GELATIN METHACRYLATE BASED ANTIMICROBIAL HYDROGELS FOR TISSUE ENGINEERED CONSTRUCTS

A THESIS SUBMITTED

BY

REVATHY.R.J.

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DECLARATION

I, **Revathy R J,** hereby declare that I had personally carried out the work depicted in the thesis entitled "**Gelatin methacrylate based antimicrobial hydrogels for tissue engineered constructs**" under the direct supervision of "**Dr. V Kalliyana Krishnan, Scientist In Charge, Dental Products Laboratory,** Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India. External help sought are acknowledged.

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CERTIFICATE

This is to certify that the dissertation entitled "**Gelatin methacrylate based antimicrobial hydrogels for tissue engineered constructs**" submitted by Revathy.R.J. in partial fulfilment for the degree of Master of Philosophy Technology in Biomedical Research to be awarded by this Institute. The entire work was done by **her** under my supervision and guidance at **Dental Products Lab**, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Thiruvananthapuram.695012.

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By

Signature

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Examiners name and designation

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List of Abbreviations

AWD	Active Wound Dressings
ATR	Attenuated Total Reflection
2D	2-Dimensional
3D	3-Dimensional
DI	Deionised
DMDM	Dulbecco's Minimal Essential Medium
DPBS	Dulbecco's Phosphate Buffered Saline
ECM	Extra Cellular Matrix
EDTA	Ethylene Diamine Tetra Acetic acid
ESEM	Environmental Scanning Electron Microscopy
FACS	Fluorescence Assisted Cell Sorting
FBS	Foetal Bovine calf Serum
FT	Fourier Transform
kDa	Kilo Daltons
Micro-CT	Micro Computed tomography
PEG	Polyethyleneglycol
RI	Refractive Index
S%	Percentage Swelling
SFM	Serum Free medium
SNP	Silver Nanoparticle

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SYNOPSIS

Hydrogels are three-dimensional networks of hydrophilic polymers which can absorb and retain high water content within their matrix. They are widely used as wound dressing material since they are considered to possess most of the ideal characteristics required such as ability to absorb exudates at the same time retaining high moisture content, non-adhesive character, vapour permeability and act as a barrier against microbial infections. Various polymeric hydrogels are used also as tissue engineering constructs for regenerative therapy. For regenerative wound healing, the dressing should be developed in such a way that it should enhance healing with cell viability after storage for a considerable period of time and should be devoid of risk of infection while application. Hydrogels composed of biological materials like gelatin, hyaluronic acid, alginate, etc are being investigated for application as tissue engineering constructs. Synthetic materials like polyvinyl alcohol, poly–2- hydroxyethyl methacrylate (pHEMA) etc also have potential tissue engineering applications in constructs for wound healing.

Gelatin, a highly bioactive material is used in methacrylamide modified form to prepare polymeric hydrogels through photocrosslinking. However, redox polymerization is the conventional polymerization technique that have been used for polymerization of synthetic materials like HEMA and these polymers have shown to possess good biocompatibility and bioactivity. The GelMA hydrogels in combination with various other materials like PEG have also shown to improve the mechanical properties. Silver nanoparticles possess broad spectrum antimicrobial characteristics with toxicities against a wide variety of bacterial species and some fungi also. Considering this background information, it can be hypothesized that

"*Copolymerization of methacrylamide modified Gelatin and HEMA in the presence of PEG protected silver nanoparticles using redox initiators can be used to develop antimicrobial tissue engineered scaffolds with appropriate strength and strong water intake characteristic which also support the attachment and proliferation of dermal fibroblasts and may be used as a wound healing dressing material*"

The objectives adopted to illustrate the hypothesis are:

- Synthesis of Gelatin methacrylate (GelMA).
- Characterization of GelMA monomer & HEMA
- Polymerization of GelMA–HEMA hydrogels with and without PEG protected silver nanoparticles.
- Characterization of hydrogels: Chemical, Swelling, Mechanical, Porosity.
- Evaluation of cytocompatibility of the synthesized hydrogels using dermal fibroblast monolayer culture.
- Evaluation of 2D attachment and proliferation of fibroblast cells on composite hydrogels with and without Ag nanoparticles.

The raw materials Gelatin, HEMA and methacrylic anhydride [MAA] used in study were characterized initially by different spectroscopic techniques, viscometry and by measuring refractive index. Gelatin Type A from porcine skin [10% (w/v)] was reacted with MAA for a period of 3 hours at 50^{o}C with constant stirring. The raw product was purified through dialysis and subsequently lyophilized. The MAA concentration was varied in order to optimize the final yield of GelMA. The monomer was characterized by spectroscopic techniques. Silver nanoparticles (20 nm) were prepared in PEG solution by a reported procedure. Different hydrogel formulations were developed through redox initiation mechanism and following characterizations were done as follows: Swelling properties, mechanical (tensile and compression), 2D surface morphology evaluation, 3D microarchitecture and porosity evaluations etc. The biological characterization of hydrogel samples were performed to analyze the cytocompatibility (viability, apoptosis, and proliferation assays) and 2D cell attachment and spreading (actin staining).

A maximum yield of 67.08% was obtained during GelMA synthesis after purification by using 2% Methacrylic anhydride concentration in reaction volume. The hydrogel samples prepared from 10% GelMA and 10% GelMA and HEMA (1:0.8) were found to posess poor mechanical, handling and degradation properties and found unsuitable for further studies. Other combinations

G200H80, G300H80 containing 20 and 30% GelMA and HEMA (1:0.8) with and without silver nanoparticles were characterized with various techniques mentioned above. The swelling characterization was done by measuring the percentage swelling, swelling ratio, and rehydration ratio. In all the cases, a gradual increase in properties was observed with increase in monomer concentration and upon addition of PEG protected silver nanoparticles. Similar results were observed for the mechanical characterization also. The silver leaching was measured to detect the quantity of residual silver present in the hydrogel sample to exert sufficient antimicrobial properties. From the results of swelling and mechanical characterization G200H80Ag formulation was selected and used for further studies.

The 2D surface morphology and 3D morphometric evaluation was performed for the hydrogel samples after and before washing using ESEM and MicroCT. The results from these studies show that porosity and pore size distribution varied with storage of hydrogel in PBS with time. The biological characterization of the samples revealed a considerable reduction in cytotoxicity after washing in PBS, since the toxic eluents were leached out. The hydrogel samples did not show any adverse effects on cell proliferation and did not induce apoptosis. The samples allowed slight surface attachment through the edges when incubated for 3days which could be observed through ESEM. The cell spreading below the hydrogel samples (when placed on monolayer culture) was confirmed by the regular network of actin visualized by Phalloidin staining.

From the results got in the short term study, it can be concluded that, the developed hydrogel sample possess good mechanical and swelling properties required for application as a wound dressing material. The cytocompatability and 2D cell attachment studies indicate that, the cell adhesion and spreading can be improved by reducing the sample thickness and using bioactive agents to enhance the proliferation.

CHAPTER 1

INTRODUCTION

BACKGROUND:

A wound is a disruption of normal anatomic structure and function of the skin. The term 'wound' specifically refers to a sharp injury which damages the dermis of the skin. Normally a cascade of complex biological processes accompanies a wound known as the 'wound healing response'. In the case of simple wounds, it is able to regain the lost structure and function of a particular tissue. However, it can sometimes produce negative outcomes such as loss of normal structure and function due to excessive collagen deposition (scar formation), development of chronic nonhealing ulcers due to concurrent disease, such as diabetes, vascular disease, renal failure, and due to other factors such as malnutrition, smoking, radiation exposure, infection, and immunocompromise. The wound healing and scar formation affects multiple parts of the body, for example, heart after myocardial infarction, healing cirrhotic liver, maxillofacial regions after traumatic injury or surgery etc. Treatment of such chronic nonhealing wounds and tissue regeneration with reduced or no scar formation is a great challenge faced by clinicians.

Wounds are generally classified into acute and chronic wounds, based on the healing response. Acute wounds are caused by trauma, burns (wounds with tissue loss) or surgery (without tissue loss) and heal within a few weeks (8 – 12) whereas chronic wounds are the outcomes of diseases such as diabetes, tumors etc and take long time to heal (more than 12 weeks). Wounds are also classified by the layers involved: a) superficial wounds involve only the epidermis, b) partial thickness wounds - involve only epidermis and dermis, and c)full thickness wounds - involve the subcutaneous fat or deeper tissue. Based on their appearance, wounds are again classified into: epithelializing (clean, medium to high exudates), granulating (clean, exudating), slough-covered and necrotic (dry) wounds. Although wound healing and tissue regeneration occurs naturally, infection, wound drying, tissue death or signs of

circulation impairment, presence of pathology etc can influence the healing time and create a 'complex wound', thus making wound care and management a great clinical challenge.

Wound healing may be defined as the *process whereby an injured tissue is repaired, resulting in regeneration of its cell lining with the reorganization of the deep tissue components into scar.* Knowledge about normal wound healing mechanism and the chemical agents involved in it **is** very important in designing a tissue engineered wound dressing as a cutaneous regenerative approach. The healing response involves consecutive cascade of biological events - Hemostasis, Inflammation, Migration, Proliferation, and Maturation.

Hemostasis:- Vasoconstriction and hemostasis are the first stages of wound healing occurring immediately after skin damage. The contraction of blood vessels (vasoconstriction) at the wound site prevents excess blood loss and the fibrin clot formation (hemostasis) stops bleeding from the wound.

Inflammation:- This phase occurs simultaneously with the hemostasis. The clot formation and increased vessel permeability allows antibodies to enter tissues through exudates. Neutrophils accumulate at the wound site within a few minutes and activate inflammation. The chemical messengers produced attract phagocytic cells such as macrophages. These long lived cells produce various growth factors that act as chemical messages for further healing process.

Migration:- In the migratory phase, outer live epithelial cells starts migrating towards injury to replace dead cells.

Proliferation:- Cellular proliferation involves three key processes, *angiogenesis, fibroblast proliferation and epithelial cell proliferation* (all require energy, protein synthesis and anabolism). Macrophages secrete a substance known as angiogenesis factor, which is felt to be a chemo-attractant for mesothelial and vascular endothelial cells which divide to form a capillary bed near the wound edge. The fibroblast begins to appear in the wound about 2 days after injury which migrate to the wound site along the fibrin and collagen strands. The fibroblasts, being metabolically active, depend on the adequacy of local O_2 supply and the adequacy of neo-vascularization

for continued proliferation. Epithelialization or proliferation of epithelial cells occurs with loss of the epidermis. The adjacent cells become reprogrammed, get detached from their basement membrane, divide, and migrate toward and across the wound, first forming a single cell layer. Various epidermal growth factors released from the macrophage and platelet, initiate the response. The re-epithelialization process can be rapid, i.e., 3 to 5 days in a superficial injury or several months, depending on the size of the defect, the nutrient supply, the number of remaining basal cells, and the wound environment. Once a single layer develops, additional layers form from mitotic division.

Maturation or Remodeling:- The wound remodeling is the result of increasing cross-linking, resulting in increased strength. This stage begins about 3 weeks after the injury and persists for months to years. At this stage fibroblasts completely cover the surface of the wound as a new layer of the skin and there is no evidence of the wound.

Wound Management: Wound management requires dressing materials and techniques that address the specific needs of the injury. Traditional wound management practices worked on 'drying up' wounds believing it prevented infection, but from late 1960s it became clear that wound healing is optimal when wound is kept moist. Epithelialisation occurs more rapidly in a moist wound environment (Alvarez, 1988). Pain in the wound is reduced or eliminated in a moist environment where nerve endings are hydrated and air is expelled. Autolytic debridement (autolytic removal of necrotic tissue by rehydration) is facilitated in a moist environment and it provides faster re-epithelialization, reduced risk of infection, reduced healing time, and better cosmetic results. Endogenous growth factors critical to healing are found in wound fluids and may be more available in a moist environment. Ability to confer an electrical gradient between the wound bed and normal skin is present in a moist wound environment, thus promoting epidermal cell migration from normal skin to wound bed.

Over the past 30 years, hundreds of dressings that help to create a moist wound environment have been developed. Many modern dressings provide several of the ideal characteristics of moist wound dressings like,

- Maintain a moist wound environment
- Absorb excess exudate
- Eliminate dead space
- Do not harm the wound
- Provide thermal insulation
- Provide a bacterial barrier.
- Maintain optimum (acidic) pH and allow gaseous exchange
- Reduced odour
- Easy to apply
- Not excessively expensive
- Should not cause increased pain
- Should not induce maceration
- Should not induce toxins, foreign particles or fibres into the wound
- Should not cause trauma on removal.
- Should have acceptable handling qualities (resistance to tear and disintegration when wet or dry), and
- Be conformable and be sterilizable.

Modern moist wound dressings providing most of these ideal characteristics include occlusive dressings such as polymer films, foams, hydrogels, alginates, hydrocolloids etc. Recent developments in wound healing include new treatments such as skin substitutes, epidermal grafts dermal replacements, composite grafts, and growth factors which have shown some particularly promising initial results in the treatment of diabetic foot ulcers.

REVIEW OF LITERATURE

For a proper wound management, an ideal wound dressing which addresses the specific requirement of the injury is needed. Inert material that does not shed fibers or compounds into the wound which may evoke a foreign-body, irritant, or allergic reaction is the desirable property of an ideal dressing composition. The development and application of wound dressing for moist healing is dependent on the health condition of patient, the nature of the wound and the phase of wound healing. The main purpose of wound dressing is highest rate of healing and best aesthetic repair of wound together with tissue regeneration. In the treatment of wounds such as severe burns, trauma, diabetic, decubitus and venous stasis ulcers, and similar tissue damages rapid and proper healing is much more important where the dressing should achieve the functions of the natural skin by protecting the area from the loss of fluid and proteins, preventing infection through bacterial invasion, and subsequent tissue damage. Also, it should improve healing by providing a support for the proliferating cells. Thus the development of a suitable wound dressing which possess the above ideal features has been a great challenge and research interest for last several years.

Most of the modern dressing materials are designed to create a moist wound healing environment which allows the wound fluids and growth factors to remain in contact with wound, thus promoting autolytic debridement and accelerating wound healing. The improved healing under wet or moist condition has been explained as the easier migration of epidermal cells over the moist wound surface instead of under a dry scab, increased partial pressure of oxygen, and the preservation of growth factors and proteinases present in fluid exudates that can hence allow to exert their potentiating effect on wound healing (Svensjo T et al., 2000). Autolytic debridement is facilitated by the enzymes produced by leucocytes accumulated at the wound site under wet wound environment. This usually occurs 72 to 96 hours after wounding; thus cleaning the wound in preparation for the repair phase. The proposed benefits to moist wound healing include (Ted S. Stashak, 2004):

- ✓ Prevention of the formation of a scab which can trap white blood cells which prevents them from participating in their important wound healing functions.
- ✓ The pH of the environment is reduced, thus adversely affecting bacteria.
- ✓ Prevention of bacterial strike through from outside the wound to the wound surface.
- ✓ More rapid epithelialization and
- ✓ The moist environment favors normal colonization of bacteria but not infection.

CLASSIFICATION OF WOUND DRESSINGS:

Large varieties of modern wound dressings available can be divided in to two broad categories: ***biological and synthetic or biosynthetic***.

Biological-derived wound dressings have ability to effectively promote granulation and epithelialization of dermal wounds and they effectively regulate evaporation and exudation and protect the wound site from bacterial infection. Collagen-based dressing made from porcine skin, sheets of collagen, laboriously harvested from sheep intestine tissue engineered acellularized human cadaver skin etc are some of the early used biological wound dressing agents. The tedious processing of tissue and risk of microbial infection and foreign body reaction on application are the major drawbacks for the use of such biological-derived wound dressings.

Synthetic wound dressings are typically inexpensive, have a long shelf life, induce minimal inflammatory reaction, and lack the risk of disease agent transmission. Such synthetics/biosynthetics include textiles, polyurethane films, foams, hydrogels, hydrocolloids, and collagen/alginate combinations. Most of these modern synthetic/biosynthetic wound dressings provide several of the ideal characteristics of the moist wound dressings, but regenerative healing becomes a major challenge with the synthetic wound dressings.

The synthetic/biosynthetic wound dressings can again be classified based on various aspects. A) Depending on their function in the wound (debridement,

antibacterial, occlusive, absorbent, and adherence dressings); B) based on type of material employed to produce the dressing (e.g. hydrocolloid, alginate, collagen); and C) the physical form of the dressing (ointment, film, foam, gel).

The major classification for the synthetic wound dressings are based on their role in wound healing and regeneration, such as, ***passive, interactive and bioactive*** wound dressings. Passive dressings simply provide a cover over the wound so that the wound can rehabilitate underneath while active or interactive dressings are believed to be capable of modifying the physiology of the wound environment. Conventional wound dressings such as gauze and tulle are examples for passive wound dressings. Interactive materials are the second class of wound dressings that control the microenvironment on the wound surface which are transparent and permeable to water vapor and atmospheric oxygen. These are good barriers against permeation of bacteria to the wound environment and include materials such as hyaluronic acid (HA), hydrogels and foamed covers. The third type is bioactive products known as active wound dressing materials (AWD) that stimulate some part of the healing cascade. This includes materials such as hydrocolloids, alginates, collagens and chitosan. The AWDs can have suitable mass transfer properties with the wound bed which provides a means to tailor and monitor the chemical state of a wound and, thus potentially, to aid the healing process.

