THE EFFECT OF SILICONE SHEETING ON THE PRODUCTION OF EXOPROTEINS BY MICROORGANISMS

JADE YUK MAI CHAN

A thesis submitted in partial fulfilment of the requirements of the Manchester Metropolitan University for the degree of Master of Science (by Research)

Faculty of Science and Engineering
School of Healthcare Science
2014

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Acknowledgements

First and foremost, thank you to Prof Val Edwards-Jones, Dr Rebecca Taylor and Chris Maryan for their patience, help and support in this project. It has been an incredible learning opportunity to work with such aspiring people.

Many thanks to Carol Winter (Consultant Maxillofacial Prosthetist & Technologist, Salford Royal Foundation Trust) and Jane M^{c}Phail (Consultant Prosthetist Burns and Plastic Surgery, St Helens & Knowsley Hospitals NHS Trust) for their experiences and advice associated with this study.

I would also like to thank Anne Leahy-Gilmartin, Gill Collier, Dr Paul Benson and Natalie Callaghan, the amazing microbiology staff at MMU for all their help, kindness, cakes and laughs.

And last but not least, many thanks to my work husband, Michail Ostrovsky. There was never a dull day in the lab!

Declaration

With any exception of any statements to the contrary, all of the data presented in this report are of my own efforts and have not previously been submitted in candidature for any other degree or diploma. No parts of this report have been copied from other sources. I understand that any evidence of plagiarism and the use of unacknowledged third party data will be dealt with in a serious manner.

Abstract

Hypertrophic scarring is common amongst burn injuries. Pressure therapy and the use of silicone sheeting is often prescribed to treat these scars, but there is weak evidence of the effect of silicone sheeting during treatment. At present, it is not known how the silicone dressings work. In this setting, it has been proposed that water transmission may play a role. The treatments provide favourable conditions for bacteria to colonize and multiply due to the sheeting being worn for at least 12 hours at a time and there are no studies investigating the microorganisms found on fully healed wounds before or after treatment with silicone. As it is unclear on how effective silicone sheeting may be in treating hypertrophic scars, there could be an efficiency factor due to microorganisms found under the dressings. This study aimed to investigate the microbiology of intact skin under silicone sheeting and to construct a model to study the *in-vitro* effects on extracellular protease production. *In-vitro* models were set up to determine bacterial numbers and protease activity. Various models were constructed using Petri dishes and universals with broth to allow organisms to permeate throughout the silicone dressings. An azocasein substrate was used to quantify total protease levels. Ten healthy volunteers were recruited into the study and one volunteer presented with considerable hypertrophic scarring and agreed to be a case study. Swabs were taken of the skin prior to application of silicone sheeting, and then the skin and sheeting were swabbed subsequently once a week over a one-month period. *In-vitro* results showed increases in bacterial growth for all organisms tested, but protease activity increase was only displayed by *S.epidermidis, S.aureus, A.johnsonii* and *C.albicans*. *A.johnsonii* showed a significant change in protease activity (P=0.020) as well as *S.aureus* (P=0.001). The volunteer study revealed variable results, which may have been due to interference with the azocasein assay. An ANOVA showed no statistical significance. The mechanism of action of silicone treatment remains inconclusive and requires further study.

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Abbreviations

- *A.johnsonii - Acinetobacter johnsonii*
- BAP – BAP SCAR CARE T dressing
- BHI – Brain Heart Infusion agar
- *C.albicans – Candida albicans*
- CBA – Columbia blood base agar
- CFU – Colony forming units
- CNS – Coagulase-negative *Staphylococci*
- *E.coli – Escherichia coli*
- ECM – Extracellular matrix
- EDTA – Ethylenediaminetetraacetic acid
- FITC – Fluorescein isothiocyanate
- HA – Hyaluronic acid
- HCl – Hydrochloric acid
- IGF – Insulin-like growth factor
- IL – Interleukin
- INF- γ – Interferon- γ
- MMP – Matrix metalloproteinase
- MRSA – Methicillin Resistant *Staphylococcus aureus*
- NA – Nutrient Agar
- NB – Nutrient broth
- ONBC – Overnight broth culture
- *P.aeruginosa – Pseudomonas aeruginosa*
- PCNA – Proliferating cell nuclear antigen
- PDGF – Platelet-derived growth factor
- PMSF – Phenyl methyl sulphonyl fluoride

- PTFE – Polytetrafluoroethylene
- *S.aureus – Staphylococcus aureus*
- *S.epidermidis – Staphylococcus epidermidis*
- *S.homini – Staphylococcus homini*
- *S.warneri – Staphylococcus warneri*
- *S.mitis – Streptococcus mitis*
- sp. – Species
- SE – Standard error
- SDA – Sabouraud dextrose agar
- SSA – Semi-solid agar
- T – Time
- TA – Technical agar
- TCA – Trichloroacetic acid
- TGF-ß – Transforming growth factor ß
- TIMP – Tissue inhibitor of metallproteinases
- TSA – Tryptone soy agar
- TSB – Tryptone soy broth
- UMHS – University of Michigan Health System

Chapter 1 – Introduction

Hypertrophic scars are formed by the excessive synthesis of collagen induced by a hyperbolic wound healing response. The aesthetics of these scars can leave patients physically impaired by hindering movements at joints and also present with hyperpigmentation and excessive growth. Patients suffer psychological distress and low self-esteem due to the appearance of the scars and they can also cause reduced independence (O'Brien and Pandit, 2008). In the UK, it is common for hypertrophic scars to be treated with silicone sheets in order to correct the aesthetic problems presented.

Management of hypertrophic scars has advanced over the years, but still remain difficult to prevent and treat (Bloemen *et al.,* 2009). It has been reported by many authors that both pressure and silicone in combination and separately are effective in treating hypertrophic scars. Within the current scientific literature, there are no definitive conclusions on the mechanisms of action on the effect of silicone sheeting on hypertrophic scarring. Some clinicians associated with the study have reported anecdotal findings that patients with poorer personal hygiene appeared to have improved scars compared to patients who had meticulous cleaning habits. It may be that increased microbial activity or numbers could be taking place under the dressings, which potentially could influence scar modulation.

1.1 Skin morphology

The skin is the largest, dynamic organ of the body and accounts for 16% of total body weight (Wickett and Visscher, 2006). The three main purposes of the skin are protection, regulation and sensation, and wounding of the skin can affect all these functions.

The primary and foremost function of the skin is its action as a physical barrier to the exterior environment, protecting against mechanical impacts, microorganisms, ultraviolet radiation and chemicals. A state of homeostasis is maintained by the skin through sweat and hair movement to regulate body temperature, peripheral circulation and fluid balance. The pH of skin is normally in the range of 4-6, creating an acid mantle, which influences barrier homeostasis and antimicrobial properties that repel pathogenic microorganisms (Ali and Yosipovitch, 2013). As an organ of sensation, the skin has an extensive network of autonomic and sympathetic nerve cells that are receptive to temperature, touch and pain. The skin is made up of multiple layers of cells, blood, lymphatic vessels and nerve endings that are linked to underlying structures by connective tissue. The three main layers of skin are: the epidermis, dermis and the subcutaneous layer (subcutis) (figure 1.).

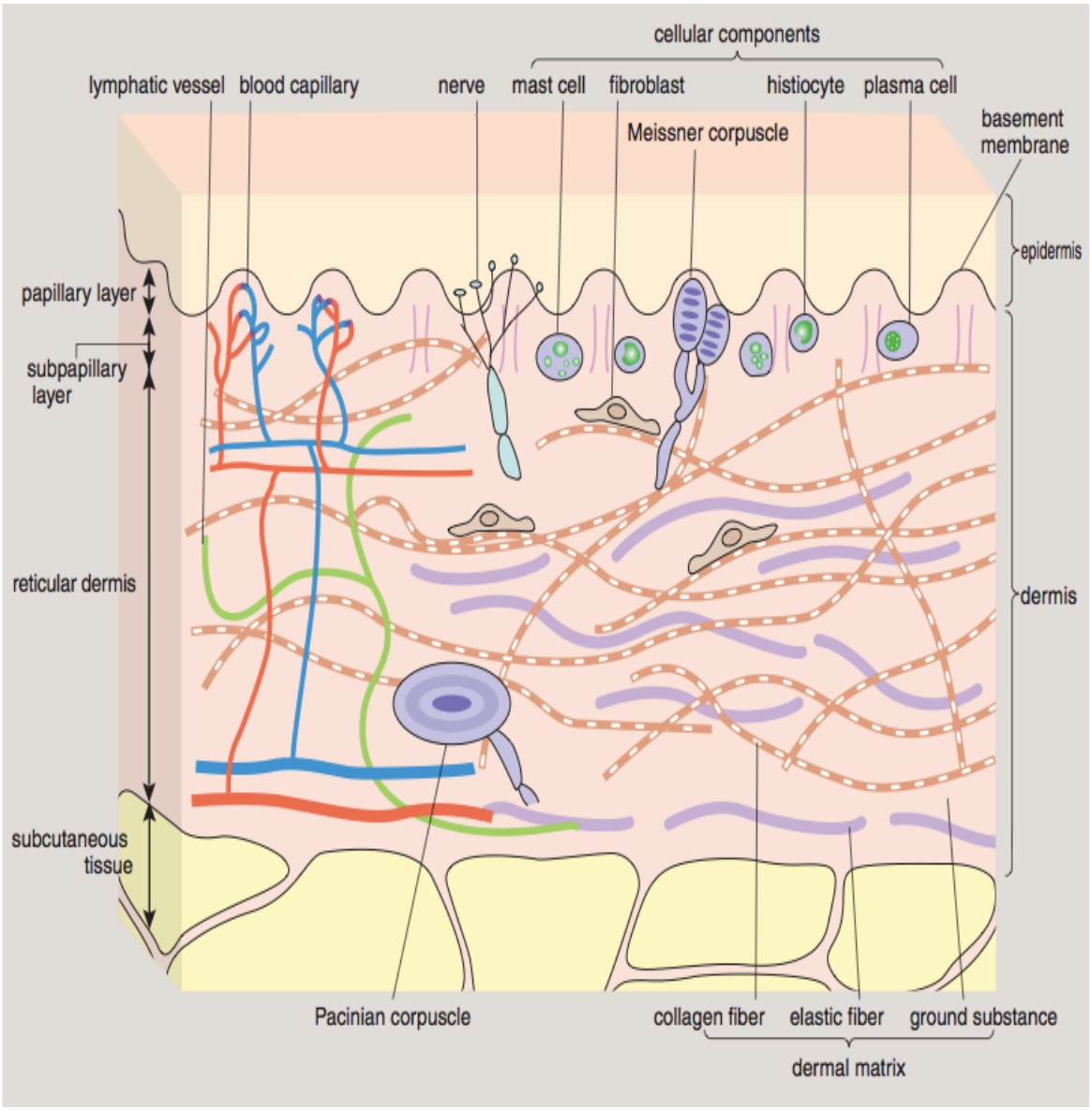

Figure 1. Cross-section of skin. Taken from Shimizu (2007)

The epidermis consists of stratified squamous epithelium and acts as semi-permeable barrier. The four layers of the epidermis are: the *stratum basale* (basal cell layer*),* *stratum spinosum* (prickle cell layer), *stratum granulosum* (granular cell layer), *stratum lucidum* and the *stratum corneum* (horny layer). The cells in all of the layers are made up of keratinocytes, except for the *stratum basale*, which consists of other cell types, such as melanocytes, Merkel cells and Langerhans cells (figure 2) (OpenStax College, 2013). Keratinocytes secrete keratin, which is a fibrous protein that provides skin its hardness and water resistance. The most superficial layer, the *stratum corneum,* is made up of a dead layer of keratinocytes that is regularly shed and replaced by rising cells from the *stratum granulosum*. This layer of dead cells protects against microorganisms entering the body and prevents dehydration of the underlying structures, as well as some mechanical protection.

The dermis is composed of supportive connective tissue and contains sweat glands, neurons, fibres and blood and lymph vessels. It is the deepest layer of the skin and provides structural support (Koziel and Potempa, 2012). A thin papillary layer and thicker reticula layer comprise the dermis. The papillary layer connects to the epidermis and is made up of thin, loosely arranged collagen fibres whilst the reticular layer which runs from the base of the papillary layer to the subcutis is made of thicker bundles of collagen. Fibroblasts also make up the dermis and produce collagen, elastin and structural proteoglycans (Gawkrodger, 2002).

The subcutis is made up of loose connective tissue and fat that serves to connect the skin to the underlying fibrous tissue of the bones and muscles. Its main function is for fat storage, insulation and cushioning (OpenStax College, 2013).

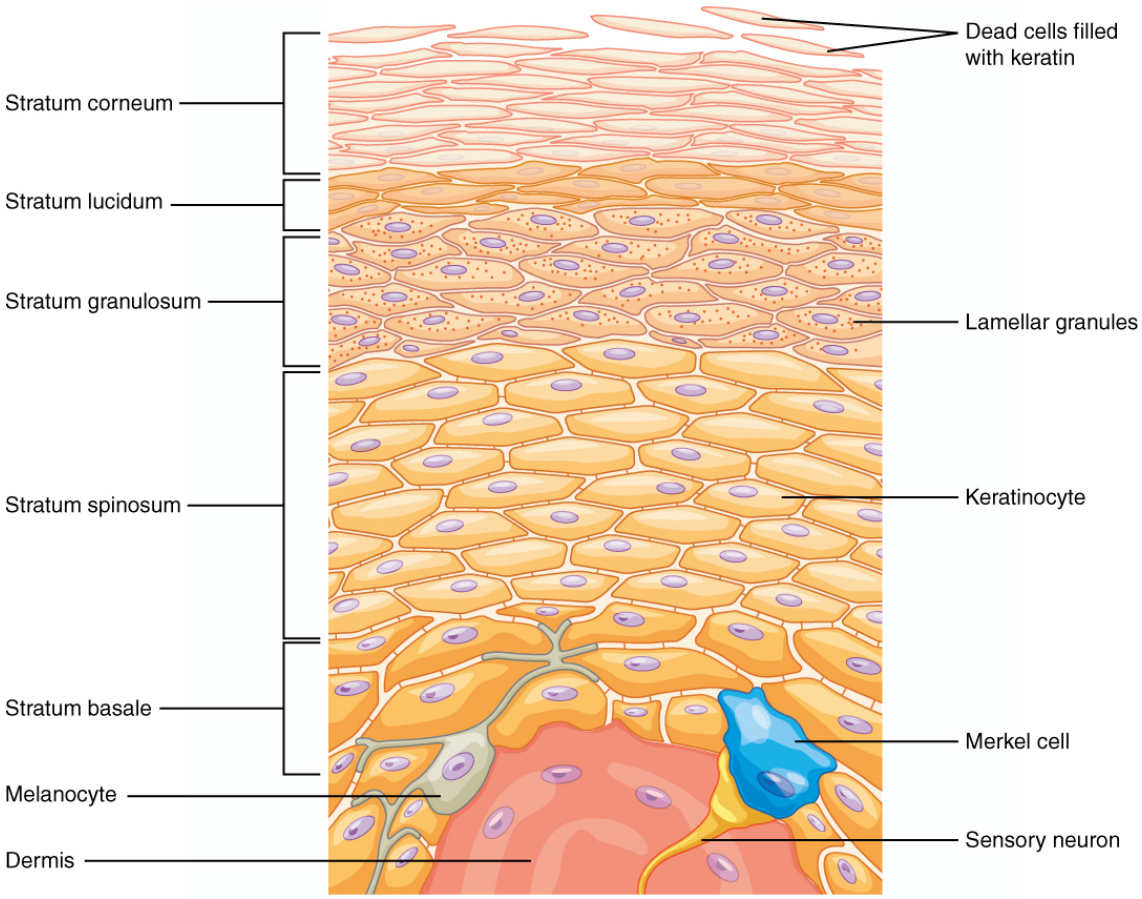

Figure 2. The layers of the epidermis. Taken from OpenStax College (2013)

Collagen is the main constituent of the extracellular matrix (ECM) that provides strength and elasticity in skin. It is a fibrous protein that consists of three polypeptide chains that are coiled in shape and linked by hydrogen bonds. With regards to wound healing, collagen is an important component that is formed during scar formation.

Verhaegen *et al.* (2009) evaluated the collagen structure in three different scar types (normotrophic, hypertrophic and keloid) and normal skin and established that clear differences in collagen morphology were demonstrated (figure 3). The collagen fibres were found to be organized in a more parallel manner in scars when compared to the fibres in normal skin, and in particular, the collagen bundles in hypertrophic scars were thinner than normal skin (Verhaegen *et al.,* 2009). These findings possibly show that the deviation from the normal phases of wound healing in skin that ultimately affects the appearance and formation of hypertrophic scars.

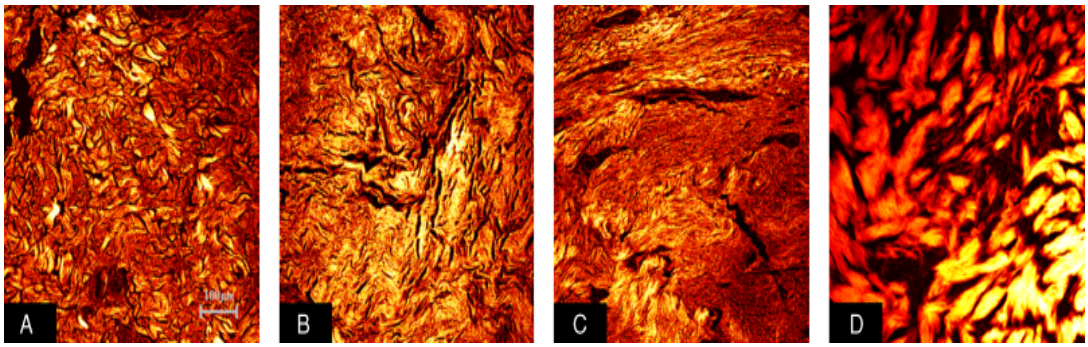

Figure 3. Collagen structure in normal skin and scars viewed by confocal microscopy. The scale bar in (A) represents 100 mm and also applies to B-D. Taken from Verhaegen *et al.* (2009)

Key:

(A) Normal skin

(B) Normotrophic scar

(C) hypertrophic scar

(D) keloidal scar.

1.2 Phases of normal wound healing

After skin trauma, there is a regular initiation of repair response, summarized as normal wound healing resulting in scar formation (Bran *et al.,* 2009). There are three phases of wound healing; the inflammation, proliferation and remodelling phases.

After initial wounding, the inflammation phase is the body's natural response to the injury. During this phase, vasoconstriction of the capillaries occurs until haemostasis has been achieved. Vasodilation and leakage of plasma from the capillaries then forms a fibrin clot. Tissue oedema, phagocytic activity and secretions of macrophages, T lymphocytes, lymphokines, collagenase and elastase also occurs (Guo and Dipietro, 2010). Figure 4 summarizes the cytokines and chemotactic agents present during the wound healing phases.

In the proliferation phase, fibroplasia and angiogenesis occurs for re-epithelialization. Fibroblasts encourage tissue growth through the production of collagen and capillary growth and there is an increase of keratinocytes, endothelial cells and growth factors (Schultz and Mast, 1999). Other matrix constituents include fibronectin and hyaluronic acid (HA) (Gauglitz *et al.,* 2011).

Remodelling phase, the initial collagen is weak and randomly distributed, but as maturation occurs the fibres cross-link and orientate to the direction of stress. Healed tissue strength never reaches the same as original tissue. An equilibrium is reached between synthesis of new components and degradation by matrix metalloproteinases (MMP) such as collagenase and gelatinase (Schultz and Mast, 1999). The healing process can take up to a year to complete.

