ABSTRACT

JINDANI, RAHIM. Requirements and Efficacy of Donor Site Wound Dressings and an Investigation on the Use of Chitin/Chitosan as Dressing Materials. (Under Direction of Martin W. King and Abdel-Fattah Mohamed Seyam.)

Burn wounds are one of the leading accidental injuries around the world. One of the surgical procedures in the treatment of burn wounds is the use of donor site skin autografts harvested from the patients themselves. Such surgical procedures create another type of injury called a donor site wound. From a nursing or clinical management point of view there does not appear to be a standardized procedure for treating donor site wounds. Sometimes they are treated in the same way as burn wounds although the wound etiology is completely different. So the initial part of our research involved developing and administering a questionnaire that asked the personnel who work in burn units about their normal clinical practices and use of various dressings for the treatment of donor site wounds. The results from this survey have improved our understanding of the clinical aspects and major issues faced by the health care team when managing donor site wounds. Based on the results from this survey we have investigated the use of chitosan and chitin together with ionic silver materials as possible dressings for donor site wounds.

We have concluded from our findings that the etiology of donor site wounds is different from that of burn wounds, and key factors that can impair or prolong healing are uncontrollable pain, excessive loss of blood and bacterial growth. While investigating alternative materials in the second part of our study we have found that chitosan along with silver provides better

hemostasis and antibacterial activity, which are suitable for donor site wounds. The optimum level of silver concentration and the extent to which it bonds to the dressing material need to be determined in future work.

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Requirements and Efficacy of Donor Site Wound Dressings and an Investigation on the Use of Chitin/Chitosan as Dressing Materials

by
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DEDICATION

I have been fortunate enough and blessed by God and want to give thanks for His blessings. My life would be incomplete without my family support. My mother, father, sister, brother, fiancé and my grandparent's exceptional love has enabled me today to be what I am. I am thankful to my advisor Dr. Martin W. King who has always been like a fatherly figure and tried to support my studies in the best possible way by going beyond his capacity to ensure fulfillment of my studies in China and US and also provided me with opportunities to experiment with my unconventional ideas academically. I Love you all for being with me always. I also want to thank to all my friends and the support I have received in Raleigh, NC throughout my studies.

BIOGRAPHY

Rahim Jindani was born into a family that loves to serve people and the community. His personal concern and awareness of being compassionate and philanthropic came from his family's teaching. In 2006 he was admitted to the Textile Institute of Pakistan. He was accepted into the program of Textile Sciences and Chemistry. He worked with Ms. Faiza Saeed and her colleagues at that time to develop an innovative way to modify the surface of cotton fibers using organic solvents to improve the dyeing of cotton fabrics. This experimental work was subsequently published by Lambert Academic in December 2012 with the title "Surface Modification of Cotton Substrates using Organic Solvents".

Soon after his graduation, Rahim worked as a management trainee for Artistic Milliners looking towards product development of denim fabric for various international brands and customers. Later the same year he was selected as a trainee auditor for the textiles sector at United Registrar of Systems. This is where he was trained and certified for auditing as per standard ISO 9001:2008 and Sustainability Reporting according to GRi3 initiatives. He was part of certification team for CSR and business reporting initiatives presented for the year 2010 by Engro and AkzoNobel Pakistan. In 2012 he had the opportunity to pursue further studies at North Carolina State University. Currently he is working on his master's degree in Textile Technology and also working towards earning certification from the Non-wovens Institute. His thesis research project has involved working in the area of donor site wounds created on burn patients and an investigation of alternative dressing materials that will promote the healing process. He has been advised by surgeons at the University of North

Carolina Chapel Hill Jaycee's Burn Unit and has worked under the guidance of Dr. Martin W. King. Rahim plans to continue his studies as a PhD candidate in Fiber and Polymer Sciences at NCSU after finishing his master's degree.

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CHAPTER 1. INTRODUCTION

1.1. Introduction:

Skin is the largest living human organ that protects the human body from various environmental stresses. When injuries occurs and the skin layer is affected either due to physical, chemical or other type of injuries then it limits the human body's ability to protect itself from environmental stresses. Since the skin barrier properties are compromised (Proksch et al., 2008) alternative measures are needed to protect the open wounds. Amongst the many different types of wounds that can occur, burns are one of the leading causes of human skin injuries across the United States. When burn injuries cause a complete loss of skin at certain anatomical sites then a common therapeutic approach is to harvest an autologous skin graft obtained from another anatomical site on the patient's own body. In such cases another type of wound called a donor site wound is created. The management and care procedure for such donor site wounds has not been well documented or standardized. The wound may be left uncovered or it may be protected with various burn wound care products or tissue engineered products if the rate of wound healing is seen to be progressing slowly. Donor sites are sometimes treated like burn wounds; although the etiology is completely different. In an attempt to learn more about current clinical practices a survey was developed and administered to clinicians, surgeons, nurses, therapists and research assistants working in burn units. The objective was to learn how donor site wounds currently are managed and which wound dressing products are preferred and found to be efficacious. Based on the results of this clinical survey, large variations in clinical procedures are currently being followed at the different burn units in the Southern Region of the USA. Some

of the key factors that were identified during the clinical survey have helped us design the functional features of an optimal dressing for donor site wounds.

In particular the result of the survey identified bacterial colonization and continuous pain associated with donor site wounds as two of the biggest challenges. Our hypothesis was therefore to develop hydrogels based on chitin and/or chitosan to be tested along with commercially available wound care dressings. Chitin and chitosan were obtained from two different biological sources of squid and were used to study the development of chitin and chitosan hydrogels which, when applied to a donor site wound, would have the capability of covering the exposed nerve endings, and may be able to control and limit the extent of pain. Previously chitosan obtained from crustaceans has been found to have the capability of covering the exposed nerve endings and hence may be found to have antibacterial and hemostatic properties. An example would be the Chitogauze ® dressing currently being marketed commercially. It was therefore planned to study chitosan from different squid sources and to compare the results with a traditional shellfish chitosan to determine how the hemostatic and antibacterial performance was influenced by the specific biological origin.

When the skin suffers from cuts or acute wounds then in such cases human keratinocytes help in protecting the skin against bacteria (Falabella et al., 2005) but in the case of third degree burns and other deep skin wounds the ability to protect against infection is compromised. Thus a number of wound dressing formulations are being developed with antimicrobial activity so as to limit bacterial infection (Gao et al., 2008). In most cases these

antimicrobial treatments involve the use of either silver or other metallic ion such as copper and zinc that are utilized because of their bactericidal activity (Aziz, et al., 2012). However if silver or another metallic ion is used in excessive quantities then possible leaching of these chemicals can result in wound toxicity and incomplete healing (Meghan E. Samberg et al., 2010).

One of the limitations of most gauze-based dressings is that they need to be changed every 2-3 days. Gauze based dressings cause pain and trauma when removed because fibers adhere to the wounds. The extent of healing can thus be compromised by dressing removal. So this question is being addressed by the use of absorbable fibers, and dressings containing such fibers are being evaluated. It is worth noting that natural chitosan is one of those materials that resorb with time in the human body. There are three different forms of chitin found in nature: $\propto, \beta\ and\ \gamma$. The β-chitin structure will be evaluated in this study as it has a more stable structure with a parallel arrangement of molecules. It is thought that the β-chitin structure will help in providing better healing of donor site wounds compared to other commercial chitosan materials and silver based dressings.

1.2. Problem Statement:

Unlike burn injuries, diabetic ulcers and other skin wounds, which differ greatly between patients, the donor site wound is a "standardized" injury created with the use of dermatome at a fixed and preplanned thickness. Given this fact one would assume that there would be an agreed and "standardized: treatment protocol that all burn unit personnel would use routinely. Since no such clinical protocol has been published or is known to exist, the first task of this study was to establish and define the most widely used donor site wound management procedure with the use of a questionnaire. If no "standardized" treatment protocol was found, then at least the questionnaire would identify which wound dressings were preferred and what their current limitations are. This will lead directly to the second task of this study, namely to develop an ideal prototype dressing for donor site wounds. This prototype needs to address the key issues surrounding donor site wounds such as i) continuous pain, ii) blood loss and iii) infection.

1.3. Goals and Objectives:

This study was divided into two phases.

1. To design, develop and administer a questionnaire that would solicit the opinions and factual information about current management practices and treatment preferences of dressing materials used for donor site wounds by clinicians, surgeons, nursing staff and therapists currently working in burn units.
2. To use the information learnt in phase 1 about the performance requirements of donor site wound dressings, so as to develop a hydrogel wound dressing material from

β −chitin and chitosan and to evaluate its performance against Hemcon ® Chitogauze Silverclear nylon based wound dressings and an experimentally electrospun chitosan fiber web with silver nanoparticles. In particular it was necessary to:

- Determine differences between the different chitin sources and to measure the soluble protein content level in two different varieties of squid chitin / chitosan.
- Prepare & test the mechanical properties of lyophilized freeze-dried chitin and chitosan hydrogel sponges.
- Compare the level of antibacterial activity of chitin and chitosan with and without silver.
- Perform a thrombin formation assay so as to measure the relative contact time prior to blood coagulation.
- Undertake SEM imaging so as to observe differences in blood cell activation caused by the different biomaterials.

1.4. Limitations:

The chitin samples were obtained from different biological sources, which are expected to be associated with variations in structure and properties. In addition, the performance will depend on how the squid pens are extracted and their storage conditions. In our case the squid pens were washed with deionized water and packaged instantaneously in airtight zip lock bags. Squid pens are located near the gut of the squid and proteins in the biological

system can interact with the chitin if the pens are not collected and washed immediately. This can affect the level of chitin coloration, its chemical and mechanical properties.

CHAPTER 2. LITRERATURE REVIEW

2.1. Anatomy of Skin:

Human skin is the largest living tissue found on the human body. It is a blended composite of three layers. These layers have different cellular structure, function and protective barrier capabilities which ensures that micro-organisms, biological organisms, harsh weather conditions, fluctuating temperatures, and pH variations do not cause harm to the human body. Skin is the most sophisticated and advanced form of protective barrier that nature has provided. Skin is able to breath, stretch, shrink, and varies according to physiological and environmental variations.

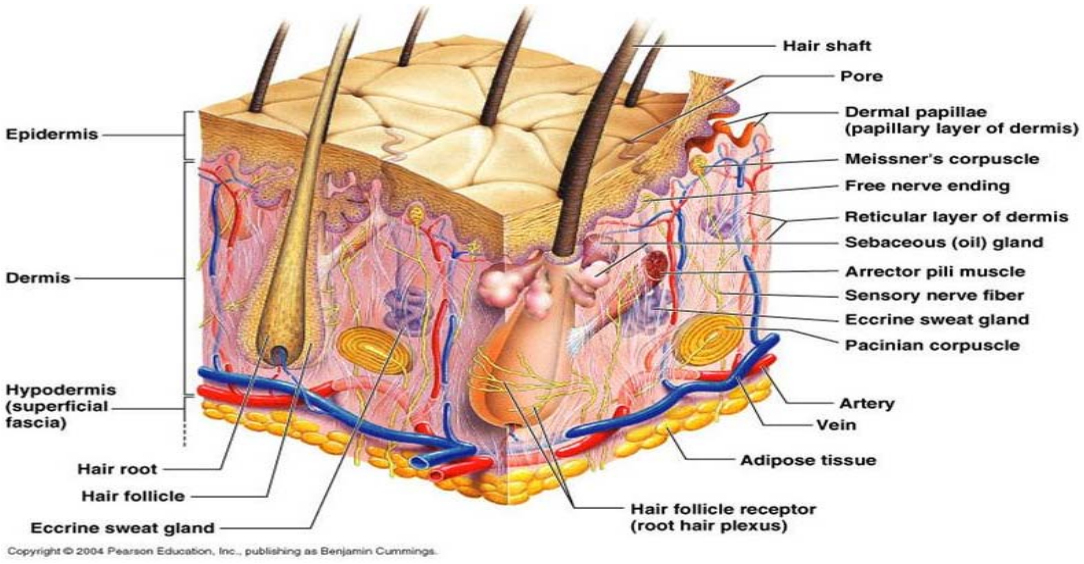


Figure 1: Complete anatomical view of skin structure ("Pearson - Laboratory Manual for Anatomy & Physiology, 3/E - Elaine N. Marieb").

2.1.1. Epidermis:

The epidermis is the outermost layer that comes in contact with the surrounding microenvironment. The epidermis is 90 to 95 % composed of keratinocytes. A healthy epidermal layer can be achieved by having a balance between keratinocyte proliferation and maturation. There are 30 different types of keratin polypeptides that have been found (Freinkel et al., 2001). Mutations in keratin expressions can cause blisters, rashes to appear on the skin and various other diseases such as epidermolysis bullosa simplex (EBS) or epidermolytic hyperkeratosis which can severely hamper integrity of the skin as seen in studies (Fuchs, Elaine et al., 1998). The epidermis usually regenerates every 4 weeks in a normal skin. The epidermis consists of stratum corneum, the outer layer, and a living inner cell layer called the rete mucosae. It is from these layers that keratinocytes arise and differentiate (Goldsmith et al, 1991). The stratum corneum or the squamous layer is made up of keratin flakes and is replaced by proliferating keratinocytes from the rete mucosae that also serve as a barrier to micro-organisms and environmental factors (Fuchs et al., 2007). The cellular structure of the stratum corneum found on the skin surface of the skin consists of hexagons, which interlock with one another like bricks. Lipid proteins are also found in between the stratum corneum. They help in ensuring that the bricks of keratinocytes form a confluent protective barrier layer which maintains moisture balance. Lipids are important to ensure the protective layer doesn't become dry, flaky and itchy (Hess et al., 2004). The epidermis also contains melanocytes responsible for pigmentation of the skin and protection from UV radiations. Langerhans cells present in epidermal skin act as immune cells to ensure protection from foreign antigens, while fatty acids and cholesterol are important to ensure the

integrity of the skin, and maintain trans epidermal water loss (TEWL). Meissners corpuscles or mechanoreceptors and the stratum lucidum, which are responsible for sensitivity to light and touch, are mostly found on the hand and toes. Meissners corpuscles are found on the hands while the stratum lucidum consists of translucent lines of cells both on the palms and the toes (Lanza et al., 2011),(Hess et al., 2004).

The permeability of the epidermal layer and its barrier function can vary depending on the skin type (Reed JT, 1995) and from person to person due to age, gender and (Gambichler et al., 2006) demographical variations. In the case of donor site grafts when partial thickness skin grafts are being harvested, usually 95 to 100 % of the epidermal layer is removed. On the other hand, in the case of full thickness skin grafts, the complete epidermal layer is removed along with part of the dermal layer (Andreassi et al., 2005).

2.1.2. Dermis:

Dermis on the other hand is the most dominant structure in skin and it comprised of 75-77 % various types of collagen. Dermis is divided into papillary dermis and reticular dermis. Type I, V and VI collagen are present at large in skin. Type I collagen is present in about 80 to 90 %, while Type III is 8 to 12 %. Collagen provides a supportive structure and provides the required strength and dimensional stability for skin (Goldsmith et al, 1991) (Freinkel et al., 2001). Papillary dermis is able to contour into the grooves and
ridges of epidermis. An extensive amount of connective tissue and collagen are loosely distributed and are found in papillary dermis. It contains hair follicles, hair roots, eccrine

sweat glands, apocrine (scent) glands, arteries and veins that ensure blood supply and transportation of nutrients. While reticular dermis contains a large number of collagen and elastin which are interconnected in a latticework. A number of polysaccharides are found in reticular dermis. Elastin provides for elasticity and is required to ensure that the skin structure is extensible. Densely interconnected fibroblast cells, extracellular matrix proteins (ECM), polysaccharides such as glycosaminoglycan's, glycoproteins and proteoglycans assist in cell adhesion, migration and the spreading of cells. Hyaluronates, chondroition-4-sulfate, dermatan sulfate and hepran sulfate are also present and each has its specific function in providing nutrients and maintaining the skin physiology in balance (Goldsmith et al., 1991). Lamellar corpuscles and other mechanoreceptors responsible for pressure and vibration (Hess et al., 2004) are also found in the dermis to plays their role of providing integrity, strength and extensibility to the skin. Physiological, chemical and environmental changes that occur in skin tissue are regulated by dermis. In 1861 Karl Langer found Langer identified the lines, which were found to provide extensibility and the components that help in the healing of wounds (Gibson et al., 1978). Langer's lines run throughout the body and usually if skin incisions are made the direction of these lines, then due to tension provided by the skin, wounds were thought to heal faster. More recently Kraissl and Borges lines have demonstrated that the tension measured by Karl Langer were only in cadaver skin. In real skin the tension along limbs and on the face were quite different to Karl Langer's proposed idea. (Gibson et al., 1978)(Silber and Then., 2013). The Karl Langer's lines provided insight into the fact that skin itself is elastic in nature. This is provided by elastin present in the

dermis, because of this it was found that wounds start to contract and turn into oval shapes over time.

2.1.3. Subcutaneous Layer (Hypodermis):

This layer is composed of fat and connective tissues beneath the skin that ensures nerve endings, blood vessels and other structural components of the skin remain intact within the tissue to ensure adequate blood supply and regulation of body temperature according to the environment (Diller, International Center for Heat and Mass Transfer, and Bio transport '98: International Symposium on Heat and Mass Transfer in Biological and Medical Engineering). Heat and mass transfer is regulated by the thickness of the subcutaneous layer which can vary depending on the anatomical site as well as gender, age, diet, physiological and environmental surroundings of a person and a number of other factors (Hess et al., 2004). If the hypodermis is removed along with the dermis and epidermal layer then it needs to be treated with a full thickness graft or skin flap because of its completely different wound etiology that needs a different mode of therapy.

2.2. Donor wound physiology:

Donor site wounds are created by harvesting skin from other anatomical sites of the patient, which can then be utilized for treating the burn injuries. For this purpose the donor site is first shaved followed by a surgical procedure in which a scalpel, knife or dermatome is used.

Dermatomes are the preferred instrument for skin grafting as they minimize the variability in thickness of the graft (Andreassi et al., 2005) (Leung and Fish., 2009).

Figure 2: Skin grafting using a dermatome [49].

Saline solutions are often injected beneath the skin prior to grafting so as to reduce perfusion and pain at the donor site. A particular surgical protocol has been recently developed and practiced at the Jaycee Burn Center, UNC-Chapel Hill. According to this treatment a tumescent solution concentration of 1 % lidocaine, (a local anesthetic drug to relieve pain, burning and itching of skin), 1 ml epinephrine solution (also known as adrenaline a neurotransmitter) together with 5ml, 0.5 % ropivaccine in 1000 ml to serve as a nerve blocker (Blome-Eberwein et al., 2013) is injected at donor site under the skin during the surgical procedure of skin grafting. It has been found that this tumescent solution perfusion

of the donor site relives pain for a number of days with no side effects. However this procedure makes no difference in terms of healing or pain relief in the long time. Another aspect that has been studied previously by (Blome-Eberwein et al., 2013)(Leun, Lowe, and Beerens., 1974) is the effect of temperature. An elevated temperature in the range of (20-43^{O}C) causes the epidermal and dermal components of skin to separate out faster and the extent of binding between the two layers is reduced. The skin grafts obtained through such a surgical procedure are then washed in saline and marked to ensure the dermal side can be distinguished from the epidermal side, which is difficult to visualize for white and pale skin patients. Some form of identification is made on the skin graft to ensure the dermal side is placed on the burn wound, which facilitates the burn wound healing in a timely fashion. The healing of a skin graft on a burn wound site takes place a process of four-stage.

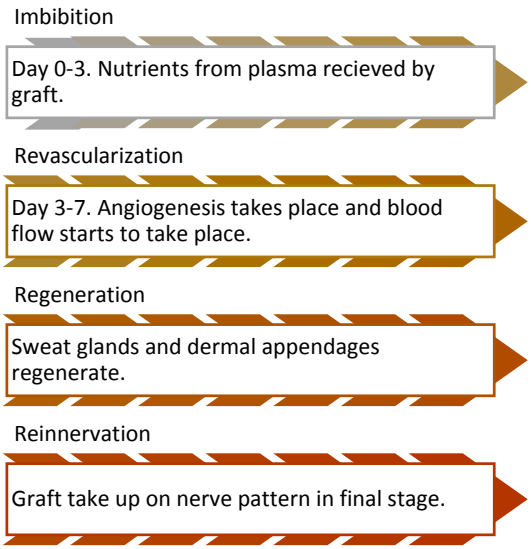


Figure 3: Stages of graft healing (Leung and Fish, 2009)

Most of burn patients who experience burn injuries to 20 % or greater of their total body surface area require surgical treatment involving either skin flaps, donor site grafts or tissue-engineered products. Since autologous skin grafts are easily accessible and available for most patient's and they do not cause any immune response or complications, they are currently considered the best surgical option. Xenograft such as bovine grafts and allogeneic grafts form other human donors can cause a negative immune response. Skin grafts are usually classified as split thickness skin grafts or as full thickness skin grafts (Leung and Fish., 2009)[49] . There is currently no other way of differentiating between them on the basis of skin thickness that has been harvested from the donor site (Table 1) (Andreassi et al., 2005).

Table 1: Skin graft relation to thickness (Andreassi et al., 2005)

Table 1 Classification of skin grafts in relation to the thickness of the explant

	Name	Thickness (mm)
Split thickness	Thin (Thiersch-Ollier)	0.15-0.3
	Intermediate (Blair-Brown)	0.3-0.45
	Thick (Padgett)	0.45-0.6
Full thickness	(Wolfe-Krause)	>0.6

Any eschar and necrotic tissue is completely removed prior to collecting an autologous skin graft. Likewise burn wounds are cleaned completely to ensure that the new tissue can integrate and heal successfully. Skin grafts are not preferred when treating facial skin burns especially split thickness skin grafts due to their poor cosmetic appearance and greater

contraction on deep wounds. The use of full thickness skin grafts has been found to be more successful for facial reconstruction (Ang., 2005).

For many years surgeons have used autologous skin grafts, but the partial thickness and full thickness wounds created at the donor site have not been cared for or managed with a specific procedure. In fact they are often considered to need the same therapy as burn wounds despite their different etiology. Thus it is important to understand the factors that need to be addressed when taking donor site skin grafts creating wounds which will be required a specific healing regimen (Ang., 2005).

2.3. Clinical Management of Donor Site Wounds:

Clinical factors and wound management issues are important to ensure the optimal healing of donor site wounds. Reconstruction of the donor site skin or any other tissue reconstruction requires that the pathology and physiology of the wound is clearly understood. There are numerous burn wound care products and various other skin care products, which are claimed and marketed for different types of wounds. Claims stating that particular dressings provide successful results on all types of patients and all types of wounds would appear to be exaggerated claims. These claims need to be clinically justified and evidence regarding such claims needs to be demonstrated prior to marketing. In actual fact, the clinical understanding of the preferred therapy for donor wound sites is not well known or widely disseminated. Wound dressings marketed currently by various companies including their construction and

active components need to be studied so as to understand which dressing materials and construction can claim to provide optimal donor site wound healing.

2.3.1. The pH of Wound dressing:

Human skin normally has a pH slightly on the acidic sides but when skin is not cleansed it attains a basic pH which encourages micro flora and organisms to survive. Similar to the stratum corneum which tends towards an acidic pH (Proksch, Brandner, and Jensen., 2008) similar acidic pH values have been reported for the growth of fibroblast cells (Falabella et al., 2005). However it has been found that diabetic patients with ulcers and other wounds tend to develop alkaline pH. Variation in pH is not associated with people's age or sex. It is based on human physiology and the type and amount of amino acids, fatty acids and keratinocytes within the body that cause a natural lactate bicarbonate buffer to move towards an acidic milieu (Aly et al., 1978). An acidic pH between 4.5 and 6 helps in ensuring that bacteria are not able to grow and proliferate. In fact our normal skin pH of 6 ensures that certain bacterial species such as staphylococcus aureus and enterobacter species are not able to thrive (Falabella et al., 2005)(Chiller et al., 2001). Since donor wound sites are created by either a split thickness skin graft or by a full thickness skin graft different layers in the remaining skin tissue are exposed to the environment. Depending on the type of skin graft and its exposure to the environment, the pH can vary and move towards either an acidic or a basic pH.

2.3.2. Anatomical site of skin graft:

Another aspect that can vary in the clinical setting is the anatomical site from where skin graft is being harvested. It has been reported previously by Langer in 1861 that there are lines of tension in cadaver skin. Later Kraissl and Borges in 1960 corrected his understanding by finding that the lines of tension in the skin run parallel to wounds created on the human body, except that these findings are different for facial skin and skin on the limbs (Gibson et al., 1978),(Hess et al., 2004). Skin wounds take on an elliptical shape during normal healing to ensure better closure of the wound contraction of the tissue and overall healing. In partial thickness wounds a similar phenomenon is observed (Armitage and Lockwood., 2011) but in the case of full thickness wounds staples and sutures are utilized to close the elliptical open wounds which can become infected.

2.3.3. Pain associated with Wounds:

It is claimed that the pain associated with donor site wounds is 6 to 10 times greater than the pain associated with burn wounds. It has been reported in several research studies that pain and discomfort associated with split thickness skin grafts greater the most excruciating pain that is experienced by a patient with third degree burns (Herndon et al., 2002), (Myers et al., 2004). Split thickness skin grafts or flash burns are the most painful because the epithelial layer is being removed exposing live nerve endings in the dermal layer to air. Split thickness partial wounds are moist and can cause blisters due to edema (Myers et al., 2004). While in the case of full thickness wounds the pain sensation is minimized since all the nerve endings have been removed during graft harvesting.

