GLUCOMANNAN BASED HYDROGELS AS DRESSING FOR BURN WOUNDS

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DECLARATION

I, **Deepa Mohan**, hereby certify that I had personally carried out the work depicted in the dissertation entitled, ***"Glucomannan based hydrogels as dressing for burn wounds",***under the direct supervision of Dr.V.Kalliyana Krishnan, Scientist G (Senior Grade), Scientist-in-Charge, Division of Dental Products and Head, Department of Biomaterials Science & Technology, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences & Technology, Thiruvananthapuram, Kerala, India except where due acknowledgment has been made in the text. No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

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The dissertation entitled

"GLUCOMANNAN BASED HYDROGELS AS DRESSING FOR BURN WOUNDS"

Submitted by

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Dedicated to

MY TEACHERS & MY FAMILY

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ABBREVIATIONS

HEMA	2-hydroxyethylmethacrylate
ASTM	American Society for Testing and Materials
TPO	2,4,6 Trimethyl Benzoyl Biphenyl Phosphine oxide
FTIR	Fourier Transform Infra Red spectrophotometer
WVTR	Water vapor transmission rate
ESEM	Environmental Scanning Electron Microscopy
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
GLU	Glucomannan
Hb	Haemoglobin
ABAM	Antibiotic antimycotic
ISO	International Organization for Standardization
IU	International Unit
Mn	Number average molecular weig
Mw	Molecular Weight
PBS	Phosphate Buffered Saline
PCL	Poly(ε-caprolactone)
PEG	Poly(ethylene glycol)
pHEMA	poly(2-hydroxyethylmethacrylate)
RBC	Red Blood Cell
SD	Standard Deviation
SEM	Scanning electron microscopy
SNPs	Silver Nano-Particles
TS	Tensile Strength
UTM	Universal Testing Machine
HDRG	Hydrogel system
DI/W	Deionised water

CHAPTER 1

INTRODUCTION

Globally millions of people suffer from skin burn injuries every year. Burns are the most intense painful injuries. Burn victims experience pain regardless of the cause, size or depth of burn with2ndand3rddegree burns being the most painful. Skin burns can result from exposure to several possible sources, including hot water or steam, hot objects or flames, chemicals, electricity, or overexposure to the sun. While the minor skin burns (1st degree) can be managed at home, serious skin burns requires evaluated, attention and treatment by a healthcare provider. Treatment for burns depends on the cause, depth and area covered by the burns. Over the years many advances on curing burn wounds, including analgesics, sedatives and topical wound therapies have evolved which resulted in more patients surviving burn injuries. Despite these developments, great variability in wound management has resulted in. Providing appropriate wound dressing with all the qualities to a burn patient is a challenge to clinicians. Because of different component and ever changing pattern overtime, it has become difficult to cure burn wounds

During the past 20 years, major advances in the production of synthetic dressings have occurred leading to a large variety of suitable polymeric materials. These materials come in the form of films,sprays, foams and gels. However, there is minor progress in the clinical acceptance and usage of these dressings as clinicians tend to be relatively conservative. Biological dressings in this period have become established as temporary (allografts and xenografts) and permanent (autograft) wound coverings. Recent research has led to a clearer indication of the required properties of a replaceable dressing. Conventional dressings are still widely used in conjunction with topical agents to control wound infection. The disadvantages associated with these dressings are the pain

associated .during frequent dressing changes, poor fluid absorption, inertness without any contribution to healing process and inability to control bacterial contamination. Many synthetic materials have been developed and assessed, and a set of wound dressing criteria has evolved from this extensive research (Davies, 1983; Quinn *et al*., 1985). Further research is required in the field of synthetic dressings to produce the 'ideal' dressing or a range of 'ideal' dressings. It may be that such dressings could be produced by combining properties of several of the dressings presently available.

Severe burn injuries causing extensive damage are notoriously complicated by loss of body fluids. More often than not, such wounds become seriously infected further aggravating morbidity. Despite advances in burn management, the mortality rate of these injuries continues to be high and the search for economical and easily available topical measures to control burn wound infection continues. Invariably, many of the different methods applied for local treatment are still controversial. Irrespectively, the main requirement in burn wound management is an economical, easy to apply, readily available dressing or method of coverage that will provide good pain relief, protect the wound from infection, promote healing, prevent heat and fluid loss, be elastic and non-antigenic and adhere well to the wound while waiting for spontaneous epithelialization of superficial partial thickness burns or for permanent coverage with autologous epithelium of deeper burn wounds. Methods for handling burn wounds have changed in recent decades. Increasingly, aggressive surgical approach with early tangential excision and wound closure is being applied. It is probably the most significant change in recent years leading to improvement in mortality rates of burn victims at a substantially lower cost. By shortening hospital stay, early wound closure reduces pain associated with local burn wound care, number of operative procedures and infective complications. It also decreases the severity of hypertrophic scarring, joint contractures and stiffness, and promotes quicker rehabilitation. Irrespective of any other consideration, early healing is paramount for good aesthetic and functional recovery. It has been clearly demonstrated that disruption of epidermal–mesenchymal communication due to a delay in epithelialization, increases the frequency of developing fibrotic conditions such as scar hypertrophy and contractures. Autografts from uninjured skin remain the mainstay of treatment for many patients and skin graft preservation for the purpose of delayed application is still a basic tool in burn treatment and plastic and reconstructive

surgery.Autologous skin, however, has limited availability and is associated with additional scarring

In this scenario the hydrogel wound dressing can play a significant role.They are usually made with any of the hydrogel which are proven to be biocompatible. The hydrogel which is proposed in this study is made with HEMA, PEG and PCL and each have its own role in the matrix. The hydrogel materials are known for its flexibility and absorptionproperty more over they provide a moist environment to the burn wound. Thus the thesis here gives an overview of the burn wounds and its dressings currently available and its limitations along with an overview on the proposed materials with its advantages explained in the second section (Chapter 2). The study proposes a hypothesis that addition of glucomannan, a biological molecule isolated from a plant which is proven to have lot of important properties in the skin care, may eventually contribute to wound healing without compromising the required qualities of the wound dressing.

In chapter 3, the various methodologies which were adapted for the making of hydrogel (PHEM+PEG+PCL) and its characterization and other biological studies are discussed which finally gives an idea of how the matrix is formed. The photopolymerization technique followed by the various characterization methods like FTIR spectroscopy, mechanical strength evaluation, biological studies etc have been described

In chapter 4, the results obtained are presented and discussed where the final outcome of the previous chapter is given. Statistical analysis has been carried out and standard deviation calculated wherever applicable.

Finally in chapter 5 the summary and the conclusion of the work is presented, which gives a brief summary of the investigation along with the conclusions of the study and directions for future research.

CHAPTER 2

LITERATURE SURVEY

2.1. INTRODUCTION

Skin is one of the prominent organs that make up the human body. In adults, it covers an area of 1.5 to 2.0 m^2 and accounts for about 15% of the body weight (Saladin, 2008). Skin is vital to our overall health and wellbeing and consists of multiple skin cells that work together to complement each other and provide essential functions such as skin barrier function and protection against the harmful ultraviolet radiation. There are structurally three layers of the skin: the epidermis, the dermis, and subcutis. Apocrine glands, sebaceous glands, sweat glands, hairs and nails are regarded as derivatives of skin. Skin is known as a dynamic organ as they are prone to constant state of change which includes the continuous shedding of cells from an outer layer which is replaced by inner cells moving up to the surface. The skin incorporates the major support systems like blood, muscle and nerves; also they have roles in ultraviolet radiation sensing, immuno-competence, psycho-emotional, endocrine function etc. (Tobin, 2005). Although structurally consistent throughout the body, skin varies in thickness according to anatomical site and the age of the individual.

2.2. SKIN ANATOMY

Skin arises from the juxtaposition of two major embryological components which includes the epidermis and the mesoderm, which originates from a surface area of the early gastrula, and the latter, which is brought into contact with the inner surface of the epidermis during gastrulation. Differentiation of epidermal structures such as hair follicle depends on the mesoderm. The adult epidermis is maintained by the influence of the

dermis. The other components like pigment cells in the skin are contributed by the neural crest (McGrath *et al.*, 2004).Skin can be histologically classified into three layers (Figure 2.1). The outer most layers are the epidermis, which act as the physical and chemical barrier between the interior body and exterior environment; the dermis is the deeper layer which provides the structural support of the skin, next to which is a loose connective tissue layer, the epidermis which is an important depot of fat.

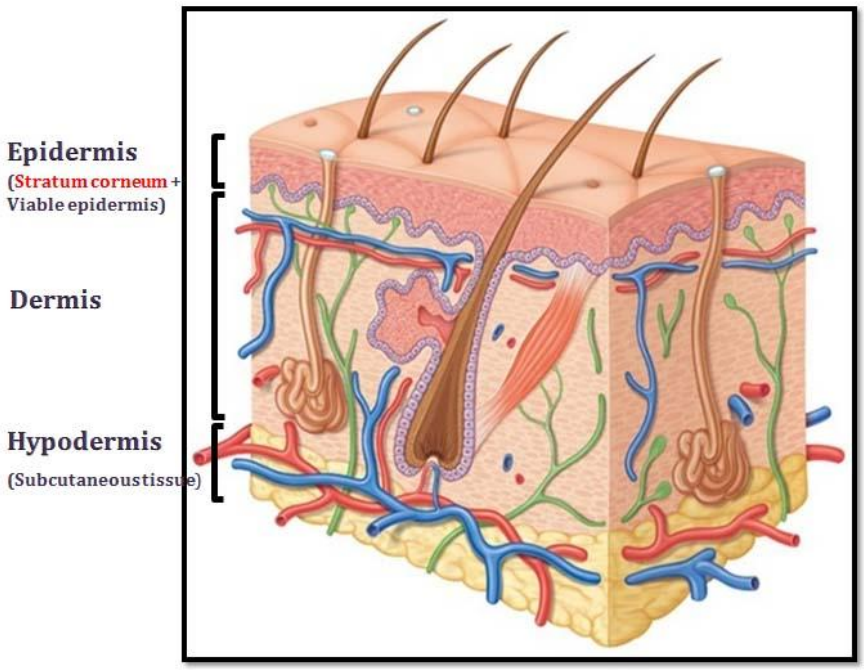


Figure 2.1. Organization of human skin(Burns *et al*., 2004)

2.2.1. Epidermis

It is made up of stratified squamous epithelium. The keratinocytes are the main cells of the epidermis. The main function of keratinocytes is to synthesize the protein keratin. Desmosomes, also known as the protein bridges, connect the keratinocytes that are further transformed from the deeper layer of the superficial skin. The epidermis further consists of four layers (i) stratum basale (basal or germinativum cell layer)- which mainly consist of keratinocytes constitutes about 15 to 20 layers (ii) stratum spinosum (spinous or prickle cell layer)- they have polyhedral keratinocytes also the intercellular bridges, the desmosomes appear as `prickles' at a microscopic level, which connect the cells, (iii) stratum granulosum (granular cell layer)- As they change to the surface cells , they appear flattened and lose their nuclei and their cytoplasm appears granular, (iv) stratum corneum (horny layer)- it consists of denucleated keratinocytes, corneocytes that

shed from skin, and it is a highly-functional outer layer of skin tissue **(**Burns *et al* ., 2004).

2.2.2. Dermis

It lies between the epidermis and subcutaneous layer. It contributes to 15-20% of the total body weight. It consists of three fibrous proteins, elastin, collagen and small quantities of reticulin and a supporting matrix or ground substance. It contains connective tissue as collagen in bulk and elastin in minimal quantities which contain a rich interwining blood supply. These type of cells located in the dermis are fibroblasts, mast cells and histocytes. Hair follicles, lymphatic vessels, nerves and sweat glands are also found in the dermal layer of skin.

2.2.3. Dermo-epidermal junction

It is a complex structure which is composed of two layers. The junction provides a physical barrier for cells and large molecules and a strong bandage between the dermis and the epidermis by macromolecular attachments. The attachment is done by parts of the epidermis that penetrate the papillary dermis, which results in large cones and rete ridges, or papillae. In epidermis the nutrients are obtained and waste disposal is done through diffusion at this junction.

2.2.4. Hypodermis

It is the lower most layers consisting of fat, which is loosely connected to the superficial dermis and provides protection from injury also produces heat and serves as a cushion for the body. It occurs almost universally over the body surface. Its thickness varies with age, sex, race, endocrine and nutritional status of the individual. They constitute about 10% of the body weight.

2.3. WOUNDS

Skin is prone to many injuries or wounds as a part of its protective function. Wounds can be defined as the disruption of tissue integrity which results in its damage, leading to the loss of function (Lazarus *et al*., 1994). The wounds can be classified as: (i) wounds without tissue loss which include surgery and (ii) wounds with tissue loss like burn wounds, wounds caused as a result of trauma, abrasions etc. Another type of classification is based on the disruption of the layers involved: Superficial wounds-where

the epidermis is only involved; partial thickness wounds-which involve epidermis and dermis; full thickness wound-involve the subcutaneous fat or deeper tissue. The time for healing of these wounds depends on the severity of each wound type **(**Balassa *et al*., 2014**).**

2.3.1. Burn wounds

An injury to the skin caused by heat or due to other factors like electricity, contact with chemicals, radiation or radioactivity is called burn. The pathophysiology of the burn wound is determined mostly by the initial distribution of heat onto the skin. Jackson explains the three zones of histopathological injury: coagulation, stasis and hyperemia (Jackson, 1953). The zone of coagulation consists of eschar or necrotic tissue and is nearest to the heat source. It is surrounded by the zone of stasis, where there is only moderate tissue damage, but the blood flow is slow and edema occurs due to capillary leakage and cell membrane disruption. The poor blood flow may lead to local tissue ischemia and necrosis. Surrounding the zone of stasis is the zone of hyperemia, where the cell damage is minimal and blood flow gradually increases that resulting in early recovery **(**Despa *et al*., 2005**).**

The burn injury can be of many types and according to which the healing of the wound occurs. The depth of the wound is one factor which determines the healing process. The superficial burn wound involves the disruption of the epidermis only hence they heal within 3-4 days without any scarring of the tissue. Mostly the treatment strategies include the use of the smoothening lotions Table 2.1. Partial thickness burns interrupt the epidermis and the papillary dermis. The main indication of this type of burn is blistering. They can be again classified as superficial or deep. The superficial partial thickness burns are pink, painful and moist and they usually heal by 2 to 3 weeks without any scarring while deep partial thickness burn wounds affects the reticular layer of the dermis and they are white, dry and variably painful. The deep partial thickness burns when not infected will heal by 3to 8 weeks. Full thickness burn is another severe form of burn wound which extend from entire dermis to the subcutaneous tissue and they appears white or black, dry and are usually painless (Monstrey *et al*., 2008).

Table 2.1 Overview of Burn Wound Management

Burn wound type	Clinical features	Management
Superficial	Erythematous, pain	Soothing, moisturizing lotions (i.e., aloe)
Partial thickness	Blistered, pink, moist, painful	Silver sulfadiazine; greasy gauze once epithelial buds are present
Deep partial thickness	Dry, mottled pink and white, less painful	Silver sulfadiazine dressings daily; surgical excision and grafting if not going to heal within 3 week
Full thickness	Dry, leathery, black or white, painless	Silver sulfadiazine; early excision and skin grafting

Burn patients are expected to be at high risk of infection as a result of the nature of the burn injury, prolonged hospital stay, the immuno compromising effects of burns, and intensive diagnostic and therapeutic procedures. Another major problem is the sepsis of the burn wound which is a serious issue that may lead to death of the victim and is one of the major factors determining the prognosis o the burn diseases. The sepsis is directly related to the extent of burn. The infection is mainly due to the impaired resistance from the disruption of mechanical integrity of skin and immune suppression (Rashid *et al*., 2017). These factors delays the burn wound healing in most cases.

2.3.2. Burns-extent and severity

Burns is a three dimensional injury. Severity of burns depends upon quantum of tissue burnt and depth. Whole body surface area is taken as 100%. Proportion of surface burn is represented as % age.

2.3.3. Estimation of surface area

There are many ways to estimate surface area burned. None of these are 100% accurate. Rule of Nine which was popularized by A.F.Wallace of Edinburgh remains the most popular method of describing the surface area burn. In this, body is divided into 11 equal parts making this 99% and 1% is given to perineum (Figure 2.2).