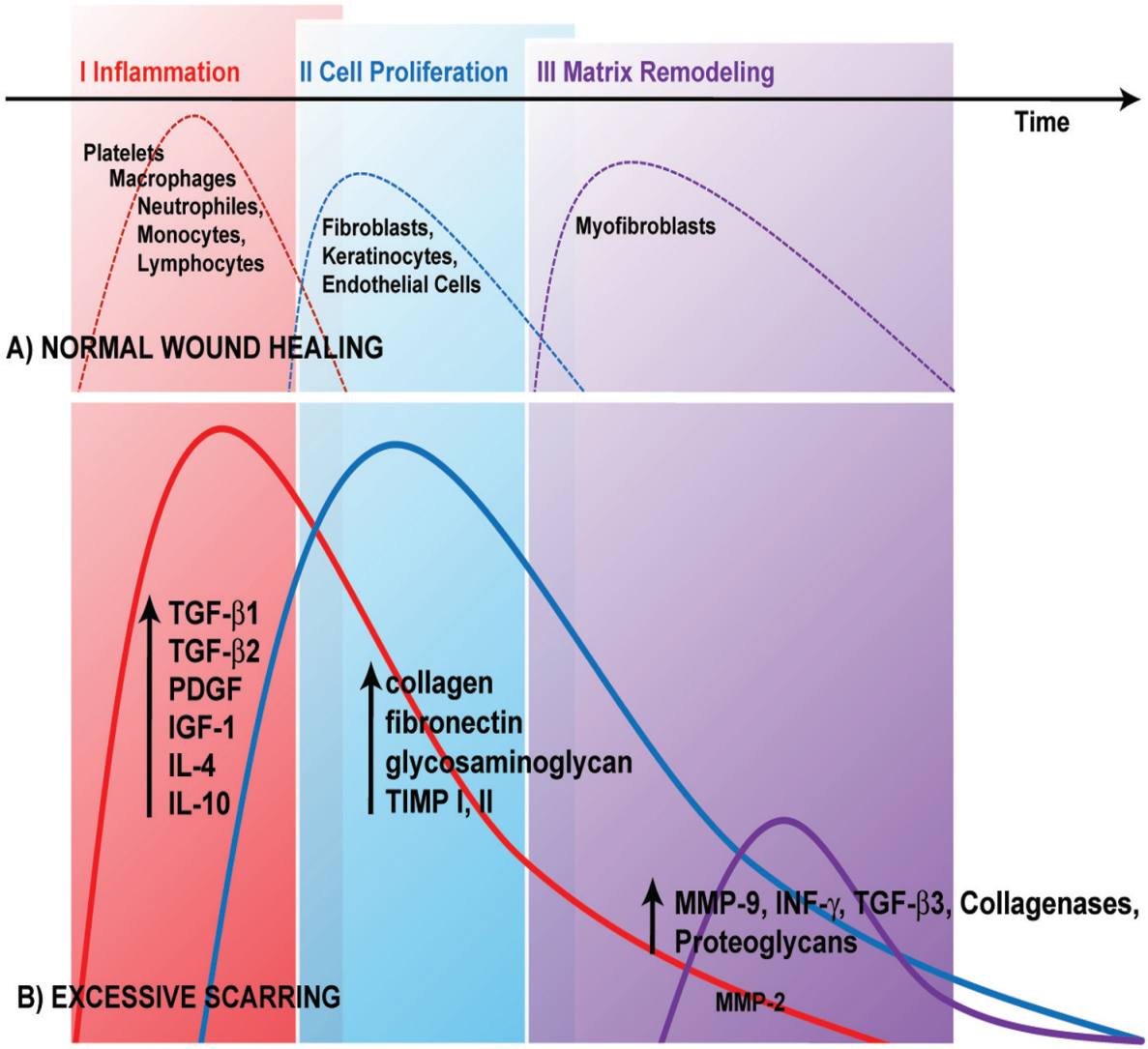

Figure 4. The difference between normal wound healing and excessive scarring. Taken from Gauglitz *et al*., (2011)

Key:
TGF-ß – Transforming growth factor ß
PDGF – Platelet-derived growth factor
IGF – Insulin-like growth factor
IL – Interleukin
TIMP – Tissue inhibitor of metallproteinases
MMP – Matrix metalloproteinases
INF- γ – Interferon- γ

1.3.1 Factors affecting wound healing

There are local and systemic factors that can influence wound healing. Local factors include oxygenation and infection. Systemic factors include age, hormones, underlying problems, medication and nutrition (Guo and Dipietro, 2010).

A disturbance in the phases of wound healing can impair the results of recovery. Chronic wounds or excess scar formation (hypertrophic scars and keloids) are usually presented where normal wound healing has been affected (Bran *et al.,* 2009). Excess scar formation can form as a result of burn injuries, lacerations, abrasions, surgery, piercings and vaccinations (Gauglitz *et al.,* 2011).

Collagenase breaks down collagen fibres and is one of three major MMPs in wound healing (Toy, 2005). It is thought that in the proliferation phase, growth factors regulate MMP activity, however excessive MMP expression is detrimental to normal wound healing if it is not controlled (Toy, 2005). According to Amălinei *et al.* (2007), MMP-1 (fibroblast collagenase) is the main collagenase that can break down collagen fibres. MMP-1 is produced by fibroblasts, keratinocytes, endothelial cells and macrophages. However, from figure 4, although there is an increase of collagenases in the remodelling phase of excessive scar formation, the graph shows a small optimum of activity compared to the excessive activity during the proliferation phase.

1.3 Hypertrophic scarring

A hypertrophic scar is a type of excessive scar formation. According to Juckett and Hartman-Adams (2009), hypertrophic scarring is defined as a scar that is raised above the skin that stays within the limits of the original wound. The scars are often unaesthetic, impair movement, itchy and painful (Bombaro *et al.,* 2003; Li-Tsang *et al.,* 2006). The redness is mainly due to increased blood flow and increased vascularity. A study by Fu *et al.* (2005) showed that in hypertrophic scars, the epidermal layer was thickened, collagen fibres were arranged as swirls and few secretory glands remained or were irregular. Histologically, there is an increase in collagen in hypertrophic scars compared to normal skin (Urioste *et al.,* 1999).

1.3.1 Pathology of hypertrophic scars

Hypertrophic scarring usually manifests within 4-8 weeks after the initial skin injury (Gauglitz *et al.,* 2011). Hypertrophic scars are caused by prolonged wound healing which leads to the excessive growth of blood vessels and collagen. Excessive scarring arises due to a deviation to the process of normal wound healing as shown in figure 4. Gauglitz *et al.* (2011) suggests that the prolonged inflammatory phase initiates an increase of fibroblast activity, and therefore forming a hypertrophic scar. In figure 4, the ratio of proliferation and inflammation is grossly distorted to the size of the remodeling phase, which would suggest that the persistence of the former two phases initiate excessive collagen synthesis, and there may be a deficiency of MMPs for degradation. There is a rapid growth phase for up to six months, and then a regression over a few years (Alster and West, 1997).

A study by Baker *et al.* (2007) reported in a retrospective study that hypertrophic scar formation might be influenced by bacterial colonization whilst the wound is healing due to differences in microorganisms found, in particular, *Staphylococcus aureus* (*S.aureus*) and *Escherichia coli* (*E.coli*). They also assert that previous studies had not been established, but the results indicated that bacterial colonization of wounds may be more important than previously believed and that aseptic burn wound environments could reduce the incidence of hypertrophic scars. The lack of association with bacterial influence in literature concerning hypertrophic scars demonstrates that some explanations to hypertrophic scar formation and healing are being overlooked.

1.3.2 Difference between hypertrophic scars and keloids

Keloids are more commonly seen in non-Caucasians and appear usually after three months (Juckett and Hartman-Adams, 2009). There may be a hereditary factor on the predisposition of keloids developing after skin injury, but it has also been linked with abnormal functioning of the hypothalamus and thyroid glands (Tsao *et al.,* 2002). Overtime, keloids do not improve like hypertrophic scars (Wolfram *et al.,* 2009).

Hypertrophic scars can often be misdiagnosed as keloids (Bloemen *et al.,* 2009) and incorrect diagnosis can result in inappropriate therapeutic treatment (Bran *et al.,* 2009). A keloid is described as an over-growth of scar issue beyond the limits of the original wound and rarely regresses over time (Berman *et al.,* 2007; Bran *et al.,* 2009). Characteristically, hypertrophic scars have bundles of collagen arranged parallel to the epidermis surface whilst in keloids the collagen bundles are large, thick and disorganized (Köse and Waseem, 2008; Gauglitz *et al.,* 2011). Hyaluronic acid (HA) is a constituent of the ECM and a major component in the early granulation tissue seen in hypertrophic scars whilst in keloids, it is only a minor component (Köse and Waseem, 2008). Keloid fibroblasts produce high levels of collagen and fibronectin and shows aberrant responses to metabolic modulators such as growth factors and cytokines. However, fibroblasts in hypertrophic scars display a moderate elevation in collagen production, but their responses to metabolic modulation are similar to those of normal fibroblasts (Erlich *et al.,* 1994). Table 1 summarizes the differences in hypertrophic scars and keloids.

	Hypertrophic scars	Keloids
Collagen bundles	Fine, well-organized, wavy parallel to epidermis	Large, thick, closely packed random to epidermis
Myofibroblast	Present	Absent
PCNA expressing	Low	High
ATP levels	Low	High
Hyaluronic acid localization	Major component papillary dermis	Thickened, granular/spinous layer Diffuse pattern
Apoptosis	Decreased	Increased/Decreased

Table 1. The biochemical and molecular differences between hypertrophic scars and keloids. Modified from Köse and Waseem (2008)

Proliferating cell nuclear antigen (PCNA) is a marker of cell proliferation.

1.4 Burn injuries

Around 250,000 people suffer burn injuries each year in the UK (Benson *et al.,* 2006) There are various ways that burns can be induced; explosion, steam, hot liquid, flames, electricity and radiation.

Burn injuries (figure 5.) are classified as: First-degree burns (superficial), no significant tissue loss, only the top layer of the epidermis is affected. An example of a superficial burn is mild sunburn. Second-degree burns (partial thickness) results in significant tissue loss, involving all of the epidermis and part of the dermis, or all. The burn site is red and blistered. Third-degree burns (full thickness), often require debridement and skin graft as there is loss that is extended further than the dermis. Full thickness burns includes damage of the epidermis, dermis and possible damage to subcutis, muscle and bone. There is no sensation in the area as nerve endings are damaged.

Scarring is a common problem post burn healing and hypertrophic scars are due to excessive growth of fibrous tissues and collagen (O'Brien and Pandit, 2008). Gauglitz *et al.* (2011) stated a 91% incidence rate of scarring following burn injury. Bloemen *et al.* (2009) indicates that there are many factors that could influence an individual's susceptibility to hypertrophic scarring, such as race, age, genetics and hormones.

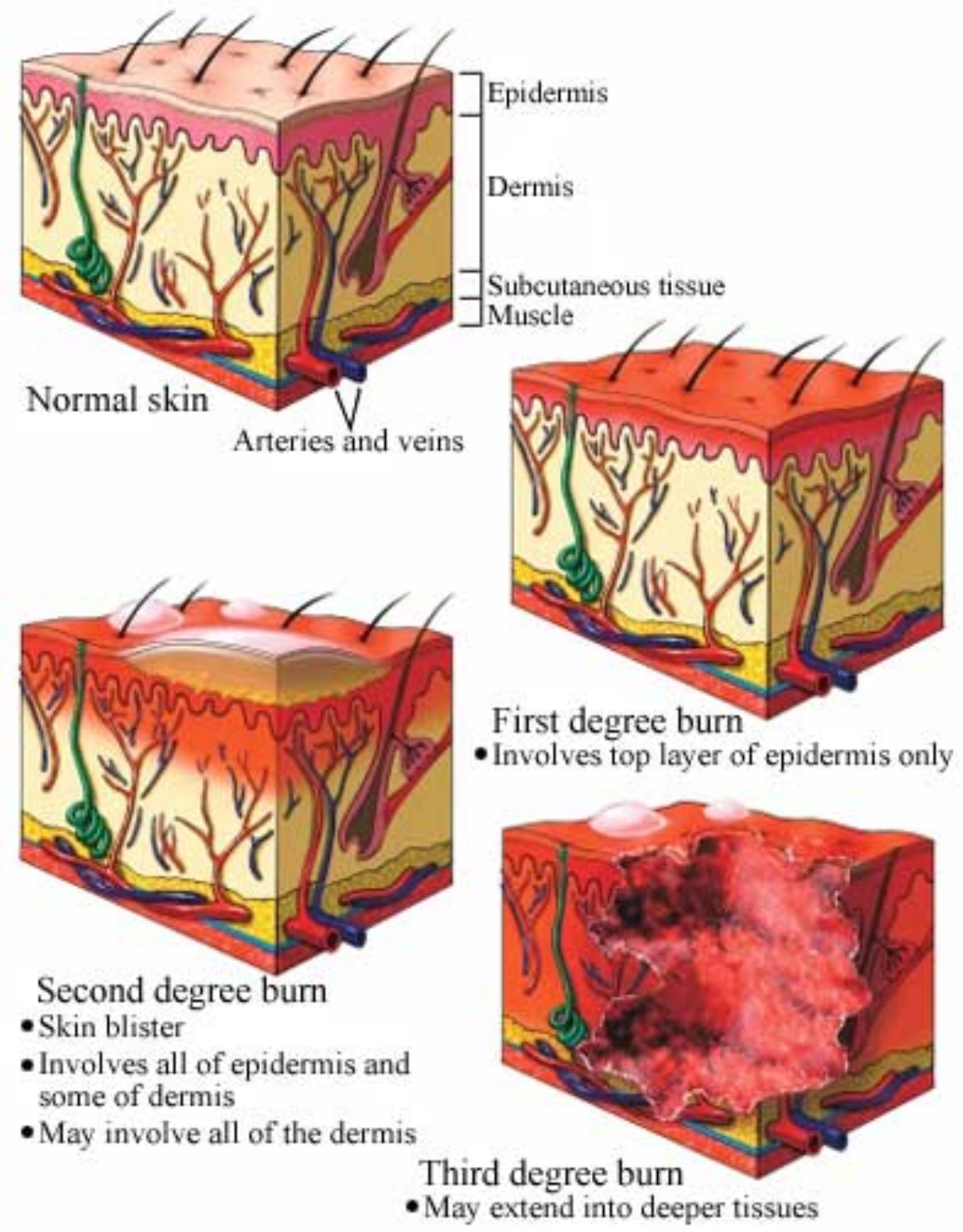

Figure 5. Classification of burn injuries cross-section. Taken from Nucleus Medical Art (2003)

1.5 Invasive treatment of burn scars

The aesthetic quality of healed tissue post burn is very important for the individual and there are a number of treatments currently used to improve the quality of scar tissue. They are varied but can be classified as invasive and non-invasive.

1.5.1 Skin grafts/surgery

Following full healing the scar can be removed and the resulting lesion covered with a skin graft (usually an allograft). The results are improved, although function is greatly restored (UMHS, 2013). There are other various surgical techniques undertaken, such as the Z-Plasty and tissue expansion, which seem to be effective as discovered by Bloemen *et al.* (2009). Surgical excision of keloids tend to worsen the scarring (Tsao *et al.,* 2002).

1.5.2 Injections

The use of intralesional corticosteroid injections is not used frequently (Bloemen *et al.,* 2009), but help to flatten and reduce the redness of hypertrophic scars by breaking down the skin's collagen (UMHS, 2013). A course of injections is usually completed over a few months. The disadvantages of this treatment are burning sensations, skin atrophy and is not suitable for extensive burn scars (Bloemen *et al.,* 2009). Collagen injections increase the volume of sunken scars, however is only a temporary solution (UMHS, 2013). A pilot study using collagenase injections by Kang *et al.* (2006) found that there was no effect on hypertrophic scar. The study used a small sample size of seven patients, however after 6 months of follow-up, there was no change in scar volume and numerous side affects were experienced.

1.5.3 Dermabrasion

The outer layers of the skin are abraded to smooth out surface irregularities. Normally, the procedure is performed with anesthetic and the skin is 'sanded' with rotary equipment (UMHS, 2013).

1.5.4 Radiotherapy

This method of treatment can be used alone, or in conjunction with excision, however it is quite a controversial topic. As highlighted by Branski *et al.* (2012), some healthcare practitioners consider radiotherapy as a last resort option, although it has been indicated that the risk of irradiation is quite low and the success rate of scar improvement is high. Wittgruber *et al.* (2012) claims that the risk of carcinogenesis is exaggerated.

1.5.5 Cryotherapy

In cryotherapy, liquid nitrogen is used to cause a defect within the cells of a scar at a cellular level by creating a freezing stimulation, initiating anoxia and tissue necrosis (Branski *et al.,* 2012). Recent keloids and hypertrophic scars show a particularly good response to this method according to Branski *et al.* (2012), and seemed to show better clinical results compared to intralesional corticosteroid injections. Cryotherapy may affect the collagen remodeling phase (Bloemen *et al.,* 2009).

1.5.6 Laser therapy

There are many types of laser systems used. Ablative lasers make scars softer and flatter, but can cause further complications in wound healing (Branski *et al.,* 2012). Treatment of scars with non-ablative lasers works well with other methods, such as the use of topical/intralesional corticosteroids. Both methods remove the outer layers of skin for smooth skin to form (UMHS, 2013). Bloemen *et al.* (2009) comments that the mechanism of laser therapy is unclear, but suggests it may influence the collagen remodeling phase and/ or angiogenesis.

1.6 Non-invasive treatment of burn scars

1.6.1 Silicone

Silicone is the main form of non-invasive treatment of hypertrophic scars (O'Brien and Pandit, 2008). Since the early 1980s, silicone has been used to treat hypertrophic scars. There are many different forms available, including silicone sheets, gel, sponges and sprays. Currently, there is no clear evidence that the silicone modifies scar tissue, but several theories for possible mechanisms include increased surface temperature, improved hydration or increased oxygen tension. A review by O'Brien and Pandit (2008) found that there was no significant difference in these stated mechanisms under silicone. However, Momeni *et al.* (2009) found that silicone sheeting was significantly more effective during treatment than a placebo from a study on 34 participants and scoring using a modified version of the Vancouver scale. A study by Li-Tsang *et al.* (2006) on Chinese patients concluded that pain, pliability, thickness and colouration were improved after using silicone sheets compared to a control group, although statistically significant differences were not demonstrated.

1.6.1.1 Silicone sheets

Prescribed silicone sheeting is often a self-adhesive and semi-occlusive sheet that is made from medical grade silicone, which is a cross-linked polydimethylsiloxane polymer (Berman *et al.,* 2007). It is suggested that the sheets should be worn for at least 12 hours a day continuously (Branski et al., 2012; Bloemen *et al.,* 2009). The postulated modes of action for silicone sheets are increased *stratum corneum* hydration, increased temperature and increased fibroblast apoptosis (Armour *et al.,* 2007).

1.6.2 Pressure therapy

Pressure therapy was established in the 70's, though the science behind the resulting reduced scar formation has still not been fully explored (Branski *et al.,* 2012). According to Bloemen *et al.* (2009), pressure therapy is believed to have an effect on the collagen-remodeling phase of wound healing. It is suggested that bespoke garments are worn for at least 12-18 hours a day for the first six months, however, the success rate is often impeded due to improper seating of pressure in the correct area or poor patient compliance (Branski *et al.,* 2012). An investigation into poor patient compliance was conducted by Ripper *et al.* (2009), which interviewed patients. The main problems stated were reduced movement/functionality, additional care effort and perceived deficiencies. Engrav *et al*. (2010) found that the flattening of scars was only statistically significant in scars that were quite thick. Van den Kerckhove *et al.* (2005) stated that a higher pressure is more effective than a lower with regards to flattening scars after using a mean of 15 mmHg pressure instead of the 10 mmHg used in other previous studies. This finding was based on a study of 60 patients with hypertrophic scars who were given pressure garments to wear during the period 1999 and 2002 and assigned a "normal" or "lower" compression class. The modes of action for pressure therapy are postulated to involve increased MMP activity and decreased collagen synthesis (Armour *et al.,* 2007).