Pain is the sensation that humans feel because of pain mediator's function by the release of growth factors, hormones and other cytokine factors. This usually promotes the healing of wounds, but certain pain mediating factors are known to slow down the healing process. For example it has been found that damaged endothelial linings and platelets release ATP mediators which cause pain in case of blisters and surface wounds as well as, acetylcholine and serotonin which is a neurotransmitter responsible for the reduction in the transmittance of pain intensity to the brain. Serotonin (5-HT) helps in the production of Type IV collagen and can up regulate those growth factors that help in the healing of wounds. Other pain mediating factors such as Bradykinin (BK) is released when fibroblast cells undergo contraction during healing and TNF-α cause's apoptotic death of certain cells and promotes inflammation. In the cases of non-healing wounds it has been found that the wound fluid has high concentrations of TNF- α, which ensures that fibrosis does not occur (Trengove et al., 2000), (Schultz et al., 2004).

A recent improvement in clinical practice at the Jaycee Burn center at UNC-Chapel Hill is the use of lidocane along with high concentrations of adrenaline (Blome-Eberwein et al., 2013), (Leung and Fish et al., 2009) for selected patients. It was found that this treatment helped reduce the number of patients complaining of pain, by limiting blood perfusion for a number of days. This is because adrenaline is a vasoconstrictor and thus it helps in ensuring localized drug affectiveness is maintained for 6 to 8 hours after surgery for the donor site wound. However there was not much difference in the long term healing performance. Another important chemical release that has a direct effect on neuro stimulation and pain is

the release of nitric oxide within the wound. Nitric oxide reacts with reactive oxygen species present in the wound and promotes the inflammatory response. Nitric oxide is also responsible for up regulating the cytokine cascade (Widgerow and Kalaria., 2012). It has been found that a person's diet has a direct effect on release of nitric oxide into the blood stream. Inhibition of nitric oxide can exacerbate the sensation of pain and cause vascular smooth muscles contraction which results in reduced blood flow while on the other hand excess of nitric oxide can be toxic to bacteria and at the same time impair wound healing by limiting collagen production (Reichner et al. 1999). An extensive study of pain mediators by (Widgerow and Kalaria., 2012) showed that wound healing in acute or chronic wounds is more of an orchestration of factors. If various pain-mediating factors are not released during wound healing in an efficient manner then normal wound healing remains impaired. One therefore needs to develop dressings that serve as scaffolds that can reduce those pain mediating factors that impair healing and promote those that play, a positive role in the inflammatory and immune responses.

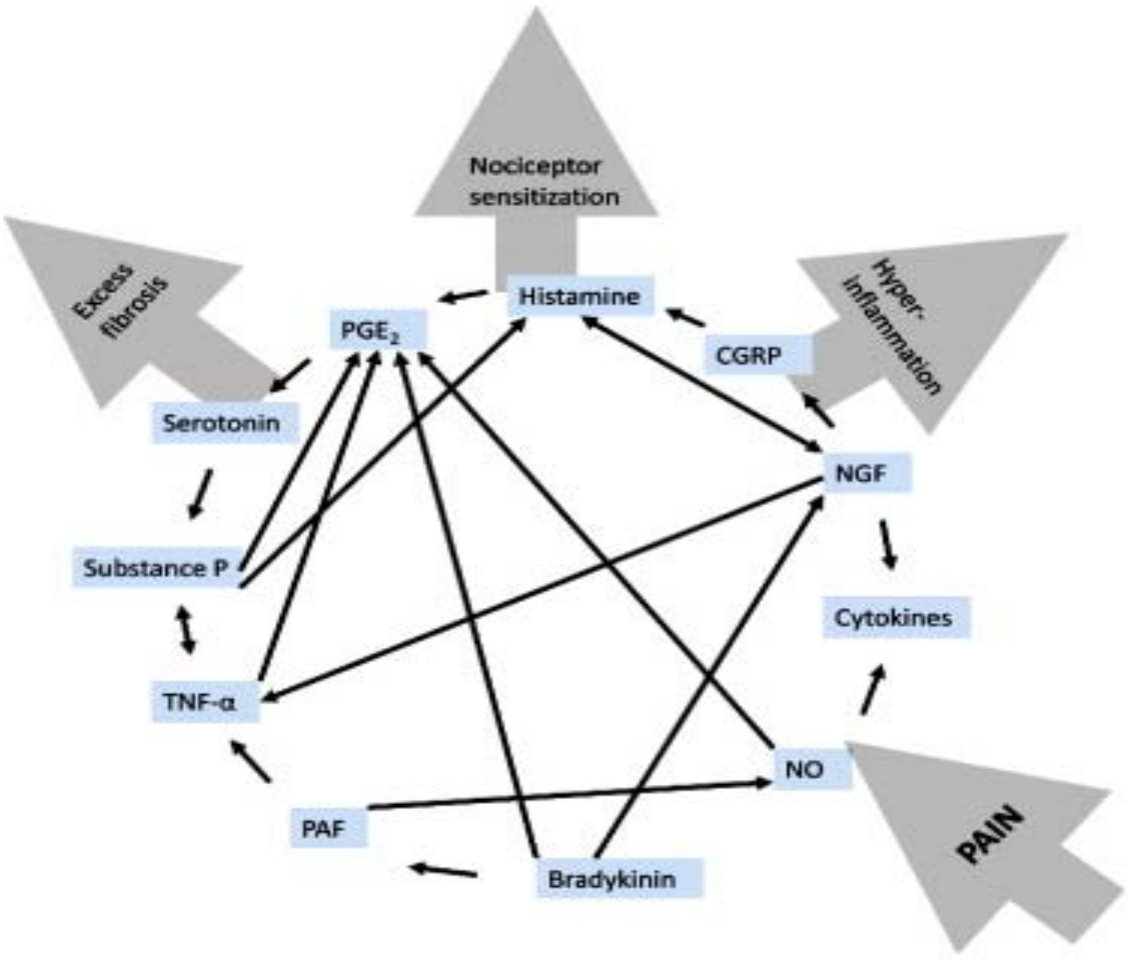


Figure 4: The orchestration of pain mediating factors (Widgerow and Kalaria., 2012)[79].

Pain also occurs at donor site wounds because the need for the dressing changes that have to be made every week or when the wound becomes infected. If the dressing material is adhering to the wound, then the frequency of dressing changes needs to be minimized (Chung and King., 2011). Open wounds require protection from environmental stresses such as microorganisms, contaminants, air, humidity, sweat, thermal changes and other factors,

especially in the case of diabetic wounds where the edges of the wound can easily be infected and can be a source of pain and delayed healing (Rakel et al., 1998). Various dressings have been tested and tried for split thickness skin grafts. Clinical studies using Suprathel ® a product from Germany containing >70% DL-Lactide, trimethylene carbonate & E-caprolactone as described in US Patent 6,706,058 B2 "Wound Dressing" was utilized to develop a nonwoven absorbable layer. The patent claims that due to this unique design removal of the dressing from the wound does not cause any discomfort or pain (Schwarze et al., 2007) Other possible reasons for the easy removal could be due to the use of biodegradable polymers which slowly degrade and dissolve over time and so does not adhere to the eschar or dry scab as described by Baser (Baser et al., 2008). A better option could be to utilize degradable material, which has antibacterial properties as it degrades. This would ensure that bacterial colonization would be minimized.

2.3.4. Moisture Vapor Transport Rate & Antibacterial properties:

Skin also breaths and sweats. The epidermal basal layer of the human skin has keratinocytes and consists of strata basale, spinosum, granulosum and corneum which ensure that vapor transmission across the skin is maintained and at the same time its barrier properties are not compromised that would allow foreign bodies to penetrate through the skin (Herndon et al., 2002). The dermal layer of human skin is attached to the epidermis via collagen Types IV and VII, but when a split thickness skin graft is harvested there is the chance that the epidermal dermal junction will be disturbed and epidermal necrolysis or steven Johnson syndrome may occur (Hess et al., 2004). This disease can be life threatening and can cause

morbidity. Once the epidermal skin is removed direct fluid loss takes place throughout the human body. In case of thick split thickness grafts and full thickness grafts large amounts of albumin are lost that can cause further systemic responses to be generated and secondary edema can occur.

Knitted, woven and gauze dressings made up of cotton cannot be used for donor site wounds because these dressings will rapidly absorb wound exudate which is likely to cause the wounds to become dry and impair epithelialization of the skin and results in possible sepsis. As a result nonwoven dressings have been developed (McCarthy et al., 2012). Fibers present in such dressings are associated with capillary action, which causes wound exudate to travel along the length of the fibers in dressings. Such structures are therefore not recommended since they would cause the wounds to be completely dry. A wound dressing needs to have some extensibility and at the same time it needs to cover and protect the wound and only allow a limited amount of moisture vapor transport. Studies done by Tarun (Tarun and N., 2012) found that various blends of calcium alginate and polyvinyl alcohol when used to develop wound dressings had a higher MVTR. The higher the proportion of calcium, the less water was lost since calcium absorbs water, but the antibacterial properties, were compromised. Thus wound-dressing materials need to have both adequate moisture vapor transport and antibacterial activity so as to ensure that an infection does not occur during healing. One of the natural materials that has been found to be able to achieve both requirements is chitin/ chitosan. It can maintain a moist environment at the same time and allow for gaseous exchange to take place. It also acts as a barrier to micro-organisms,

removes excess exudate, and does not cause any toxicity to human skin. The material is non-allergenic and non-adherent and has antimicrobial activity. In addition the dressings developed using chitosan can be removed easily without causing any trauma.

2.4. Wound Dressings

2.4.1. Dressing requirements for donor site wound healing:

In current clinical practice it has been found that dressings such as Opsite ®, Tegaderm ™ (Wiechula., 2003) which are effective medicated films and used for healing wounds usually adhere to donor site wounds. When dressings adhere to dry wounds it causes pain, trauma and in certain cases skin that has been healing is completely removed. As a result clinicians do not prefer to use these dressings as their first choice. While on the other hand EZ-Derm (Rakel et al., 1998), (Wainwright., 1995) a product by AM Scientific Limited developed from porcine xenograft helps in healing the wound without pain once the graft has been accepted covers the wound. Negative immune responses have been reported in some instances with the use of xenografts. Currently marketed xenografts are washed and cross-linked prior to usage (Courtman et al., 2001). Xenograft skin allows for moisture to pass through and at the same time does not adhere to the wound site, hence reducing the incidence of pain. In a survey reported by the Plastic, Aesthetic and Reconstructive Surgery Department of Surgery, Hospital in Vienna, Austria it was found by reviewing literature and taking opinions of various burn surgeon's that an ideal dressing would be non-adherent, non-

traumatic at the wound interface and would not be bulky (Selig et al., 2012). These proposed properties for an ideal wound dressing for burn wounds could equally apply to the wound created by a split thickness skin graft with the addition of antibacterial properties. Materials such as bovine collagen and porcine collagen peptides have been linked to silicon or have been utilized as part of wound dressings such as in the case of Biobrane ® and other researched products.

Interestingly chitosan has antibacterial properties and it resorbs slowly with time. Based on these findings hydrogel's have been prepared using chitin or chitosan in Japan, China, India and the US Their applications and success have been reviewed by Jayakumar (Jayakumar et al., 2011). A number of hydrogels have been prepared with different formulations with the use of silver nanoparticles, zinc, collagen and other materials to achieve antimicrobial properties. Various scientists have prepared chitosan hydrogels and sponges, but the method discussed by Tamura used (Tamura et al., 2006) milder conditions to develop his chitin hydrogels which have gained much interest since the milder chemical formulation did not produce cytotoxic effects. It was found that $CaCl_2$ when utilized with methanol was effective in developing 94-96 % (w/v) chitin hydrogels that were suitable for medical applications. It has also been discussed in various reviews and research articles that β-chitin and chitosan from these structures have a parallel arrangement that resorbs much more faster and allows wound dressing scaffold materials to help in healing and improving the wound-restructuring mechanism. (Jayakumar et al.,2011), (Habibi et al., 2012). The antimicrobial activity of β-

chitin extracted from the jumbo squid (Dosidicus gigas) found in the Gulf of California. (Hoving et al., 2013).

The following list indicates a number of requirements that would be considered desirable for a donor site wound dressing. This list was generated after studying the literature and looking into the clinical perspectives/ for wound dressings.

1. Dressings should have a moisture vapor transport rate equivalent to skin or greater than skin (Baser et al., 2008).
2. Dressing should be easy to remove
3. Dressing needs to be inherently antimicrobial or have some kind of antibacterial component added.
4. Dressing should not adhere to the wound (Selig et al., 2012).
5. Dressing should adhere to the surrounding skin (Schwarze et al., 2007).
6. Dressing should allow the wound to be hydrated but not moist.
7. Slightly acidic pH for cellular growth and proliferation.

Keeping these factors in mind the following section reviews the currently available dressings on the United States market. This review attempts to evaluate how these various types of dressings might interact with a donor site wound.

2.4.2 Review of types of Dressings:

2.4.2.1. Topical Ointments:

In the past Argyrols antiseptic ointments based on silver nitrate were commercialized and widely used for wound care. More recently silver sulfadiazine cream was developed in 1970's and is still used today in burn centers to keep wounds moist. However it does have some disadvantages (Maillard et al., 2012) such as causing the development of rashes and slowing down epithelialization (Falabella et al., 2005). It was reported by (Aziz, Abu, and Chong et al., 2012) that topical treatments with silver sulphadizine was least effective in comparison to other silver based dressings and ointments currently available on the market. Silver sulphadizine has been shown to cause contact dermatitis in various patients and has also been reported to cause argyria (a skin disorder) especially in the case of diabetic patients if used continuously over an extended period (Fisher, Marsh, and Lazova et al., 2003).

Mafenide acetate (Sulfamylon ®) an FDA approved product is a bacteriostat that is effective against Gram positive and Gram-negative microorganisms including E. coli pseudomonas aeruginosa. Adverse reactions have been reported with Sulfamylon ® such as rashes, metabolic acidosis and pain. Zinc neomycin (Bacitracin/ polymyxin) has also been used previously as a successful topical treatment but it has been reported to cause delayed contact dermatitis (Katz and Fisher). Other topical treatments that have been reported include the use of cadexomer iodine. All these treatments focus on covering the wound completely and introducing some antibacterial activity so as to minimize bacterial growth and provide some

reduction in pain. However, most of these treatments have long-term side effects and can introduce toxicity if used continuously (Falabella et al.,2005).

2.4.2.2. Films and meshes-semi permeable:

Semi-permeable acrylic adhesive films such as Opsite ® by Smith and Nephew has been used to ensure that wounds can be protected from the surrounding environment and the wound site can be kept dry. Opsite ® is largely used for donor site or burn wounds if the perfusion of drugs or other medications are required. Cellulose acetate dressings, made up of Adaptic ® have been in use for quite a long time. Since Adaptic ® is an open mesh structure, it allows for wound exudate to pass through it. Due to its open structure it also allows for moisture vapor to pass through. A common problem associated with meshes and films is that either the moisture vapor transport rate (MVTR) is too high or too low (Wiechula et al., 2003). In the case of too high a moisture vapor transport rate the wound can become dry quickly that leads to the occurrence of trauma along with discomfort and pain. Alternatively, if the moisture vapor transport rate is too low then wound exudate will accumulate leading to perforation of the barrier dressing and increase the risk of infection.

2.4.2.3. Silver incorporated Dressings:

Back in the 1920's the FDA approved the use of ionic silver as an antimicrobial agent. Silver has been utilized in various forms and ionic silver particles have been shown to be much more effective over the long term when utilized in various forms, and ionic silver particles

have been shown to be much more effective over the long term when impregnated in hydrocolloids or textile products. It has been found that the activity of silver against bacteria is due to its ionic charge. Ionic silver solutions with concentrations in the range of 10^{-9} to 10^{-6} mol/L are effective, and at the same time ensure that the bacterial load is minimized (Maillard and Hartemann et al., 2012). It has been demonstrated that there are certain bacteria that develop a resistance to ionic silver. Silver nanoparticles are able to show effectiveness when their size is less than 80 nm. Above this size the particles are not able to penetrate the bacterial cell membrane thus limiting their resistance against certain bacteria. Some mutant strains of E.coli bacteria have developed resistance against silver ions and this prolongs burn wound healing. Currently there are some new products with a mixture of ionic silver and pure silver metal coated on various substrates that ensure the silver can reduce the bacterial burden. By incorporating other biomaterials in combination with silver can ensure that E. coli bacteria do not develop a resistance.

Silver toxicity is a problem that needs to be addressed. Hydro fiber ®Aquacel Ag dressings by Convatec have been quite popular in treating burn wounds, skin graft wounds, venous stasis, ulcers, pressure ulcers, diabetic wounds and even other wound etiologies (Coutts and Sibbald., 2005). Hydro fibers have been developed using sodium carboxy methylcellulose hydrocolloid polymers (Dornseifer et al., 2011). It has been observed that Aquacel ® Ag dressings release silver ions to entrap the bacterial burden in a gel form. Aquacel ® ensures that moisture is entrapped, and the release concentration of silver ions is prolonged so as to ensure that the bacterial burden is minimized and inflammation of the wound is prolonged in

the case of burn wounds (Coutts and Sibbald., 2005), (Rigo et al.). Studies have shown that the rate of silver ions released from Aquacel ® Ag dressings more rapid in serum and saline solutions than in pure water. The greater concentration of Cl^- ions and other pathogens in serum has been shown to cause this shift in silver ion release. In the case of saline solution the chloride concentration causes AgCl salt to redissolve and form anionic chlorocomplexes (Rigo et al., 2013), thus reducing the antibacterial effect. Dressings such as Therabond ® wraps utilize spacer fabric structures that have been successful in healing ulcerations and burn wound injuries. Therabond ® wraps are good and effective but their removal from the wound site without trauma poses major challenges. Nursing staffs at various burn and trauma centers complain that these dressings stick to large exuding wounds due to the greater bio burden. Thus care needs to be taken on their removal together with the use of saline water to ensure that trauma is reduced.

2.4.2.4. Composite Dressings:

Wound healing is such a challenging task that it requires a number of factors to be dealt with at the same time. Currently certain commercial dressings are referred to as composite dressing materials with healing components for the skin and materials that can interact with the outside microenvironment. Mepilex Ag ® a Molnlycke healthcare product developed in Ireland by a number of scientists (Elefsen, Gudnason, and Sigurjonsson., 2008) is one such product. It utilizes etched-silicon using "Safetac technology as claimed in Patent US 7910793 B2. It ensures that the etched silicon completely penetrates into the wound which does not allow any air pockets to form. The "Safetac" technology allows the etched silicone gel to

bind on one side with a non-woven polyurethane foam which is impregnated with silver ions and activated carbon, while the silicon sheeting on the other side provides protection from microorganisms. This allows the wound dressing to be able to absorb wound exudate and also bind with donor site wounds ensuring complete coverage of the nerve endings. The top silicon sheeting ensures that the wound dressing adheres to the surrounding skin. The soft silicon used for Mepilex ® dressing is vinyl substituted and hydride containing silicone as discussed in patent US 7910793 B2. Vinyl substitution allows for various moieties to be attached to the silicon sheeting. The Mepilex ® product shows that a successful dressing needs to have a barrier layer to protect against environmental stresses. Mepilex ® utilizes silver in the form of silver sulphate and this helps in ensuring that bacteria such as MRSA and E. coli do not grow quickly. Mepilex® Ag utilizes "Safe Tac ®" technology. The silicon sheet is etched to develop grooves, which can ensure capillary action takes place. Also silicon conforms to the shape of the wound site in such a way that there are no air pockets left between the wound and the dressing. This ensures that pain is minimized and the nerve endings are covered. The silicon grooves are placed 0.5 mm apart from one another and have a depth of 4 to 5 mm. The amount of silver release was measured in ultra-pure water to be (50 %) and in human serum substitute (67 %) within 5 minutes of exposure. In the case of normal saline solution the release rate was (15 %) and was consistent and constant for 7 days (Rigo et al., 2013). These results suggest that in alkaline conditions Mepilex ® Ag would have better silver release than under neutral or acidic pH condition when the release profile for silver could vary and make the dressing's antibacterial properties questionable.

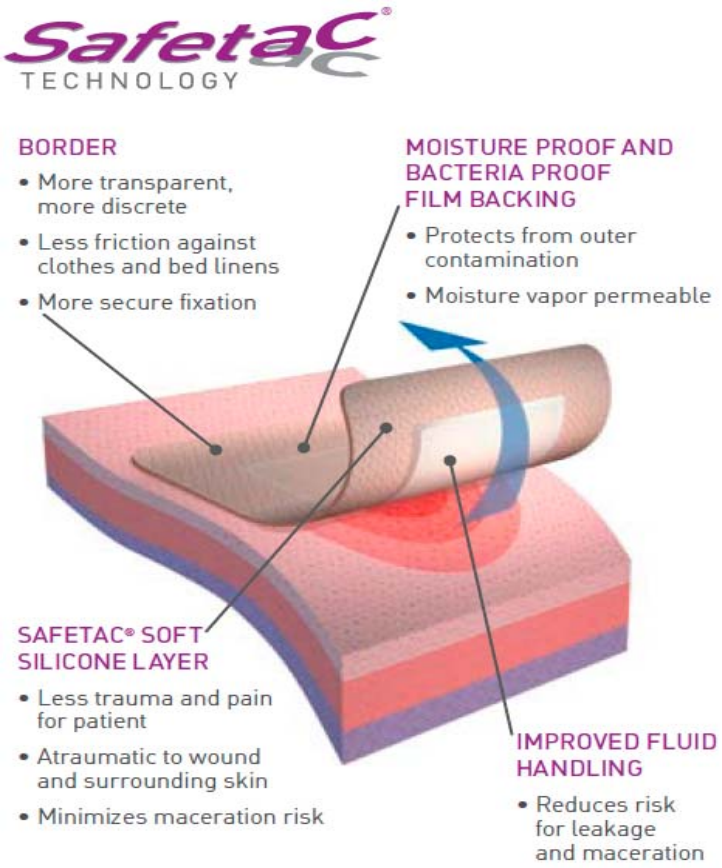


Figure 5: Mepilex ® dressing with Safetac ® technology.

2.4.2.5. Other dressings/ Techniques:

Xeroflo ®, Integra ®, Acell ®, EZDerm ®, Tegarderm ® and Adaptic ® have been reported in the literature to be applied to split thickness skin graft (STSG) wounds. Sometimes antimicrobial creams such as zinc, neomycin (Bacitracin ® / polymyxin ® are applied to donor site wounds to ensure that the wounds are kept moist and not allowed to dry out. It is reported in the literature that using bacitracin ®, hydrogels or synthetic dressings based on silicon or hydrocolloid dressings reduces the sensation of pain compared to paraffin based

wound dressings such as those fabricated from cellulose or cellulose acetate in a mesh structure and gauze dressings, which can adhere to the wound site and cause trauma (Eskes et al., 2011).

An important aspect regarding skin grafting concern from which part of the anatomy is the skin being harvested (Reed, 1995). In addition to considering the extent and location of the burn injury, other factors such as age, gender, demographics and skin thickness of the patient need to be taken into account (Gambichler, 2006; Giacomoni, 2009).

The literature reports that in most cases if available skin is grafted from the waist of males (Falabella et al., 2005) and from the thighs and hips in the case of women. The reasons behind selecting these anatomical site is because fat is usually stored in these areas. Other possible anatomical sites include the thigh, buttock, instep, forearm, upper arm, scalp, back, chest, flank, abdomen, calf and shin.

Skin thickness on the chest and back is slightly greater compared to other parts of the body. It has also been found that skin thickness depends on gender in certain cases and males usually have thicker skin than women (Gambichler, 2006). It is well known that epidermal thinning of skin occurs with age as a natural phenomenon, but in certain demographic situations it has been found that skin actually becomes thicker with age (Sauermann et al., 2002) because there is variation in skin thickness depending on demographics and age, and because there is variation in the thickness of skin graft due to performing the surgery with different

dermatomes, scalpels and knifes it is difficult to predict the rate of healing of donor site wounds.

Another aspect regarding donor site wound management relates to whether or not it should be kept moist. The literature reports that in certain clinical studies (Field et al., 1994) wounds, which were kept moist and hydrated, healed faster with fewer complications compared to those wounds that were allowed to dry out and form a scab over time.

It is also important from surgical point of view to know that when wounds are exposed to the surrounding environment bacterial growth and infections are a major concern particularly for diabetic and pediatric patients. If bacterial infections persist underneath a dressing, then healing will be delayed. Various methods are employed to take microbiological samples while maintaining a protected healing environment. Punch biopsies, visual inspection, swab tests, serological tests, CBS (Complete blood count), CRP (C-reactive protein), ESR (Erythrocyte sedimentation rate) and curettage methods are currently utilized at different burn centers (Falabella et al., 2005). As a result various antibacterial treatments are used to ensure effective protection at the donor site. The use of metallic ions and other antibacterial and antimicrobial agents can effectively reduce the bacterial burden, but they do not eliminate the need for skin biopsies or microbiological testing. Researchers at the University of Bath in the UK have developed an innovative way to identify the presence of (Zhou Jin et al., 2011) bacterial colonization in the wound. The bacteria react with a carboxyfluorescein

dye in the dressing, which then changes color. This color change directs the nursing staff to change the dressing.