The modern wound dressings are mainly of ***occlusive or semi-occlusive type*** which occlude or retain moisture within the wound bed. This moisture prevents desiccation and additional trauma, thus preventing the generation of obstacle for the migration of new epidermal cells across the wound surface. The occlusive dressings have been reported clinically in association with a faster healing rate of partial-thickness abrasions than without any dressings (Beam JW, 2008).

Main classes of occlusive dressings are: Polymer Films, Foams, Hydrocolloids, Alginates and Hydrogels.

Polymer films:

The effects of polymer films for moist wound healing were first investigated by Winter (Winter GD, 1962) and Hinman and Maibach (Hinman CD, Maibach H, 1963) in 1960s. Film dressings were originally made from nylon derivatives supported in an adhesive polyethylene frame which made them occlusive. Later it has been modified to semipermeable dressings composed of polyurethane or polyethylene with an adhesive coating on one side. Since the films are transparent, direct inspection of wound is possible. They possess good elastic properties that accounts for its conformity, and provide resistance to shear and tear. Films are permeable to water vapor, oxygen, and other gases, but are impermeable to water and bacteria. The films are usually indicated in the treatment of superficial abrasions, blisters, cuts, and minor burns.

One drawback to semipermeable dressings is that they are nonabsorbent; therefore, they are not advised for heavily draining wounds. Channels often form in the dressing, allowing wound fluid to leak out. Leaking compromises the dressing's barrier capabilities and the fluid accumulation may lead to maceration of the wound surroundings. The pealing out of newly formed epithelium together with the adhesive and further damage to the healing wound during removal is another drawback of the polymer films.

Foams:

These dressings consist of porous polyurethane foam or polyurethane foam film, sometimes with adhesive borders. Foam dressings maintain a moist environment round the wound, provide thermal insulation and are convenient to wear. They are highly absorbent, absorbency being controlled by foam properties such as texture, thickness and pore size. The open pore structure also gives a high moisture vapour transmission rate (MVTR). The porous structure of the dressings, make them suitable for partial- or full-thickness wounds with minimal or moderate drainage, to

highly absorbent structures for heavily exuding wounds. Foam dressings are also indicated for granulating wounds where they are reported to help treat over granulation.

Foam dressings are not suitable for dry epithelialising wounds or dry scars as they rely on exudates unlike the polymer films, to achieve an optimum wound healing environment. They are opaque, and visual monitoring of wound is not possible. It needs frequent changing of the dressings, usually every 1-3 days and may cause undesirable drying effect on inadequately exudative wounds.

Hydrocolloids:

Hydrocolloids are dressings in which a hydrophilic gellable mass is applied in a semisolid form to a flexible semi permeable carrier (Thomas S, 2008). Those dressings 'melt' over the wound to form a mobile gel. They limit or prevent water loss and are capable of maintaining the hydration status in a wound without too much absorption that could dry up the wound. By trapping wound exudates, they create a moist environment that softens and lifts dry eschars, favours granulation tissue formation and re-epithelialization, promotes angiogenesis and stimulates macrophages (Atiyeh BS, et al., 2002). In their intact state, hydrocolloid dressings are impermeable to water vapour but on absorption of wound exudate, a change in physical state occurs with the formation of a gel covering the wound. They become progressively more permeable to water and air as the gel forms. The hydrocolloid dressings are most commonly applied in the treatment of chronic wounds (Harding KG, et al., 2000). However, these dressings have also been shown to improve healing rates in partial-thickness wounds such as burns, donor sites and superficial traumatic injuries. Hydrocolloids are primarily used for lightly to moderately exudating wounds, which are reported to reduce wound pain and improve the quality of healed wound. They are typical gel forming agents such as carboxymethylcellulose (CMC), gelatin and pectin in an adhesive mass usually made of polyisobutylene.

Hydrocolloid dressings generally have an occlusive outer cover that prevents water vapour exchange between the wound and its surroundings. This can be

disadvantageous for infected wounds that require a certain amount of oxygen to heal rapidly. Maceration of the surrounding tissue and leakage of excess exudates are other undesirable results by the application of hydrocolloid dressings. These dressings may leave an odorous residue of adhesive mixed with wound exudate upon removal of the dressing (known as 'Gel and smell' phenomenon). Bacterial colonization under occlusive dressing is also reported but will less report on increased infection.

Alginates:

Alginate dressings are natural polysaccharides derived from algae or kelp (seaweed). The non woven composite of fibers from calcium alginate, are produced from the calcium or sodium salts of alginic acid, a polymer composed of mannuronic and guluronic acid residues, extracted from seaweed (Atiyeh BS, et al., 2002). It is insoluble in water, but an ion exchange between the Calcium within the alginate fibers and the Sodium from the blood or wound exudate occurs up on application. Since the dressing is hydrophilic, it can absorb up to 20 to 30 times its weight in wound fluid. This process converts the initial dry felt like material into a hydrophilic gel on the wound surface that is easily removed. The calcium ion released from the dressing is known to promote the activation of prothrombin in the clotting cascade (Turner TD, 2001). Additionally some alginate containing dressings have the potential to activate macrophages within a chronic wound bed and have the ability to generate a pro-inflammatory signal which promotes granulation tissue formation (Thomas A, et al., 2000). Also, some alginates have the ability to kick start the healing cascade by causing lysis of mast cells resulting in release of histamine and 5-hydroxytryptamine (5HT). Because of these attributes, calcium alginate dressings are considered bioactive. The alginate dressings can entrap the bacteria in the gels and can be removed during dressing change, thus providing an indirect antibacterial property (Fanucci D, et al., 1991**).** The other advantage of alginate dressings include solubility in saline providing easy removal by irrigation and are biodegradable without any adverse effects.

Unpleasant odour and need of frequent dressing changes are the main drawbacks of alginate dressings.

Hydrogels:

Hydrogels are a three-dimensiueonal network of hydrophilic polymers with water content between 90 and 95%. They can swell in aqueous solutions upto equilibrium. Usually hydrogel dressings are made of synthetic polymers such as polyhydroxymethacrylates and polyvinylpyrrolidone. They are also made of biological materials like gelatin or polysaccharide crosslinked with a polymer. Hydrogel dressings are available in the form of amorphous gels or sheets or films. Because of their water holding capacity, the hydrogels can act as 'moisture donor' at the wound site. The hydrogel dressings are thus considered to be having most of the ideal characteristics of moist wound dressing.

They are suitable for cleansing of dry, sloughy or necrotic wounds by rehydrating dead tissues and enhancing autolytic debridement by increasing the moisture content of the necrotic tissue and increasing collagenase production (Flanagan M, 1995). Due to high water content, the hydrogels exert a cooling effect on application to wound and results in almost immediate reduction in pain. This can be explained as the reduction in inflammatory process due to cooling of wound surroundings.

The amorphous gel dressings need a secondary coverage when applied on the wound. However, the sheets do not need a secondary dressing since they can easily adhere and fit on to the wound site. Also the hydrogel sheets are highly flexible and can be cut into required size based on the wound size. They never leave any residue on wound surface and have shown to improve re-epithelialization of wounds (Debra JB, et al., 1998). The hydrogel dressings, even though considered occlusive, are able to absorb considerable amount of wound exudates into the polymer matrix and have comparable water vapour permeability to semipermeable membrane (Atiyeh BS, et al., 2002). These dressings are easily removed from the wound bed because the moist interface between dressing and the wound prevents dressing adherence.

Hydrogels are indicated in the treatment of blisters and minor lacerations, but may also be used for more severe wounds such as deep abrasions and second-degree burns because of the dressing's cooling ability and exudate absorbency (Mertz PM, 1990). Although hydrogels possess high water retention capacity, the dressings cannot be left in wound place for more than 1 to 2 days because of their tendency to dry out. This reduces the application of hydrogel dressings for lightly exuding wounds. Hydrogel dressings contain significant amounts of water (70–90%) and as a result they cannot absorb much exudate, and hence they are used for light to moderately exuding wounds. Also, the hydrogels may support the growth of microorganisms in the presence of absorbed wound exudates (Turner TD, 1985). This fluid accumulation can also lead to skin maceration and further bacterial proliferation leading to a foul smell in infected wounds, which necessitate the frequent changing of the wound dressings. In addition, hydrogels have low mechanical strength and therefore difficult to handle and this has been noted to affect patient compliance (Morgan DA, 1999).

Tissue engineering for wound healing:

The occlusive dressings, even though provide proper wound management, cannot assure the regenerative approach of wound healing. The use of bioactive wound dressings like collagen, hyaluronic acid, chitosan, alginates and elastin dressings together with the growth factors like Platelet derived growth factor (PDGF) have demonstrated a 25% increase in closure or healing rates in chronic wounds (Ehrenreich and Ruszczak, 2006). But the growth factor must be applied daily forcing the patients to change their bandages during each application. Thus ideally the goal would be to regenerate tissues such that both the structural and functional properties of the wounded tissue are restored to the levels before injury, regardless of the specific advanced wound care product. The advancements in our understanding of the cellular and molecular processes involved in acute wound healing and in the pathobiology of chronic wounds have enabled to analyze the applicability of regenerative wound healing through tissue engineered wound dressings.

Tissue engineered wound dressings are primarily developed to overcome the difficulties with autografts and allografts such as the risk and expense of surgery, donor site morbidity, and rejection of the transplanted tissue and also to get permanent healing of wound. The initial studies on tissue engineering for cutaneous wound healing involve the application of in vitro cultured autologous epidermal cells isolated through punch biopsies. Although these cells were efficient in small burn wounds (Lancet, 1981), they could not produce satisfactory results in deeper burns and full thickness wounds (Williamson JS, et al., 1995). The use of hyaluronan derivatives for keratinocyte delivery has demonstrated the application of bioactive materials in wound healing (Campoccia D, et al., 1998).

The dermal reconstruction using bioactive agents, another suitable approach for skin regeneration, was initially developed as a cell free construct (Burke JF,et al., 1981) but later the cell containing constructs with cells in cryopreserved live state (Marston WA, et al., 2003) were developed. These dermagrafts were found not stimulating immune rejection and thus considered as a suitable dermal substitute. However, it was then found suitable only as a delivery vehicle for growth factors and ECM produced by the implanted human foreskin fibroblast since these cells die within a few weeks after implantation (Ehrenreich and Ruszczak, 2006).

For full thickness wound regeneration, the dressing material should substitute both the dermal and epidermal part. A bilayered complete skin transplant, Apligraf, was the first to be marketed as an organotypic skin substitute for full thickness wounds. It consisted of type I bovine collagen matrix with live allogeneic human neonatal foreskin fibroblasts and keratinocytes and was found suitable for accelerated wound healing (up to 25%) of chronic non-healing venous stasis ulcers when compared to standard compressive therapy (Falanga et al., 1998). Although Appligraf does not produce any immunological rejection, the rate of infection was higher when compared to standards of wound care and the donor cells do not remain viable beyond 4–8 weeks. Thus for a regenerative wound healing, the dressing should be developed in such a way that it should enhance healing with cell viability after storage for a

considerable period of time and should be devoid of risk of infection while application.

Selection of material:

To design an ideal wound dressing, the selection of biomaterial would depend on the ease of availability, minimal processing, fitting for proper sterilization and storage, and the ability to retain, after sterilization and storage, the biological characteristics that promote wound healing. A biologic dressing made of such a material would incorporate both the advantages typical of synthetic dressings (low cost, long shelf life, and low risk of immunological reaction) and those typical of biological-derived dressings (regulated fluid flow, increased resistance to bacterial contamination, and enhanced wound healing).

Polymeric hydrogels:

Hydrogels are super absorbent polymeric materials which have significant roles in wound treatment / protection. This is due to their hydrophillicity, biocompatibility, non-toxicity, and biodegradability. Hydrogels are a class of polymer materials that can absorb large amounts of water without dissolving due to physical or chemical crosslinkage of the constituting hydrophilic polymer chains. The water holding capacity of the hydrogels depends on the amount of hydrophilic groups such as amino, carboxyl and hydroxyl groups, in the polymer chains. Hydrogels can hold 10 to thousands of times of its dry weight or xerogel weight which is the most desirable property of an ideal wound dressing material. This is mainly due to the availability of free hydrophilic groups which in turn depend on the crosslinking. The increased crosslinking can reduce equilibrium swelling since the hydrophilic groups are crosslinked (M. M. Alam, et al., 2003) and also there is a subsequent increase in the hydrophobicity and a corresponding decrease in the stretchability of the polymer network. Thus through controlled crosslinking of the polymer chains, the swelling, hydrophilicity and mechanical properties can be tailored. The biocompatibility of hydrogels is also associated with the hydrophilic nature, which helps in washing off the toxic and un-reacted chemicals during synthesis.

Hydrogels with biomedical applications are made from both synthetic (pHEMA, pMMA) and biopolymers (chitosan, alginate, hyaluronan, gelatin etc.). Because of the ease of preparation, desirable mechanical and biological properties of hydrogels, they are extensively used in drug delivery, wound healing, and as tissue engineering scaffolds in regenerative medicine. Hydrogels possess ECM-like viscoelastic and diffusive transport characteristics (Lutolf, 2009; Slaughter et al., 2009) and high water holding capacity that make them suitable for tissue engineering scaffolds and as wound dressings for skin regeneration. Since hydrogels mimic the ECM and their chemistry, cross-linking density and response to environmental stimuli (e.g., heat, light, electrical potential, chemicals, and biological agents) may be manipulated, they are ideal for producing tailored 3D cellular microenvironments.

During wound healing, the cells multiply by degrading and reorganizing the ECM by themselves and any wound dressing material must, therefore, be conductive to rapidly recruit host tissue cells and inductive to stimulate invading cells to proliferate, synthesize new ECM, and, if required, differentiate. Various studies have linked the mechanical and chemical properties of hydrogels to cell behavior. For example, the introduction of RGDS (Arg, Gly, Asp, Ser) sequence in PEG hydrogels have shown attached growth and spreading by endothelial cells (He et al., 2010). Thus hydrogels have greater potential to apply as a dressing material that accelerates healing with tissue regeneration.

Hydrogels have been found to promote fibroblast proliferation by reducing the fluid loss from the wound surface and protect the wound from external environment necessary for rapid wound healing. Hydrogels help in maintaining a micro-climate for biosynthetic reactions on the wound surface necessary for cellular activities. Fibroblast proliferation is necessary for complete epithelialisation of the wound, which starts from the edge of the wound. Since hydrogels help to keep the wound moist, keratinocytes can migrate on the surface. Hydrogels may be transparent, depending on the nature of the polymers, and provide cushioning and cooling/ soothing effects to the wound surface. The main advantage of the transparent hydrogels includes monitoring of the wound healing without removing the wound

dressing. The process of angiogenesis can be initiated by using semi-occlusive hydrogel dressings, which is initiated due to temporary hypoxia. Angiogenesis of the wound ensures the growth of granulation tissue by maintaining adequate supply of oxygen and nutrients to the wound surface.

Gelatin based hydrogel systems:

To develop a hydrogel as wound healing dressing, the most attractive materials would be biological materials or natural polymers that can mimic the ECM and thus enhance host cell attachment and proliferation. The various biological polymer hydrogels studied for wound healing include collagen, hyaluronic acid, chitosan, alginates and elastin. These biomaterials play an active part in the wound healing process and so sometimes referred to as 'bioactive dressings'. These materials are biodegradable and some play an active part in normal wound healing and new tissue formation. For example, collagen is the major component of skin and bone and represents approximately 25% of the total dry weight of mammals. It is the major constituent of the connective tissue that interacts with the cells and play definite role in wound healing and tissue regeneration. This makes it a suitable material for wound healing and tissue engineering scaffold for tissue regeneration.

Collagen hydrogels are extensively used as collagen matrix or in combination with other ECM components (Srivastava S, et al., 1990) for reconstruction of liver (Kaufmann PM, et al., 1997), skin (Auger FA, et al., 1998), blood vessel (Seliktar D,et al., 2000), and small intestine (Voytik-Harbin SL, et al., 1998). Collagen's high tensile strength, low extensibility, fiber orientation, controllable cross linking, low antigenicity, and effects on wound healing and blood coagulation are of principal importance which makes them important in biomedical application. However, collagen expresses antigenicity in physiological condition and, due to its scarce solubility, is difficult to process into a scaffold. Because of the compact triple-helix structure, the crosslinking of collagen is not easy as they lack free hydrophilic ends. Also the crosslinked collagen are found to be immunogenic due to the presence of traces of crosslinking agents like gluteraldehyde and the heterogenicity of collagen fibres from batch to batch reduces the consistency of cell response which makes it

not suitable for clinical applications. The availability and cost of production may also hinder the use of collagen.

The denaturated-type collagen, gelatin is derived from the partial hydrolysis of native collagen breaking the triple-helix structure of collagen into single strand molecules. Gelatin is composed of a variety of aminoacids forming a linear polymer with molecular weight varying between 15,000 and 250,000 Da. Based on the type of hydrolysis, ie, acidic or alkaline, gelatin is of two types: A and B. Type A gelatin has higher bloom number which directly correlates with the mechanical strength of the formed gel. Gelatin has a gel-sol transition temperature is approximately 30ºC. It dissolves in hot water (below 60ºC) and forms gel upon cooling. Gelatin has been extensively used as a wound dressing in medicine because of its self assembling, nontoxic, biodegradable, inexpensive and nonimmunogenic nature.