1.6.3 Combination of silicone sheeting and pressure therapy

A study by Harte *et al.* (2009) found that pressure and silicone improved hypertrophic scars after 24 weeks, but could not determine which factor had the greater influence because of the small sample size used. Steinstraesser *et al.* (2011) conversely found that the use of multimodal treatment (pressure and silicone) yielded equivalent results to pressure therapy alone.

1.6.4 Topical application of natural ingredients

Singh (2013) claims that some studies found that numerous herbal extracts and essential oils can be effective in reducing the appearance of keloids and preventing their formation. The natural remedies include: Aloe vera gel, apple cider vinegar, calendula oil, coconut oil, jojoba oil, lavender oil and vitamin E oil. Mustoe *et al.* (2002) however states that the reports are anecdotal and there is not adequate published information to evaluate the efficacy of these natural extracts, but more recently, Muangman *et al.* (2011) and Hosnuter *et al.* (2007) found that treatment using dressings with a combination of silicone and onion extract yielded indicative results that dressings incorporated with natural remedies may be effective.

1.6.5 Massage

Massaging aims to reduce scar thickness by loosening the structure and is thought to be most ideal for smaller scars (Branski *et al.,* 2012). A case study by Chen *et al.* (2012) using an allograft acellular dermal matrix as treatment of a hypertrophic scar included exercise motions and massage as part of the therapy. Branski *et al.* (2012) state that studies on scar massages are rare, and are only regarded as supportive therapy, although some patients did experience some improvement of aesthetics, movement and a reduction of itching and overall pain.

1.7 Skin microbiology

Commensal bacteria and opportunistic pathogens inhabit the skin. Predominantly, Gram-positive organisms such as staphylococci, micrococci and diphtheroids are found and can be dense or sparse in population, depending on the area (Davis, 1996). The following genera, *Propionibacterium, Corynebacterium, Brevibacterium, Acinetobacter* and *Pityrosporum* are also considered part of the normal skin flora, as well as some transients and contaminants (Noble, 1984). According to Koziel and Potempa (2012), both commensals and pathogens express extracellular enzymes such as proteases, but proteases secreted by commensals contribute to bacterial coexistence whilst pathogens use proteases as virulence factors to colonize the skin and break the epithelial layer. Some common organisms such as *Staphylococcus aureus* (*S.aureus*) and *Pseudomonas aeruginosa (P.aeruginosa)* have been found on the skin and shown to express proteases that can cause connective tissue destruction by targeting elastin, fibrinogen and collagen (Koziel and Potempa, 2012).

1.7.1 Staphylococci

Staphylococci are Gram-positive cocci that form in clusters (Foster, 1996). They are traditionally separated into two groups; those that are coagulase-positive and those that are coagulase-negative (Foster, 1996). Coagulase-negative staphylococci (CNS) are common commensals of the skin, such as *Staphylococcus epidermidis (S.epidermidis), Staphylococcus warneri (S.warneri)* and *Staphylococcus hominis (S.hominis)* (Foster, 1996). Noble (1984) describes that some species have unexplained preferences for different habitats and further explains that *S.epidermidis* and *S.hominis* are mainly found on skin, with *S.epidermidis* more likely to be found on the upper body. According to Foster (1996) and Noble (1984), *S.epidermidis* is the most important of the species as it is the main cause of infection associated with medical devices and catheters (Otto, 2009).

1.7.2 Proteases

Proteases can be categorized as mainly cysteine, serine or metalloproteases. The proteases can then be further classified as endoproteases or exoproteases (Monod *et al.,* 2002). Only a limited number of proteases can decompose collagen. Collagenolytic proteases secreted by bacteria are mainly MMPs and have been effectively used in wound healing (Watanabe, 2004). *S.aureus* is the most studied Gram-positive organism. Cysteine proteases referred to as Staphopains are secreted by *S.aureus,* and have been found to degrade collagen and fibrinogen (Ohbayashi *et al.,* 2011). *S.epidermidis* largely secretes cysteine and serine proteases, which results in the degradation of fibrinogen and fibronectin (Dubin *et al.,* 2001, cited in Koziel and Potempa, 2012). It has been shown that *Candida albicans (C.albicans)* secretes proteases that can degrade the *stratum corneum* and fibronectin (Monod *et al.,* 2002). This implies that tissue modulation and or destruction may be taking place, although whether this has an effect under silicone dressings is unknown.

1.8 Mechanisms of action by silicone treatment

Many authors have cited different mechanisms of action from the use of silicone treatment for hypertrophic scars. The main mechanisms that are suggested the most are temperature, hydration and oxygen transmission.

1.8.1 Surface temperature

Musgrave *et al.* (2002) indicates that higher temperatures encourage collagenase activity, therefore suggesting that the silicone raises the surface skin temperature to aid the breakdown of collagen. Berman *et al.* (2007) stated that the surface temperature of 18 hypertrophic scars increased from 29°C to 30.7°C in 16 patients when treated with silicone sheeting. Enzymes in human cells normally have an optimal temperature for catalytic activity of 37.5°C (body temperature). A temperature below this means less kinetic energy, leading to slower functioning.

1.8.2 Skin hydration

Sawada and Sone (1992) cited in a previous study by Sawada and Sone (1990) that hydration and occlusion are the likely mechanisms of action when treating scars with silicone cream, however, in the 1992 study with a non-silicone cream, all scars seemed to have improved, raising the question as to whether silicone has an essential role. It is suspected that hydration of the skin may cause eventual maceration of the scar (Sawada and Sone, 1992). Collagen production is thought to decrease through the inhibition of the fibroblasts as the *stratum corneum* is hydrated, thus aiding in remodelling collagen fibres on the surface of the skin (Chan *et al.,* 2005; Li-Tsang *et al.,* 2006). Suetak *et al.* (2000) conversely found that the hydration of the *stratum corneum* over 7 days decreased considerably with the application of a silicone sheet, but Borgognoni (2002) concluded that hydration is an important factor for the treatment of hypertrophic scars with or without silicone use.

1.8.3 Oxygen tension

Bermen *et al.* (2007) found that after treatment with silicone, the *stratum corneum* was more permeable to oxygen, therefore causing an increase in oxygen tension in the epidermis. The increased tension inhibits a hypoxia signal, which results in angiogenesis being prevented and stopping new tissue growth (Bermen *et al.,* 2007).

Borgognoni (2002) states that although silicone has been reported to improve hypertrophic scars faster than pressure therapy, the results obtained from these studies are not due to pressure, temperature or oxygen tension. As there is still inconclusive evidence of the mechanisms of action when silicone treatment is used for hypertrophic scars, it may be viable that there could be a microbial influence. Greenwood *et al.* (2012) extrapolated findings from research to explore the possible mechanisms of silicone in burn scars and open wounds, but established that further investigation is required. Baker *et al.* (2007) concluded that bacteria may have an influence on the formation of hypertrophic scars during wound healing after stating that there was an absence of bacterial studies on the potential likeliness to form these scars. This could possibly be the missing piece of information that needs to be investigated to determine the efficacy of silicone sheeting.

1.9 Aim

The aim of this study was to investigate the microbiology of intact skin under silicone sheeting and to construct a model to study the *in-vitro* effects on extracellular protease production.

1.10 Objectives

The objectives were to develop an *in-vitro* model to estimate bacterial numbers and extracellular enzyme production, to evaluate the effect of silicone sheeting on the production of extracellular enzymes (protease) *in-vitro* and to isolate microorganisms found on intact skin during the application of silicone sheeting.

Chapter 2 – Materials and Methods

2.1 Bacterial strains used

- *P.aeruginosa* NCTC 6749
- Methicillin Resistant *Staphylococcus aureus* (MRSA) clinical isolate
- *E.coli* NCTC 9001
- *C.albicans* ATCC 10231
- CNS clinical isolate
- *S.epidermidis* NCTC 11047
- *S.aureus* NCTC 8532
- *S.warneri* clinical isolate
- *Streptococcus mitis* NCTC 12261
- *Acinetobacter johnsonii* NCTC 12154

2.2 Dressings examined

Mepiform®	(Mölnlycke Health Care, Gothenburg, Sweden) – Self-adherent scar dressing with Safetac technology.
KerraPro™	(Crawford Healthcare Ltd., UK) – Silicone pressure reducing pad
Oleeva® Fabric	(Bio Med Sciences, Inc., Allentown, USA) – Self-adhesive sheet with fabric backing.
Silon-SES®	(Bio Med Sciences, Inc.) – Silicone elastomer sheeting polytetrafluoroethylene (PTFE).
Dermatix®	(Valeant Pharmaceuticals Ltd., Basingstoke) – Transparent 0.1mm thin sheet that can be used for up to five weeks.
Oleeva® Clear	(Bio Med Sciences, Inc.) – Transparent self-adhesive sheet.
Silon-TEX®	(Bio Med Sciences, Inc.) – Silicone bonded textile fabric that is washable.
BAP SCAR CARE T	BAP Medical BV, the Netherlands - Self-adhesive, thin and transparent silicone patch.

Table 2. Scar dressings used in this study.

2.3 Preservation of bacterial cultures

The bacterial cultures were subcultured for purity and then inoculated onto nutrient agar (NA) (Oxoid, Basingstoke, UK) slopes, which were stored at room temperature. Fresh streak plates of each organism were subcultured every week on NA plates and maintained at 4°C.

2.4 Preparation of inoculum

10 ml of tryptone soy broth (TSB) (Oxoid, Basingstoke, UK) was inoculated with 1-2 colonies of bacteria from purity plates. This was then incubated at 37°C overnight to form an overnight broth culture (ONBC). The ONBC was diluted 1/100 from approximately 10^8-10^9 colony forming units per ml (CFU/ml) to 10^6-10^7 CFU/ml prior to testing.

2.5 *In-vitro* models

Different techniques were used to attempt to create a suitable *in-vitro* model to simulate the dressing being applied to a surface that bacteria can inhabit.

At first, different sponges were tested with varying amounts of TSB in petri dishes to simulate a dressing lying on top of a structure like skin. It was found that the sponges used did not leave enough residues to perform any tests.

Subsequently, a large 150 ml petri dish of tryptone soy agar (TSA) with small holes bored into the set agar was used to accommodate the TSB and allow sufficient residual fluid to be obtained to determine a viable count and protease activity (figure 6.). The TSA was used as a stability medium to balance the dressing above TSB to allow contact between the TSB and dressing without submersion. Unfortunately, after 48 hours of incubation at 37°C, no liquid remained.

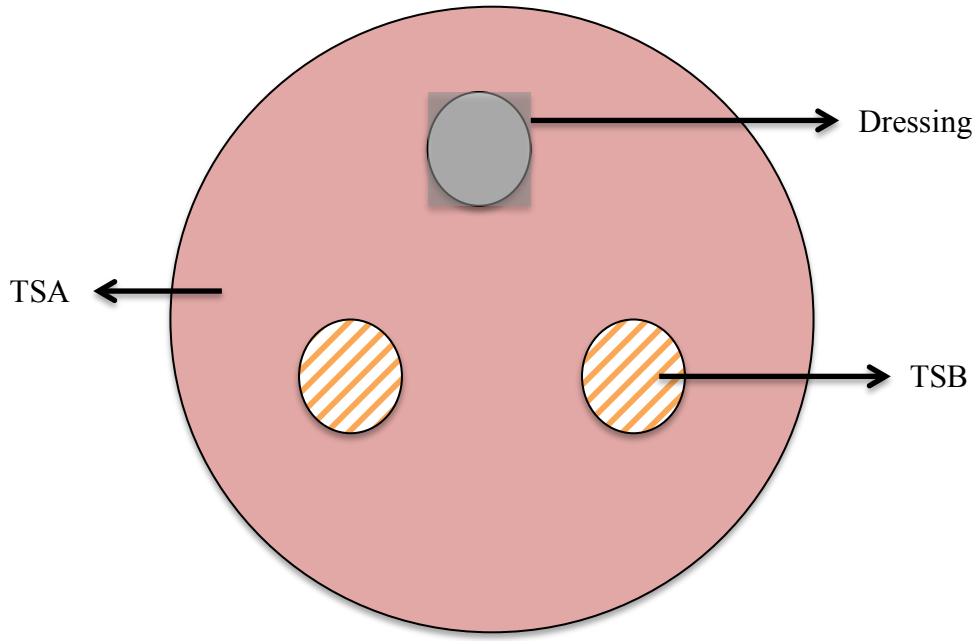

Figure 6. *In-vitro* model using TSA and TSB in a 150 ml petri dish.

Following a similar model to figure 6, semi-solid agar (SSA) (0.2% w/v TA in nutrient broth (NB)) was prepared and poured into wells made in 50 ml petri dishes of set TSA (figure 7.). The SSA was used in an attempt to allow organisms to move throughout the liquid whilst in contact with the dressing, and hopefully prevent too much residue from being lost. The TSA acted as the stability medium to hold the dressing on top of the SSA. The plates were then sealed with parafilm to prevent evaporation of any residue. After incubation at 37°C for seven days, the SSA was transferred to a sterile universal and centrifuged for 5 minutes to harvest the liquid residue from the agar. A sufficient amount of residue could not be obtained as the SSA still experienced some evaporation of liquid, despite using the parafilm.

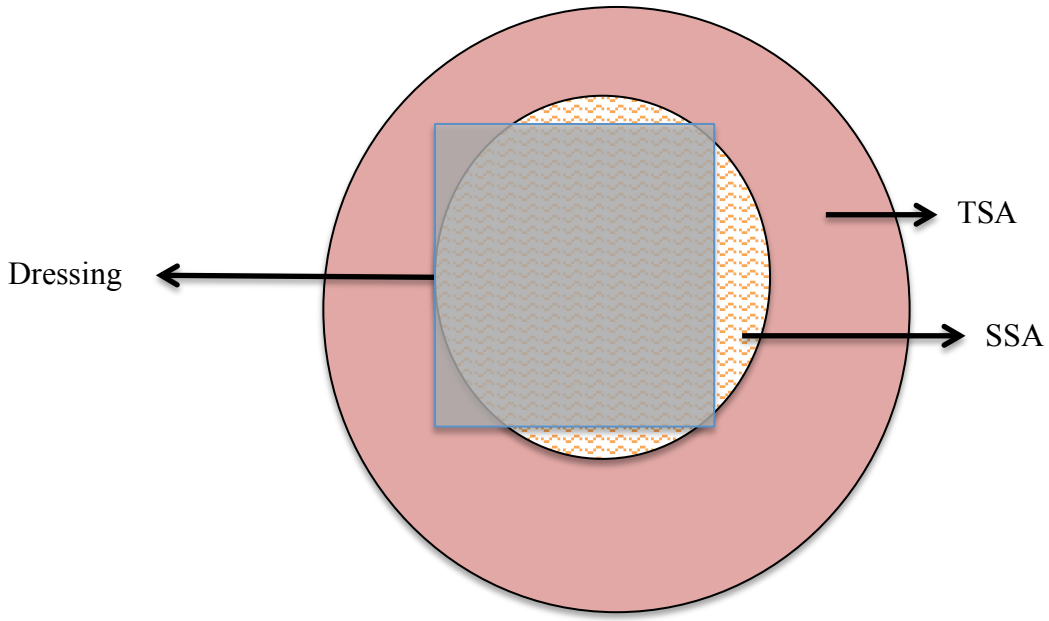

Figure 7. *In-vitro* model using SSA.

Due to the evaporation of residue, containers with screw top lids were tested filled with TSA and a central cavity left to hold TSB (figure 8.). The lids prevented evaporation of TSB and the TSA acted as the stability medium to hold the dressing above the TSB. This model seemed to hold the dressing in place and left sufficient residue for testing, so was used for preliminary testing of the different dressings in quadruplicate.

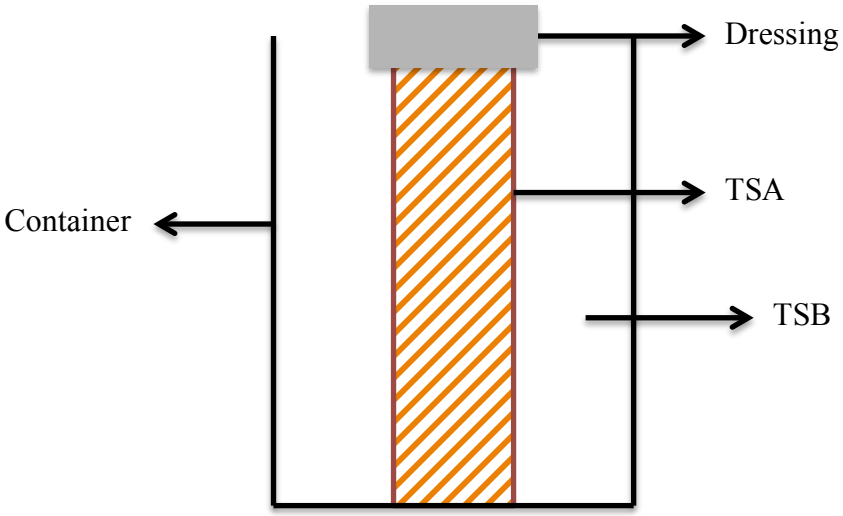

Figure 8. *In-vitro* model using a container with a central reservoir for TSB.

Although the model in figure 8 fulfilled some of the criteria to be successful, a lot of media and space was required. The dressings also had to be modified to 1 cm^2 sizes which was difficult to achieve aseptically. After the initial testing of the various dressings, 10 ml of TSB in universals containing 3 cm^2 dressings were used for subsequent tests (figure 9.).

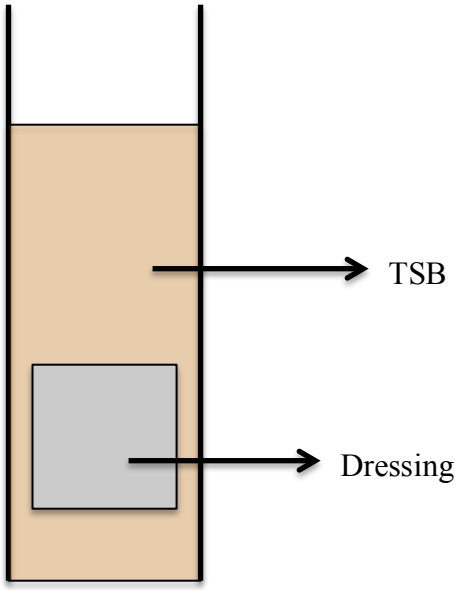

Figure 9. *In-vitro* model using a universal with TSB.

2.6 Determination of viable numbers of microorganisms in a broth

The viable count was determined using a method described by Miles and Misra (1938). The inoculum was diluted 10 fold until a dilution of 10^{-8} was reached. NA plates were divided into 8 segments and 50 µl drops of each dilution were dropped in duplicate and allowed to absorb into the medium in each section and then the plates were incubated at 37°C for 48 hours. The section where counts of approximately 20-60 colonies per drop were observed was used to calculate the amount of colony forming units (CFU) determined by:

CFU/ml=N/VD

Where:

N= mean number of colonies per drop

V= volume plated (0.05 ml)

D= dilution

2.7 Supernatants

Supernatant fluids were harvested by centrifugation of Eppendorfs containing the broth culture at 10,000 xg for 5 minutes and separated from the pellet. The supernatant fluids were stored at -20°C until used in further assays.

2.8 Protease determination

Initial screening methods were undertaken to confirm protease activity was exhibited and could be measured.