Other dressings that have been utilized for healing donor site wounds and other burn wounds are tissue engineered dressings such as dressings made by Integra ® which is a matrix of purified bovine collagen mixed with glycosaminoglycan along with polysiloxane. Integra® dressings have been utilized for the healing of ulcers, burn wounds and even full thickness and partial thickness donor site wounds (Wainwright et al., 1995). Integra ® a cross-linked matrix has been quite popular in wound care for a long period. Currently another tissue engineered product called Strata graft ® is being developed and researched by Strata tech Corp under the guidance of Dr.Hoffmann. This patent (US 746244 B2) (Conrad, Ivarie, and Allen-Hoffmann) talks about utilizing genetically modified human keratinocytes, which are utilized to culture allogeneic skin grafts. Genetic modification allows the cultured keratinocytes and skin graft to be free from any immune response. Currently clinical trials of genetically modified keratinocyte skin graft substitutes have already had success and have been approved by the FDA under the orphan drug category. The burden of utilizing skin grafts from autologous donor sites for the treatment of burn wounds is definitely going to be reduced in the future. Another technique called cellular spray therapy was developed by Gerlach (Gerlach et al., 2011). It requires a punch biopsy to be taken from patient and the harvested kerinocytes and stem cells are then cultured and allowed to grow for 2 to 5 hours. These cultured cells are then sprayed on the wound using a spray gun. Currently this technology is being practiced by McGown Institute of Regenerative Medicine and by Avita

Medical Inc. under the name of Recell ® (Wood et al., 2012). Cell spraying has been successful and positive results can be seen even in the case of second-degree burn patients within 24 hours.

2.4.2.6. Future of Wound Dressings:

Since there is an exhaustive list of wound care products that are being created from scratch and are currently being developed along with tissue-engineered products, this creates challenges for clinicians and wound care staff. With the advent of biological and tissue engineered products currently being developed one can expect the use of biomaterials more as hybrid materials which will support cellular component growth and proliferation, and at the same time will allow for antibacterial and antimicrobial properties. Biomaterials, which are non-toxic and can be obtained from biological sources, need to be tested for this purpose along with other materials to develop future wound care products. These products need to be developed keeping in mind the conditions of storage and the cost associated with the shelf life of the dressing. Otherwise skin grafting will continue despite the reported issues and problems.

2.5. Chitin and chitosan:

Chitin is the second most abundant natural polysaccharide after cellulose. Chitin is a hard and rigid component found in various forms of living organisms, animals, insects and plants. For example as the material of crab shells, it supports and protects organisms. The first ever

discovery of chitin took place in 1811 when derived from mushroom it was named "fungine". Later it was discovered in plants and insects and was termed chitine (Habibi et al., 2012).

2.5.1. Chitin and Chitosan Chemical Structure and Properties:

Chitin is a mucopolysacharide with chemical formula of poly β-(1-4)-2 acetamido-2-deoxy-D-glucopyronose (Ravi Kumar et al., 2000) , (Roberts et al., 1992). Chitin and cellulose have similar structural forms (Figure 6, Rinaudo etal., 2006). While chitosan is a derivative of chitin and has been reported to have amine groups that give it an added advantage of antibacterial properties and hemocompatibitiy.

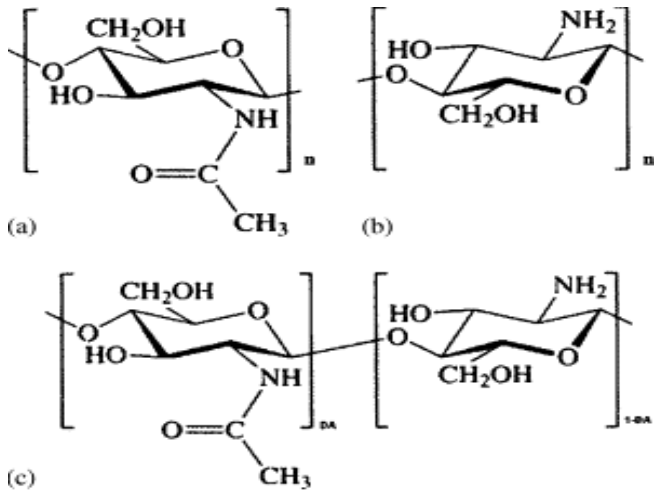
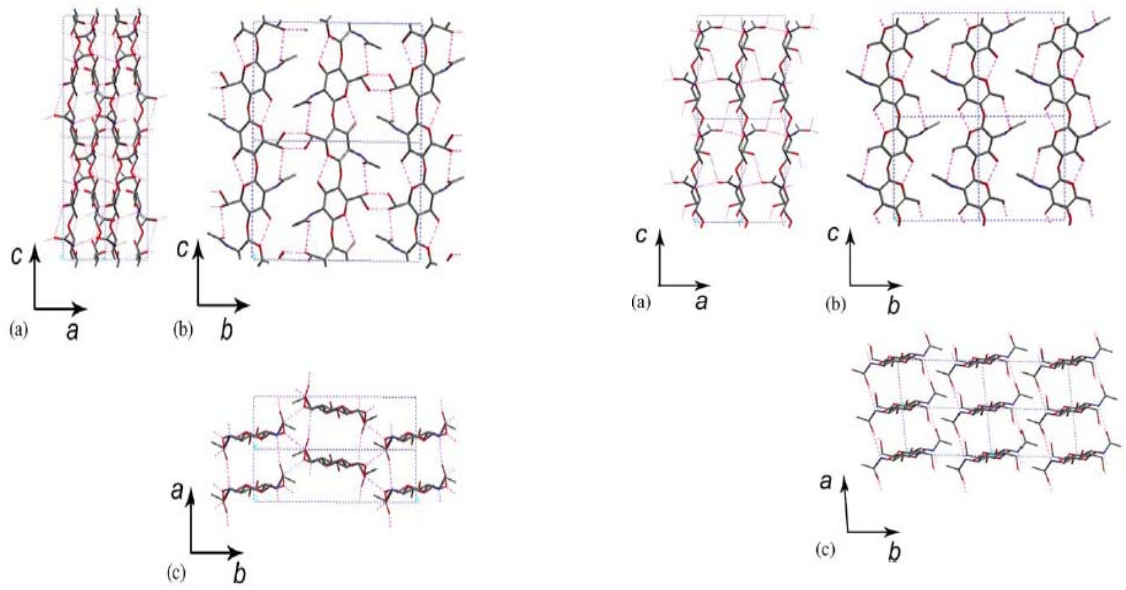


Figure 6: Chemical structure of (a) Chitin poly (N-acetyl-β-D-glucosamine, (b) chitosan (poly(D-glucosamine) and (c) partially acetylated chitosan (Rinaudo et al., 2006).

The chitin structure found in nature can be either in the $\propto, \beta \; or \; \gamma$ form. The ∝-chitin structure is the hardest and most stable form of chitin found in nature. Its rigidity is due to the extensive amount of intermolecular hydrogen bonding (Figure 7a). The $\propto -$chitin has a crystalline structure, which has been described in previous studies (Rinaudo et al., 2006). The γ- structure has been found to have similarities to the β-chitin. The β-chitin structure has an anti-parallel arrangement to chitin chains in a structure that allows for weak intermolecular hydrogen bonding between chains (Figure 7b). β –chitin is found in squid pens, also known as squid exoskeleton or backbone. The parallel arrangement of chains in β-chitin is different from that of γ-chitin chains which having an arrangement with two chains up and one chain down (Roberts et al., 1992) provides a more flexible and stable structure as compared to α-chitin. β -chitin is an easier form of chitin to dissolve but if harsh chemistries are utilized along with boiling temperatures then the β-chitin structure reverts to ∝-chitin, which is the more stable structure. β and γ structures are unstable when treated with 6 M HCl or formic acid. In both cases the structure changes from β or γ chitin to α chitin arrangement if treated for an extended time. It has been reported that once the α-chitin arrangement has been achieved it is irreversible (Roberts et al., 1992).



(i) (ii)

Figure 7: Structure of α-chitin (i) and β-chitin (ii)

Chitin is utilized for a number of applications. It is a good chelating agent and has a much higher amount of nitrogen content in comparison to cotton. Chitosan is a derivative of chitin extracted after N-deacetylation. Due to the importance and value of chitosan in agriculture, filtration and medical applications, different chemical reactions have been tried to derive chitosan from chitin structure (Roberts et al., 1992), (Ravi Kumar et al., 2000). Since β-chitin structure has better flexibility and weaker intermolecular hydrogen bonding then α-chitin, milder chemistries such as calcium chloride and methanol treatments (Tamura et al., 2006) have been utilized to develop β-chitin/chitosan hydrogels. This approach usually ensures that the chain structure is not lost and the chemical and mechanical properties and shape of the

structure are retained. Chitosan has been used traditionally for the development and reconstruction of soft tissues and products for medical usage because of its biodegradable properties, biocompatibility, hemostatic effect, antimicrobial activity and anti-inflammatory activity (Habibi et al., 2012).

2.5.2. Medical related usage:

Chitosan is used as a biomaterial for various applications. As an anti-inflammatory drug it is used at large for healing of intestinal wounds and for the construction of scaffolds for bone, cartilage and nerve regeneration as well as wound dressings and drug delivery systems (Habibi et al., 2012), (Yao et al., 2012).

Tissue engineering is an aspect where chitosan usage has been reported. Commercial products that utilize chitosan as a biomaterial and have reached the world market are:

1. Chitoflex and Chitogauze developed by Hemcon ® (Patent US 8,269,058 B2) (McCarthy et al., 2012) utilizes lyophilized chitosan extracted from crab shells along with a mesh backing made up of an absorbable polyester layer that is woven. Lyophilization of chitosan is done under extreme pressure.
2. Tegasorb is a 3M, product developed in the early 90's (Patent US 6864245 B2) (Holm et al., 2013). It has polyurethane backing, which is joined to the chitosan with an acrylic adhesive.
3. Syvek and mRDH are two dressings that contain chitosan (poly-N-acetyl glucosamine) and chitin (poly-N-glucosamine) developed by Marine Polymer Technologies Inc, USA. These dressings have a nonwoven foam backing that is also lyophilized and is quite similar to the Hemcon ® dressing (Habibi et al., 2012).
4. Chitopack C ® utilizes chitosan from crabs. It is manufactured by Eisai Co ltd Japan and is used largely for orthopedic implants and products for veterinary therapy.

There are additional products being developed commercially and extensive research continues to develop products utilizing chitosan.

2.5.3. β- Chitin/ Chitosan Structure:

Since a number of commercial products are available for tissue engineering there is a preference to utilize the β-chitin/chitosan structure with its greater ease of solubility. Theoretically the β-chitin structure undergoes chain scission more easily compared to α and γ chitin **(Figure 7a & b)**. It is predicted that with global demand for chitosan will continue of rise as it is a sustainable polysaccharide. It is important to understand which of these alternative structures of chitin are obtained from various sources and species. β-chitin/ chitosan can be obtained from the exoskeleton of loligo and Dosidicus gigas squid. It has been reported that β-chitin is able to retain a greater amount of moisture compared to α-chitin. It is important to analyze the proteins associated with the different sources of chitin found in nature to understand how biomaterial interaction takes place. The antibacterial activity and initial thrombin formation of chitin also will be studied to see how variations in properties are achieved by different sources of the same structure. For example, the sulfation of chitin has been reported to increase the rate of thrombin formation (Tokura et al., 1994).

CHAPTER 3: RESEARCH METHADOLOGY AND EXPERIMENTS

The initial purpose of this study was to identify and record clinical perspectives and differences relating to the management of donor site wounds. This led to a study of commercial chitosan and silver based wound dressings, which were compared with the prototype chitin/chitosan sponges from different squid sources. An experimental approach was taken to determine which chitin biomaterial could be used for a donor site wound dressing.

To achieve these objectives our study was divided into two phases:

Phase I:

1. We developed and designed a survey that solicits information about donor site etiology and management practices to treat donor site wounds. The survey questionnaire was administered to clinical personal and surgeons in person, which generated a return rate of 98 %.
2. We analyzed the data obtained from the questionnaire so as to identify which wound dressing materials were most popular and used most often.

Phase II:

1. We evaluated currently available commercial chitosan and silver based wound dressing materials.

2. We extracted, purified and deproteinised chitin/chitosan derived from two different squid sources.
3. We developed chitin/ chitosan hydrogels and tested their structure and mechanical performance.
4. We evaluated the hemostatic properties of the hydrogels.
5. We evaluated the antibacterial properties of hydrogels and compared them to commercial products.
6. We defined and proposed the future use of resorbable hydrogel materials together with smart materials to address the problems pertaining to donor site wound etiology.

3.1. Survey Instrument Design and Preparation:

Donor site wounds or split thickness skin graft wounds have a specific wound etiology that needs to be understood so that the appropriate therapeutic conditions and clinical management protocols can be established for optimum healing. At the present time no optimal or standardized therapeutic practice has been established, and there is no clear consensus in terms of what type of dressing should be used for donor site wounds. For this reason a survey questionnaire was designed to identify the range of different therapeutic approaches practiced at different burns unit in the USA. The questionnaire instrument was designed on Adobe Acrobat forms using the central online resource at www.acrobat.com/formscentral/en/home.html. The survey instrument was divided into a total of 9 questions. Most of these questions were subdivided into parts. The questions were interconnected to one another so if a participant skipped a question then the questionnaire

would not allow the participant to move forward with the next question. Questions were asked about donor site wounds and the preference for different types of wound dressings being used on donor site wounds (Appendix A: Survey Donor site wounds and dressings).

The survey was designed to be completed by healthcare personnel who have direct experience working with burn patients. Nurses, doctors, surgeons, therapists participated in the survey. The questionnaire instrument encouraged the participating volunteers to include their comments, opinions and favorable outcomes with the use of open-ended questions.
It was important to know which wound dressings are currently used for donor site wounds so as to completely satisfy the patients clinical requirements. Participants were questioned about which dressing options they preferred. They were provided with a number of commercial options and asked to suggest other dressings other than the ones mentioned. The survey instrument included other questions related to the preferred anatomical location for harvesting skin grafts from men, women and children. It was also asked whether or not the donor site wounds should be kept moist and hydrated, and it asked the respondents to indicate their level of concern about the risk of infection occurring and what preventive measures they would take to avoid bacterial contamination.

In conjunction with this question respondents were asked how they would modify the management of donor site wounds for specific populations such as the elderly, children, diabetic and immunosuppressive patients.

It was anticipated that these questions would provide important and relevant information about current practices in the management of donor site wounds. At the same time the results will provide useful directives for identifying the specific requirements for the dressings rather than claim that generic wound care products are effective in treating all burn injuries as well as donor site wounds. The survey instrument is attached in Appendix A.

3.2. Administration of Survey and Evaluation of Results:

Prior to administering the survey, the questionnaire was reviewed by several members of the Department of Surgery and the Jaycee Burn Center, at the University of North Carolina – Chapel Hill, by the Director of the Burns Unit at Wake Forest Baptist Health Center and by the Clinical Trial Consortium. The Institutional Ethical Review Board at the University of North Carolina Chapel Hill gave its approval to the working of the survey instrument before it was administered.

The questionnaire was administered at the 25th Annual Southern Regional Burn Conference held in Norfolk, VA between November 15-18, 2012. Participants were from various burns and trauma centers located in hospitals and clinics from Florida to Washington, DC. There was representation from 14 States, with 31 burn and trauma surgery units participating in this study. The respondents were from various backgrounds, ranging from nurses, surgeons, interns, therapists, researchers and physicians, but they all had direct personal experience of working with the burn patients. Out of the 92 questionnaires that were distributed, 80 surveys

were returned and, 79 complete surveys were complete. This corresponds to a voluntary response rate of over 98 %.

Each completed questionnaire was entered into Adobe software, which collated the results and generated the average data. Other personal comments extracted from the surveys and opinions were collated using Excel software.

3.2.1. Development of Prototype Wound Dressing:

The results from the survey provided insight into the key requirements for a wound dressing suitable for donor site wound care. From among the various biomaterials that would be considered suitable for fabricating a prototype donor site wound dressing the use of chitin and chitosan were considered attractive candidates.

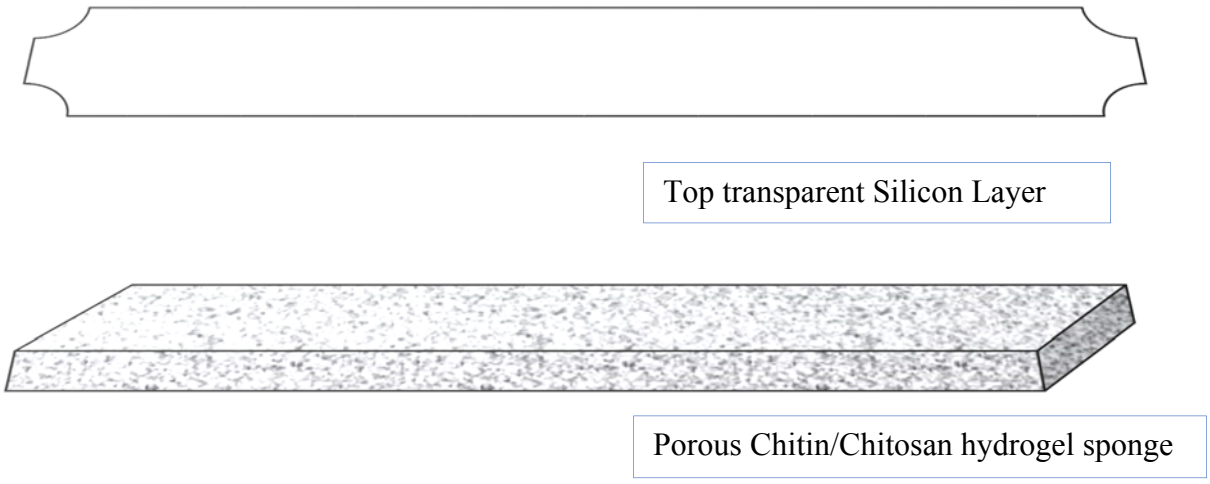


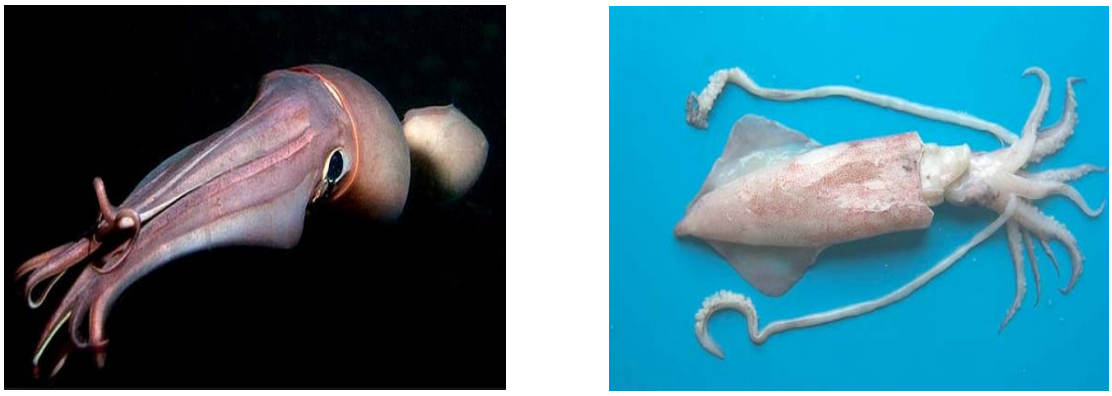
Figure 8: Ideal Donor site wound dressing.

In the current circumstances and limited by the availability of material chitin/chitosan sponges were created and tested thoroughly while polydimethylsiloxane and other siloxanes in combination will be tested later.

3.2.2. β-Chitin Raw Materials:

For our experiments two different sources of chitin were used. They were obtained from two very different types of squids. First were pens from the Humboldt squid also known as dosidicus gigas or giant squid the other pens were obtained from the loligo squid. Dosidicus gigas squids vary from 4 meters to even 40 meters in length (Figure 9a). Stanford Marine Station researcher Dr. Gilly, provided dosidicus gigas pens taken from experimental animals caught along the California coast during the December 2012 – January 2013 catch season. These are one of the most amazing creatures found in the oceans. Giant squids can live deep down below 2500 meters in the ocean and can survive at low critical oxygen levels. These creatures only come on the ocean floor in the evening to eat. Humboldt squid pens are slender structures that extend from the gills to the stomach of the squid. As far as we know no previous research on Humboldt squid chitin pens has been performed. The second source of chitin was obtained from loligo squid pens. Loligo squid are smaller in size ranging from 0.5 to 1.5 meters in length, and the animals we studied were obtained by Dr. Joel Sohn of the Department of Biology, Harvard University, Boston from the sea off Japan and Thailand (Figure 9b). Dr. Sohn explained how analysis of the structure from pens can show the age of the squid. Once the chitin pens have been extracted from the squid similar to tree rings could be observed along the squid pen under an optical microscope. These lines are formed each

day and are associated with their nutritional regime hence they help to determine the age of the loligo squid.



(a) (b)

Figure 9: Showing (a) Humboldt squid and (b) Loligo squid

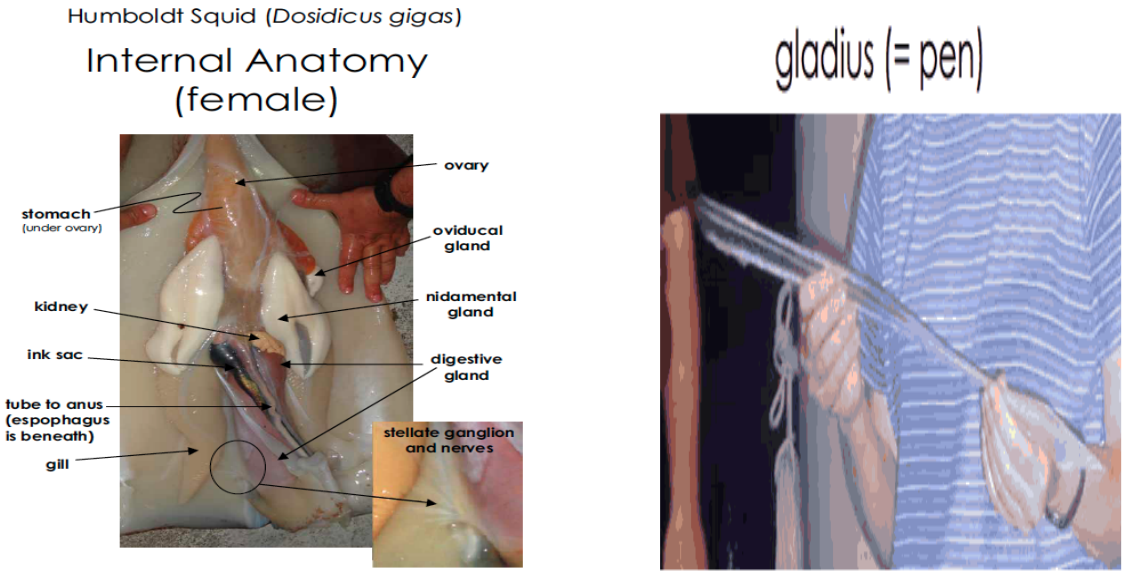


Figure 10: Anatomy of Squid and Squid gladius (pen) on removal.

On removal of the pens from the Humbodlt squids they were washed in 70 % aqueous ethanol solution and dried in a fume hood. The pens were packaged and stored in sealed plastic bags. The loligo squid pens were washed first in saline and then in deionized water before drying. Initial visual assessment of the loligo and Humbodlt squid pens showed that the loligo pens were hydrophilic aqueous transparent structures (Figure 11a), while the Humbodlt squid pens appeared to have a slightly brownish color (Figure 11b.)

(a) (b)

Figure 11: Image of Loligo Squid Pens (8X) and (b) Humbodlt Squid Pens (8X)

The following section describes how the chitin structures obtained from the loligo and Humbodlt squid species were solubilized, characterized and their properties compared.

3.2.3. Commercial Wound Dressing Materials:

The prototype chitin/ chitosan materials prepared from the squid pens were tested and compared against three other wound dressings Hemcon ® Chitogauze dressing was developed by freeze drying dissolved chitosan and compressing it under pressure to generate a sponge structure (Figure 12a.). The second material was a nylon spandex warp knitted fabric with an antimicrobial silver treatment developed by Silverclear ® (Figure 12b). The third material was a nano fiber mat made by electrospinning a mixture of chitosan and silver nanoparticles by Abdelrahman Abdelgawad at the College of Textiles, North Carolina State University The average diameter of the fibers was 120 nm and the ratio of chitosan to silver was 60:40 (Figure 12c.).

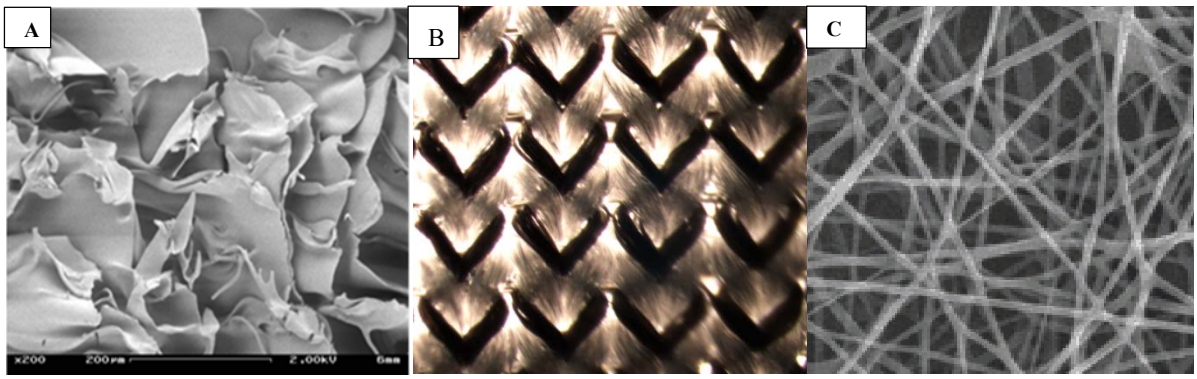


Figure 12: Commercial Products and research products (A) Hemcon ® Chitogauze, (B) Silverclear dressing, (C) Chitosan fiber web mat with Ag-nanoparticles.