Gelatin is composed of a polypeptide chains formed from the denaturation of collagen. It is a heterogeneous mixture of single or multi-stranded polypeptides, each with extended left-handed Proline helix conformations and containing between 50 - 1000 amino acids. The triple helix of type I collagen extracted from skin and bones, as a source for gelatin, is composed of two α1(I) and one α2(I) chains, each with molecular mass ~95 kD, width ~1.5 nm and length ~0.3 μm. Gelatin consists of mixtures of these strands together with their oligomers and breakdown (and other) polypeptides. The basic aminoacid composition of a gelatin macromer is given in Fig.1. Gelatin contains many glycine (almost 1 in 3 residues, arranged every third residue), proline and 4-hydroxyproline residues. A typical structural unit is -Ala-Gly-Pro-Arg-Gly-Glu-4Hyp-Gly-Pro-. The free primary amino group containing residues can be chemically modified to enable production of chemically crosslinked Gelatin hydrogels.

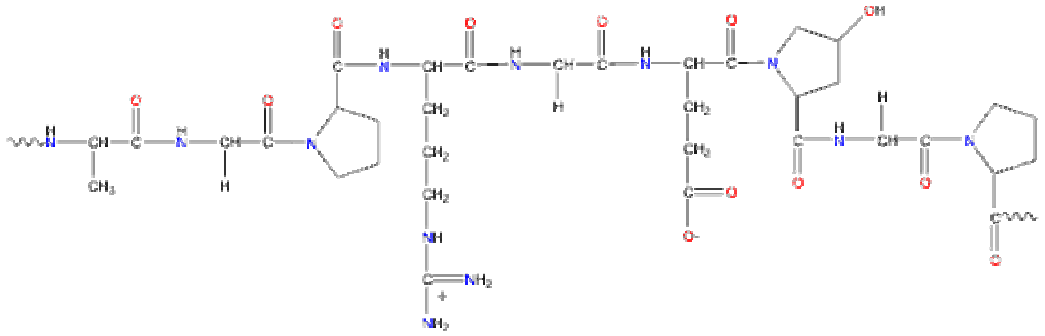


Fig. 1: Gelatin Structural Unit

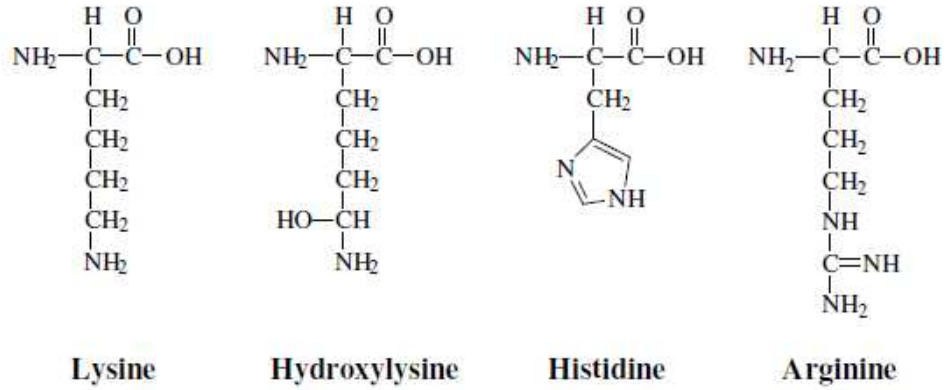


Fig. 2: Primary amino group containing residues of Gelatin

Gelatin easily forms physical hydrogel in water with poor mechanical properties. But to use as a wound healing dressing, it should possess mechanical properties similar to that of ECM. Solutions undergo coil-helix transition of the chains followed by aggregation of the helices by the formation of collagen-like right-handed triple-helical proline/hydroxyproline rich junction zones. Higher levels of these pyrrolidines result in stronger physically crosslinked gels. It has very low melting point and these physically crosslinked gelatin are not stable at body temperature (37ºC). So gelatin strands are chemically crosslinked to get stable sheets of hydrogels under body temperature. Gelatin hardening through chemical cross-linking typically utilizes bifunctional reagents such as glutaraldehyde (Jayakrishnan A, et al., 1996) and diisocyanates (Olde Damink LH, et al., 1995), as well as carbodiimides (Ofner CM, et al.,1996), polyepoxy compounds (Sung HW, et al.,

1996), genipin (Sung HW, et al., 1999), and acyl azides (Petite H, et al., 1990). Glutaraldehyde is by far the most widely used agent, due to its high efficiency to stabilize collagen-based biomaterials. But their role in local cytotoxicity and calcification of long-term implants necessitates an alternate for chemical crosslinking.

Gelatin is chemically modified using methacrylic anhydride to produce methacrylamide - modified gelatin or Gelatin methacrylate (GelMA) (An I. Van Den Bulcke, et al., 2000). Upon reaction with methacrylic anhydride, the ε-amino groups of Gelatin are modified to form methacrylamide linkages. The primary amino group containing residues of Gelatin like Lysine, Hydroxylysine, and Arginin are thought to be modified with methacrylate groups. This modified gelatin is photopolymerized to get the gelatin hydrogel with high storage modulus. By modifying the degree of substitution, polymer concentration, initiator concentration and uv irradiation conditions, the structural and rheological properties of gelatin-based hydrogels can be controlled.

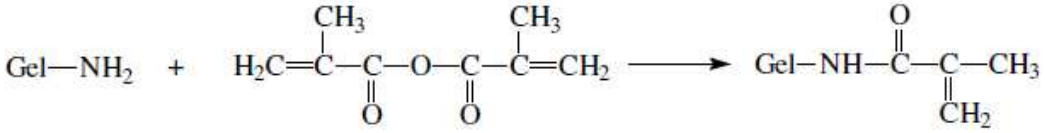


Fig. 3: Methacrylamide modification of Gelatin.

Photocrosslinking of GelMA monomers under uv irradiation to prepare cell encapsulated gelatin based hydrogels have shown to support fibroblast growth and proliferation and there were no need of alternate sterilization methods since uv irradiation functioned also as a sterilizing agent (Nichol JW, et al., 2010). However, the genetic stability of the cells after uv irradiation cannot be assured and the toxicity of monomers, due to the presence of acrylamide groups may harm considerable number of cells before polymerization. So to develop a cell encapsulated hydrogel material for tissue engineering and wound healing, alternative polymerization technique should be adopted.

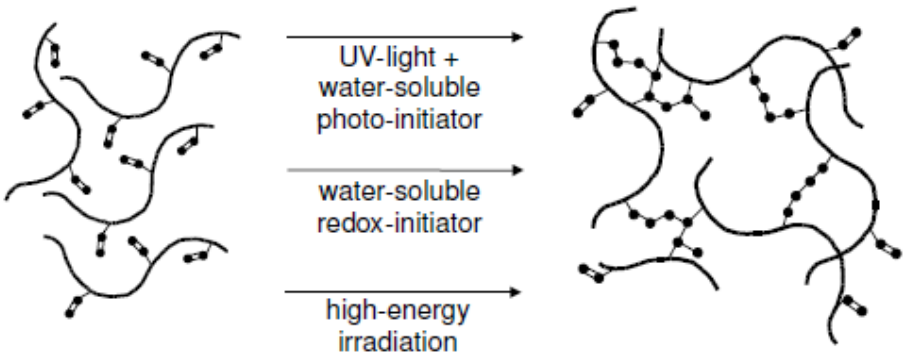


Fig. 4: Chemical crosslinking of GelMA.

Radical polymerization is the most common technique used for the preparation of acrylamide based hydrogels. The use of redox initiators, Sodium metabisulfite and potassium persulfate, are widely used in preparation of hydrogels from synthetic biomaterials. Radical polymerization of GelMA is found to be superior to other polymerization techniques like enzymatic polymerization. Radically polymerized GelMA hydrogels have shown to possess a defined pore structure with interconnections and as a consequence, it was more suitable for hepatocytes colonization (Barbetta A, et al., 2006). However, the lesser biocompatibility compared to the enzymatic crosslinking can be overcome by the incorporation of ECM components like Chondroitin sulfate and hyaluronic acid during radical polymerization (Andrea Barbetta, et al. 2008).

GelMA hydrogels are used as tissue engineering scaffolds for a wide variety of cell types. TGF-β1 incorporated GelMA hydrogels had shown good performance to support chondrocyte growth and maintain chondrocytic phenotype in vitro with improved bioactivity because of TGF-β1 in terms of cell proliferation and extracellular matrix secretion (Xiaohong Hu, et al., 2009). The GelMA hydrogels are also applied as an in vitro model for investigating cell and tissue morphogenesis in 3D, as well as for creating tissue constructs with microscale control of 3D cellular alignment and elongation that could have great potential for the engineering of functional tissues with aligned cells and anisotropic function (Aubin H, et al., 2010).

Photocrosslinked TGF-β1 incorporated GelMA hydrogels were also investigated for promoting aortic valvular interstitial cell (VICs) function in vitro (Benton JA, et al., 2009). GelMA as a tissue engineering scaffold for a variety of human cells were investigated by tuning the porosity. Also, GelMA hydrogels have a concentration dependent tunability of cell responsiveness to endothelial cells in both 2D culture and 3D encapsulation. The compressive strength of the hydrogel also increases with increased concentration of the gel (Nichol JW, et al., 2010). Three dimensional scaffolds that retain the enzymatic degradation property of ECM have been prepared with GelMA that support porcine mesenchymal stem cell adhesion, proliferation and differentiation (Ovsianikov A, et al., 2011). A modified cryogenic preparation of GelMA hydrogels (Vlierberghe SV, et al., 2007) and its cell interaction studies points out the use of GelMA hydrogels for long term culture studies of human cells (Dubruel P, et al., 2007).

Poly HEMA hydrogels:

Poly(2-hydroxyethyl methacrylate), or poly(HEMA), is one of the most important hydrogels in the biomaterials world since it has many advantages over other hydrogels. These include a water-content similar to living tissue, inertness to biological processes, resistance to degradation, permeability to metabolites, resistance to absorption by the body and biocompatibility. It can be easily manufactured into many shapes and forms, and be easily sterilized. The most common example of poly(HEMA) is its use as contact lenses.

Poly(HEMA) hydrogels were developed by Wichterle and Lim (Wichterle O., and Lim D., 1960) which was primarily used as a soft contact lens and, to a more limited extent, as a general biomedical polymer. After the development of this synthetic hydrogel, more attention was gained for the application of synthetic hydrogels as wound dressing. The Biomedical Polymers Technology Unit at Chiang Mai University has previously studied synthetic hydrogels based on HEMA for use as wound dressings (Siriwittayakorn T, et al., 2001; Prasitsilp M, et al., 2003). Regarding the low-fouling property, polyHEMA prevents non-specific protein adsorption (Lord MS, et al., 2006) and cell adhesion (Cook AD, et al., 1993). A

copolymer of polyHEMA and PEG-MA hydrogels were shown to have increased resistance towards non-specific protein adsorption and bacterial adhesion than polyHEMA alone (Oh Hyun Kwon, et al., 2003). Furthermore, quantitative hydroxyl functional groups of polyHEMA can be chemically modified to facilitate immobilization of molecules (Martin SM,et al., 2003) such as collagen, that are capable of modulating and enhancing wound healing. The surface modification with collagen and other ECM proteins have shown to be stable in physiological solutions and enhanced the adhesion and growth of mesenchymal stromal cells and astrocytes (Brynda E, et al., 2009).

Antibody loaded polyHEMA hydrogels have been investigated as antibacterial wound dressings with sustained antibacterial effect upto 12 days (Tsou TL, et al., 2005). PolyHEMA hydrogels are nonbiodegradable and this property reduces its use as a tissue engineering scaffold. But several studies have proven to produce biodegradable hydrogels with specific crosslinking mechanisms with peptides (Casadio YS, et al., 2010). Also, the techniques for preparation of polyHEMA hydrogels with controlled pore architecture have been developed (Studenovská H, et al., 2008) indicative of the increased interest of polyHEMA for tissue engineering and regeneration.

Antimicrobial wound dressings:

An ideal wound dressing should enhance the wound healing as well as should prevent infections at the wound site. The delivery of antibiotics to local wound sites may be a preferred option to systemic administration for several reasons. Antibiotic doses needed to achieve sufficient systemic efficiency often results cumulative cell and organ toxicity like in the ears and kidneys (Patrick BN, et al., 2006). The use of dressings to deliver antibiotics to wound sites can provide tissue compatibility, low occurrence of bacterial resistance and reduced interference with wound healing. The use of lower antibiotic doses within the dressings also reduces the risk of systemic toxicity considerably. In addition, local delivery from dressings can overcome the problem of ineffective systemic antibiotic therapy resulting from poor blood circulation at the extremities in diabetic foot ulcers.

The purpose of applying antibiotics and other antibacterials is mainly to prevent or combat infections especially for diabetic foot ulcers, surgical and accident wounds where the incidence of infections can be high due to reduced resistance resulting from extreme trauma. Common antibiotics incorporated into available dressings for delivery to wounds include dialkylcarbamoylchloride which is incorporated into Cutisorb a highly absorbent cotton wool dressing, povidone-iodine used with fabric dressing and silver used with most of the modern dressings. Other antibiotics delivered to wounds include gentamycin from collagen sponges, ofloxacin from silicone gel sheets and minocycline from chitosan film dressings. Treatment of dermal depth burn wounds using antimicrobial releasing silicone gel sheets which promotes epithelisation of superficial burns has been described by Sawada et al (Sawada Y, et al., 1990).

Incorporation of antimicrobial agents to dressings has also being investigated for their role in preventing infection without affecting wound healing (Phaneuf MD, et al., 2005). Antiseptic agents like Iodine, Borax etc have also been investigated for preventing infections during wound healing. The use of topical antiseptics came under severe criticism in 1980s and 1990s with the main target being those antiseptics that were identified as cytotoxic in vitro not only to micro-organisms but to the host's own cells.

Silver containing dressings are one such new group of dressings and are available in a variety of forms including foams, hydrofibres and hydrocolloids, all containing free silver ions as the active ingredient. Silver has long been recognized as a powerful antimicrobial and was used as early as 1895 for dressing surgical wounds and minimizing post-operative infection (Lansdown 2003). ***Silver is an effective broad spectrum antimicrobial agent due to its ability to bind to the DNA of bacteria and bacterial spores, reducing their ability to replicate (Ballard 2002) and is reported to be effective against almost all known bacteria including fungi and some viruses.*** The silver binds to the cell membranes causing significant damage and preventing replication (Ballard 2002). Silver salts such as silver nitrate and silver sulphadiazine were commonly applied to burns (Bowler 2003). Silver can be

delivered to a wound in a number of chemical formulations including metallic, nanocrystalline or ionic silver. A preparation may have a high concentration of silver but the availability of the silver to the wound may vary. For this reason studies may use the term 'free' silver to describe the amount of bioavailable silver from the product. The amount and rate of free silver released onto the wound surface will impact on its antimicrobial activity and Lansdown has reported that levels in excess of 20 mg/l demonstrate the best results (Lansdown 2002). However, a cost-effective use of silver incorporated wound dressing is to be developed.

The silver nano particles (SNPs) have gained much attention for its antimicrobial effect at very less concentrations itself, which is not harmful for the mammalian cells. 0.1% (w/w) SNPs incorporated into PCL scaffolds, for example, are found to be effective as antimicrobial agent and nontoxic to endothelial cells (Ragaseema V, et al., 2011). For burn wound applications, silver nanoparticle dressings are found applicable (Chen J, et al., 2006). Also, SNPs show anti-inflammatory effect on wound healing in thermal injury, diabetic wound, and chronic wound models (Tian J, et al., 2007).

Copolymers of Gelatin and synthetic biomaterials:

Gelatin is applied in combination with several synthetic biomaterials to prepare suitable scaffolds to exploit the cell binding sites of Gelatin and the mechanical properties of synthetic biomaterials. polyHEMA – Gelatin composite polymers have shown to possess improved ability for cell culture than each one alone (Santin M, et al., 1996). Also, combination of PEG to GelMA hydrogel have improved its mechanical properties and the composite hydrogels had tunable properties like, strength, hydration, enzymatic degradation, 2D attachment and growth of fibroblast as well as 3D encapsulation (Hutson CB, et al., 2011). Interpenetrating Networks of GelMA hydrogel – elastomer (polyurethane –HydroThane) composite biomaterial with increased tensile properties have shown the applicability of GelMA in combination with synthetic biomaterials as a wound dressing (Peng HT, et al., 2008).

Mechanical strength of a hydrogel directly correlates with its handling properties. The GelMA hydrogel strength directly depends on the crosslinking and which in turn depends on the methacrylamide substitution. So the mechanical strength can be tailored by increasing the degree of substitution. However, the gelatin hydrogels have an intrinsic property of fast degradation under physiological conditions irrespective of the degree of substitution and crosslinking and methacrylamide substitution. The combination of GelMA with a synthetic nonbiodegradable polymer like cross linked polyHEMA can improve its tensile properties and reduce degradation, through increased crosslinking with hydrophilic moieties of polyHEMA. Because gelatin is derived from collagen (i.e., the main constituent of the ECM), the developed materials mimic the cellular microenvironment from a chemical point of view. In addition, for an antimicrobial wound healing dressing, incorporation of Silver nanoparticle is preferred in controlled quantity that is nontoxic to fibroblast cells. The PEG protected SNPs can provide additional advantage to the GelMA-HEMA composite as evident from the previous literatures.