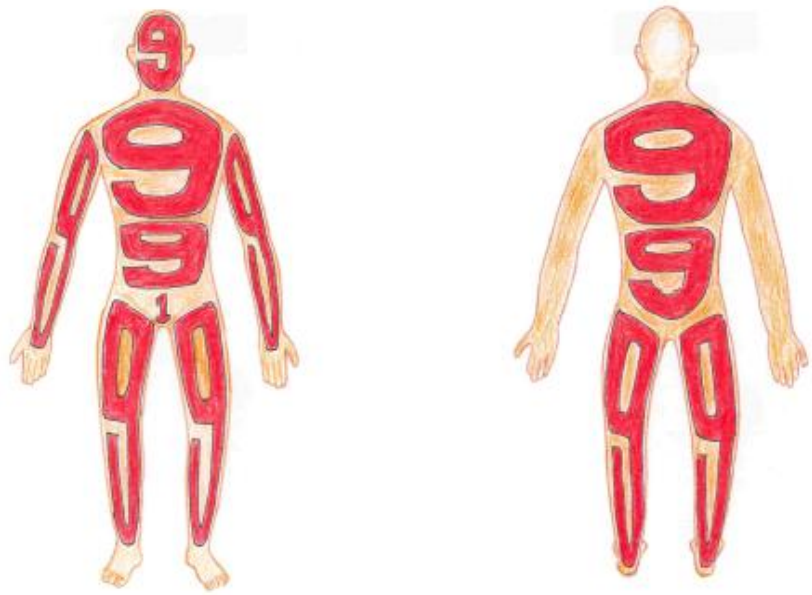
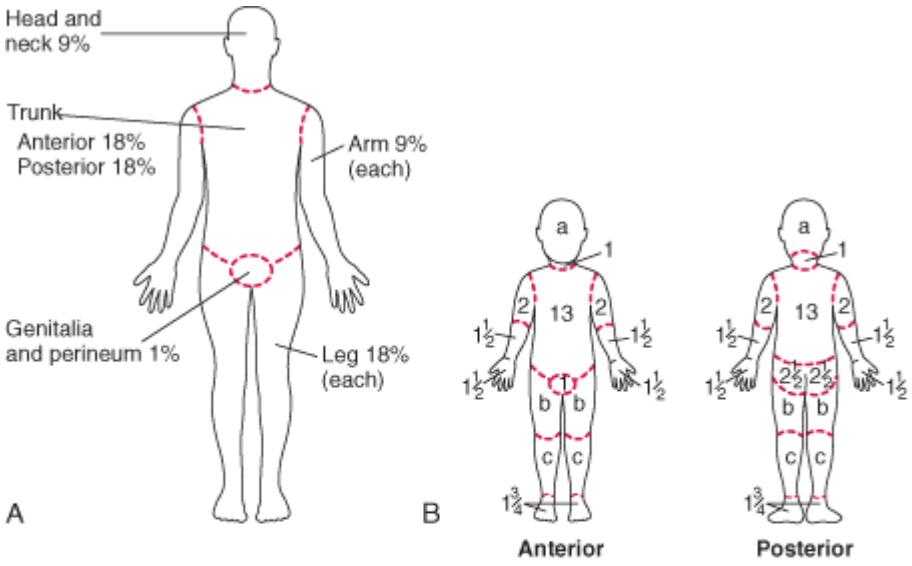


Figure 2.2. 'Rule of Nine' for estimation of TBSA burned (Ref-Sarabahi, 2010)

In new borns and children, because of the larger size of head and small body surface area of limbs, the Rule of Nine is not applicable. Lund and Browder chart simplifies the calculation of total body surface area burn in children. This takes into account the variation in the body surface area of different parts of the body in different age group. Also, it is common to see patchy burns making calculations a difficult proposition. For this, more elaborate chart was proposed by Lund and Browder which also takes in consideration the patient's age for calculation of surface area of burns involved. This appears to be the most accurate method so far available but it requires availability of well written charts and is difficult to remember. (Figure 2.3).

One closed hand of an individual is equal to his 1% body surface area. This hand must be of the person concerned who sustains burns. A hand consists of all the fingers and thumb brought together in extended position, which include palm and all the fingers. This is applicable universally in every age group. This is popularly known as the 'Rule of Palm'. (Figure 2.4)



Relative percentage of body surface area (% BSA) affected by growth

	Age				
Body Part	**0 yr**	**1 yr**	**5 yr**	**10 yr**	**15 yr**
a = 1/2 of head	9 1/2	8 1/2	6 1/2	5 1/2	4 1/2
b = 1/2 of 1 thigh	2 3/4	3 1/4	4	4 1/4	4 1/2
c = 1/2 of 1 lower leg	2 1/2	2 1/2	2 3/4	3	3 1/4

Figure 2.3. Lund & Browder chart (Ref-Orgil, 2009)

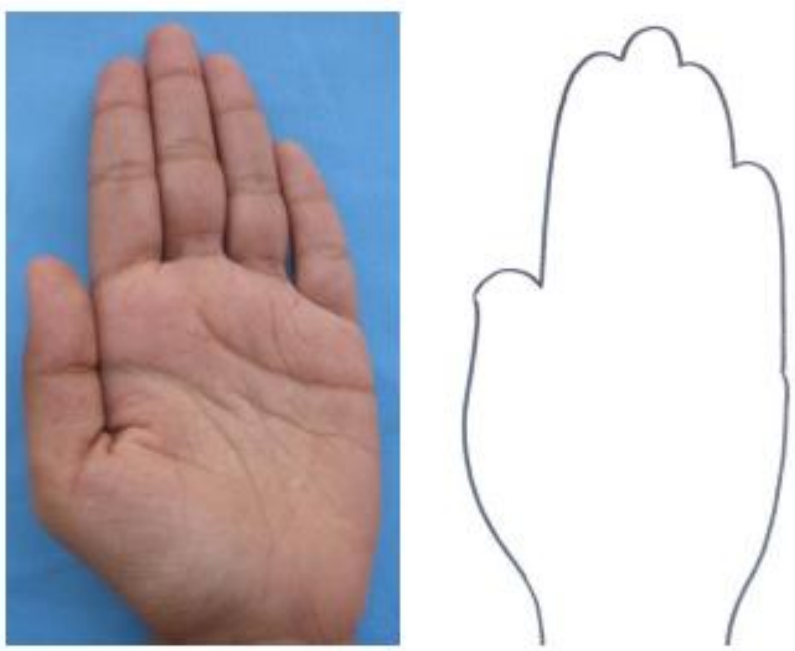


Figure 2.4. Rule of Palm (1%) (Ref-Marella, 2011)

2.4. BURN WOUND HEALING

Wound healing is a complex and dynamic process that is dependent on a number of interrelated factors in the wound environment changing with the shifting health of an individual. Wound treatment should be based on a pre-knowledge on normal tissue repair

and factors affecting the process. The normal wound healing mechanism includes mainly four phases: hemostasis, inflammation, granulation and maturation that provide a framework for understanding the basic principles of wound healing. By this information the healthcare professional can develop the skills required to care wound and tissue repair. Certain wounds are not cured effectively and such wounds give the space for the doctors to pursuit certain hidden causes. Wounds that does not heal by it easily requires considering the factors including holistic, inter professional, patient-centered, collaborative, cost-effective and evidence-based care **(**Kerstein, 1997**).**

2.4.1. Principles of burn wound healing

Any thought of wound healing process should always consider the general principles of wound healing, which consists of four phases (Table 2.2). The fundamental, molecular and biological events after the dermal injury cannot be separated and treated in an obvious manner while it has been a practice to separate the repair procedure into main four overlapping phases of hemostasis, inflammation, proliferation and re-modeling (Van *et al* ., 2001).

Table 2.2 The four phases of wound healing

Phase of healing	Time post injury, days	Cells involved in phase	Function/activity
Hemostasis	Immediate	Platelets	Clotting
Inflammation	1-4	Neutrophils Macrophages	Phagocytosis
Proliferation (granulation and contraction)	4-21	Macrophages, Lymphocytes, Angiocytes, Neurocytes, Fibroblasts, Keratinocytes	Fill defect, Re-establish, Skin function Closure
Remodeling (maturation)	21-730	Fibrocytes	Develop tensile strength

2.4.1.1. Hemostasis

The first step in this phase is the sealing of the damaged blood vessels to avoid further blood flow which is done by the platelet cells. After any injury the blood vessels constrict to resist the further blood flow and gradually they relax as the healing continues. The platelets secrete vaso-constrictive substances to form a stable clot that aid the sealing of damaged vessels. The damaged tissue has adenosine diphosphate leaking from them which influence the platelets to adhere to the exposed type 1 collagen. Thus the type 1

collagen gets activated and secretes adhesive glycoprotein, leading to platelet aggregation. Moreover they also secrete factors that interact with the intrinsic clotting cascade via the production of thrombin, which in turn initiates the formation of fibrin from fibrinogen. The platelet aggregation is strengthened by the fibrin mesh which ultimately results in a stable haemostatic plug. Thus these platelets secrete growth factors -platelet derived growth factor which is one of the first factors in initiating the subsequent healing steps. These growth factors recruit many other cells like neutrophils and monocytes which in fact stimulate epithelial cells and fibroblasts. Hemostasis occurs within minutes of the initial injury unless the particular patient has some clotting disorders (MacLeod, 1981).

2.4.1.2. Inflammation

The tissue damage followed by the activation of clotting factors stimulates the release of inflammatory mediators such as histamine and prostaglandins from mast cells. The inflammatory mediators cause blood vessels near to the wounded area to become more vasodilate and permeable. The inflammatory response can be easily identified by the presence of swelling, discomfort, erythema, localized heat, and functional disturbance. Some of the major indicators of inflammation are increased blood flow to the wounded area and the accumulation of fluid in the soft tissues. Due to the increased permeability of capillary membranes, the wound exudates are produced in this stage of healing (Hutchinson, 2000). These exudates have high content of proteins and nutrients including growth factors, enzymes which facilitate easy wound healing. They cleanse the wound bed and act as a growth medium for phagocytic cells. Neutrophils arrive at the wound within a few hours of injury and are the first type of white blood cell to be attracted into the wound. They provide initial protection against microorganism at the wound site. After a few days macrophages arrives at the wound site and they mainly cleanse the wound bed and also produce various substances that regulate wound healing. The new tissue formation in wound bed won't occur until the macrophages stimulate the proliferative phase by the growth factors; also the wound bed has been cleansed by the inflammatory process. Hence the changes between the inflammatory phase and proliferative phases of healing are controlled by the macrophages. (Katz *et al*., 2011)

2.4.1.3. Proliferation

The formation of ECM and the beginning of angiogenesis acts as a marker for proliferative phase. The cells involved in this phase include the fibroblast and endothelial cells. These cells are proliferated by the action of the growth factors and cytokines which are released from the macrophages or platelets or from the fibrin clot. Fibroblasts releases collagen and glycosaminoglycan in response to macrophage released growth factors. The collagen forms the new extracellular matrix that develops the granulation tissue which eventually fills the wound. Angiogenesis allows nutrients and healing factors to enter the wound space. It is also very essential for the growth of granulation tissue. The principle growth factors that regulate angiogenesis are FGF (Fibroblast growth factor) and VEGF (Vascular endothelial growth factor) which are released by the keratinocytes and macrophages.

2.4.1.4. Maturation

It begins after 3 weeks of injury and can take up to years to complete. Usually the collagen fibers which are newly formed are randomized and disorganized.

2.4.1.5. Remodeling

Here the remodeling and realignment of the collagen are done so as to acquire the maximum strength to the so formed tissue. Moreover, cell and capillary density decrease. The main cell which is involved in this step is the fibroblast. The remodeling can take more than 2 years after a wound occurs. (Sussman *et al*., 2007)

2.5. FACTORS AFFECTING WOUND HEALING

The host and local factors play a major role in wound healing. The main host factors include age, nutritional intake, body condition and concurrent disease. Larger wounds keep animals in a catabolic state and thus the calorie and protein intake must be increased so that the nutritional requirements are met. Hypoproteinemia and diets deficient in protein leads to delaying of wound healing and decrease in wound strength. Many other conditions like metabolic disorders such as diabetes mellitus also delays wound healing, also medications like chemotherapy can also led to delayed wound healing. Non-steroidal anti-inflammatory drugs are also under investigation with regard to their effects on wound healing. But a significant effect of these components is not observed yet (Cornell, 2012).

Local wound factors known to impact healing include tissue perfusion, tissue viability, infection, presence of hematoma and/or seroma, and mechanical factors (tension, motion, wound debris, etc.). The presence of debris, dirt, hair, suture, and necrotic or devitalized tissue act as foreign material, leading to an intense inflammatory reaction that prolongs the inflammatory phase and delays the repair phase (Franz, 2008). Accumulation of fluid in the wound bed, as with a hematoma or seroma, inhibits fibroblast migration, encourages infection, and leads to wound ischemia, delaying wound healing and strength formation. Infection of the wound negatively impacts the process of wound healing as well. It is generally recommended that a wound contaminated with greater than 105 organisms per gram of tissue not be primarily closed, as the incidence of infection is increased. Other mechanical factors that impede normal healing include tight bandage placement, tension, and motion, all of which may lead to impaired blood supply, tissue ischemia and necrosis, increased risk of infection, and dehiscence. Following Halsted's surgical principles like gentle tissue handling, strict aseptic technique, sharp anatomic dissection, meticulous hemostasis, and obliteration of dead space, avoidance of tension, preserved vascularity, and careful approximation of tissues will reduce negative local factors in wound healing. (Amsellem, 2011).

2.6. BURN WOUND MANAGEMENT

A wide variety of dressings is available for the treatment of partial thickness burn wounds, but none has strong evidence to support their use (Wasiak, 2013). Understanding the key principles of dressing selection will help to simplify the process. Traditional dressings include a combination of paraffin-impregnated gauze and an absorbent cotton wool layer (Hudspith, 2004). However, these simple dressings tend to adhere to the wound surface. Advances in dressing technology has lead to a wider range of dressing options, some of which may offer advantages over traditional products in terms of time to healing, pain experienced and frequency of dressing changes (Alsbjörn, 2007). The characteristics of a good burn wound dressing have been described as:

- ❖ Maintains a moist wound environment
- ❖ Non-adherent to protect delicate skin
- ❖ Retains close contact with the wound bed
- ❖ Protects against infection
- ❖ Contours easily
- ❖ Painless on application and removal
- ❖ Easy to apply and remove
- ❖ Cost-effective

A simple non-adhesive wound contact layer with a secondary absorbent layer is effective for most non-complex superficial dermal burns. Pain is also an important consideration and, where possible, non-adherent products (e.g. incorporating soft silicone) should be considered. These can remain in place for a few days, allowing the wound bed remain undisturbed. The secondary absorbent layer can be changed more often to manage exudates (Atiyeh, 2005).

2.7. WOUND DRESSING

Wounds are to be considered carefully and treated accordingly and the first step towards it includes a proper wound dressing. Dressing is designed to be in contact with the wound, which is different from a bandage that holds the dressing in place. Historically, wet-to-dry dressings have been used extensively for wounds requiring debridement. In 1600 BC, Linen strips soaked in oil or grease covered with plasters was used to occlude wounds. Clay tablets were used for the treatment of wounds by Mesopotamian origin from about 2500 BCE. They cleaned wounds with water or milk prior to dressing with honey or resin. Wine or vinegar usage for cleaning the wounds with honey, oil and wine as further treatment was followed by Hippocrates of ancient Greece in 460- 370 BCE. They used wool boiled in water or wine as a bandage (Daunton, 2012). There was a major breakthrough in the antiseptic technique during the 19th century, antibiotics were introduced to control infections and decrease mortality. Modern wound dressing arrival was in 20th century. When the wound is closed with dressing they are continuously exposed to proteinases, chemotactic, complement & growth factors, which is lost in the wound exposed. So during late 20th century, production of occlusive dressing began to protect and provide moist environment to wound. These dressings helps in faster re-epithelialization, collagen synthesis, promotes angiogenesis by creating hypoxia to the wound bed and decreases wound bed pH which leads to decrease in the wound infection (Sujatha, 2012). Woven absorbent cotton gauze was used in 1891. Until the mid 1900's, it was firmly believed that wounds healed more quickly if kept dry and uncovered whereas 'closed wounds heal more quickly than open wound' written in an

Egyptian medical text -Edwin smith surgical papyrus in 1615 BC. In the mid 1980's, the first modern wound dressing were introduced which delivered important characteristics providing moisture and absorbing fluids (e.g. polyurethane foams, hydrocolloids, iodine-containing gels). During the mid 1990's, synthetic wound dressings expanded into various group of products which includes hydrogels, hydrocolloids, alginates, synthetic foam dressing, silicone meshes, tissue adhesives, vapor-permeable adhesive films and silver/collagen containing dressing.

2.7.1. Traditional wound dressing

Traditional wound dressing products including gauze, lint, plasters, bandages (natural or synthetic) and cotton wool are dry and used as primary or secondary dressings for protecting the wound from contaminations. Gauze dressings made out of woven and non woven fibres of cotton, rayon, polyesters afford some sort of protection against bacterial infection. Some sterile gauze pads are used for absorbing exudates and fluid in an open wound with the help of fibres in these dressings. These dressings require frequent changing to protect from maceration of healthy tissues. Gauze dressings are less cost effective. Due to excessive wound drainage, dressings become moistened and tend to become adherent to the wound making it painful when removing. Bandages made out of natural cotton wool and cellulose or synthetic bandages made out of polyamide materials perform different functions. For instance, cotton bandages are used for retention of light dressings, high compression bandages and short stretch compression bandages provide sustained compression in case of venous ulcers. Xeroform™ (non-occlusive dressing) is petrolatum gauze with 3% of Bismuth tribromophenate used for non-exudating to slight exudating wounds. Tulle dressings such as Bactigras, Jelonet, Paratulle are some examples of tulle dressings commercially available as impregnated dressings with paraffin and suitable for superficial clean wound. Generally traditional dressings are indicated for the clean and dry wounds with mild exudate levels or used as secondary dressings. Since traditional dressings fail to provide moist environment to the wound they have been replaced by modern dressings with more advanced formulations (Boateng, 2008).

