annexure 5**) and Witwatersrand (Animal Ethics Screening Committee) (2018/01/06/C) (**Annexure 6**) approved the experimental protocols.

4.2 Materials

Jelonet® dressing was purchased from Smith and Nephew, Hull, UK, while collagen was extracted from porcine abdominal skin (refer to **Section** Error! Reference source not found.) and the lauroyl chitosan conjugate (LCs_{10}) was synthesised at the Council for Scientific and Industrial Research (CSIR) (**Chapter 2).** All drugs and material required for wounding and biopsies were provided by the Wits CAS.

4.3 Animals

Four “large white” female pigs weighing +/- 32 kg were sourced from a single breeding farm in Pretoria and were kept in a large pen for 14 days for acclimatisation and humanising prior to the study.

4.4 Methods

4.4.1 Co/LCs_{10} paste preparation

Lyophilised collagen and lyophilised LCs_{10} were weighed and mixed at the ratio 1:4 (Co:LCs_{10}). Thereafter the Co/LCs_{10} powder was dispersed in distilled water 10% w/v to form a thick gel-like solid paste. The paste was stirred and manipulated well until smooth. The pH was adjusted to between 5 - 6 with 0.5 M NaOH before transferring into 15 ml centrifuge tubes.

4.4.2 Sample sterilisation

Samples were sterilised using gamma rays at a dose of 5.52 kGy/hour for 4 hr 15 min. No significant physical changes in the colour or consistency of the paste were observed.

To confirm full sterilisation, aliquots of sterilised samples were suspended in DMEM and incubated at 37 °C for 72 hr and no bacterial or fungal growth was seen when observed under a light microscope nor any change in medium colour.

4.4.3 Animal acclimatisation

On arrival, the pigs were housed in large pens at the Wits CAS for fourteen days for acclimatisation. The pigs were all exposed to 12 hr light/dark cycle in their individual cages. Their diet consisted of commercial feed (Pig Growth and Sow, Epol, Johannesburg) once daily and water *ad libitum*. The investigators tended the animals while constant monitoring was provided for by the trained staff from Wits CAS. The pigs got regular physical contact, and as outlined in the facility's standard operating procedures, their straw bedding was sufficient and allowed for "snuffling" for food pellets or raisins.

4.4.4 Surgical procedures

The surgery was performed by a qualified veterinarian at Wits. Prior to surgery, the animals were anaesthetised using injectable Dormicum® (Midazolam) (0.3 mg/kg) and AnaketV (11 mg/kg). Isofluor was used to maintain anaesthesia during surgery.

All procedures were carried out in a theatre under sterile conditions. The initial procedures were to sterilise the complete dorsal area using chlorhexidine gluconate (Hibiscrub surgical scrub) and finally with F10 antiseptic fluid. This was followed by marking the skin with a

permanent marker using a template cut from a used X-ray plate. Sixteen full skin thickness wounds (1 cm^2 each) were created using a scalpel blade (1.1 x 1.1 cm) on the dorsum of 4 pigs, 8 on each psoas muscle ridge (**Figure 4.1**). The wounds were created to a depth of the dermal layer but not extending into the subcutaneous layers. For pain management post-operatively, the analgesic Temgesic (buprenorphine) (0.3 - 0.6 ml) and Flunixin meglumine (2.2 mg/kg i.m.) were administered.

4.4.5 Platelet-rich plasma preparation

While under anaesthesia, 16 ml whole blood was drawn from the jugular vein of each pig using 8 ml sodium citrate containing CPT vacutainer tube and a gauge 22 needle and "needle holder device". No negative physiological effects resulting from the phlebotomy were observed for any of the animals. To isolate the PRP, the blood was centrifuged at 1800 x *g* for 20 min using a Beckman Allegra 22 centrifuge. Three layers; PPP, PRP and blood cells were obtained after centrifugation. The PRP layer was collected using a plastic pipette and transferred to a tube with Co/LCs_{10} paste at a concentration of 1:5 PRP:Co/LCs_{10} (v/v) and mixed with a sterile wooden tongue depressor. This mixture was kept at ambient temperature (22°C) until used. The paste formed was applied within 30 min of PRP separation.

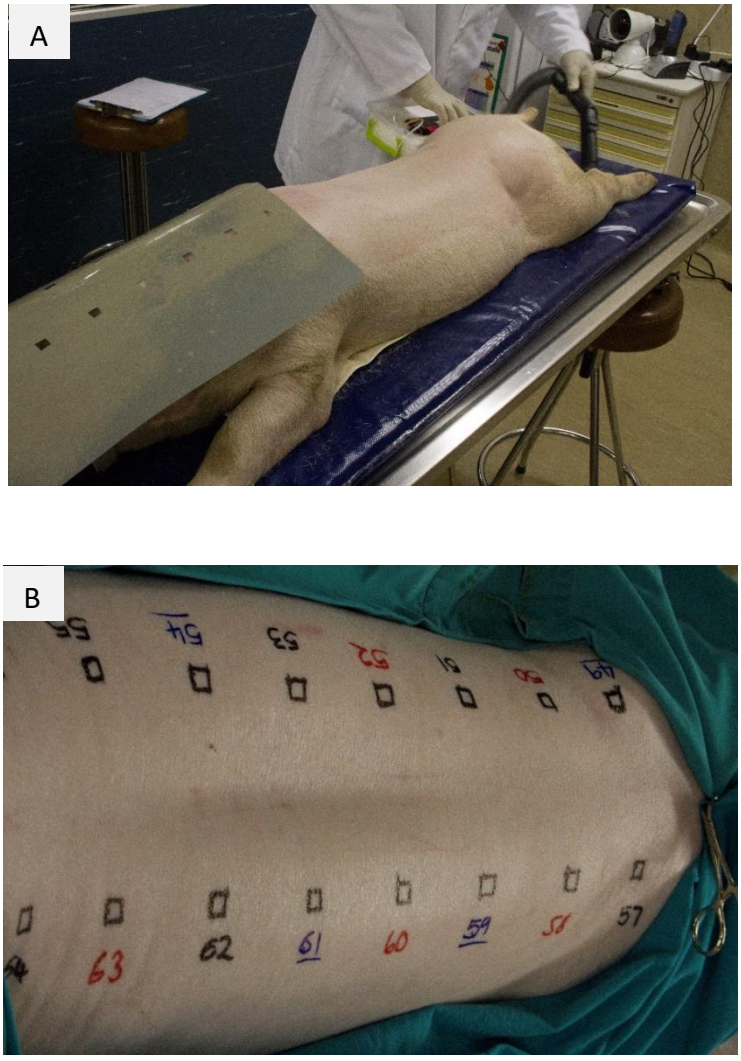

Figure 4.1 Wound creation

(A) Custom-made template for wounds used to (B) mark 1 cm^2 wounds with a uniform distance between each wound. Each colour marking represented a different treatment.

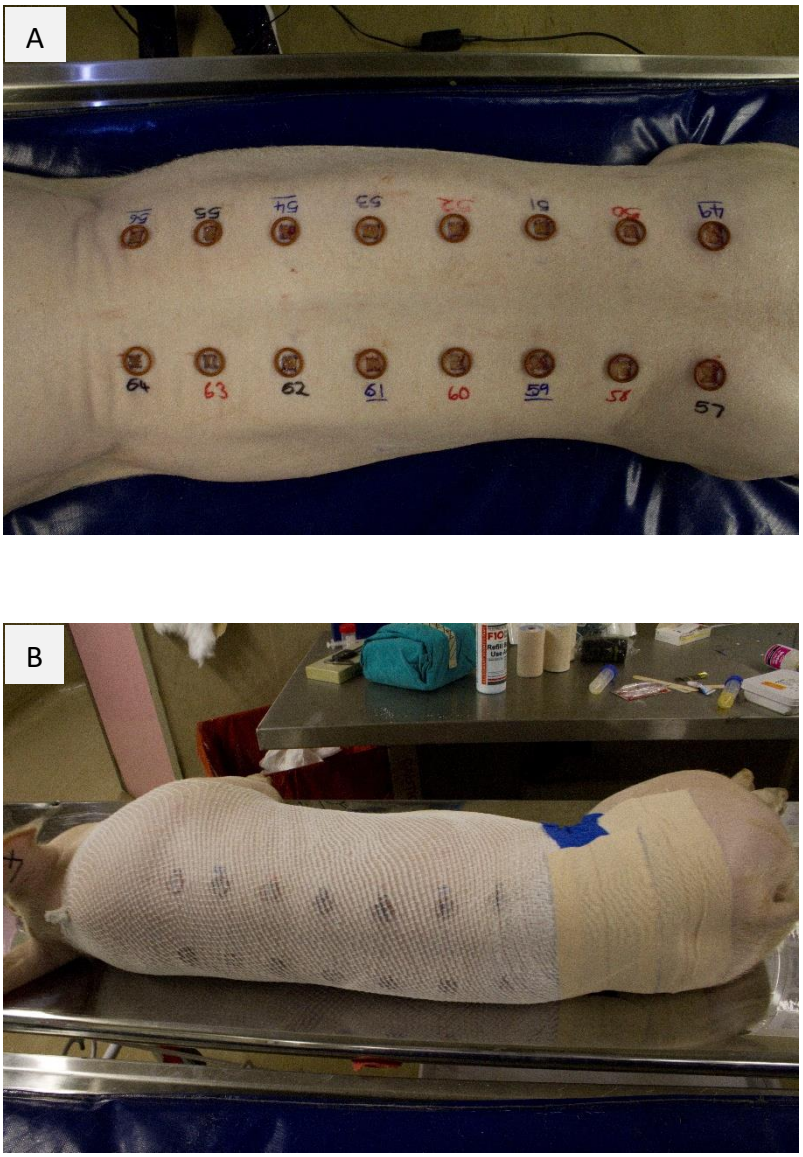

Figure 4.2 Dressed wounds

(A) Wounds protected with O-rings to prevent direct contact of OpSite® dressing with the dressing material inserted into the wound and (B) wound sock protecting dressed wounds from further trauma.

4.4.6 Wound dressings

Immediately after surgery, the wounds were compressed with sterile gauze to stop the bleeding. Once bleeding had ceased the wounds were dressed with application of either Jelonet® dressing, LCs_{10} plus collagen paste (Co/LCs_{10}) or the Co/LCs_{10} supplemented with PRP (Co/LCs_{10}/PRP). These treatments were also coded: black, blue and red; where black was the Jelonet®, blue the Co/LCs_{10} and red the Co/LCs_{10}/PRP. These codes were used for the duration on the animal study.

To allow undisturbed infiltration of cells into the dressing material until the wounds had healed, wound dressings were not renewed. Renewal of the dressings was only performed where the animals had rubbed the dressing and the dressing had dislodged. Additional matching dressing material was applied into the biopsy punch site immediately after the biopsy was made during the wound healing assessments. After each wound assessment time, the wounds were covered with OpSite® and animal's torsos were covered with a sock to protect the wounds. (**Figure 4.1(B)**)

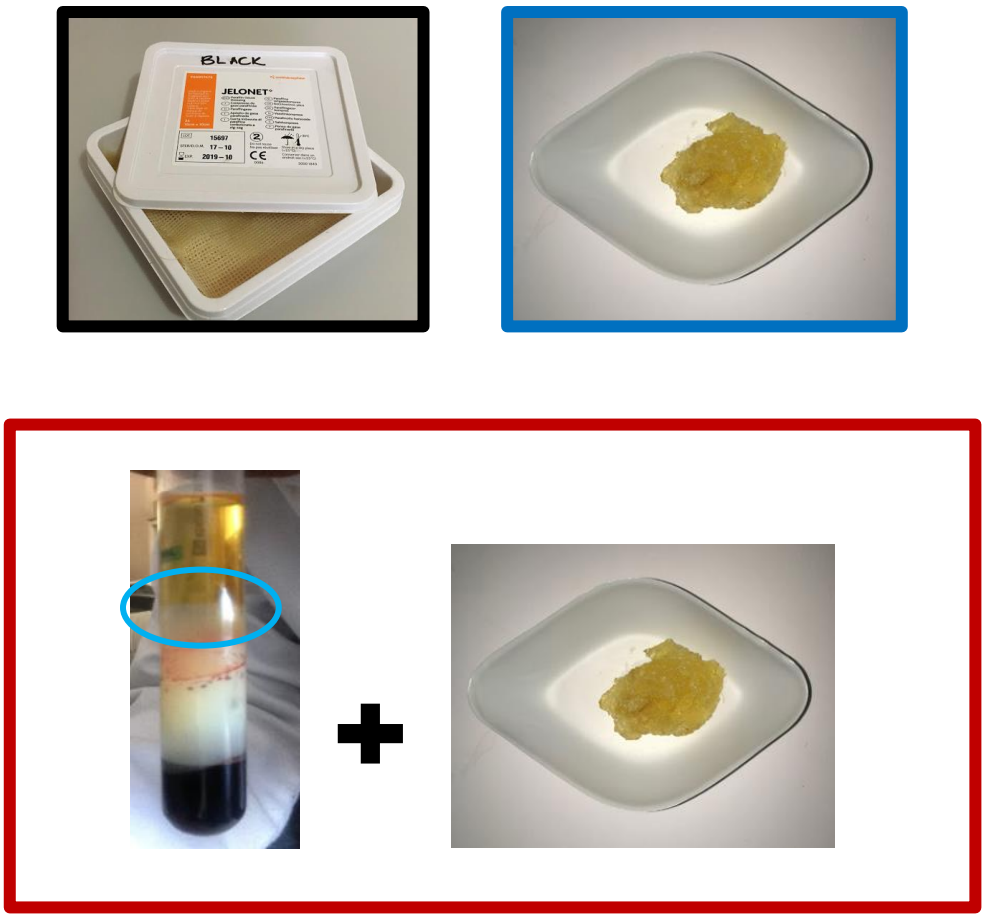

Figure 4.3 Wound treatments used (Black: Jelonet®; Blue: Co/LCs$_{10}$; Red: Co/LCS$_{10}$/PRP.
After applying the dressing material, the wounds and surrounding areas were covered with OpSite® then covered with a stretchable sock to protect the wounds).

Table 4.1 Day to day schedule of procedures

Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Blood collection for PRP preparation	**X**																		
Dressing change (if necessary)			**X**		**X**														
Digital photograph	**X**		**X**		**X**			**X**		**X**		**X**			**X**		**X**		**X**
PRP application (Group C-Day 1 and all biopsy days)	**X**		**X**		**X**			**X**		**X**		**X**							
Biopsy (full-thickness wound)			**X**		**X**			**X**		**X**		**X**							

4.4.7 Housing after surgery

Each pig was housed in its own pen to prevent mutual interference with the wound sites of the other animals. They each had visual contact to stimulate social interaction. Their straw bedding was cleaned daily and they also had overhead infrared heating lamps. Animal enrichment items were used to keep the animals busy and treats like raisins were buried in the bedding to enhance their interest.