HYPOTHESIS:

"Copolymerization of methacrylamide modified Gelatin and HEMA in the presence of PEG protected Silver Nanoparticles using redox initiators can be used to develop antimicrobial tissue engineered scaffolds with appropriate strength and strong water intake characteristic which also support the attachment and proliferation of dermal fibroblasts and may be used as a wound healing dressing material."

OBJECTIVES:

- Synthesis of Gelatin methacrylate (GelMA).
- Characterization of GelMA monomer & HEMA
- Polymerization of GelMA–HEMA composite hydrogels with and without PEG protected Ag nanoparticle.
- Characterization of hydrogels: Chemical, Swelling, Mechanical, Porosity.
- Evaluation of cytocompatibility of composite hydrogels using dermal fibroblast monolayer culture.
- Evaluation of 2D attachment and proliferation of fibroblast cells on composite hydrogels with and without Ag nanoparticles.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND INSTRUMENTATION

The materials and instruments used are listed in tables below:

Table: List of Materials used

Sl. No.	Name of Chemical	Grade	Source
1	Gelatin Type A (from Porcine skin)	Laboratory Grade	Sigma Aldrich, USA
2	Methacrylic anhydride	Laboratory Grade	Sigma Aldrich, USA
3	Sodium chloride	GR	Merck, Germany
4	Potassium chloride	AR	SD Fine Chemicals, India
5	Potassium dihydrogen phosphate	GR	Merck, Germany
6	Disodium hydrogen phosphate	GR	Merck, Germany
7	Potassium bromide	Spectroscopic Grade	Merck, Germany
8	2-hydroxyethylmethacrylate [HEMA]	LR	Merck, Germany
9	Polyethylene Glycol 200	Laboratory Grade	Merck, Germany
10	Polyethylene Glycol 3400	Laboratory Grade	Sigma Aldrich, USA
11	Sodium metabisulphite	AR	SD Fine Chemicals, India
12	Potassium persulfate	AR	SD Fine Chemicals, India
13	Conc. Hydrochloric acid	AR	SD Fine Chemicals, India
14	Iscove's modified Dulbecco's Minimal Essential Medium – F12	Cell culture grade	Invitrogen, USA
15	Antibiotic-antimycotic solution	Cell culture grade	Invitrogen, USA
16	Foetal Bovine Calf Serum	Cell culture grade	Invitrogen, USA
17	Typsin-EDTA	Cell culture grade	Invitrogen, USA
18	Texas red - Phalloidin	Cell culture grade	Molecular Probes, USA

19	Trypan Blue	Cell culture grade	Sigma Aldrich, USA
20	Vibrant apoptosis assay kit	Cell culture grade	Molecular Probes, USA
21	Anti-PCNA antibody	Cell culture grade	Molecular Probes, USA
22	Formaldehyde	Laboratory Grade	Merck, Germany
23	Dialysis tubing	Laboratory Grade	Sigma Aldrich, USA

Table: List of Equipments used

Sl. No.	Name of Equipment	Model	Make
1	FTIR Spectrometer	FT/IR-6300	JASCO, Japan.
2	FT Raman Spectrometer	RFS 100/S	Bruker, Germany.
3	UV-Vis Spectrophotometer	UV-1800	Shimadzu, Japan.
4	Micro Computed Tomography	μCT-40	Scanco Medical AG, Switzerland
5	Universal Testing Machine	Instron-3365	Instron, USA.
6	Environmental Scanning Electron Microscope	Quanta 200	FEI, Netherlands
7	Phase contrast microscope	DMIRB	Leica, Germany
8	CO_2 incubator	Thermo	Thermo electron Ltd. USA
9	Flow cytometer	FACS ARIA	BD Biosciences, USA
10	Lyophilizer	Alpha 1-4 LD	Christ, Germany.
11	Deep freezer	Model 902	Thermo Scientific, USA.
12	Hot air Oven	JC-018	Pyrodevices, India.
13	Incubator	151	NSW, India.
14	Rotary evaporator (Rotavap)	RV06-ML, H4 Basic	IKA, Germany
15	Abbe Refractometer	3T	ATAGO, Japan
16	Improved Neubauer Counting chamber	Bromma S16126	BROMMA, Sweden
17	Schott Automatic Viscosystem	CT-62	Schott, Germany

METHODS

I. MOLECULAR WEIGHT DETERMINATION OF GELATIN:

Viscosity average molecular weight of Type A Gelatin (from Porcine skin) was determined using the Ubbelohde viscometer and Schott automatic viscosystem apparatus . 100mL Stock Gelatin solution (Concentration = 0.01g/mL) was prepared in deionised (DI) water with continuous stirring for two days at 40 ºC. Four working standards with concentrations 0.02g/dL, 0.03g/dL, 0.04g/dL and 0.06g/dL were prepared in 25mL standard flasks from the stock. The rate of flow of standards and DI water (Blank) were measured, taking 10 readings per sample, in the Ubbelohde viscometer at 25ºC. The average rate of flow was used to calculate the specific viscosity, η_{sp} using the equation:

$$\eta_{sp} = (t-t_0) / t_{0,}$$

Where, t is the average rate of flow of standard and t_0 is the average rate of flow of DI water blank. The reduced viscosity was calculated as (η_{sp} / c), where c is the concentration of standard. The Y-intercept of the straight line graph obtained after plotting Reduced viscosity Vs Concentration was taken as the Intrinsic viscosity (η).

The viscosity average molecular weight of Gelatin was determined using the Mark – Houwink equation.

$$[\eta_{sp} /c]_{c\rightarrow o} = KM^a,$$

Where, K and 'a' are Mark – Houwink constants and M is the viscosity average molecular weight (Mv). From literatures (W.Brooke Zhao, 1999), the values for K′ and 'a' were taken as (1.10×10^{-4}) and 0.74 respectively.

II. SYNTHESIS OF GELATIN METHACRYLATE MONOMER:

Type A porcine skin gelatin was mixed at 10% (w/v) into Dulbecco's phosphate buffered saline (DPBS) at 50ºC and stirred until fully dissolved.

Methacrylic anhydride was added dropwise at a rate of 0.5mL/min to the gelatin solution under stirred conditions (200 rpm) at 50ºC until the target volume was reached and allowed to react for 3 hrs. Following a 5x dilution with additional warm (40ºC) DPBS to stop the reaction, the mixture was dialyzed against de-ionized water using 12 - 14 kDa cutoff dialysis tubing for 1 week at 40ºC to remove salts and unreacted methacrylic acid. It was then distilled under vacuum at 40ºC for 3 hrs to remove excess water. The viscous solution obtained was snap frozen in liquid Nitrogen (-192ºC) and lyophilized for 3 days to get an off-white solid of GelMA monomer and stored at -50ºC until further use. The reaction set up is shown in Fig 5.

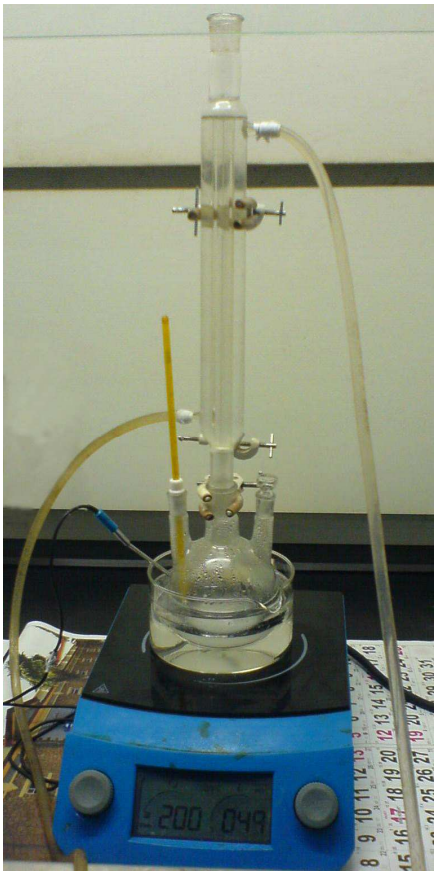


Fig.5: Synthesis of Gelatin Methacrylate monomer (GelMA)

The yield of GelMA production was calculated as:

Yield (%) = [Dry weight of GelMA / (Weight of Gelatin + Weight of MA)] x 100

III. CHARACTERIZATION OF MONOMERS:

1. FTIR Spectroscopy:

1.1. KBr Pellet method:

Fourier Transform infra red spectroscopy was used to characterize the GelMA monomer using KBr pellet technique. Samples were powdered fine by grinding a small amount (~ 0.1 mg) of GelMA with anhydrous KBr (1:300) in a smooth agate mortar. The fine powder was then made into a thin transparent disk using the hydraulic hand press under 10 ton pressure for about 3 minutes. KBr without monomer was used as the control.

After warming up the instrument for 20 minutes, the background measurements were taken with the transparent pressed – disk of KBr. The sample disks were monitored after background measurements using the "Spectra manager" software. Spectra were collected in the range 400 – 4000 cm^{-1} using at least 16 scans at a resolution of 4 cm^{-1}. The obtained FTIR spectra were processed for baseline corrections, CO_2 peak removal, smoothening and labeling of peaks using the software.

1.2. NaCl Cell Method:

To measure the FTIR spectra of liquid samples such as HEMA and SNP solution in PEG, NaCl Cell was used. A drop of sample was placed in between the cells to get a uniform thin film of the sample. The cell containing the sample was then placed in the sample chamber and its spectrum was measured after the background scan. The NaCl cell alone was used for the background measurements. The spectrum was processed using the "Spectra manager" software. Spectra were measured in the range of 400 – 4000 cm^{-1} using at least 16 scans at a resolution of 4 cm^{-1}.

2. FT Raman Spectroscopy:

Dried GelMA was subjected to FT-Raman spectroscopic studies.The sample was filled into the sample cup and placed it on the sample stage which is controlled by a XYZ motor to focus the LASER. The sample was placed facing to the optical system and using XYZ motor, the reference LASER (He-Ne LASER, 1064 nm wavelength) was focused in the area of interest of the sample. OPUS software package was used to measure FTRaman spectrum at 150mW LASER power (Nd-YAG LASER) with the use of a pre-cooled Germanium detector. The interferometric set up associated with the optical system provides interferogram at the output which is converted to spectra (spectral range - 3600-50 cm^{-1}) in terms of wave numbers using Fourier transformation.

3. UV spectroscopy:

The UV spectra of GelMA monomers were taken under 190 – 350nm range in a UV-VIS spectrophotometer. 1% solution of GelMA (w/v) in DI water was used for recording the spectra. DI water alone was used for baseline corrections. Using "UV Probe" software associated with the spectrophotometer, the peaks were labeled.

4. Refractive Index:

The purity of distilled HEMA was checked by measuring the Refractive Index using refractometer. A drop (about 1mm diameter) of the sample was placed on to the polished surface of the test piece, after wiping the main prism surface and test piece clean, and fixed to the centre of main prism so that the drop of sample spreads into a thin film. Upon illumination and viewing through the eyepiece, the colour compensator knob was turned to get a 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.

IV. PREPARATION OF SILVER NANOPARTICLES (SNPs):

PEG protected **s**ilver nanoparticles were prepared as per a reported procedure (Ragaseema V et al., 2010)**.** PEG 3400 (3.88g) was dissolved in 10mL of PEG 200 at 80ºC with stirring. After dissolution, temperature of the solution was decreased to 40ºC followed by the addition of silver nitrate (0.1g) with mild stirring so that a clear brown solution was obtained.

V. PREPARATION OF HYDROGEL:

10%, 20% and 30% GelMA (w/v) solutions were prepared by dissolving adequate amounts of lyophilized monomer in DI water/DPBS by heating at 40ºC with stirring. 80% (w/w) distilled HEMA (containing 0.03% EGDMA) was added to this. PEG protected silver nanoparticle solution was added to get 0.1% (w/w) of the total monomer weight and kept stirring for 15min at 40ºC.

From this stock solution, required volumes of monomer and initiators were aliquoted in the ratio of 5:1:1. 3% (w/v) Sodium metabisulphite and Potassium persulfate were used as the redox initiators. After adding the initiators, the gels were cast on to the required mold and incubated for 5-8 minutes for gelation and removed from the mold and washed in DI water/DPBS and used for further studies.

VI. CHARACTERIZATION OF HYDROGEL:

1. Chemical characterization:

1.1. Attenuated Total Reflection (ATR):

To characterize the hydrogels, ATR method was used, in which, the hydrogel sample of 7cm length, 1.5cm width, and 2mm thickness was placed on to the Zinc Selenide horizontal flat plate sample holder ATR assembly of a FT-IR spectrometer. Background measurements were done without the sample. The spectra were measured between 650 – 4000 cm^{-1} range with 72 scans per sample at a resolution of 4 cm^{-1}.

1.2. FT Raman spectroscopy:

The hydrogel samples were air dried and placed on to a clean glass slide. The slide was then placed on the sample stage and recorded the FTRaman spectra as mentioned earlier.

2. Swelling Studies:

Six different formulations were used to prepare the hydrogel as provided in the following table.

Sl No.	Name	Components		
		GelMA (mg/mL)	HEMA (µL/mL)	PEG-SNP (µL/mL)
1	G-10%	100	-	-
2	$G_{100}H_{80}$	100	80	-
3	$G_{200}H_{80}$	200	153	-
4	$G_{300}H_{80}$	300	224	-
5	$G_{200}H_{80}Ag$	200	153	57.1
6	$G_{300}H_{80}Ag$	300	224	85.7

G: GelMA; H: HEMA; Ag: silver; SNP: silver nanoparticle.

Six samples (7cm x 1.5 cm x 0.2 cm LxWxT) each from the different hydrogel compositions were prepared and placed in DI water at 37ºC for 24 hrs. Samples were removed from DI water and blotted with tissue paper to remove the residual liquid and the swollen weight was recorded. It was lyophilized at -60^{o}C under vacuum for nearly 4 hours and weighed to get the dry weight of polymer. The mass swelling ratio was then calculated as the ratio of swollen hydrogel mass to the mass of dry polymer.

Mass Swelling Ratio = Initial swollen weight / Dry weight

The dried hydrogels were put in DI water again for 48 hrs and weighed again to check the rehydration. The rehydration ratio was calculated as the ratio of rehydrated weight to the initial swollen weight.

Rehydration Ratio = Rehydrated weight / Initial swollen weight

The percentage swelling, S%, was calculated from the following equation:

$$S\% = [(M_t - M_0)/M_0] \times 100$$

Where M_0 is the mass of the dry gel at time 0 and M_t is the mass of the swollen gel at time t.

3. Mechanical testing:

Mechanical properties of hydrogels were determined using Universal Testing Machine (Instron 3365). Hydrogel samples with 7cm length, 1.5 cm breadth, and 2mm thick were prepared and incubated in DI water for 24hrs prior to testing. The samples were preconditioned by keeping for 1hr in the room temperature where the test is to be conducted.

3.1. Tensile Strength:

Six samples per group were prepared and pre conditioned as mentioned above for measuring the tensile strength. To test the tensile strength, the samples were blotted surface dry, both ends of the rectangular samples were clamped into the gigs and the test was conducted at a cross head speed of 10 mm/min using a 10 N load cell. The ultimate stress and strain parameters were calculated, respectively, as the force (load) at break divided by cross-sectional area, and as the elongation at break divided by the initial length (Gauge length = length in between the clamping heads) of the hydrogel sample.

3.2. Compressive strength:

Rectangular pieces of samples were prepared as mentioned above and preconditioned. From these samples, 8mm diameter discs were punched using stainless steel punch and eight discs per group were used for measuring compressive strength. The disc was blotted dry and compressed at a rate of 20% strain/min using

load cell 10N. The compressive modulus was determined as the slope of the linear region corresponding with 0%–10% strain.

4. Morphology:

Morphology of the hydrogel samples were studied using Environmental scanning electron microscope (ESEM). The dried and wet hydrogel samples were placed on the Aluminium stub and observed using low vacuum mode of the ESEM. The images were acquired at different magnifications. Fibroblast cells spread on the hydrogel were also observed using ESEM.

5. Porosity and 3-dimensional microarchitecture:

Lyophilized hydrogel samples were used for the porosity measurements using micro-computed tomography. The samples were placed in the sample holder (PMMA tube) for detecting the X-ray attenuation and scanning was performed with 45kV X-ray energy, 177µA intensity and 10µm resolution (2D slice thickness). Two dimensional reconstructions was done using Cone beam algorithm. Region of interest in the 2D slices were contoured and 3D evaluation was performed by setting appropriate threshold value of X-ray attenuation to actual 3D image of the sample. Pore and sample thickness distribution images and its histograms were also generated.