2.7.1.1. Honey dressings

The use of honey as an antibacterial is well established in modern wound care, with medical-grade honey used in a variety of commercially available dressings. These

dressings provide antimicrobial and anti-inflammatory properties through autolytic debridement and maintenance of a moist wound environment while inhibiting bacterial growth, stimulating wound healing and deodorising the wound, although research trends are mixed in regard to their overall efficacy (Dumville *et al*., 2011). Honey is bactericidal and antifungal against approximately 70 bacterial strains, both gram-positive and gram-negative, and some yeasts (Molan, 2001) and is often used to control bacterial strains resistant to conventional antibiotics. Antimicrobial action is both mechanical and enzymatic. Like sugar pastes, honey can inhibit bacterial growth through its osmolarity, where the high concentration of sugars causes water to be drawn from the local wound environment. This also maintains a moist wound environment by stimulating fluid transfer from surrounding tissues (Cooper, 2008). Whilst this action dilutes the honey, its antibacterial effects remain (Kwakman *et al*., 2012). Honey is applied topically to a wide range of wounds in the form of an ointment, for packing cavities, or impregnated within a hydrogel or alginate dressing (Dutta *et al*., 2011). When used as an ointment, the honey will rapidly dilute due to absorption of wound exudates as well as increase in fluidity upon warming to body temperature and may, therefore, require frequent dressing changes in order to maintain efficacy.

2.7.1.2. Aloe vera

Aloe vera Linn, synonym: *aloe vera* barbadensis Mill. is in family Liliaceae, which is a tropical plant easily grown in hot and dry climates including Thailand. Numerous cosmetics and medicinal products are made from the mucilaginous tissue, called aloe vera gel, located in the center of the aloe vera leaf. Aloe vera gel has been used for many indications since the Roman era or even long before. In Mesopotamia, clay tablets dated 1750 B.C.E. showed that Aloe vera was being used in a pharmaceutical manner. Egyptian books from 550 B.C.E. mentioned that infections of the skin could be cured by the application of Aloe. In 74 C.E., a Greek physician, Discordes, wrote a book entitled De Materia Medica in which he stated that Aloe could treat wounds, heal infections of the skin, cure chapping, decrease hair loss, and eliminate hemorrhoids.' Aloe was used predominantly for eczema around 1200 C.E (Coats *et al*., 1979). Because Aloe was used mostly as a cathartic medicine, little thought was given to its other uses. Previously reported applications of Aloe vera, which are not well substantiated, include seborrheic dermatitis, thermal burns and sunburn, cystic acne, peptic ulcers, amputation stump ulcers, lacerations,^ colds, tuberculosis, gonorrhea, asthma, dysentery, and

headaches. It has also been used as an insect repellent and as a laxative. Burn wound healing is one of major indications of aloe vera gel use in many countries (Cole *et al*., 1993). In Thailand, aloe vera gel was included in the Thai Herbal Fundamental Public Health Drug List as burn wound therapy. Several studies suggested that aloe vera, or one or more of its constituents, promote wound healing in various animal models. In 1851, it was discovered that the potency of Aloe was the result of aloin, a bitter juice that dried to a yellow powder and functioned as a cathartic medicine (Gottlieb, 1990). It is synonymous with barbaloin, 10-(r, 5'-anhydroglucosyl)-aloe-emodin-9-anthrone, which is a glycoside. Anthraquinone derivatives include anthracenes such as aloe-emodin, which is 1,8-dihydroxy-3(hydroxymethyl)-9,10-anthracenedione. These water-soluble glycosides were separated from the water-insoluble resinous material (Klein *et al*., 2008). The different species of Aloe have different chemical compositions. The leaves of the Aloe vera plant contained 99.5% water and 0.013% protein**.** Glucomannan (GLU) and acemannan are the other two components of aloe vera which are responsible for the wound healing activity in aloe vera gel (Reynolds, 1999).

2.7.1.3. The use of sugar for wound treatment

Sugars such as granulated sugar, starch (amylose), honey and cellulose have the same chemical structure of $C_6H_{12}O_6$ with differences in molecular weight and stereochemistry. The use of sugar in medicines to treat wounds and ulcers has been reported extensively in the last few centuries by Scultetus in 1679 and Zoinin in 1714 (Pieper *et al*., 2003). Sugars help to reduce exudation and edema, enhance nutrients in healing tissue, stimulate granulation tissue, lower the wound pH, induce a bacteriostatic effect, and encourage growth of epithelial tissue (Knutson *et al*., 1981). Silvetti claimed that a high molecular weight polysaccharide prevented the growth of bacteria in wounds by lowering the water content below 0.65% through osmosis. (Silvetti, 1981). The binding of water creates a template suitable for cell migration and proliferation (Brenda *et al*. 1995), encouraging the production of hyaluronic acid from glucose and simultaneously suppresses the formation of fiber forming collagen that contributes to scarring. Specific interactions of sugar with different cell types also encourage the excretion of essential cytokines and growth factors that can improve conditions of wound healing (Topham 2002).

2.7.2. Modern wound dressing

Modern wound dressing have been developed to facilitate the function of the wound rather than just to cover it. These dressings are focused to keep the wound from dehydration and promote healing. Based on the cause and type of wound, numerous products are available in the market, making the selection a very difficult task. Modern wound dressings are usually based on synthetic polymers and are classified as passive, interactive and bioactive products. Passive products are non-occlusive, such as gauze and tulle dressings, used to cover the wound to restore its function underneath. Interactive dressings are semi-occlusive or occlusive, available in the forms of films, foam, hydrogel and hydrocolloids. These dressings act as a barrier against penetration of bacteria to the wound environment (Strecker, 2007).

2.7.2.1. Semi-permeable film dressings

These dressings are composed of transparent and adherent polyurethane which permits transmission of water vapor, O_2 and CO_2 from the wound and it also provides autolytic debridement of eschar and impermeable to bacteria (Debra, 1998). Initially, films were made from nylon derivatives with an adhesive polyethylene frames as the support which made them occlusive. Originally nylon derived film dressings were not used for highly exudating wounds due to their limited absorption capacity and caused maceration of the wound and the healthy tissues around the wound. But, these dressings are highly elastic and flexible, and can conform to any shape and do not require additional tapping. Inspection of wound closure is also possible without removal of wound dressing because of transparent films. Hence these dressings are recommended for epithelializing wound, superficial wound and shallow wound with low exudates, e.g. Opsite™, Tegaderm™, Biooclusive™. Commercially available film dressings differ in terms of their vapour permeability, adhesive characteristics, conformability and extensibility (Thomas, 2008).

2.7.2.2. Semi-permeable foam dressings

Foam dressings are made up of hydrophobic and hydrophilic foam with adhesive borders sometimes. The hydrophobic properties of outer layer protect from the liquid but allow gaseous exchange and water vapor. Silicone-based rubber foam (silastic) molds and contours to wound shape. Foam has capability of absorbing varying quantities of wound drainage depending upon the wound thickness. Adhesive and non adhesive foam

dressings are available. Foam dressings are suitable for lower leg ulcers and moderate to highly exudating wounds, also indicated for granulating wounds. They are generally used as primary dressings for absorption and secondary dressings are not required due to their high absorbancy and moisture vapour permeability (Marcia, 2002). Disadvantage of foam dressing is requiring frequent dressing and is not suitable for low exudating wounds, dry wounds and dry scars as they depend on exudates for its healing e.g. Lyofoam™, Allevyn™ and Tielle™.

2.7.2.3. Hydrocolloid dressing

Hydrocolloid dressings are among the most widely used interactive dressings and are consist of two layers, inner colloidal layer and outer water- impermeable layer. These dressings are made up of the combination of gel forming agents (carboxymethyl cellulose, gelatin and pectin) with other materials such as elastomers and adhesives. Hydrocolloids are permeable to water vapor but impermeable to bacteria and also have the properties of debridement and absorb wound exudates (Thomas, 1998). They are used on light to moderately exudating wounds such as pressure sores, minor burn wounds and traumatic wounds. These dressings are also recommended for pediatric wound care management, as they do not cause pain on removal. When this hydrocolloids contact with the wound exudate they form gels and provide moist environment that helps in protection of granulation tissue by absorbing and retaining exudates. Granuflex™, Comfeel™, Tegasorb™ are available in the form of sheets or thin films. Disadvantage of hydrocolloids are they are not indicated for neuropathic ulcers or highly exudating wounds, also they are mostly used as a secondary dressings.

2.7.2.4. Alginate dressing

Alginate dressings are made from the sodium and calcium salts comprising mannuronic and galuronic acid units. Absorbent and biodegradable alginates are derived from seaweed. Absorption capability is achieved by strong hydrophilic gel formation, which limits wound exudates and minimizes bacterial contamination. Even though some studies have reported that alginate inhibits keratinocytes migration, Thomas *et al*., have reported that alginates accelerate healing process by activating macrophages to produce TNF-α which initiates inflammatory signals. Once alginate dressings are applied to the wound, ions present in the alginate are exchanged with blood to form a protective film. Alginate dressings are suitable for moderate to heavy drainage wounds and not suggested

for dry wound, third degree burn wound and severe wounds with exposed bone. Also these dressings require secondary dressings because it could dehydrate the wound which delay healing. Sorbsan™, Kaltostat™, Algisite™ are some alginate dressings commercially available.

2.7.2.5. Bioactive wound dressings

The last type of modern wound dressing is bioactive dressings and is produced from biomaterials which play an important role in healing process. These dressings are known for their biocompatibility, biodegradability and non-toxic nature and are derived generally from natural tissues or artificial sources such as collagen, hyaluronic acid, chitosan, alginate and elastin. Polymers of these materials are used alone or in combination depending on the nature and type of wound. Biological dressings are sometimes incorporated with growth factors and antimicrobials to enhance wound healing process. Collagen, a major structural protein has been discussed by many researchers for their active role in natural healing process (Ishihara *et al*., 2002) Collagen initiates fibroblast formation and accelerates endothelial migration upon contact with wound tissue. Hyaluronic acid (HA) is a glycosaminoglycan component of extra cellular matrix (ECM) with unique biological and physicochemical features. Similar to collagen, HA also biocompatible, biodegradable and lack immunogenicity naturally (Supp, 2005). Chitosan promotes the formation of granulation tissue during the proliferative stage of wound healing. When compared to other dressings, biological dressings are reported to be more superior to other types of dressings.

2.7.2.6. Medicated dressings

Medicated dressings incorporated drugs plays an important role in the healing process directly or indirectly by removal of necrotic tissues. This has been achieved by cleaning or debriding agents for necrotic tissue, antimicrobials which prevents infection and promotes tissue regeneration. Some commonly incorporated compounds include antimicrobial agents, growth factors and enzymes. Commercially available antimicrobial dressings include Cutisorb™. Silver impregnated dressings available are Fibrous hydrocolloid, Polyurethane foam film and silicone gels. Antiseptic Iodine dressing acts on bacterial cells via oxidative degradation of cell components by interrupting the function of protein, which is widely effective against pathogen. Prolong usage of iodine leads to skin irritation and staining (Liesenfeld *et al*., 2009). The purpose of antimicrobials is

mainly to prevent or combat infections especially for diabetic foot ulcers. Normal tissue repair process in the body is controlled by cellular activities caused by growth factors that are naturally present in our body. In case of chronic wounds, growth factors and cells are arrested in the wound bed within the clots that affects the healing process. So exogenous application of growth factors benefits the wound healing process and this was proved by numerous studies. Among the different growth factors, platelet derived growth factor (PDGF) is the most commonly used growth factor which promotes chemotactic recruitment and proliferation of cells and increasing angiogenesis. Besides, PDGF, fibroblast growth factor (FGF), epidermal growth factor (EGF), and autologous platelet thrombin are also studied extensively for their application in healing process. Among which, PDGF and EGF are approved by FDA for human application. Enzymatic debridement of necrotic tissues without harming healthy tissue is also a crucial part to promote normal healing process. Papain and collagenase based ointments are currently used to digest necrotic tissue. Collagenase acts on the collagen by attacking native collagen and gentle on viable collagen by gradual breakdown of tissue whereas papain attacks cysteine residue and associated with inflammatory response. Debridace^{TM} is a commercially available dressing which increases proteolytic action.

2.7.2.7. Composite dressing

Composite dressings are versatile and convenient for both partial and full thickness wounds. A composite or combination dressings has multiple layers and each layer is physiologically distinct. Most of the composite dressings possess three layers. Composite dressings may also include an adhesive border of non-woven fabric tape or transparent film. They can function as either a primary or a secondary dressing on a wide variety of wounds and may be used with topical medications. Outer most layer protect the wound from infection, middle layer usually composed of absorptive material which maintains moisture environment and assist autolytic debridement, bottom layer composed of non adherent material which prevents from sticking to young granulating tissues. Composite dressings have less flexibility and they are more expensive (Laurie, 2011).

2.7.2.8. Hydrogels dressing

Hydrogels are insoluble hydrophilic materials made from synthetic polymers such as poly (methacrylate) and polyvinyl pyrrolidine. The high water content of hydrogels (70-90 %) helps granulation tissues and epithelium in a moist environment. Soft elastic

property of hydrogels provides easy application and removal after wound is healed without any damage. Temperature of cutaneous wounds is decreased by hydrogels providing soothing and cooling effect. Hydrogels are used for dry chronic wounds, necrotic wounds, pressure ulcers and burn wounds. Morgan has reported that except infected and heavy drainage wounds, hydrogel dressings are suitable for all four stages of wound healing. Hydrogel dressings are non irritant, non reactive with biological tissue and permeable to metabolites. Many researchers have reported that hydrogel dressings are used to treat chronic leg ulcers. Difficulties of hydrogel dressings are exudates accumulation leads to maceration and bacterial proliferation that produces foul smell in wounds. Besides, low mechanical strength of hydrogels making it difficult to handle. Some examples of hydrogels are Intrasite™, Nu-gel™, Aquaform™ polymers, sheet dressings, impregnated gauze and water-based gels (Pal *et al* ., 2009).

2.8. HYDROGELS

There are a variety of hydrogel dressings available. They are hydrogel materials which are often supplied on an impermeable polymeric backing sheet. The presence of the backing sheet prevents the hydrogel from dehydrating and desiccating the underlying wound. Hydrogels are supplied in a partially hydrated form, which allows for subsequent absorption and further hydration by absorption of wound exudates. Gels are similar to hydrocolloid dressings in their management of exudates as these materials have little or no vapor transport capacity (Talaat *et al*., 2008). Once they reach the maximum absorptive capacity they can no longer manage exudates and maintain an optimum microenvironment. Due to the fact that hydrogels are supplied in a partially hydrated state, they-are not supplied with a pressure sensitive adhesive coating. Further taping or application of a secondary dressing is therefore required to assure adequate attachment (Don *et al*., 2008). In addition, hydrogel dressings, after absorption of moderate quantity of exudate undergo swelling and change their physical parameters. The swelling results in the dressing moving away from the wound bed and providing potential air spaces or pockets in which bacteria may proliferate (Qunyi *et al*., 2005). In some instances, manufacturers recommend that the impermeable backing sheet be removed during the healing sequence on heavily exuding wounds. The removal of the sheet encourages the dehydration of the hydrogel, which moderately increases the dressing's ability to handle levels of exudates. During dehydration, however, the hydrogel becomes noncompliant and often results in damage to the underlying wound. Lastly, hydrogel wound dressings

generally do not dissolve and contaminate the wound in a fashion similar to hydrocolloids.

2.8.1. Hydrogel technical features

An ideal hydrogels for wound dressings should meet the many functional features. It should be high on absorption capacity (maximum equilibrium swelling) in saline, absorbency under load (AUL), durability and stability in the swelling environment and during the storage, biodegradability without formation of toxic species following the degradation. It should be colorless, odorless and absolutely non-toxic and should have desired rate of absorption depending on the application requirement.

It is impossible that a hydrogel sample would simultaneously fulfill all the above mentioned required features (Kabiri *et al*., 2003). In fact, the synthetic components for achieving the maximum level of some of these features will lead to inefficiency of the rest. Therefore, in practice, the production reaction variables must be optimized such that an appropriate balance between the properties is achieved. For example, a hygienic products of hydrogels must possess the highest absorption rate, the lowest re-wetting, and the lowest residual monomer, and the hydrogels used in drug delivery must be porous and response to either pH or temperature (Varshney *et al*., 2007).

2.8.2. Hydrogels as wound dressings for superficial and partial-thickness burns

Following a burn injury, the wound healing process, as well as the time required for healing, will basically depend on the thickness of the injured dermis layer. Appropriate burn care appears crucial for optimal healing and final appearance of the scar, since burn depth might dangerously increase if the wound dries or become infected and scar synthesis and contracture typically worsen for delayed wound closure (Odland *et al*., 2008). A number of specialized primary wound dressings are currently available for the treatment of partial-thickness burns (both superficial and deep partial-thickness burns) and other types of wounds, with low to high levels of wound exudates. Such dressings are designed to absorb the wound exudates, thus keeping a moist environment to facilitate debridement of the necrotic tissue and spontaneous re-epithelialization of the skin, (Winter *et al*., 1962) while providing temporary and prompt wound coverage and a mechanical barrier to infections. Several dressings might also include specific antibiotics or different antibacterial agents (e.g. silver ions) in their formulation, in order to further

protect the wound bed from undesired microbial contaminations. Due to their hydrophilic nature and soft tissue-like properties, polymeric hydrogels emerge over different types of biomaterials as prime candidates for the development of wound dressings for the treatment of burns and other skin lesions (Falanga, 1988).