4.4.8 Wound healing

Macroscopic appearance of the wounds was assessed and wounds were photographed as scheduled on **Table 4.1**. Standardised photographs were taken using a Canon EOS 7D camera that was modified with a light tube with an internal ruler and evenly spaced light-emitting diode (LED) lighting to give a fixed uniform focal distance and scale and for all wounds. Autofocus at a focal distance of 55 mm was used for all images.

To quantitatively measure wound size, images were assessed using Image J software. The change in wound area due to both re-epithelialisation and contraction was measured using Image J software. To calculate percent change in wound area, the following equation was used:

$$\text{Percent wound area} = \left(\frac{Day\ x\ wound\ area}{Day\ 1\ wound\ area}\right) * 100$$

4.4.9 Biopsy sampling

For biopsy sampling, the animals were anesthetised using Dormicum® (0.3 mg/kg) and AnaketV (11 mg/kg). Isofluor was used to maintain anaesthesia during surgery. To manage pain post-operatively, Temgesic (0.3 – 0.6 ml) and Flunixin meglumine (2.2 mg/kg) were administered intramuscularly. The body sock and dressings were removed and the wounds photographed. The skin was sprayed with 70% alcohol to ensure sterility and wiped with sterile gauze. Three wound biopsies were obtained from each pig (one from each treatment group) using a 4 mm biopsy punch. The area of each biopsy included approximately half healthy skin at the edge of the wound with half from the adjacent wound area. The specimens were immediately snap-frozen in liquid nitrogen and stored at -80 °C until required for use.

4.4.10 Histology

For the preparation and staining of histology samples, the samples were kindly sent to the Pathology Section, Faculty of Veterinary Sciences, University of Pretoria, Onderstepoort. Full-thickness skin biopsies were fixed in 10% buffered formalin for 48 hr at room temperature. Care was taken to ensure that the tissues were completely immersed in the fixative. Fixed biopsies were trimmed into appropriate size and shape and placed in embedding cassettes in the appropriate orientation. The following paraffin embedding schedule was used; 70% ethanol, two changes of 1 hr each; 80% ethanol, once, 1 hr; 5% ethanol, once, 1 hr; 100% ethanol, three changes, 1.5 hr; xylene or xylene substitute (i.e. Clear Rite 3), three changes, 1.5 hr each; paraffin wax (58 - 60 °C), two changes, 2 hr each. Tissues were then embedded into paraffin blocks. The paraffin blocks were trimmed as necessary and cut at 4 µm sections from epidermis to subdermal layers through the centre of the wound biopsy. The paraffin wax ribbon was floated in a water bath at about 40 – 45 °C and individual sections mounted onto glass microscope slides and allowed to air-dry for 30 min followed by baking in a 45 - 50°C oven overnight. Adjacent 4 µm sections were stained with either Haematoxylin and Eosin (H & E) or Masson's trichrome (MT).

4.4.10.1 H & E staining

For close to a century H & E staining has been used to differentiate morphologic changes in tissues and the various tissue types. The H & E stains nuclei blue and cytoplasm pink. Briefly, the adjacent sections were dewaxed in xylene for 5 min followed by staining for 1 min each in ethanol; 100%, 96%, 70% and finally brought to distilled water. Thereafter, the sections were stained in Haematoxylin for 10 min followed by rinsing in tap water. The sections were then differentiated using one dip in Acid Alcohol. The samples were left to turn blue in running tap water for 10 min, followed by 70% alcohol for 3 min. thereafter, the samples were counterstained in Eosin for 2 ½ min and finally dehydrated for 3 min each in alcohol; 96% and 100%, respectively. The samples were cleared in Xylol and mounted in Entellan.

4.4.10.2 Masson's trichrome staining

As inferred by the name, the MT method employs three dyes which selectively stain collagen blue, cell nuclei black, and the cytoplasm, muscles, erythrocytes and fibrin red. The principle of this method is the selective colouring of the tissue based on their porosity and the size of the dye molecule. For the purposes of the current study, the sections were dewaxed in xylene for 5 min followed by staining for 1 min each in alcohol; 100%, 96%, 70% and finally, brought to distilled water. They were then stained in filtered Celestine blue solution for 15 min followed by rinsing three times in distilled water. Thereafter, the samples were stained in Lily-Mayer Haematoxylin for 1 min and placed in blue tap water for 10 min. Next, they were stained in Biebrich Scarlet Fuchsin solution for 15 min followed by rinsing in distilled water. This was followed by mordanting in Phosphotunstic-phosphormolybdic acid for 3 min and then dehydrated in 96% and 100% alcohol respectively. Finally, the samples were cleared in xylene and mounted in Entellan.

4.4.10.3 Microscopic images

Images of all stained sections were taken using bright field microscopy (Olympus BX51; Olympus, Tokyo, Japan) and measurements made at x10 and x20 and x40 magnification. Epithelialisation, collagen deposition, fibroblast proliferation, neovascularisation, and collagen deposition were assessed. Digital images with the magnifications were collected using the microscope camera and stored on an external hard drive for later reassessment by an independent observer.

4.5 Results and discussion

The preparation of a wound filler paste provided a matrix that was expected to promote cellular and vascular infiltration into the wound area and promote collagen deposition and myofibroblast driven wound contraction. Lauroyl chitosan was combined with micronized collagen in the ratio 4:1 (LCs_{10}:Collagen). The consistency of Co/LCs_{10} was a thick paste (**Figure 4.4**). After the paste was prepared it was exposed to high dose gamma irradiation to ensure sterility. Gamma irradiation sterilisation utilises the high energy of gamma rays to disrupt bacterial DNA; thus, inhibiting bacterial division and decontaminating the samples.[351] The irradiation required for sterilization has the potential to also degrade the structure of proteins and polysaccharides, thus PRP was added to the already sterilised Co/LC_{10} paste.

The gamma irradiation dose utilised in this study theoretically disrupts the physicochemical properties of collagen and chitosan[352], however, physical examination of the paste did not show significant changes in the morphology and viscosity of the material.[353] In line with a study by Singh *et al*, the benefits of using gamma irradiation were predicted to outweigh the costs. Singh *et al* reported that radiation sterilised amniotic membranes favoured epithelialisation when applied to burn wounds.[353]

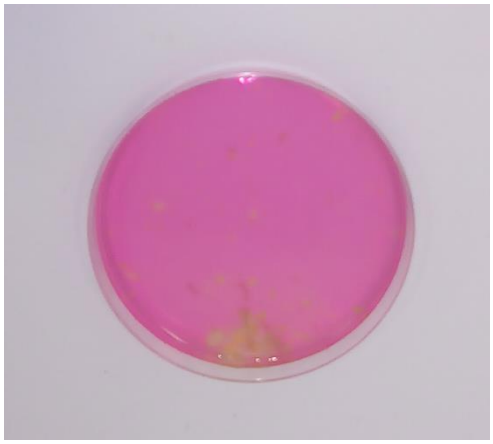

Figure 4.4 Sterilised Co/LCs_{10} paste (beige area in lower centre) after 72 hr incubation in DMEM at 37°C.

The main objective of the animal study was to evaluate the wound healing properties of the collagen/lauroyl chitosan (Co/LCs_{10}) paste and Co/LCs_{10} paste supplemented with PRP (Co/LCs_{10}/PRP) in the treatment of full-thickness wounds in the well described porcine

model. The wound dressings were not changed from initial application until the end of the study. The expectation was to have epidermal cells infiltrate the paste with the paste providing a scaffold for the growth of new tissue. Collagen and chitosan have independently shown wound healing properties. Collagen is as versatile as chitosan also with diverse applications in tissue engineering. However, despite its biocompatibility, collagen's rapid biodegradation rate and low mechanical strength are the major limitations of using collagen alone as a wound dressing material.[354] Collagen and chitosan were thus combined to increase the biostability of the material while potentiating the wound healing effect. Previous wound healing studies using collagen and chitosan composites have shown increased mechanical strength of the final wound dressing. In one study, a collagen/chitosan (9:1) porous scaffold cross-linked with glutaraldehyde was invented by Ma *et al* to increase its tensile strength by functioning as a crosslinking bridge in the crosslinking treatment with glutaraldehyde.[354] Chen *et al* fabricated composite nanofibrous membranes (NFM) from type I collagen, chitosan, and polyethylene oxide by electrospinning. To prevent the solubility of the NFM in water, they were further crosslinked using glutaraldehyde vapour.[355] This NFM showed excellent skin regeneration potential when compared to gauze and commercial collagen sponge.[355] Analysis of these two studies reveal that the low ratio of chitosan to collagen and the solubility of the underivatised chitosan and collagen in water obliged the addition of a crosslinking material to further stabilise the material. In another study, the physical, thermal and biological properties of a collagen/chitosan gel resulted in a novel type of composite gel prepared by Li *et al* to enhance wound healing. This gel contained collagen/chitosan supplemented with a cell-penetrating peptide (oligoarginine) (collagen/chitosan/CPPs). The result was improved antibacterial activity, while histopathological examination showed that collagen/chitosan/CPPs improved granulation tissue formation, promoted angiogenesis and augmented collagen deposition in the wound tissue.[356] In yet another study, collagen and chitosan were enzymatically conjugated using microbial transglutaminase to get a product with superior antioxidant activity compared to carboxymethyl chitosan.[272]

While composites of collagen and chitosan have been extensively subjected to wound healing analysis, LCs has been used for many other applications but has not been applied expansively in wound healing. LCs' haemostatic properties have however been reported.

Shelma and Sharma successfully developed an amphiphilic derivative (lauroyl sulphated chitosan, LSCS) that significantly improve chitosan's haemo-compatibility. [275]

Some of the characteristics of an ideal wound dressing include maintaining moisture, removing excess exudate, provision of an impermeable barrier to bacteria, protection of the wound from further trauma, comfortable and shape conforming.[145] These parameters were used as markers of the success of the wound dressings in the management of the wounds.

For histological assessments, samples of the skin immediately after cutting with the dermatome were snap-frozen and stored at -80 °C until required for histological analysis. Other samples were collected at predetermined time points were examined by H & E and MT staining. The MT stained collagen fibres blue, the cytoplasm in pink, the nuclei very dark blue, and the red blood cells in cherry red. The H&E stained collagen fibres pale pink, the cytoplasm purple, the nuclei in blue, and the red blood cells cherry red.[84] MT stained histological samples could show the presence of inflammatory cells in the skin samples immediately upon wounding, even before the wound dressings were applied.

Approximately 10 min after applying the dressings, the Jelonet®-treated wounds appeared to have continued haemorrhaging after removal of the compressive gauze, when comparing to the wounds treated with Co/LCs_{10} and Co/LCs_{10}/PRP used on day one. The first response to an injury within the first few minutes is haemostasis. This study supported the fact that chitosan is a haemostatic agent with several FDA approved haemostatic chitosan based dressings already in use.[213-214] Furthermore, the rapid arrest of the haemorrhaging especially in the Co/LCs_{10}/PRP group was attributed to PRP's haemostatic and tissue sealing properties. Platelet-rich plasma is a concentrated source of platelets, which after aggregation and activation, achieves haemostasis through the formation of a fibrin clot. According to the normal wound healing process, platelets play a pivotal role during the haemostasis phase. In this case, the platelets, activated by chitosan, were understood to have released chemotactic mediators, cytokines and growth factors which facilitated vasoconstriction, coagulation and the conversion of soluble fibrinogen to a network of insoluble fibrin fibres. The result was a stable chitosan based plug that had fibrin exposed surfaces due to the activated platelets.[59] The plug functioned as both a tissue sealant and growth factor delivery

system with chemotactic and mitogenic properties as was seen during the subsequent phases of wound healing.

Within 10 min of wounding, inflammatory cells had already infiltrated the tissue directly around the wound edges. During early inflammation, platelets release alpha granule proinflammatory cytokines and growth factors while vascular endothelial cells, keratinocytes and fibroblasts in the lesion, release cytokines such as TNF-α. These cytokines promote chemotaxis of leukocytes to the injury site. Neutrophils are the dominant leukocytes in the wound area during the early stages and facilitate elimination of debris, bacteria and damaged tissue[61] but also produce cytokines that attract and activate various inflammatory cells in the wound. However, prolonged neutrophil infiltration was observed in chronic wounds.

By the 3rd day of treatment (**Figure 4.5**), wounds treated with Co/LCs_{10} and Co/LCs_{10}/PRP material had formed a distinct scab that reduced the volume of exudate. Wounds treated with Co/LCs_{10} had a dark, almost dry scab covering the wound while the Co/LCs_{10}/PRP group showed a tan coloured scab.

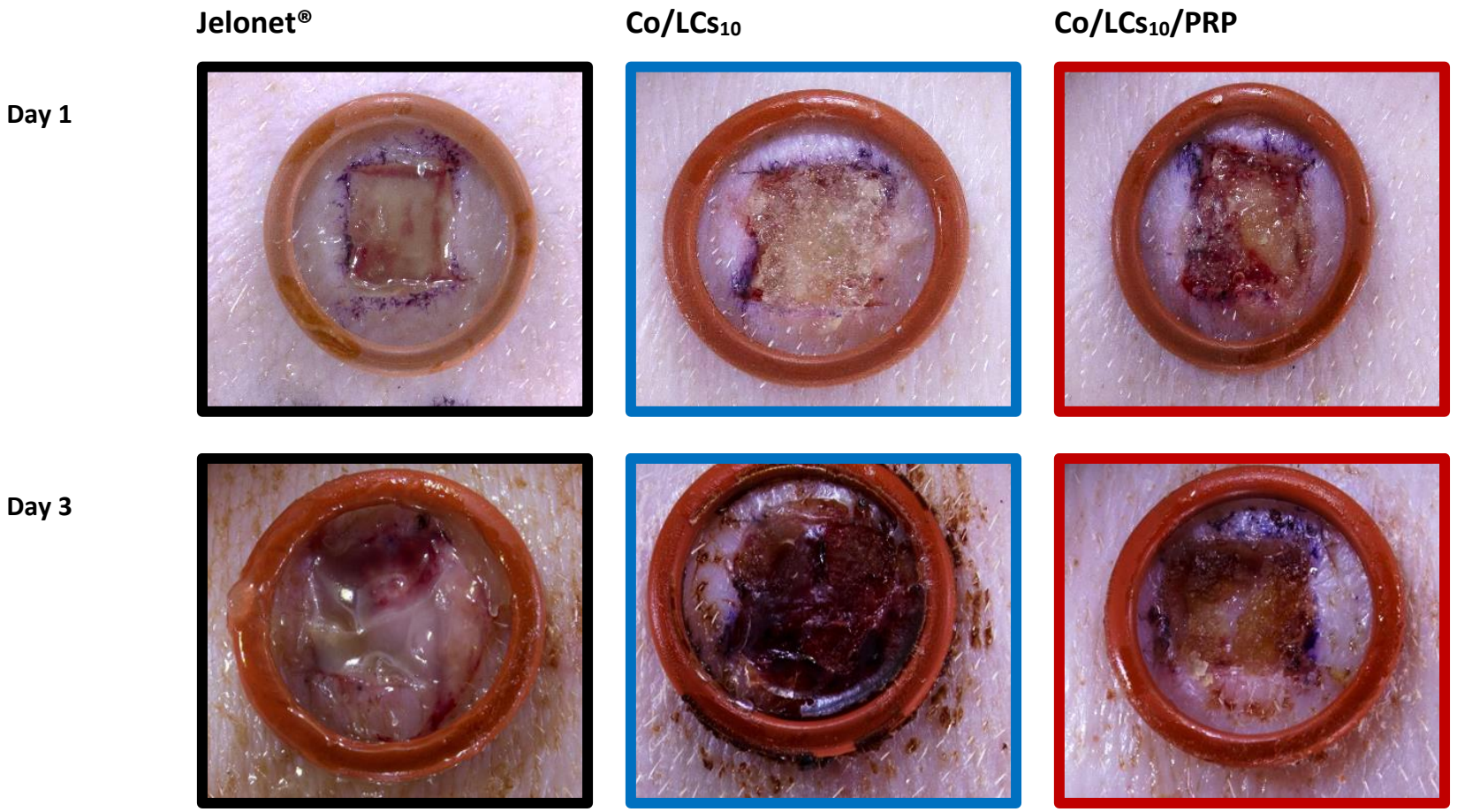

Figure 4.5 The same wounds photographed on Day 1 and Day 3 of wound healing.
Notice the macerated Jelonet-treated wounds on Day 3 while the other treatment groups already had scab formation.