6. Silver leaching Evaluation:

To check the leaching of SNPs into the medium, silver incorporated hydrogel samples were incubated with DI water for different intervals with complete change of water after every 24 hrs. SNP on reaction with 1N HCl showed turbidity correlated to the concentration of nanoparticles, and which was measured turbidometrically as % transmission at 480nm using UV-Vis spectrophotometer. With known concentrations of SNP, a calibration plot was drawn and was used for the quantification of silver leaching into water by mixing 3mL of the collected water at each time period with 3mL of 1N HCl and then measuring the turbidity.

VII. BIOLOGICAL EVALUATION OF HYDROGEL:

1. Cell culture:

Isolated sheep dermal fibroblast cells were used for biological evaluation of the hydrogel. The cells were cultured in 35mm diameter culture dishes using DMEM-F12 medium containing 10% (v/v) Foetal BovineSerum (FBS) and and 1% (v/v) Antibiotic-antimycotic solution. The seeded culture dishes were incubated at 37ºC with 95% humidity and 5% CO_2 in CO_2 incubator. The medium was changed every day and images were taken using phase contrast microscope after the culture became confluent monolayer. At this stage trypsinization was performed as follows:

The cells were washed with serum free medium (SFM) after removing the culture medium from the dish. 1mL of Trypsin-EDTA (0.05%) solution was added and incubated for 5 minutes at 37ºC. The plates were then observed under a phase contrast microscope to confirm the detachment of cells from the dish and 1mL of serum containing culture medium was added to block trypsin activity. It was then centrifuged at 500g for 5 minutes to pelletize the cells. Supernatant was discarded and the pellet was resuspended in 3mL fresh culture medium. From this, 1mL each was used for seeding into three new 35mm culture dishes (1:3 split ratios).

2. Cytocompatibility:

For cytocompatibility studies direct contact method was used. Hydrogel samples used for biological evaluation were cast, under sterile conditions, into 1.5 cm diameter discs with 2mm thickness in the 24 well culture plates. The discs were then washed in sterile PBS for 3 days with intermittent changes of PBS. Discs were then sterilized by washing with 70% ethanol for 15-20 minutes. It was again washed three times in sterile PBS and soaked in it for 1hr. It was then conditioned by soaking in SFM medium for three days with changing the medium every day.

The hydrogel discs conditioned in the SFM were then placed onto the sub-confluent monolayer culture in 35mm culture plates and incubated for 24-48 hrs at 37ºC in CO_2 incubator with 95% humidity and 5% CO_2 to observe the changes in morphology and cell death through phase contrast microscope.

Following experiments were carried out to study the cytocompatibility of hydrogel samples.

2.1. Cell Viability assay:

Trypan blue exclusion assay was used to quantify the cell viability in presence of hydrogel. The cells cultured in presence of hydrogel for 72hrs as mentioned above were harvested by trypsinization after removing the gel from the dish. The cell pellet was then resuspended in 1mL sterile PBS. Trypan Blue stain was diluted in 1:9 ratio with DPBS and mixed 90µL stain to 10µL cell suspension. About 20µL was taken into the Neubauer counting chamber and counted under 10X magnification of microscope.

The number of cells in the sample was calculated using the equation:

No. of cells = (Total no. of cells counted / No. of squares counted) x Dilution x 1 x 10^4

The percentage viability was calculated as following:

$$\text{Viability \%} = \frac{\text{Total No. of viable cells per mL of sample}}{\text{Total No. of cells per mL of sample}} \text{ X } 100$$

2.2. Apoptosis Assay:

The Apoptosis of fibroblast cells in presence of hydrogel were quantified using Vibrant Apoptosis Assay kit. The cells grown in the presence of hydrogel were harvested by trypsinization and stained according to the manufacturer's protocol. After staining, the cells were washed, resuspended in annexin binding buffer and analyzed using flow cytometer. The percentages of live, apoptotic and necrotic cells were estimated using Diva software associated with flowcytometer.

2.3. Cell Proliferation by Immunostaining:

The fibroblast cells grown in the vicinity of hydrogel samples were harvested by trypsinization. The pellet was washed in PBS and fixed by incubating in 3.7%

formaldehyde for 30 minutes. The cells were then washed in PBS and stained with FITC-tagged antibodies against proliferating cell nuclear antigen (PCNA) (1:200 dilution in 0.5% BSA). The cells were then analyzed using a flow cytometer to distinguish PCNA-positive cells and their percentage was calculated using Diva software.

3. Two Dimensional Cell Attachment:

The washed hydrogel discs were placed onto 35mm culture plates and seeded with trypsinized dermal fibroblast cells on the gel and culture dish. After incubation for about 48hrs, the gels were taken out and placed onto a new sterile culture dish and added 1mL fresh culture medium and incubated for 24hrs. The plates were then visualized through phase contrast microscope to observe morphology of attached cells.

5. Cell adhesion and spreading:

The fibroblast cells incubated for 48hrs with hydrogel disc were used for visualizing actin filament assembly during cell adhesion and spreading.The plates were washed with PBS three times after removing the culture medium. Cells were fixed in 3.7% Formaldehyde for 30minutes and washed again for three times with PBS and treated with 0.2% Triton X100 (Permeabilizing agent). They were incubated for 5-7minutes and washed three times again in PBS. Texas red Phalloidin stain was diluted in 1:500 ratio in 0.5%BSA and added to the dish under dark. It was then incubated under dark at room temperature for 1 hr and washed in PBS three times and soaked in PBS for 1hr to remove excess stain at 4ºC. Fluorescence microscope was used to observe the stained cells using green filter.

CHAPTER 3

RESULTS AND DISCUSSION

I. Raw material characterization:

Viscosity average molecular weight of Gelatin Type A from porcine skin, used in the study was determined using viscometric technique and calculated using the Mark – Houwink equation ($\eta = KM^a$), after measuring the specific and reduced viscosities. The intrinsic viscosity (Y-intercept) was deduced from the Reduced viscosity Vs Concentration graph. The molecular weight of Gelatin was obtained as 2.51×10^5. Gelatin was also characterized using FT-IR spectroscopy as shown in Fig. 6.

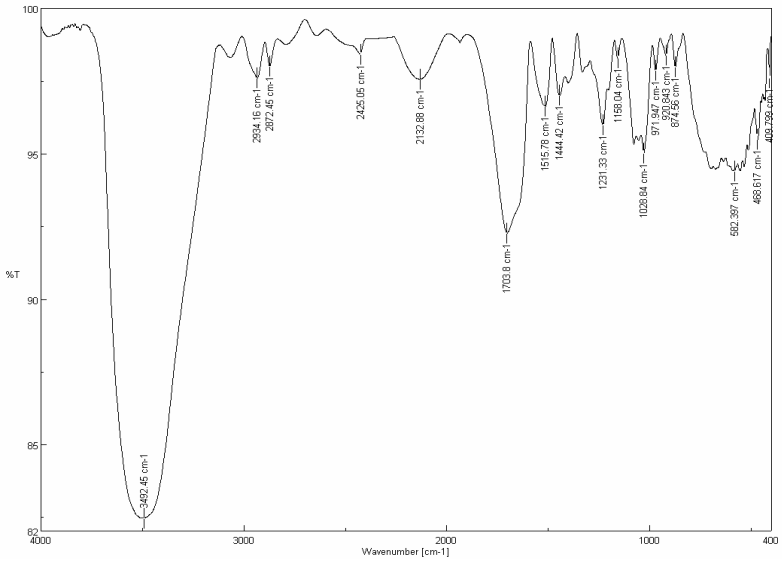


Fig.6: FT-IR spectrum of Gelatin.

Methacrylic anhydride (MAA) used for the conversion of Gelatin to GelMA was used as received and characterized using refractometry and FT-IR spectroscopy before use. A value of 1.4530 was obtained as the RI of MAA which compared similarly with reported values. FTIR spectrum of MAA showed characteristic absorption peaks (Fig 7) and compared well with reported spectrum.

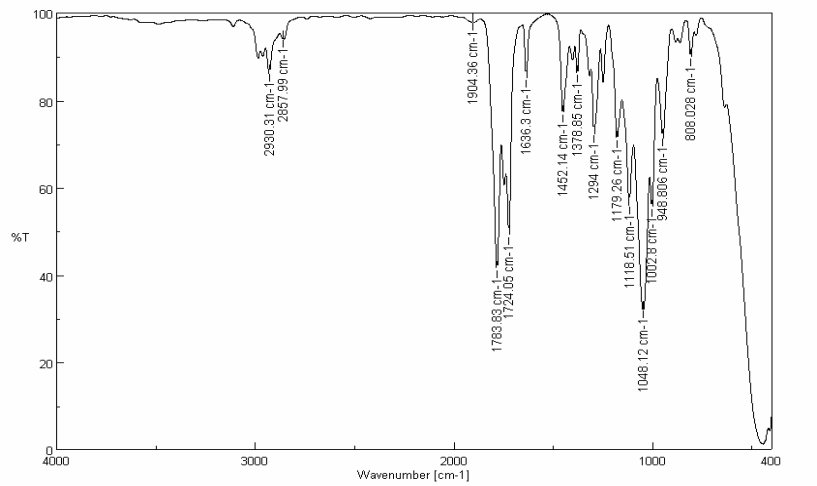


Fig.7: FT-IR spectrum of MAA

II. Synthesis of Gelatin Methacrylate (GelMA):

Gelatin was chemically modified with methacrylate groups from methacrylic acid formed by the hydrolysis of methacrylic anhydride upon stirring at 50ºC under physiological pH (7.4). Concentrations of MAA were varied from 15 ml to 0.5 ml with gelatin concentration remaining constant (5 gm in 50 ml PBS) in the reaction mixture and the yield of GelMA was calculated from each batch. Yield was plotted against the concentration of MAA as given in Fig 8. Maximum yield of 67.08% was obtained was at 2% (v/v) MAA in the reaction mixture which is represented as a peak in the graph.

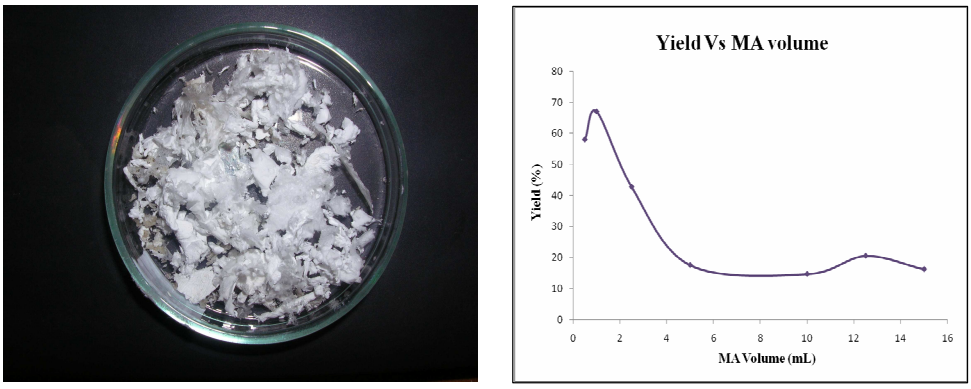


Fig.8: Standardization of GelMA sysnthesis for maximum yield (right) and dried GelMA monomer (left).

III. Characterization of Monomers:

The monomers, GelMA and HEMA and PEG protected silver nanoparticles used in the study were characterized using different spectroscopic techniques as given below:

GelMA was chemically characterized for the presence of functional groups and specific bonds using FT-IR and FT Raman spectroscopy (Figs.9 and 10). An examination of the IR & Raman spectra shows the disappearance of the unsaturated groups of MAA at 1636 cm^{-1}(Fig 7) and the formation of the amide linkage characterized by the 1658-1662 cm^{-1} peak (Fig 9). The UV spectrum showed absorption maxima for GelMA at 254 nm and 284 nm characteristic of the chromophores present in gelatin methacrylate (Fig 11).

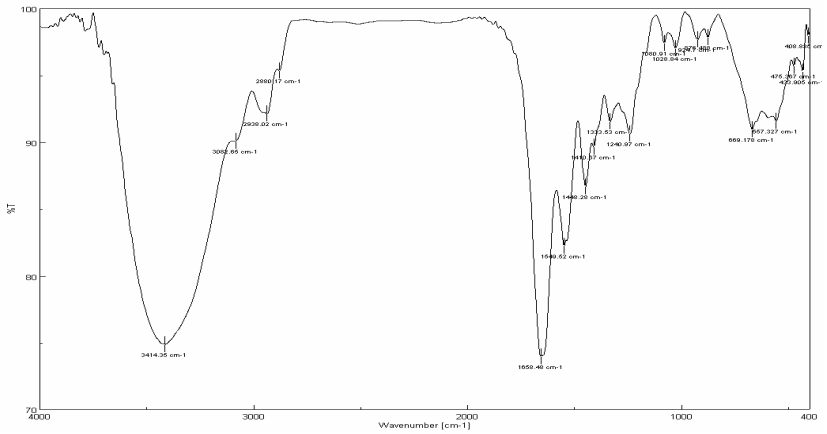


Fig.9: FT-IR spectrum of GelMA.

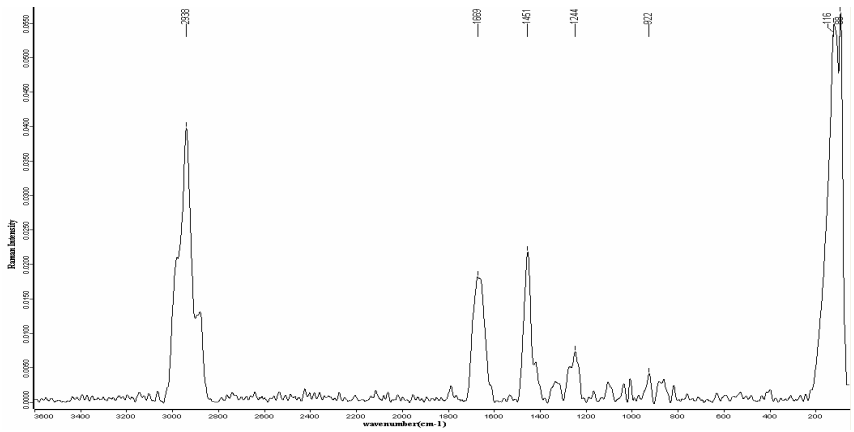


Fig.10: FT Raman spectrum of GelMA.

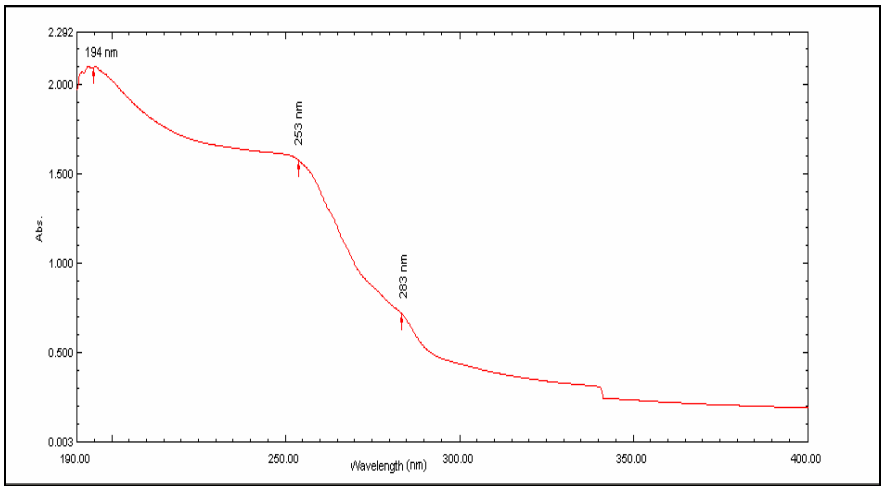


Fig.11: UV absorption spectrum of GelMA.

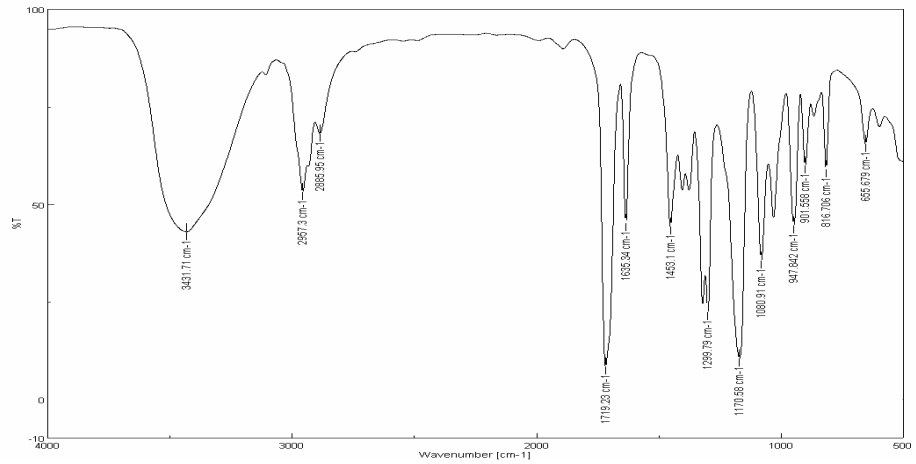


Fig.12: FT-IR spectrum of HEMA.