3.2.4. Solubilizing Chitin/ Chitosan:

The squid pens received from both sources required purification since the chitin obtained from the exoskeleton has minerals such as $CaCO_3$ (calcium carbonate) and other attached proteins that need to be removed prior to being used for medical purposes. A series of chemicals, such as KOH, NaOH, $NaHCO_3$, Na_3PO_4, have been utilized to deacetylate and perform deproteinisation of chitin (Roberts). Impurities were removed by treating 5 grams of Humbodlt squid pens and loligo squid pen samples separately in 100 ml by 4 % NaOH solution at 80°C for 30 minutes. Low concentrations of NaOH were utilized to obtain chitin and observe its antibacterial and hemostatic properties. Samples were initially washed in 100 ml of distilled water to remove any adhering proteins. Later 1 % HCl (hydrochloric acid) was added to 100 ml of distilled water and the samples were kept overnight to ensure that any minerals adhering to the samples were removed. The samples were recovered from the bath next day and were washed with distilled water. The washed samples were collected and dried in an air dry oven for 2 hours to remove all the moisture present in the partially deacetylated chitin. The moisture content along with protein contents was obtained by performing protein analysis. Harsh chemical treatments that have been utilized previously to dissolve chitin were not utilized in this case.

The chitosan solution obtained from 5 % acetic acid was more viscous than with 10 % acetic acid and formed a more viscous solution with a slight yellow color. The dried pens from the loligo and Humbodlt squids were ground in an industrial grinder to obtain powdered chitin. It

was observed that the Humboldt squid had lower density compared to the loligo squid chitin sample. It was also observed that chitosan easily dissolved in 5 % - 90 % acetic acid.

An alternative solvent involving a calcium chloride methanol solution was prepared by adding 15 grams of calcium chloride dihydrate to 100 grams of methanol in a soxhlet reflux system and heating them to 60-80°C for 20 minutes (Tamura et al., 2006). The solution was stored overnight after filtering to ensure the impurities and undissolved particulates were removed. Two grams of chitin obtained from loligo squid and two grams of chitin derived from Humboldt squid were dissolved in this alternative solvent. Each sample was refluxed for 30 minutes at 60°C. It was observed during this experiment that the Humbodlt squid chitin turned to a dark brown color. It was thought that this could be due to inorganic ash content that was associated with the Humbodlt squid chitin. For example if chitin was heated beyond 60°C then it was treated carefully to ensure that excessive heating did not occurred. The chitin samples were then subjected to dialysis using Spectra/pore© membranes that had a molecular weight cut off of 6000-8000 Daltons. A 100 ml of sample was placed inside the closed membrane. Membranes were then placed in 2-liter beakers of deionized water to allow for the methanol and calcium chloride particles to move out through the membrane over time. The dialysate (distilled water) was replaced every 2 hours with fresh water. This procedure was repeated for 24 hours to ensure removal of low molecular weight impurities. The chitin solutions obtained at the end of this experiment were 50 % transparent and 50 % milky white in appearance.

3.3. Characterization of Chitin and Chitosan:

A series of characteristic properties of chitin and chitosan were measured to determine whether or not there were differences between the two different sources of chitin.

These properties included bulk density, pH, viscosity, FTIR spectroscopy, x-ray diffraction and protein analysis. Samples of chitin, chitosan solutions and dried chitin powder were analyzed. The chitin samples were centrifuged prior to lyophilization to ensure that the water content of the solution was reduced to 15 to 20 %.

3.3.1. Bulk Density Measurements:

The density of the chitin and chitosan samples was determined using Archimedes principle by measuring the amount of water displaced by the squid pens or chitin before deproteinisation compared with after deproteinisation. One gram of squid pen or chitin was added to a 100 ml volumetric flask which was completely filled with distilled water and weighed on a scientific balance to the nearest 0.1 mg. The amount of distilled water displaced was collected in a 150 ml beaker and weighed.

The density of the samples was calculated using the following equation.

$$Density = \frac{mass(g)}{volume(cm^3)} \qquad (1)$$

3.3.2. pH Measurements:

The pH values of various concentrations of chitin and chitosan samples were measured using a Thermo Scientific Orion Star pH meter. The readings were taken at 21.1($\pm$0.06) °C once the pH electrode had been calibrated.

An important reason to know the pH of the chitin/chitosan is because these biomaterials are known to have antibacterial and anti-inflamatory properties within a certain pH range on the acidic side. At too high or too low a pH these biomaterials will provoke an unacceptable inflammatory response and cause irritation. In addition chitosan has significant conductivity at high and low pH values. As seen in Figure 13 this property is lost at neutral pH.

At high pH values in the presence of alkali or in the presence of acid the hydroxyl groups control the properties of chitosan (Lim, S., 1971), (Kulicke et al., 2004). The pH of biomaterials can also impact the healing of skin (Schmid-Wendtner et al., 2006).

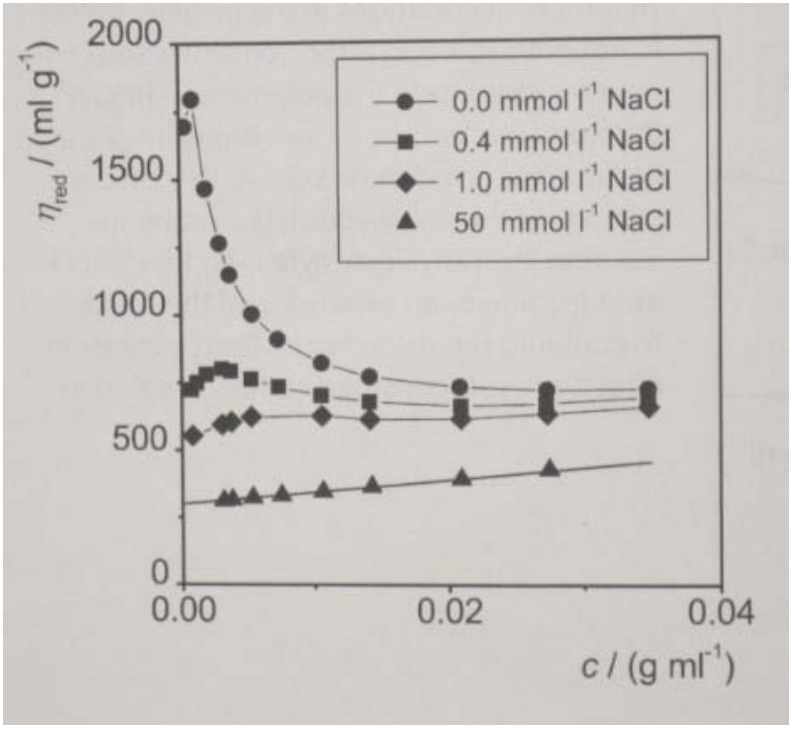


Figure 13: Conductometric titration curve for chitosan at varying concentration of Salt (Kulicke et al., 2004).

3.3.3. Viscosity Measurements:

Viscosity measurements of the chitin solutions were performed using a Cannon Ubbelohde C7-100 viscometer. Whereas a Cannon Ubbelohde 1B-L459 viscometer was used to measure the chitosan solutions. Sample solutions were first filtered through Millipore© filter (pore size: 0.4 um) in order to remove undissolved chitin particles that might cause blockage of the capillary. Ten ml of these filtered solutions were transferred to the appropriate viscometer

and the time of flow readings were taken at different dilutions. Measurements were performed at room temperature, 20.0 ±0.5°C.

Humboldt and loligo chitin samples were successfully dissolved in the calcium chloride/ methanol solvent system at 60°C. The solutions obtained were fairly viscous and transparent. However as soon as chitin solutions were brought to room temperature or to 37°C, the samples turned opaque as if the chitin was precipitated out at these lower temperatures. In addition, when these chitin samples were dialyzed, it could still be seen that the samples were opaque. However the chitin/chitosan samples did dissolve completely in acetic acid and the solutions were found to be transparent and more viscous and stable at 37°C. Measurements were performed using the Ubbelohde viscometer and the intrinsic viscosities of the solutions were calculated using the Huggin's and Kraemer equations. The Huggin's method involves extrapolating the following equation to zero concentration (Huggins, M., 1942), (Kraemer, 1938), (Kasaai et al., 2007).

$$\frac{\eta_{sp}}{c} = [\eta] + k_H[\eta]^2 c \qquad (2)$$

Where η_{sp} indicates the specific viscosity, which is calculated by dividing the difference between the efflux time at a certain concentration and the efflux time of the solvent, by the efflux time of the solvent. The concentration is noted as c, and the intrinsic viscosity is

represented by $[\eta]$. k_H is known as the Huggins constant and can be used to describe an ideal solvent. The Kraemer equation utilizes η_r the relative viscosity, which is calculated by dividing the efflux time at a certain concentration by the efflux time of the solvent (Curvale, Rolando, 2008), (Huggins, M., 1942).

$$\frac{\ln(\eta_r)}{c} = [\eta] + k_K[\eta]^2 c \qquad (3)$$

The Huggin's and Kraemer equations utilize the linear relationship between the concentrations of dilute polymers and the viscosity of their solutions. Intrinsic viscosities were found in both cases by extrapolating the equation to zero concentration (Chen, Rong Huei, 2009), (Curvale, 2008). Attempts to measure the viscosity of chitin/chitosan solutions dissolved in calcium chloride/ methanol solvent generated large variations in times and thus their viscosity could not be measured with confidence.

3.3.4. FTIR Spectroscopy:

Fourier Transform Infrared Spectroscopy was performed on 1-gram specimen of loligo and Humboldt squid chitin and chitosan. A Thermo Electron FTIR spectrophotometer with a Nexus 470 bench and continuum microscope was used to perform qualitative analysis. The infrared scans obtained were analyzed by comparision with the Spectra ^{TM} Database software for the presence of amine and amide groups. The presence of primary and secondary amine

group's was determined by analyzing the peaks at different wavelengths. In addition two characteristic amide bands were studied. The Amide I peak for C=O stretching vibration lies between 1695 – 1630 cm^{-1} and the amide II band of N-H has a stretching vibration in the 1560-1500 cm^{-1} range. It was anticipated that the chitosan derivative compound would be differentiated using infrared analysis. Deacetylation of chitin causes the formation of chitosan and the amount of deaccetylation is followed by the reduction and eventual disappearance of the amide I band. Also a primary NH bending band appears at 1650-1590 cm^{-1} because of the acetyl group conversion into amine groups.

3.3.5. X-Ray diffraction:

X-ray diffraction was performed to see how the chemical treatment of the chitin affected the order and crystallinity of the molecular structure. The unstable β – chitin structure has been known to experience modifications as it changes its structural form to α-chitin. X-ray diffraction was performed at the Analytical Instrumentation Facilty (AIF) at NC State University using Bragg-Brentano XRD – Rigaku Smart Lab.

3.3.6. BCA Protein analysis:

BCA (Bicinchoninic acid) protein assay is a standard test for identifying the protein content present in biomaterials and food products. The protein content is identified using the BCA protein assay in two ways. First one can use colorimetric variation, and second one can quantify the total protein content found in the substrate using a spectrophotometer. Two

different reagents are involved. Reagent A (Sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate) and Reagent B (4% cupric sulphate) are combined which causes reduction of Cu^{+2} to Cu^{+1} under alkaline conditions. An albumin standard with various dilutions is included so as to establish a calibration curve. The presence of any of the four amino acids (cysteine, cysteine, tryptophan and tyrosine, can cause a color change to purple. The BCA protein analysis was performed according to the protocol defined by Pierce ™ (Appendix B). The solutions were first filtered to remove any undissolved particles. The chitin/ chitosan solutions were then diluted to bring the change in color to within the range of the spectrophotometer. The procedure is summarized in Figure 14.

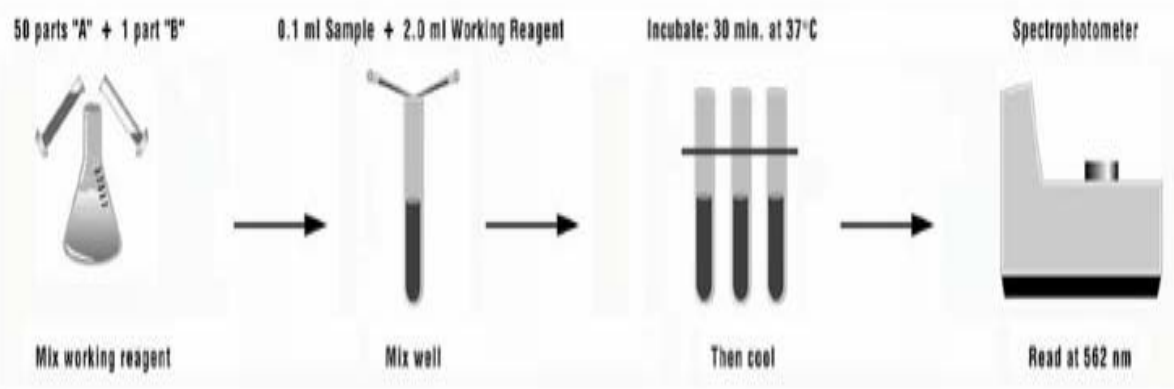


Figure 14: Preparation of BCA reagent and suspension of Samples.

Figure 15: BSA protein on left compared with protein content for chitin samples.

No previous studies have been found in the literature where chitin/chitosan have been analyzed for their protein content.

3.4. Preparation of Chitin/ Chitosan Hydrogels:

Hydrogel prototype samples containing a mixture of chitin/chitosan were prepared from loligo and Humboldt squid pens by first dissolving them in NaOH and later in calcium chloride / methanol solvent system. The excessive solvents were removed using Spectra pore™ dialysis tubing. Since the hydrogels prepared this way were both milky white and transparent at the same time, it was believed that this was due to the presence of both chitin and chitosan in hydrogels.

The next section describes how the loligo and Humboldt squid chitin pens using calcium chloride / methanol solvents originally described by (Tamura et al., 2006) were prepared from obtained chitin using a 4 step modified recipe.

3.4.1. Removal of impurities:

Any impurities and proteins found on the loligo and Humbodlt squid were washed off by soaking 15 grams of pens in a 4 % NaOH in a covered bath at 80°C for 30 minutes. The washed samples were then rinsed in distilled water and left to soak in distilled water with 1 % H_2SO_4 over night to neutralize the effect of the alkalinity and to remove other colored proteins. After rinsing in distilled water both varieties of pens were dried for 2 hours in a hot air oven at 50°C. Care was taken while drying not to overheat the samples and avoid the chitin charring and turning dark brown in color. The dried samples were then ground into a fine powder using an industrial grinder. They were collected and stored in separate sealed vials.

3.4.2. Dissolving Chitin/Chitosan:

Samples of chitin/chitosan in powder form were dissolved in a solvent mixture containing $CaCl_2$ $2H_2O$ and methanol combined in a ratio of 1:1. A 10 gram specimen of chitin/ chitosan powder was refluxed for an hour just above the boiling point of methanol within the temperature range of 70-80°C. This was repeated for each chitin/chitosan sample. When a volume of 100 ml solution was collected it was then pored into Spectra / Por ® Membranes

with a molecular weight cut off in the range of 6000-8000 Daltons. These Membranes with a 32 mm diameter had a capacity of 8-ml/cm length. Dialysis was performed by placing the tubing in distilled water in a 2-liter beaker with a magnetic stirrer to ensure transport of the solvent and impurities through the tubing. Effective dialysis was achieved by changing the distilled water every 2 hours. After 24 hours of dialysis a purified chitin/chitosan solution was obtained.

3.4.3. Freeze Drying:

Chitin/chitosan hydrogels were prepared from the dialysed solution by freezing them at -70°C in liquid nitrogen, and freeze-drying them by two different methods. In the first method directional freezing was achieved by pouring 50 ml vials of chitin/chitosan solution into beakers containing liquid nitrogen (Figure 16b.) (Qian and Zhang, 2011). The frozen chitin/chitosan solution in the 50 ml vials was then lyophilized for 60 to 72 hours in Forest Biomaterial Natural Sciences laboratory as shown in (Figure 17.) In the other method chitin/chitosan solutions were freeze dried after adding 10 % acetic acid. It was found that freeze-drying of chitin/chitosan along with acetic acid required lyophilization for a longer time.

Figure 16: Freeze-drying a) Freeze dryer used for freeze-drying, b) Liquid nitrogen used to freeze dry chitin/chitosan samples prior to freeze-drying.

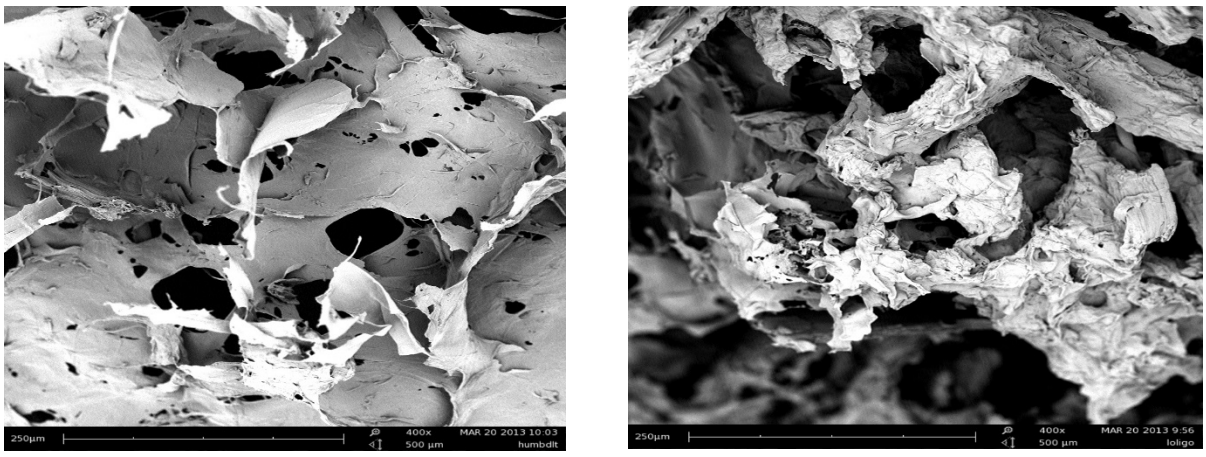


Figure 17: Showing Humbodlt and loligo squid chitin/chitosan hydrogel prepared by freeze-drying with 10% acetic acid prior to freezing

3.5. Performance of Hydrogels:

3.5.1. Thickness, Compression Resistance and Recovery:

The porous prototype hydrogels were evaluated for their ability to compress when under external pressure such as a supporting bandage or an applied film. A thickness gauge (SDL. International, Stockport, England) together with a vernier caliper was utilized to measure the loss of thickness under a series of applied pressures (Figure 18). Forces ranging from 0 to 500 gf/cm^2 were applied and released to observe compression, recovery and possible failure within a specific time period. If the sample recovered within a specific time after the weights had been removed then compression resistance value was noted. If recovery did not occur, or the samples collapsed then failure was noted. The thickness of samples was measured in mm using vernier calipers, which was able to provide experimental reading to the nearest 0.01 mm. Strain was measured in terms of percentage loss of thickness. The relationship between applied force and percentage loss of thickness has been plotted for the different samples. Five readings were taken from 5 specimens and the mean values were calculated for the each sample.

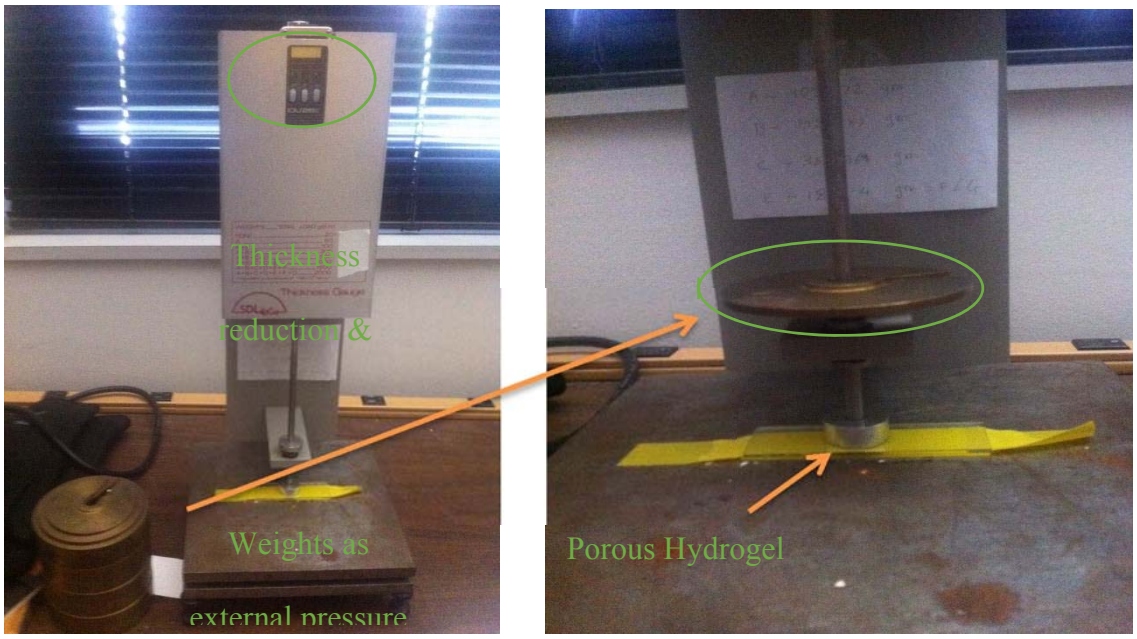


Figure 18: Thickness gauge (SDL, International, Stockport, England) with carrying weights being used to measure initial thickness compression resistance and recovery.

3.5.2 Antibacterial Activity:

The antibacterial activity was measured for both types of chitin/ chitosan hydrogel prototypes as well as three other controls using the zone of inhibition test as per AATCC-100 Standard for Antibacterial Textiles. Three control dressings such as Chitogauze ®, Silverclear ®, a silver antibacterial treated nylon dressing, and a chitosan mat with silver nanoparticles, were included so as to understand the bacteriostatic (growth inhibiting) effect. Details of this test method are described in the flow chart presented by Wiegand (Figure 19.) (Wiegand et al, 2008). The organisms used for this test was Escherichia Coli (E. Coli) bacterial strain (ATCC 25922). 1 ml of E.Coli bacteria were mixed with 9 ml of agar broth and kept shaking

overnight for 12 hours at 37°C. Agar plates were prepared using agar broth and poured onto 9 mm petri dishes. Next day E. coli cultures were checked for turbidity. 100 µl of E.coli was cultivated in 9 ml of broth and 1 ml of solution after suspension was spreaded using L-shaped glass rod onto agar plate to check initial bacterial concentration after culturing for 12 hours. The initial concentration was 35 * 10^8 CFU/ml, which was measured using a laboratory colony counter. 1 ml of bacterial solution from this stock bacterial solution was added to 9 ml of sterilized nutrient broth solution (0.8%). Dilutions were performed till a concentration of 35 *10^6 CFU/ml was obtained. 1ml of bacterial/nutrient solution was spread onto agar plates and incubation for 6 to 8 hours. Visualizing and measuring the zone of inhibition around each sample on Day 1, 2 and 5 was undertaken. This test was performed to understand the bacteriostatic effect of the different samples. Previously it has been shown that certain metal ions at an appropriate concentration can provide a bacteriostatic effect (Du et al, 2009).

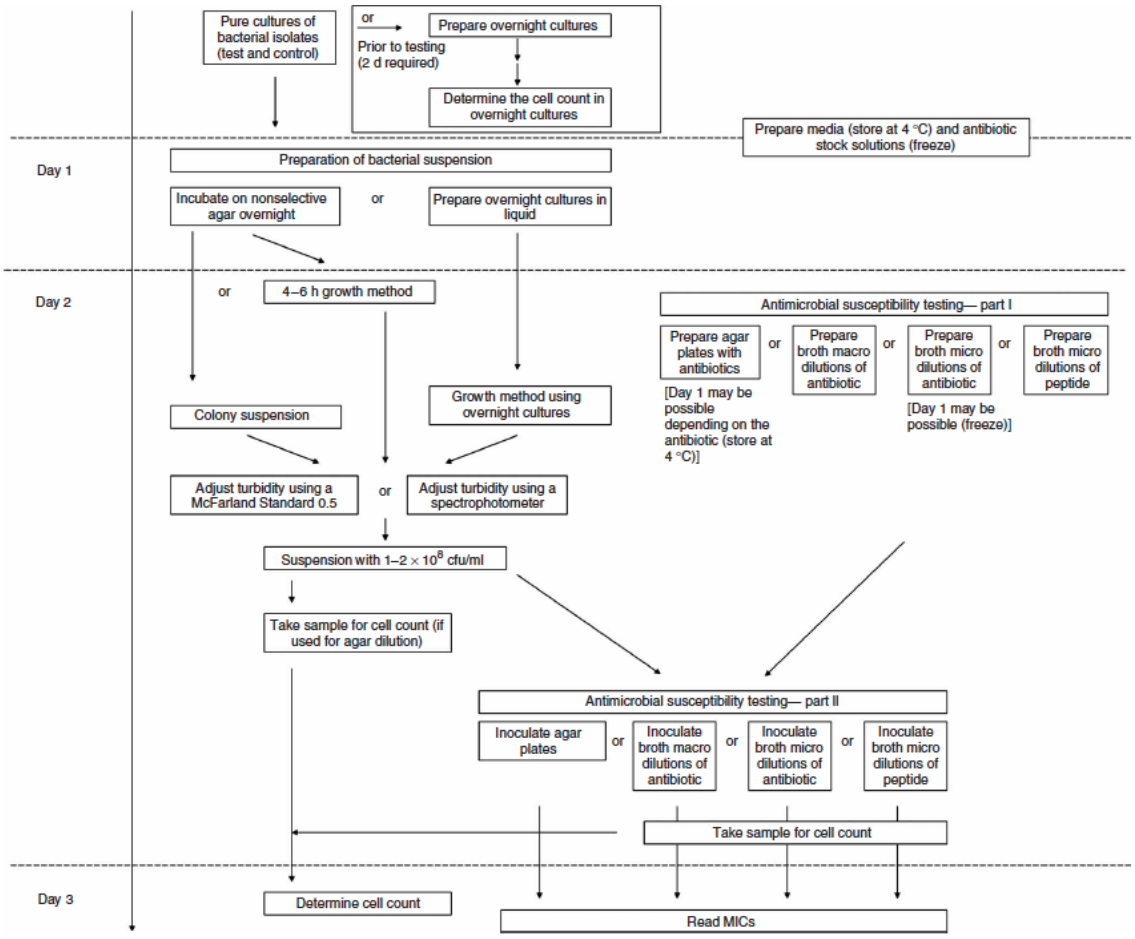


Figure 19: Flow chart on how to perform antibacterial culture assay (Wiegand et al, 2008).