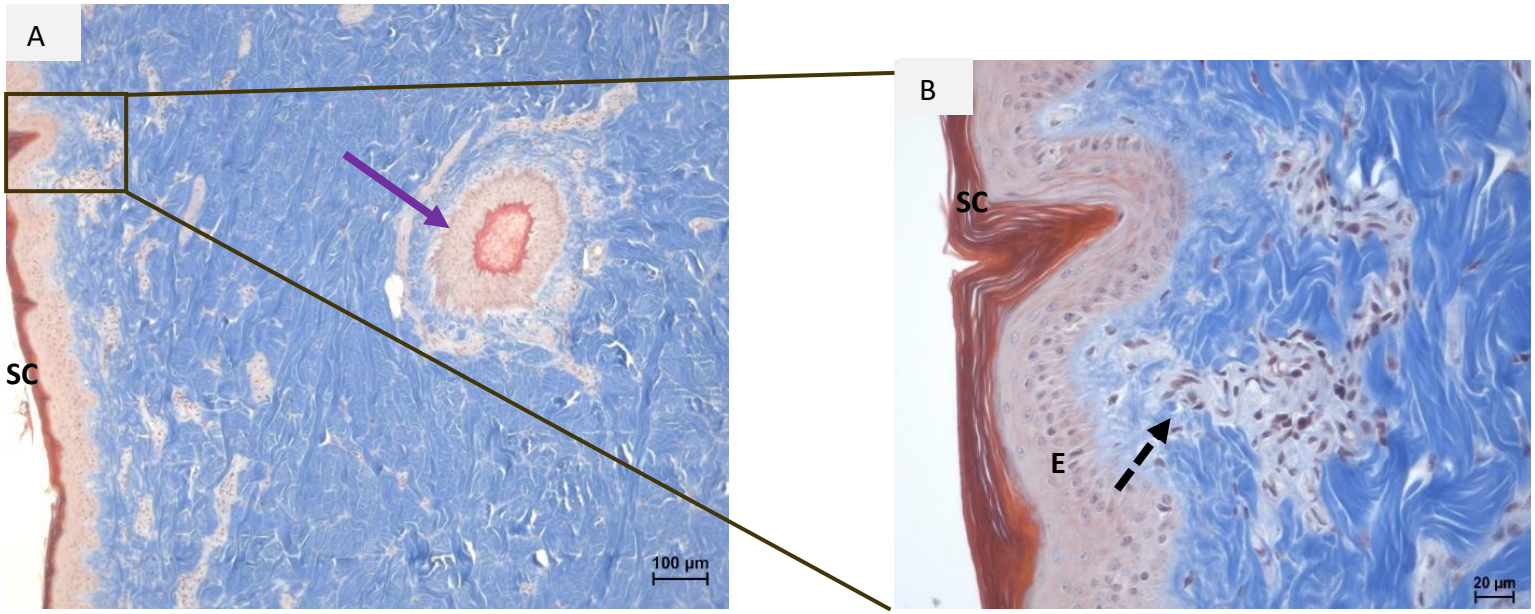

Figure 4.6 (A) Day 1, immediately after wounding and applying wound dressings, skin cut using dermatome

Note the hair follicle (purple arrow), inflammatory cell accumulation, well-formed epidermal layers. (B) Magnified view of the area indicated in A: Accumulation of inflammatory cells could be seen within minutes after wounding. Inflammatory cells also infiltrated healthy skin. Notice the well organised structure of the stratum corneum (SC) and epidermis (E), the accumulation of inflammatory cells (dotted black arrows), and the arrangement of the mature collagen fibres in the dermal layers of the skin.

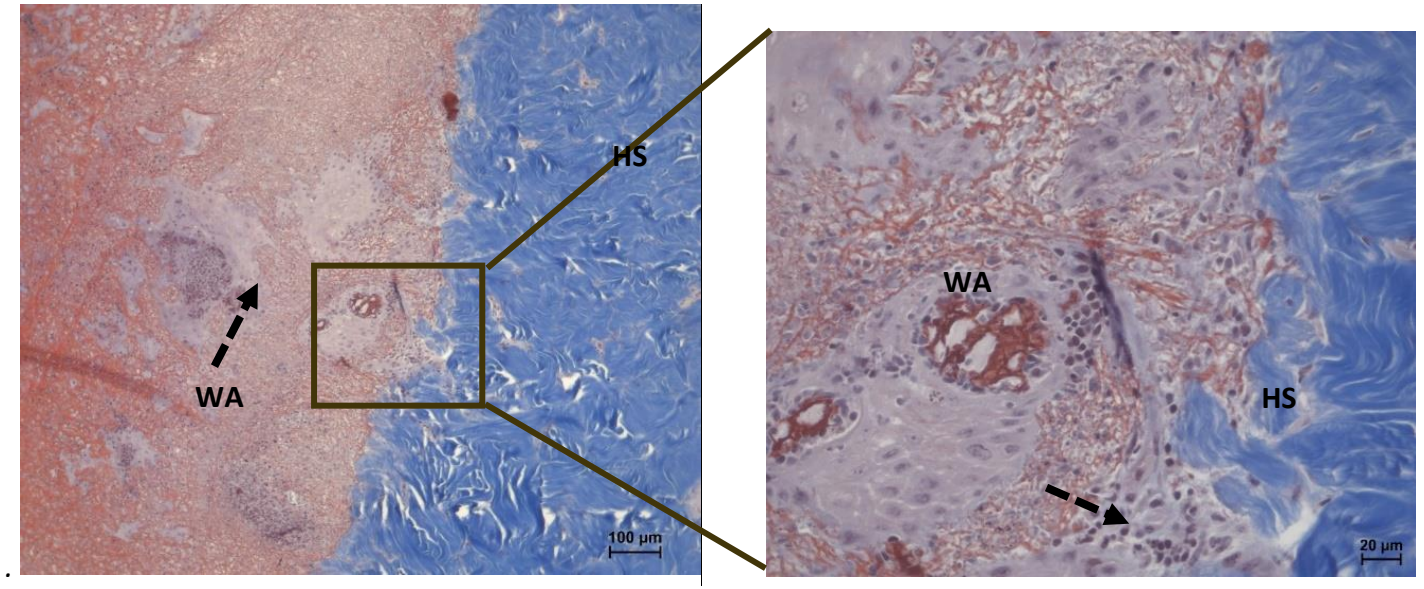

Figure 4.7 Microscopic images of a Jelonet®-treated wound (black) on Day 3

Notice increased accumulation of inflammatory cells (dotted black arrows) in wound area (WA) compared to healthy skin (HS). Note the lack of any visible collagen within the WA

A moist wound environment allows cell migration and proliferation providing an overall optimum environment for healing.[27] However, an equilibrium needs to be established for optimum moisture to prevent maceration of surrounding tissues. In acute wounds, wound exudate is produced because of vasodilation during inflammation, which is an important factor for healing. Conversely, a prolonged inflammatory phase leads to delayed and complicated wound healing. Exudate from chronic wounds contains proteolytic enzymes capable of breaking down ECM proteins and these enzymes are absent in acute wounds. Proteolytic enzymes are required in early stages of wound healing to degrade proteins and clear the wound environment of debris during the healing processes. Chronic wound exudate decreases cell proliferation and migration and may result in maceration of surrounding tissue. On **Day 3**, moderate exudate which resulted in low grade maceration was observed in the Jelonet®-treated wounds (**Figure 4.5**). This was coupled with bleeding that was not completely arrested on the first day. Overall, this resulted in loose attachment of Jelonet® on the wound. All the wounds from this control group were carefully cleaned to remove excess exudate and blood that had clotted within the rubber ring with gauze dipped in sterile saline followed by reapplication of Jelonet®. The same procedure was followed for all other wounds that appeared macerated and had lost the dressing material. On Day 3, only three wounds from the Co/LCs_{10}/PRP required redressing, while the Co/LCs_{10} group had five wounds. From **Day 5** only the Jelonet® group required cleaning and retreatment and from **Day 8** none of the wounds had to be cleaned or redressed.

The lower volume of exudate in the Co/LCs_{10} group and Co/LCs_{10}/PRP group was attributed to the LCs_{10} in the dressings. The air permeable and moisture absorbing LCs_{10} in the dressings provided a sterile, moist environment under a dry scab protecting the wound from dehydration and contamination thus enhancing the healing conditions.[177] Chitosan reduces inflammation by absorbing the secreted of proinflammatory cytokines; TNF-α and IL-6.[217] Inflammatory cells rid the wound area of debris during the inflammation phase of wound healing; thereafter, inflammatory cells play a minor role. The anti-inflammatory properties of lauric acid are known. It thus confirmed that the Jelonet®-treated wounds seemed to have been in a prolonged inflammatory phase when compared to the other treatment groups. The Jelonet®-treated wounds had higher concentrations of inflammatory cells until **Day 8** as seen from the histological analysis (**Figure 4.7** and **Figure 4.8**), where inflammatory cells

were visible on **Day 3** and even on **Day 5** compared to the Co/LCs_{10} and the Co/LCs_{10}/PRP wounds. The inflammatory cells also appear to invade the healthy tissue adjacent to the wounds. The scab covering the Co/LCs_{10} and Co/LCs_{10} treatment groups was also highly concentrated with inflammatory cells and erythrocytes (**Figure 4.8**). It is believed that the Co/LCs_{10} scab remains chemotactic to the inflammatory cells and thus facilitated the removal of inflammatory cells from the wound area. The chemokine properties of chitosan to neutrophils are reported to be via firstly, the stimulation of persistent IL-8 secretion by fibroblasts which also results in increased angiogenesis.[357] The second mechanism is through the binding and subsequent activation of blood complement proteins resulting in a surface that promotes neutrophil chemotaxis.[293] This poses a paradoxical effect for the use of chitosan as a wound healing material as high levels of inflammatory cells in the wound may result in a chronic wound. Nonetheless, Park *et al* elucidated this topic further as they showed that a decrease in the degree of deacetylation leads to increased hydrophobicity and a shift from a positive charge to a negative charge which may have been the reason for the increased secretion of IL-8 following exposure to their acetylated chitosan.[358]

According to Diegelmann, chitosan is said to be chemo-attractive to PMN cells.[359] It is possible that the cells seen in the scab on **Day 3** were PMN cells. The inflammatory cells; PMN cells together with neutrophils migrate into the wound area during the early stages of the inflammation phase.

The Co/LCs_{10} and Co/LCs_{10}/PRP pastes were intended to form a provisional matrix that would promote collagen deposition and myofibroblasts driven wound contraction. Contrary to this hypothesis, all the wounds appeared to heal from the dermal interface beneath the scab without much infiltration into the chitosan-based dressing material. (**Figure** 4.8). The scab which consisted mostly of entrapped dry blood and the inflammatory cells did not incorporate into the new tissue as visible in the histology by **Day 5** (**Figure 4.8**). The MT stained slides showed a clear distinction between new epithelium and the scab in all the healing wounds.

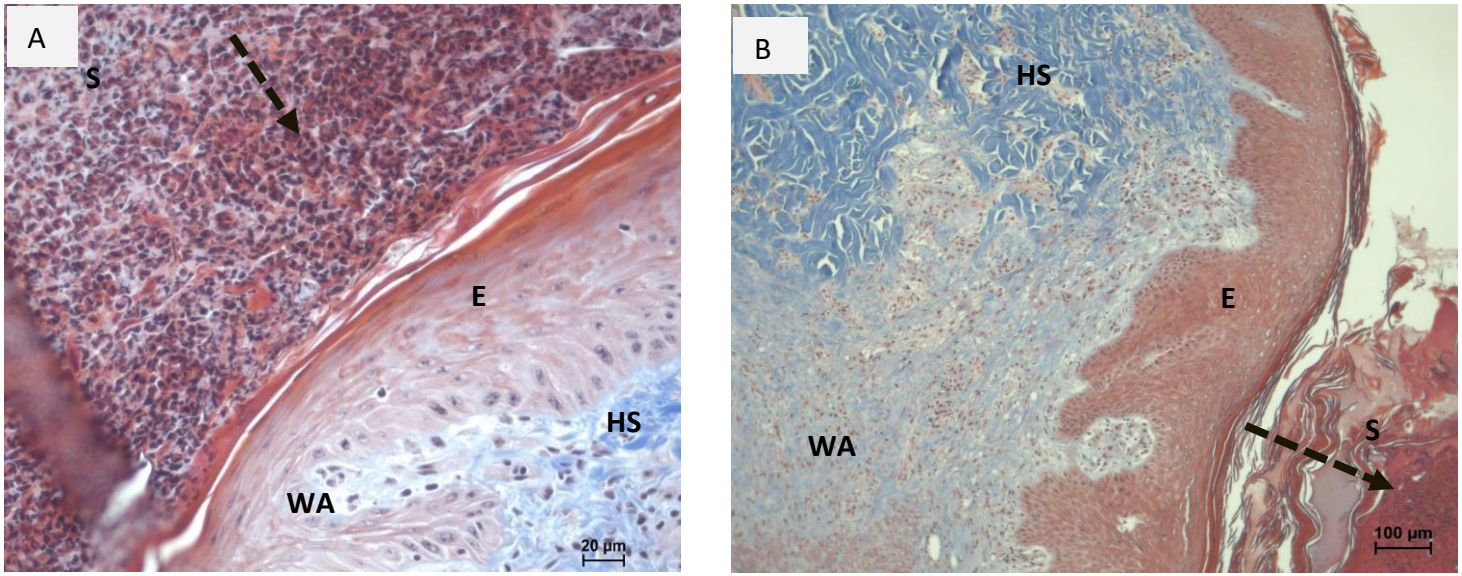

Figure 4.8 Masson's trichrome stained biopsy of a Cs/LCs$_{10}$/PRP treated wound on Day 5

(A) High concentration of inflammatory cells and erythrocytes (dotted black arrow) in the scab (S). (B) Notice separation between scab and wound area and the lower concentration of inflammatory cells in the wound area compared to the scab. (B) Day 12 showing a distinct epithelial layer with a thin layer of stratum corneum already formed. Notice that the dressing material has not been incorporated and the thin collagen fibres in the wound area (Lower left).