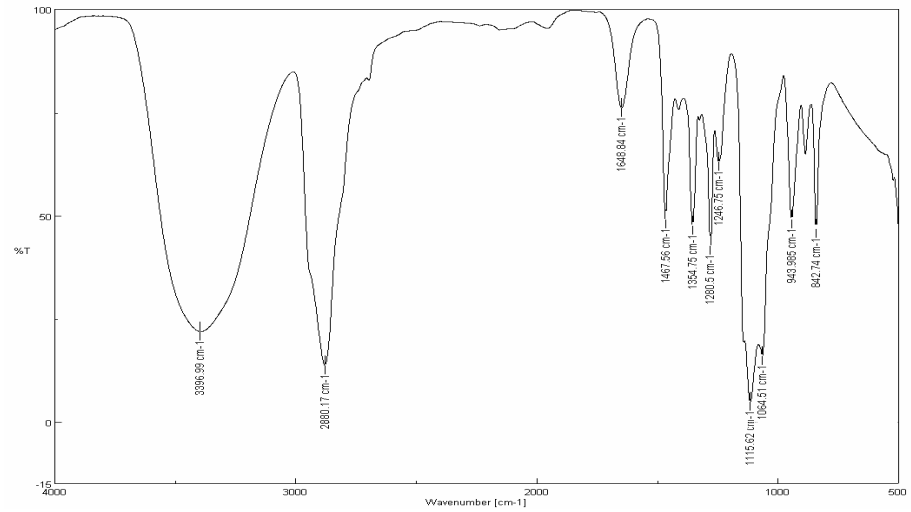


Fig.13: FT-IR spectrum of PEG protected Silver Nanoparticles.

Figs.12& 13 show the infrared spectra of distilled HEMA and PEG protected silver nanoparticles. HEMA was vacuum distilled to remove the inhibitor present. HEMA shows characteristic strong hydroxyl absorption peak at 3431 $cm^{-1,}$ ester peak at 1719 cm^{-1}, unsaturated C=C peak at 1635cm^{-1}. PEG also exhibits its absorption maxima 3396 cm-1 and -C-H stretching at 2880 cm^{-1}. Both of them compare well with reported absorption peaks for the monomers. The purity of distilled HEMA was also checked by measuring the refractive index. At 25ºC, RI for HEMA was determined as 1.4498 and is found comparable with the reported value (1.453 at 20ºC).

IV. Synthesis and characterization of Hydrogel polymer

Redox Polymerization of 10% GelMA monomer alone produced a hydrogel with very poor mechanical properties as evidenced by the extremely high water absorption characteristics (nearly 1117.5%), difficulty in handling and the tendency for the hydrogel to disintegrate into smaller fragments when stored at pH 7.4. Copolymerizing 10% GelMA along with HEMA monomer in the ratios 1:0.8, 1:0.4 and 1: 0.2 also yielded hydrogels with varying degradation times and poor mechanical properties which was difficult to measure. But it was observed that with increasing concentration of HEMA, the degradation times increased as shown in Fig 14.

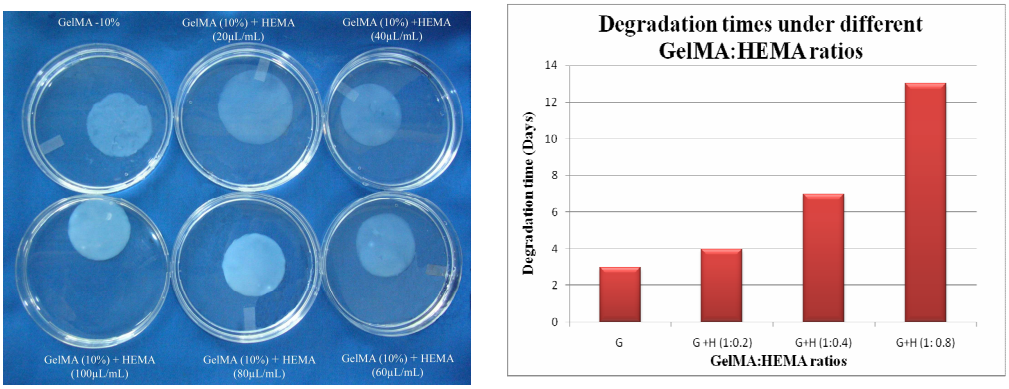


Fig.14: 10% GelMA and HEMA composite hydrogels in different proportions (left) and increased degradation time with increased HEMA in the composite hydrogel (right).

In order to improve the properties, the monomer content in the polymerization mixture was increased and hydrogel samples were prepared at different formulations, shown in fig.15 and as mentioned in Table 1.

Sl No.	Name	Components		
		GelMA (mg/mL)	HEMA (µL/mL)	PEG-SNP (µL/mL)
1	G-10%	100	-	-
2	$G_{100}H_{80}$	100	80	-
3	$G_{200}H_{80}$	200	153	-
4	$G_{300}H_{80}$	300	224	-
5	$G_{200}H_{80}Ag$	200	153	57.1
6	$G_{300}H_{80}Ag$	300	224	85.7

G: GelMA; H: HEMA; Ag: silver; SNP: silver nanoparticle.

Table 1: Formulations of hydrogel samples used in study.

In all the formulations used, the hydrogel samples had a 1:0.8 GelMA:HEMA monomer ratio though the total monomer concentration varied from 20 to 30% and all the gels were found to polymerize within 5 minutes. Incorporation of PEG protected silver ions to induce antimicrobial property also tended to improve the mechanical properties as evidenced by the Fig 20.

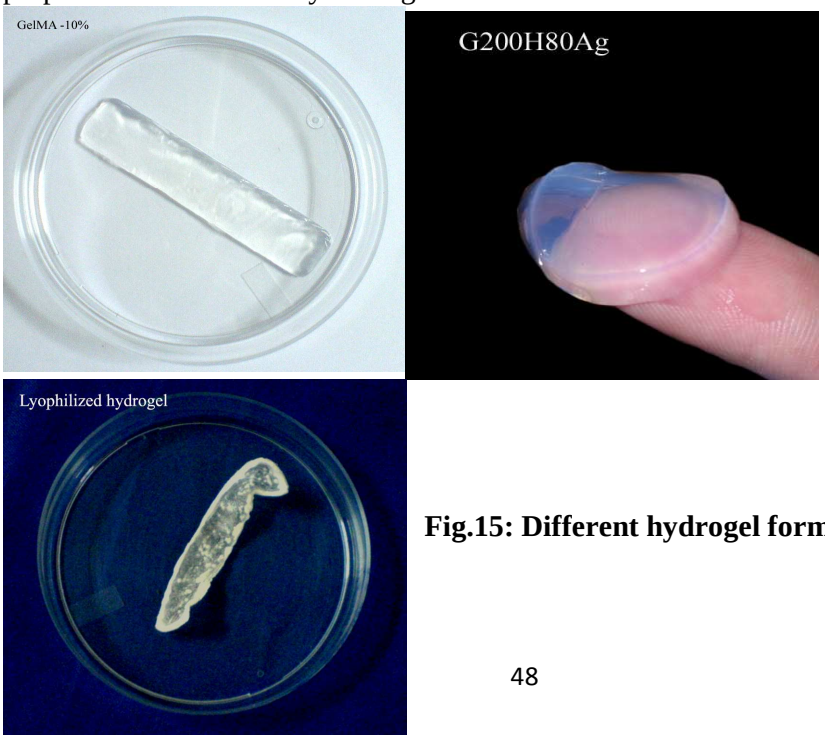


Fig.15: Different hydrogel formulations

The prepared hydrogel samples were characterized using spectroscopic techniques, swelling studies and mechanical testing. After analyzing the results, $G_{200}H_{80}Ag$ formulation was selected for biological characterization after evaluating its morphology, porosity and 3-dimensional micro architecture.

1. Chemical characterization:

Spectroscopic characterization of hydrogel samples was carried out using FT-IR spectroscopic technique as shown in fig.16. Conversion of monomers to hydrogel was monitored by disappearance of the unsaturated olefinic linkage present at 1636cm^{-1} using ATR-IR spectroscopy. While HEMA and GelMA formed primary chemical bonds by polymerizing through their respective double bonds by a redox free radical mechanism, the PEG protected Ag nanoparticles was bound only be secondary forces and by the interpenetrating network.

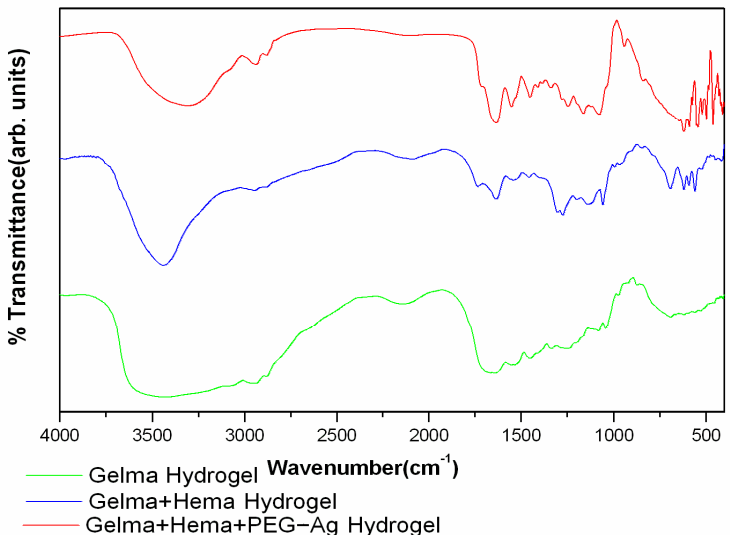


Fig.16: Comparison of FTIR Spectra of Hydrogel formulations.

2. Swelling Studies:

Hydrogel swelling studies were performed for the selected four formulations of hydrogel samples. The percentage swelling, swelling ratio and rehydration ratio of the four formulations were compared (Figs 17 & 18).

The percentage swelling (S%) represents the water absorbability of hydrogels. The GelMA hydrogels in combination with HEMA showed significant swelling characteristics as shown in Fig. 17. From the graph, it is evident that, the swelling percentage or water absorbability is almost similar for both the concentrations of monomers without PEG-SNPs and with SNPs. But, the addition of PEG protected silver nanoparticles is found to reduce the water intake property considerably as exhibited by the lower swelling % values which may be attributed to increased hydrophobic character of the gel after addition of the nanoparticles.

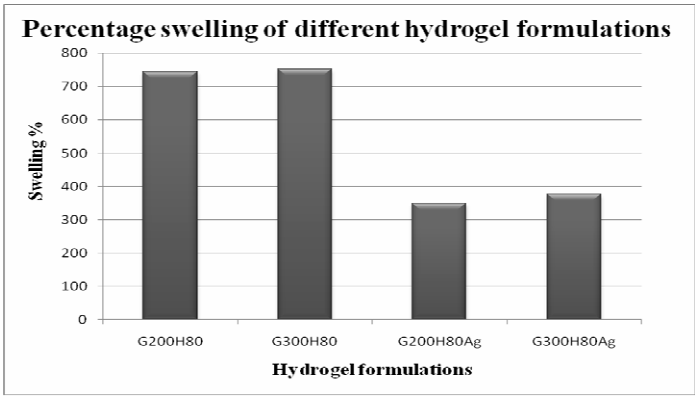


Fig.17: Percentage swelling of hydrogel

The swelling characteristics of hydrogels can greatly affect the pore size and diffusive and mechanical properties. The swelling ratio (initial swollen weight/dry weight) represents the amount of water that a hydrogel can absorb within initial 24hrs. Similar to percentage swelling, the swelling ratio also shown almost similar values for both the concentrations in two cases (with and with out PEG-SNP). Here also, a significant decrease in swelling ratio was observed by the incorporation of PEG-SNPs.

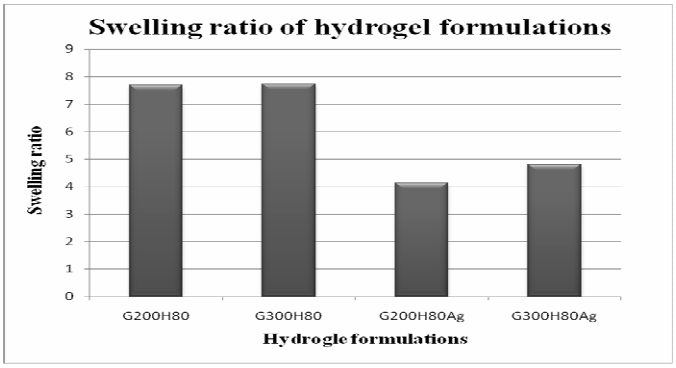


Fig.18: Swelling ratio of different hydrogel formulations

The rehydration of hydrogels is important as it can give an idea about the reproducibility of water absorption capacity and retention of properties like morphology, porosity, mass transport, and mechanical properties. The rehydration ratio was calculated as the ratio of rehydrated swollen weight to initial swollen weight after incubating the dried hydrogel samples in water for equilibrium swelling (48hrs). The rehydration ratios of the two monomer concentrations without incorporation of PEG-SNPs did not show much difference as evident from the Fig.19. But the incorporation of PEG protected SNPs to the $G_{200}H_{80}Ag$ samples shown maximum rehydration, whereas the other formulation with higher monomer concentraion ($G_{300}H_{80}Ag$) showed very poor rehydration.

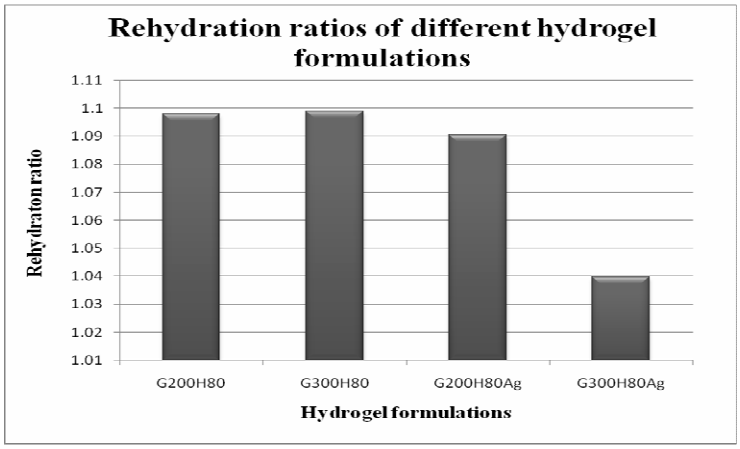


Fig.19: Rehydration ratio of hydrogel formulations

3. Mechanical characterization:

The mechanical properties of hydrogels are well documented to influence cellular behavior, function, and differentiation since they act as the extracellular matrix (ECM) in vitro. The tensile and compression studies of the hydrogel samples were caried out to determine the mechanical properties of the hydrogel samples. The 10% GelMA and $G_{100}H_{80}$ samples showed poor mechanical and handling properties and were found unsuitable for further studies in spite of the fact that HEMA is likely to contain traces of dimethacrylate monomer Ethylene glycol dimethacrylate (EDMA) which facilitates crosslinking. The other four formulations were tested for tensile and compression strength and the results are discussed below.

3.1. Tensile properties:

Tensile properties were characterized by measuring the modulus of elasticity (Young's modulus), Tensile strength (Stress at maximum load) and elongation (strain at maximum load). The modulus of elasticity (Youngs modulus) was found to increase with increasing monomer concentration and with the addition of PEG protected nano particles(Fig 19). An interesting aspect in this study has been the observation of increasing modulus and strength and decrease in strain values by addition of SNPs which facilitated easier handling and corroborated the application side. The formulation G200H80Ag was chosen for further studies because of its improved mechanical properties and rehydration property compared other three batches.

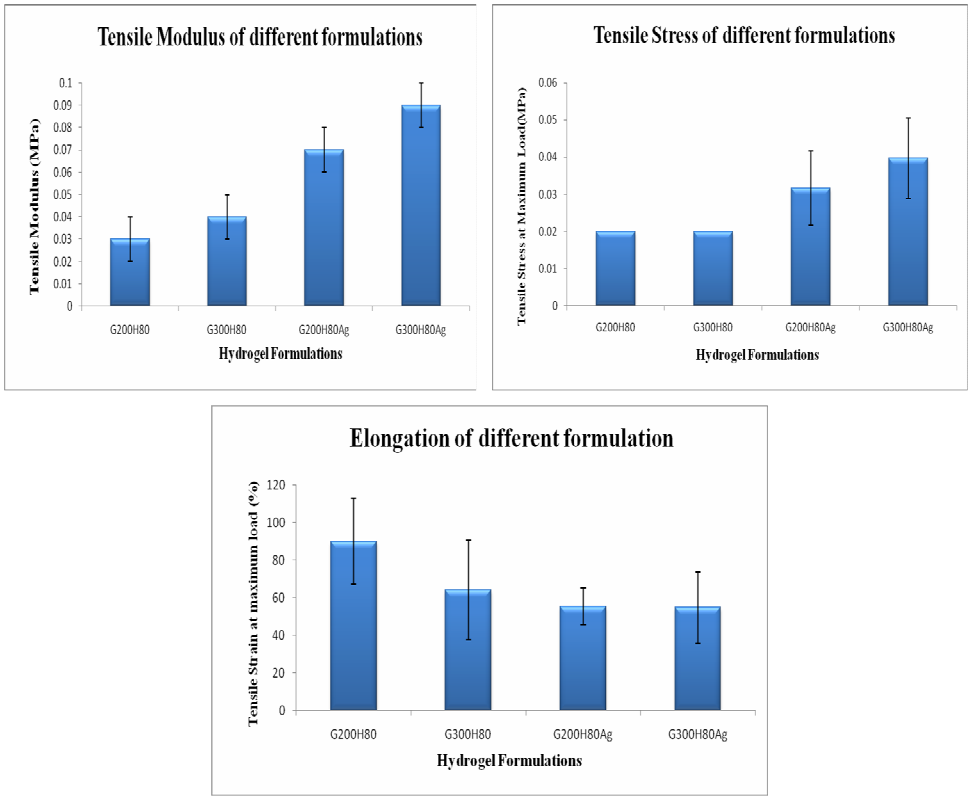


Fig. 20 : Tensile properties of Hydrogel formulations.