2.8.1 Milk agar preparation (method 1)

Milk agar plates were prepared following a modified method described by Sokol *et al.* (1979). Brain Heart Infusion agar (BHI) (Oxoid, Basingstoke, UK) and 3% (w/v) skimmed milk solution were prepared and autoclaved separately. Sterile solutions of each were mixed in equal volumes at 60°C. The milk agar was distributed into 15 ml volumes in Petri dishes and allowed to set. Wells of 1 cm diameter were bored into the plates and 100 µl of supernatant were pipetted into the wells. The supernatants were harvested after 3 days of incubation following the method of 10 ml TSB in universals with the dressings. The organisms tested were *P.aeruginosa,* MRSA, *E.coli* and *C.albicans* with Mepiform® and KerraPro™. The preliminary testing of the milk agar plates resulted in areas of hydrolysis of casein around the wells of 1 cm in diameter. The plates were incubated at 37°C for 48 hours. The extent of hydrolysis of casein was measured radially around the wells with a ruler. Experiments were carried out in quadruplicate.

2.8.2 Milk agar preparation (method 2)

Another method was followed as described by The Microbial Ecology Group (no date). Skimmed milk agar was prepared with 1 g technical agar (TA) (Oxoid, Basingstoke, UK) mixed with 5 g skimmed milk powder in 100 ml distilled water at pH 7.2. Plates were poured in volumes of 15 ml and inoculated with one streak of inoculum. Plates were incubated at 37°C for 48 hours in quadruplicates. The method from 2.4 was also followed for inoculation. These methods showed that the organisms tested produced enough protease to be detected, albeit not quantifiable.

2.9 Azocasein assay

Azocasein is a substrate consisting of casein conjugated with an azo dye. It is used to determine proteolytic enzyme activity as the release of colour is caused by the degradation of casein.

2.9.1 Azocasein method

A modification to the method described by Lincoln and Leigh (1994) was used. In Eppendorf tubes, 100 µl supernatants were mixed with freshly made 1 ml azocasein (Sigma, 5 mg/ml in 0.1 M Tris-HCl, pH 7.2) and incubated at 37°C in the dark for 90 minutes. Of the mixture, 250 µl was then mixed with 1 ml 5% (w/v) trichloroacetic acid (TCA) (Sigma) and allowed to precipitate for 30 minutes at 37°C in the dark. The samples were then centrifuged at 10,000 x g for 10 minutes to separate the precipitate from the sample. Absorbance was read using Model 6305 Spectrophotometer (Jenway, Essex, UK) at 328 nm. Samples with distilled water following the method outlined above were used to calibrate the spectrophotometer. *P.aeruginosa* was initially tested with the dressings to see if there was a change in protease production if different dressings were used that were also used for hypertrophic scar treatment. An incubation period of 7 days was used. The azocasein assay was repeated, incubating Mepiform® with MRSA, *E.coli, C.albicans* and a CNS isolate using the model with universals in section 2.5. This time, incubation periods of 1, 5 and 7 days were tested to observe if there was an optimum incubation length for total protease production. Common skin organisms *S.epidermidis, S.aureus, S.warneri, S.mitis, A.johnsonii* and *C.albicans* were tested with a 7 day incubation interval. An assay was set up to determine if the size of a dressing to the amount of broth affected the levels of total protease production. Mepiform® of 2 cm^2 and 4 cm^2 was incubated with an ONBC of an *S.epidermidis* clinical isolate in 10 ml TSB at 37°C for 7 days. A viable count was also undertaken.

2.9.2 Protease inhibitors

To inhibit metalloproteases, 0.025 mol l^{-1} ethylenediaminetetraacetic acid (EDTA, Sigma) in distilled water was mixed in equal amounts with the samples and incubated at 37°C for 10 minutes prior to the assay. Controls contained the solvent alone.

To inhibit serine proteases, 0.025 mol l^{-1} phenyl methyl sulphonyl fluoride (PMSF, Sigma) in ethanol was mixed in equal amounts with the samples and incubated at 37°C for 10 minutes prior to the assay (Gudmundsdottir, 1996). Controls contained the solvent alone.

To inhibit cysteine proteases, E-64 (Calbiochem, Germany) in distilled water was mixed in equal amounts with the samples and incubated at 37°C for 10 minutes prior to the assay. Controls contained the solvent alone.

The inhibited results were subtracted from a set of results with no inhibiters added to show the final amount for each protease.

2.10 Collagenase activity

The method of preparation for gelatin agar to aid the detection of collagenase was followed as described by Vermelho *et al.* (1996). BHI agar was supplemented with 1% w/v gelatin (powdered collagen).

2.11 Hyaluronidase activity

A turbidimetric assay of hyaluronidase as described by Sigma was followed where 750 µl of supernatant was mixed with 250 µl enzyme diluent (20 mM Sodium Phosphate and 77 mM Sodium Chloride with 0.01% (w/v) Albumin, Bovine, pH 7.0, 37 °C) and equilibrated to 37°C for 10 minutes. Next, 1 ml of 0.03% (w/v) hyaluronic acid solution (0.3 mg/ml in phosphate buffer pH 5.35, 37°C) was added, mixed and incubated at 37°C for 45 minutes. After incubation, 500 µl of each test was mixed with 2.5 ml acidic albumin solution (24 mM Sodium Acetate, 79 mM Acetic Acid, 0.1% (w/v) Albumin, Bovine, pH 3.75, 25°C). The mixture was allowed to stand for 10 minutes before reading the % transmittance (%T) at 600 nm. The blank contained enzyme diluent alone. The uncorrected %T for each test needed to be between 130-170% to be valid.

2.11.1 Calculation for hyaluronidase activity

Units/mg enzyme = (%T test - %T blank)(df)/(14.84)(0.75)

Where: df = dilution factor of enzyme

14.84 = Sigma Determined Extinction Coefficient

0.75 = Volume (in ml) of enzyme used in reaction

2.12 Statistical analysis

The independent t-test and ANOVA was calculated using IBM SPSS Statistics software version 21. Differences where $P<0.05$ were considered statistically significant.

Chapter 3 – *In-vitro* Investigation of Protease Production in the Presence of Silicone

3.1 Introduction to *In-vitro* investigations

Hypertrophic scars form due to abnormal volumes of deposition of collagen and other ECM constituents after injuries such as a burn or surgical incisions. A common non-invasive treatment of these scars uses silicone sheets, which are worn for up to 24 hours to achieve aesthetic results. The reasons that this treatment proves successful are yet to be identified fully, however, in the current literature there are no publications on the influence of microbial flora and activity under the silicone dressings. Numerous methods are available in the scientific literature for protease determination and quantification.

A common substrate used to determine protease activity is casein, derived from milk and forms an opaque suspension that causes the white colour of milk. Proteolytic proteases hydrolyse proteins into smaller peptide fractions and amino acids by the addition of water between carboxyl and amino groups (Microbial Ecology Group, no date). Casein reveals the bacteria capable of hydrolysing the substrate by secretion of caseinase (Leboffe and Pierce, 2011). Therefore, bacteria that produce casease appear to have clear halos around the colonies on agar plates. Agar plates are an easy form of detecting activity. Sokol *et al.* (1979) clarifies that the proteases expressed by *P.aeruginosa* are capable for hydrolysing casein, therefore skimmed milk agar was used to detect protease production. They further explained that currently established assays required a 48-hour incubation period and failed to identify weak protease-producing strains of *P.aeruginosa,* leading to the development of another milk medium that was more sensitive. However, for metagenomic proteases, Morris *et al.* (2012) claimed that

there is an issue with false positives whilst using skimmed milk for proteolytic activity. Azocasein is a substrate variation of casein that is a conjugate of casein and an azo dye that releases colour upon reaction with casein. This substrate has been used by several authors for protease determination and results are normally read using a spectrophotometer or a micro plate reader. Azocasein is used where a more sensitive assay is required for detecting smaller amounts of protease. A variation to this type of substrate by Sigma is casein labeled with fluorescein isothiocyanate (FITC), following similar principles where upon reaction, fluorescence of the resulting solution is measured.

Other substrates have been determined to be able to detect protease activity. Vermelho *et al.* (1996) conducted a study that described a qualitative method for detection of extracellular proteases on agar plates using gelatin, bovine serum albumin and haemoglobin as substrates and yeast extract or BHI agar as the culture media. The study showed that the most preferential substrate to exhibit proteolytic hydrolysis was BHI supplemented with gelatin.

More specific assays are available to identify certain proteases, such as collagenase and hyaluronidase. Enzymes known as hyaluronidase degrade HA. HA is a polysaccharide that contributes to wound healing, particularly in the proliferation phase (Voigt and Driver, 2012). Since HA is hydrophilic, it can absorb up to 3000 times its own weight in water, making it an important component in hydrating agents for injections (Voigt and Driver, 2012). The aim of this investigation was to determine the protease activity of microorganisms when in contact with silicone sheeting.

3.2 *In-vitro* Results

3.2.1 Determination of protease activity using milk agar plates

In table 3, *P.aeruginosa* showed protease activity when grown with and without the silicone dressings Mepiform® and KerraPro™, but the area of hydrolysis could not be measured as the whole plate was cleared. Protease activity was detected in each condition for MRSA, but a slight increase in protease activity is shown with KerraPro™ present. No protease activity was detected for *E.coli* except when KerraPro™ was present. The control for *C.albicans* showed no activity, however, the presence of both silicones presented a larger amount of activity than any of the other organisms tested.

Some results were found to be insufficiently discriminatory and were omitted from the values in table 3. Overall, four replicates for each organism were read.

	Average diameter of hydrolysis (mm)
***P.aeruginosa* Control**	Unmeasurable
***P.aeruginosa* Mepiform®**	Unmeasurable
***P.aeruginosa* KerraPro™**	Unmeasurable
MRSA Control	11 (0.75)
MRSA Mepiform®	12 (1.03)
MRSA KerraPro™	16 (2.78)
***E.coli* Control**	0
***E.coli* Mepiform®**	0
***E.coli* KerraPro™**	9 (6.75)
***C.albicans* Control**	0
***C.albicans* Mepiform®**	20 (1.85)
***C.albicans* KerraPro™**	21 (0.29)

Table 3. Shows the average diameter of hydrolysis of casein. Results are the mean of four replicates. The *P.aeruginosa* plates were unmeasurable as the clearing around the wells was greater than the diameter of the petri dish. Values in ( ) represent SE.

3.2.2 Effect of various dressings on protease activity using the azocasein assay

The *in-vitro* model as shown in figure 8 was used and tested as the most successful to obtain a residue without submerging the dressings to follow for the azocasein assay.

Some results were omitted where *P.aeruginosa* expressed a pigment that affected the absorbance readings. The control, Mepiform® and Dermatix® results showed very similar values in protease expression of 65, 64 and 64 arbitrary units respectively (figure 10.), whilst with the other dressings it would appear there was a reduction of protease activity. The dressings Oleeva® fabric and Silon-TEX® showed statistically significant reductions in protease activity compared to the control sample, P=0.001 and P=0.004 respectively.

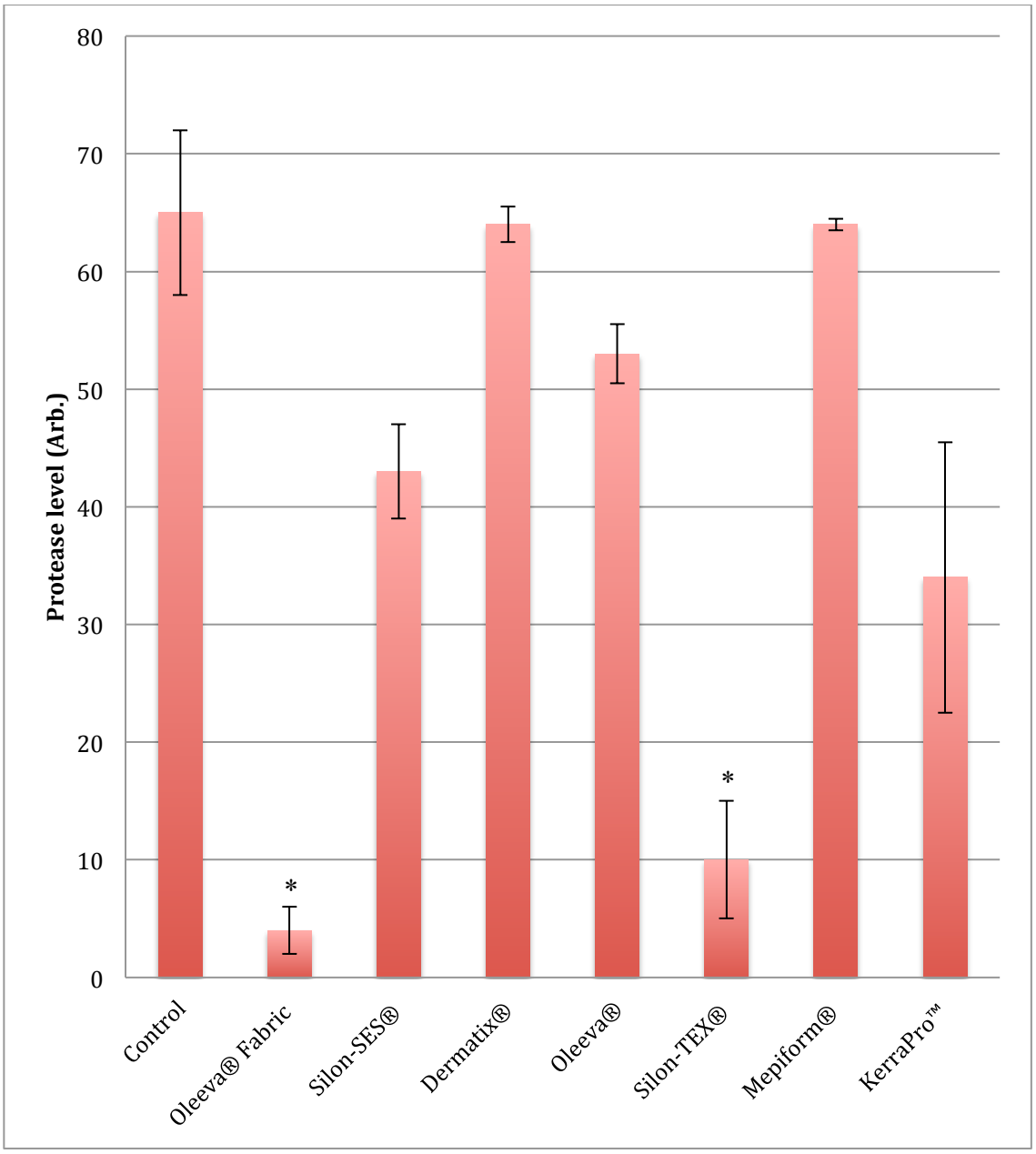

Figure 10. Shows the effect of various scar dressings on the level of total protease activity of *P.aeruginosa* after a 7 day incubation period. Levels are expressed in arbitrary units where 1 unit of activity refers to an absorbance of 1000 at 328 nm. Results are the mean of four replicates. Error bars represent the standard error (SE). Results with * show statistical significance to control test ($P<0.05$).

3.2.3 Determination of optimal incubation period for protease activity

From incubation periods of 5 and 7 days, higher protease levels were seen (figure 11). The MRSA control and Mepiform® results showed an optimal protease expression at 5 days of incubation, but both also exhibited large amounts of variance for each incubation period. *E.coli* control exhibited optimal protease expression at 5 days of incubation. Interestingly, when *E.coli* was incubated with Mepiform® it seems that the protease activity decreased as the length of incubation increased. After 1 day of incubation to 7 days, there was a five-fold reduction in protease activity. The optimal incubation period for *C.albicans* seemed to be after 1 day of incubation for both the control and when incubated with Mepiform®. Protease production peaked at day 5 of incubation for the CNS isolate.

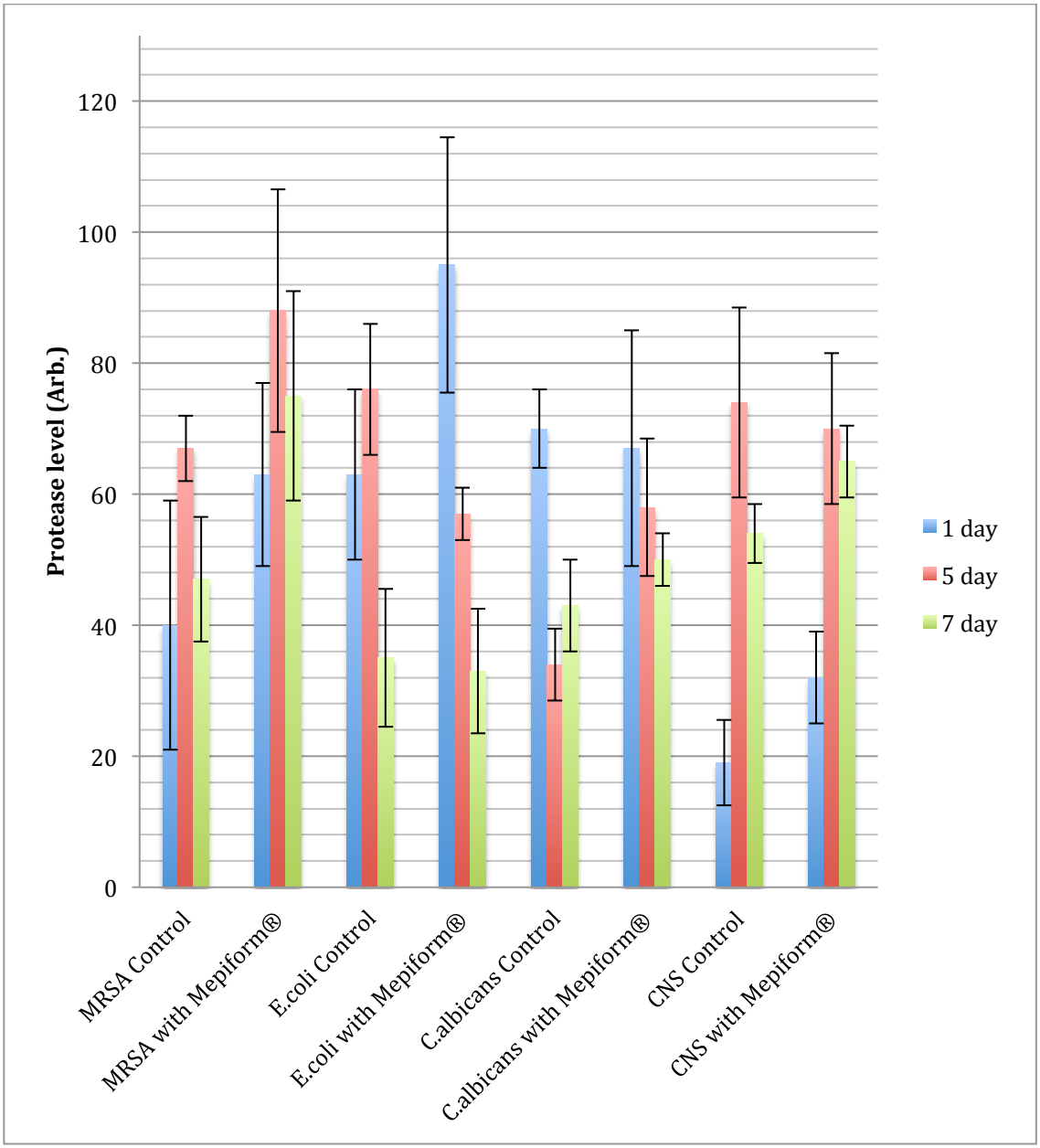

Figure 11. Shows the effect of Mepiform® on the level of total protease activity after 1, 5 and 7 day incubation periods. Levels are expressed in arbitrary units where 1 unit of activity refers to an absorbance of 1000 at 328 nm. Results are the mean of four replicates. Error bars represent the standard error (SE). Results with * show statistical significance to control test ($P<0.05$).