On **Day 5**, the Jelonet®-treated wounds still showed a high volume of cloudy yellow/brown exudate exhibiting the presence of fibrin strands as a result of inflammation **(Figure 4.9)**. Despite this exudate, the histological images shown in **Figure 4.10** revealed increased collagen deposition in all three treatments, but with a higher abundance of collagen deposition in the Co/LCS_{10}/PRP treated wounds. The number of inflammatory cells had decreased in the two treatments and the wound was clearly in the proliferative phase. Consistent with what happens in the proliferative phase, capillaries and fibroblast cells were now clearly visible. Fibroblasts synthesise the ECM components; glycosaminoglycans, proteoglycans, fibronectin and collagen which support granulation tissue formation.[70-71]

Diegelmann's study did not give promising results from the use of chitosan in subcutaneous wounds in rats. This was a result of the chitosan sponge significantly delaying the appearance of macrophages and also diminishing the infiltration of fibroblasts, capillaries, and the deposition of mature collagen fibres compared to the control.[359] In contrast with these findings, studies by Ueno *et al* in a dog model[203] and Ishihara *et al in a* mouse model[130] showed that chitosan promoted granulation tissue formation, fibroblast infiltration and collagen deposition which was consistent with the results found in this study.

The explanation for the current findings was initially thought to lie in the lauric acid incorporated into the chitosan used for dressings. The increased collagen deposition and fibroblast infiltration may be due to the micronized collagen incorporated into the dressings. Collagen has been demonstrated *in vitro* to exhibit a chemotactic response to fibroblast cells[252] while fibroblast cells synthesise the new collagen.

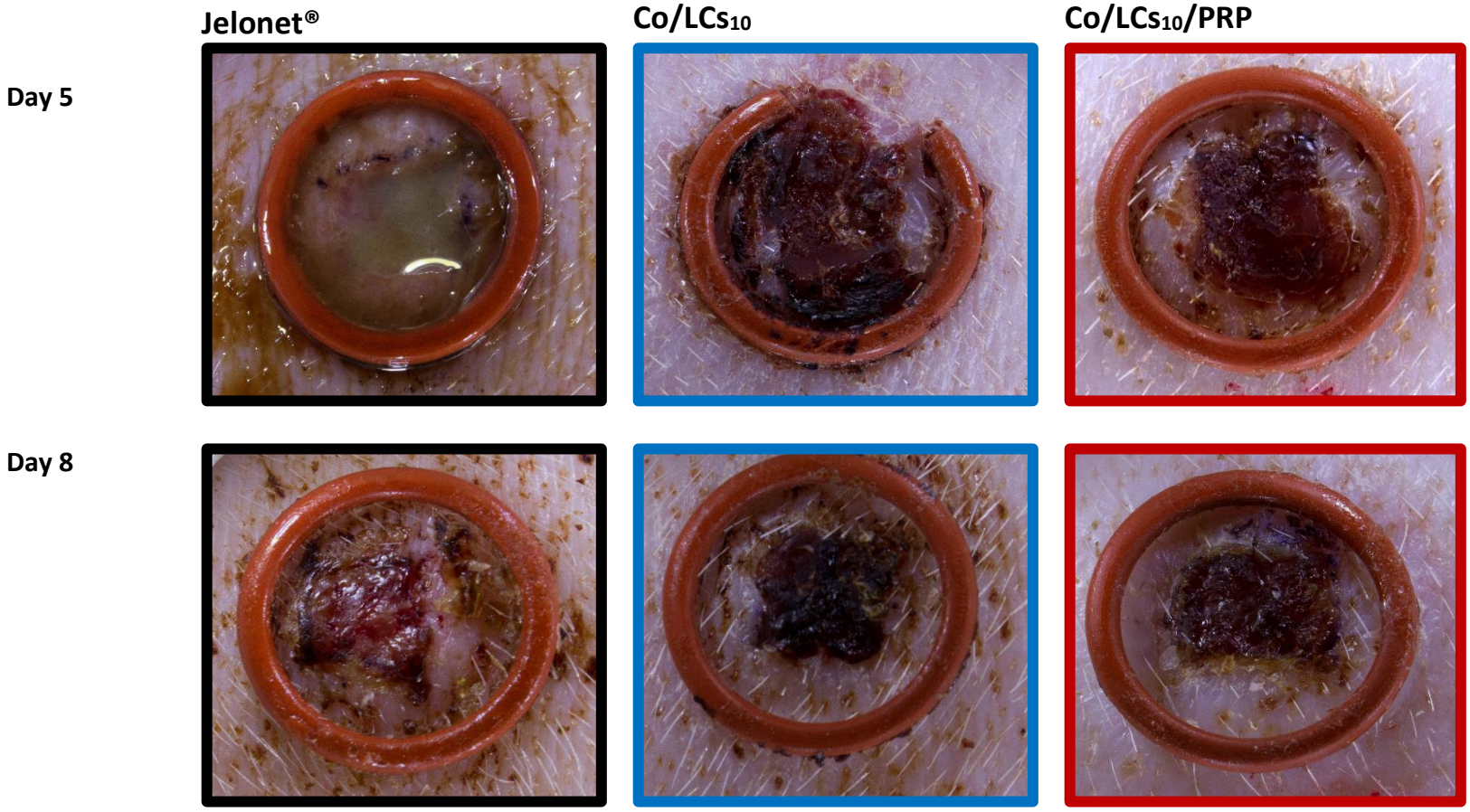

Figure 4.9 Photographs of the three treatment groups on Days 5 and 8.
Jelonet®-treated wounds were still exuding on Day 5 and only showed granulation tissue formation on Day 8. The Co/LCs10 and Co/LCs10/PRP treatment groups both mentioned the scab throughout the treatment period.

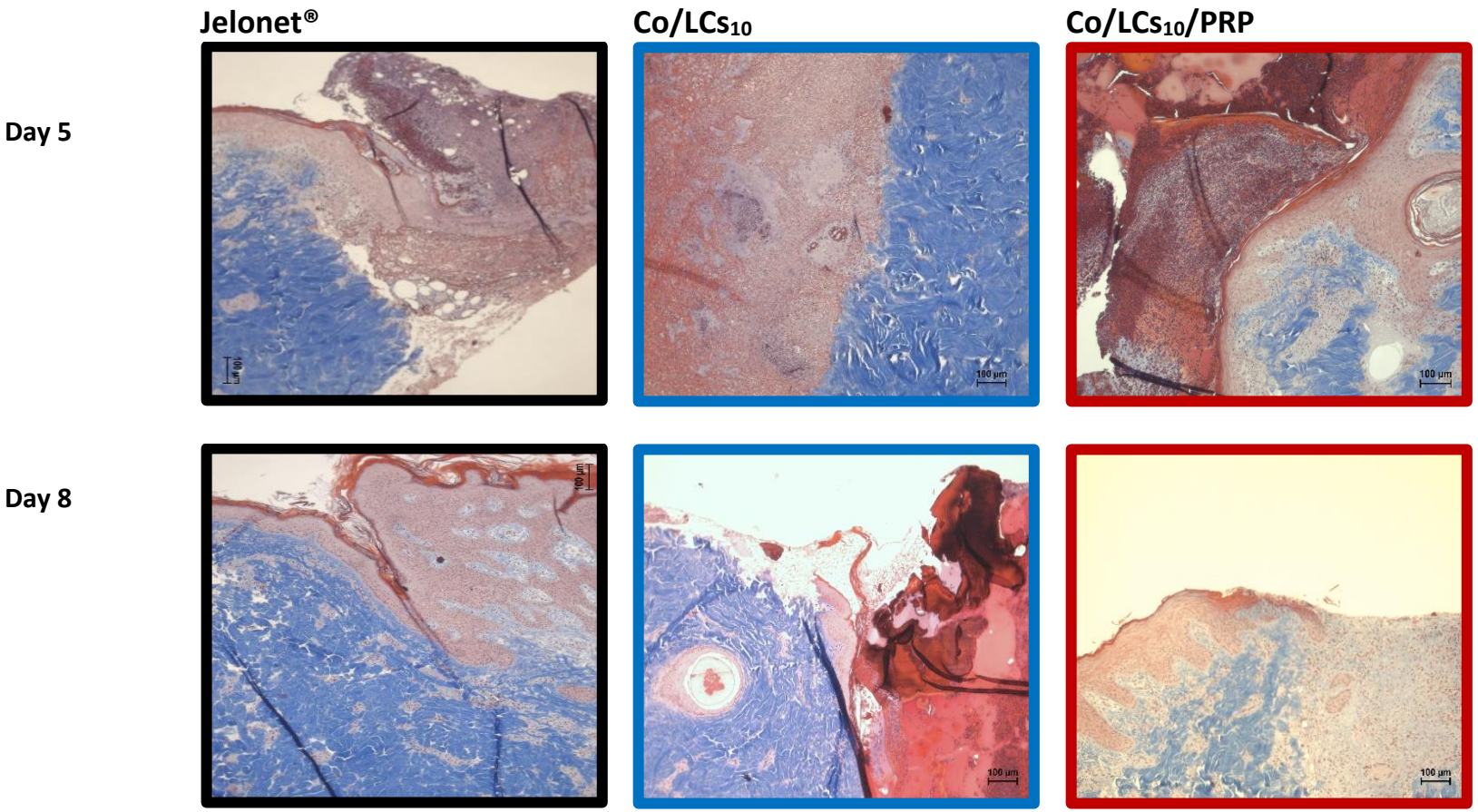

Figure 4.10 Masson's Trichrome stained biopsy images of the treatment groups on Days 5 and 8.

Fibronectin, a large major glycoprotein is found throughout all phases of wound healing; from the fibrin clot, the papillary dermis, to the newly synthesised collagen in the granulation tissue.[70-71] Granulation tissue is also composed of collagen and fibroblasts that are involved in the migration of cells into the wound area and overall in the contraction of the wound. In addition to this, granulation tissue also has monocytes and macrophages that are active inflammatory cells and newly formed blood vessels. There was no visible granulation tissue formation in Jelonet®-treated wounds until **Day 8** (**Figure 4.9**). The granulation tissue appeared grey brown, shiny and granular as a result of the new capillaries; thus, indicating a healthy wound. Histological analysis with the MT stain (**Figure 4.10**) showed fibroblast infiltration and collagen deposition was more pronounced on **Day 8**. It was then postulated that despite the two chitosan based treatment groups (Co/LCs_{10} and the Co/LCs_{10}/PRP groups) showing scab formation by **Day 3**, which is commonly regarded as a signal that granulation tissue formation has been initiated, the Jelonet®-treated wounds possibly only started contracting around the 8th day when visible granulation tissue formation could be seen.

By **Day 10** it was evident that the wounds were in the tissue remodelling phase. In this phase, the temporary matrix formed in the proliferative phase is replaced by thicker fibres and stronger more organised collagen matrix.[73] The dense collagen observed from **Day 10** showed fibres that were possibly remodelling from the more gel-like type III collagen to more fibrous type I collagen. Furthermore, PRP is known to enhance cell proliferation while promoting angiogenesis.[360] The presence of fully-formed blood vessels on **Day 10** in the Co/LCS_{10}/PRP treated wounds, substantiated already published data. PRP was postulated to have promoted angiogenesis in the Co/LCs_{10}/PRP treatment group as a consequence of the continuous slow release of exogenous growth factors in the dressings.

On **Day 10**, after biopsy sampling, one of the pigs was found dead in its pen. Autopsy results (

Annexure 7) revealed an intestinal torsion, which was a result of the handling of the pigs for each procedure.

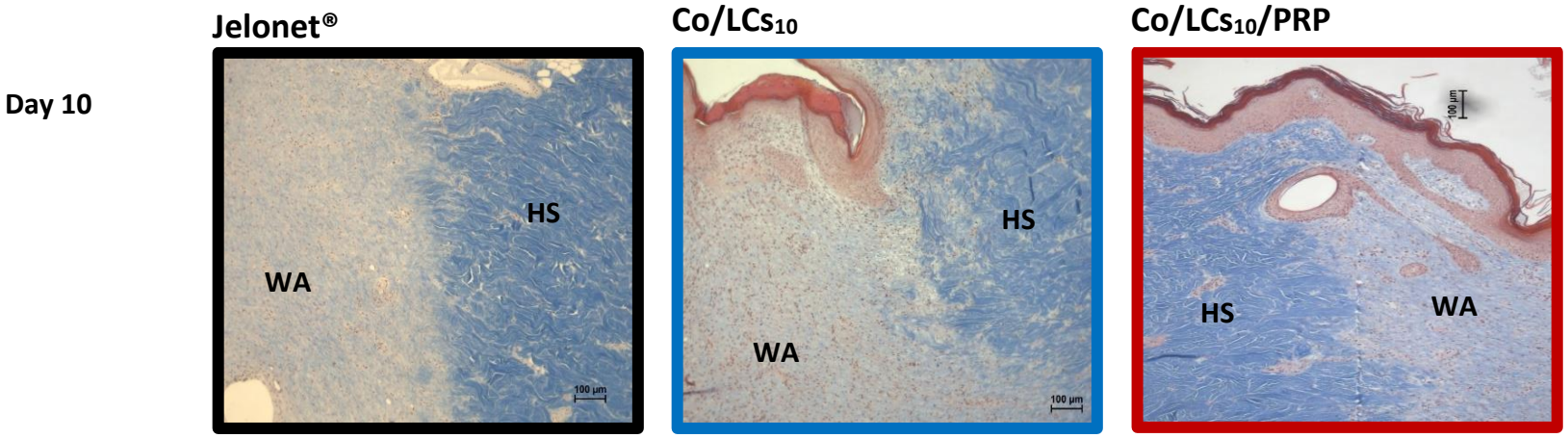

Figure 4.11 Masson's Trichrome histology images of the wounds on Day 10.

Wound area (WA) shows increased collagen density especially in the Co/LCs$_{10}$/PRP treatment. Collagen fibres were still not fully mature as in the HS.