3.2. Compression properties:

Compression test gives an idea about the ability of the samples to retain the morphology after compression and that directly correlate with the ability of a dressing material to persist any compressive forces upon application, under use and while changing of the dressing. Also, the compressive strength is very important to support the growth and proliferation of cells in vitro and in vivo.

The compression test was performed for the samples with and without SNPs at the two monomer concentrations. 8mm diameter with 2mm thickness discs were used for the study and the results are given in Fig.21 below. By increasing the monomer concentration and by the addition of PEG-SNPs a gradual increase in the compressive modulus was observed.

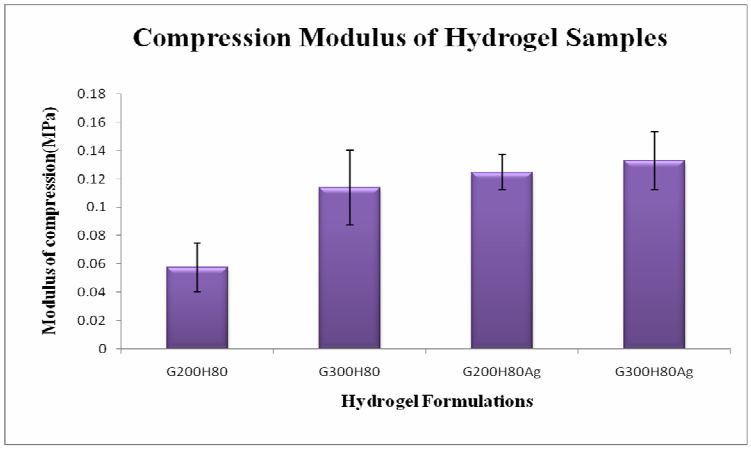


Fig. 21: Compression properties of hydrogel foumulations

From the above results, the $G_{200}H_{80}Ag$ formulation was selected for further studies such as 2D surface and 3D morphology, porosity and 3D micro architecture, silver leaching evaluation and biological characterization. The Gels were washed in PBS for three days prior to conditioning in medium before cell culture studies. The gels before and after washing in PBS were used for morphology and porosity evaluations.

Fig.22: $G_{200}H_{80}Ag$ sheet with 1mm thickness in PBS.

4. Surface Morphology of $G_{200}H_{80}Ag$:

Morphology of $G_{200}H_{80}Ag$ samples before and after washing in PBS were studied using Environmental scanning electron microscopy. The SEM images showed considerable amount of pores in the lyophilized samples where as the swollen samples were observed to possess smooth surface (Fig.23). The washed samples showed pores distributed throughout the hydrogel more uniformly when compared with the unwashed samples.

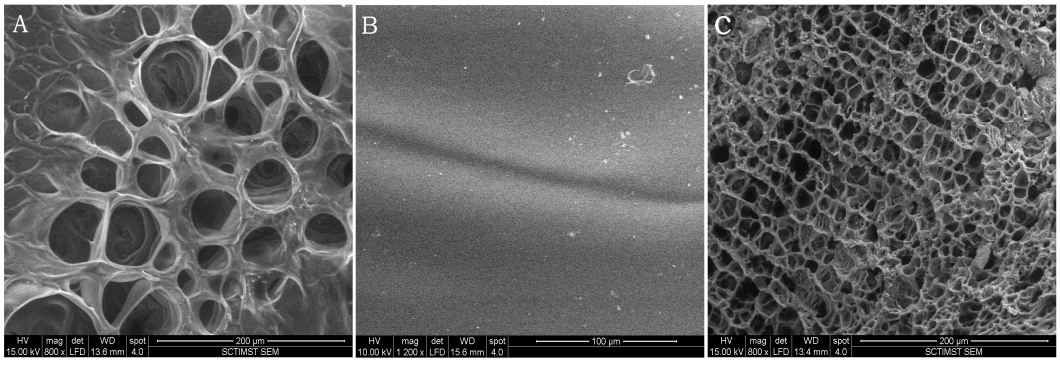


Fig.23: $G_{200}H_{80}Ag$ sample surface morphology before and after washing in PBS.

5. Porosity and 3D micro architecture:

Porosity and 3D microstructure of $G_{200}H_{80}Ag$ before and after PBS washing were analyzed using micro computed tomography. 3 D morphometric analysis was carried out for $G_{200}H_{80}Ag$ sample before and after washing with PBS and subsequent lyophilisation. Sample thickness distribution and porosity distribution (Figs. 24 & 25) were estimated. When the lyophilized unwashed sample is stored in PBS, the hydrogel swells up after absorbing water thereby expanding the network structure and this swollen polymer is lyophilized after deep freezing. Higher porosity percentage which was observed is likely due to the migration of PEG molecules or unreacted monomers from the hydrogel matrix.

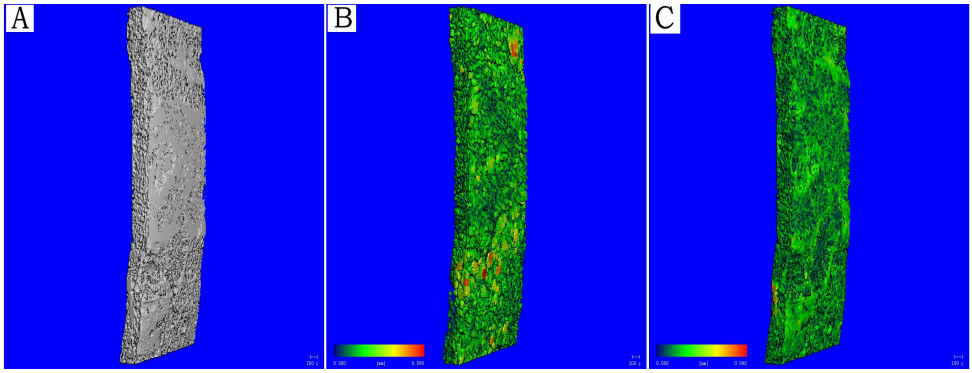


Fig.24: $G_{200}H_{80}Ag$ samples before washing: A) 3D morphology image. B) Pore size distribution image and C) Thickness distribution image.

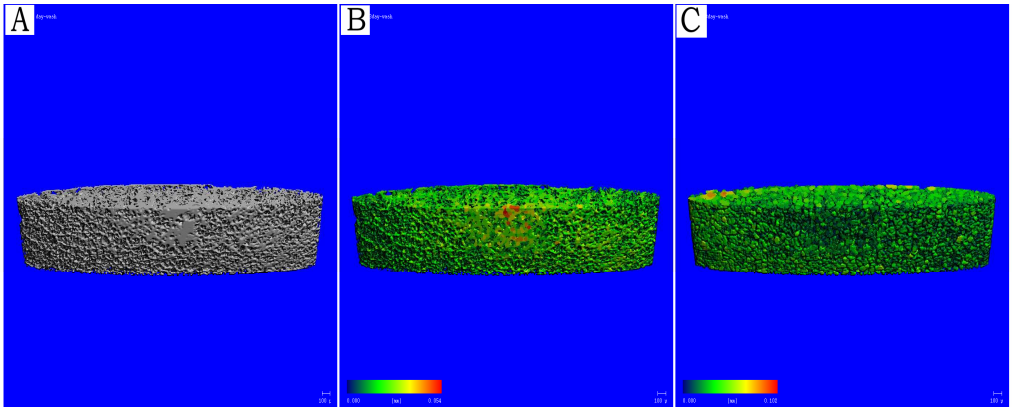


Fig. 25: $G_{200}H_{80}Ag$ samples after washing: A) 3D morphology image. B) Pore size distribution image and C) Thickness distribution image.

Micro CT parameter	G200H80Ag Before washing	G200H80Ag After washing
Total volume, mm^3	2.5124	2.2356
Scaffold Volume, mm^3	1.5173	0.8272
Scaffold Volume / Total Volume	0.6039	0.3700
Porosity Volume (%)	39.61	63.0

An examination of the porosity of the $G_{200}H_{80}Ag$ samples before and after PBS wash are obtained as 39.6% and 63% respectively. The average pore sizes of the two samples are found to be 25.3µ and 32.8µ for unwashed and washed samples.

Pore size distribution histogram obtained for the two samples are shown in Fig.26. The samples before washing had a maximum of 34.18% contribution by pore size 24µ and after washing, it had changed to a maximum of 30.397% contribution by pore size 30µ. From all these results it can be interpreted that, washing in PBS can increase the porosity of the hydrogel to a small extent, which may be due to the swelling phenomena and dissolution of PEG in PBS.

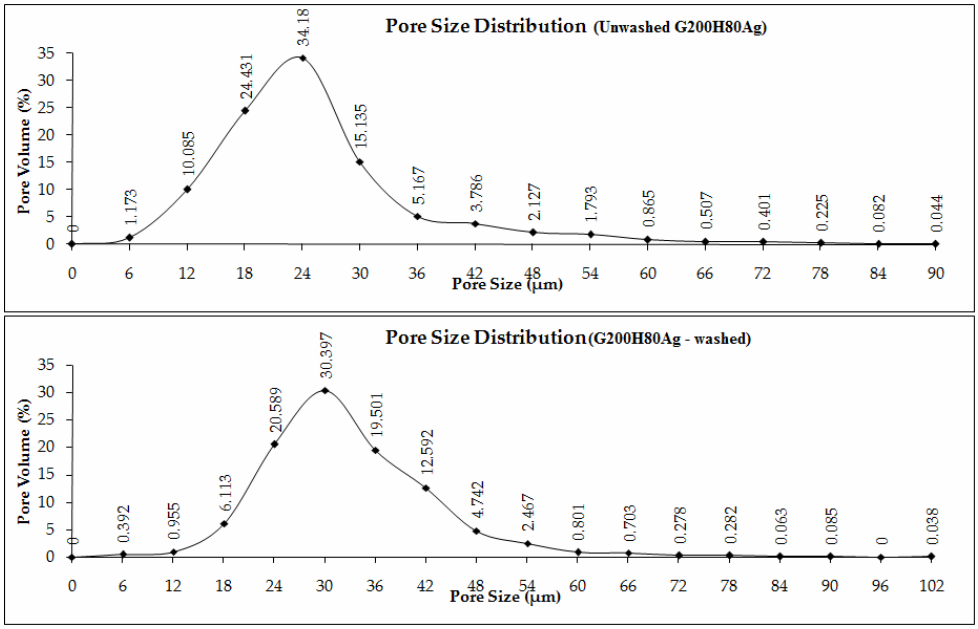


Fig.26: Pore size distribution in $G_{200}H_{80}Ag$ samples, before and after washing in PBS.

6. Silver release studies:

It has been reported that, the PEG protected silver nanoparticles can easily be washed out from the polymers by incubating in water (Ragaseema V, et al., 2010). Because of this property, any toxic effects of silver and its oxides up on long term contact to tissues or cells can be avoided. The leaching of silver from the $G_{200}H_{80}Ag$ samples after incubation with DI water were measured turbidometrically as % transmission of the silver chloride (at 480nm) formed upon reaction with 1N HCl which directly correlate to the concentration of silver in the solution. The leaching for three consecutive days were measured and shown in Fig 27.

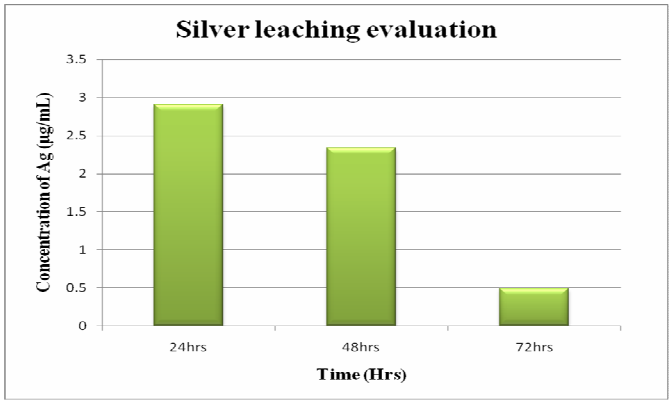


Fig.27: Silver release up on incubation in DI water.

By the silver leaching study, the total amount of silver released from G200H80Ag sample is 5.73µg. Thus, from the known concentrations of SNPs incorporated in hydrogel (127.08 µg), the residual SNP amount supposed to be present in the hydrogel after three days washing is about 121.35µg.

V. Cytocompatibility of $G_{200}H_{80}Ag$:

Cytocompatibilty of the hydrogel sample was tested using sheep dermal fibroblast cells isolated in the laboratory. The cells were maintained in DMEM F12 medium containing Foetal Bovine Serum (FBS, 5%) and antibiotic-antimycotic solution (1%). 1.5cm^2 discs of $G_{200}H_{80}Ag$ samples with 2mm thickness were used for cytocompatibility evaluation.

1. Cytocompatibility evaluation:

Compatibility to sheep dermal fibroblast cells was studied by direct contact method. The hydrogel discs were placed on the monolayer cultures after sterilization of disc using 70% ethanol with final wash for three hour in PBS. When cells were observed under phase contrast microscope, 24h after putting the disc in fibroblast monolayer, severe cytotoxicity with change in pH of the culture medium and rounded dead cell morphology were seen Fig. 28 (A). The pH change indicated release of residual monomers present in the hydrogel polymer. Therefore, prolonged washing of gel in PBS for 24h and preconditioning in serum free medium reduced the cytotoxicity to a moderate level as evident by the morphology evaluation Fig.28 (B). Washing of gel for 24 also prevented pH change of medium. To further improve cytocompatibility, gels were immersed in PBS with intermittent change of wash buffer for three days. When these thoroughly washed gels were placed on fibroblast monolayer, complete removal of cytotoxicity was obtained. These cultures were viewed under phase contrast microscopy and observed the normal spindle shaped morphology of the fibroblast cells, around the disc and underneath the hydrogel disc Fig. 29. It may be appreciated that due to the transparency of the material, cells that grow below the gel could be viewed easily. In all the three set of experiments described, washing with PBS was followed by an equilibration with culture medium for three days. For further culture and analysis, 3 days washing followed by three days equilibration was employed. There was no reduction in size of the gel after the entire process of washing, equilibration and culture for three days. No visible degradation was noted.

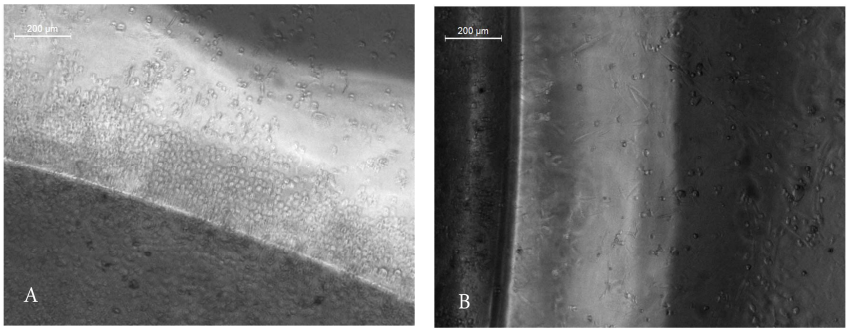


Fig. 28: Initial toxicity images: A) severe cytotoxicity before gel washing and B) moderate cytotoxicity after 24hr washing of gel in PBS

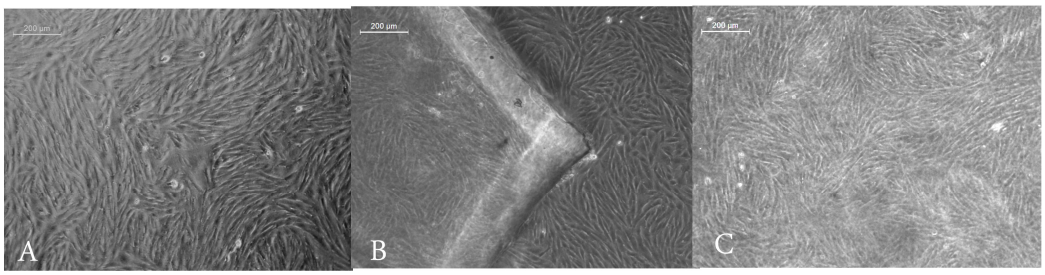


Fig. 29: Phase contrast micrographs showing cytocompatibility of hydrogels after washing and conditioning. A) Control cells grown on tissue culture dishes, B) Cells incubated with hydrogel for 72hrs and C) Spindle shaped fibroblast cells seen through the transparent hydrogel.