Hydrogels are macromolecular networks, stabilized by means of chemical or physical interactions among the polymer chains, which are able to retain large amounts of water in their mesh structure. The dissolution of the polymer in the solvent is indeed prevented by the cross-linking nodes existing among the macromolecules. Due to this peculiar structure, hydrogels show a hybrid behavior, with mechanical (elastic) properties similar to those of solid, but diffusive properties matching those of a liquid. Remarkably, hydrogels can absorb and release water in a reversible manner, in response to specific environmental stimuli, e.g. temperature, pH and ionic strength (Madden *et al*., 2007). Such a smart behavior towards changes of physiological variables suggests their use in a variety of biomedical applications. The intrinsic potential of hydrogels to promote skin healing has been increasingly investigated and applied in the clinical setting since the early eighties. First of all, hydrogels absorb and retain the wound exudate, thus promoting fibroblast proliferation and keratinocyte migration, which are both necessary for complete epithelialization of the wound. Furthermore, the tight mesh size of hydrogels (in the order of 100 nm in the swollen state) prevents bacteria from reaching the wound, (Agris *et al*., 1979) while still allowing for an efficient transport of bioactive molecules (e.g. antimicrobial agents and pharmaceuticals) to the wound bed. Such molecules can be easily entrapped in the polymer network during the gelling process, in order to be gradually released to the wound as the hydrogel absorbs the exudate and swells. The peculiar and tunable mechanical properties of hydrogels also provide them with suitable elasticity and flexibility to adapt to wounds located in different body sites. Wearing comfort and immediate pain relief are likely the most advantageous features of hydrogels to the patients, if compared to traditional bandages, pads or gauzes (Turner, 2005) In case of burns, if clean running water is not available for first aid, application of hydrogels onto the wound is the only way to cool the wound, in order to minimize the extent of damage and reduce pain. The high water content of hydrogels makes them particularly cooling and soothing on the wounded area. Hydrogels are also non-adhesive, since cells do not readily attach to highly hydrophilic surfaces. This implies that hydrogel dressings do not stick to the wound, highly facilitating the change of the dressing by causing less pain and

discomfort to the patient. Hydrogel transparency, which may depend on the crosslink density of the polymer network, is an additional advantage over traditional bandages, as wound healing can be constantly monitored without removing the primary dressing. An enormous range of hydrogel dressings is commercially available for the treatment of minor burns and other skin wounds, in the multiple forms of amorphous gels, gel-impregnated gauzes, sheets or plasters (Lawrence, 1985). While amorphous gels are preferred for cavity wounds, sheets and gel-impregnated gauzes find application mainly in the treatment of superficial burns. Plaster-like hydrogel dressings (e.g. MySkin®) are particularly attractive for their easy use and removal, as they can be correctly positioned onto the wound without the need for additional dressings (adhesives and bandages). In spite of the various hydrogel-based products already on the market, the development or optimization of advanced hydrogel dressings still represents a very active research field, with the aim of further improving skin healing in relation to specific clinical applications. In particular, there is a growing tendency in the development of hydrogel formulations that encompass multiple materials, in an attempt to simultaneously address different aspects of wound healing (epithelialization, collagen synthesis, vascularization, contraction) and wound management (e.g. infection control, dressing flexibility) (Patti *et al*., 2006) In situ forming gels and radiation cross linked gels are also appealing for the development of novel wound dressings.

2.8.3. Poly (hydroxyethyl methacrylate)

Poly (hydroxyethyl methacrylate) (polyHEMA, PHEMA) is one of the most important and most widely applied hydrogel biomaterials. The poly (HEMA) copolymers have also been used to improve the biocompatibility of other materials to enhance their biomedical applications such as tissue engineering, controlled drug release and separation of bio-macromolecules (Hussain *et al*, 1983). The presence of the hydroxyl and carboxyl groups makes the polymer compatible with the water while the hydrophobic entity methyl group and back bone provide hydrolytic stability and mechanical strength to the polymer. PHEMA is easy and painless to apply and reduces the pain; the dressing is translucent and flexible, although the tensile strength is relatively low. Furthermore, the free hydroxyl group of the polymer system can be used to facilitate immobilization of other molecules such as collagen, which have a greater role in wound healing (Martin *et al*., 2003). As the PHEMA is permeable, the dressing can be loaded with antimicrobial agent, thereby reducing the infections. The PHEMA is mixed with a water-soluble solvent, a

suitable plasticizer and optionally antibacterial topical agent to obtain a burn wound dressing.

2.8.4. Hydrogel loaded with various factors

The new trend of designing wound dressing is by incorporating various factors which eases the wound healing capacity of the material. The factors like antimicrobials, anti inflammatory agents, growth factors are found to be incorporated in wound dressings. The medicated dressings have significantly increased the wound healing ability of the material.

2.8.4.1. Hydrogel loaded with antimicrobials

Antimicrobial dressings claim to manage the bio burden of the wound: they are thought to reduce the risk of invasive infections by minimizing the bacterial colonization of wounds. Antimicrobial hydrogels are extremely attractive materials for use as wound dressings and fillers. Due to their high water content, gels provide a moist, heavily hydrated environment to the wound area, facilitating cellular immunological activity essential to the wound healing process. However, this same hydrated environment can also facilitate microbial infection. Thus, gels capable of imparting antimicrobial action in addition to serving their primary functional role (e.g. wound healing, drug, delivery, etc) are hence desirable (Sibbald *et al*, 2011). Antimicrobials like ciprofloxacin, gentamicin, teicoplanin, and amoxicillin have each been used to prepare active gels.

2.8.4.2. Hydrogels loaded with silver nanoparticles

Silver nanoparticles (SNPs) have potential use in biomedical applications given their known antimicrobial properties against a broad range of bacteria and fungi Although their mechanism of antimicrobial action is not completely understood it seems to involve the generation of reactive oxygen species and binding to bacterial cell membranes, leading to membrane damage. Additionally, silver ions released from the NP can also exert antimicrobial action independently. The incorporation of silver NPs into a given hydrogel allows the formation of hybrid materials that display antimicrobial properties and are advantageous for biomedical applications. Hydrogels of distinct composition have been used to prepare antimicrobial hydrogel-silver NPs systems (Ana *et al*, 2013). These include hydrogels derived from synthetic polymers such as PVA, PVP,

and poly (acrylamide-co-acrylic acid), as well as natural polymers such as gelatin and alginate.

2.8.4.3. Hydrogels loaded with Growth factors

Growth factors (GF) have been shown to promote wound healing in acute wounds by stimulating the growth of keratinocytes. Since the process of wound healing depends on the mitosis and migration of keratinocytes, reduction of GF represents a key factor for chronification of wounds. Application of GF implemented into topical wound dressings represents one possible attempt to face this problem. The in vivo use of a bilayer wound dressing containing epidermal growth factor- (EGF-) loaded microspheres decreased wound areas in a rabbit model in a dose-dependent manner much faster than wound dressings without EGF (Ulubayram *et al* .,2001). Granulation tissue could be increased by stimulation of fibroblast proliferation and collagen production and healing time of diabetic ulcers was reduced by 32%, nearly 6 weeks faster than placebo gel (Smiell *et al*, 1999). Beyond certain remarkable case of adverse events in the use of GF, the application of purified GF via a wound dressing raises technical difficulties with low bioavailability and high manufacturing costs, and the fact that wound healing requires a complex mixture of growth factors. But there are still developments to improve bioavailability of single or multiple growth factors in wound dressings, for example, by incorporating them into biomaterial based dressings like silk fibers or with release in a time-dependent manner.

2.8.4.4. Glucomannan for promoting wound healing

Carbohydrates may be utilized both topically and by ingestion for optimal skin health (AlGhazzewi and Tester, 2014). In topical formulations, specific carbohydrates can be applied to the skin to promote the growth of beneficial skin flora (Krutmann, 2009). Mannan-containing carbohydrates (e.g. depolymerised Konjac glucomannan) are able to exert a positive impact on skin health by reducing infections such as acne vulgaris and by inducing a ‘glow’ (Bateni, 2013). Glucomannan have been reported to promote accumulation of fibroblasts and stimulate the production of collagen in skin wounds (Shahbuddin *et al*., 2013). Similarly, Shahbuddin, 2014 reported that Konjac glucomannan has the ability to stimulate fibroblast metabolites and the migration of both fibroblasts and keratinocytes. Aloe vera leaf extracts have been utilized for wound care historically where their mannans ‘acemannans’ promote healing (Tizard, Busbee, Maxwell, & Kemp, 1994). Glucomannan from Konjac is widely used by Chinese and

Japanese as traditional medicine in the treatment of asthma, coughs, hernia, burns, and dietary fiber in the treatment of diabetes and obesity. Glucomannan helps to decrease total cholesterol and low density lipoprotein and to maintain high density lipoprotein and increase insulin sensitivity from the pancreas.

Glucomannan can be extracted from either *Amorphophallus Konjac C* .Koch, also known as Konjac, devil's tongue or elephant yam or from Aloe vera. The main source of glucomannan is Konjac. It is a nonionic, linear polysaccharide formed by units of 1, 4-linked-b-D-mannopyranose and b-D-glucopyranose, with acetyl substitutes. The backbone chain of Glucomannan is branched about every 68 monosaccharide units. The branch chains, length of 3-4 monosaccharides, are linked to either the C-3 hydroxyl group of glucose or the C-6 hydroxyl group of glucose/mannose on the main chain (Chen *et al*. 2013). The ratio of mannose to glucose is 1.6:1 (Katsuraya *et al*. 2003) and the monosaccharide residues may be randomly distributed. The molecular weight of Glucomannan varies from 200 to 2,000 kDa, depending on cultivars, origin, producing methods and storage time. It is soluble in water, despite its strong inter-chain association that normally renders a polymer water-insoluble. Its water solution is extremely viscous and is considered the most viscous among other natural colloidal solutions (Wang *et al*. 2011). Although Glucomannan has both hydrophilic (i.e. hydroxyl groups) and hydrophobic groups (i.e. acetyl groups), it is insoluble in organic solvents such as methanol, ethanol, acetone or ether. Its rheological properties and safety make glucomannan a popular emulsifier and stabilizer in the food industry (Chen *et al*. 2006). In the biomedical field, glucomannan is widely used for drug delivery and, in particular, for the design of colon-specific drug delivery system (CSDDS) and in enhancing wound healing.

2.9. GAP AREA

Briefly, the literature described above indicates that there are several limitations with the currently available wound dressing. The major problem with currently available wound dressings is the adherence of the wound dressing to the burn wound which leads to sprouting of another serious wound which causes pain, discomfort to the patient especially when the dressings are changed. Another equally important concern is the exorbitant cost making them unaffordable to common people who often suffer from burn wounds. Because most of the dressings are prone to hold a huge amount of exudates from

the wound, infection is a major problem which retards wound healing. In the cases where antimicrobials are loaded into the dressing, sudden burst of the active components into the wound initially may cause toxic effects and is not favorable for wound healing (Boateng *et al*., 2007). Furthermore, some of the medicated dressings with antibiotics may produce antibiotic resistance at the site. The other problems include the lack of strength of the wound dressing which is obtained when the material is a hydrogel. Deactivation of growth factor within the polymeric matrix during the formulation procedure is a concern in the development of growth factor releasing wound dressings. Hence the designing of a burn wound dressing is difficult as there are many factors which should be considered; including depth variations, size and location of the burn wound. Nature of healing may be affected by factors such as infection, patient's age, other health conditions such as diabetes, nutritional status, associated injuries etc. Therefore, this thesis proposes development of a wound dressing using unique approach to produce a cost-effective hydrogel based dressing incorporated with certain molecules that can promote wound healing.

2.10. HYPOTHESIS

Incorporation of commercially available glucomannan(normally extracted from *aloe vera* or Konjac plant) which has been widely used in skin care, with in-house developed hydrogel system comprising PHEMA, PCL and PEG to result in an improved non-adhesive bioactive wound dressing with antimicrobial and wound healing properties.

2.11. OBJECTIVES

1. To prove nontoxic behavior of glucomannan by testing the material as per ISO10993-5 for in vitro cytotoxicity (test on contact)
2. To synthesize the PHEMA-PCL.-PEG hydrogel and characterize its physicochemical characteristics
3. To incorporate glucomannan into the hydrogel and characterize its physicochemical properties
4. To test the effect of glucomannan in promoting fibroblast proliferation
5. To test the antimicrobial effect of glucomannan when incorporated in the hydrogel

CHAPTER 3

MATERIALS AND METHODS

3.1. DEVELOPMENT OF PHEMA-PCL-PEGHYDROGEL MATRIX

3.1.1. Materials used

2-hydroxyethylmethacrylate [HEMA], 2,4,6-Trimethyl Benzoyl Biphenyl Phosphine oxide (TPO), Poly (ε-caprolactone) (PCL, M_w 14,000, M_n 10,000) and Ofloxacin (OFL, R&D grade) were all purchased from Sigma-Aldrich Chemical Company Inc., USA. Polyethylene glycol (PEG, MW 200) was procured from Merck, Germany. Glucomannan (GLU) was purchased from Biovea, USA.

3.1.2. Purification of 2-hydroxyethylmethacrylate monomer

2-hydroxyethylmethacrylate [HEMA] was purified by continuous vacuum distillation at 150-160^{o}C (in oil bath) at reduced pressure in a fume hood (Figure 3.1).

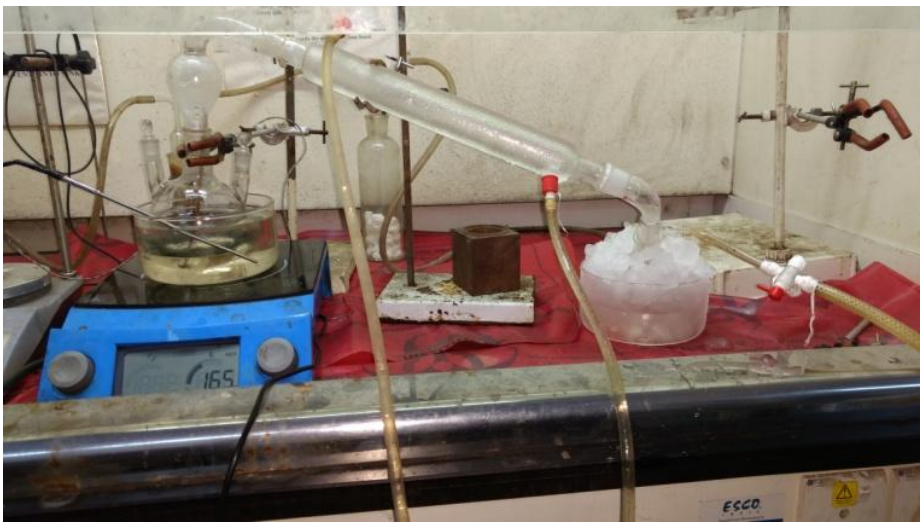


Figure 3.1. Vacuum distillation of 2-hydroxyethylmethacrylate

Temperature of the reaction was monitored using a thermometer. The distilled monomer was then collected in a round bottom flask kept in an ice bath and stored in refrigerator at 4ºC.

3.1.3. Refractive Index of 2-hydroxyethylmethacrylate monomer

The purity of distilled HEMA was checked by measuring the refractive index using a refractometer (NAR-3T, ATAGO, Japan) (Figure 3.2). A drop of the monomer was placed onto the polished surface of the test piece, after wiping the main prism surface and the test piece clean, and fixed to the centre of the main prism so that the drop of sample spreads into the thin film. Upon illumination and viewing through the eyepiece, the color compensator knob was rotated to get the boundary line between the upper red and lower blue regions. The boundary line was then aligned with the intersection of the crossed lines in the refraction field of vision and scale reading was noted as the refractive index of the sample at room temperature.

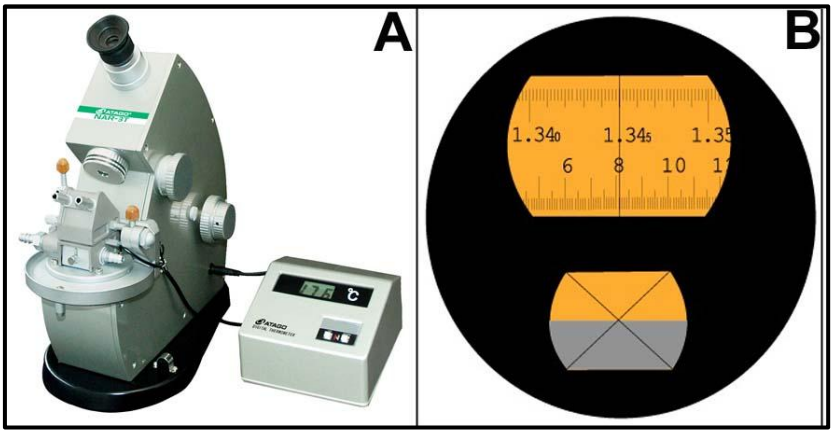


Figure 3.2. Image of (a) refractometer and (b) scale

3.1.4. Preparation of the hydrogel matrix

Vacuum distilled HEMA monomer was used to prepare the hydrogel matrix using photopolymerization method. Specific amounts of PCL and PEG were added to the HEMA monomer (Table3.1) and warmed to 70-75ºC in a water bath to obtain a homogenous mixture.

Table3.1 Formulations of hydrogel

Sample Code	**Wt%**		
HDRG	HEMA	PCL	PEG
	85	10	5

The step-wise preparation of HDRG is given in (Figure 3.3).