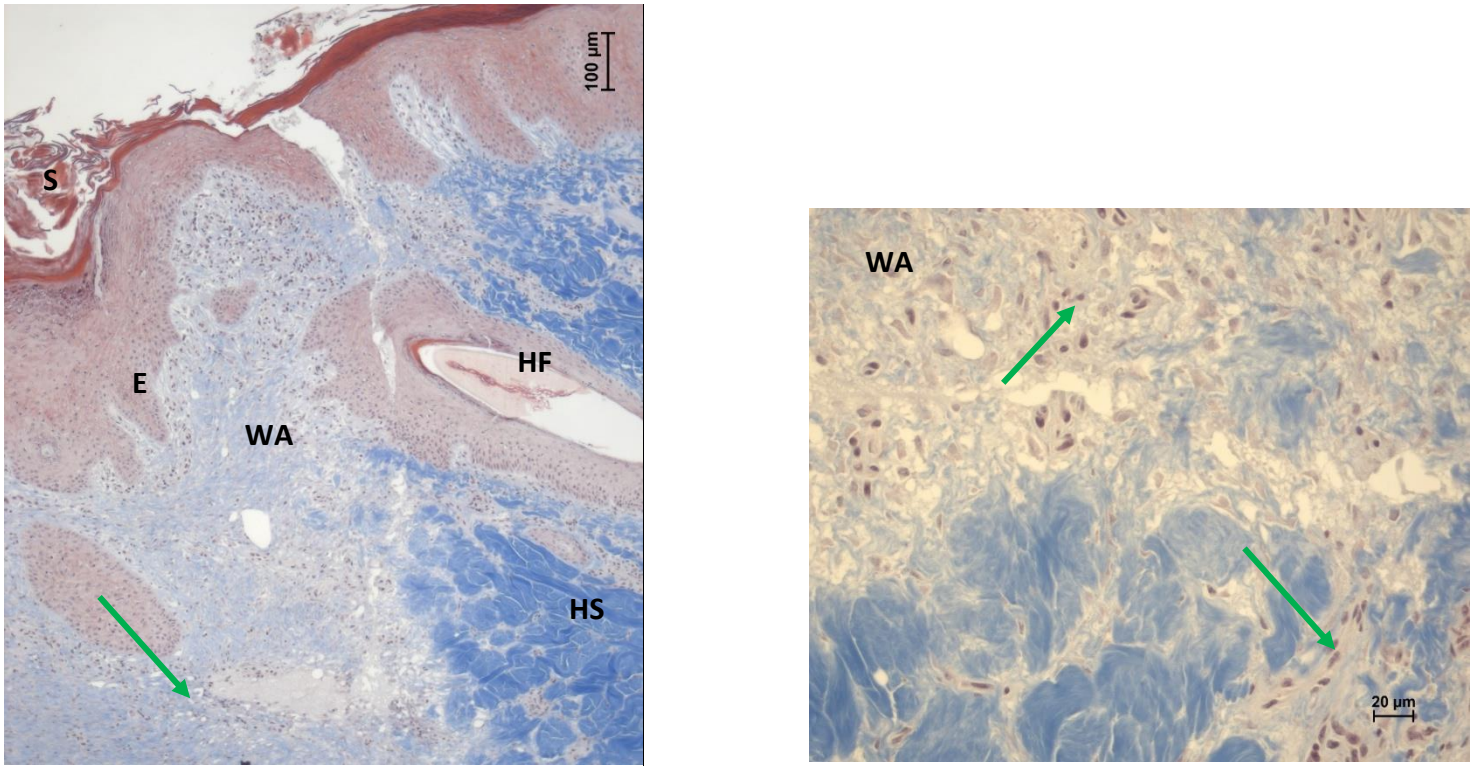

Figure 4.12 Wound 5 (black) on Day 12 – Collagen deposition, fibloblast cells (green arrows) visible, little inflammation.
Notice collagen deposition in the wound area (WA)and the large hair follicle (HF).

It was only on **Day 12** that clear wound healing was observed after gentle removal of the loose scabs from wounds. The scabs that were semi-detached or falling off revealed healed skin with only minor scarring. Regrowth of hair was observed macroscopically as well as microscopically as indicated by the hair shafts in **Figure 4.12.**

During the entire duration of the study, the outward appearance of $CoLCs_{10}$ and Co/LCs_{10}/PRP treated wounds did not change except for the darkening and slight reduction in the size of the scab as the wounds progressed through the healing phases. These wounds appeared not to be reducing in size macroscopically for the entire 15 days despite seeing that the scab was becoming loose at the edges. Histological analysis gave a clearer view of what was happening under the dry scab.

When all the wounds were observed on Day 19 not much scarring was observed (**Figure 4.13**). Co/LCs_{10} paste was observed to have aided in preventing the formation of dysfunctional scars.

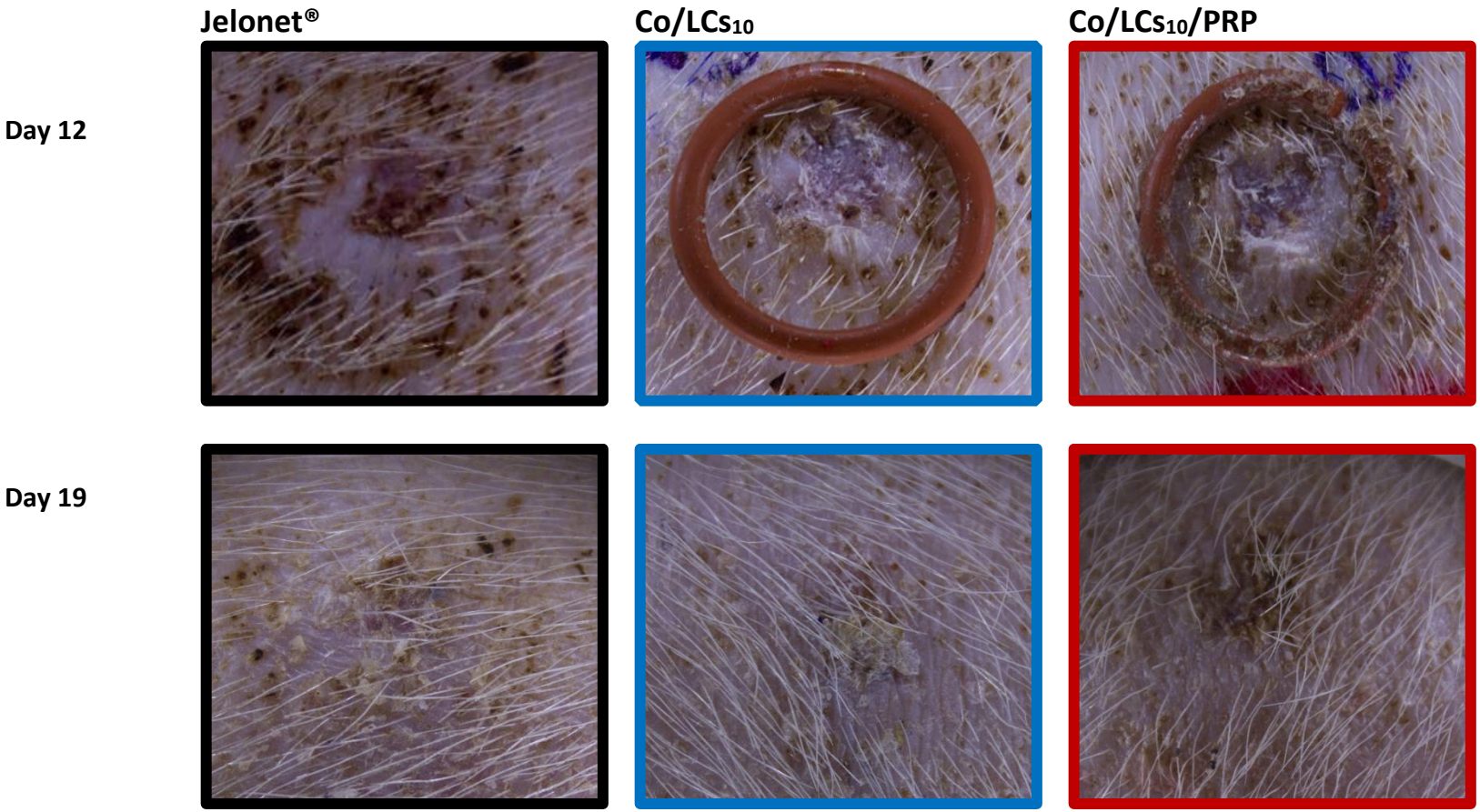

Figure 4.13 Photographs of wounds on Days 12 and 19 after wounding.
All wounds healed with minimum scarring. Notice the faster regrowth of hair in the CO/LCs$_{10}$/PRP treatment group.

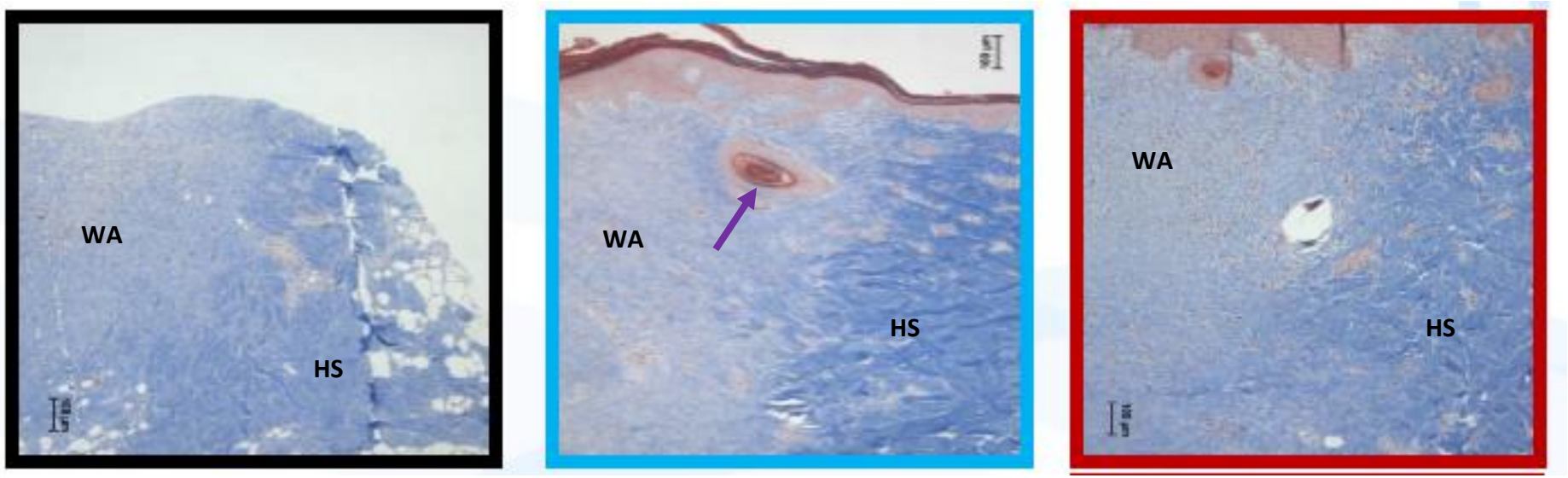

Figure 4.14 Microscopic images of MT stained wounds at 10x magnification on Day 15

Wound area (WA) in the Co/LCs$_{10}$ (Blue boarder) had a well-developed HF (purple arrow). Jelonet®-treated wounds (Black boarder) and Co/LCs$_{10}$/PRP wounds (Red boarder).

Consistent with the findings of Agyingi *et al* who simulated the process of epithelialisation and angiogenesis using the Eden model, it was found that as the wounds healed, they lost the original square shape (**Figure 4.15**) as a result of migration of epidermal cells from the wound edges propagating towards the wound centre.[361] The areas with the highest curvature in wounds heal first[362] as seen by the initial contraction of the wound corners (**Figure 4.15**). It was thus apparent that wounds in the porcine model heal by simultaneous re-epithelialisation and contraction. However, on microscopic examination, the wounds in this study healed more by re-epithelialisation from the bottom of the wound bed up rather than migration from the wound edges. Therefore, the wound measurements taken gave an indication of the healing effect due to contraction, which in this case could have been hidden under the well-established scab.

When measuring the wound area, it appeared as if the Jelonet®-treated wounds were decreasing in wound area more rapidly than the other treatments **(Figure 4.19)**. This was because of the scab covering the two treatment groups that obstructed wound measurements. When the scabs eventually fell off, the two treatment groups showed the most favourable healing outcomes. These included little or no scarring, quick return of skin to its original colour and morphology and functionality. Histologically this was confirmed by the presence of more mature and thicker collagen fibres and angiogenesis as explained above.

Overall, the Co/LCs_{10} treated wounds on macroscopic examination did not appear to show great healing progress due to the scab that masked the underlying changes. Microscopic examination showed wounds progressing well through each of the phases of wound healing with a high concentration of inflammatory cells initially which then decreased during the later stages of healing as collagen was deposited **(Figure 4.16)**. In summary, the wounds showed a similar healing pattern (healing from the bottom of the wound bed up) with an overall higher and more mature collagen deposition in the Co/LCs_{10}/PRP treatment group.

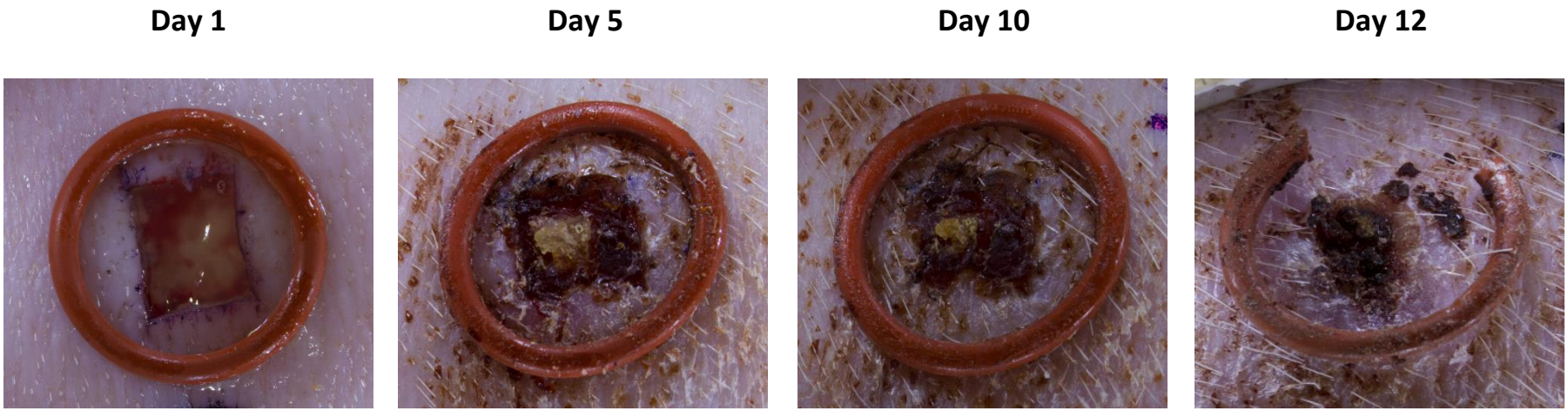

Figure 4.15 Images of a Jelonet®-treated wound taken on different days showing the loss of square shape as wounds re-epithelialised as a result of the scab breaking up in a non-symmetrical way.