3.2.4 Azocasein assay of skin organisms with Mepiform®

Viable counts were obtained and observed to determine if the higher protease levels were due to increased bacterial numbers, rather than an effect on actual protease production. For all of the organisms, when incubated with the dressing there were more CFU/ml than without the dressing present (table 4). *S.epidermidis, A.johnsonii* and *C.albicans* expressed noticeable protease activity. Only *S.*aureus (P=0.001) and A.*johnsonii* with Mepiform® (P=0.020) showed a difference in protease level compared to it's control that was statistically significant. All organisms tested showed a significant difference in CFU/ml compared to the relative controls except for *C.albicans.*

	CFU/ml ($x10^6$)	% change between control and dressing	(P) values	Total protease production (Arb.)	% change between control and dressing	(P) values
***S.epidermidis* Control**	76(2.16)	12		6(6.70)	320	
***S.epidermidis* with Mepiform®**	86(0.85)		P=0.047	19(7.71)		P=0.303
***S.aureus* Control**	257(1.73)	97		24(3.04)	-100	
***S.aureus* with Mepiform®**	507(2.43)		P<0.001	0		P<0.001
***S.warneri* Control**	66(3.04)	700		0	0	
***S.warneri* with Mepiform®**	462(4.50)		P<0.001	0		-
***S.mitis* Control**	58(1.86)	48		0	0	
***S.mitis* with Mepiform®**	86(1.56)		P=0.001	0		-
***A.johnsonii* Control**	0.78(2.40)	9200		0	100	
***A.johnsonii* with Mepiform®**	72(0.75)		P<0.001	36(7.23)		P=0.020
***C.albicans* Control**	0.88(1.94)	13		22(2.25)	114	
***C.albicans* with Mepiform®**	1(1.34)		P=0.944	25(6.30)		P=0.416

Table 4. Shows the viable counts of skin organisms after a 7 day incubation period with and without (control) Mepiform® compared with total protease production. Mean CFU/ml was calculated from the mean of four replicates; two at 37°C and two at 25°C. Values in ( ) represent SE.

3.2.5 Protease inhibition of skin organisms with Mepiform®

Metalloproteases, serine proteases and cysteine proteases were inhibited and compared to the total protease produced using the same organisms in 3.2.6. Variable amounts of each protease were seen in all organisms, which did not total the amount produced without any inhibitors (figure 12.). *S.epidermidis* with serine inhibition (P=0.01), *S.aureus* without inhibition (P=0.01) and *A.johnsonii* with metalloprotease inhibition (P=0.046) showed statistically significant differences.

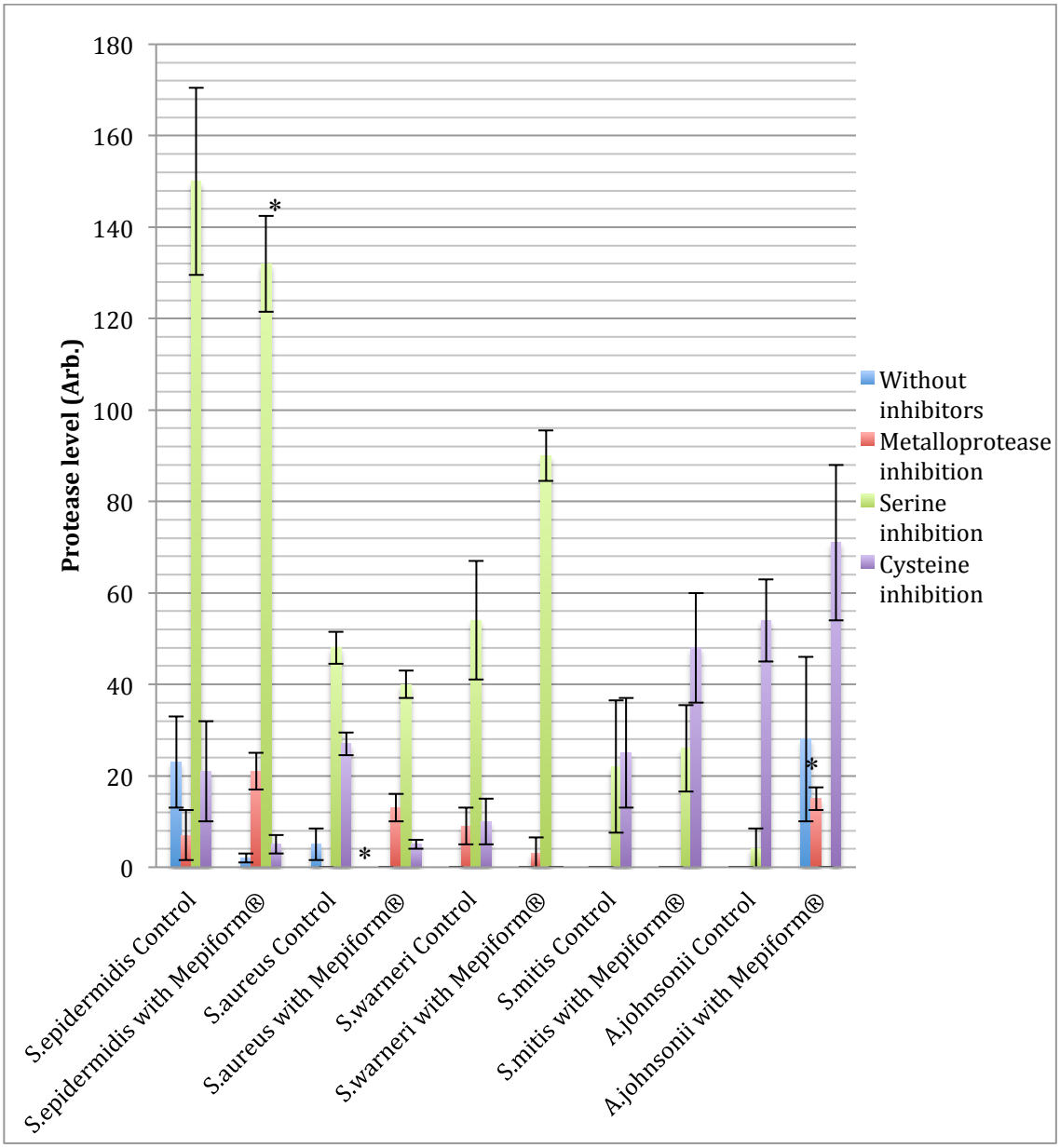

Figure 12. Shows the inhibition of metalloprotease, serine and cysteine in skin organisms with and without incubation with Mepiform®. Levels are expressed in arbitrary units where 1 unit of activity refers to an absorbance of 1000 at 328 nm. Results are the mean of four replicates. Error bars represent the standard error (SE). Results with * show statistical significance to control test ($P<0.05$).

3.2.6 Dressing to broth ratio

The size of the dressings used in the TSB altered the levels of total protease production slightly (figure 13), but the difference was not significant. There was no significant difference in bacterial numbers or protease production. The 2 cm^2 and 4 cm^2 dressings resulted with $3.2x10^7$ and $3.4x10^7$ CFU/ml respectively, where mean CFU/ml was calculated from the mean of two replicates. Therefore, it was concluded that the size of the dressing for these experiments does not affect protease activity or bacterial growth.

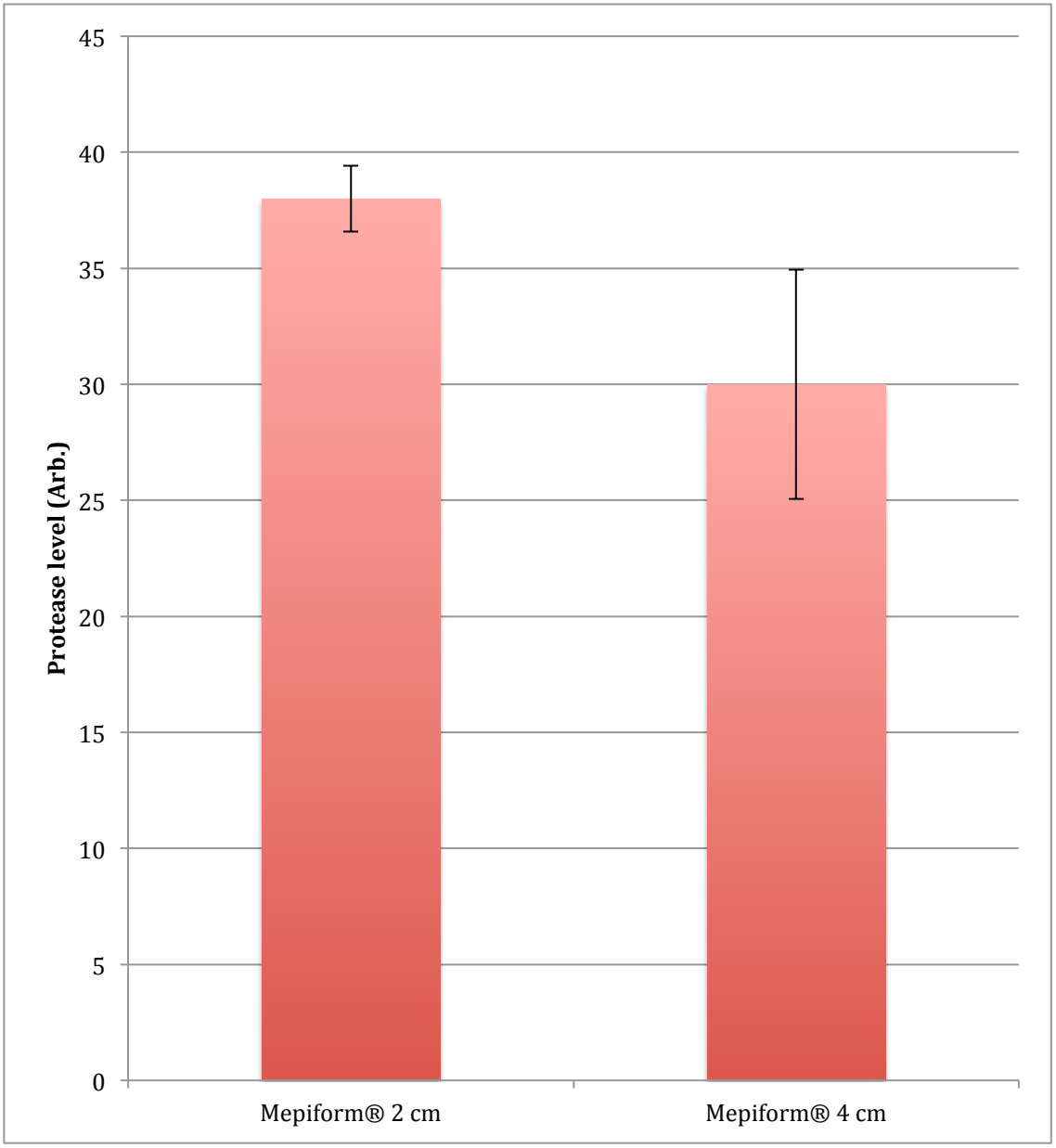

Figure 13. Shows the effect of Mepiform® on the level of total protease activity with different sized sheets after a 7 day incubation period. Levels are expressed in arbitrary units where 1 unit of activity refers to an absorbance of 1000 at 328 nm. Results are the mean of 4 replicates. Error bars represent the standard error (SE).

3.2.7 Collagenase activity

Unfortunately, no results were measurable using this method.

3.2.8 Hyaluronidase activity

The results were not valid as all were below 130%T.

3.2.9 Summary of findings

The model that used universals with 10 ml TSB produced results with the most protease production following the azocasein assay. The milk agar plates produced results, but levels of protease were not quantifiable. Different incubation periods were utilised, and it was determined that the most precise and optimal results were obtained after 7 days of incubation due to the smaller SE variation seen. The organisms that were tested were *P.aeruginosa,* MRSA, *E.coli, C.albicans,* CNS isolate, *S.epidermidis, S.aureus, S.warneri, S.mitis, A.johnsonii* and an *S.epidermidis* isolate. All organisms expressed protease detected by the azocasein assay apart from *S.warneri* and *S.mitis,* however, there were some mixed results with *P,aeruginosa* due to pigment release. A general trend seen was that incubation with Mepiform® present caused a higher level of protease production for all organisms that expressed protease.

3.3 *In-Vitro* Discussion

3.3.1 Preliminary detection of protease activity

Results for all the bacteria tested with Mepiform® and KerraPro™ were positive for protease activity when the two milk agar methods were conducted, but there was some difficulty measuring the levels of protease to a degree of accuracy. The inability to distinguish where the borders lay when measuring the rings of hydrolysis was the main drawback. This could be rectified by placing less testing zones on one plate, however, these methods using skimmed milk fulfilled the primary objective of detecting protease activity.

A preliminary fluorometric method using a protease fluorescent detection kit by Sigma was attempted to compare the results with the azocasein assay. The technique was very similar to the azocasein methods, whereby fluorescein isothiocyanate (FITC) was used as the substrate to detect protease activity, instead of azocasein. FITC is orange in colour with an absorption maximum at 495 nm. Upon excitation it emits a yellow-green colour with an emission maximum at 525 nm. The results were read using FLUOStar Omega (BMG LABTECH Ltd., Aylesbury Bucks, UK) and expressed in ng of trypsin. This was a relatively easy method to perform, as it was quite similar to the azocasein assay and designed under the same principle, but some readings could not be determined as the assay was too sensitive and could not be used. Some slight alterations to the method could have been made to obtain readings, but unfortunately due to time restraints this was not possible and the equipment was not available at all times.

3.3.2 Testing of various dressings

The addition of a variety of other dressings to test could have allowed some comparison between brands as all were promoted to be used for hypertrophic scar treatment. Unfortunately, the dressings received were not sterile, which potentially may have affected the accuracy of the results in figure 10. *P.aeruginosa* was used in this analysis, as it was well known to produce a significant amount of protease that can be detected using the azocasein assay described. However, two of the samples when grown in the presence of the organism caused release of a brown pigmentation, which affected the absorbance readings of the protease levels. The standard error (SE) for Silon-SES®, Dermatix®, Oleeva® and Mepiform® were small, but the other dressings showed a larger SE which may have been affected by a minimal amount of pigment expressed by *P.aeruginosa* that was not detected by eye. However, this variation could also be due to some interference from the assay or the way the model was constructed. Therefore, other organisms that did not produce interfering substances were further tested.

However, for the purposes of testing the use of the *In-vitro* model (figure 8.), the model seems to work well as an *in-vitro* method, but it required a lot of medium to be prepared and took a considerable amount of room for incubation as the model was much larger than petri dishes, which led to the development of using figure 9 as a more suitable model. A study by Ip *et al.* (2006) utilized a similar bacterial broth method using 1 cm^2 squares of silver dressings in 3 ml of TSB measuring the inhibition of bacterial growth and stated that the method was consistent and reproducible

3.3.3 The azocasein assay

During the initial assay optimisation, it was seen that the samples with silicone dressings present seemed to have an effect on total protease production. In order to optimize the assay, different incubation times were assessed for the co-incubation of supernatant and azocasein. The times tested were 1, 1.5 and 3 hours to observe any differences in protease production. After 1 hour there was some protease production, and at 3 hours there was a marked increase, but due to time limitations it was optimized at 1.5 hours.

A study was also conducted to determine whether the incubation period of the organisms affected the level of protease production. 1, 5 and 7 day incubations were tested in figure 11. It was noted that after 1 day of incubation, the protease production was low and increased as the incubation time increased to 5 and 7 days, except for *E.coli* with Mepiform® and *C.albicans* with and without Mepiform®. The results obtained for *E.coli* with Mepiform® seem to indicate that longer exposure to the dressing caused the protease levels to decrease. When comparing the 1 day samples between *E.coli* with and without the dressing, there was a spike in protease activity with the dressing present, which may have been caused by a response from the bacteria due to a change in environment, but this can only be seen as a minor speculation. *C.albicans* displayed in decrease in protease activity after 1 day, perhaps affected by the choice of broth used in this assay as C.albicans grow optimally in Sabouraud Dextrose media. The largest difference in protease activity was seen with CNS, from 1 day to 5 days of incubation by more than double the absorbance, but this was not statistically significant. Overall, figure 11 showed that 5 days of incubation produced the most protease activity to be detected, but further tests were assayed using 7 days because smaller SE values were achieved more with this incubation period than 5 days for precision.

Very little protease was produced by the selection of skin organisms tested, however, there was production observed by the commonest skin colonizer, S.*epidermidis.* The viable counts for this assay were also noteworthy. From table 4, the bacterial growth did not increase as the protease levels did, which suggests that the dressings are feasibly affecting total protease production. Adams *et al.* (2012) conducted a study to analyze if there were antimicrobial properties of silicon oil *in-vitro* and studied the effect on similar bacteria used in this project. It was reported that the silicon oil did not show inhibitive properties, but argued that *in-vivo* the silicon oil may cause depravation of nutrition for bacteria in eye treatments, as the *in-vitro* results did not confirm the substance as antimicrobial. The results from table 4 would suggest that the silicone dressing might encourage bacterial growth, which was seen with statistically significant increases from the control sample for all the skin organisms tested, apart from *C.albicans*. However, these studies may not correlate with each other, with one material being a viscous liquid and the other a dressing, but both are derivatives of siloxane.

A review by Willcox (2013) focused on the adhesion of bacteria to silicone hydrogel lenses, which reported that within the literature, the *in-vitro* studies generally showed that bacteria adhered to the silicone lens in higher numbers than a comparative soft lens as the silicone lens generally has a more hydrophobic surface. Biofilms were also covered in this review, but was not focused on the silicone lens alone. It is possible that a biofilm would have formed on the Mepiform® during incubation, and the viable counts may not reflect a true representation of the bacterial growth, even though the samples were agitated for at least 10 seconds. *S.epidermidis* is a notorious biofilm former and often problematic for medical devices (Mack *et al.,* 2004). The change of 12% in bacterial growth for *S.epidermidis* would suggest that there might not have been enough agitation to release the cells from the biofilm into the broth as a strong adhesion

was formed with the dressing. The other organisms tested also resulted with smaller percentage changes, but *S.aureus* showed a 527% increase in growth. Despite this growth, the protease levels decreased by 100% for *S.aureus,* but increased by 300% for *S.epidermidis*. The results do not fit any trends when comparing the organisms to each other, so it seems plausible that a biofilm formation could affect the absorbance readings as a biofilm would increase the turbidity of a sample. This should not be a possible problem, but it may be fortuitous that the centrifugation speed was insufficient and remnants of cell material were still present in some samples.

S.epidermidis is more commonly found on the skin than *S.*aureus, and it was interesting to find that *S.epidermidis* seemed to respond more to the presence of silicone .The increases in protease levels, especially from *S.epidermidis* could conceivably cause some skin modulation as the homeostasis of the surface of skin could be affected by this increase in protease. The increased levels of protease may have an affect on collagen modulation, which is an important component in scar formation, however no evidence can be substantiated from this assay.

Metalloproteases, serine proteases and cysteine proteases were inhibited and compared to the total proteases found. The results suggested that when serine or cysteine proteases are inhibited, the others flourish. In contrast, when metalloprotease was inhibited, there does not seem to be a difference or increase of the other two proteases. However, this may not be the case as the supernatants should not contain any cells, and therefore the levels of each protease should not show huge differences when individual proteases are inhibited. This could be due to interference between the inhibitor chemicals used and the azocasein, or as mentioned before that the speed of the centrifuge was lacking, but insufficient time meant that this could not be further investigated.

3.3.4 Testing the dressing size to broth ratio

A question raised when conducting these assays was whether the protease production was only seen to be higher due to an increase in bacteria, thus per CFU the amount of protease was not increased, or were the dressings causing an actual increase per CFU. As seen in figure 12, there did not seem to be a significant difference in protease activity or bacterial growth when the size of dressing was added to 10 ml of TSB. This assay was investigated because although the dressings were cut to size as similarly as possible, there was a possibility that there were slight differences in size due to human error. Also, when the initial testing was conducted with KerraPro, the dressing for this brand was considerably thicker than Mepiform®. Testing just Mepiform® to look at the size ratio, there did not appear to be noticeable differences, so the results were possibly due to the presence of the dressings.

3.3.5 Collagenase and hyaluronidase detection

It was very unfortunate that the hyaluronidase assay did not yield any usable results as all the absorbance readings were below 130%T. This was a disappointment as it has been specified that at least *S.aureus* is known to express hyaluronidase.