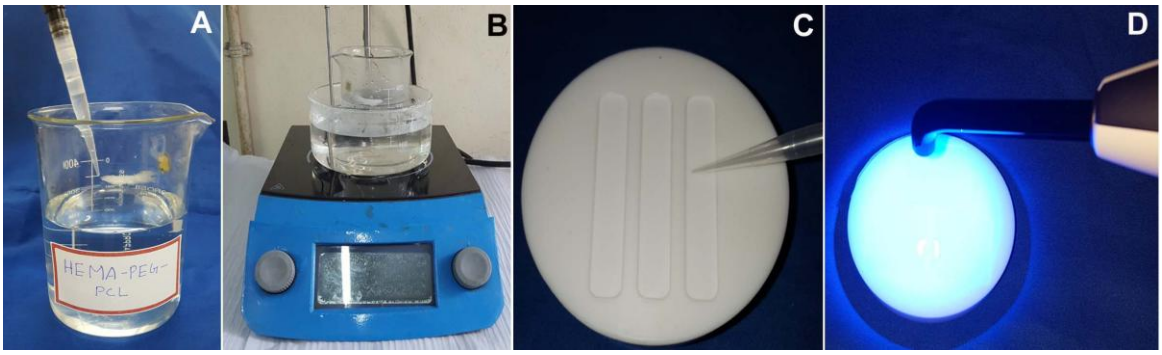


Figure 3.3. Photographs showing stepwise preparation of HDRG

Photo-initiator (TPO, 1wt%) was added to this mixture under constant stirring until complete dissolution. The solution was then poured into a teflon molds (60 mm x 10 mm x 1 mm) and exposed to a dental LED light source (Bluephase N., Ivoclar Vivadent Inc., Austria)having a wavelength 390-430nm to initiate the polymerization. Light exposure was provided for a maximum of 3-5min. After polymerization, the mold containing the cured polymer was immersed in distilled water until the cured polymer was detached as hydrogel sheets from the teflon mould. The hydrogel sheets were stored in distilled water at 37ºC. The structure of photoinitiator (TPO) and radicals formed upon photo-initiation is given in (Figure 3.4a). A picture of light cured polymer is shown in (Figure 3.4b). The PCL/PEG/HEMA system will hereafter be referred to as HDRG.

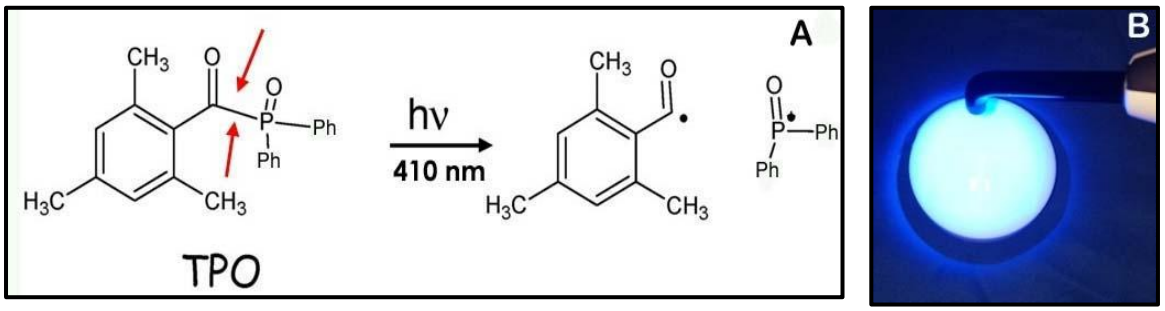


Figure 3.4.(a) Structure of TPO and radicals formed following photo-initiation (b) Light curing of hydrogel

3.1.5. Preparation of the Glucomannan-incorporated hydrogel matrix

HDRG was prepared as per the method described in section 3.1.4. TPO was added to HDRG and subjected to partial polymerization. Thereafter, a layer of Glucomannan of concentrations (0.1, 0.3, 0.5, 0.7 and 0.9) wt. % was uniformly coated on the under-polymerized hydrogel surface and exposed to the dental light for a maximum of 3-5 minor until complete curing was commenced. The final hydrogel systems thus obtained

are abbreviated as HDRG1 (0.1wt% GLU), HDRG3 (0.3wt% GLU), HDRG5 (0.5wt% GLU), HDRG7 (0.7wt% GLU) and HDRG9 (0.9wt% GLU).The teflon moulds with the cured polymer were immersed in distilled water and polymeric strips were removed (Figure 3.5).

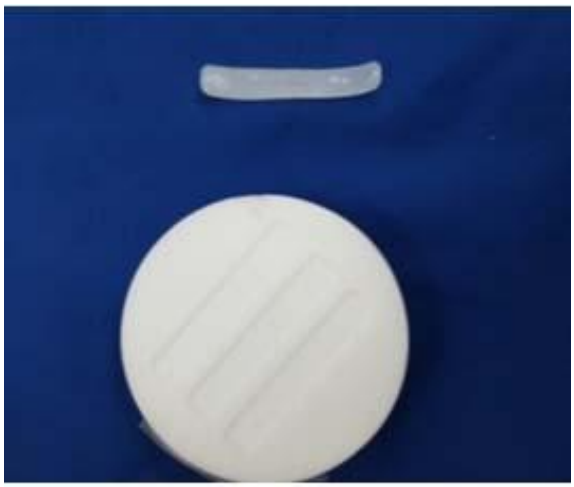


Figure 3.5. Teflon mould for casting polymers and the cast polymer strip

3.1.6. Preparation of the Ofloxacin-incorporated hydrogel matrix

HDRG was prepared as per the method described in section 3.1.4. TPO and 0.5 wt% of antibiotic drug Ofloxacin (OFL) was added to HDRG under constant stirring. The solution was then poured into a Teflon molds (60 mm x 10 mm x 1 mm) and exposed to the dental light source to initiate the polymerization. Light exposure was provided for a maximum of 3-5 min. After polymerization, teflon moulds with the cured polymer were immersed in distilled water and polymeric strips were removed. The hydrogel system thus obtained is abbreviated as HDRG_OFL.

3.1.7. Preparation of the Glucomannan-Ofloxacin incorporated hydrogel matrix

HDRG_OFL was prepared as per the method described in section 3.1.6. The mixture was subjected to partial polymerization, and was coated with a layer of Glucomannan. Then the light exposure was provided for a maximum of 3-5 min for complete curing. After completion of polymerization, teflon moulds with the cured polymer were immersed in distilled water and polymeric strips were removed. Only 0.1% glucomannan was used as it was found from previous experiments that maximum swelling resulted at this concentration itself. The hydrogel system thus obtained is abbreviated as HDRG1_OFL.

3.2. CHARACTERIZATIONAND EVALUATION OF HYDROGEL SYSTEMS

3.2.1. Structural characterization

Fourier Transform infrared spectroscopy (Cary 600 Series FTIR Spectrometer, Agilent Technologies, Malaysia) was used to characterize Glucomannan powder, using KBr pellet technique, while to characterize the molded hydrogel (bare and glucomannan incorporated), Attenuated total reflection (ATR) method was used.

In KBr method, the glucomannan was powdered fine by grinding a small amount (0.1mg) of Glucomannan with anhydrous KBr (1:300) in a smooth agate mortar. The fine powder was made into a thin transparent disk using the hydraulic hand press under 10-ton pressure for about 3 minutes. KBr without glucomannan was used as the control

After warming up the instrument for 20 min, background measurements were made with control KBr pellet. CO_2 and moisture peak removal was made effective after this recording. The sample discs were monitored after the background measurements using the software *Resolutions Pro*. The spectra was collected in the range 4000-400 cm^{-1} with 40 scans at a resolution of 4 cm^{-1} .The obtained FTIR spectra were processed for baseline corrections, smoothening, and labeling of peaks using the software.

In Attenuated total reflection method, the samples of 6cm length and 1cm width with 1mm thickness was placed onto the zinc selenide (ZnSe) horizontal flat plate sample holder ATR assembly of a FTIR spectrometer. Background measurements were done without the sample. The spectra were measured between 4000-400cm^{-1} ranges with 40 scans per sample at a resolution of 4 cm^{-1}.

3.2.2. Surface topography analysis

The surface topography of HDRG and HDRG1were analyzed using an environmental scanning microscope (ESEM, FEI Quanta 200, Netherlands). For ESEM, the matrix was fixed on aluminium stubs and scanned at a voltage of 15 kV. The images were acquired at various magnifications. The dried hydrogel samples were coated with gold, placed on the aluminum stubs and observed under vacuum using a SEM(Hitachi S2400, Japan).

3.2.3. Swelling studies

The hydrogel systems HDRG, HDRG1, HDRG3, HDRG5, HDRG7 and HDRG9 were subjected to swelling studies. Six samples (6cm x 1cm x 1mm) of each hydrogel compositions were prepared, freeze-dried (CHRIST, Model Alpha 1-4 LD, Germany) and weighed (Wd) in an analytical balance (Model CP224S, Sartorius, Germany). The samples were then immersed in PBS (pH=7.4) and incubated at 37ºC for 24h. At the end of 24h, the samples were removed from PBS; surface blotted with tissue paper to remove the surface adherent water and the swollen weight (Ws) was recorded. The percentage swelling was calculated using the equation:

$$\%\, Swelling = \frac{Ws - Wd}{Wd} \times 100$$

3.2.4. Determination of Mechanical Properties

The tensile stress at maximum load of HDRG, HDRG1, HDRG3, HDRG5, HDRG7 and HDRG9were determined using a Universal Testing Machine (UTM, Instron 3365, UK,Figure 3.6).Rectangular samples with dimension (6cm x 1cm x 1mm) were employed for tensile test. The test was conducted uniaxially at crosshead speed of 1mm/min using a 10N load cell. Maximum load (N) is obtained from the load extension curve and knowing the sample cross sectional area, tensile strength of matrix was calculated. The ultimate stress and strain parameters were calculated, respectively using the following. The load at break and elongation were noted and stress and strain were calculated using the following equations. Mean and SD were also calculated.

$$Tensilestress(MPa) = \frac{Load\ (N)}{Cross - sectionalarea\ (mm^2)}$$

$$Tensilestrain(\%) = \frac{Final\ length - initial\ length}{Initiallength} \times 100$$

Figure 3.6 Tensile property evaluation using UTM

Sample size was six per group, and the mean and standard deviation of the samples were calculated. Statistical analysis (ANOVA single factor) was carried out to determine significant changes in stress. Figure 3.6shows the tensile property evaluation by UTM.

3.2.5. Water Vapor Transmission Rate (WVTR) Studies:

The WVTR was measured according to the ASTM E96/E96M-14 and the published methods (Adekogde & Ghanem, 2005). Briefly, a test dish (2.0 cm in diameter) was filled with distilled water, leaving a space of 19±6 mm from top of the samples to the water level. HDRG and HDRG1 to HDRG9 were firmly glued to mouth of the evaporating dish as shown in Figure 3.7. A test dish without sample was kept as control. The sample and the test dish were weighed using a top loading balance (Sartorius CP224S, Germany) and placed in an incubator at 37^0C and 35% humidity for 3 days. The weight loss was plotted against elapsed time to get a slope with units of g/day. To calculate the WVTR, the slope was divided by the evaporating dish mouth area.

$$WVTR = \frac{Weightloss/time}{Areaofspecimen} \times \frac{24g}{m^2}/day$$

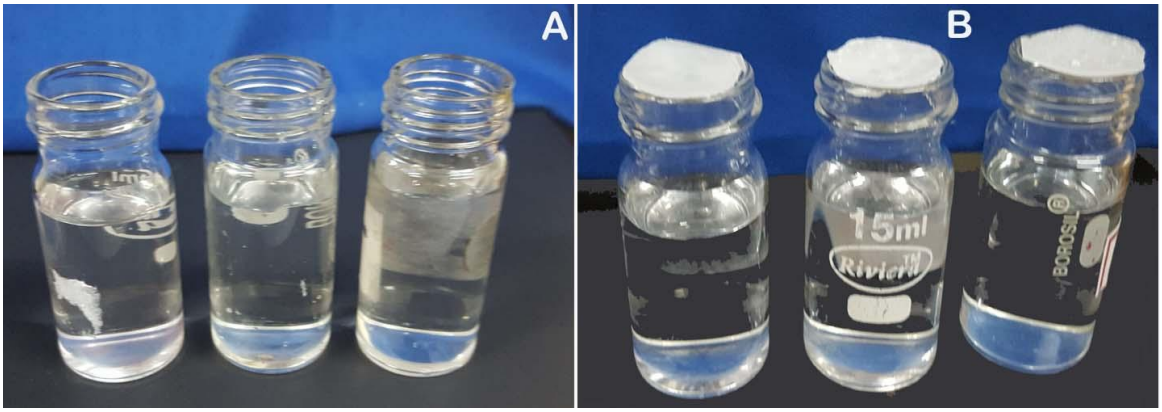


Figure 3.7 Experimental set up for WVTR. (A) without and (B) with HDRG

3.2.6. Release studies of glucomannan

The elution studies were carried out for 48h HDRG, HDRG1, HDRG3, HDRG5, HDRG7 and HDRG9 using rectangular samples of dimensions (6cm x 1cm x 1mm). The hydrogels were lyophilized and then the dry weight of the samples was taken. The dry samples were immersed in equal volume of PBS (pH-7.4) and incubated at 37ºC. At the end of 24h, the samples were lyophilized, weighed, immersed in fresh PBS for 24h. At the end of 48h, the samples were lyophilized and dried weights taken.

3.2.7. Percentage Hemolysis Test (In vitro hemocompatibility of materials)

Hemocompatibility testing of hydrogel systems was carried out broadly on the basis of ISO 10993-4:2002 (E) selection of tests for interaction of materials with blood. The test is mainly aimed at finding the extent of hemolysis caused by the sample. Blood from a healthy human volunteer was collected and the anticoagulant acid citrate dextrose (ACD). The test materials (6mm x 1mm) was placed in the polystyrene culture plates and agitated with phosphate buffer saline (PBS). After 5 min, PBS was withdrawn immediately for analysis and remaining 1ml was exposed to the matrix for 30minutes under agitation at 75 ±5 rpm using Environ shaker (Labline instruments Inc, USA) at 35 ±2ºC Four empty culture wells exposed to blood were taken as reference. The total hemoglobin in the initial samples was measured using an automatic hematology analyzer (Sysmex-K4500, Sysmex Corporation, Japan). Platelet-poor plasma was prepared from blood exposed for 30 minutes. Free hemoglobin (Hb) liberated into the plasma after exposure was measured for each sample using a Diode array spectrophotometer (HP 8453, Hewlett Packard, Germany) and the percentage hemolysis calculated using the equation:

$$\% \, Hemolysis = \frac{FreeHb}{TotalHb} \times 100$$

3.3. *IN VITRO* EVALUATION GLUCOMANNAN

3.3.1. Commercial reagents:

Dulbecco's Modified Eagle's (DMEM: F12) medium, antibiotic-antimycotic, foetal bovine serum (FBS), trypsin-EDTA (Gibco BRL, USA), MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide) (Sigma Aldrich, USA) were obtained from the respective sources mentioned.

3.3.2. Isolation and culture of human skin dermal fibroblasts:

Human foreskin biopsies were obtained from patients (age group between 6 months - 5 years) after taking informed consent from the patient's relatives and institutional ethics committee approval. The skin samples were washed Hank's balanced salt solution (HBSS) with 5x antibiotic-antimycotic. The tissue was then transferred to a culture dish using a sterile forceps and washed with betadine solution and then again with HBSS. The samples were cut into $1cm^2$ pieces. These pieces were then incubated with dispase solution (1.8 IU/mL) and kept at 4ºC overnight. After 12h incubation, the skin samples were removed from the dispase solution and the epidermis was peeled off from the dermis using fine forceps. Then the epidermis and dermis was washed separately using Hank's balanced salt solution containing antibiotics. The epidermis was used for the keratinocyte isolation and the dermis was used for the fibroblast isolation.

The dermis separated from the epidermis as described above was used for the isolation of the fibroblast. The dermis was minced with mincing scissor and 10 ml of 1.5 mg/ml collagenase was added to it and this tissue suspension was transferred into a sterile 50 ml tube and incubated for 45-560 min in shaving incubator at 37^0C. The resultant cell suspension was filtered through nylon meshes to get single cell suspension. This suspension was centrifuged at 400g for 6minutes and the pellet was resuspended in DMEM; F12 medium containing 10% foetal bovine serum and antibiotic-antimycotic solution and seeded onto tissue culture polystyrene dishes.

The cells were found to be attached after two hours of seeding. The medium was changed on next day to removed unattached and dead cells. The cells become confluent on the 4^{th} day of seeding. The light microscope image of the confluent fibroblast is shown

in Figure 3.8. The fibroblast cells after 4th passage was used for the biocompatibility evaluation of the matrix.

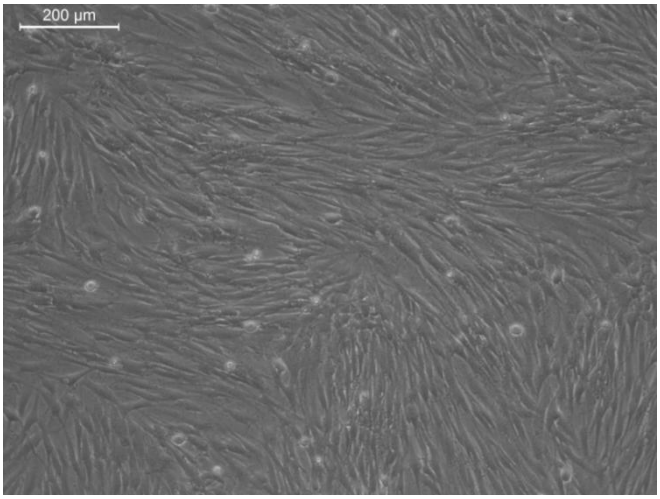


Figure 3.8 Optical micrograph showing confluent layer of fibroblasts

3.3.3. Direct contact assay

Cytotoxicity test was evaluated using direct contact method as per ISO 10993-5 procedure. Bare glucomannan of two different concentrations of 2 mg (0.1%) and 6 mg (0.3%) were used for cell culture analysis after ethanol sterilization. The sterilized samples were dispersed in DMEM: F12 medium and added on to the top of the confluent layer of fibroblast cells. The cells were incubated with the samples at 37 ± 2 ºC for 24 ± 2 h and cell culture was examined for its morphology of cells under phase contrast optical microscope for cellular response around test and control samples.