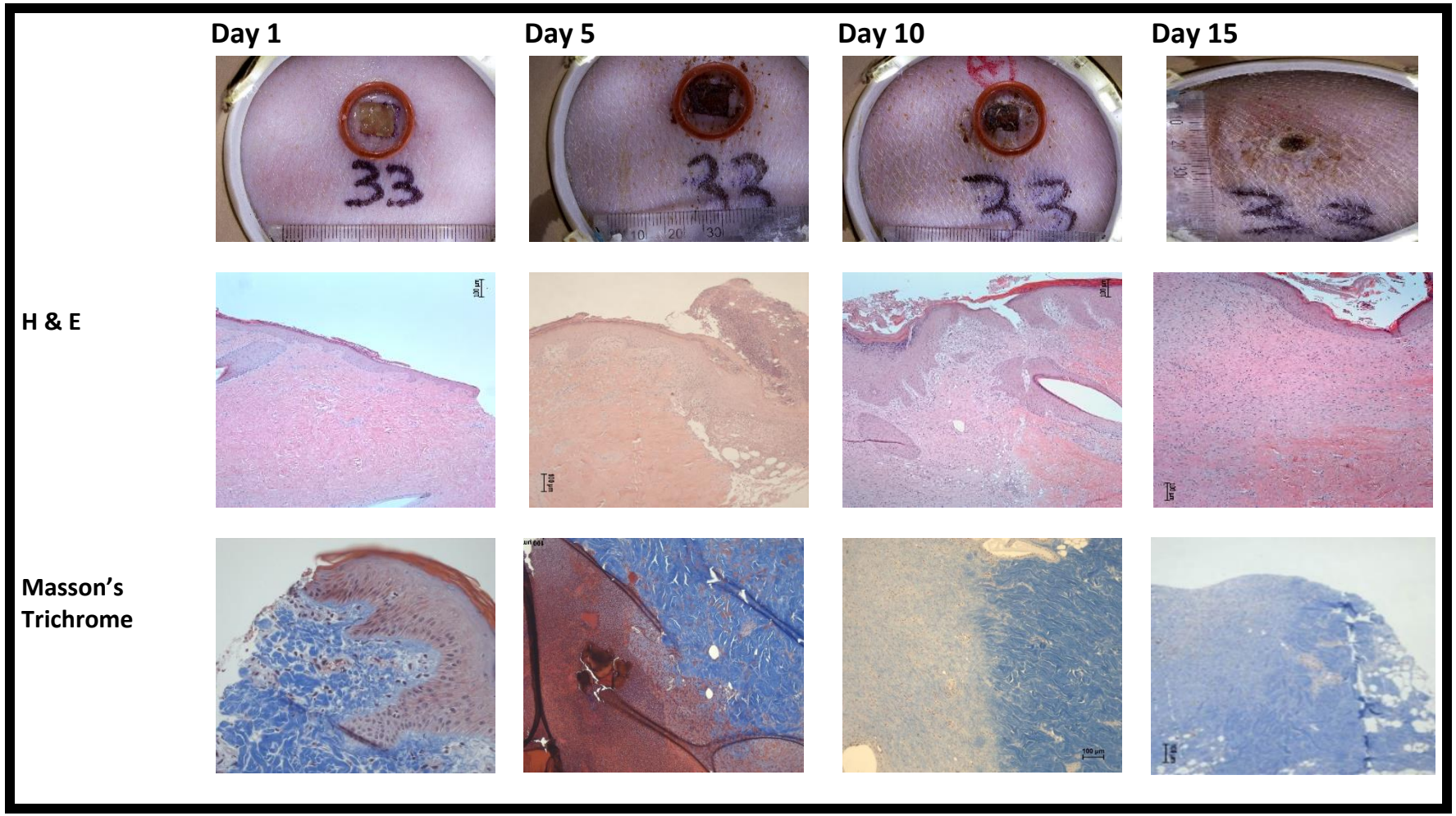

Figure 4.16 Progression of Jelonet®-treated wounds from Days 1 to 15.
First row showing the macroscopic appearance of the wounds, second row showing H & E stained wounds and third row showing MT stained wounds.

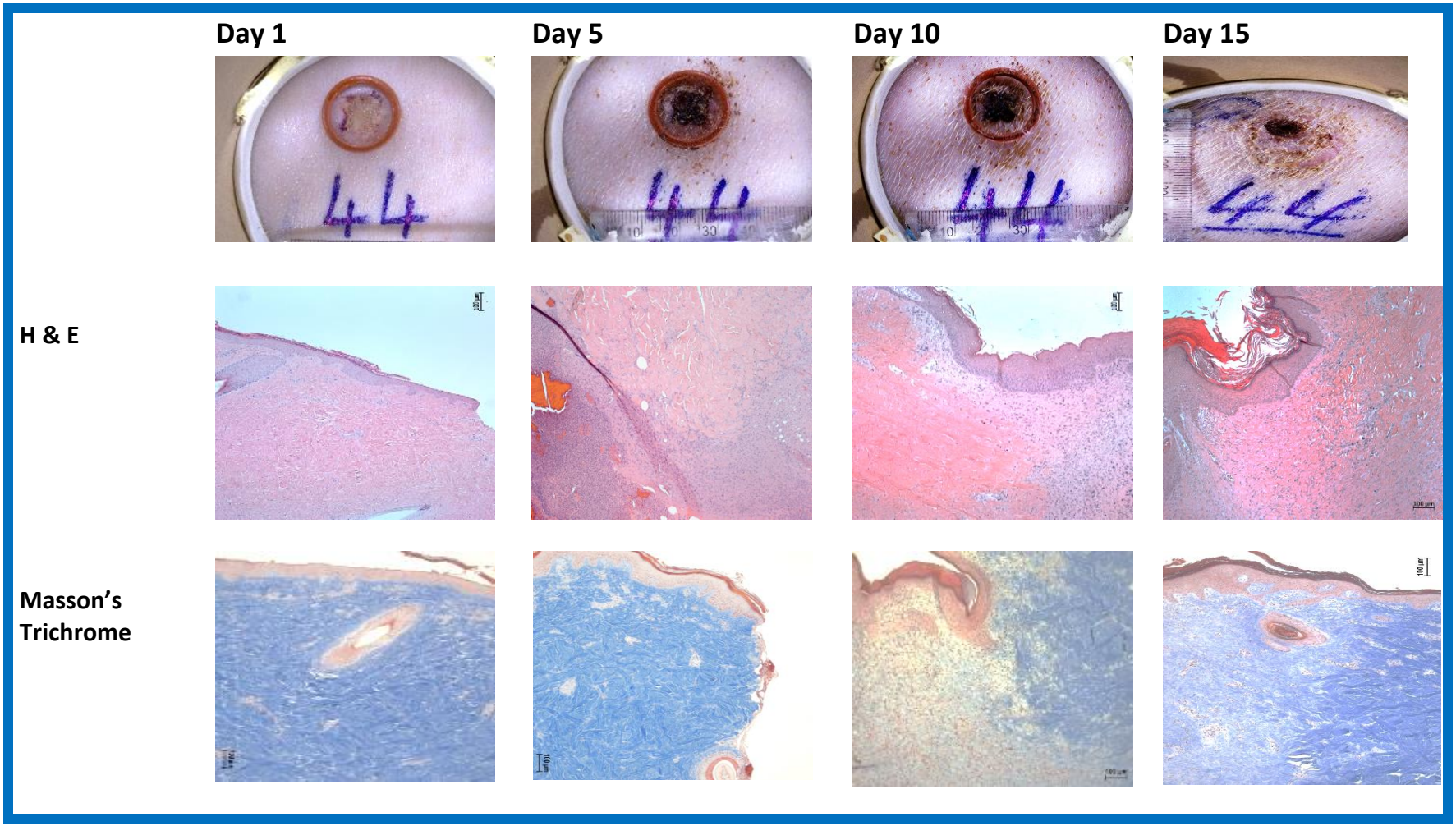

Figure 4.17 Progression of Co/LCs$_{10}$ treated wounds from Days 1 to 15.

First row showing the macroscopic appearance of the wounds, second row showing H & E stained wounds and third row showing MT stained wounds.

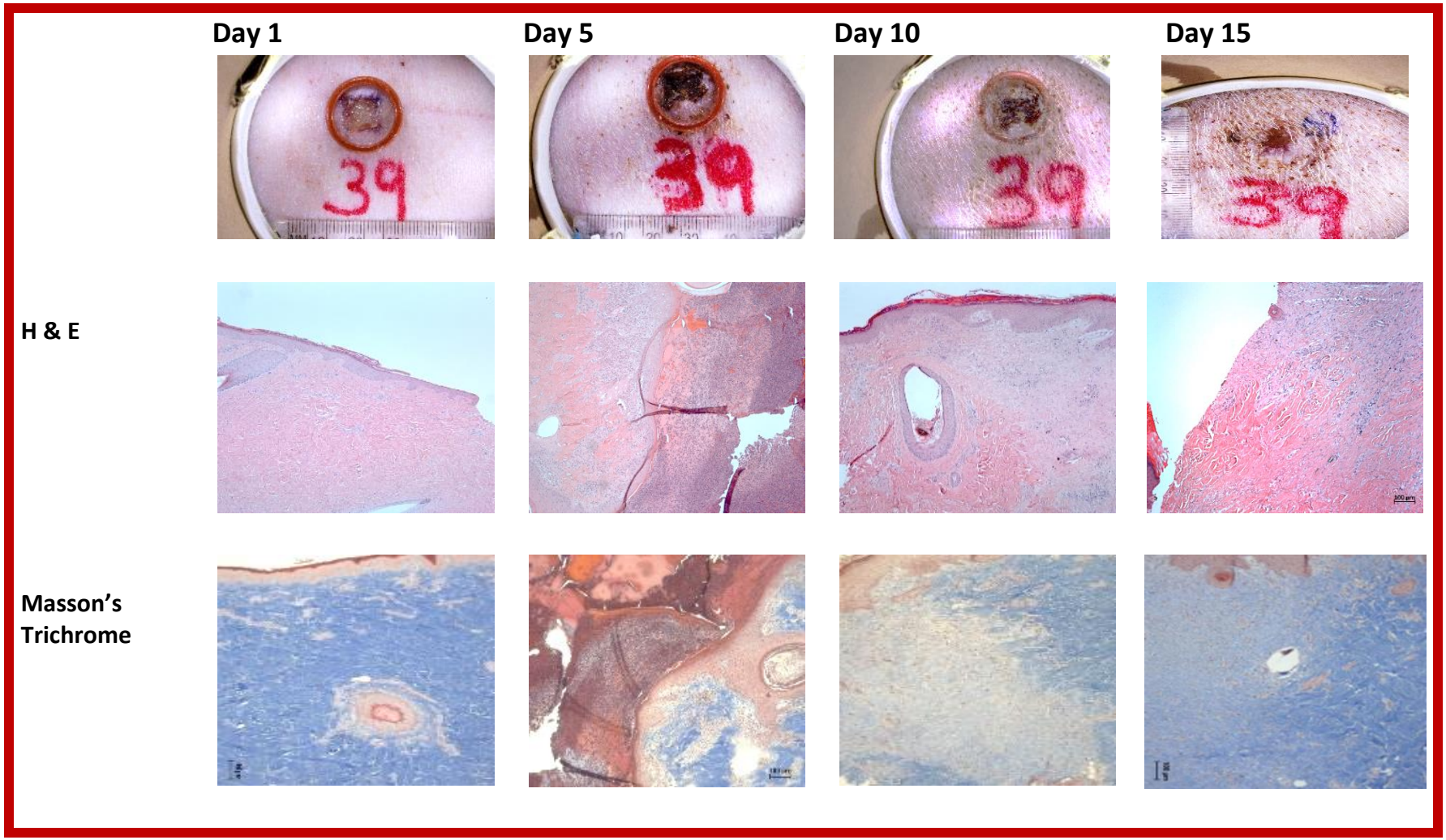

Figure 4.18 Progression of Co/LCs$_{10}$/PRP treated wounds from Days 1 to 15.

First row showing the macroscopic appearance of the wounds, second row showing H & E stained wounds and third row showing MT stained wounds.

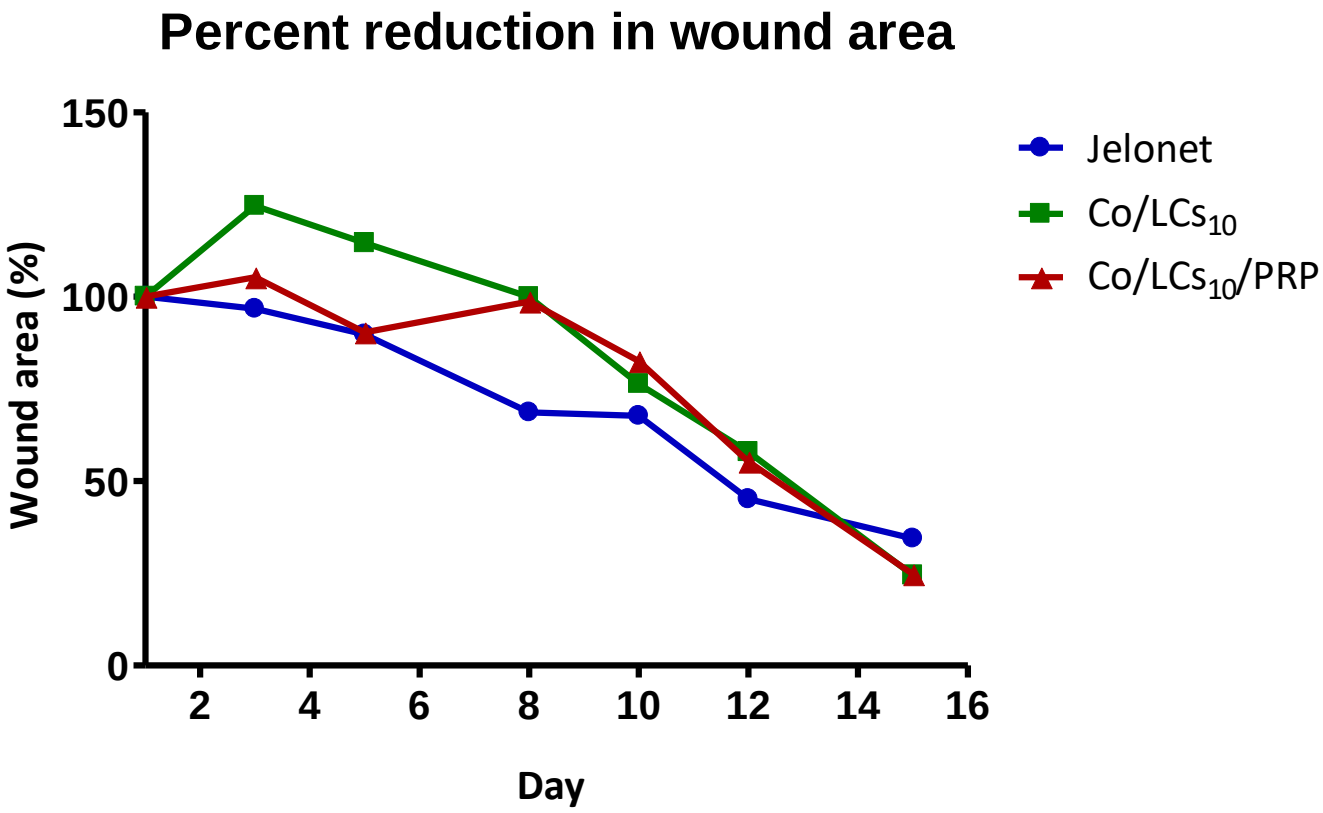

Figure 4.19 Percent wound area Days 1 up to 15.
It initially seemed as if the Jelonet® dressing was decreasing in size significantly faster than the test dressings because of the well-established scab hiding the true wound edge in the Co/LCs$_{10}$ and Co/LCs$_{10}$/PRP groups. It was only after Day 11 that Co/LCs$_{10}$/PRP had a greater decrease in size.