1.1. Viability assay:

The detailed analysis of the culture included estimation of cell death, cell survival and proliferation in presence of hydrogel. Preliminary evaluation was done using trypan blue dye exclusion assay by counting cells in Neubauer counting chamber within 5 min of treating the harvested cells with Trypan Blue. A graphical representation of data (average ± S.D.) from 3 independent experiments is shown in the Fig 30. From the graph the dead cell and live cell numbers are comparable in control (culture well without hydrogel) and test (with hydrogel). Therefore, this data indicates that no incompatibility may be assigned to the hydrogel.

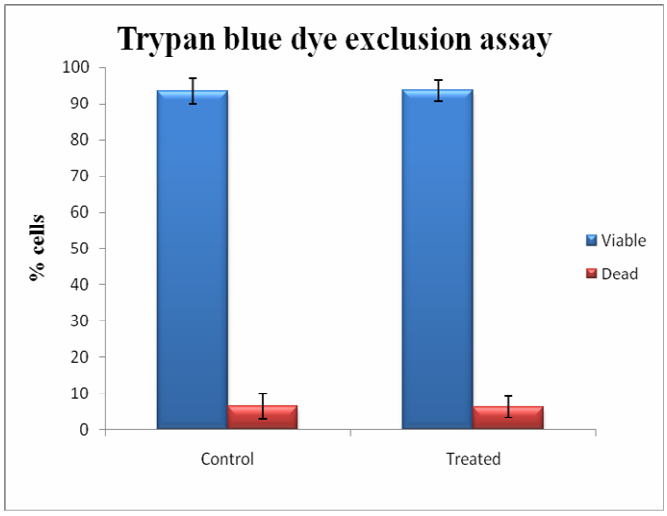


Fig.30: Graphical representation of viability assay

1.2. Flow cytometry for estimation of apoptosis:

In order to confirm the cell viability, apoptosis assay was performed using the vibrant apoptotic assay kit. It contained the FITC-conjugated Annexin which specifically bind to the phosphatidyl serine of the apoptotic cell membrane. The dead cells are differentiated in the assay, using Propidium iodide which intercalates the DNA of dead cells. The viable cells were counted as unstained cell population. The number of apoptotic cells, dead cells and viable cells were estmated with the help of using DIVA software associated with flow cytometer.

Three samples were studied for both control and the treated group (cells grown in presence of hydrogel). A graphical representation of compiled data (average ± S.D.) obtained from the flow cytometric examination of the stained cells are represented in Fig 31. It is evident from the graph that, the number of viable cells was similar and comparable for both the control and treated group. There was no significant increase in number of dead or apoptotic cells for the test group as compared to the control. The apoptosis assay correlated well with the results of trypan blue dye exclusion assay. This indicates the absence of induction of apoptosis in presence of hydrogel.

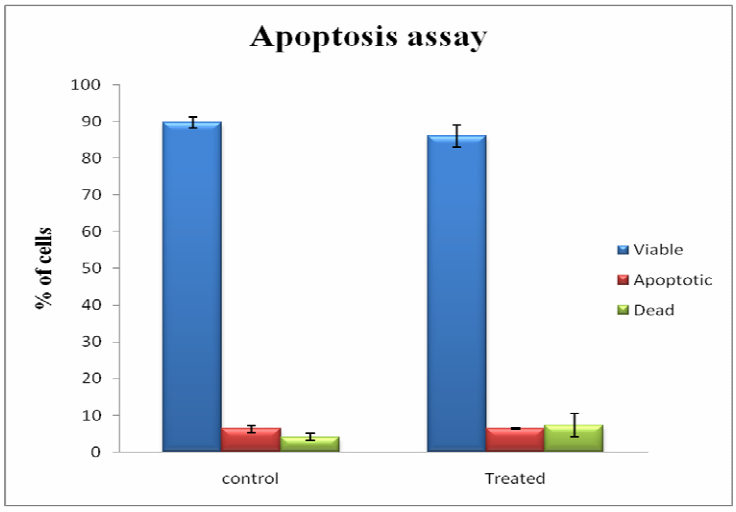


Fig. 31: Graphical representation of apoptosis assay

1.3. Cell proliferation assay:

Cell proliferation was analyzed by immunostaining for proliferating cell nuclear antigen (PCNA) to study if the hydrogel sample or its leachants exerts any influence on proliferation of dermal fibroblast cells. The PCNA stained cells, from both control and test were then analysed using flowcytometer. The histograms of flowcytometric data is shown in the figure. Unstained cells are used as negative control (Fig.32.A), the cells grown on polystyrene dishes without also analyzed as control (Fig.32.B). The proliferating cells were found in both the control and test. This data suggest that the hydrogel did not exert any negative influence on the proliferation of fibroblasts. It was found that the number of proliferating cells were higher in test sample (Fig.32.C), when compared with control (Fig.32.B). The increase in number of proliferating cells may be due to increased surface area because of hydrogel. It is possible that cells grew over the gel and could not be distinguished due to the gel transparency whether it grew above, below or on the sides of the gel. This can be confirmed only if analysis is done using environmental scanning electron microscope (ESEM).

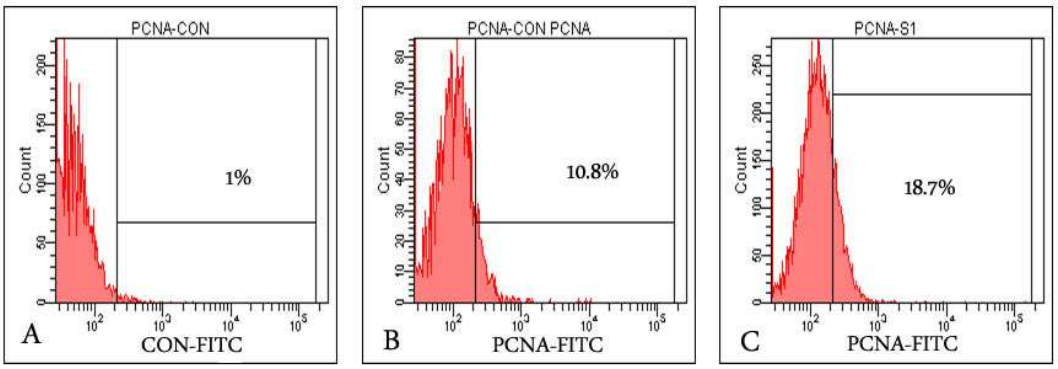


Fig. 32: Histograms of PCNA expression. A) Unstained control, B) Stained control cells grown on culture dishes and C) Cells in presence of hydrogel.

2. Two dimensional cell attachment:

To check the ability of $G_{200}H_{80}Ag$ samples for supporting the attachment and growth of fibroblasts, cells were seeded on to the gels. But, the gel placed on the

monolayer culture showed cell attachment through the edges which was confirmed by transferring the gel alone to a new culture environment supplemented with serum containing medium. Upon observation under environmental scanning electron microscope, well spread morphology of fibroblast cells could be observed on the edges of the disc Fig.33 the long term culture may allow cells to grow over the hydrogel (not studied).

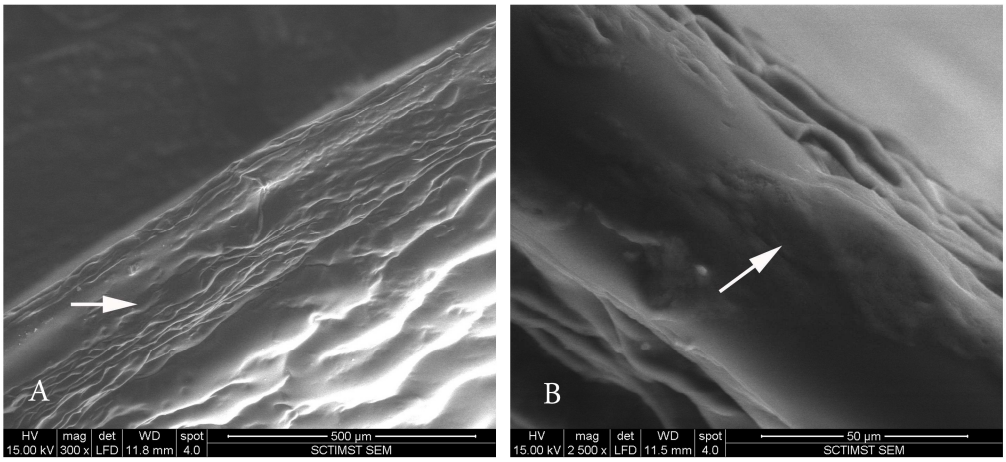


Fig. 33: ESEM images of fibroblast cell spreading on to edges of $G_{200}H_{80}Ag$ hydrogel at different magnifications.

3. Cell adhesion and spreading:

Fibroblast cell adhesion and spreading in presence of hydrogel sample was tested by actin staining. The control cells (grown in culture dish) and the cells grown in the vicinity of the hydrogels were found to possess ability to spread and grow and represented by the well spread network of actin filaments as shown in Fig.34.

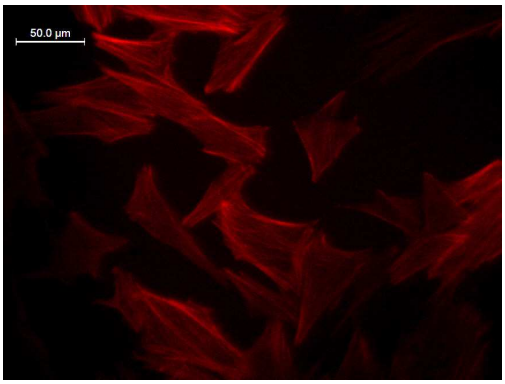


Fig. 34: Actin staining of cells grown below the disc.

In summary, fibroblast did not grow in presence of hydrogel immediately after casting the gel. Extensive washing step improved the cytocompatibility when fibroblasts were cultured in the presence of hydrogel. The gel supported cell attachment, spreading, and proliferation. The cells survived for a period of 3 days (till the end of present study) without any significant cell death. Cell attachment and spreading was demonstrated using phase contrast microscopy and ESEM. Survival of cells and ability to proliferate were demonstrated using flow cytometry.

CHAPTER 4

SUMMARY AND CONCLUSION

Gelatin methacrylate (GelMA) based hydrogels are widely used for analyzing the 2D and 3D cell interactions and as tissue engineering constructs in regenerative therapy. Microengineered GelMA hydrogels are reported to tune the *in vitro* 3D cell elongation and migration in different cell types like, fibroblasts, endothelial cells, cardiac muscle cells, adipocytes etc. In all the cases, photopolymerization technique has been used earlier for the polymerization of GelMA. Also, GelMA in combination with different hydrophilic and biocompatible materials have been reported as a tissue engineering construct to study the *in vitro* cell behavior. Redox polymerization of GelMA in combination with HEMA is a novel approach to produce composite tissue engineered constructs for wound healing application. In such a scenario, the incorporation of an antimicrobial agent can provide additional advantage in application level.

In present study, a GelMA hydrogel modified with HEMA and PEG protected silver nanoparticle was developed and characterized with the future aspect of wound healing dressing. The raw materials used for the synthesis of monomer GelMA: Gelatin Type A from porcine skin and Methacrylic anhydride were first characterized spectroscopically for the presence of characteristic peaks and the molecular weight of Gelatin was calculated viscometrically (2.51×10^5). The synthesis of GelMA monomer was standardized to get maximum yield of about 67.08% at 2% (v/v) MA concentration. The synthesized monomer, distilled HEMA, and PEG-SNPs were also characterized spectroscopically and compared with the polymer spectrum.

Different formulations of hydrogels were developed with different GelMA: HEMA ratios and the formulations with good handling, mechanical and degradation properties were selected for further studies. The selected formulations ($G_{200}H_{80}$, $G_{300}H_{80}$, $G_{200}H_{80}Ag$ and $G_{300}H_{80}Ag$) were characterized for swelling, mechanical and biological properties. The results obtained from swelling studies gave a clear idea of the water uptake capacities of different formulations and the comparative differences due to increased monomer concentrations and the addition of PEG-

SNPs. The mechanical properties of hydrogels were also found to be influenced by the increasing monomer concentration and SNP addition. From these two studies, the $G_{200}H_{80}Ag$ formulation was observed to possess good rehydration properties and tensile and compressive strengths compared to the other three formulations. Thus it was selected for further studies.

For biological evaluation of the hydrogels, removal of any eluting monomer or other toxic materials prior to use is critical. Thus continuous 3 day storage in PBS was carried out and the samples were used for 2D surface characterization (ESEM) and 3D morphometry and porosity evaluation (MicroCT) to analyze any significant changes. The leaching of silver nanoparticle is important in the case of a wound dressing material, as the long exposure to it may cause toxicity to the surrounding tissue.

The 2D surface morphology evaluation of the lyophilized samples using ESEM, revealed the presence of surface porosity which was in a uniform pattern in the gels stored in PBS compared to the ones which were kept in dry condition. Three dimensional morphometry and porosity evaluation using micro CT have shown that, the porosity and pore size distribution has a tendency to increase after storage in PBS. Silver leaching for three days was also measured to detect the amount of residual SNP present in the scaffold after storage, which is thought to provide the required antimicrobial property.

Biological characterization of the scaffold was carried out by cytocompatability and 2D cell attachment and spreading of sheep dermal fibroblast cells. The various cytocompatability tests revealed that the hydrogel scaffold developed ($G_{200}H_{80}Ag$) is non-cytotoxic after washing and has no adverse effects on the proliferation. It is also found not to induce apoptosis in fibroblasts. Upon incubation of hydrogel samples on the subconfluent monolayer culture, it did not produce any unfavorable effects on cell spreading as evident by actin elongation and network formation in cells lied underneath the gel. The 2D cell attachment was observed only through the edges of the hydrogel after incubation of about 3 days in the confluent monolayer culture and it could be visualized through the ESEM. This result suggests that, the disc (1.5cm diameter and 2mm thickness) used was of excess thickness and optimizing the thickness of samples may improve cell attachment and proliferation.

From the results obtained, it can be concluded that, the $G_{200}H_{80}Ag$ hydrogel formulation possess good mechanical and swelling properties, adequate porosity and cytocompatability which are comparable with that of a tissue engineering constructs for wound healing application. As a wound healing dressing, the hydrogel scaffold should support the growth and proliferation of fibroblast cells. The cell attachment and proliferation can be improved by the incorporation of additional biological agents like fibrin. The incorporation of fibrin can produce additional advantage for the wound healing dressing, as it enhances hemostasis and cell attachment. The hydrogel scaffold can also be modified by incorporation of growth factors that promote the cell proliferation and thus enhancing wound healing response and thus improving the quality of wound healing dressing. Considerable study needs to be done further to optimize the wound healing property of the novel hydrogel material developed as this study was only paving the way to explore the potential use of this excellent hydrogel for further skin tissue engineering applications

APPENDIX I – List of Reagents

1. Dulbecco's Phosphate buffered saline (DPBS):

    NaCl – 8g

    KCl – 0.2g

    KH2PO4 – 0.2g

    Na2HPO4 – 1.7g

    DI Water – 1000 mL

    pH – 7.4

2. Initiators:

    Sodium metabisulphite (3%) – 0.3g in 10mL DI water.

    Potassium persulfate (3%) – 0.3g in 10mL DI water.

3. Fibroblast culture medium (50mL):

    DMEM-F12 – 45mL

    Foetal Bovine Calf Serum (FBS) – 5mL

    Antibiotic-antimycotic solution - 500µL

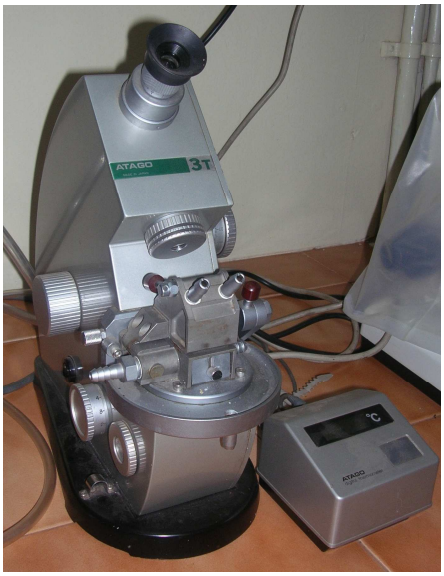
4. 3.7% Formaldehyde (10mL):

    Formaldehyde (37%) – 1mL

    DPBS – 9mL

APPENDIX II – Images of Equipments Used

FT Raman Spectrometer



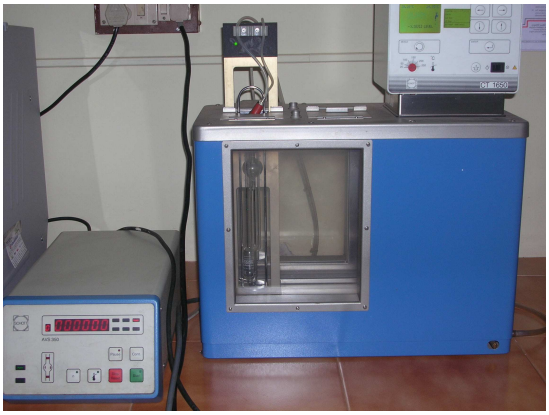
Abbe Refractometer

UV VIS Spectrometer

Universal Testing Machine

Micro Computed Tomography (Micro CT)

FTIR Spectrometer



Schott Automatic Viscosystem

Environmental Scanning Electron Microscope

Flow Cytometer (FACTS Aria)