3.3.4. MTT assay

The cell viability evaluation of glucomannan was done at 3 h, 6 h and 24 h time period 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT assay is a colorimetric indicator of cell number or viability by means of mitochondrial dehydrogenase activity according to method of Nik and Otto (Nik and Otto 1990). The cells were gently washed with PBS and 1.0 mL of MTT solution was added per well. Then the plates were incubated with for 2h at 37 °C, 5% CO_2 in a humidified atmosphere. During this time, mitochondrial dehydrogenase activity will reduce MTT into an insoluble purple-colored formazan product that can be eluted using dimethyl sulfoxide (DMSO).After 2h, the MTT solution was subsequently removed and 200 µl of DMSO was incubated for 5minto elute the formazan product from the cells. 200 µl of the DMSO was then transferred into a 96 well plate and read using micro-plate reader at 575 nm.

3.3.5. Scratch wound assay

Confluent monolayer cells are fragile and can be easily disrupted mechanically, leaving an area devoid of cells. This opening in the cell monolayer is then repopulated; because the cells on the fringe of the damage are no longer in contact-inhibited thus the cells move into the available space. This mechanical disruption is often done deliberately in a "wound healing" assay as a means to assess the migration of the cells. In such assays, scratch is made in the cell layer and viewed under the microscope to monitor the advance of the cells into the wound. The rate of migration of fibroblast cells in the presence of glucomannan at two concentrations (0.1%, 0.3%) were determined by creating a scratch wound in the cell monolayer. The wound closure rate was observed for 24 hours after treatment.

3.3.5.1. Wound creation and application of glucomannan

Uniform monolayer cultures of fibroblast cells were used for the development of wounds by scratch method. Wounds are created when the cultured cells covered the full surface area of the 6 well culture plates. Carefully using 200 µl yellow tip scratch was made on the confluent monolayer. The wounded cells were rinsed thrice with filter sterile PBS of pH 7.4 to remove the detached cells.

After the wash, the wounded cells were incubated with 1.9 ml of medium. To the medium 0.1% and 0.3% of glucomannan were added. The experiment was done in duplicate wells for each trial. The plates were then gently swirled and incubated at 37^0C in 5% CO2 incubator for next 24 hours to analyze the wound healing property.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. PREPARATION OF PHEMA-PCL-PEG HYDROGEL MATRIX

Based on the methods of preparation, hydrogels may be classified as (1) homo-polymer (2) copolymer (3) semi-interpenetrating network and (4) interpenetrating network. If one polymer is linear and penetrates another cross-linked network without any other chemical bonds between them, it is called a semi-inter penetrating network. In this study, a polymer system is prepared by making use of monomer (HEMA), which is photo polymerized to a hydrogel polymer (PHEMA) in presence of PCL and PEG.

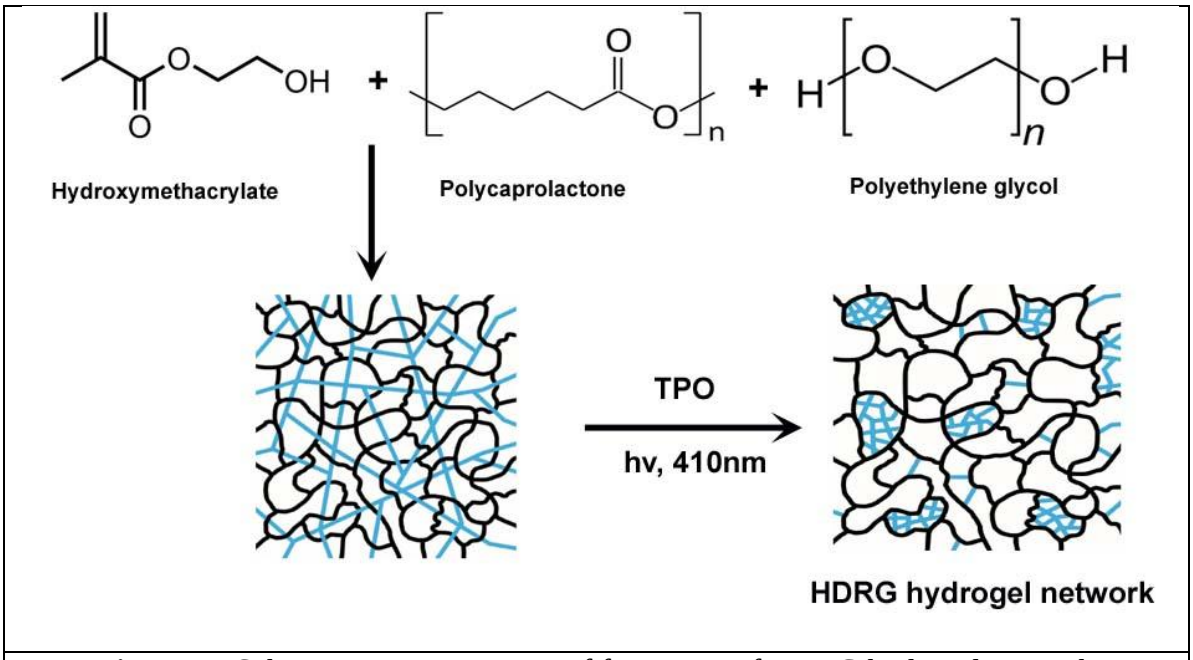


Figure 4.1 Schematic representation of formation of HDRG hydrogel network

The polymerization of HEMA to PHEMA is carried out in presence of PCL above its melting point. A third entity, PEG, is also added for imparting a plasticizing effect.

During polymerization, HEMA monomer, which is physically dispersed in PCL, polymerizes and forms cross-linked network. As a result PCL gets entrapped in PHEMA network resulting in hydrogels that are mechanically strong. Presence of PEG imparts flexibility to this hydrogel system. The formation of PHEMA-PCL-PEG hydrogel matrix is schematically depicted in Figure 4.1.Various concentration of GLU was incorporated into HDRG to obtain the final hydrogel compositions.

4.2. CHARACTERIZATION OF THE HYDROGEL SYSTEMS

4.2.1. Structural characterization using FTIR spectroscopy

Bare and glucomannan incorporated hydrogel systems (HDRG, HDRG1, & HDRG3) were successfully synthesized using photopolymerization technique by adding TPO as the initiator. Glucomannan was added in two concentrations (0.1%, 0.3%) in the hydrogel. All the hydrogel were then characterized using FTIR.

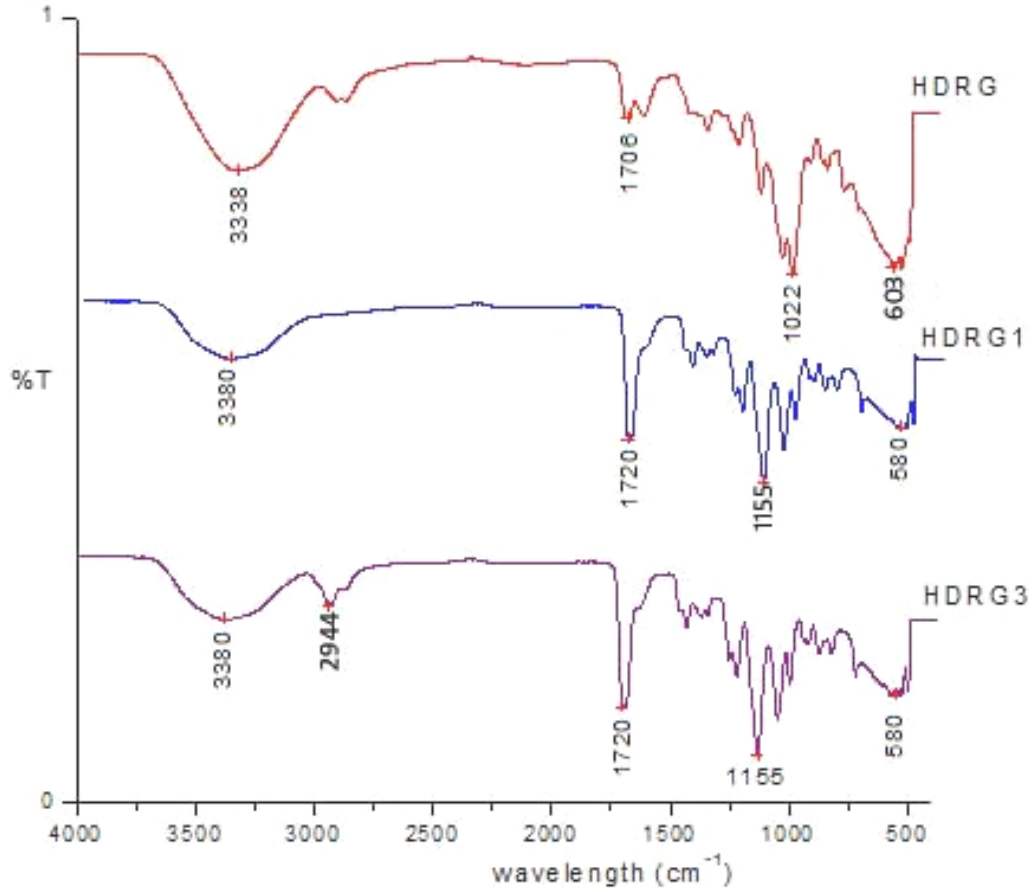


Figure 4.2 FTIR spectra of HDRG, HDRG1 and HDRG3

Fourier Transform Infrared (FTIR) is a useful tool in the investigation of molecular structure of polymers by the measurement of the changes in the dipole moment

of vibrating molecules when exposed to IR (Griffiths and De Haseth, 2007). FTIR recorded the changes in the confirmation of chemical structures which are sensitive to environmental changes. The hydrogel systems showed absorption at 1706 cm^{-1} and 3338 cm^{-1} which indicated C-O stretching and O-H stretching respectively. HDRG2 and HDRG3 showed 1720 cm^{-1}, 1155 cm^{-1} and 580 cm^{-1}peaks which indicates strong C=O stretching, strong S=O stretching. All three polymers display the peaks of hydroxyl stretching (3380 cm^{-1}) and the incorporation of glucomannan is significantly shown to have included other peaks in glucomannan incorporated hydrogel matrix.

The most frequently used IR spectral region in carbohydrate analysis for glucose and mannose is the anomeric region at 950-700 cm^{-1} (Mathlouthi and Koenig 1986) where the α and β conformers of glucose, galactose and mannose can be distinguished using the 2α and 2β bands at 870-840 cm^{-1} and 890 cm^{-1} , respectively (Mathlouthi and Koenig 1986). Here in this study the analysis with FTIR technique with attenuated total reflectance (ATR) was used to detect the structural features of the hydrogels and glucomannan incorporated hydrogels. Stretching vibration modes of O-H groups is a broad band and occurs at about 3380 cm^{-1}.

4.2.2. Swelling studies

Water retaining ability is important for the hydrogels intended for wound healing applications. The swelling capacity is often considered as the measure of water retaining ability of a hydrogel. The images of HDRG, HDRG1, HDRG3, HDRG5, HDRG7, and HDRG9 at 0h and after swelling in PBS (pH-7.4) for 24h are shown in Figure 4.3. It is clear from the figures that all the samples studied underwent an enlargement in their dimensions, indicating that they have the capacity to fluid uptake and retention.

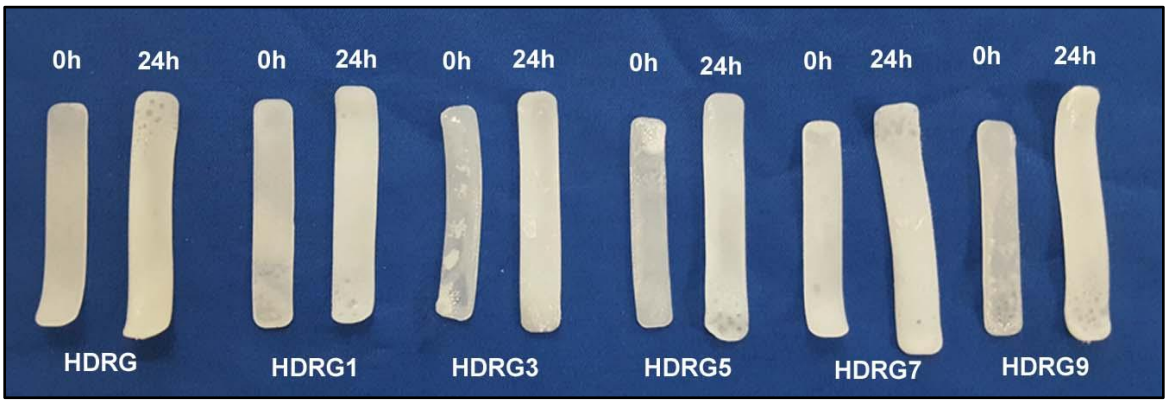


Figure 4.3 Schematic representation of formation of HDRG hydrogel network

The swelling capacity was also calculated for all of the hydrogel systems for 24h, 48h and 72h (Figure 4.4). All hydrogel systems were found to possess swelling capacity up to 50%. The highest value of swelling was exhibited by HDRG (50 ± 1%), while HDRG3 showed lowest value (45 ± 2.3%).

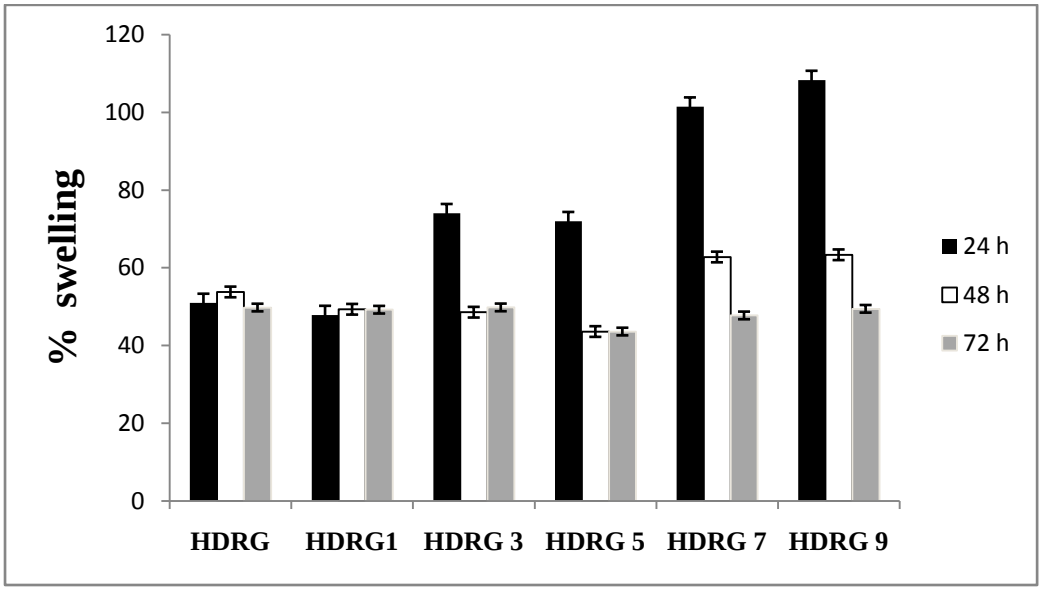


Figure 4.4 Swelling capacity of HDRG hydrogel network

Swelling characteristics can affect the pore size and its diffusive and mechanical properties. The water absorption ability of the hydrogel matrix reflects its capability to hold aqueous medium, which is necessary for wound healing (Yamaguchi *et al*., 2005 & Luangbudnark *et al*., 2012). A moist environment enhances cell migration and chemokine mobility across the wound area resulting in increased epithelialization and reduced scarring (Pankajakshan *et al*., 2009). Therefore, this hydrogel matrix by itself may enhance wound healing by providing a moist environment.

4.2.3. Scanning electron microscopy

The morphology of dry and wet samples of HDRG and HDRG1 are shown in Figure 4.5. Lyophilized HDRG was found to be more porous when compared to its wet counterpart. This is expected because when a polymer matrix swells in a liquid (PBS, in the present study), pore volume increases with subsequent decrease in pore size.