3.5.3. Hemostatic Properties:

The hemostatic properties of two chitin/chitosan hydrogel prototypes and the three control materials, which include Chitogauze® dressing foam, Silverclear® nylon based dressing and chitosan nanofiber web containing silver nanoparticles, were measured in terms of their thrombin formation time **(**Chirag et al, 2011**)**. A 96 well plate was used with 1 gram of specimen in each well. The plate was blocked using 1:4 25% human albumin serum and citrated solution. 150 µl of the blocking solution was pipetted into each well plate (Appendix F) and the plates were sealed with parafilm and kept for 24 hours at 37°C. The next day the Parafilm ® and the solution were removed. Fresh blood was obtained the next day from the NCSU pig farm and the albumin was extracted by centrifugation. The Gen5 ™ (Biotek) plate reader was used to measure the time it took to cause initial thrombin formation. According to the coagulation cascade it requires a number of clotting factors to interact simultaneously to cause thrombin formation and the creation of a blood clot (A.S. Wolberg, 2007), (K.A. Tappenden, 2007).

3.5.4. SEM imaging of Coagulated Blood:

Scanning electron microscopy was performed on all five samples to illustrate how the material interacted with fresh blood. Fresh blood was dropped onto the surface of the samples at 37°C and to ensure maximum preservation of cells the treated samples were then fixed in glutaraldehyde solution and dried in a series of ethanol solutions (Appendix D) The prepared samples were then viewed under a scanning electron microscope JEOL JSM-5900LV (JEOL USA, Inc. Peabody, MA, USA) at an accelerating voltage of 1500 – 2000

KV so as to image of cell interactions and to observe how the clotting of blood took place on different biomaterials.

CHAPTER 4: RESULTS AND DISCUSSION

4.1. Survey Analysis:

The survey questionnaire was administered at the 25th Annual Southern Region Burn Conference to healthcare professionals who worked directly with burn patients. Participants from thirty-one different hospitals spread across fourteen different states covered in the Southern Region participated in this study. Eighty survey forms were distributed out of which 79 completed responses were received. A response rate of > 98 % was achieved. The survey questionnaires were distributed amongst conference attendees on the last day of the conference right after a session focused on burn patients healing experiences. The survey was completed by four different types of professionals: surgeons (30%), registered nurses (46%), therapists (10%) and others such as research assistants (14%) (Figure 20).

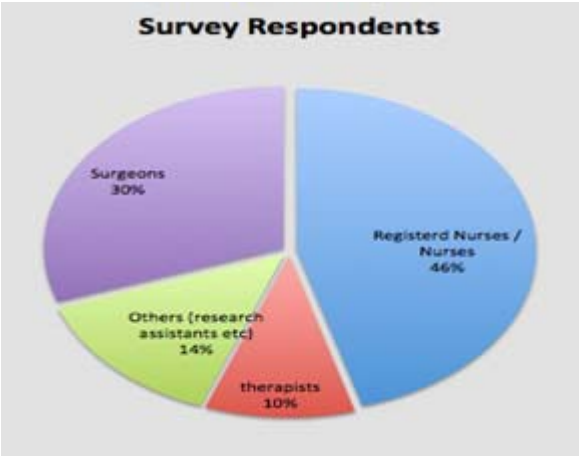


Figure 20: Breakdown of survey respondents

The survey respondents were asked whether or not they were satisfied with current treatments for donor site wounds that were being provided to burn patients who needed skin grafts. It was found that the response with regards to satisfaction and dissatisfaction with respect to current wound dressings varied widely (as shown in Table 2) amongst different types of professionals.

Table 2: Response with regards to satisfaction/dissatisfaction with current commercial dressings for donor site wounds.

Responses	**Surgeons**	**RN/Nurses**	**Therapists**	**Other Research Assistants etc.**
Satisfied	14 (58.3%)	24(66.7%)	5(62.5%)	8 (72.7%)
Dissatisfied	8 (33.3%)	11(30.6%)	2 (25.0%)	2(18.2%)
Neither	1	1	1	1
Total	24	36	8	11

Further, respondents were asked about their preference for dressings. It was learned from the various responses that there were in general two categories of dressings: dressings with metallic component such as silver or zinc, and dressings with non-metallic components, such as silicon and alginate. It was found that 67% of the respondents were in favor of silver based

dressings while dressings without silver or zinc were only preferred by 33 % of those surveyed (Figure 21).

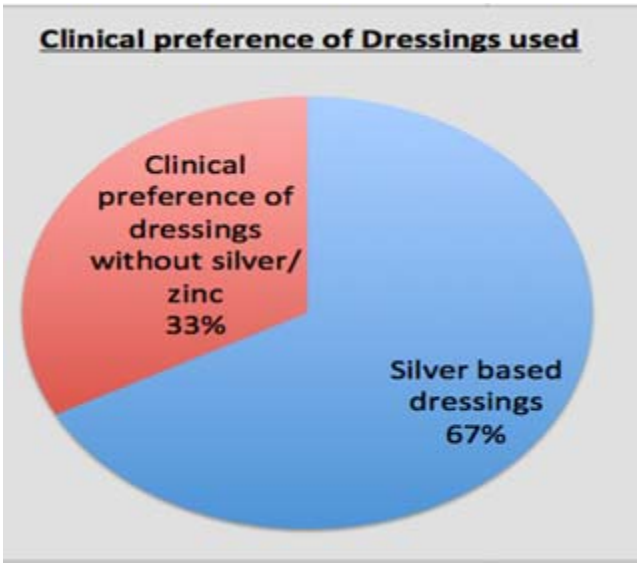
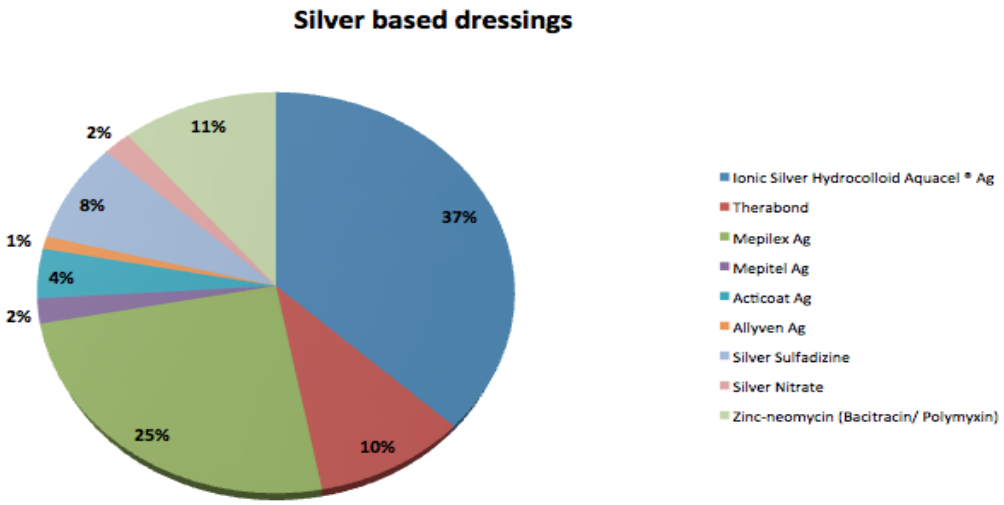
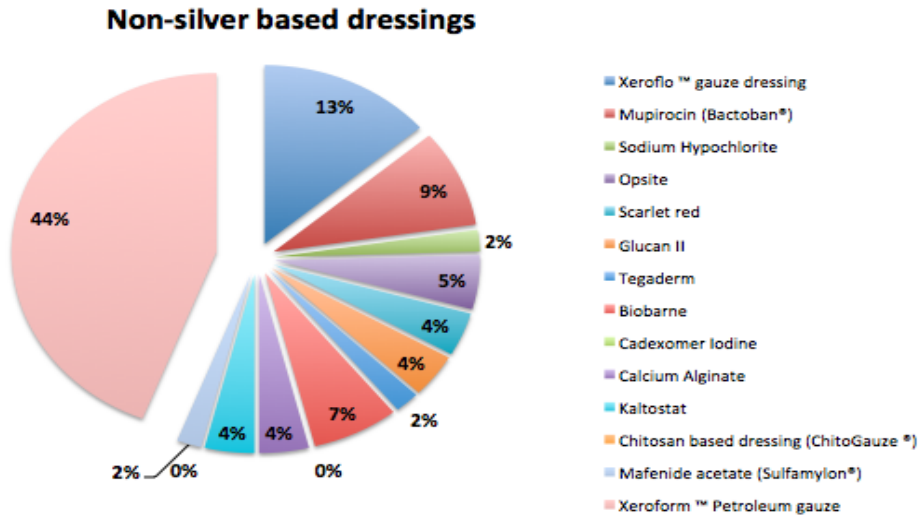


Figure 21: Dressing Preference.

It was found that amongst the silver based dressing's the ionic silver hydrocolloid Aquacel ®Ag dressing and the Mepilex Ag ® dressing were the most preferred. While amongst the non-silver based dressings the Xeroform ™ and Xeroflo ™ dressings were the most preferred.



(a)



(b)

Figure 22: a) Breakdown of preferred silver based dressings and (b) Breakdown of non-silver based dressings.

The survey then asked specifically about the harvesting of skin grafts taken from the patient's body. The responses confirmed that skin grafts were taken based on sex, age and the available anatomical site. Based on this information questions were asked about the preferred anatomical site for harvesting skin grafts from adults and children.

It was found that in the case of adults (Figure 23. a) and children (Figure 23. b) the preferred skin grafting site was the thighs followed by the back. The back was not preferred as a suitable anatomical site for children in most cases because of the greater movement of children, which could impair the healing of the donor site wounds. But if there were no other alternative sites available, then the back was recommended for skin grafting.

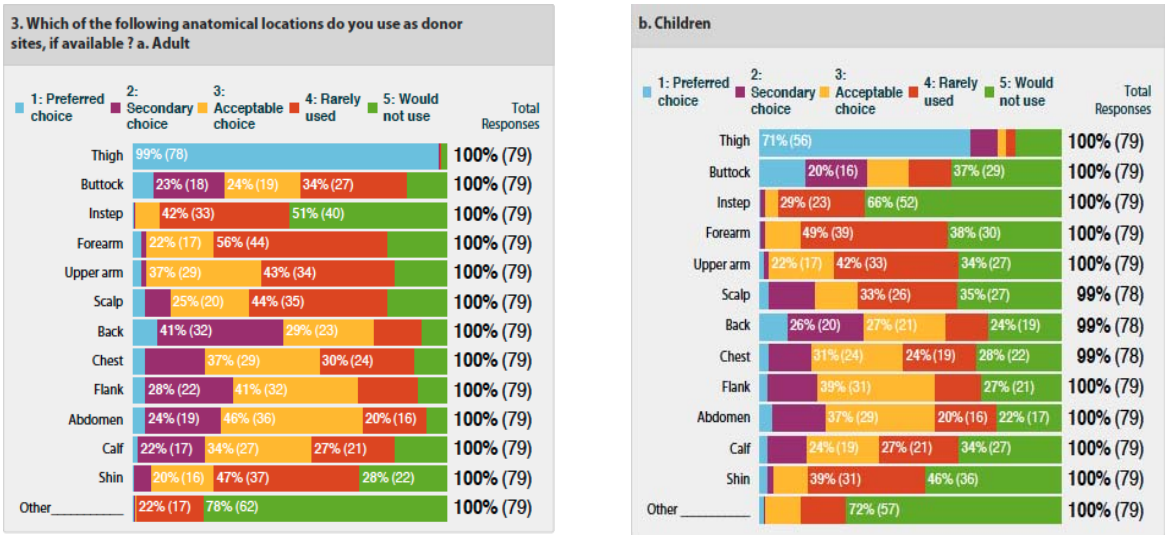


Figure 23: a) Respondents preferred anatomical site for skin grafting of adults, b) Preferred anatomical site for children.

It is important to understand the details of the wound physiology if one needs to develop a wound dressing for a particular type of wound. Since split thickness skin grafts vary in their thickness, and different practitioners harvest different thicknesses of skin grafts, some practitioners use thick skin flaps rather than grafts while others prefer medium to thin grafts. Thus respondents were asked about their preference with regards to the thickness of the skin graft that should be taken using a dermatome to ensure minimal damage to the donor site. Skin graft thicknesses were divided into three categories based on different thickness ranges: thin (0.125 – 0.275 mm, 56%), medium (0.275- 0.4 mm, 37%) and thick (0.4-0.75 mm, 7%) skin grafts (Figure 24).

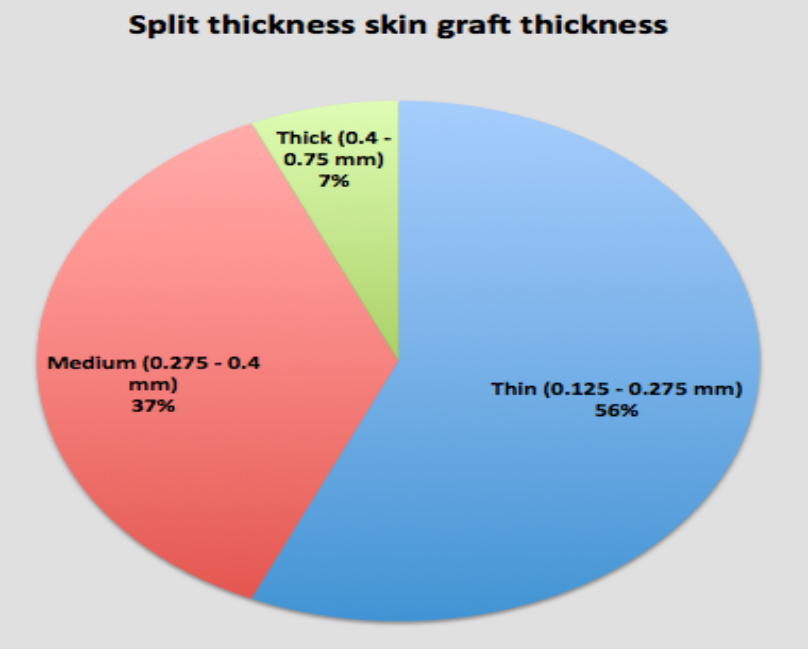


Figure 24: Shows split thickness skin graft variations.

Acute skin injuries and large wounds cause the production of exudate. The presence of exudate keeps wounds moist but at the same time it can become the breeding ground for bacteria and other microorganisms. Wounds can be kept dry with the absorption of liquid exudate but in such cases management of the dressing and its removal become complicated by the risk of tissue trauma. Thus a balance is required whereby the wound remains hydrated, but is not completely moist or dry. Respondents were asked about their preference for maintaining donor site wounds in a hydrated or dry condition.

Table 3: Response with regards to keeping donor site wounds hydrated or dry

Responses	**Surgeons**	**RN/ Nurses**	**Therapists**	**Other (research assistants etc.)**
(Hydrated) Yes	14 (58.33%)	14 (38.88 %)	5 (62.5 %)	6 (54.54%)
No	10 (41.66%)	22 (61.11%)	3 (37.5%)	5 (45.45 %)
Neither	-	-	-	-
Total respondents (79)	24	36	8	11

**Conflicting responses were recorded in this regard especially between surgeons and nurses (Table 3).*

It was interesting to note that surgeons mostly considered donor site wounds should be kept hydrated while nurses preferred these wounds to be kept dry.

The respondents were then asked about their experience with infections and the likelihood of the donor site wound becoming infected or not. The majority of the respondents to this question indicated that donor site wounds rarely (68%) become infected, followed by responses such as occasionally (19%) and never (13%). None of the responses said that wounds always or frequently become infected which suggests that infection of wounds can vary and is related to wound management procedures.

Skin type, demographics and various diseases have an impact on skin permeability, health and thickness. Respondents were asked if for specific populations, such as children, the elderly, diabetic patients or patients on immunosuppressive drugs required different care practices and regimens. The responses indicated that with regards to changing treatment options and regimens this did apply to children and the elderly, but in the case of immunosuppressive and diabetic patients the regular treatment regimens were usually maintained (Figure 25).

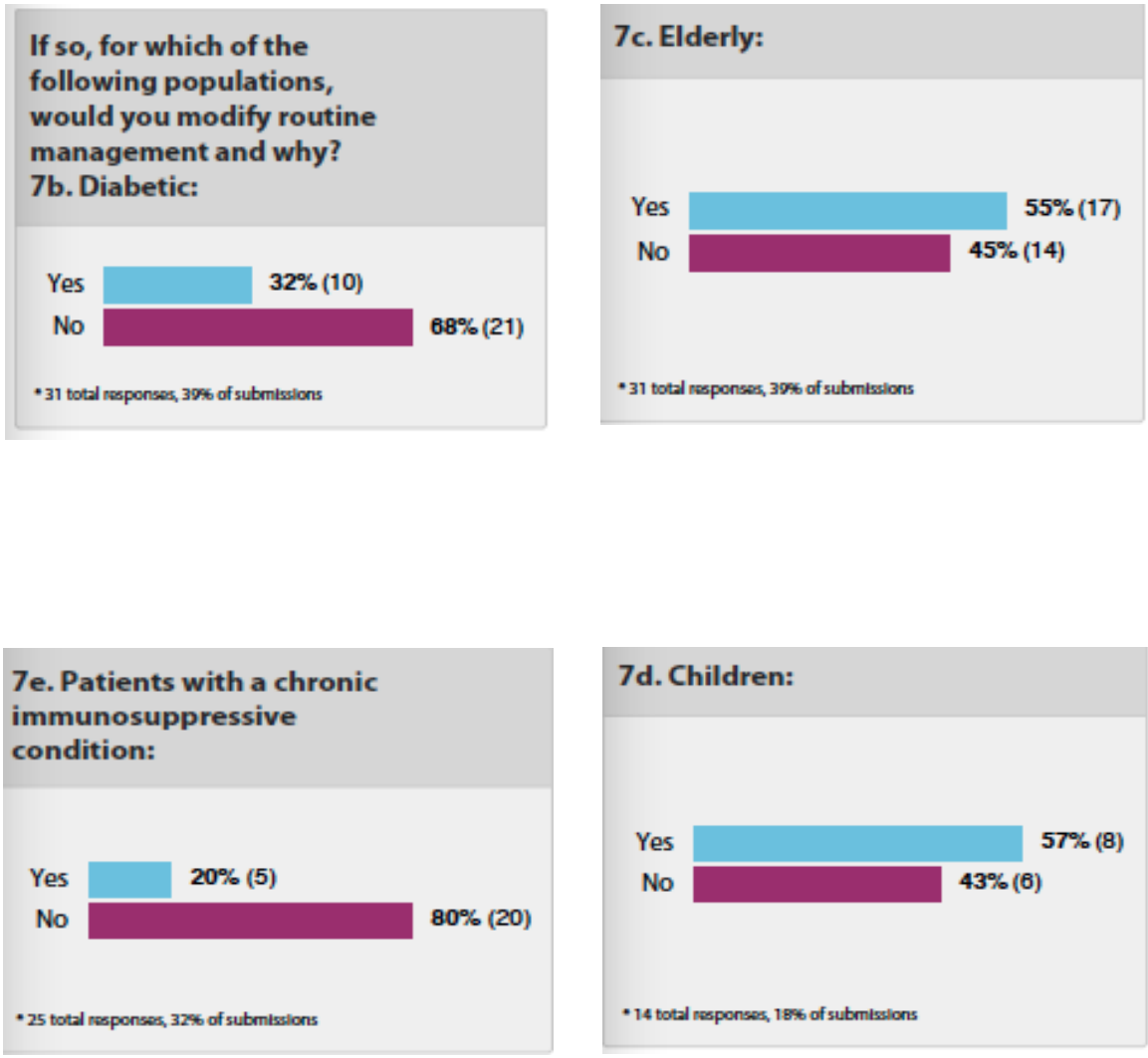


Figure 25: Responses on treatment modification for special populations

The respondents were asked about the key factors that inhibit healing of donor site wounds, and 11 different factors were identified. Amongst all the factors that were mentioned pain

was identified as the primary factor followed by other secondary factors. The other factors included hemostasis and bacterial colonization (Figure 26).

Concerns related to hemostasis, bacterial colonization, management of the wound site and the frequency of dressing changes did not appear in Figure 26 to be concerns as expected but factors related to pain were of major concern.

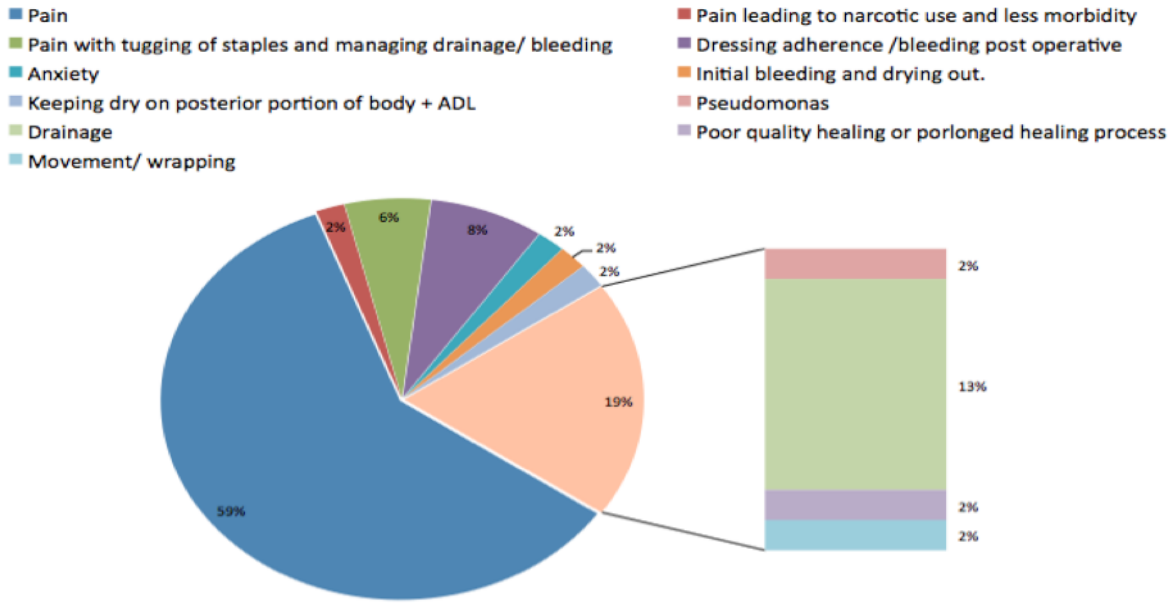


Figure 26: Factors inhibiting healing of donor site wounds

From our understanding of the literature review and from our knowledge of the various commercial dressings available on the market we listed some of the factors that may be

important to the successful function and performance of a wound dressing. In the survey the respondents were asked to rank characteristics based on their experience from most important to least important in terms of designing a donor site wound dressing (Figure 27). It was clear from the survey responses that pain was significantly highlighted as one of the biggest challenges, followed by the need for antibacterial properties; non-adherence preventing wound desiccation and having hemostatic properties.

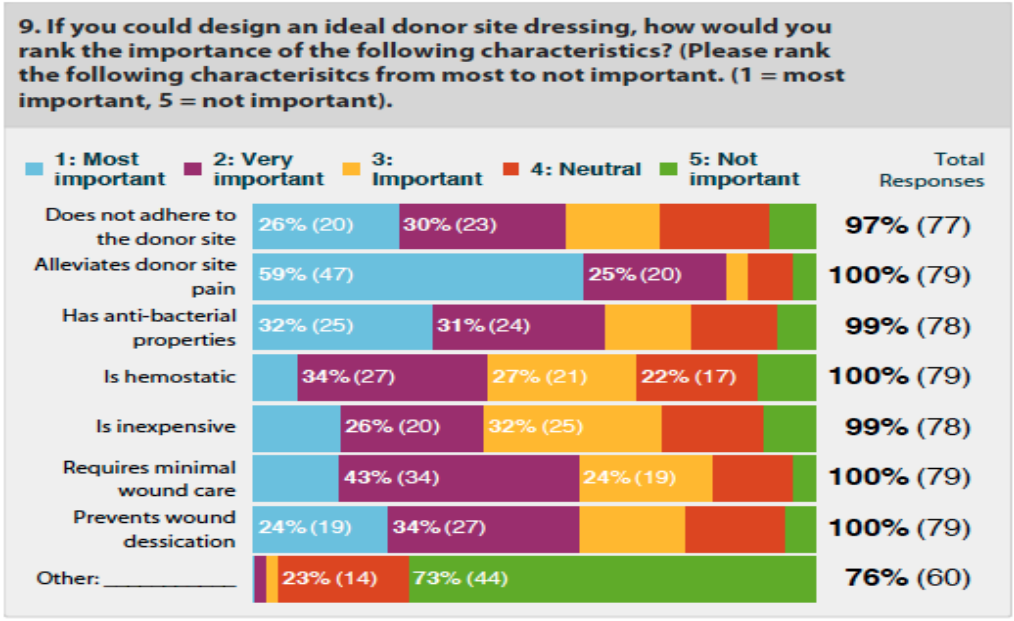


Figure 27: Characteristics and their importance for donor site wound dressing.

