# Modulation of Wound Microenvironment via Hydrogel Patch for Potential Application in Wound Management

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# Modulation of Wound Microenvironment via Hydrogel Patch for Potential Application in Wound Management

A thesis submitted in partial fulfilment of the requirements for the degree of

MS Biomedical Sciences and Engineering

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***Asad Abdullah Shahid***

*“Dedicated to dark humor. Thanks for existing.”*

# Abstract

Wound repair is a time taking process and the demands of perfect regeneration are never quite met. Many resistant bacterial species can cause infection through wounds. Severe infections can lead to anything from tissue morbidity to sepsis. Perfect wound regeneration demands that the local and systemic environment of the wound be optimized. We synthesized Chitosan-Gelatin based hydrogels with varying compositions and investigated their wound healing potential. Hydrogel variants were of two categories, Acetic Acid Variants and Polymer Ratio Variants. It was observed that with an increase in Acetic Acid, gelation time, free swell absorptive capacity, antimicrobial effects and pore sizes increased significantly. However, with an increase in Chitosan amount, gelation times and pore sizes decreased significantly whereas free swell absorptive capacity and antimicrobial effects decreased only on a minute scale. Scaffolds were shortlisted on their properties observed and were subject to degradation under PBS and 2% Ascorbic Acid. It was observed that by tweaking the Acetic Acid and Chitosan content the degradation rate could be tailored. Similarly, Ascorbic Acid was also found to increase the degradation rate to a great extent.

**Key Words:** Chitosan, Gelatin, Acetic Acid, Ascorbic Acid, Wound Healing, Burns, Wounds, Free Swell Absorptive Capacity, Degradation

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# List of Abbreviations

| | |
|---|---|
| CG | Chitosan/Gelatin |
| SS | Silver Sulfadiazine |
| ECM | Extracellular Matrix |
| WHO | World Health Organization |
| MRSA | Methicillin resistant Staphylococcus *aureus* |
| TGF-β1 | Transforming growth factor – Beta1 |
| IGFII receptor | Insulin-like Growth Factor-II Receptor |
| FDA | Food and Drug Administration |
| $NaHCO_3$ | Sodium bicarbonate |
| NaOH | Sodium hydroxide |
| USP | United States Pharmacopeia |
| SEM | Scanning Electron Microscopy |
| FTIR | Fourier Transform – Infra Red |
| PBS | Phosphate Buffer Saline |

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## *CHAPTER*

# *1 Introduction*

Hydrogels are three-dimensional structures that maintain important quantities of water depending on polymer networks. Most hydrogels in essence are biocompatible and biodegradable and can be used as scaffolds for various biomedical applications such as tissue creation, tissue engineering, drug delivery, etc. Nanofibrous formation and viscoelastic characteristics enable the hydrogels to imitate the extra cellular matrix. This hydrogel properties allows it great for scar dressings as it facilitates cell viability, migration, cellular proliferation and cellular differentiation (Slaughter et al., 2009).

Many resistant bacterial species can cause infection through wounds. Severe infections can lead to anything from tissue morbidity to sepsis. Also wound repair is a time taking process and the demands of perfect regeneration are never quite met (Carlson 2011).

Research and technological advances have shown that Staphylococcus *aureus* is the most prevalent source of skin and soft tissue disease in wounds including burns. (Miller et al., 2011). In terms of prevalence it is followed by Pseudomonas *aeruginosa* (Sydnor, 2011). These are resistant to most frequently used antibiotic drugs, and present research focuses on alternate routes and substances that demonstrate antimicrobial ability. A number of successful products have been revealed to date (Murphy et al., 2012).

Perfect tissue regeneration requires optimizing the wound's local and internal microenvironment. In order to provide a pathogen-free, safe and moist region for regeneration, many distinct products have been created to impact this wound microenvironment. In the wound healing cascade, new medicines are presently being used to substitute or increase different substrates (Murphy et al., 2012) Hydrogels are presently being studied for improved wound healing in conjunction with antimicrobial products. (Gulrez et al., 2011). Chitosan is an organic polymeric compound with a structure similar to that of cellulose, and vast applications (Bhattarai et al., 2010). It is currently being used for hydrogel formations for wound care. Certain vitamins are also reported to have positive effects on wound healing (Sinno et al., 2011). Depending upon their structure, some can potentially be used as linking molecules in hydrogels. Vitamin C, also known as L- Ascorbic acid has come out to be of particular interest because of its antioxidant properties and its role in early resolution of inflammation and collagen synthesis. Gelatin, is a denatured form of Collagen, a complex protein structure constituting the extracellular matrix. It has been vastly used in a number of wound care products because of its established effects in terms of wound healing. (Rodríguez et al., 2019).