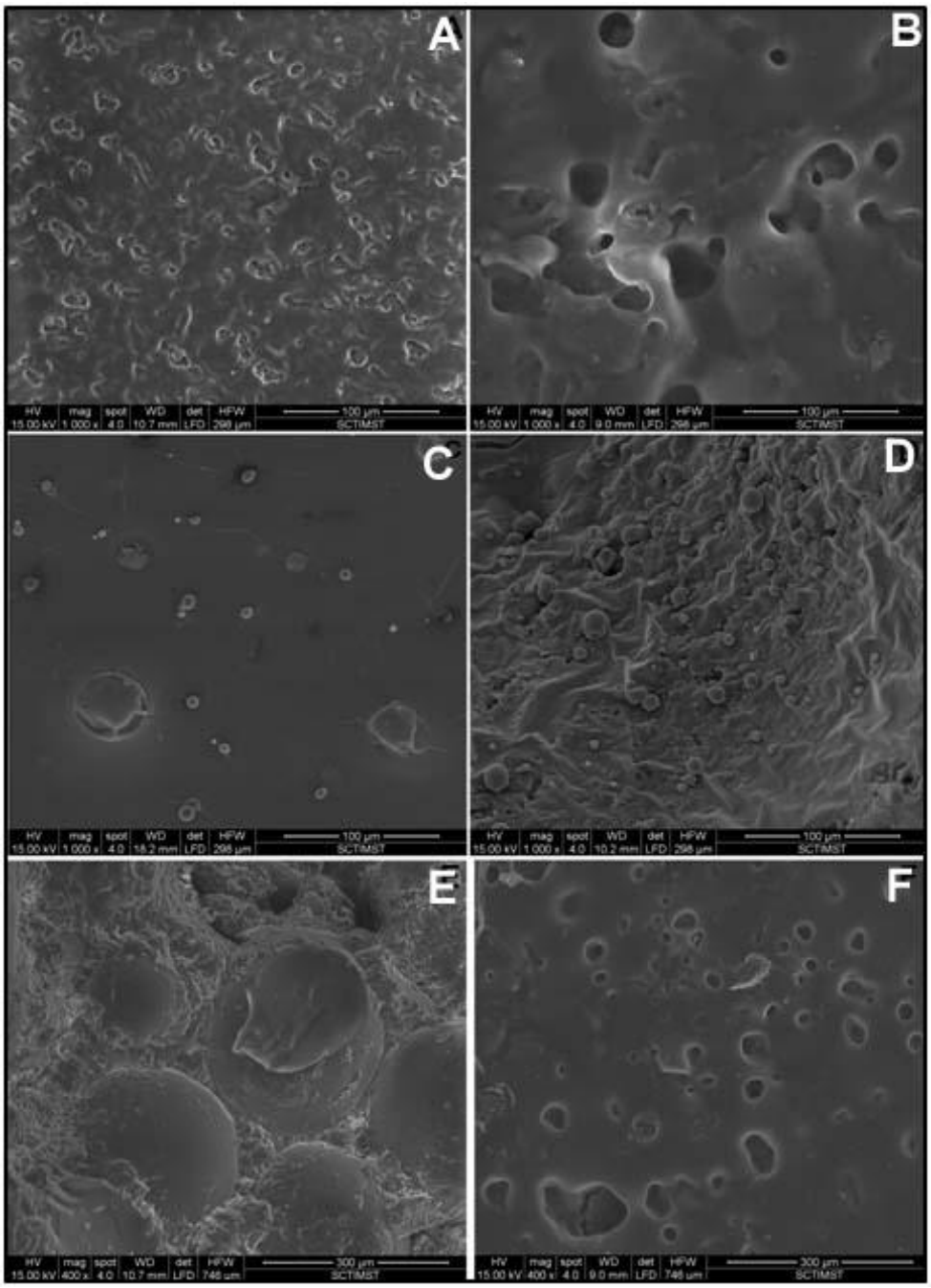


Figure 4.5 Scanning electron micrograph of HDRG and HDRG1(A) Dry HDRG; (B) wet HDRG; (C) dry HDRG1; (D) wet HDRG1; (E) wet HDRG1 (higher magnification) and (F) wet HDRG (higher magnification)

The porous structure of HDRG is highly affected with the incorporation of GLU (Fig). The porosity of HDRG is decreased when 0.1 wt. % of GLU is introduced. Also GLU is found to be dispersed in HDRG matrix. This indicates that GLU does not chemically bind to HDRG and is mechanically anchored to HDRG, which is evident from the SEM of the fractured surface of HDRG1 (Figure 4.5E). Furthermore, during fracture the HDRG matrix underwent sufficient ductile deformation indicating that the mechanical

anchorage between GLU and HDRG is quite strong. Traces of HDRG on dispersed GLU further strengthen this argument.

4.2.4. Mechanical properties

The mechanical properties of HDRG, HDRG1, HDRG3, HDRG5, HDRG7, and HDRG9 were evaluated to asses if the hydrogel systems were mechanically strong for the intended application. PHEMA hydrogels are known to be mechanically weak. In order to combat this, PCL was incorporated with the aim of obtaining hydrogels with improved mechanical properties. The variation of tensile stress of HDRG, HDRG1, HDRG3, HDRG5, HDRG7, and HDRG9 are presented in Figure 4.6.

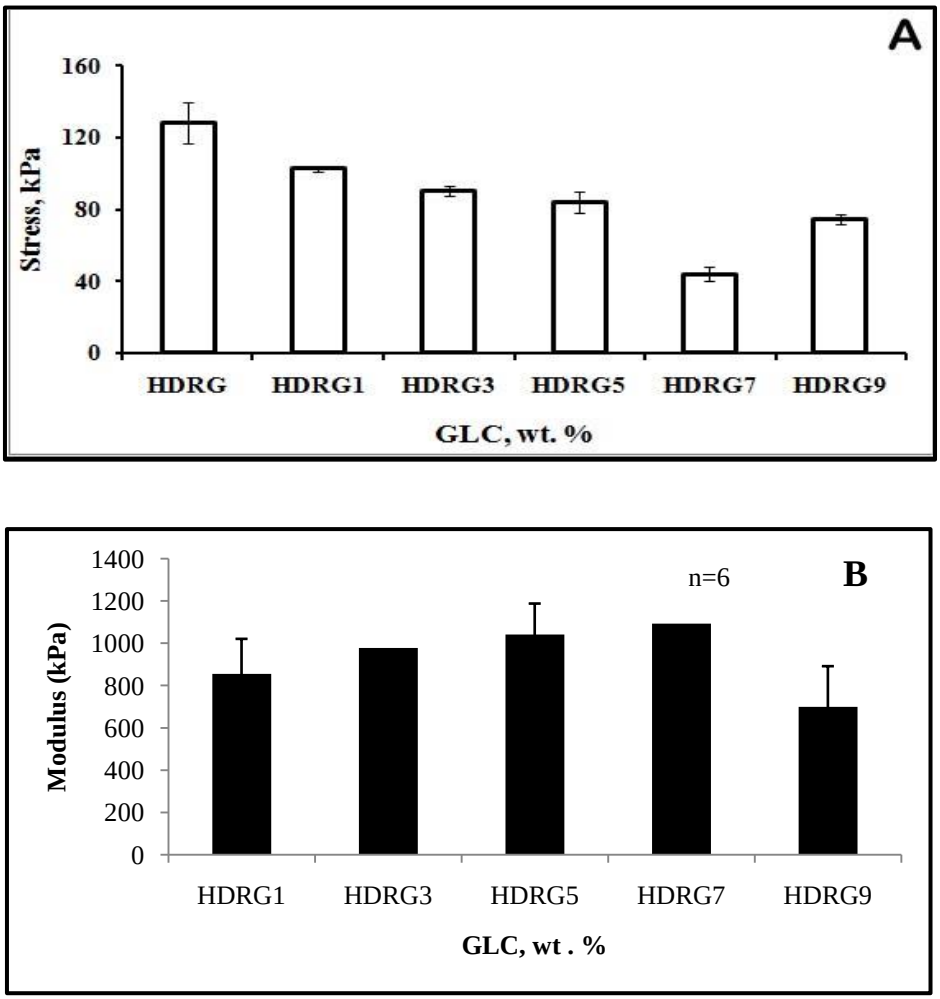


Figure 4.6 Variation of (A) tensile stress and (B) modulus of HDRG at various concentrations of GLU.

It is clear from the figure that incorporation of GLU leads to a decrease in the values of tensile stress, indicating that GLU does not reinforce the HDRG matrix, but

instead acts as the stress concentration points causing the sample to fail under stress. However, GLU does not cause catastrophic decrease in the tensile stress. Mechanical properties of polymers are highly dependent on the morphology and extent of loading of fillers. In the case of non-reinforcing fillers, sharp and pointed surfaces of fillers are more detrimental to the polymer matrix, when compared to fillers with smooth surfaces. The SEM image of GLU incorporated HDRG shows that GLU dispersed as smooth round particles in HDRG matrix. Hence, even when GLU does not contribute to improvement in mechanical properties of HDRG, its morphology and gel-like nature does not harm the hydrogel matrix. The modulus values of these hydrogel matrixes also support this argument. It is interesting to note that the inclusion of PCL in HEMA (20 kPa) resulted in 60 fold increase in tensile stress of HDRG (120 kPa).

The values of tensile stress did not decrease significantly for compositions HDRG3, HDRG5, HDRG7 and HDRG9 indicating that beyond 0.3 wt. % loadings, GLU does not influence the mechanical properties of the base hydrogel matrix (HDRG).

4.2.5. Water vapour transmission rate

Water vapour transmission is one of the most important properties of wound dressing material, as it controls the wound dehydration to a great extent. Skin has control over the evaporative water loss at an optimal rate.

The rate of water transmission for normal skin is 204 g/m^{-2}/day. Water vapor transmission rate for injured skin can range from 279 ±26 g/m^{-2}/day for first-degree burns to 5138 ± 202 g/m^{-2}/day for a granulating wound. It has been recommended that WVTR of 2000–2500 g/m^{-2}/day would provide an adequate level of moisture without wound dehydration (Lamke *et al*., 1977). An advantage of the porous nature of the hydrogel matrix is good WVTR. In the present study, WVTR was calculated as the gradient of weight loss with time.

Figure 4.7 shows the loss of water vapour through HDRG1, HDRG3, HDRG5, HDRG7, and HDRG9 when placed in a moist environment, in comparison to normal skin, first degree burn wound and granulated wound.All the hydrogel compositions incorporated with GLU showed values of WVTR well above that of normal skin, first degree burn and granulating wounds. This is very encouraging and proves that GLU incorporated HDRG hydrogels could be promising candidates as burn wound dressings.

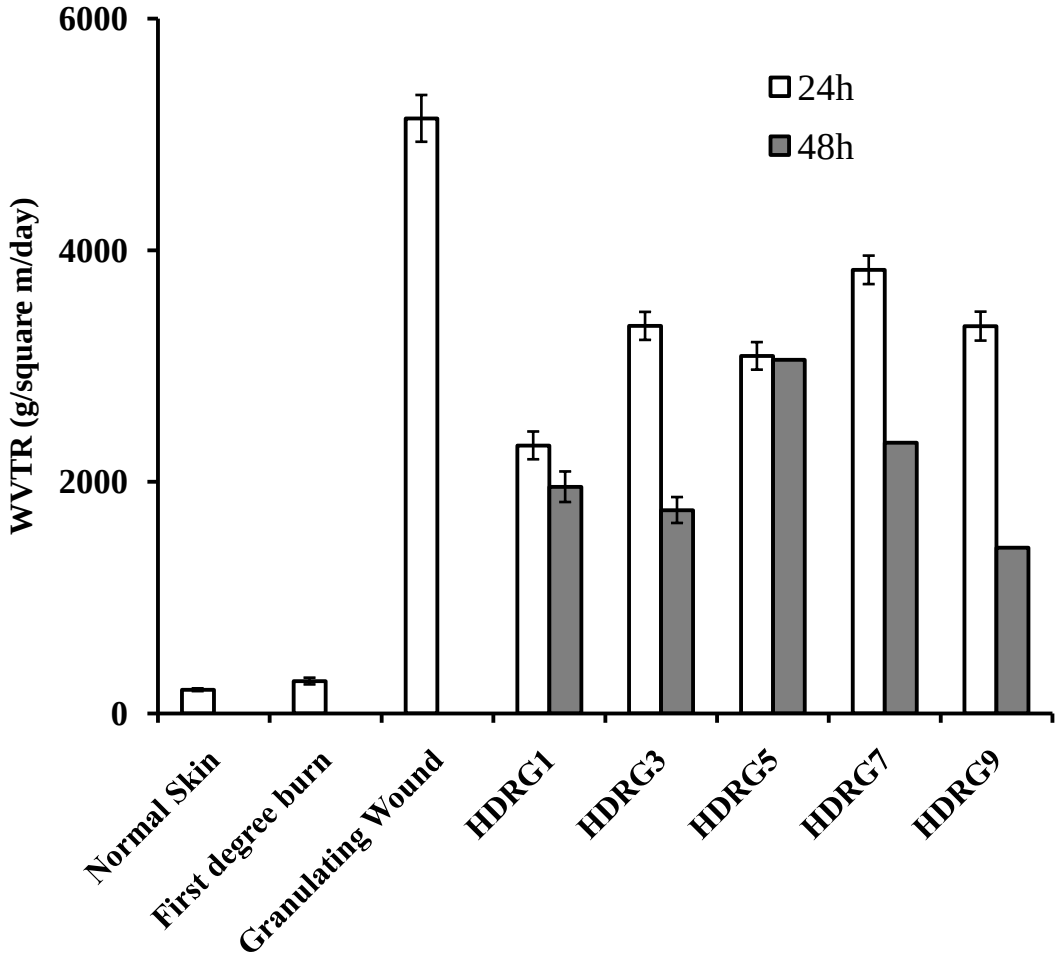


Figure 4.7 Dependence ofwater vapour transmission rateHDRG at various concentrations of GLU.

4.2.6. Release studies of glucomannan

The amount of GLU released from HDRG1 and HDRG3 at the end of 24 and 48h were examined (Figure 4.8). Glucomannan was released from all the hydrogel matrices at both the time points studied. The release of GLU at the end of 24h, however, remained almost constant. Glucomannan is adhered to HDRG through mechanical anchorage and does not reinforce the base hydrogel matrix. This has been confirmed from the mechanical properties and SEM analysis. Hence the release of GLU from HDRG is expected.

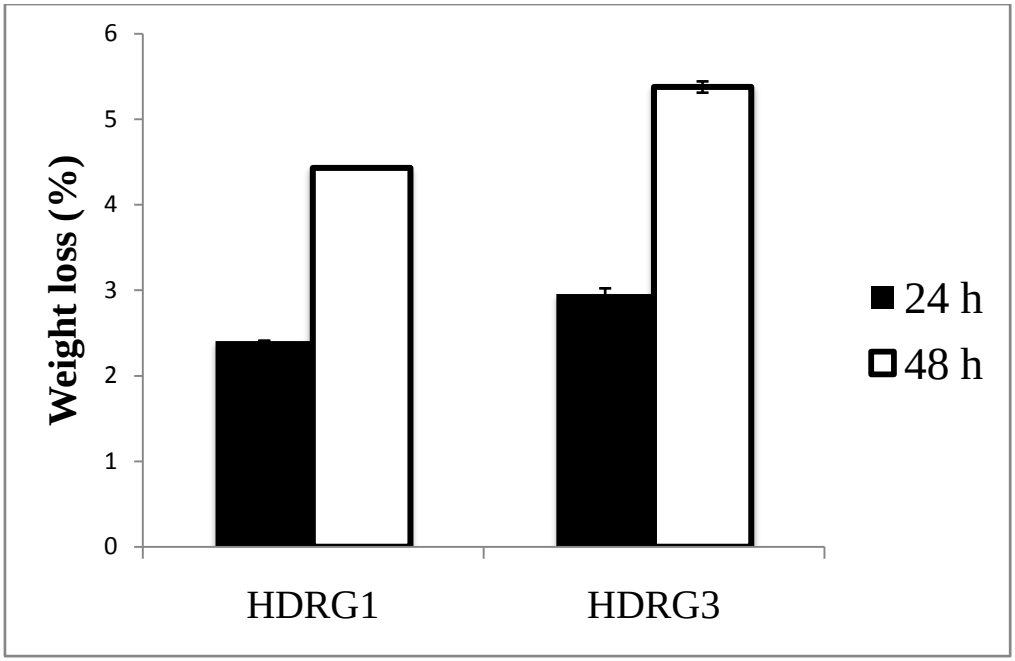


Figure 4.8 Elution of GLU from various hydrogel matrices at the end of 24 and 48h

The release profile, however, changed significantly at the end of 48h.It was found that release rate was dependent on the extent of GLU loading with larger amount of GLU being released from HDRG1 and HDRG3.

4.3. EVALUATION OF ANTIMICROBIAL ACTIVITY

Agar disc diffusion method was used to analyze the antimicrobial property of the Ofloxacin and Glucomannan incorporated hydrogel system. The antimicrobial property of the HDRG samples was evaluated against gram-positive (*S.aureus* ATCC 25923) and gram negative (*E.coli* ATCC 25922) bacteria (bacterial count 10^4/ml). HDRG with Glucomannan alone doesn't showed any antimicrobial effect against both gram positive and gram negative bacteria while HDRG incorporated with both Glucomannan and Ofloxacin showed antimicrobial effect against both the bacteria. Ofloxacin disc was used as the positive control**.**

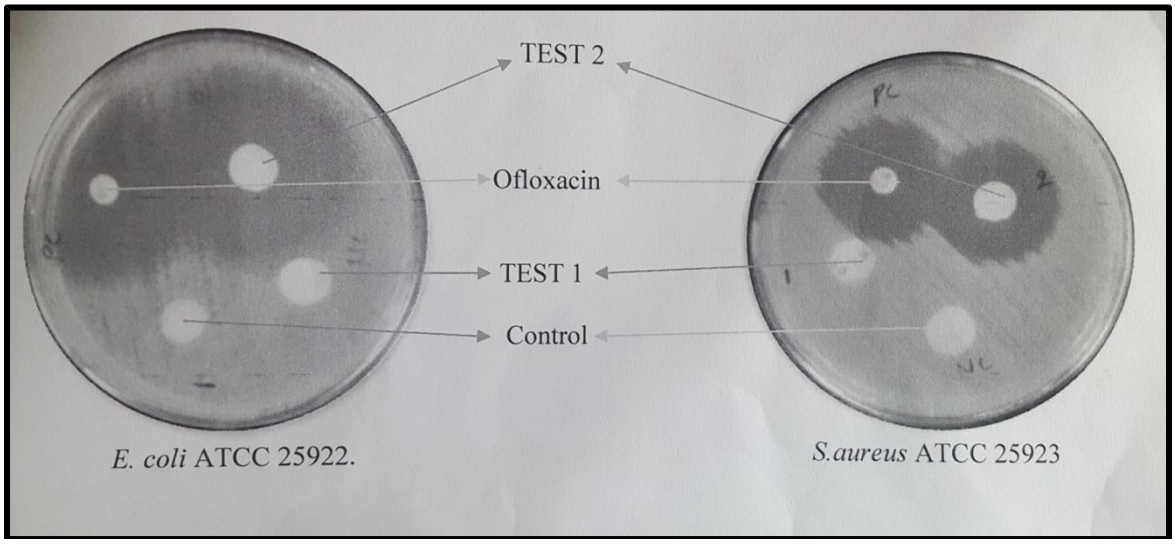


Figure 4.9 Representative patterns of zone of inhibition assay of HDRG with glucomannan and HDRG with glucomannan and Ofloxacin against *E.coli* and *S. aureus*

4.4. *IN VITRO* EVALUATION OF GLUCOMANNAN

4.4.1. Hemolysis

Hemolysis estimation has been used as simple and reliable technique for estimating blood compatibility of materials. The percentage hemolysis of the samples was evaluated to determine the hemolytic property of the material for wound applications (Figure 4.9). In this study, empty polystyrene dishes were used as a reference generating negligible hemolysis <0.1%. As per ISO 10993-4:2002 (E), for material to be non-hemolytic, the percentage hemolysis should be less than 0.1%. All tested samples displayed less than 0.1% hemolysis and was found to be hemocompatible appropriate for wound healing application.