4.2. Characterization of Chitin and Chitosan:

In order to fabricate prototype chitin/chitosan hydrogels or sponges with the objective of designing a donor site wound dressing, samples of chitin/chitosan were obtained from the pens of Humboldt and loligo squid. In this section we report a number of the important characteristics that we measured in terms of bulk density, pH, viscosity, FTIR spectroscopy, X-Ray diffraction and protein analysis. We measured the compression resistance, the morphology / antibacterial activity and hemostasis of the two freeze dried hydrogels prepared from the Humbodlt and loligo squid pens.

4.2.1. Bulk Density of Chitin/Chitosan Samples:

The average bulk density of the chitin/chitosan samples obtained from the loligo squid pens was significantly greater than that measured on the Humbodlt squid (Table 4). The density for both samples was reduced after deproteinisation suggesting that surface proteins were washed away which may have decreased the mass of the squid pens.

Table 4: Average density values of chitin before and after deproteinisation.

Sample	**Density (g/ml^3)**
Humboldt squid Pen	1.1436 g/ml^3
Loligo squid Pen	1.3276 g/ml^3
Humboldt squid pen after deproteinisation	0.9814 g/ml^3
Loligo squid pen after deproteinisation	1.1266 g/ml^3

4.2.2. pH Values for Chitin/Chitosan Samples in Solution:

It is important to know the pH reading of the dissolved chitin/chitosan samples in solution since at extremely acidic or basic conditions these biomaterials cannot be used for medical purposes due to their cytotoxicity and unacceptable inflammatory response. It can be seen in table 5 that 5% and 10% acetic acid conditions produced chitin/chitosan solutions with pH values in the range of 3-4. These pH values on the acidic side are likely to contribute to the antibacterial activity of the chitin/ chitosan samples.

Table 5: pH measurements of various samples at 21.1°C.

Sample	**pH**
10% Acetic acid	2.35
1 g Loligo chitin/chitosan in 10% acetic acid	3.00
5% Acetic acid	2.45
1 g Humbodlt chitin/chitosan in 10% acetic acid	3.33
90% Acetic acid	0.06
1 g Humboldt chitin in 90% acetic acid	0.69
1 g Loligo chitin in 90% acetic acid	0.39
1 g Humboldt chitin in $CaCl2/CH_3OH$*	4.20

4.2.3. Intrinsic Viscosity of Chitin/chitosan Samples:

In Figures 28 one can observe that the relationship between viscosity and different concentrations for the loligo chitin in 90% acetic acid does not follow the linear Huggins and Kramer plot. This is because of the polyelectrolyte effect that occurs under both highly acidic and highly basic conditions (Kulicke et al., 2004) [46]. On the other hand one can see in Figures 29 and 30 that the values for intrinsic viscosity do coincide with linear Huggins and Kramer relationship. The average intrinsic viscosity values listed in Table 6 indicate the chitosan derived from the Humbodlt squid has higher viscosity values than that obtained from the loligo squid. This may point to a higher average molecular weight for the Humbodlt squid derived chitosan.

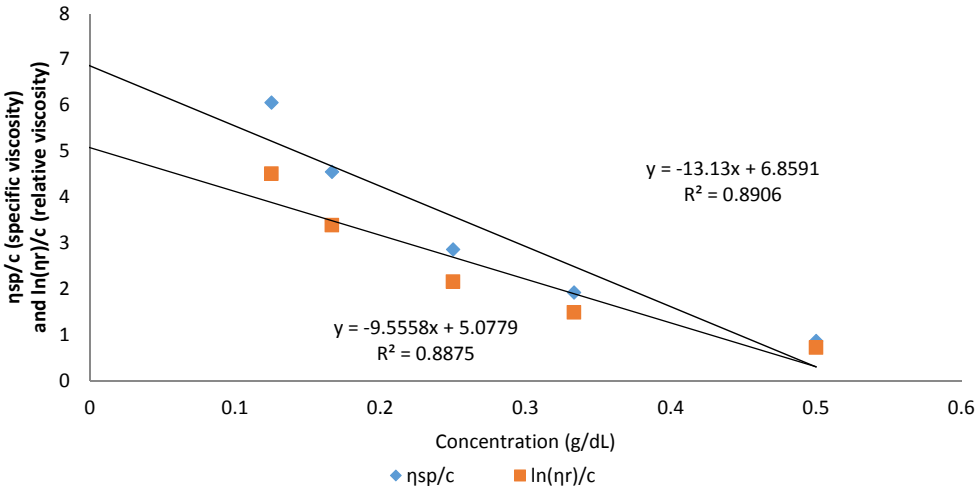


Figure 28: Huggins and Kraemer viscosity plot for Humbodlt Chitin in 90% acetic acid

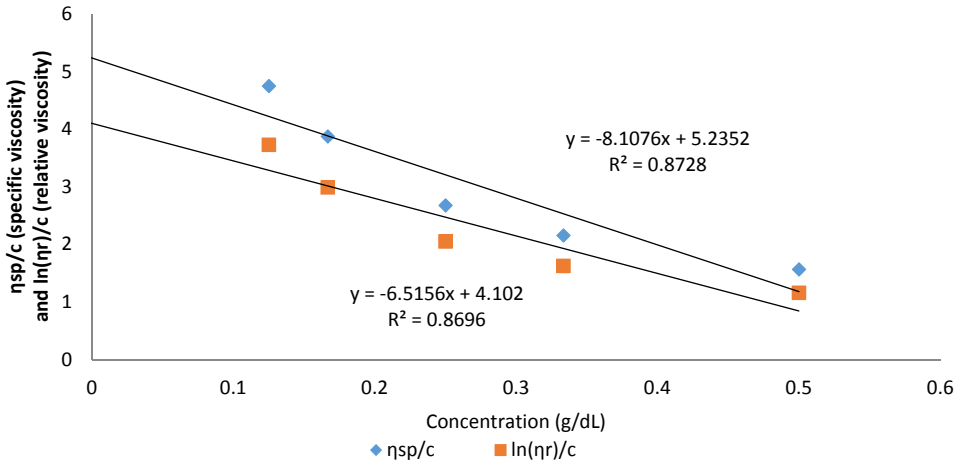


Figure 29: Huggins and Kraemer viscosity plots for loligo Chitin in 90% acetic acid

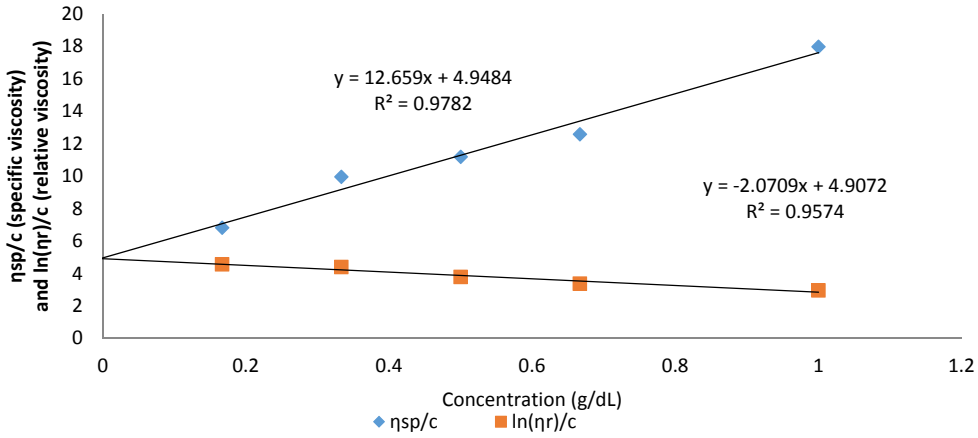


Figure 30: Huggins and Kraemer plots for Loligo Chitosan in 5% acetic acid

Table 6: Intrinsic viscosities and Huggins and Kraemer Constants for Various Samples

Sample	**Average [η]**	**Std. Deviation**	k_H	k_K	$k_H + k_K$
Loligo chitin in 90% acetic acid	5.969	0.891	0.279	-0.371	-0.092
Loligo chitosan in 10% acetic acid	4.669	0.567	-0.296	-0.387	-0.683
Humboldt chitosan in 10% acetic acid	4.928	0.021	0.517	-0.086	0.431

- *Measurements were taken prior to performing dialysis of solvents*.

4.2.4. FTIR Spectra of Chitin and Chitosan Samples:

Differences in chemical structure can be seen between the chitin samples derived from Humbodlt and loligo squid pens by comparing the FTIR spectra B and C in Figure 31. The Humbodlt and loligo squid chitin show the presence of amine peaks in the 1650-1580 cm^{-1} range, but their precise location and intensity are different primarily pointing to a difference in chemical structure. In spectrum A the loligo squid chitosan shows the presence of both amine and amide peaks. Amide peaks can be observed in the range of 3460-3400 cm^{-1}.

Primary amine peaks in the range of 1650-1580 cm^{-1} are also visible for all three different samples.

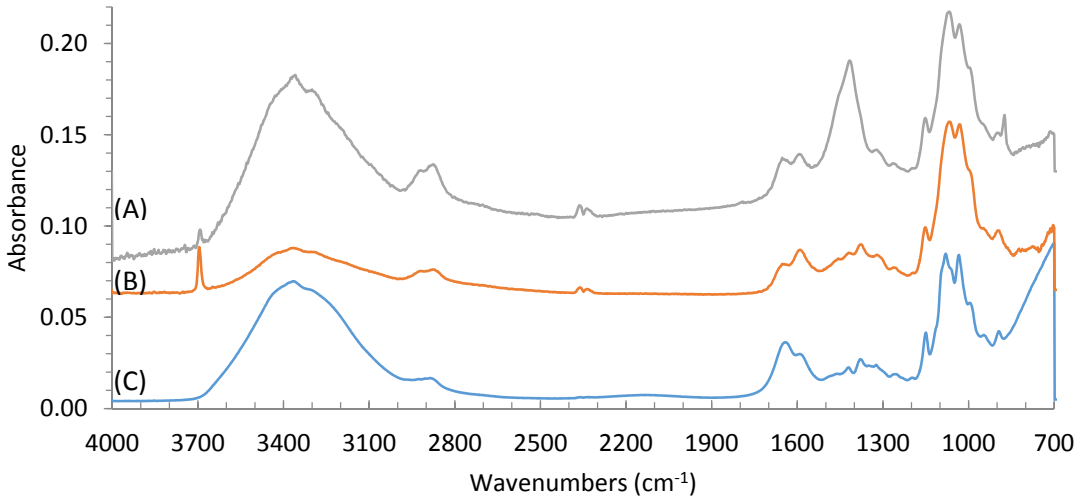


Figure 31: Typical FTIR of (A) Loligo squid chitosan, (B) Humbodlt squid chitin, (C) loligo

4.2.5. X-ray Diffraction:

The X-ray diffraction spectra for the two-chitin/chitosan samples derived from the Humbodlt and loligo squid are presented in Figures 32 and 33. From the relative position and width of the peaks these data indicate differences in the crystallinity and molecular order of these two original samples as received.

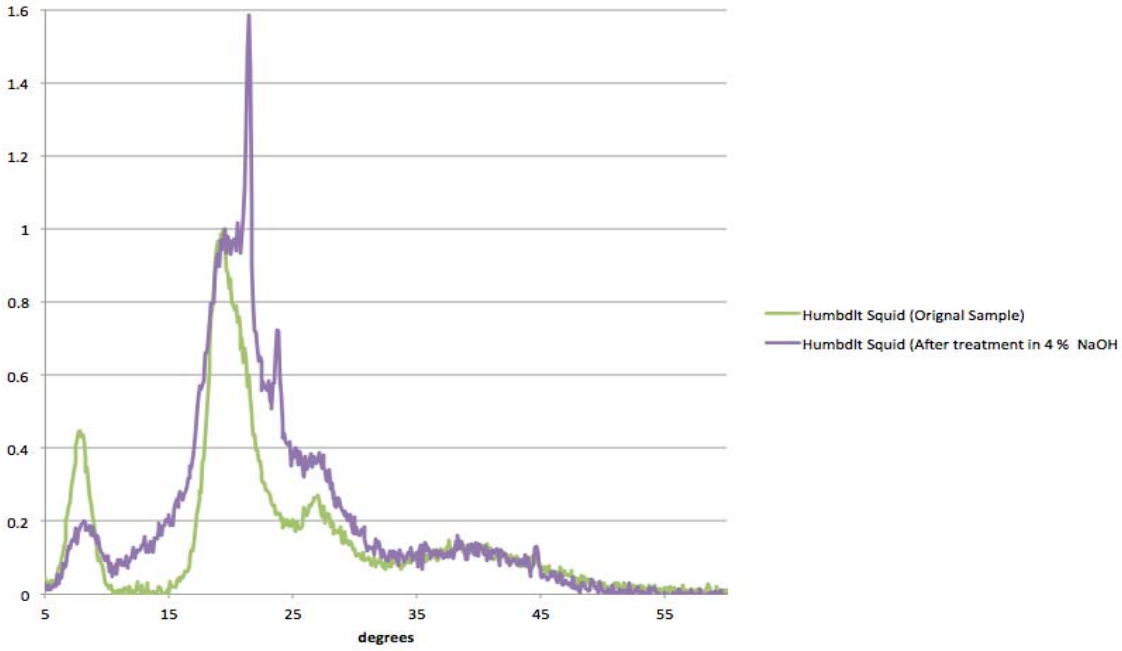


Figure 32: XRD spectra for Humboldt squid chitin/chitosan before and after NaOH treatment.

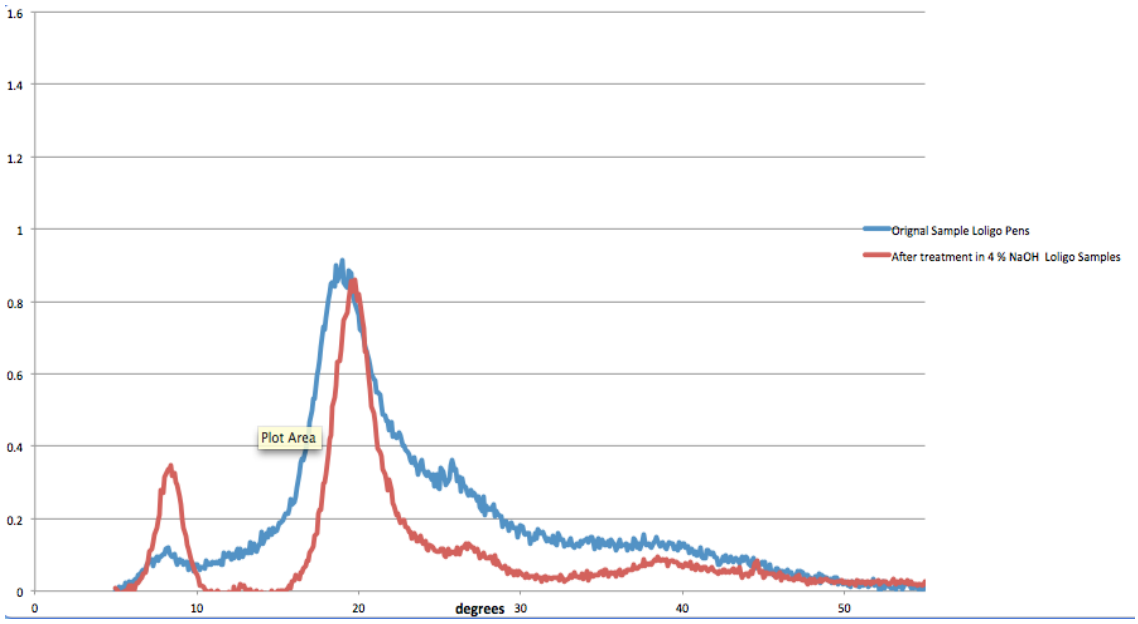


Figure 33: XRD spectra for loligo squid chitin/chitosan before and after NaOH treatment.

4.2.6. Protein Analysis of Chitin/Chitosan by BCA Assay:

This BCA analysis was an attempt to understand how the different washes and chemical treatments that were undertaken before dissolving the chitin/ chitosan could remove the inherent protein content associated with the chitin material. The protocol for performing the BCA assay is reported in Appendix B. A bovine serum albumin standard was utilized as the reference protein for establishing a protein calibration curve. The standard results and constructed calibration curve are presented in Table 7 and Figure 34 respectively.

Table 7: Bovine Albumin Standard Measurement to create calibration curve

Standard BSA	Micrograms of Protein	Absorption 1	Absorption 2	Average Absorption	Micrograms of Protein
0 µl	0 µg	0.073	0.071	0.0722	1.0515
25 µl	50 µg	0.823	0.794	0.8085	11.8083
50 µl	100 µg	1.355	1.376	1.3655	19.9434
75 µl	150 µg	1.855	1.958	1.9065	27.8449
100 µl	200 µg	2.355	2.377	2.366	34.5560

The Loligo and Humbodlt squid chitin/chitosan samples were washed in a four stage process in an attempt to remove all remaining protein. The first wash was performed using 4 % NaOH on 2 grams of chitin to remove the colored proteins at 60^{o}C for 30 minutes. Samples were then rinsed in deionized (DI) water to remove any caustic soda. The third wash was performed by immersing the samples in DI water with 1% HCl overnight to remove any remaining proteins and to neutralize the pH. The next morning the samples were rinsed for the last time in DI water.

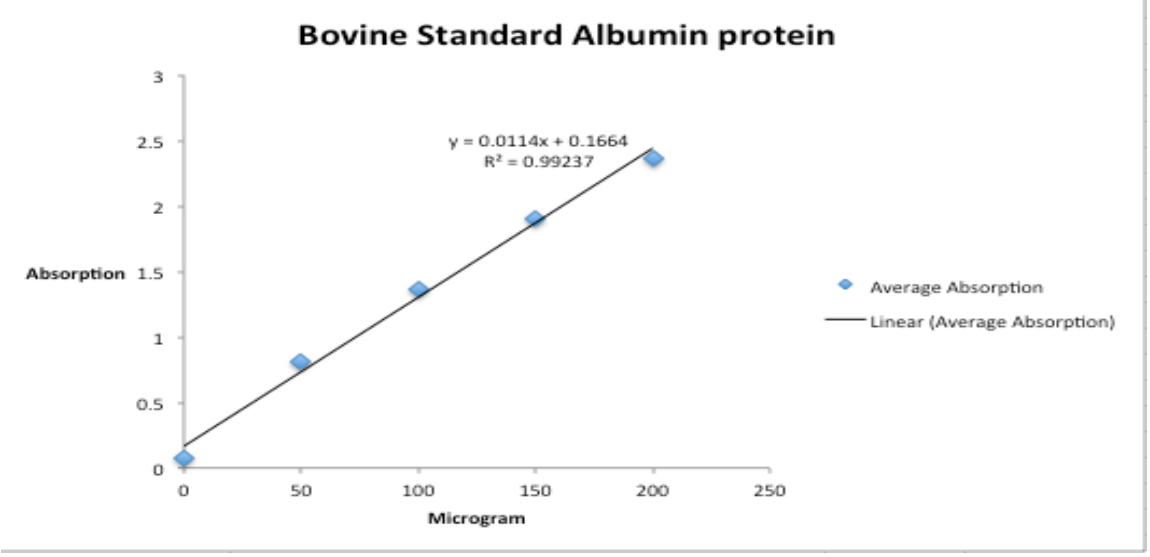


Figure 34: Albumin Standard Calibration Curve

The BCA assay generated a colored reaction with an intensity that was a direct function of the protein content. The intensity of the absorbance for each chitin/chitosan sample was measured and compared to the calibration curve. The protein concentration of the sample was obtained, by plotting the absorbance value recorded by the spectrophotometer as the y-axis variable in the equation y =bx+c (Figures 35 and 36).

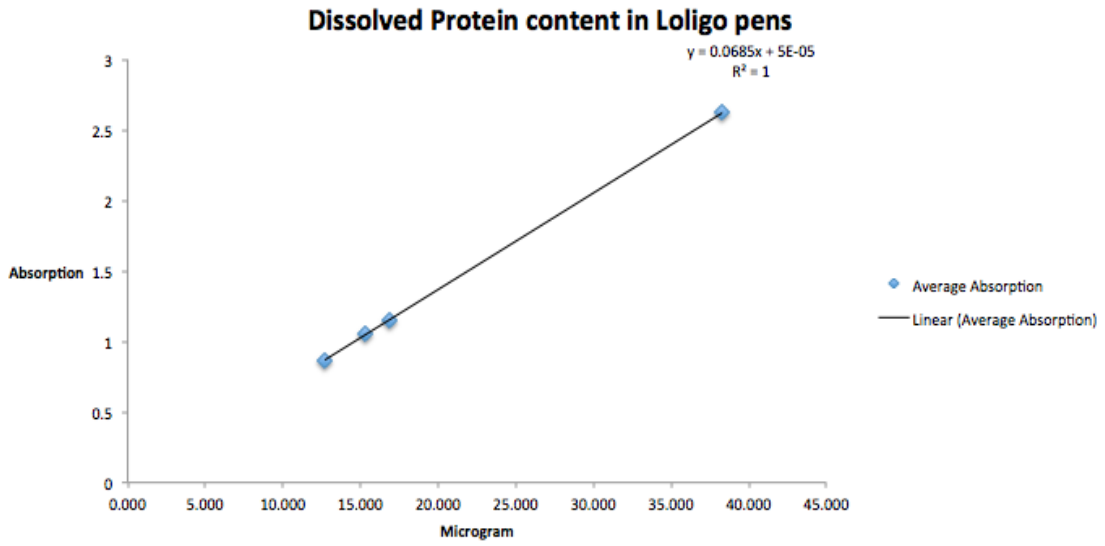


Figure 35: Dissolved protein content absorption curve for Humbodlt squid pen chitin/chitosan sample using the bicinchoninic acid assay

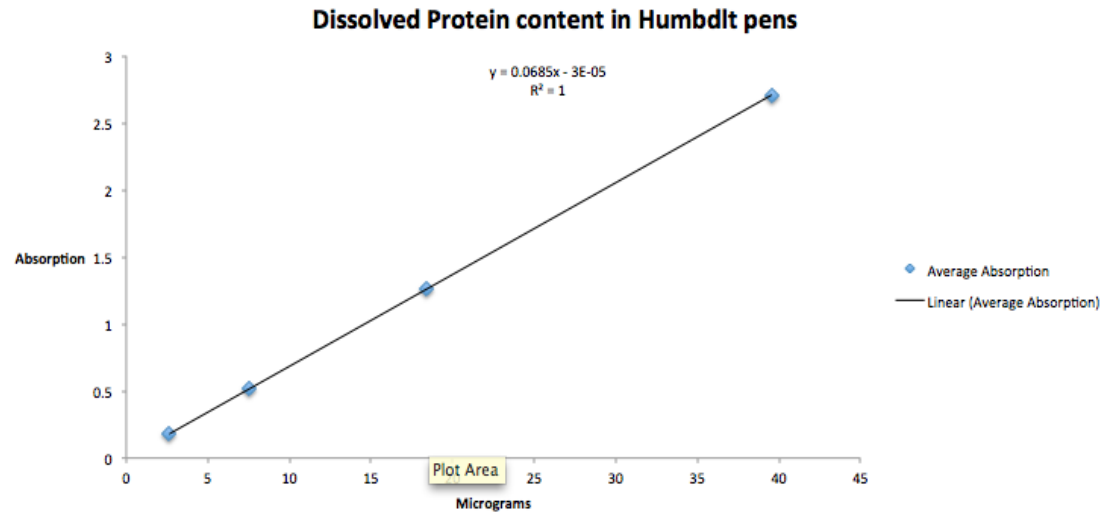


Figure 36: Dissolved protein content absorption curve for loligo squid pen chitin/chitosan sample using the bicinchoninic acid assay.

Figure 37 presents the amount of dissolved protein found for the different samples after each stage of washing. It is evident that the various washing steps removed some of the associated protein from both the Humboldt and loligo squid chitin samples but that even after Wash 4, there was still some residual protein remaining.

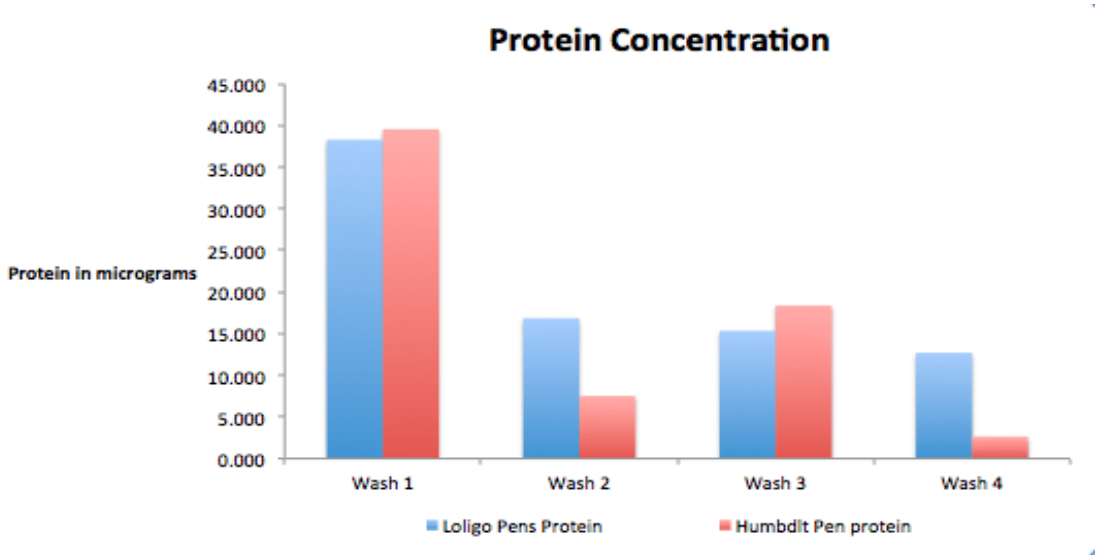


Figure 37: Dissolved protein content in chitin sample following different wash treatments

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4.2.7. Compression Resistance of Prototype Chitin/Chitosan Hydrogel Sponges:

The mechanical stress/strain results representing the amount of thickness loss (strain) when increasing compression loads (stress) were applied to the chitin/chitosan hydrogel sponge samples are shown in Figure 38.