Overall, all wounds healed by re-epithelialisation as evidenced by the infiltration of cells from the bottom of the wound bed. The Co/LCs$_{10}$ paste seemed to have provided a suitable biomaterial-based filler that initially accelerated the haemostasis and promoted growth of new tissue by providing protection of the wound beds from further trauma and a matrix that absorbed excess exudate from the wound area.

General observations from the macroscopic assessments of all the wounds during the entire duration of the study showed that none of the wounds studied became infected. Scarring was observed from the 15th day. Generally, wounds treated with Co/LCs$_{10}$/PRP and Co/LCs$_{10}$ showed less pronounced scarring compared to the Jelonet®-treated wounds. All wounds had hair growth over them indicating superior healing.

4.6 Conclusion

The use of a wound filling paste containing collagen, lauroyl chitosan and platelet-rich plasma resulted in improved healing outcomes compared to those treated with Jelonet®. Within 10 min after applying the different treatments on the wounds, it was seen that the wounds treated with Co/LCs_{10} and Co/LCs_{10}/PRP had stopped bleeding while the Jelonet®-treated wounds continued bleeding slowly and had a red tinged exudate. By **Day 3**, this bleeding caused high moisture content of the Jelonet® treatment group resulting in macerated wounds while the LCs_{10} and Co/LCs_{10}/PRP treatment groups appeared to have a solid healthy-looking scab, thus showing the haemostatic effect of chitosan. Furthermore, the two chitosan-based treatment groups appeared to encourage accumulation of inflammatory cells and erythrocytes on the dressing material surface yet reduced inflammation. It was evident that the Co/LCs_{10} paste allowed adsorption of cells onto its surface as seen from the MT histology of these scabs.

The time for re-epithelialisation between topically applied Co/LCs_{10} and Co/LCs_{10}/PRP pastes compared to the Jelonet®-treated wounds was not significantly different; however, the Co/LCs_{10}/PRP treatment increased collagen deposition considerably more than the Co/LCs_{10} without the PRP. The scab on the two chitosan-based treatment groups was shown to completely cover and to some extent distort the appearance of the wounds, thus accurate measurement the wound size as the wound healing progressed was not possible.

Two pigs died, one each on the 10^{th} and 16^{th} days of the study. This was a surprising event and the animals were sent for autopsy to determine the cause of death. The first pig was found to have died from an intestinal torsion that was possibly due to the handling procedures used when transferring the pigs while under anaesthesia. The second pig died of pulmonary distress. It was concluded from the autopsies that none of the deaths could be attributed to the wound dressings used. However, the handling method of the pigs was identified as a causative factor and further handling procedures were all performed on the same mattress for the remaining pigs for the rest of the study.

The wound areas from the Co/LCs_{10} and Co/LCs_{10}/PRP groups quickly appeared similar to the surrounding normal skin, with hair growth compared to the Jelonet®-treated wounds.

Chapter 5

5.1 General discussion and conclusion

The focus of the present study was on full-thickness wounds. These wounds present with excessive tissue loss, minimum growth factor activity and impaired angiogenesis. Dysfunctional scarring emanating from impaired healing in full-thickness wounds is inexorable unless some skin substitutes are used. A wound filler that promotes tissue growth, angiogenesis and combats infection was considered a viable option for treating these wounds.

Thus, it was hypothesised that wound management using hydrophobically modified chitosan-based dressings combined with collagen and platelet-rich plasma would enhance the wound healing rate and minimise dysfunctional scarring of full-thickness wounds when measured using the porcine wound model.

The field of wound healing is diverse and well researched, however hydrophobically modified chitosan is under-researched as a wound dressing. In this study, chitosan was hydrophobically modified to give three different loadings of lauric acid conjugated chitosan, which were chemically and physically characterised together with *in vitro* assays to determine the product with the best physicochemical properties for use in a wound dressing. The chosen conjugate was then tested on a full-thickness wound model for efficacy as a wound filling dressing material. This study thus provides an important foundation for further studies into the efficacy of hydrophobically modified chitosan in improving full-thickness wound dynamics including platelet delivery, growth factor release, exudate absorption, wound contraction and avoiding dysfunctional scarring.

First, hydrophobic modification of chitosan using lauric acid was completed successfully using an addition-elimination type reaction. This proceeded through the formation of amide linkages between the primary amine groups of chitosan and the carboxylic acid functional group of lauric acid. Initially, lauric acid was conjugated to chitosan using carbodiimide chemistry, with the EDC salt activating the carboxylic group of lauric acid. However, a second method using the highly reactive lauroyl chloride was used. This method significantly reduced the reaction time from 72 hr to just 24 hr. Characterisation of the LCs conjugates using NMR

and FT-IR confirmed the conjugation with the appearance of the aliphatic proton peak at 0.9 ppm in the NMR spectrum, and the carboxylic group at 1655 cm^{-1} and 1722 cm^{-1} in the FT-IR. The FT-IR absorption peaks at 2842 cm^{-1} and 2932 cm^{-1}, were attributed to symmetric and asymmetric C-H stretching, respectively. These stretching vibrations accounted for the methyl, methylene and methine groups in $NHCOCH_3$, CH_2OH and CH in the pyranose ring, respectively.[273]

The degree of lauroyl chain conjugation to chitosan demonstrated increased hydrophobicity as the molar concentration of lauric acid increased. When compared to the underivatised chitosan using the drop shape analysis, it appeared that as the molar concentration of lauric acid increased, the contact angle also increased. In the swelling tests, LCs_{34} had the highest swelling capacity. However, despite this good characteristic, LCs_{34} was physically rigid, hard and plastic which reduced its processability. Consequently, LCs_{34} could not provide a good malleable wound fitting matrix for space filling and absorption of excess exudate and for this reason was eliminated as a possible wound healing material. The swelling index studies also showed the LC_{s20} scaffold almost completely dissolving into a gel within 2 hr and no further readings could be taken beyond this time point. This was another reason why LC_{s10} was selected for the wound healing study.

In preliminary studies, platelet adhesion to the conjugated chitosan was done by coating of the wells of tissue culture plates with the different chitosan conjugate material and adding PRP with aspiration of the non-adherent platelets after an hour. The adherent platelets were then quantified microscopically by platelet counting. After realising the impracticality of complete uniform aspiration of non-adherent platelets, and the difficulty in counting the adhered platelets accurately, this method was discontinued and the colorimetric acid phosphatase assay was followed. The findings from the acid phosphatase assay showed that as hydrophobicity of the sample increased, platelet adhesion decreased. Chitosan adhered the highest platelet concentration, even higher than the collagen positive control. The lauroyl substitution of chitosan resulted in a negatively charged polymer surface that repelled the negatively charged platelets. Increased hydrophobicity is also known to reduce the “shear stress” of a surface, decrease the surface area available for adhesion and reduce the overall concentration of adherent platelets.[333] Fatty acids induce platelet aggregation, yet fatty acids also form a smooth surface that repels platelets. The platelet adhesion assay validated the

concept that the higher molar loading of lauroyl groups in LCs_{34} and in this case also the LCs_{20} were not the best candidates to increase platelet delivery to wound sites and thus based on these findings it was decided that LCs_{10} gave the most suitable characteristics for a wound healing dressing and was therefore further investigated in the animal study.

Next, an ELISA kit was used to assess PDGF-AB release over 24 hr. The positive control, collagen type 1 extracted from porcine skin was characterised using FT-IR. The FT-IR peaks corresponded with that found in literature. The other determinant for the collagen was its ability to form a gel in 1% aqueous acetic acid.[354] Since LCs_{10} gave superior swelling and platelet adhesion properties, it was assayed for platelet adhesion and growth factor release using the ELISA. After binding platelets, the LCs_{10} conjugate gave a sustained release of PDGF-AB over 24 hr. The release of PDGF-AB from native chitosan and collagen displayed a burst release within the first hour. This was attributed to the platelet activation by these two materials. Hydrophobic modification of Cs resulted in a material with reduced platelet-activating properties.

Finally, a wound filler paste comprising of 20% w/w collagen in LCs_{10} (Co/LCs_{10}) was prepared and sterilised using gamma irradiation. The energy released during gamma irradiation causes damage to biological products like PRP while potentially causing physico-chemical changes to collagen and chitosan. To reduce the potential damage to PRP which would alter its efficacy, the $CoLCs_{10}$ was sterilised and stored before adding PRP. PRP was only added to the paste just before applying to the wounds on the day of treatment. The Co/LCs_{10} and Co/LCs_{10} with PRP (Co/LCs_{10}/PRP) were compared to Jelonet®-treated controls to compare wound healing rates using the full-thickness porcine wound model. On the 3rd and 5th days after applying these dressings, macroscopic observations showed Co/LCs_{10}/PRP to have superior haemostatic properties compared to the Co/LCs_{10} treatment. The Jelonet®-treated wounds appeared to have macerated edges because of high exudate levels. Chitosan's haemostatic properties were a contributing factor to the low exudate levels in the two chitosan-based treatments with PRP adding further benefit to this property in the Co/LCs_{10}/PRP treatment. Histologically, the MT stain showed prolonged inflammation on Jelonet®-treated wounds continuing up to the 8th day. However, inflammatory cells on Co/LCs_{10} and Co/LCs_{10}/PRP treated wounds were adsorbed into the chemoattractant LCs_{10} scab; thus facilitating the removal of inflammatory cells from the wound bed.

Contrary to the aim of the study, which set out to provide a matrix for the infiltration and growth of new cells within the wound bed, all the wounds appeared to heal from the dermal interface beneath the scab without significant cell infiltration into the chitosan-based dressing material. The scab which consisted mostly of entrapped dry blood and the inflammatory cells also incorporated the LCs_{10} material which did not incorporate into the newly formed granular tissue as clearly visible in the biopsy histology by Day 5. The MT stained slides showed a clear distinction between newly formed epithelium and the scab in all the healing wounds. The wounds healed within 12 to 16 days with minimal scarring and the histology of the edge biopsies indicated extensive new collagen deposition within the wound bed and re-establishment of the characteristic skin structure with clear dermal and epidermal layer.

In summary, three hydrophobically modified LCs derivatives were synthesised using the acyl halide chemistry method. They were then physically and chemically characterised followed by *in vitro* analysis to determine which of these three derivatives would give optimum platelet binding and growth factor release and overall give the best-improved wound dressing. The LCs_{10} derivative proved to have optimum *in vitro* properties as shown especially in the platelet binding assay. It was further assayed using the ELISA where it showed sustained release of the PDGF-AB. Finally, LCs_{10} was combined with collagen and PRP to provide a wound filler paste that was tested on full-thickness wounds in the porcine model. The wounds treated with Co/LCs_{10} and Co/LCs_{10}/PRP formed a plug immediately after application, which proved to be haemostatic compared to the Jelonet®-treated wounds. In the later stages of healing, The Co/LCs_{10}/PRP treated wounds appeared to have healed more rapidly with less scarring than the Jelonet®-treated wounds. Finally, histological analysis using the MT and H & E showed highest and most mature collagen deposition in the Co/LCs_{10}/PRP treatment group.

Based on the findings from this study, the Co/LCs_{10}/PRP paste used as a space filling wound dressing material provided a protective scaffold that prevented wound constriction and provided a sustained release of growth factors from the added platelet-rich plasma, was haemostatic and absorbed excess exudate from the wound while providing a moist wound environment with fewer inflammatory cells thus preventing chronic inflammation in wounds. The use of PRP appeared to have an additional benefit of increased collagen density and growth of new epithelial cells. Further research on the Co/LCs_{10}/PRP is recommended to validate its efficacy as a wound filler.

5.2 Limitations and recommendations

Blood from only ten participants was used to determine the blood parameters and the average platelet count. On average, platelet concentrations increased five-fold in the PRP compared to the platelet count in whole blood. This platelet count was lower than the average eight-fold recorded platelet counts in literature. This could possibly be improved by decreasing the final volume of plasma harvested per sample and confirmed by increasing the number of study participants.

Due to cost limitations of the PDGF-AB kit, only the LCs_{10} conjugate compared to the collagen and native chitosan controls were assayed. Further PDGF-AB release assays on all the conjugates would give a good comparison of the impact of increased hydrophobicity on growth factor release.

The platelet adhesion assay used a blank well surface treated with 1% acetic acid. Future studies using albumin-coated surfaces for the blank would give a better comparison.

The SEM and TEM images from the fibroblast infiltration assay did not show clear infiltration of the fibroblasts in the images. One limitation was the adherence of fibroblasts onto the culture plates with proliferation on the plate surface rather than growing into the scaffolds. This could be prevented in future by prior coating of the plate surface with agarose to prevent adhesion of the fibroblasts.

The pH is an important factor when preparing wound dressings as it has both a direct and indirect effect on all biochemical reactions taking place. During the different stages of preparing the $CoLCs_{10}$ paste, different pH values were used. For example, the swelling index was done at pH of 7.4 while the Co/LCs_{10} paste was adjusted to a pH range of 5 – 6. This impacted accuracy in reporting the efficacy of the final product. Future studies should consider consistency with the pH range at every step.

During the course of the animal study, two pigs died. Full autopsies were performed on these two animals and it was found that the first pig had died due to an intestinal torsion, potentially due to the handling procedures of the pigs during the anaesthesia and transfers onto the theatre table and transfer trolley. The second pig appeared to have died due to undetermined pulmonary causes. The autopsy reports (see

Annexure 7) also determined that death was highly unlikely to have been due to the wounds or any of the material applied on the wounds. After the death of the first pig, it was recommended immediately that the handling of the pigs change from picking up and rolling onto the operating table to keeping the pig in the same position while transporting onto the operating table. It is also recommended that future animal studies reduce the frequency of the anaesthesia for biopsy purposes as this increased the handling frequency.

This being one of the first studies on the use of hydrophobic chitosan as a wound dressing material, the above-mentioned recommendations should be considered for further preclinical studies on these compounds.

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Annexure 1

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences
Department of Pharmacology
6th floor, Basic Medical Sciences Building
9 Bophelo Road
Prinshof Campus
Pretoria

Patch test using modified chitosan

Participant consent forms for skin sensitivity test using chitosan

Principal investigator: Bongai Khathide

Supervisor: Prof AD Cromarty

Co-supervisor: Dr MO Balogun

PATIENT PARTICIPATION AND INFORMED CONSENT

Introduction

My name is Bongai Khathide. I am a PhD student at the University of Pretoria (Pharmacology Department). I would like to invite you to participate in my research study entitled: Patch test using a modified chitosan. Please note that it is not compulsory to take part in this study. You will need to read and understand this document as it explains all you will need to know about this study. Before you participate you will need to sign informed consent forms.

Purpose of the study

A modified dressing will be made using chitosan to improve the healing of diabetic foot ulcers. Chitosan is a material found in the outer skeleton of shellfish including crab, shrimp, and lobster. It is used for many things including treating obesity and high cholesterol. In this study it will be used to make a new dressing that will increase the healing of diabetic foot ulcers.