The collagen agar plate was only attempted once and did not yield any results., despite having been successfully reported by Vermelho *et al.* (1996), however, it may have been an anomaly as it was not repeated due to time restraints.

Chapter 4 – Volunteer Study

4.1 Introduction to the volunteer study

4.1.1 Mepiform®

A common brand of self-adhesive silicone sheeting commonly prescribed is Mepiform®. It is specifically designed for the prevention and treatment of scars (Mölnlycke Health Care, no date). The benefits of using Mepiform®, as claimed by Mölnlycke Health Care (no date) are that it flattens, softens and fades red and raised hypertrophic scars, and is easy to use. The sheet should be worn 24 hours a day, but removed once a day for inspection and hygiene reasons and then reapplied for up to 7 days as long as the sheet still adheres (Mölnlycke Health Care, no date). A series of case studies were performed by Saulsbery *et al.* (2007) to evaluate the efficacy on scar treatment and characteristic performance of the dressing. The study consisted of ten patients that were followed on a regular basis for six months. The major point that was emphasized by Saulsbery *et al.* (2007) was that after wearing Mepiform®, all of the patients reported the scar as feeling moist and more pliable, and overall satisfaction. However, Mölnlycke Health Care endorsed this study, so there could be a degree of bias in the results. From this study it may be logical to imply that the clinical success from the patients could be due to the mechanism of skin hydration, but this cannot be concluded as the study only reported clinical aspects.

4.1.2 BAP SCAR CARE T

Another similar self-adhesive product that is intended for use on fresh and old scars is BAP SCAR CARE T (BAP) by BAP Medical. It is claimed that it is convenient and easy to use, invisible, has excellent skin adherence and moulds to the skin (BAP Medical BV, no date). Similarly to Mepiform®, BAP should be worn 24 hours a day and does not need to be cleaned between applications.

4.1.3 Case study

An Asian male volunteer presented with keloid scars that were over 5 years old. The same procedure for swabbing as healthy skin volunteers was followed and the microbiology of his skin was monitored over a one month period.

The aim of the sampling was to observe if there were any trends on bacterial numbers and protease levels when the dressings Mepiform® and BAP were applied and worn on healthy skin for a one month period.

4.2 Volunteer study: materials and methods

A random selection of 10 volunteers with healthy skin were recruited into the study. There were two males and eight females. Three were Asian and seven Caucasian. Participants were asked to read the participant information sheet and sign the consent form to proceed with the study. Ethical approval was sought and granted for this study (See appendix).

At the first visit, a swab was taken from the participant's upper arm in a 4 cm^2 area prior to the application of a 4 cm^2 piece of Mepiform®. The same procedure was followed for the application of the BAP dressing. Each week in a one-month period the skin under the dressings and the dressing itself were swabbed. Control swabs were also taken from an area adjacent to the dressings to correlate the normal flora with the skin under the dressings.

4.2.1 Preparation of media and culturing

The swabs taken were used to inoculate two Columbia blood agar base (CBA) (Oxoid, Basingstoke, UK) supplemented with 5% (v/v) horse blood plates. One was incubated at 37°C and the other at 25°C. Sabouraud Dextrose Agar (SDA) (Oxoid, Basingstoke, UK) plates were also inoculated to detect any potential yeasts. These were also incubated at 25°C. Universals containing 10 ml TSB were inoculated with the swab, and an immediate viable count carried out along with an immediate supernatant harvested (T=0 days).

4.2.2 Bacterial numbers and diversity logging

From the plates, bacterial counts were assessed manually where possible, after which the viable counting method described in section 2.5 was used. The viable counts were undertaken in duplicate on NA plates incubated at 37°C and 25°C and SDA plates at 25°C. The microbial flora on each participant was determined throughout the study for each dressing worn.

4.2.2.1 Identification of bacterial species

Colonies of each type were identified by Gram staining and testing for catalase. Bacteria identified as *Staphylococcus sp.* were then further tested for coagulase and an API Staph test (bioMérieux, France) following the manufacturer's instructions set up to confirm the species.

4.2.3 Total protease levels

The azocasein assay described in section 2.8 was followed in triplicate to determine the total protease levels produced under the dressings.

4.3 Volunteer study results

Volunteers 7 and 11 (V7 and V11) results were excluded from the figures due to poor adherence of both dressings. However, the results obtained can be found in the appendix.

4.3.1 Skin under Mepiform®

This dressing had poor adherence for four of the volunteers and three experienced a mild tingling sensation whilst wearing the dressing. For all volunteers, *S.epidermidis* and *Micrococcus sp.* were identified and seen as the most prevalent organisms for this study, therefore, table 5 only shows the changes for these organisms. Another notable organism found was *S.homini,* but was only seen present before application of the dressing in small numbers for some volunteers. Control swabs that were taken mainly showed negligible or no growth, with a maximum growth of $10x10^2$ CFUs for *S.epidermidis*, which were observed when the weather was hotter. Control swab results were not analyzed and can be found in the appendix.

Of the volunteers, 50% had *Micrococcus sp.* present during the one month period, but the change in numbers only changed 10-fold between weeks at most (table 5.). Conversely, for *S.epidermidis,* 40% of volunteers showed up to a 1000-fold increase in bacterial growth. In general for all the volunteers, *S.epidermidis* growth increased with wearing Mepiform®. After the first week of wearing Mepiform®, 80% of volunteers showed an increase in *S.epidermidis* growth, and 90% had a decrease in *Micrococcus sp.* or no growth at all. Of the 10 volunteers, 20% had some fungus or yeasts present throughout the study but were not included in the data as there were not significant numbers of growth.

	After 1 week		After 2 weeks		After 3 weeks		After 4 weeks	
	S.epi	*Micro*	*S.epi*	*Micro*	*S.epi*	*Micro*	*S.epi*	*Micro*
V1	^^	v	-	^	-	^	-	v
V2	^^^	0	-	0	-	0	-	0
V3	-	v	-	-	-	v	^	v
V4	^	0	^^	0	^	0	v	0
V5	-	-	v	^	^	v	^	0
V6	^	v	^^^	0	-	0	v	^
V8	^^	v	^^	0	v	^	-	^
V9	^^^	0	-	^	v	-	^	^
V10	^^^	0	-	0	-	0	-	^
V12	^^	0	v	0	^	0	^	0

Table 5. Shows the change in microbial flora of the skin under Mepiform® over a one month period. Results are the mean of two direct CBA plates, incubated at 37°C and 25°C.

S.epi = *S.epidermidis*

Micro = *Micrococcus sp.*

^ = 10-fold increase from the week before

^^ = 100-fold increase from the week before

^^^ = 1000-fold increase from the week before

- = No change from previous week

0 = None present

v = 10-fold decrease from the week before

= Changed/lost dressing

Prior to dressing application, all volunteers except for volunteer 3 did not show more than 0-9x10^2 CFUs, however after the first week of wearing Mepiform®, 60% of volunteers showed an increase in bacterial growth (table 6.). Protease levels were also increased in 60% of volunteers in the first week.

In the second week 50% of the volunteers had no significant changes in the viable counts, and 40% had a further increase. Contrariwise, for protease production, there was a reduction of activity in 70% of volunteers. 80% changed or lost the dressing during this week.

After 3 weeks of wearing the dressing, 60% did not have a change in bacterial counts, but 50% had an increase in protease production and 30% showed no activity.

In the final week, the bacterial counts did not vary, with 50% showing either an increase or decrease. 40% of the volunteers had an increase in protease production in this week and 30% showed no activity present.

The dressing was only changed once after two weeks for 60% of volunteers whilst volunteer 5 needed a new dressing applied every week.

An ANOVA was performed, showing no statistical significance in difference in protease activity (P=0.903) or viable counts (P=0.250) per week.

		Skin prior dressing	Skin under dressing after 1 week	Skin under dressing after 2 weeks	Skin under dressing after 3 weeks	Skin under dressing after 4 weeks
V1	CFUs	0	$10x10^2$	$10x10^2$	$10x10^2$	$10x10^2$
	Protease (Arb.)	3(2.89)	12(6.93)	2(3.46)	0	0
V2	CFUs	0	$100x10^2$	$100x10^2$	$100x10^2$	$100x10^2$
	Protease (Arb.)	0	0	0	0	0
V3	CFUs	$10x10^2$	$10x10^2$	$10x10^2$	$10x10^2$	$100x10^2$
	Protease (Arb.)	0	10(6.35)	0	0	0
V4	CFUs	0	0	$10x10^2$	$100x10^2$	$1x10^2$
	Protease (Arb.)	13(6.35)	0	1(1.15)	22(14.43)	42(16.17)
V5	CFUs	0	0	$1x10^2$	$1x10^2$	$10x10^2$
	Protease (Arb.)	19(3.46)	16(4.62)	1(1.73)	27(0.58)	10(4.62)
V6	CFUs	0	0	$100x10^2$	$100x10^2$	$10x10^2$
	Protease (Arb.)	31(5.77)	9(9.81)	73(27.14)	31(2.89)	29(7.51)
V8	CFUs	0	$10x10^2$	$1000x10^2$	$100x10^2$	$100x10^2$
	Protease (Arb.)	0	16(8.66)	0	7(5.77)	9(6.93)
V9	CFUs	0	$100x10^2$	$100x10^2$	$10x10^2$	$100x10^2$
	Protease (Arb.)	0	15(6.35)	7(3.46)	19(0.58)	11(7.51)
V10	CFUs	0	$100x10^2$	$100x10^2$	$100x10^2$	$100x10^2$
	Protease (Arb.)	0	8(1.73)	3(2.31)	12(1.73)	23(4.62)
V12	CFUs	0	$10x10^2$	$1x10^2$	$10x10^2$	$100x10^2$
	Protease (Arb.)	17(1.73)	18(1.73)	16(6.35)	3(0.58)	20(4.04)

Table 6. Shows the mean viable counts of bacteria and protease production from a 4 cm^2 area of skin swab where Mepiform® was applied over a one month period. Results for the viable counts are the approximate mean of two direct CBA plates, incubated at 37°C and 25°C. Protease results are the mean of triplicates and expressed in arbitrary units where 1 unit of activity refers to an absorbance of 1000 at 328 nm. Where 0 is quoted for CFUs, actual values are between 0-$9x10^2$ CFUs. Values in ( ) represent SE.

= Increase in protease/bacterial growth from previous week

= Decrease in protease/bacterial growth from previous week

Underline = Shows where dressings were changed/lost

4.3.2 Mepiform® dressing

Of the results that were available during the second week of wearing Mepiform® (table 7.), 86% of the dressings showed an increase in bacterial growth, but 57% had a reduction in protease activity. In the third week, bacterial growth did not alter for 50% of the volunteers and 50% also had no change in protease production, but 40% did have an increase in protease. In week four of the data available, 38% still presented an increase in bacterial growth, however 50% experienced a decrease in protease production.

An ANOVA was performed, showing no statistical significance in difference in protease activity (P=0.133) or viable counts (P=0.152) per week.

		Week 1	Week 2	Week 3	Week 4
V1	**CFUs**	$10x10^2$	-	$1x10^2$	$10x10^2$
	Protease (Arb.)	4(2.89)	-	0	0
V2	**CFUs**	$10x10^2$	$1000x10^2$	$10x10^2$	$100x10^2$
	Protease (Arb.)	0	0	0	0
V3	**CFUs**	0	0	$1x10^2$	$1x10^2$
	Protease (Arb.)	10(1.73)	0	0	6(1.73)
V4	**CFUs**	0	$10x10^2$	$10x10^2$	-
	Protease (Arb.)	3(9.24)	0	30(15.01)	-
V5	**CFUs**	$1x10^2$	-	$1x10^2$	$10x10^2$
	Protease (Arb.)	17(4.04)	-	21(4.04)	10(8.08)
V6	**CFUs**	$1x10^2$	$100x10^2$	$10x10^2$	-
	Protease (Arb.)	9(26.56)	9(6.93)	21(2.89)	-
V8	**CFUs**	$1x10^2$	$100x10^2$	$100x10^2$	$10x10^2$
	Protease (Arb.)	17(2.31)	0	28(7.51)	0
V9	**CFUs**	$1x10^2$	$10x10^2$	$10x10^2$	$10x10^2$
	Protease (Arb.)	11(4.62)	22(6.35)	21(2.31)	8(6.35)
V10	**CFUs**	$10x10^2$	$100x10^2$	$100x10^2$	$10x10^2$
	Protease (Arb.)	8(1.15)	4(3.46)	11(2.31)	18(4.62)
V12	**CFUs**	$1x10^2$	-	$10x10^2$	$10x10^2$
	Protease (Arb.)	12(2.31)	-	7(1.73)	5(3.46)

Table 7. Shows the level of total protease activity and mean viable counts of bacterial growth from the fitting side of the dressing Mepiform® over a one month period. Results for the viable counts are the approximate mean of two direct CBA plates, incubated at 37°C and 25°C. Protease results are the mean of triplicates and expressed in arbitrary units where 1 unit of activity refers to an absorbance of 1000 at 328 nm. Where 0 is quoted for CFUs, actual values are between 0-$9x10^2$ CFUs. Values in ( ) represent SE. Where – is shown, data was unavailable.

= Increase in protease/bacterial growth from previous week

= Decrease in protease/bacterial growth from previous week

Underline = Shows where dressings were changed/lost

4.3.3 Skin under BAP SCAR CARE T

This dressing had very poor adherence for two of the volunteers and three experienced a mild tingling sensation whilst wearing the dressing. Similar organisms were found as in 4.3.1. Control swabs that were taken mainly showed negligible or no growth, with a maximum growth of $10x10^2$ CFUs for *S.epidermidis*, which were observed when the weather was hotter. Control swab results were not analyzed and can be found in the appendix.

Of the volunteers, 100% had *Micrococcus sp.* present at some point during the one month period, but the change in numbers only changed 10-fold between weeks at most (table 8.). For *S.epidermidis,* 30% of volunteers showed up to a 1000-fold increase in bacterial growth after the first week of wearing BAP. In general for all the volunteers, *S.epidermidis* growth increased with wearing BAP After the first week of wearing BAP, 80% of volunteers showed an increase in *S.epidermidis* growth, and 90% had a decrease in *Micrococcus sp.* or no growth at all. Of the 10 volunteers, 20% had some fungus or yeasts present throughout the study but were not included in the data as there were not significant numbers of growth.

	After 1 week		After 2 weeks		After 3 weeks		After 4 weeks	
	S.epi	*Micro*	*S.epi*	*Micro*	*S.epi*	*Micro*	*S.epi*	*Micro*
V1	^^^	^	-	-	-	v	x	x
V2	^^	0	-	0	-	0	-	^
V3	-	v	^	v	v	^	^	v
V4	-	v	^	^	x	x	x	x
V5	^	v	^	v	^^	0	-	^
V6	^^	v	-	-	x	x	x	x
V8	^^	0	-	^	-	^	^	v
V9	^^^	v	v	^	^	0	v	^
V10	^^^	v	-	^	-	0	v	0
V12	^^	0	^^	0	vv	^	^	0

Table 8. Shows the change in microbial flora of the skin under BAP over a one month period. Results are the mean of two direct CBA plates, incubated at 37°C and 25°C.

S.epi = *S.epidermidis*

Micro = *Micrococcus sp.*

^ = 10-fold increase from the week before

^^ = 100-fold increase from the week before

^^^ = 1000-fold increase from the week before

- = No change from previous week

0 = None present

v = 10-fold decrease from the week before

vv = 100-fold decrease from the week before

x= No data available

= Changed/lost dressing

Prior to dressing application, all volunteers except for volunteers 3, 6 and 8 did not show more than 0-9x10^2 CFUs. After the first week of wearing BAP, 70% of volunteers showed an increase in bacterial growth (table 9.). Protease levels were also increased in 50% of volunteers in the first week.

In the second week 60% of the volunteers had no significant changes in the viable counts, and 30% had a further increase. For protease production, there was an increase of activity in 50% of volunteers. 80% changed or lost the dressing during this week.

After 3 weeks of wearing the dressing, two volunteers were unable to finish the study. 50% of the remaining volunteers did not have a change in bacterial counts, but 63% had an increase in protease production and 39% showed a decrease.

In the final week, 42% of the remaining volunteers had an increase in CFUs. 71% of the volunteers had decrease in protease production in this week

The dressing was only changed once for 60% of volunteers and 30% had a new dressing applied twice whilst volunteer 5 needed a new dressing applied every week.

An ANOVA was performed, showing no statistical significance in difference in protease activity (P=0.561) or viable counts (P=0.246) per week.

		Skin prior dressing	Skin under dressing after 1 week	Skin under dressing after 2 weeks	Skin under dressing after 3 weeks	Skin under dressing after 4 weeks
V1	CFUs	0	$100x10^2$	$100x10^2$	$100x10^2$	-
	Protease (Arb.)	0	0	1(5.20)	8(1.15)	-
V2	CFUs	0	$100x10^2$	$100x10^2$	$100x10^2$	$100x10^2$
	Protease (Arb.)	0	0	0	7(4.62)	0
V3	CFUs	$10x10^2$	$10x10^2$	$100x10^2$	$10x10^2$	$100x10^2$
	Protease (Arb.)	40(32.33)	56(27.14)	48(4.04)	39(3.46)	0
V4	CFUs	0	0	0	-	-
	Protease (Arb.)	2(3.46)	3(2.31)	19(4.62)	-	-
V5	CFUs	0	0	$10x10^2$	$100x10^2$	$100x10^2$
	Protease (Arb.)	3(4.04)	32(4.04)	25(6.35)	20(5.20)	18(2.31)
V6	CFUs	$10x10^2$	$100x10^2$	$100x10^2$	-	-
	Protease (Arb.)	27(2.31)	40(5.77)	13(2.31)	-	-
V8	CFUs	$10x10^2$	$100x10^2$	$100x10^2$	$100x10^2$	$1000x10^2$
	Protease (Arb.)	0	0	3(2.89)	16(4.62)	40(9.24)
V9	CFUs	0	$100x10^2$	$10x10^2$	$100x10^2$	$10x10^2$
	Protease (Arb.)	8(1.15)	35(4.62)	21(4.04)	35(6.35)	34(4.04)
V10	CFUs	0	$100x10^2$	$100x10^2$	$100x10^2$	$10x10^2$
	Protease (Arb.)	0	0	4(6.93)	23(4.62)	6(0.58)
V12	CFUs	0	$10x10^2$	$1000x10^2$	$10x10^2$	$100x10^2$
	Protease (Arb.)	0	0	18(5.77)	17(4.62)	17(5.77)

Table 9. Shows the mean viable counts of bacteria and protease production from a 4 cm^2 area of skin swab where BAP was applied over a one month period. Results for the viable counts are the approximate mean of two direct CBA plates, incubated at 37°C and 25°C. Protease results are the mean of triplicates and expressed in arbitrary units where 1 unit of activity refers to an absorbance of 1000 at 328 nm. Where 0 is quoted for CFUs, actual values are between 0-$9x10^2$ CFUs. Values in ( ) represent SE.

= Increase in protease/bacterial growth from previous week

= Decrease in protease/bacterial growth from previous week

Underline = Shows where dressings were changed/lost

4.3.4 BAP dressing results

Of the results that were available during the second week of wearing BAP (table 10.), 89% of the dressings showed an increase in bacterial growth and 56% had an increase in protease activity. In the third week, bacterial growth increased for 57% of the volunteers and protease production decreased. In week four of the data available, 43% presented no change in bacterial numbers and 43% had decrease, however 71% experienced a decrease in protease production.

An ANOVA was performed, showing no statistical significance in difference in protease activity (P=0.548) or viable counts (P=0.740) per week.