From the figure it is evident that the loligo chitin/chitosan sponge could not resist the applied compression load as well as the Humboldt derived sponge. The reason why the Humboldt chitin/chitosan hydrogel was better able to resist compression is believed to be due to its different porous structure (See Section 4.4).

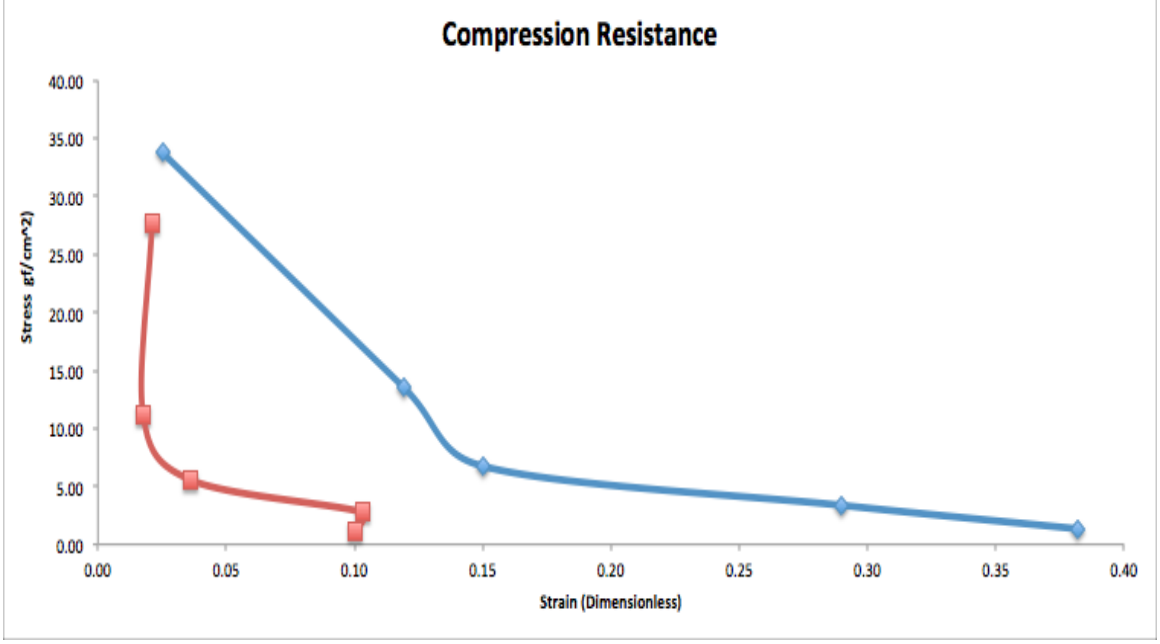

Figure 38: Average compression resistance results measured on chitin/chitosan hydrogel sponges.

4.3. Morphology of Chitin/Chitosan Freeze Dried Samples:

The SEM images of the freeze-dried samples are presented in Figures 39 and 40. Image analysis of the average pore size generated values for the Humbodlt squid chitosan to be 14.7 ±4.0 μm whereas for the loligo squid chitosan the pore size was 58.3± 87.6 μm. Clearly there was a large amount of variability observed for the loligo squid samples.

i. **Loligo Squid Chitin/chitosan:**

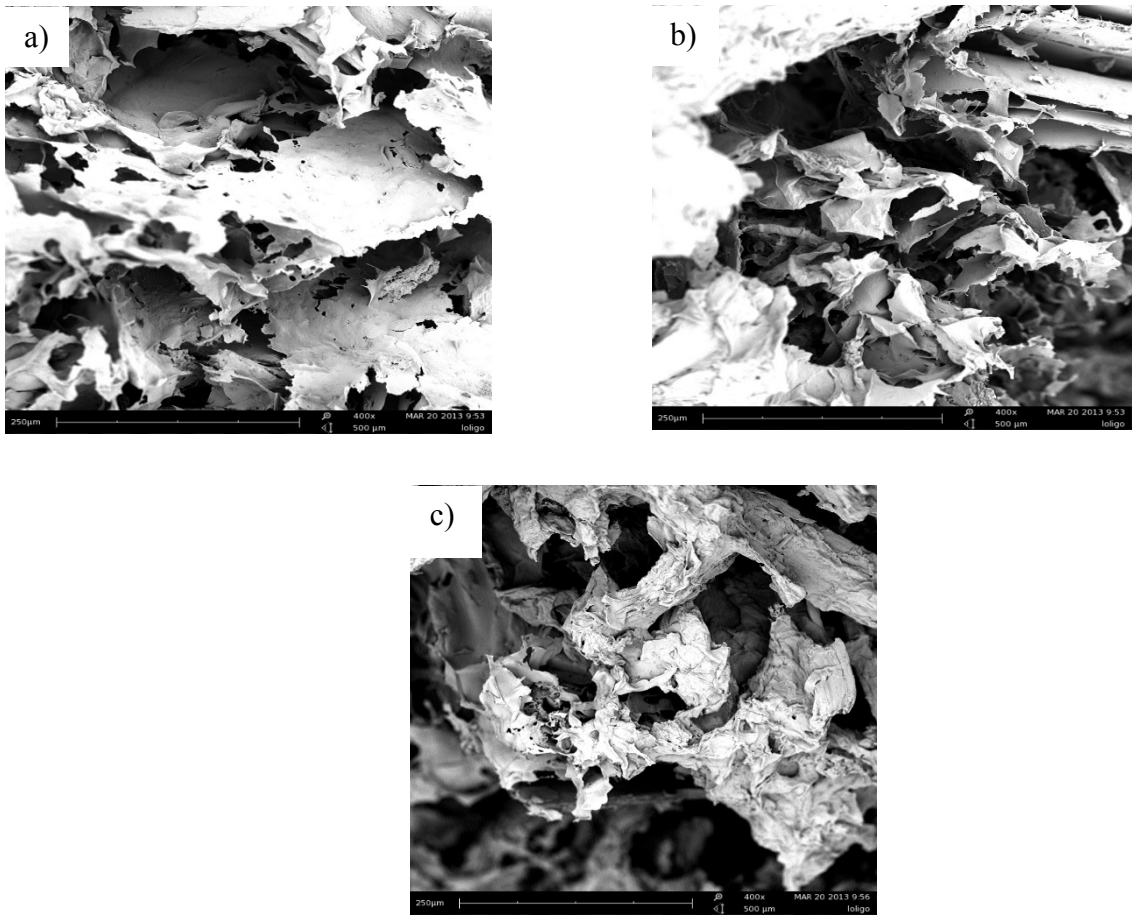

Figure 39: (a, b, c) Showing SEM images of freeze dried loligo squid chitin/chitosan sponge samples at (400X)

ii. <u>Humboldt Squid Chitin/chitosan:</u>

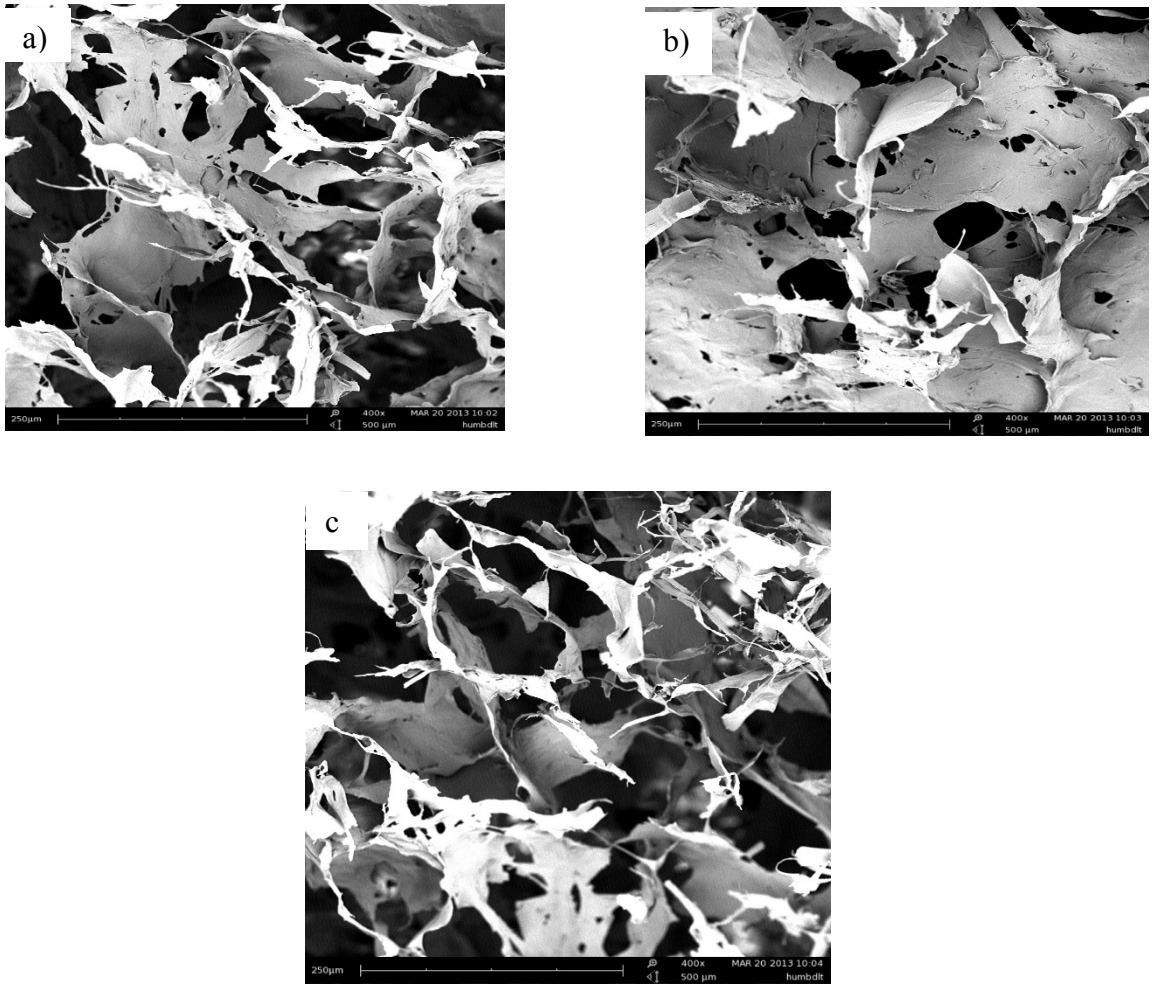

Figure 40: (a,b,c) Showing SEM images of freeze dried Humbodlt squid chitin/chitosan sponge samples at (400X).

4.4. Antibacterial Activity:

The average zones of inhibition showing the extent of antibacterial activity were measured using vernier calipers and are presented in Figure 41. Both the Humbodlt and loligo squid chitin/chitosan sponges provided similar antibacterial activity for Day 1 and 3. By the Day 5 they were both beginning to lose their effectiveness. Neither prototype freeze-dried sponge was able to compete with the three-control wound dressing samples. It is believed that the inclusion of silver is particularly effective in maintaining strong antibacterial activity over an extended period.

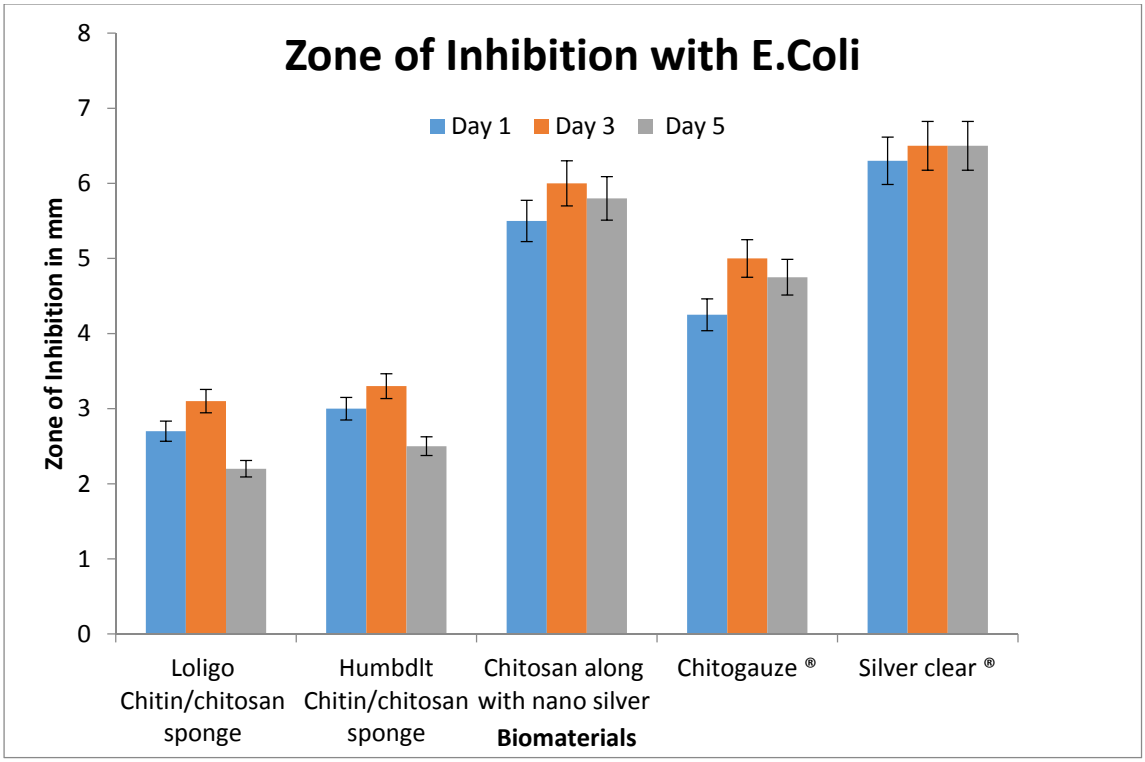

Figure 41: Zone of inhibition Results for the chitin/chitosan prototype hydrogel sponges and the control dressings.

4.5. Hemostasis:

Hemostasis has been identified as an important issue for donor site wound dressings, particularly in the case of thick skin grafts where blood oozing needs to be stopped immediately. Often saline injections are utilized prior to grafting to reduce blood loss, but for thick skin grafts saline injections prior to surgery do not always control bleeding.

The results of the initial thrombin formation assay for each of the prototype samples and the control dressings are presented in Figure 42. A fluorogenic substrate called Z-Gly-Gly-Arg-AMC was used with a calibrated automated thrombogram (CAT) to generate a fluorescent intensity that corresponds to the amount of thrombin generated with time according to the protocol described in the Appendix F. Figure 42 indicates that the time required to initiate a blood clot differed between the Humbodlt and loligo squid chitin sponges, but they gave similar thrombin times to the other commercial and control dressings.

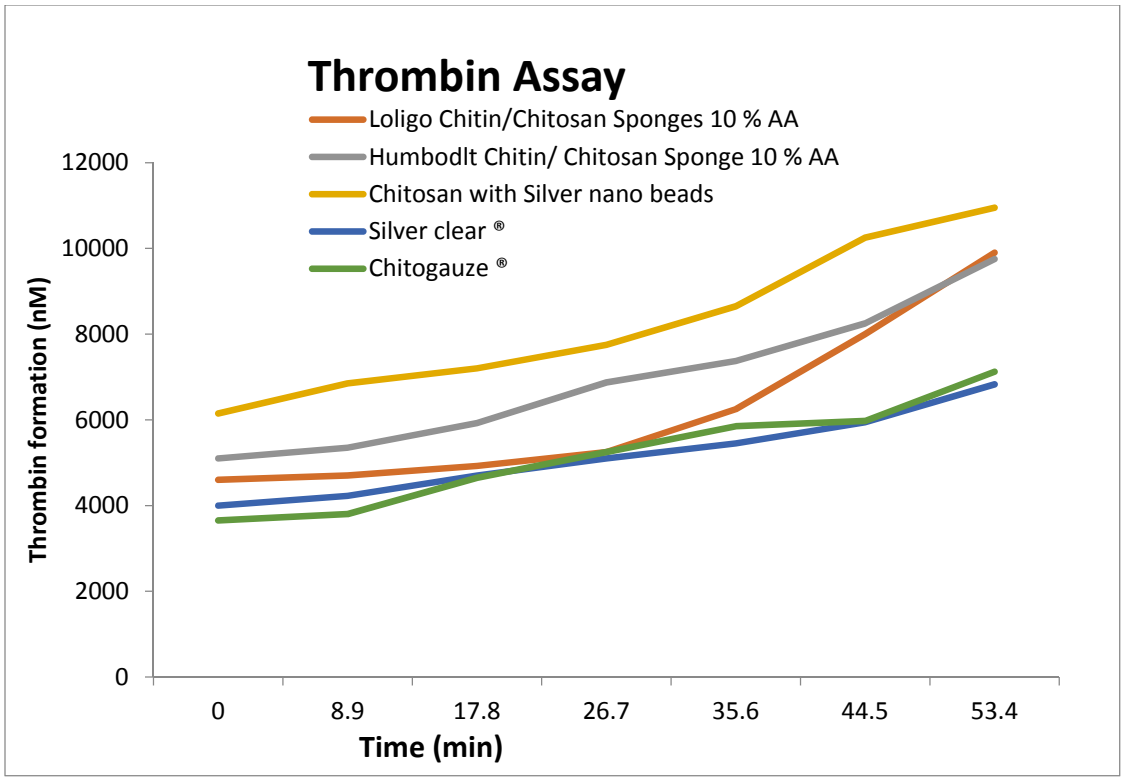

Figure 42: Thrombin assay results for chitin/chitosan prototype hydrogel sponges and the control dressings.

4.6. SEM Images Showing Initial Blood Contact:

The prototype chitin/chitosan sponge samples along with other control dressings were exposed to fresh whole blood obtained from the NCSU Veterinary College pig farm. The morphology of the blood cells was viewed by SEM by immediately fixing, preserving and drying the samples according to the protocol described in Appendix E. The following images Figures 43-47 show how the blood cells were activated by the various chitin/chitosan prototype sponges as well as the control dressings.

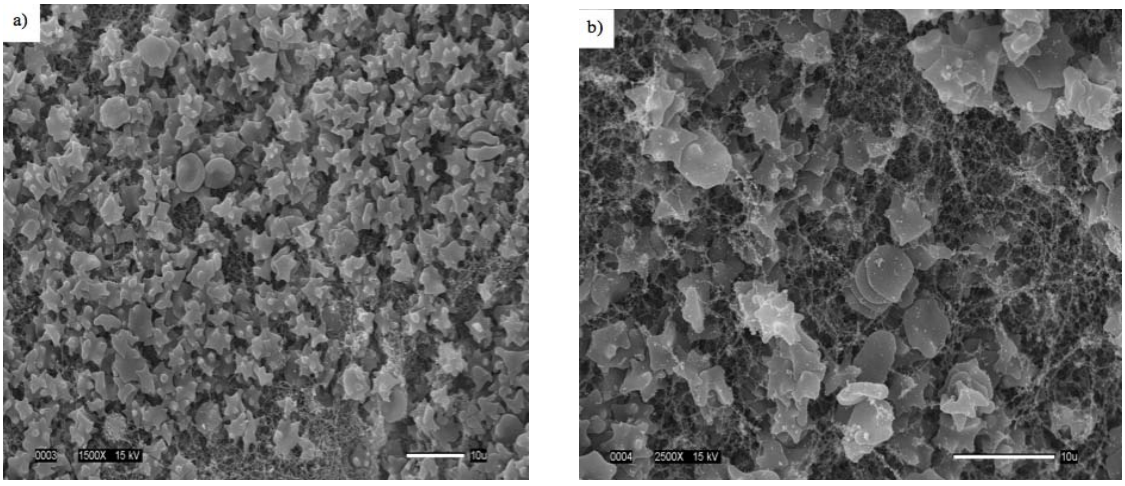

Figure 43: SEM images with loligo chitin/chitosan showing a) leukocytes and red blood cells (RBC's) interacting (1500X) and b) RBC's with leukocytes activated interacting with the chitin/chitosan (750X).

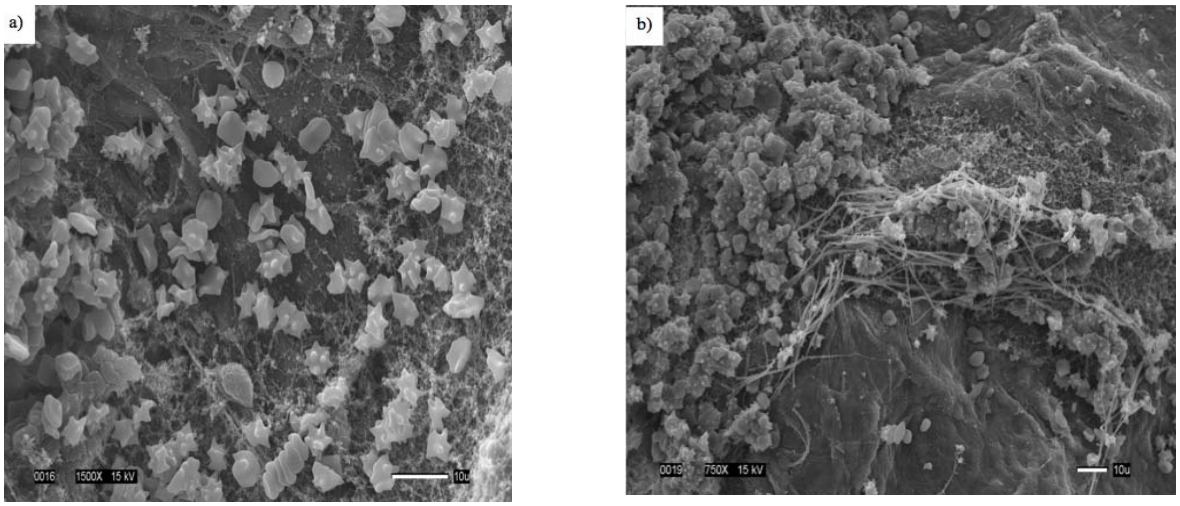

Figure 44: SEM images of with Humboldt squid chitin/chitosan showing a). Red Blood cells and leukocytes with fibrin formation (1500X), b). Clot formation with RBC's and leukocytes (750X).

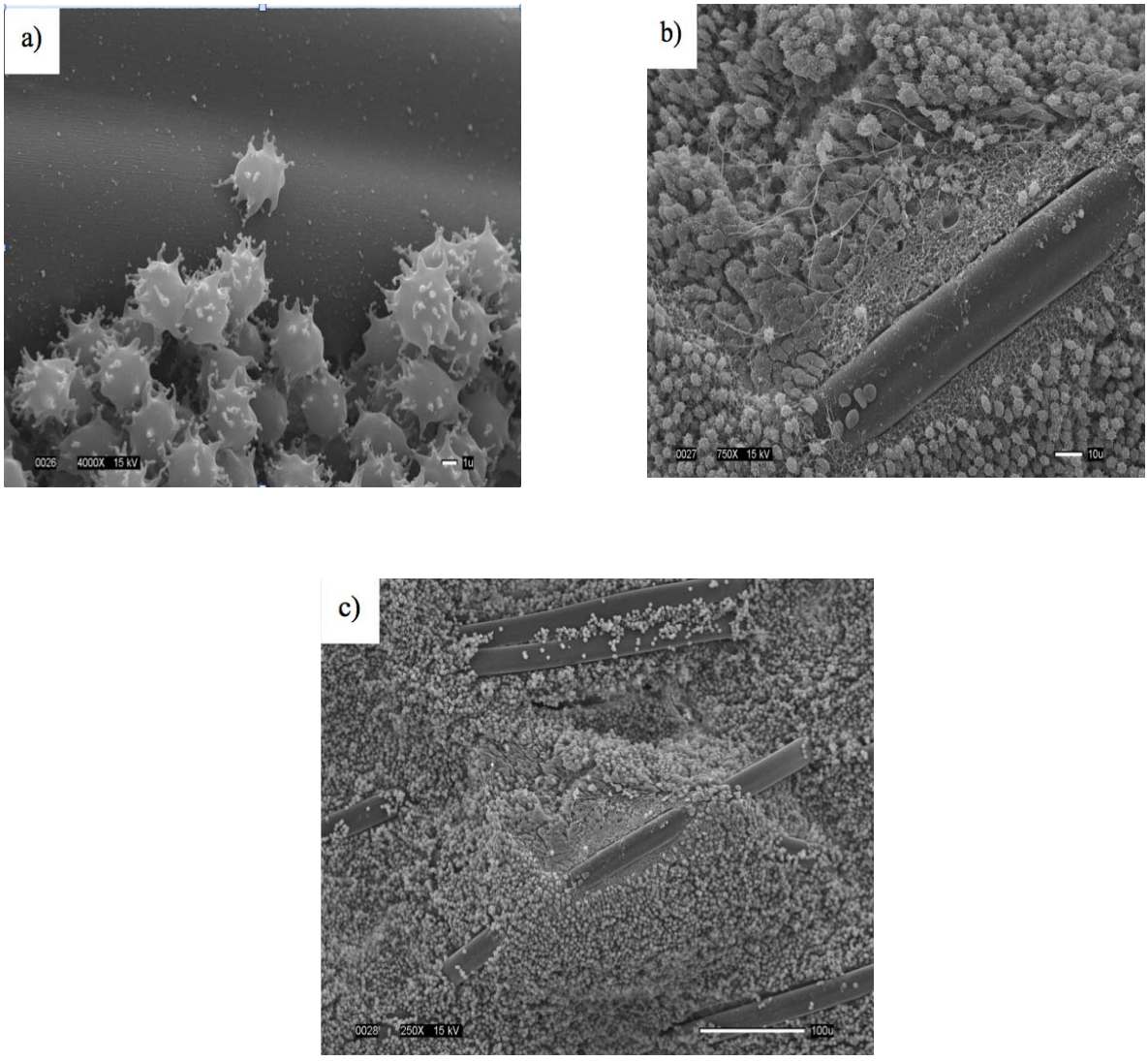

Figure 45: SEM images of with Silverclear ® commercial dressing. a). Showing activated leukocytes attached to the dressing (4000X), b) RBC's and leukocytes with fibrin formation (750X) and c). Fibrin formation with RBC's and leukocytes covering the nylon yarn (250X).