The purpose of this is to document the safety of these two compounds before a dressing is made. No adverse reactions are expected.

Length of the study and the number of participants

The duration of this study is 3 days. This study will have 15 participants, from the University of Pretoria.

Procedures

- Within 3 days before the beginning of the study, you will be screened to make sure you can take part in the study. You will be assigned a study number so that your identity will be kept confidential.
- We will expect you to come back after 3 days. A chitosan foam will be applied to your skin. It will be covered with OpSite® Flexifit.
- A photograph of your skin before the foam is applied and after the 2-day exposure will be recorded so we can be able to track any reactions to the chitosan.

Risks

No known risks have been recorded with the use of both the OpSite® dressing and chitosan. Chitosan has been used orally and thus is safe. However, if you experience any adverse effects at any time of the day, feel free to call me as soon as possible on this number: 078 018 6511. If I am not available, you can call Prof AD Cromarty on 073 306 4220. The University of Pretoria will cover insurance of each participant.

Benefits

The benefits of using chitosan include:

1. It is non-toxic.
2. It provides material that keeps wounds moist.
3. It has antibacterial properties.
4. It has antifungal properties.

Participants' rights

It is your right to drop out of this study at any point. We would like to know if the reason is due to the chitosan paste or not.

Confidentiality

You will be assigned a unique study number that will assure that all the information we gather from you is kept confidential. Only the team I am working with and I will have access to your information. If any of the data is published in scientific journals, your identity will not be used. All the information we obtain from you will be held with strict confidence.

Emergency care and hospitalisation

If you happen to be hospitalised for any reason or visit any health care practitioner, please tell them that you are involved in this study.

Ethical approval

This clinical trial Protocol was submitted to the Faculty of Health Sciences Research Ethics Committee, University of Pretoria, telephone numbers 012 354 1677 / 012 354

1330. Ethical approval was obtained for this patch test. The study has been structured in accordance with the Declaration of Helsinki (last update: October 2013), which deals with the recommendations guiding doctors in biomedical research involving human subjects. A copy of the Declaration may be obtained from the investigator should you wish to review it.

Do you have any questions regarding the study?

Yes/No

If yes, please record them below:

1.

2.

3.

4.

5.

INFORMED CONSENT

1. Participant

I ……. hereby confirm that I have read and understood the contents of this document and therefore understand the benefits, risks, and the nature of this clinical study.

- I also understand that the data collected from this research will be published in a scientific journal. This will not include personal information.
- I agree for a photograph to be taken of the area where the study material is applied.
- I understand that I can withdraw from participating in this study at any stage.
- I have asked all the necessary questions.

Signature: Date:

2. Study Doctor

I Dr……confirm that the above participant has been fully informed about the nature, conduct and risks of this study.

Signature: Date:

3. Principal Investigator

I Bongai Khathide confirm that I have explained fully the nature of this study and the risks/ benefits.

Signature: Date:

4. Witness

Name:

Signature: Date:

Annexure 2

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences
Department of Pharmacology
6th floor, Basic Medical Sciences Building
9 Bophelo Road
Prinshof Campus
Pretoria

Research title: Assessing modified chitosan wound dressings to enhance wound healing in the porcine model

Participant consent forms for blood donations from healthy voluntary participants at the University of Pretoria

Principal investigator: Bongai Khathide

Supervisor: Prof AD Cromarty

Co-supervisor: Dr MO Balogun

PATIENT PARTICIPATION AND INFORMED CONSENT

Introduction

My name is Bongai Khathide. I am a PhD student at the University of Pretoria (Pharmacology Department). I would like to invite you to participate in my research study entitled: Assessing modified chitosan wound dressings to enhance wound healing on the porcine model. Please note that it is not compulsory to take part in this study. You will need to read and understand this document as it explains all you will need to know about this study. Before you participate you will need to sign informed consent forms.

Purpose of the study

A modified dressing will be made using chitosan to improve the healing of deep wounds. Platelets obtained from blood will then be added to the modified dressing to maximize the wound healing effect. Chitosan is a material found in the outer skeleton of shellfish including crab, shrimp, and lobster. It is used for many things including treating obesity and high cholesterol. Platelets are small cells in the blood that are involved in forming a clot to stop bleeding. Platelets are also rich in growth factors that have been shown to play a big role in wound healing.

This study will have the first phase where the material will be tested in the laboratory before being tested on wounds on pigs. Thus, we ask for you to donate blood so we can prepare the platelet-rich plasma required for the laboratory tests.

Who can take part in this study?

- If your age is between 18 and 60.

Who cannot take part in this study?

- If you are anaemic. (*In the event that you do not know if you are anaemic or not, you will be tested by having your finger pricked and testing the blood droplet density)*
- If you are pregnant
- If you are a participant in another clinical study
- If you are on medication that interferes with platelet function i.e. aspirin, heparin

Procedure

- The World Health Organisation has guidelines for drawing blood and these will be followed.
- Blood samples will be drawn from 10 healthy participants. A needle and two blood collection tubes of 8 ml each will be used to draw blood from a vein in ***your arm***.
- A trained person such as a nurse or a doctor will draw the blood. A restriction cuff will be placed on your upper arm, the area cleaned with a sterilising solution tissue and a needle will be inserted into your vein near the inside of your elbow. Your blood will be drawn into the two blood tubes and this will take about 5 minutes.
- The blood samples will only be used to isolate platelets for testing how they bind to the wound healing material. Any leftover blood will be discarded in the biohazard containers in the lab.

Risks

Slight pain and bruising in some cases will be felt from the needle prick. It is possible to feel lightheaded just after donating the blood.

Benefits

This study has no direct benefits for you; however it is part of a bigger study that will assist in getting an improved wound dressing.

Participants' rights

It is your right to drop out of this study at any point as you will be donating blood voluntarily.

Confidentiality

Your blood sample will be assigned a unique study number that will assure that all the information we gather from you is kept confidential. Only the team I am working with and I will have access to your information. If any of the data is published in scientific journals, your identity will not be used. All the information we obtain from you will be held with strict confidence.

Emergency care and hospitalisation

If you happen to be hospitalised for any reason or visit any health care practitioner, please tell them that you are involved in this study.

Ethics approval

This Protocol was submitted to the Faculty of Health Sciences Research Ethics Committee, University of Pretoria, telephone numbers 012 3541677 / 012 3541330. Ethical approval was obtained for obtaining this blood and for the experimental work. The study has been structured in accordance with the Declaration of Helsinki (last update: October 2013), which deals with the recommendations guiding doctors in biomedical research involving human subjects. A copy of the Declaration may be obtained from the investigator should you wish to review it.

Do you have any questions regarding the study?

Yes/No

If yes, please record them below:

1.

2.

3.

4.

For any further questions or queries you can contact any one of these people:

Principal investigator: Bongai Khathide

Cell: 078 018 6511

Email address: bonghie14@gmail.com

Supervisor of Study: Prof AD Cromarty

Tel: 012 319 2622

Email address: duncan.cromarty@up.ac.za

Co- supervisor: Dr M Balogun

Tel: 012 841 2340

Email address: mbalogun@csir.co.za

INFORMED CONSENT

1. **Participant**

I ……. hereby confirm that I have read and understood the contents of this document and therefore understand the benefits, risks and the nature of this study.

- I also understand that the data collected from this research will be published in a scientific journal. This will not include personal information.
- I understand that I am allowed to withdraw from participating in this study at any stage.
- I have asked all the necessary questions.

Signature: Date:

2. **Principal Investigator**

I Bongai Khathide confirm that I have explained fully the nature of this study and the risks/ benefits.

Signature: Date:

3. **Witness**

Name:

Signature: Date:

Annexure 3

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 20 Oct 2016.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 22/04/2017.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

26/05/2016

Approval Certificate
New Application

Ethics Reference No.: 99/2016

Title: A modified platelet-rich plasma dressing to enhance the healing of diabetic foot ulcers

Dear Mrs Bongai Manyakara

The **New Application** as supported by documents specified in your cover letter dated 29/04/2016 for your research received on the 17/05/2016, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 25/05/2016.

Please note the following about your ethics approval:
- Ethics Approval is valid for 2 years
- Please remember to use your protocol number (**99/2016**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:
- The ethics approval is conditional on the receipt of **6 monthly written Progress Reports**, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

Additional Conditions:
- Approved this study as a pilot study to the PhD study

We wish you the best with your research.

Yours sincerely

Dr R Sommers; MBChB; MMed (Int); MPharMed,PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

012 356 3085 fhsethics@up.ac.za http://www.up.ac.za/healthethics
Private Bag X323, Arcadia, 0007 - Tswelopele Building Level 4-59, Gezina, Pretoria

Annexure 4

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 03/14/2020.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

26/04/2018

Approval Certificate
New Application

Ethics Reference No: 173/2018

Title: Assessing modified chitosan wound dressings to enhance wound healing in the porcine model

Dear Bongai Manyakara

The **New Application** as supported by documents specified in your cover letter dated 16/04/2018 for your research received on the 18/04/2018, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 25/04/2018.

Please note the following about your ethics approval:
- Ethics Approval is valid for 1 year
- Please remember to use your protocol number (**173/2018**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:
- The ethics approval is conditional on the receipt of **6 monthly written Progress Reports**, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

Additional Conditions:
- Approval is conditional upon the Research Ethics Committee receiving Animal Ethics Committee approval

We wish you the best with your research.

Yours sincerely

*** Kindly collect your original signed approval certificate from our offices, Faculty of Health Sciences, Research Ethics Committee, Tswelopele Building, Level 4-60*

Dr R Sommers; MBChB; MMed (Int); MPharMed,PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health).

☎ 012 356 3084 deepeka.behari@up.ac.za / fhsethics@up.ac.za http://www.up.ac.za/healthethics
✉ Private Bag X323, Arcadia, 0007 - Tswelopele Building, Level 4, Room 60 / 61, 31 Bophelo Road, Gezina, Pretoria

Annexure 5

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Assessing modified chitosan wound dressings to enhance wound healing in the porcine model**
PROJECT NUMBER	**H009-17**
RESEARCHER/PRINCIPAL INVESTIGATOR	**B Manyakara**

STUDENT NUMBER (where applicable)	**U_15412068**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SPESIES/SAMPLES	**Porcine**
NUMBER OF ANIMALS	**4**
Approval period to use animals for research/testing purposes	**May 2018-May 2019**
SUPERVISOR	**Prof AD Cromarty**

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 28 May 2018
CHAIRMAN: UP Animal Ethics Committee	Signature

S4285-15

Annexure 6

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2018/01/6/C

APPLICANT: Professor D Cromarty

SCHOOL: University of Pretoria - Department of Pharmacology
DEPARTMENT:
LOCATION:

PROJECT TITLE: Assessing modified chitosan wound dressings to enhance wound healing in the porcine model

Number and Species

4X 32 +-2kg female large white Porcine

Approval was given for the use of animals for the project described above at an AESC meeting held on 2018/01/30. This approval remains valid until 2020/06/11.
Unreported changes to the application may invalidate the clearance given by the AESC
An annual progress report must be provided

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and is subject to any additional conditions listed below:

Signed: _____ (Chairperson, AESC) Date: 12/06/2018

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ (Registered Veterinarian) Date: 12 June 2018

cc: Supervisor: N/A
Director: CAS

Works 2000/lain0015/AESCCert.wps

Annexure 7

POST MORTEM EXAMINATION: (to be completed by Veterinarian):

PI NAME: Prof. A.D. Cromarty	AREC#: 2018/01/06/C	DATE: 07/11/2018
BODY CONDITION: Good body condition score. Bloated abdomen.		
BODY MASS: Click or tap here to enter text.		

PM FINDINGS & SAMPLES TAKEN:

Skin: Dorsum, iatrogenic incisional wounds to assess wound healing. The extremities and snout cyanotic.
Muscle: No abnormalities grossly discernable
Small intestine: purple discoloration of serosal surface with petechiae. Mucosal surface bloody and lumen filled with blood and gas
Large intestine: purple discoloration of serosal surface with petechiae. Mucosal surface bloody and lumen filled with blood and gas
Stomach: Distended with gas and ingesta. Normal mucosal and serosal surfaces.
Liver: Congested
Spleen: Enlarged and congested
Pancreas: No abnormalities visible
Kidneys: Normal serosal surface and on cut surface.
Reproductive organs: No abnormalities detected.
Lungs: No abnormalities grossly discernable
Heart: No abnormalities grossly discernable
Trachea: Normal mucosal surface
Brain: Not examined.
Other comments: Abdominal cavity filled with straw coloured fluid. Torsion of main mesenteric vessels at the root.

SAMPLE PROCESSING: Skin collected for histology as part of study REPORT ID: Click or tap here to enter text.
ANATOMICAL PATHOLOGICAL DIAGNOSIS: Acute torsion of intestines compromising patency of the mesenteric blood vessels.

REPORT PREPARED BY: K.H. Erlwanger		DATE: 08 November 2018

DG* ☐

DP#☐ I ☒ E ☐ N ☐ X ☐

Disease | Incidental | Experimental | No Macroscopic Pathology | No PM done

Developed by: M Costello Checked by: Add Name Click or tap to enter a date. Version #: Choose an item. AREC Approval: Click or tap to enter a date.

Annexure 8

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

POST MORTEM EXAMINATION: (to be completed by Veterinarian):

PI NAME: D Cromarty	AREC#: 2018/01/06C	DATE: 13/11/2018

BODY CONDITION: BCS 3
BODY MASS: 45.6 Kg

PM FINDINGS & SAMPLES TAKEN:

Skin: NAD other than wounds created for study purposes on dorsum
Muscle: NAD
Small intestine: Severe congestion of mesenteric blood vessels. No torsion evident but small section of SI loop loosely entrapped between a distal segment with gas dilation on either side.
Large intestine: Gas distension
Stomach: Dilated with ingesta, congested gastric vessels
Liver: Dark in colour (cyanotic) and severely congested, oozing blood on cut surface
Spleen: Dark and congested
Pancreas: NAD
Kidneys: NAD
Reproductive organs: NAD
Lungs: Congestion and pulmonary oedema, rounded lung lobe edges and distention of the interlobular septa. Multifocal areas of cranioventral consolidation. Oedematous fluid , blood tinged on cut surface
Heart: Coronary blood vessel congestion
Trachea: NAD
Brain: Not examined
Other comments: Euthanased due ARDS and inability to maintain normal oxygen saturation .

SAMPLE PROCESSING: Click or tap here to enter text. REPORT ID: Click or tap here to enter text.

ANATOMICAL PATHOLOGICAL DIAGNOSIS: Acute respiratory distress syndrome as a result of severe pulmonary compromise

REPORT PREPARED BY: Dr K Jardine	K Jardine	DATE: 13 November 2018

DG ☐

DP#☒ I ☐ E ☐ N ☐ X ☐

Page 1

Developed by: M Costello Checked by: Add Name Click or tap to enter a date. Version #: Choose an item. AREC Approval: Click or tap to enter a date.