		Week 1	Week 2	Week 3	Week 4
V1	**CFUs**	**$10x10^2$**	**$10x10^2$**	**$10x10^2$**	-
	Protease (Arb.)	9(4.62)	13(4.62)	2(1.73)	-
V2	**CFUs**	**$10x10^2$**	**$1x10^2$**	**$10x10^2$**	**$10x10^2$**
	Protease (Arb.)	0	0	8(0.58)	0
V3	**CFUs**	**$10x10^2$**	**$10x10^2$**	**$10x10^2$**	**$10x10^2$**
	Protease (Arb.)	25(9.81)	54(3.46)	43(7.51)	0
V4	**CFUs**	-	-	-	-
	Protease (Arb.)	-	-	-	-
V5	**CFUs**	**0**	**0**	**$10x10^2$**	**$100x10^2$**
	Protease (Arb.)	42(6.35)	18(5.77)	7(4.04)	0
V6	**CFUs**	**$100x10^2$**	**$100x10^2$**	-	-
	Protease (Arb.)	29(1.15)	26(2.31)	-	-
V8	**CFUs**	**$10x10^2$**	**$10x10^2$**	**$100x10^2$**	**$10x10^2$**
	Protease (Arb.)	8(4.04)	19(16.74)	42(12.12)	28(6.93)
V9	**CFUs**	**$10x10^2$**	**$10x10^2$**	**$10x10^2$**	**$1x10^2$**
	Protease (Arb.)	23(2.31)	72(2.89)	35(5.20)	30(4.04)
V10	**CFUs**	**$10x10^2$**	**$10x10^2$**	**$100x10^2$**	**$10x10^2$**
	Protease (Arb.)	12(5.77)	0	3(1.15)	6(3.46)
V12	**CFUs**	**$10x10^2$**	**$10x10^2$**	-	**$10x10^2$**
	Protease (Arb.)	0	14(2.31)	-	12(2.89)

Table 10. Shows the level of total protease activity and mean viable counts of bacterial growth from the fitting side of the dressing BAP over a one month period. Results for the viable counts are the approximate mean of two direct CBA plates, incubated at 37°C and 25°C. Protease results are the mean of triplicates and expressed in arbitrary units where 1 unit of activity refers to an absorbance of 1000 at 328 nm. Where 0 is quoted for CFUs, actual values are between 0-$9x10^2$ CFUs. Values in ( ) represent SE. Where – is shown, data was unavailable.

= Increase in protease/bacterial growth from previous week

= Decrease in protease/bacterial growth from previous week

Underline = Shows where dressings were changed/lost

4.3.5 Case study results

The skin with BAP applied displayed higher protease production levels over month than Mepiform®, except after the dressings were worn for three weeks (figure 14.). The highest protease level produced was seen after BAP was worn for one week at 31 arbitrary units, but the highest level was measured at 21 arbitrary units for Mepiform®. The skin swabs prior to the application of both dressings did not show any activity for protease production. As with figure 14, figure 15 which shows the protease levels found on the fitting side of the dressings also showed that the higher levels of protease were seen in week one of wearing the dressing for BAP and week three for Mepiform®.

The bacterial counts for the area of skin under Mepiform® stayed at a constant level of around $10x10^2$ CFUs each week ,whereas the fitting side had a maximum of $1x10^2$ CFUs for weeks two and four. BAP also showed an average of around $10x10^2$ CFUs on the skin and $1x10^2$ CFUs on the fitting side only in the first week of wearing the dressing.

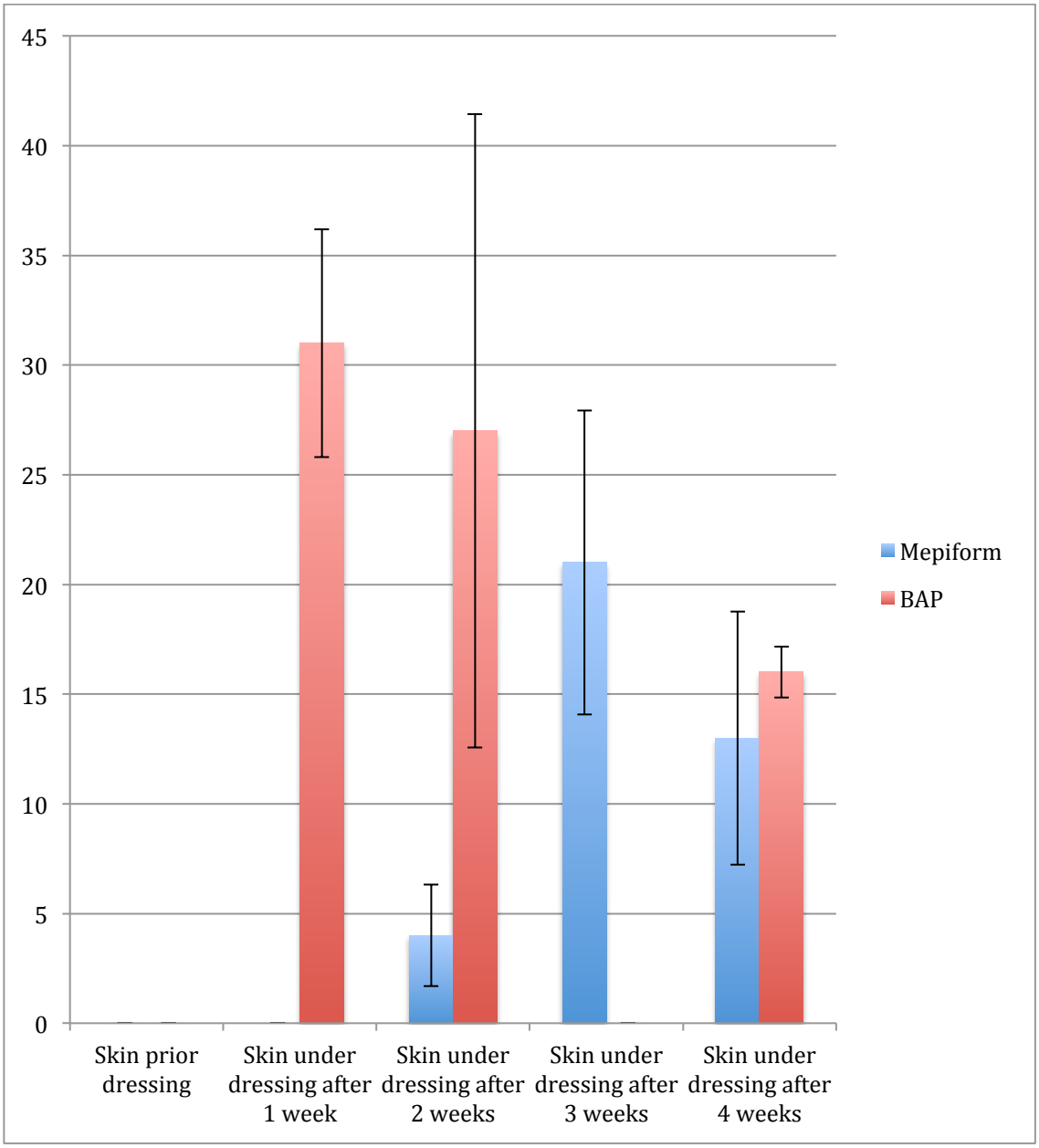

Figure 14. Case study: Shows the level of total protease activity of the skin swab where a 4 cm^2 dressing was applied. Levels are expressed in arbitrary units where 1 unit of activity refers to an absorbance of 1000 at 328 nm. Results were the mean of three replicates. Error bars denote SE.

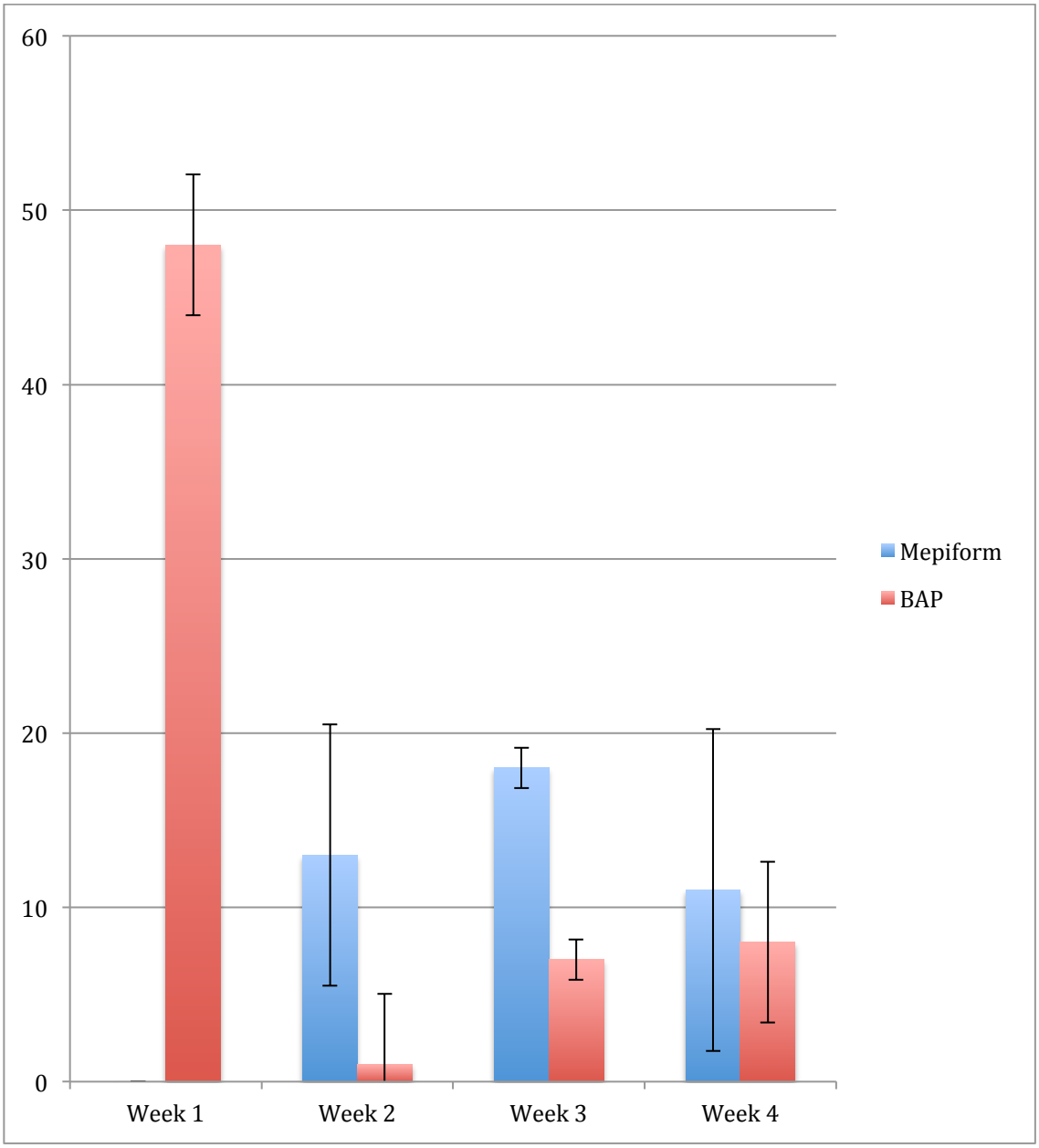

Figure 15. Case study: Shows the level of total protease activity of the fitting side of the 4 cm^2 dressings worn. Levels are expressed in arbitrary units where 1 unit of activity refers to an absorbance of 1000 at 328 nm. Results were the mean of three replicates. Error bars denote SE.

4.4 Volunteer study discussion

Overall, 12 volunteers were recruited in the study as some were poor candidates or the dressings did not adhere very well and were lost. The majority of volunteers, however, managed to wear a dressing continuously before a new dressing was required due to lack of adhesion or the dressing was no longer aesthetic. There was a lot of variability with the results, the most interesting of which was that one volunteer did not seem to produce any protease, whilst others produced huge amounts, with 73 arbitrary units as the highest in table 6. It was also interesting to see that the case study showed varying amounts of protease production, but the bacterial growth was quite low compared to the healthy volunteers. This could be due to the lack of sweat glands, which were inevitably damaged by the burn injury. Fu *et al.* (2005) found that there were less secretory glands on the skin where there was a burn injury, thus the skin is probably much drier than healthy skin, and is not an ideal area for bacteria to flourish. The lack of sebum secreted by sweat glands would also affect the pH balance of the skin (acid mantle), which acts as protection against fungal and bacterial infection. From these results, it is difficult to distinguish whether an increase in protease production would suggest better healing of hypertrophic scars, or the increase in bacterial numbers simply has an effect for other reasons.

Both tables 5 and 8 displayed growth increase in *S.epidermidis* in the first two weeks of the dressings being worn. Current usage procedures suggest wearing the dressings for 7 days, therefore from this study it could be suggested that if the increase in protease modulates the skin to improve scars, it would be beneficial to advise patients to keep the same dressing on longer.

Table 5 showed the increases and decreases in numbers of *S.epidermidis* and *Micrococcus sp.* over the one month of wearing Mepiform®. At most, *Micrococcus sp.* only showed variations in numbers by 10-fold increases or decreases, unlike *S.epidermidis, which* had up to 1000-fold increases in numbers. Both organisms are part of common skin flora, so it was not surprising to find these Gram-positive organisms in abundance compared to Gram-negative organisms like *P.aeruginosa, which* is more commonly found in wounds. The only other organism found in this study was *S.homini*, but in small numbers on the skin prior to application of a dressing or from the control swabs. The lack of diversity from the 4 cm^2 area on the arm could be due to the arm being a generally dry habitat for bacteria to colonize, and therefore is not most preferable for bacterial growth. However, Koziel and Potempa (2012) stated that *S.epidermidis* plays a probiotic role by preventing other pathogenic organisms from colonizing the skin, such as *S.aureus.* A study by Ariani *et al.* (2012) investigated microbial biofilm formation on facial prostheses that are made by a similar silicone elastomer. Healthy facial skin was swabbed and the diversity of bacteria found CNS, *P.aeruginosa* and *Bacillus spp.* as the most prominent organisms. The fitting side of the facial prosthesis revealed ten organisms, which does not seem similar to the results to this study apart from the CNS organisms, but the area swabbed is a different ecological habitat of the body to this study. Ariani *et al.* (2012) explain that the enrichment of some organisms found on the prosthesis could be related to the surface roughness and hydrophobicity of the material. Mepiform® and BAP are both self-adhesive dressings, and it could be possible that the technique swabbing of the fitting side could not pick up some organisms strongly adhered to the adhesive which may account for the lack of diversity of bacteria, but could also explain the lower CFUs numbers of the fitting side (table 7.) compared to the CFUs found on the skin under the dressings (table 6.).

The volunteers of particular peculiarity when wearing Mepiform® were volunteers 2 and 6. Volunteer 2 showed no protease activity at all (table 6.), but the viable count showed that the growth under the dressing increased 1000-fold. Volunteer 6 was found to have high protease levels after 2 weeks of wearing the dressing with 73 arbitrary units, however, the viable count was approximately the same as volunteer 2 for that week. The skin under BAP for volunteer 3 showed the highest level of activity at 56 arbitrary units (table 9.), but with only $10x10^2$ CFUs. It is difficult to conclude if the amount of bacterial growth affected the absorbance readings as heavier growths resulted in greater optical density. Yellow producing pigments which show on growth plates as yellow colonies characterize some strains of *S.aureus* and *Micrococcus* sp., which may have caused some interference with the azocasein assay. *S.aureus* produces carotenoid pigments (Liu and Nizet, 2009), which could affect the absorbance readings, as it would be read at the same wavelength as the azo pigment. It could also be concluded that different strains of *S.epidermidis* may have lead to variations in absorbance. A study by Brown (1966) investigated the absorbance and reflectance of different strains of *S.epidermidis* isolated from cows. It was implied that spectral absorbance varied between the different strains of *S.epidermidis* by purity and brightness. Though the samples were centrifuged for ten minutes, there is a possibility that perhaps the speed or length of time was insufficient and remnants of the bacteria remained in the broth culture, and affected the absorbance readings. The results for the case study (figures 13. And 14.) did not reveal any discernable trends apart from an initial spurt of protease activity after one week of wearing each of the dressings.

The swabs were incubated in 10 ml of TSB for 7 days after the initial results were taken (T=0) to investigate if similar values were seen as in the *in-vitro* studies, but this was not the case. Some values for the viable counts and protease production did not seem in line with the values at T=0. This might be due to the variability of skin flora that may have changed upon incubation, but this was not explored owing to time restrictions. As was discussed in Chapter 3, more accurate results may have been obtained following the method of fluorescence to quantify proteases.

Although the data for volunteer 11 was excluded due to poor compliance, the results showed a higher level of protease activity compared to other volunteers before dressings were applied, and then it seems that the protease activity stopped after wearing the dressings. This suggests that protease modulation may be affected by silicone, but varies between people, which could explain why some patients present better results than others. From this study, conclusions cannot be drawn that silicone dressings affect protease modulation as a mechanism for the healing of hypertrophic scars. Although it has been accepted that skin organisms like *S.epidermidis* and *S.aureus* are known to secrete proteases that degrade collagen and fibrinogen, noticeable effects are probably only seen in wounds where the proteolytic activity of *P.aeruginosa* are used for pathogenicity in chronic ulcers (Koziel and Potempa, 2012). Seven of the volunteers did comment that the skin under the dressings had more moisture than the skin adjacent, which could suggest that the mechanism of action is more likely hydration of the skin. As mentioned in Chapter 1, Berman *et al.* (2007) identified a temperature increase in patients wearing silicone sheeting that varied between 29-30.7°C. The volunteers in this study quoted moistness, which coupled with temperature increase, it is very likely these conditions increased the growth rate of bacteria. It would be rational to suggest that this would also increase protease production, but this study did not prove this.

Chapter 5 – General Discussion

Hypertrophic scars are formed where there is an abnormality during the process of normal wound healing. The scars normally present as hyperpigmented, tight and raised lesions that are not aesthetic (Engrav *et al.,* 2007). Treatments using silicone are still relatively new (O'Brien and Pandit, 2008). Many authors who have conducted studies all over the world have debated the exact mechanism of how the sheets work. Some suggest that hydration is the main factor (Sawada and Sone, 1992; Borgognoni, 2002), whilst others claim it is the raising of the surface skin temperature (Musgrave *et al.,* 2002), occlusion or oxygen transmissivity (Bermen *et al.,* 2007). Interestingly, during the participant study, six of the volunteers commented that the skin surface under the dressing felt damp after wearing it and four experienced a mild tingling sensation when pressure was applied in the area where the dressing was present. This may infer that the dressings do have an effect on hydration, and possibly occlusion, but this cannot be proven. The protease activity could be responsible for the tingling sensation as bacterial proteases are normally excreted mainly to increase its own pathogenicity. Although this may not seem like an advantage, but if particular proteases such as collagenase and hyaluronidase are secreted, the healing mechanism of the silicone dressings could be due to the changes of the amount or type of proteases released by the skin flora from a change in environment. In theory, collagenase and hyaluronidase could be ideally the best explanation of hypertrophic scar breakdown as the scars are formed from an excess of collagen.

The aim of this project was to investigate the microbiology of intact skin under silicone sheeting and to construct a model to study the *in-vitro* effects on extracellular protease production.

The first part of this study investigated the detection of bacterial proteases and the effect of the presence of silicone in a broth culture. One of the main problems experienced with this assay was measuring the absorbance of the TSB. The initial assays showed no problems, with average level of absorbance being relatively the same across each assay, but during the volunteer study, there was a considerable amount of difference in numbers and may have skewed the results slightly for this part of the study. An overall average of absorbance for TSB was calculated and used for the volunteer part. The change in absorbance reading could have been due to a different batch of TSB being used as the project was conducted over an eight-month period. There is also the possibility that there may have been some interference of the assay between the azocasein and the broth, or the pigments produced by certain species of bacteria. Overall, it was concluded that the silicone dressing Mepiform® instigated increased bacterial growth for all organisms tested, but the effect on protease activity seemed to affect *S.epidermidis* noticeably.