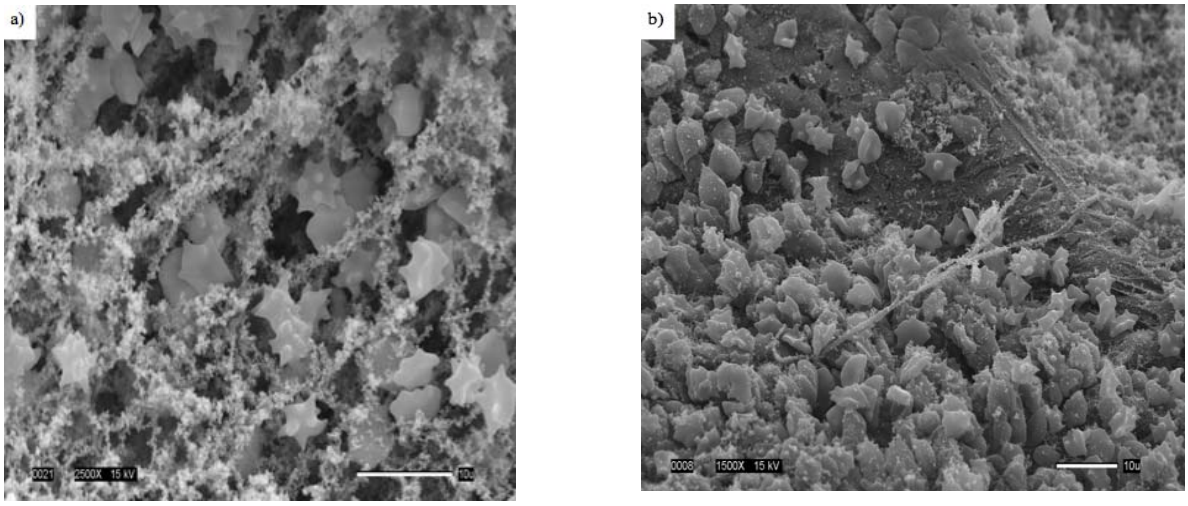

Figure 46: a). SEM images showing a chitosan nanofibers with leukocytes and red blood cells (2500X) and, b). Red blood cells along with leukocytes and other factors forming fibrin strands (1500X).

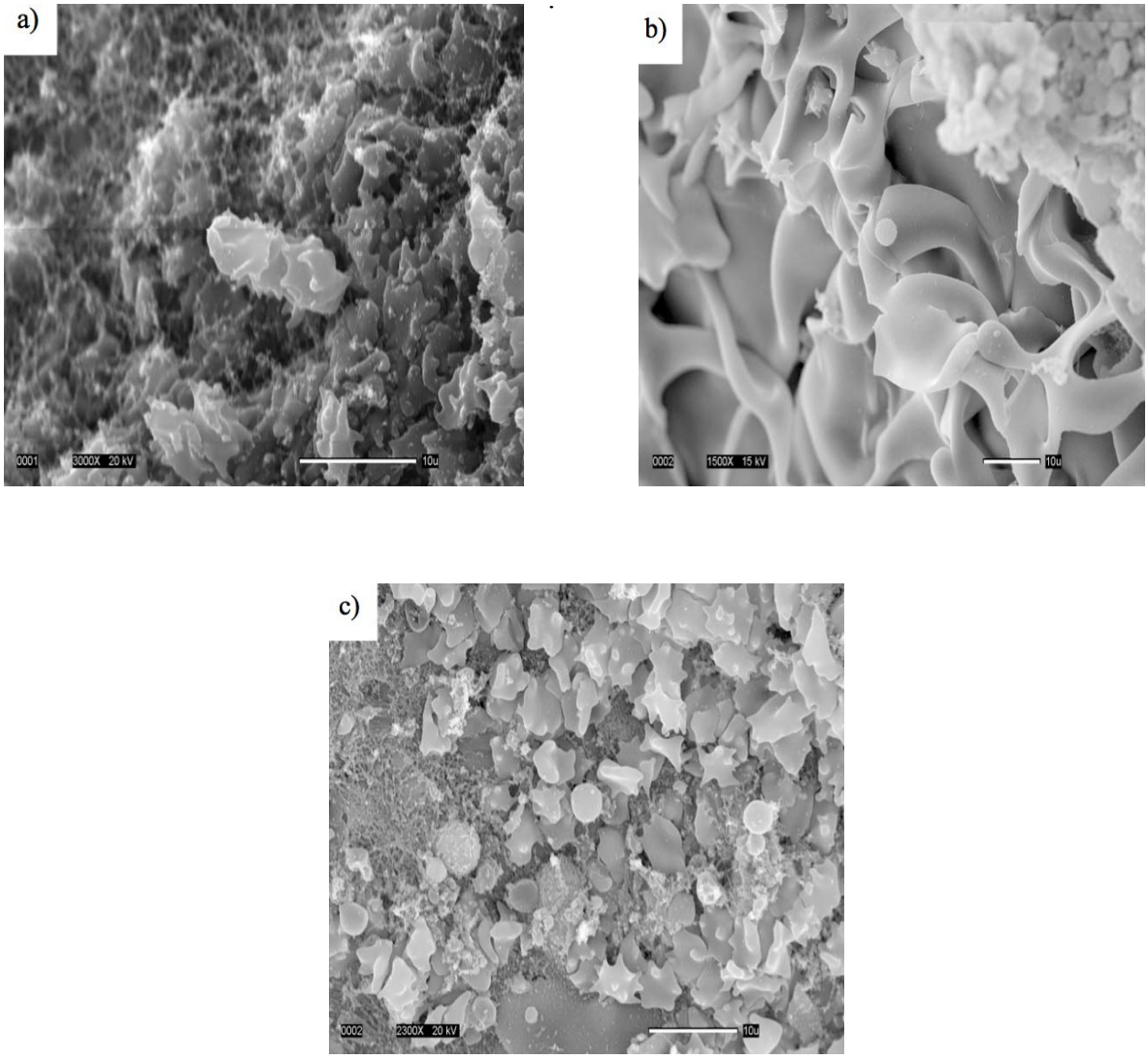

Figure 47: SEM image showing leukocytes and red blood cells attached to the chitosan dressing Chitogauze ® with mostly leukocyte cells along with dense chitosan and red blood cells at different magnifications, a). 3000X, b). 1500X and c). 2300X.

CHAPTER 5: CONCLUSIONS AND FUTURE WORK

5.1. Conclusions:

The goal of this study were to improve our understanding of the issues and concerns related to donor site wound injuries and to take some initial steps in designing an effective and functional dressing for donor site wounds. These two major goals were further divided into six objectives. In this section we report our six conclusions, one for each objective.

5.1.1. Our survey highlighted that a donor site wound is a completely different type of wound that has different requirements to burn wounds in general. The survey also found that reduction of pain, hemostasis and antibacterial activity were the three main requirements for healing of donor site wounds. This means that in developing a wound dressing for donor site wounds, these are three key performance indicators that need to be integrated into the product design. These findings are based on results obtained from our survey that was administered specifically to healthcare professionals who worked directly with patients who have suffered burns and trauma. The results from the survey also underline the current lack of consensus amongst healthcare professionals as to the preferred management protocol and the optimal type of dressing that should be used for donor site wounds. This survey was the first of its kind among healthcare professionals and for this reasons the results are medically relevant and clinically important.

5.1.2. Initial steps have been taken to select β −chitin/ chitosan as the prime candidate material for dressing a donor site wound dressing with a porous hydrogel structure, samples of maritime chitin/chitosan have been obtained from Humbodlt and loligo squid pens. They have been cleaned, extracted, deacetylated, purified and freeze dried and their key functional properties have been evaluated and compared against three controlled dressings; (i). The Silverclear ® nylon warp knitted dressing with ionic silver treatment. (ii). Chitogauze ® a commercial chitosan based dressing manufactured by Hemcon ®, (iii) Nanofiber chitosan web with ionic silver.

5.1.3. The protein content for loligo squid and Humboldt squid chitin/chitosan samples has been measured for the very first time. The measured soluble protein content and for Humbodlt squid chitin/chitosan washed away more easily than for the loligo squid chitin/chitosan. The presence of soluble proteins is believed to effect the color and biological performance of the wound dressing material.

5.1.4. Prototype samples of porous hydrogel lyophilized chitin/chitosan were prepared by freeze-drying and their porosities and mechanical performance were measured. There was a significant difference in total porosity for the Humbodlt squid chitin/chitosan hydrogel as compared to the loligo squid hydrogel. It was found that the Humbodlt squid chitin/chitosan hydrogel had smaller pores with less variation compared to loligo squid hydrogel. It was also found that the compression resistance of the Humbodlt squid chitin/chitosan was much greater than the lyophilized loligo squid hydrogel. In fact significant differences were found

in terms of the sponge's ability to support applied stresses. For example, the Humbodlt chitin/chitosan sponge's hydrogel had a superior mechanical performance to the loligo chitin/chitosan hydrogel sponge. This was particularly true in terms of the compression recovery properties. On removal of the applied compression load the Humbodlt sponge hydrogel recovered well, but the loligo chitin/chitosan sponge failed to recover.

5.1.5. The results of the antibacterial activity test demonstrated that improvements appear to be achieved due to the presence of ionic silver. It was observed that chitosan mats with silver and Silverclear ® dressing retained their bacteriostatic effect over 5 days of incubation while Chitogauze ® dressings and squid chitin/chitosan hydrogels failed to retain their antibacterial activity over this period of time.

5.1.6. The findings of the initial thrombin formation assay showed that there were different rates of thrombin formation for different sources of chitin/chitosan. Thrombin formation was observed to be fastest for chitosan webs that contain ionic silver, which can help ensure improved hemostasis for donor site wounds. In addition, the Humbodlt squid chitin/chitosan hydrogel had a significantly faster thrombin formation time than the loligo squid chitin/chitosan hydrogel.

5.1.7. When the dressing samples were in contact with fresh blood scanning electron microscopy was used to monitor the cell activation and the formation of fibrin threads as part of the coagulation cascade. It was clear that the formulations containing silver promoted the

fastest rate of fibrin formation, and Silverclear ® and chitosan web with silver nanoparticles, caused the most rapid activation of leukocytes. Possible reasons could be because of the high charge density created by the ionic silver. With respect to chitin/chitosan dressings activation of the white blood cells could be seen to occur slowly and cause clot formation by an apparently different mechanism. It was also found that the hemostatic properties improved significantly with the use of a chitosan based dressing with silver.

5.2. Future Work:

Pain associated with donor site wounds has been reduced to some extent by the use of epinephrine solution as reported in recent, clinical trials that were being performed at University of North Carolina – Chapel Hill, Jaycee Burn Center (Sigrid Blome-Eberwein, MD, et al)[8]. Possible alternatives in future could be to test this method along with silver and chitosan based dressings.

Another option could be the use of silicon permeable based materials, as coverings for donor site wounds to ensure that the wounds are completely covered to ensure nociceptors and other nerve endings are not exposed to air and the surrounding environment.
Moisture is an important aspect with respect to the healing of donor site wounds and the amount of moisture can vary based on the thickness of skin being harvested. Silicon based materials such as PDMS have proven to provide a moisture permeable barrier to the wound sites so as to ensure exudates from wounds do not form crusts that are hard to remove. In

addition they can cause dressings to adhere to the wound site, which is also one of the reasons that they can cause pain and trauma.

It will be important in future to know the maximum concentration of silver in the form Ag^{+2} that can be used without causing any cytotoxic effects and can provide a slow continuous delivery of ionic silver to the wound.

The usefulness of chitosan/chitin hydrogels and sponges has been demonstrated in this study. In the future possible alternatives could be to develop nanofiber web or sponge made from chitosan/chitin and impregnated or chemically bonded with silver ions. One needs to ensure that the leaching of silver can be limited while at the same time maintaining presence of silver can be beneficial.

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APPENDICES

Appendix A

Survey on donor site care and dressings

1. Are you satisfied with the current options for donor site dressings?

- Yes
- No

2a. In order to minimize bacterial colonization and infection of your donor sites, which of the following antimicrobials, if any, do you use? (Please select all applicable options).

	1: Preferred choice	2: Secondary choice	3: Acceptable choice	4: Would not use	5: Not available in our clinical setting
Silver sulfadiazine					
Mafenide acetate (Sulfamylon®)					
Silver nitrate					
Zinc-neomycin (Bacitracin/ polymyxin)					
Xeroform™ petrolatum gauze					
Xeroflo™ gauze dressings					
Mupirocin (Bactroban®)					
Sodium hypochlorite					
Cadexomer iodine					
Ionic silver (e.g., hydrocolloid Aquacel® Ag dressings)					
Chitosan based dressings(e.g., Chito Gauze®)					
Manuka honey (e.g., Medihoney® dressing)					
None					
Other: _____					

If "other," please specify:

2b. What makes your "preferred choice" your number one option?

3. Which of the following anatomical locations do you use as donor sites, if available ?

a. Adult

	1: Preferred choice	2: Secondary choice	3: Acceptable choice	4: Rarely used	5: Would not use
Thigh	O	O	O	O	O
Buttock	O	O	O	O	O
Instep	O	O	O	O	O
Forearm	O	O	O	O	O
Upper arm	O	O	O	O	O
Scalp	O	O	O	O	O
Back	O	O	O	O	O
Chest	O	O	O	O	O
Flank	O	O	O	O	O
Abdomen	O	O	O	O	O
Calf	O	O	O	O	O
Shin	O	O	O	O	O
Other_____	O	O	O	O	O

If "other" selected, please specify location:

b. Children

	1: Preferred choice	2: Secondary choice	3: Acceptable choice	4: Rarely used	5: Would not use
Thigh	0	0	0	0	0
Buttock	0	0	0	0	0
Instep	0	0	0	0	0
Forearm	0	0	0	0	0
Upper arm	0	0	0	0	0
Scalp	0	0	0	0	0
Back	0	0	0	0	0
Chest	0	0	0	0	0
Flank	0	0	0	0	0
Abdomen	0	0	0	0	0
Calf	0	0	0	0	0
Shin	0	0	0	0	0
Other _____	0	0	0	0	0

If "other," please specify location:

4. How thick do you harvest your donor sites for Split Thickness Skin Grafts (STSGs)?

5. Do you believe that donor sites should be kept moist and hydrated?

- Yes
- No

6a. How often do donor sites become infected?

- Always
- Frequently
- Occasionally
- Rarely
- Never

6b. How do you manage infected donor sites?

7a. Do you modify the routine management of donor sites in special populations?

- Yes
- No

If so, for which of the following populations, would you modify routine management and why?

7b. Diabetic:

- Yes
- No

Explain:

7c. Elderly:

- Yes
- No

Explain:

7d. Children:

- Yes
- No

Explain:

7e. Patients with a chronic immunosuppressive condition:

- ○ Yes
- ○ No

Explain:

7f. Other: _____

Explain:

8a. What is your biggest challenge in the management of donor sites?

8b. In addition to your answer to question 8a, what other issues do you encounter in the management of donor sites?

9. If you could design an ideal donor site dressing, how would you rank the importance of the following characteristics? (Please rank the following characterisitcs from most to not important. (1 = most important, 5 = not important).

	1: Most important	2: Very important	3: Important	4: Neutral	5: Not important
Does not adhere to the donor site	O	O	O	O	O
Alleviates donor site pain	O	O	O	O	O
Has anti-bacterial properties	O	O	O	O	O
Is hemostatic	O	O	O	O	O
Is inexpensive	O	O	O	O	O
Requires minimal wound care	O	O	O	O	O
Prevents wound dessication	O	O	O	O	O
Other: _____	O	O	O	O	O

If "other," please specify and explain:

Additional comments:

Demographic Information

Position/ Title

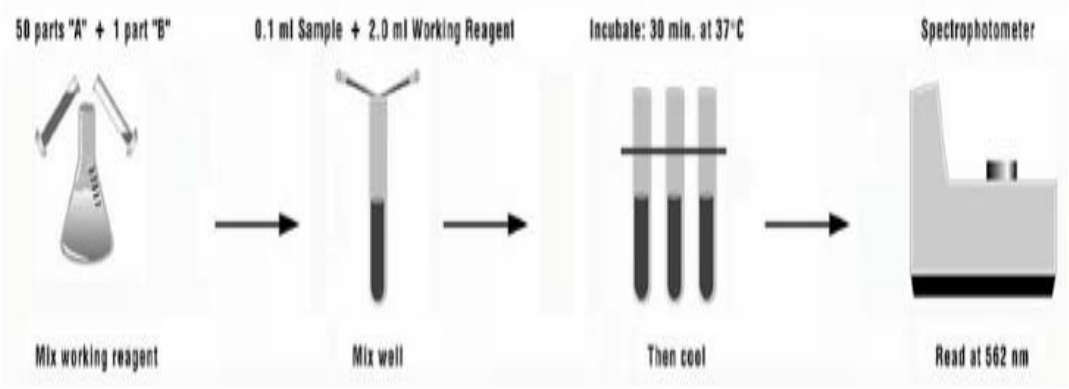
Responsibilities

How many years have you worked in burn care?

Name of your affiliated institution (Optional):

Appendix B

BCA Assay Protocol: BCA protein assay is detergent compatible based on bicinchoninic acid (BCA) for colorimetric detection and quantification of total protein. Cu^{+2} to Cu^{+1} by protein in a alkaline medium where by color change occurs to purple color on presence of increased protein concentration which can be measured using absorbance in the visible wavelength of 562 nm using a spectrophometer. The absorbance is linear with increasing protein concentrations over a (20-2,000$\mu g/ml$). BCA method



Final color is not achieved using BCA at very instance and color change occurs. (Cysteine, cysteine, tryptophan and tyrosine) cause change of color formation with BCA method thus burette is kept in shaker for 30 minutes before analyzing the absorption and color change for solutions.

Working procedure to perform this experiment is as under:

- Prepare working solution:

- Mix reagent A (in general the blue bottle in a BCA Kit) and reagent B with ratio of A: B – 1:20 for enough volume of using.

- Pipette working solution into micro plate wells (100 μl/well)

- Add gradient volume of standard protein (2mg.ml) to each well (5 or more) followed by adding of sample protein (5 μl/well)
- Mix wells thoroughly with pipet.
- Cover the plate and incubate at 37°C for 30 minutes.
- Cool down the plate to RT.
- Measure the absorbance at or near 562 nm in a plate reader.
- Make a standard curve using data of standard protein. Calculate concentration of sample protein from the standard curve.

Loligo Squid Pens:

Washes	Average Absorption	Protein in µg
Wash 1 (4% NaOH)	2.6215	38.288
Wash 2 (DI water)	1.154	16.854
Wash 3 (DI water & 1 % HCl	1.0495	15.328
Wash 4 (DI water)	0.8695	12.699

Humboldt Squid Pens:

Washes	Average Absorption	Protein in µg
Wash 1 (4% NaOH)	2.708	39.551
Wash 2 (DI water)	0.515	7.522
Wash 3 (DI water & 1% HCl)	1.2585	18.381
Wash 4 (DI water)	0.181	2.644

NaOH, Dilute water and HCL were also tested for its average absorption. Results showed no interaction of reagent in either way to limit or increase dissolved protein content for samples.

Appendix C

Stress/ Strain (Compression Resistance):

Loligo chitin pen								
S.No.	Load (gf)	Thickness (cm)	Change in thickness (cm)	Stress	Strain			
1	20	1.543	0.155	1.109	0.101	Diameter	2.179	1.089 cm
2	50	1.392	0.159	2.774	0.103	Area	18.021	cm^2
3	100	1.233	0.056	5.549	0.036	Thickness	15.43 mm	0.015 m
4	200	1.177	0.027	11.098	0.017			1.543 cm
5	500	0.904	0.0326	27.745	0.211			

Humbdlt Squid Chitin/Chitosan sponges								
S.No.	Load (gf)	Length (cm)	Change in thickness (cm)	Stress	Strain			
1	20	1.466	0.560	1.349	0.381	Diameter	1.938	0.969
2	50	0.906	0.425	3.372	0.289	Area	14.825	cm^2
3	100	0.743	0.220	6.745	0.150	Thickness	14.66 mm	1.466 cm
4	200	0.6	0.175	13.490	0.119			
5	500	0.425	0.037	33.726	0.025			

Antibacterial Activity:

Zone of Inhibition with E.coli at concentration of 35 * 10^6 CFU/ml				
S.No.	**Samples**	**Day 1**	**Day 3**	**Day 5**
1	Loligo Chitin/chitosan sponge with 10 % AA	2.7 mm	3.1 mm	2.2 mm
2	Humboldt Chitin/chitosan sponge with 10 % AA	3 mm	3.3 mm	2.5 mm
3	Chitosan along with nano silver	5.5 mm	6 mm	5.8 mm
4	Chitogauze ®	4.25 mm	5 mm	4.75 mm
5	Silver clear ®	6.3 mm	6.5 mm	6.5 mm

Appendix D

SEM FIXATION PROTOCOL FOR ANIMAL TISSUE:

1. Place chitosan fabric squares (or dots) into a multi-well culture plate; Incubate with blood until formation of clots occurs (you provide this part of the protocol). NOTE: clotted blood must NOT be allowed to air dry; keep it moist at all times.

2. Carefully transport the samples to the EM Center for the remainder of the processing.

3. Decant any remaining liquid and fix with 3.0% glutaraldehyde in 0.1M PBS pH 7.4, at 4 at least 48 hours.

4. Wash in 0.1M PBS pH 7.4, 3 changes of 30 minutes each at 4

5. Post-fix in 2% osmium tetroxide in 0.1M PBS, pH 7.4, for 2 hours at 4

NOTES:

a. This step and the subsequent wash may be omitted in some instances.

b. Osmium tetroxide is necessary if the tissue will be used for TEM later.

c. Osmium tetroxide may help cut down on charging.

6. Wash in 0.1M PBS pH 7.4, 3 changes of 30 minutes each at 4

7. Dehydrate in 30% and 50% ethanol, 30 minutes each change at 4

8. Change to 70% ethanol and hold for final dehydration at 4

9. Continue dehydration in 95% ethanol for 30 minutes at 4

10. Dehydrate in 100% ethanol, 3 changes of 30 minutes each; start with cold 100% ethanol and remove from the ice to allow it to come to room temperature; the second and third changes are done with room temperature ethanol.

11. Critical point dry for 15 minutes at critical point.

12. Remove from critical point dryer and secure samples to stubs, which have been prepared in advance with carbon dots, cut side up.

13. Sputter coat on the angle supports with gold/palladium; 25Å on each of 4 sides using the angle supports plus 25Å on top.

14. Store in the desiccator provided in the lab.

15. Examine in the JEOL 5900LV at 20 kV.

Appendix E

Operating Image J for measuring porosity:

Once Image J software has been installed on computer.

1 Open the appropriate SEM file (Choose best image possible keeping in mind, we are able to measure the fibers) file-> Open-> Locate the file

2. Choose (----)Line tab, its located beneath “Image tab” in Image J.

3. SEM image has the scale at the bottom, start at the start point and drag it till the end point.

4. Go to Analyze function (5 th) in Image J software.

5. Choose Set scale

6. Type your known distance (Seen in left hand bottom corner of SEM image)

7. Click Ok

8. Use magnifier function to magnify the image, so that edges of fibers are clearly visible.

9. Click inside the image, it should magnify the image.

10. Click the “Line (------) tab” in imageJ. If not clicked, it can only magnify.

11. From left hand corner, draw a line across the edges of fiber (it’s the diameter), click “M”.

12. Another window should open titled “Results”

13. Start from top to bottom in the image so we don’t miss any fibers.

14 After choosing each fiber, click “M”. It will automatically add fiber diameter and other information to window titled “Results”

15. When done with fiber measurements, go to “Results window”, Choose Results (4 th option), Click “Summarize”, it will automatically compute the summary (Mean, SD, Min max values, these are preset)

16. It can be “saved as” excel work sheet, where we can further compute the statistics we like to.

Appendix F

Thrombin Assay

Coaster 96-well plate are blocked using a solution of 1:4 25 % Human Serum Albumin and Citrated Saline. 150$\mu L$of the blocking solution is pipetted into each well plate. The plate is then sealed by stretching the Parafilm over the plate and incubated overnight at 37°C. Para film is then removed and the solution is emptied. Plate lid is replaced and the edges of the plates sealed with Para film. Sealed plates are placed in refrigerator for storage until later use. Running the plate in Gen5® Biotek Plate Reader:

1) Click and set the test for thrombin assay as per the protocol on Gen5®.
2) Check for correct filters (excitation – 390 nm and Emission – 460 nm)
3) Click Start Measurements
4) Select the protocol developed for thrombin assay
5) Place 96-weil plate in plate holder with A1 in top corner.
6) Click start button
7) Once test is finished, click next and save data.
8) Click on back button and then data view button.
9) Click Excel export to get the data in Excel file.

Copy data from Excel into Excel template to measure max-slope of the substrate florescence curve for each and every sample.