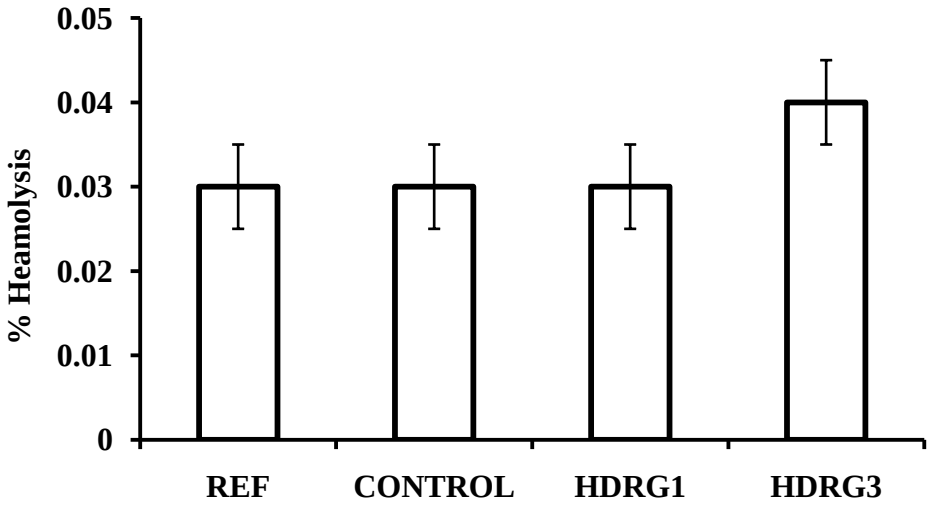


Figure 4.9 Percentage hemolysis to the reference, control, HDRG1 and HDRG3

4.4.2. Direct contact assay

Glucomannan (0.1%, 0.3%) were placed on monolayer culture for 24h and observed under phase contrast microscope. Direct contact assay demonstrated that glucomannan did not induce any toxic effects and it was found to be cyto-compatible. The normal spindle-shaped morphology of dermal fibroblast cells was maintained when cultured in presence of both glucomannan concentrations which is shown in Figure 4.10

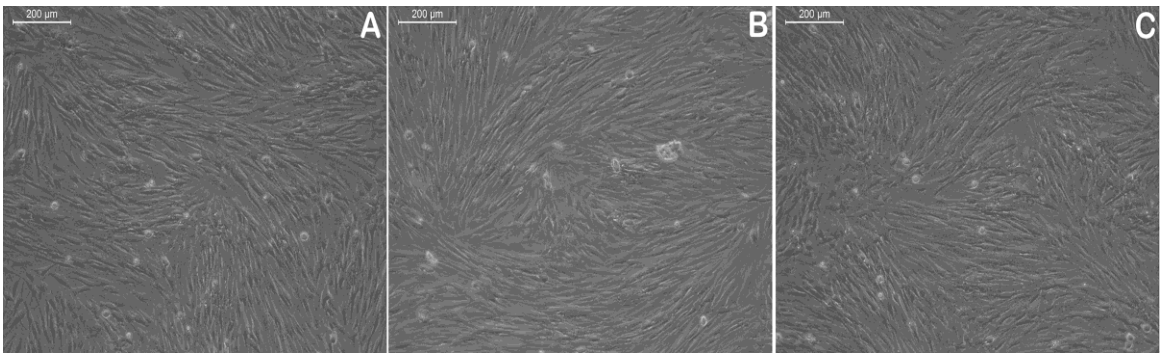


Figure 4.10 Response of human fibroblasts to various concentrations of glucomannan at the end of 24 h. (A) 0%; (B) 0.1% and (C) 0.3%

4.4.3. MTT assay

MTT cell assay was used for the determination of cell viability based on the reduction of the yellow colored water soluble Tetrazolium dye (4, 5-dimethyliazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) to formazan crystals (Figure 4.11). It is used here to determine the effect of glucomannan in the cells. The rate of cell proliferation was

visually observed. When analyzed, the cells treated with 0.1%, 0.3% of glucomannan showed good proliferation. It was also noted that 0.1% after 24 h showed 92% cell viability while 0.3% showed 97% cell viability. After 48h it was noted that the cell viability of 0.1% GLU showed 106% while 0.3% only showed 99 %, hence we concluded that the even with the increase in amount of GLU, the cell viability doesn't had sufficient variation . Hence the glucomannan is non cytotoxic to cells.

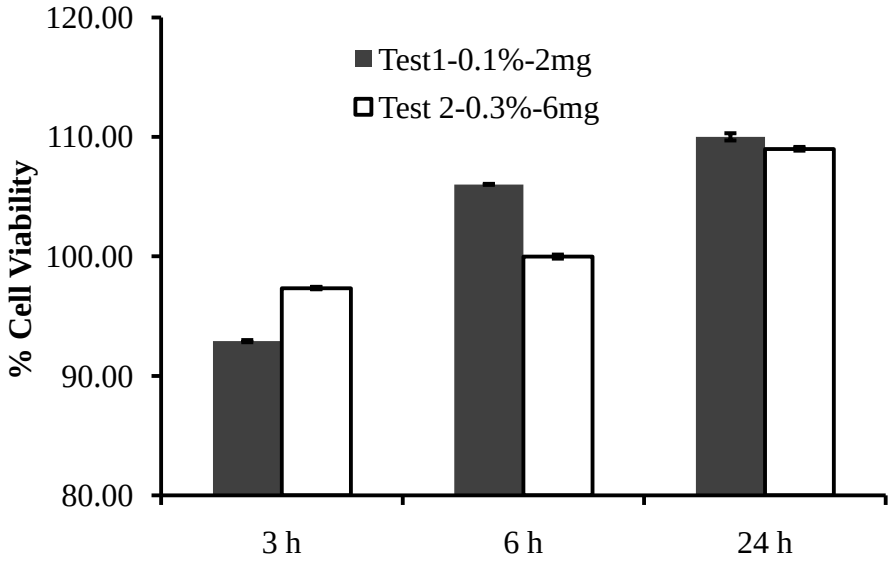
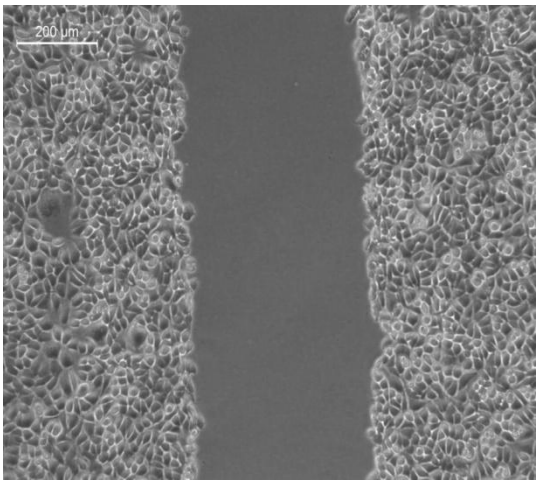


Figure 4.11 Response of human fibroblasts to various concentrations of glucomannan at the end of 24 h. (A) 0%; (B) 0.1% and (C) 0.3%

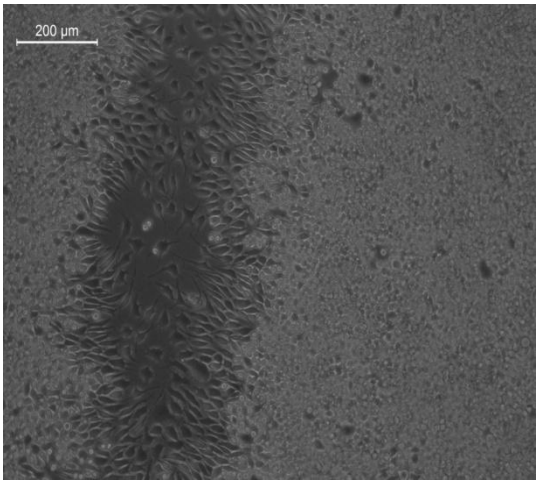
4.4.4. Scratch wound assay

The wounded monolayer was treated with freshly prepared 0.1% and 0.3% of glucomannan along with culture medium (0.5% FBS) in corresponding wells. The cells around the wounded area were observed for certain responses including survival of cells from acute damage at the wound edge, re-attachment of cells to the substrate, extension of cells to the wounded area and enhanced migration and proliferation rate to the wound site in 12 – 24 hours post treatment. The migration rate was monitored every 12 hours of post treatment. After the 24 hours, it was observed visually that glucomannan (0.3%) treated cells showed higher rate of proliferation towards the wounded area under inverted phase contrast microscope (Fig 4.12 e&f). When compared to glucomannan (0.3%) treated

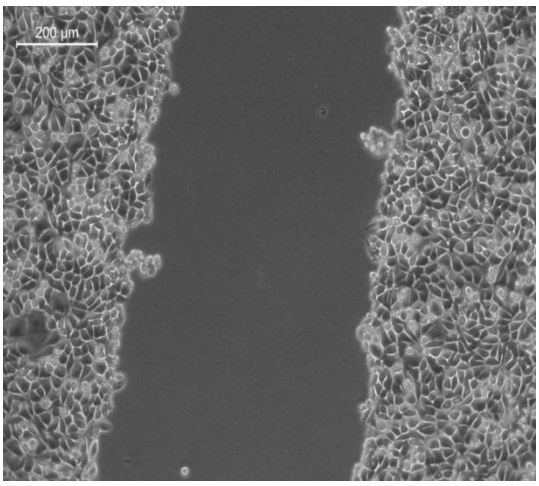
cells, glucomannan (0.1%) treated cells showed only moderate level of proliferation towards the wounded area (Fig. 4.12 c&d). Whereas, in case of control cells, the rate of proliferation was observed to be very slow (Fig 4.12 a&b). After incubation when the cells were visualized it was seen that the ccontrol cells, did not have any significant wound closure, whereas in glucomannan (0.3%) 90% of wound closure was visualized under inverted phase contrast microscope. The glucomannan (0.1%) treated cells also showed an enhanced level of wound closure when compared to the control cells. The wound closure observed in the cells after treatments may be achieved due to both cell proliferation and migration**.**



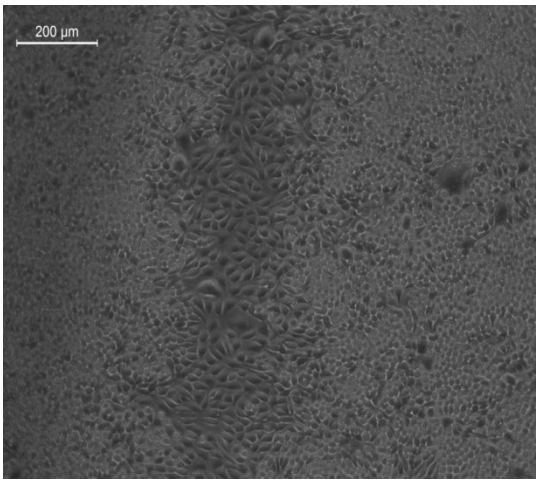
(a) Fibroblast cells at 0h



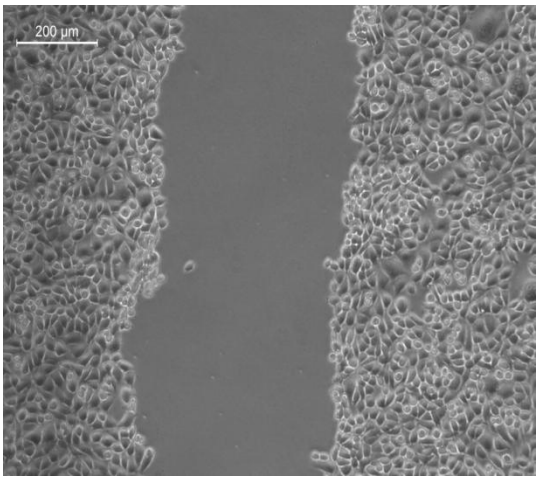
(b) Fibroblast cells after 24h



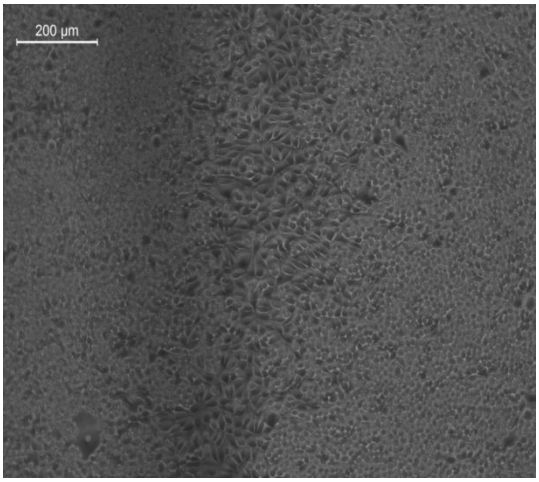
(c) Fibroblast cells with glucomannan (0.1%) at 0h



(d) Fibroblast cells with glucomannan (0.1%) at 24h



(e) Fibroblast cells with glucomannan (0.3%) at 0h



(f) Fibroblast cells with glucomannan (0.3%) at 24 h

Figure 4.12 Fibroblast cells (a) control at 0h (b) control at 24 h (c) Fibroblast cells treated with glucomannan (0.1%) at 0h (d) Fibroblast cells treated with glucomannan (0.1%) at 24h (e) Fibroblast cells treated with glucomannan (0.3%) at 0h (f) Fibroblast cells treated with glucomannan (0.3%) at 24 h

Chapter 5

SUMMARY, CONCLUSIONS AND FUTURE OUTLOOK

4.5. SUMMARY

Hydrogel matrix (HDRG) based on PHEMA, PEG; PCL was prepared using photo-polymerization as a non-adhesive wound dressing for burns. Glucomannan (GLU), a compound derived from *aloe vera* or Konjac plant was then added at various concentrations to HDRG. Both the HDRG and GLU modified HDRG were subjected to physic-chemical and biological characterization.

The FTIR results showed the presence of the HEMA, PCL and PEG within the hydrogel matrix, also the incorporation of the GLU into the hydrogel systems were evident from the FTIR data. The surface morphology examined using SEM, showed the hydrogel system as sufficiently porous with glucomannan dispersed in the HDRG matrix. Hydrogel systems were mechanically strong enough for a burn wound dressing. Upon immersing these hydrogel systems in PBS, they swelled up to 50% indicating good swelling capacity. Water vapour transmission rate of the hydrogel system was comparable to that of wound dressings already available and were within the limits of currently available wound dressings. The GLU release studies showed that adequate amount of glucomannan was released from hydrogel matrix over a period of 48h. The percentage of hemolysis of GLU was within the limits and the material was found to be hemocompatible. Direct of contact assay of glucomannan showed to human fibroblasts showed cell viability above 90% even at increase in GLU concentration. It is hence concluded that glucomannan is non cyto-toxic to the fibroblasts. Moreover the scratch

wound assay reveals the wound healing ability of glucomannan. The glucomannan treated fibroblast cells showed more migration towards the wound area when compared to the control fibroblast cells which are untreated. The fibroblast cells treated with glucomannan (0.3%) showed higher proliferation and migration rate when compared to the control cells. Hence this experiment clearly validate the wound healing ability of glucomannan

4.6. CONCLUSIONS

Formation of a hydrogel by photopolymerization of HEMA monomer incorporated with PCL and PEG was confirmed by FTIR. TPO was found to be an efficient photo-initiator for the above system. The synthesized hydrogel system was found to have physical and chemical properties showing the requirements of temporary wound dressing materials. The non-cytotoxic, non-hemolytic nature of the hydrogel system substantiated its selection as a potential wound dressing material. The addition of glucomannan in the hydrogel matrix doesn't have many changes in physico chemical properties of the hydrogel matrix. Moreover, the glucomannan showed cell viability and they were non cytotoxic to primary cells. The glucomannan wound healing property was showed by the scratch wound assay. Hydrogel systems based on PHEMA-PCL-PEG with glucomannan incorporated were developed successfully with potential application as a short-term burn wound dressing material.

4.7. FUTURE OUTLOOK

Further *in vivo* studies need to be evaluated in animal models to understand the wound healing properties of the glucomannan in hydrogel wound dressing *in vivo*, which may be followed by the clinical trials for use of hydrogel burn wound dressing in humans.

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