The second part of the study sampled volunteer skin to research bacterial diversity, counts and protease activity. This part of the study remains inconclusive as to the effect of silicone dressings on hypertrophic scar healing effectiveness, as protease activity was variable throughout the month. However, it was concluded that bacterial growth increased upon wearing the dressings. An ANOVA did not reveal any statistical significance for the volunteers as a whole for viable counts or protease activity. *S.*epidermidis and *Micrococcus sp.* were mainly found, especially after wearing the silicone dressings. The problems outlined above could have resulted in this variability, and needs to be further studied.

To further explore the findings in this study, a larger sample size would be ideal to conclude whether there may be a trend in protease production and individual people. The small sample size was insufficient to make any solid conclusions. It would also be valuable to administer a social questionnaire to correlate the protease results with the possibility of whether or not protease levels may be influenced by a person's predisposition to form hypertrophic scars. An additional investigation into bacterial isolates from certain people that may heal better could perhaps help find if the application of such isolates could be beneficial to those who do not heal from hypertrophic scars as easily. Although the main organisms found in this study from healthy volunteers were *S.epidermidis* and *Micrococcus sp.,* examination of a larger array of skin organisms could be looked at in future work. *Micrococcus sp.* should be investigated, but various strains of CNS organisms should also be tested, as it could be possible that some strains possibly secrete more protease than others that could have an effect on hypertrophic scar treatment. Additional results that would complement this project would be temperature and pH measurements of skin under the dressings to determine actual environmental factors that can then be replicated in *in-vitro* analyses.

Further investigation should be followed on other dressings that are used on hypertrophic scars, but it would also be interesting to see if there would be an effect on a dressing without silicone incorporated. There could be the possibility that the silicone in the dressings may not be the reason scars improve, but is merely serving as a placebo. It was found by de Oliveira *et al.* (2001) that there were no significant differences in effective treatment between silicone and no silicone dressings, but only 26 patients were recruited.

A lot of focus around the mechanisms of action of silicone on scars revolves around the epidermis, such as surface temperature and epidermal hydration. Although this pilot study cannot prove that protease definitely modulates the skin, which could improve scar formation, examining effects on the dermis where the collagen fibres lie could further this investigation. Collagen is an important component in the formation of hypertrophic scars and it would be likely that maybe the action of silicone cannot be found because clinical studies examine the epidermis mainly. If protease indeed does have an effect, there should be some further study into the absorption of components into the skin. Some cosmetic skin primers contain silicone to create a pore free complexion that hydrates the skin. While pores cannot physically open or close, silicone primers achieve this complexion by covering and possibly filling up inside the pore channels. Since the sweat glands are likely to be damaged in burn patients, any remaining pore channels may not fill with sebum, allowing a passage for silicone to travel through, or even proteases. This could be a viable way that silicone or proteases reaches the dermis via pore channels. A review on the function of silicone in the dermis or the effect on collagen could be researched.

To broaden the *in-vitro* studies, it may be suitable to assess the effect of hypertrophic scar dressings on a pig model, using pieces of pork. The pig model could serve as a medium to simulate bacterial growth on the skin in similar conditions as a volunteer. A study was conducted by O'Shaughnessy *et al.* (2009) on rabbits, which were purposefully wounded to create hypertrophic scars. Tandara and Mustoe (2008) constructed a rabbit ear model and deduced that at early onset of treatment with silicone, epidermal thickness was reduced and suggested that hydration was the key stimulus. A model developed by Birch *et al.* (2005) could be adapted to mimic burn wounds and skin grafts to try and simulate human clinical situations. Testing the growth of bacteria and protease production in different broths would be an ideal supplementary piece of work to improve the laboratory *in-vitro* studies. In this study, only TSB was used, but it would be interesting to observe if other broths such as Brain Heart Infusion, nutrient and Mueller Hinton affected protease production. Although this may be of certain relevance in a laboratory environment, this may not be of beneficial significance from a clinical perspective as an important question raised in this study, was better healing linked to higher protease levels.

In conclusion, there is no conclusive evidence that exoproteins from microorganisms modulate the skin in anyway under the influence of silicone sheeting. Further investigation is needed to determine the mechanism of action of silicone sheeting on scars.

Chapter 6 – References

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Appendix

FACULTY OF SCIENCE AND ENGINEERING

Manchester Metropolitan University

M E M O R A N D U M

TO Jade Chan

FROM AnneMarie Walsh

DATE 23rd January 2013

SUBJECT Application for Ethical Approval **(SE121311)**

On the 24/05/2013 the Head of Ethics for Science & Engineering considered your application for Ethical Approval (SE121311) entitled "The Effect of Silicone Sheeting on the Production of Exoproteins by Microorganisms". The application has been granted Favourable Opinion and you may now commence the project.

MMU requires that you report any Adverse Event during this study immediately to the Head of Ethics (Prof Bill Gilmore) and the Administrator (AnneMarie Walsh). Adverse Events are adverse reactions to any modality, drug or dietary supplement administered to subjects or any trauma resulting from procedures in the protocol of a study.

An Adverse Event may also be accidental loss of data or loss of sample, particularly human tissue. Loss of human tissue or cells must also be reported to the designated individual for the Human Tissue Authority licence (currently Prof Bill Gilmore).

Regards

AnneMarie Walsh
Research Degrees Group Officer
All Saints North

Mepiform® volunteer average sample plate results

Results for each table are the mean of two CBA plates, one at 37°C and one at 25°C. The tables show only the growth of the two major organisms that were found. Where yeasts were present, the results were of one SDA plate.

Key:

- + = Approx. 100 CFU
- ++ = Approx. 1000 CFU
- +++ = Approx. 10000 CFU
- ++++ = Approx. 100000 CFU
- - = No data available

Volunteer 1	*S.epidermidis* (CFU/4cm^2)	*Micrococcus sp.* (CFU/4cm2)
Skin prior to Mepiform®		11
Skin under Mepiform® after 1 week	++	1
Mepiform®	++	4
Skin under Mepiform® after 2 weeks	++	14
Mepiform®	-	-
Skin under Mepiform® after 3 weeks	++	18
Mepiform®	+	5
Skin under Mepiform® after 4 weeks	++	+
Mepiform®	++	+

Volunteer 2	*S.epidermidis* (CFU/4cm^2)	*Micrococcus sp.* (CFU/4cm^2)
Skin prior to Mepiform®	6	4
Skin under Mepiform® after 1 week	+++	
Mepiform®	++	
Skin under Mepiform® after 2 weeks	+++	
Mepiform®	++++	
Skin under Mepiform® after 3 weeks	+++	
Mepiform®	++	
Skin under Mepiform® after 4 weeks	+++	
Mepiform®	+++	

Volunteer 3	*S.epidermidis* (CFU/4cm^2)	*Micrococcus sp.* (CFU/4cm^2)
Skin prior to Mepiform®	++	++
Skin under Mepiform® after 1 week	++	+
Mepiform®	2	5
Skin under Mepiform® after 2 weeks	++	+
Mepiform®	30	8
Skin under Mepiform® after 3 weeks	++	14
Mepiform®	+	
Skin under Mepiform® after 4 weeks	+++	5
Mepiform®	+	+

Volunteer 4	*S.epidermidis* (CFU/4cm^2)	*Micrococcus sp.* (CFU/4cm^2)
Skin prior to Mepiform®	2	
Skin under Mepiform® after 1 week	4	
Mepiform®	22	
Skin under Mepiform® after 2 weeks	++	
Mepiform®	++	
Skin under Mepiform® after 3 weeks	+++	
Mepiform®	++	
Skin under Mepiform® after 4 weeks	+	
Mepiform®	-	-

Volunteer 5	*S.epidermidis* (CFU/4cm^2)	*Micrococcus sp.* (CFU/4cm^2)	White yeast (CFU/4cm^2)	Pink yeast (CFU/4cm^2)
Skin prior to Mepiform®	16	4	4	4
Skin under Mepiform® after 1 week	15	4	15	12
Mepiform®	+	3		1
Skin under Mepiform® after 2 weeks	6	+	7	6
Mepiform®	-	-	-	-
Skin under Mepiform® after 3 weeks	+	6	21	7
Mepiform®	17	2	2	2
Skin under Mepiform® after 4 weeks	++		5	2
Mepiform®	+		1	

Volunteer 6	*S.epidermidis* (CFU/4cm^2)	*Micrococcus sp.* (CFU/4cm^2)	White yeast (CFU/4cm^2)	Pink yeast (CFU/4cm^2)
Skin prior to Mepiform®	2	18		
Skin under Mepiform® after 1 week	18	3	2	
Mepiform®	+	+	7	
Skin under Mepiform® after 2 weeks	+++			
Mepiform®	+++		2	
Skin under Mepiform® after 3 weeks	+++		2	
Mepiform®	++		2	
Skin under Mepiform® after 4 weeks	++	3	9	1
Mepiform®	-	-	-	-

Volunteer 7	*S.epidermidis* (CFU/4cm^2)	*Micrococcus sp.* (CFU/4cm^2)
Skin prior to Mepiform®	3	+
Skin under Mepiform® after 1 week	6	23
Mepiform®	+	+
Skin under Mepiform® after 2 weeks	3	
Mepiform®	26	
Skin under Mepiform® after 3 weeks	2	3
Mepiform®	-	-
Skin under Mepiform® after 4 weeks	-	-
Mepiform®	-	-

Volunteer 8	*S.epidermidis* (CFU/4cm^2)	*Micrococcus sp.* (CFU/4cm^2)
Skin prior to Mepiform®	4	15
Skin under Mepiform® after 1 week	++	4
Mepiform®	+	
Skin under Mepiform® after 2 weeks	++++	
Mepiform®	+++	
Skin under Mepiform® after 3 weeks	+++	3
Mepiform®	+++	5
Skin under Mepiform® after 4 weeks	+++	13
Mepiform®	++	2

Volunteer 9	*S.epidermidis* (CFU/4cm^2)	*Micrococcus sp.* (CFU/4cm^2)
Skin prior to Mepiform®	14	2
Skin under Mepiform® after 1 week	+++	
Mepiform®	+	
Skin under Mepiform® after 2 weeks	+++	2
Mepiform®	++	
Skin under Mepiform® after 3 weeks	++	2
Mepiform®	++	
Skin under Mepiform® after 4 weeks	+++	+
Mepiform®	++	

Volunteer 10	*S.epidermidis* (CFU/4cm^2)	*Micrococcus sp.* (CFU/4cm^2)
Skin prior to Mepiform®	7	9
Skin under Mepiform® after 1 week	+++	
Mepiform®	++	
Skin under Mepiform® after 2 weeks	+++	
Mepiform®	+++	
Skin under Mepiform® after 3 weeks	+++	
Mepiform®	+++	
Skin under Mepiform® after 4 weeks	+++	1
Mepiform®	++	

Volunteer 11	*S.epidermidis* (CFU/4cm^2)	*Micrococcus sp.* (CFU/4cm^2)
Skin prior to Mepiform®	9	1
Skin under Mepiform® after 1 week	30	
Mepiform®	-	-
Skin under Mepiform® after 2 weeks	+++	
Mepiform®	+++	
Skin under Mepiform® after 3 weeks	-	-
Mepiform®	-	-
Skin under Mepiform® after 4 weeks	-	-
Mepiform®	-	-

Volunteer 12	*S.epidermidis* (CFU/4cm^2)	*Micrococcus sp.* (CFU/4cm^2)
Skin prior to Mepiform®	4	2
Skin under Mepiform® after 1 week	++	
Mepiform®	+	
Skin under Mepiform® after 2 weeks	+	
Mepiform®	-	-
Skin under Mepiform® after 3 weeks	++	
Mepiform®	++	
Skin under Mepiform® after 4 weeks	+++	
Mepiform®	++	

Case study 1	*S.epidermidis* (CFU/4cm^2)	*Micrococcus sp.* (CFU/4cm^2)
Skin prior to Mepiform®	++	20
Skin under Mepiform® after 1 week	++	+
Mepiform®	-	-
Skin under Mepiform® after 2 weeks	++	2
Mepiform®	+	1
Skin under Mepiform® after 3 weeks	++	1
Mepiform®	20	2
Skin under Mepiform® after 4 weeks	++	+
Mepiform®	+	2

BAP SCAR CARE T volunteer average sample plate results

Results for each table are the mean of two CBA plates, one at 37°C and one at 25°C. The tables show only the growth of the two major organisms that were found. Where yeasts were present, the results were of one SDA plate.

Key:

- + = Approx. 100 CFU
- ++ = Approx. 1000 CFU
- +++ = Approx. 10000 CFU
- ++++ = Approx. 100000 CFU
- - = No data available

Volunteer 1	*S.epidermidis* (CFU/4cm^2)	*Micrococcus sp.* (CFU/4cm^2)
Skin prior to BAP		
Skin under BAP after 1 week	+++	+
BAP	++	2
Skin under BAP after 2 weeks	+++	+
BAP	++	1
Skin under BAP after 3 weeks	+++	5
BAP	++	
Skin under BAP after 4 weeks	-	-
BAP	-	-

Volunteer 2	*S.epidermidis* (CFU/4cm^2)	*Micrococcus sp.* (CFU/4cm^2)
Skin prior to BAP	+	+
Skin under BAP after 1 week	+++	
BAP	++	
Skin under BAP after 2 weeks	+++	
BAP	+	
Skin under BAP after 3 weeks	+++	
BAP	++	
Skin under BAP after 4 weeks	+++	19
BAP	++	6

Volunteer 3	*S.epidermidis* (CFU/4cm^2)	*Micrococcus sp.* (CFU/4cm^2)
Skin prior to BAP	++	++
Skin under BAP after 1 week	++	8
BAP	++	
Skin under BAP after 2 weeks	+++	5
BAP	++	
Skin under BAP after 3 weeks	++	+
BAP	++	5
Skin under BAP after 4 weeks	+++	3
BAP	++	

Volunteer 4	*S.epidermidis* (CFU/4cm^2)	*Micrococcus sp.* (CFU/4cm^2)
Skin prior to BAP	5	2
Skin under BAP after 1 week	5	1
BAP	-	-
Skin under BAP after 2 weeks	6	3
BAP	-	-
Skin under BAP after 3 weeks	-	-
BAP	-	-
Skin under BAP after 4 weeks	-	-
BAP	-	-

Volunteer 5	*S.epidermidis* (CFU/4cm^2)	*Micrococcus sp.* (CFU/4cm^2)	White yeast (CFU/4cm^2)	Pink yeast (CFU/4cm^2)
Skin prior to BAP	12	9	8	1
Skin under BAP after 1 week	26	5	9	1
BAP	5		5	4
Skin under BAP after 2 weeks	+	4	10	
BAP	12		3	1
Skin under BAP after 3 weeks	+++		7	4
BAP	++		1	
Skin under BAP after 4 weeks	+++	4	5	3
BAP	+++		3	2

Volunteer 6	*S.epidermidis* (CFU/4cm^2)	*Micrococcus sp.* (CFU/4cm^2)	White yeast (CFU/4cm^2)	Pink yeast (CFU/4cm^2)
Skin prior to BAP	+	13	11	2
Skin under BAP after 1 week	+++			
BAP	+++			
Skin under BAP after 2 weeks	+++			
BAP	+++			
Skin under BAP after 3 weeks	-	-	-	-
BAP	-	-	-	-
Skin under BAP after 4 weeks	-	-	-	-
BAP	-	-	-	-

Volunteer 7	*S.epidermidis* (CFU/4cm^2)	*Micrococcus sp.* (CFU/4cm^2)
Skin prior to BAP	4	22
Skin under BAP after 1 week	18	6
BAP	-	-
Skin under BAP after 2 weeks	-	-
BAP	-	-
Skin under BAP after 3 weeks	5	4
BAP	-	-
Skin under BAP after 4 weeks	-	-
BAP	-	-

Volunteer 8	*S.epidermidis* (CFU/4cm^2)	*Micrococcus sp.* (CFU/4cm^2)
Skin prior to BAP	+	9
Skin under BAP after 1 week	+++	
BAP	++	2
Skin under BAP after 2 weeks	+++	1
BAP	++	
Skin under BAP after 3 weeks	+++	11
BAP	+++	6
Skin under BAP after 4 weeks	++++	2
BAP	++	22

Volunteer 9	*S.epidermidis* (CFU/4cm^2)	*Micrococcus sp.* (CFU/4cm^2)
Skin prior to BAP	4	3
Skin under BAP after 1 week	+++	2
BAP	++	
Skin under BAP after 2 weeks	++	10
BAP	++	3
Skin under BAP after 3 weeks	+++	
BAP	++	1
Skin under BAP after 4 weeks	++	2
BAP	+	

Volunteer 10	*S.epidermidis* (CFU/4cm^2)	*Micrococcus sp.* (CFU/4cm^2)
Skin prior to BAP	10	9
Skin under BAP after 1 week	+++	4
BAP	++	
Skin under BAP after 2 weeks	+++	9
BAP	++	10
Skin under BAP after 3 weeks	+++	
BAP	+++	
Skin under BAP after 4 weeks	++	
BAP	++	

Volunteer 11	*S.epidermidis* (CFU/4cm^2)	*Micrococcus sp.* (CFU/4cm^2)
Skin prior to BAP	1	
Skin under BAP after 1 week	+++	
BAP	+++	
Skin under BAP after 2 weeks	++++	
BAP	+++	
Skin under BAP after 3 weeks	+++	
BAP	++	
Skin under BAP after 4 weeks	-	-
BAP	-	-

Volunteer 12	*S.epidermidis* (CFU/4cm^2)	*Micrococcus sp.* (CFU/4cm^2)
Skin prior to BAP	7	12
Skin under BAP after 1 week	++	
BAP	++	
Skin under BAP after 2 weeks	++++	
BAP	++	
Skin under BAP after 3 weeks	++	6
BAP	-	-
Skin under BAP after 4 weeks	+++	
BAP	++	

Case study 1	*S.epidermidis* (CFU/4cm^2)	*Micrococcus sp.* (CFU/4cm^2)
Skin prior to BAP	+	
Skin under BAP after 1 week	++	4
BAP	+	1
Skin under BAP after 2 weeks	+	16
BAP	17	
Skin under BAP after 3 weeks	++	1
BAP	13	2
Skin under BAP after 4 weeks	++	3
BAP	16	

Control swab volunteer average sample plate results

Results for each table are the mean of two CBA plates, one at 37°C and one at 25°C. The tables show only the growth of the two major organisms that were found. Where yeasts were present, the results were of one SDA plate.

Key:

- + = Approx. 100 CFU
- ++ = Approx. 1000 CFU
- +++ = Approx. 10000 CFU
- ++++ = Approx. 100000 CFU
- - = No data available
- W. Yeast = White yeast
- P. Yeast = Pink yeast

	After 1 week		After 2 weeks		After 3 weeks		After 4 weeks	
	S.epi	***Micro***	***S.epi***	***Micro***	***S.epi***	***Micro***	***S.epi***	***Micro***
V1	0	0	++	11	1	0	++	+
V2	0	0	0	0	5	1	8	+
V3	++	+	++	+	+	14	+	+
V4	1	0	0	0	14	1	0	0
V5	0	0	3	4	32	3	8	1
V6	0	0	5	0	0	0	0	0
V7	5	+	8	10	0	2	-	-
V8	4	3	++	+	+	7	1	7
V9	3	2	1	3	17	1	3	+
V10	6	15	1	1	13	+	2	2
V11	2	0	3	0	1	0	-	-
V12	6	1	2	0	16	10	1	0
C1	++	+	+	0	+	0	++	++

	After 1 week		After 2 weeks		After 3 weeks		After 4 weeks	
	W. Yeast	P. Yeast	W. Yeast	P. Yeast	W. Yeast	P. Yeast	W. Yeast	P. Yeast
V5	0	0	5	3	7	6	16	0
V6	0	0	2	1	0	0	1	0