*CHAPTER*

# *2 Literature Review*

## 2.1 Burn Wounds

Burns are injuries of the skin that occur mainly by contact with heated materials or chemicals. Less common causes include friction, radiation and electric contacts. Based on the cause of injury, burns are divided into thermal and non-thermal wounds. Thermal wounds include burns caused by flames, hot solids and liquids whereas non-thermal wounds include burns caused by radiation, friction, electric and chemical contacts. (Bailey et al., 2008)

World Health Organization (WHO) statistics show that globally around 1,80,000 deaths are due to burns. A major percentage of this number occurs in the middle-income and low-income countries. In the year 2004, WHO published that about 11 million people suffered burn wounds severe enough that they required medical attention. Considering some more local figures, in Pakistan, 17 percent children suffer temporary disability due to burn injuries and 18 percent suffer permanent disability. Majority of burns are caused by flames or hot substances while radiation and cold burns are the rarest. According to the Global Wound Dressings Market 2018-2022 report published in January 2014 by Cancer Research UK, each year, more than 305 million acute, traumatic and burn wounds are recorded and treated globally; more

than nine times the total number of people around the world living with cancer. In U.S., cost for wounds is conservatively estimated at $31.7 billion.

### 2.1.1 Degrees of burn wounds

Burns are categorized on the basis of the degree of damage that occurs. Burn categorization is summed up in table 2.1 below.

| **Type** | **Skin Layer Involved** | **Appearance** |
|---|---|---|
| First-degree (superficial) burns | Epidermis | Red, painful, dry, and with no blisters. Long-term tissue damage is rare |
| Second-degree (partial thickness) burns | Epidermis and part of the dermis | Red, blistered, and may be swollen and painful |
| Third-degree (full thickness) burns | Epidermis and dermis (may involve hypodermis) | The burn site may appear white or charred |
| Fourth degree burns | All the way to the underlying bones, muscles, and tendons | There is no sensation in the area since the nerve endings are destroyed |

### 2.1.2 Pathophysiology of burns

Skin being the main target organ of burn injuries is the source of many pathophysiological manifestations of burns (Dzhokic et al., 2008). Networks of inflammatory cascades are activated following a burn injury. Initially, protein molecules in the vicinity of the burned area are denatured which activates the complement system. Mast cells degranulate and coat the denatured proteins. Complement system attracts the neutrophils, degranulation of which increases the free radical quantity and releases proteases into the burned area. Mast cells then release tumor necrosis factor alpha (TNFα) which attracts inflammatory cells to the wound site. Release of inflammatory cytokines by the inflammatory cells alters vascular permeability, producing a net flow from intravascular space to the extravascular space. In large burns, this inflammatory phase is prolonged and reduces the cell-mediated immunity in individuals (Schwacha, 2003; Dreifke et al., 2015).

Hemostasis initiates the healing process with the formation of a blood clot over the wound site. This provides a temporary matrix that secretes a number of essential cytokines and growth factors to make the wound progress towards and through the different phases of healing, as well as provides stability to the underlying vasculature. This process of hemostasis occurs in the first 2 hours of wound formation (Scheraga and Laskowski, 1957; Blomback et al., 1978; Reinke and Sorg, 2012; Shah et al., 2012).

Infection and/or cellular damage initiates the inflammatory response that marks the first main phase of wound healing. It starts as different blood components get distributed around the wound site. These are mainly the blood plasma carrying various entities involved in the innate immune mechanisms of the body, including antibodies, macrophages and leukocytes etc. (Vinay et al., 2003; Majno and Joris,

2004). This inflammatory phase takes place in the next 1-2 days following wound formation and exhibits the textbook symptoms of inflammation including redness, swelling and pain etc. (Busti et al., 2005). Following hemostasis, the key cells of the inflammatory response such as neutrophils, macrophages, and lymphocytes assemble into the wound site and then release a number of cytokines and growth factors to initiate the inflammatory phase (Broughton et al., 2006; Campos et al., 2008; Gosain and DiPietro, 2004). Proliferative phase continues from 2 days after till around 2 weeks and sees the proliferation of cells and the laying out of a new matrix following the synthesis of collagen.

Remodeling is the final stage of wound healing that is accomplished up to a year after the original wound injury. This stage is defined by contraction of the wound surface. Fresh epithelium forms during this stage as the granulation tissue transforms into a mature scar. Capillary formation initiated in the proliferative phase is reduced as the capillaries combine to form larger vessels (MacKay and Miller, 2003; Sethi et al., 2002).

Severe wounds exhibiting an impaired mechanism of healing are frequently characterized by persistent injury and prolonged inflammation, excessive proteolysis and an impaired macrophage function and angiogenic response (Maruyama et al., 2007; Galiano et al., 2004). Due to inflammation extended over a period that are longer than normal, the large number of neutrophils inducted to the wound site will incur an excessive recruitment of inflammatory cells into the wound site. Neutrophils are known to be able to remove damaged tissue from the wound site's temporary matrix and deter microbial infection. However, on the other side, the unmanageable capacity of neutrophils to kill pathogens can also contribute to excessive protease manufacturing that can cause tissue damage at the wound site that is detrimental to

wound healing as it causes degradation of the extracellular matrix and growth factors. (Peplow, 2005). In addition, the inefficient proliferation of cells within the wound owing to ECM molecule degradation leads to impaired angiogenesis indicating further degradation of the wound bed and impaired healing. Preventing extended inflammation is therefore a target approach in serious wound treatment to overcome this problem.

The entire process is summarized in the figures below adapted from Dreifke et al., 2015.

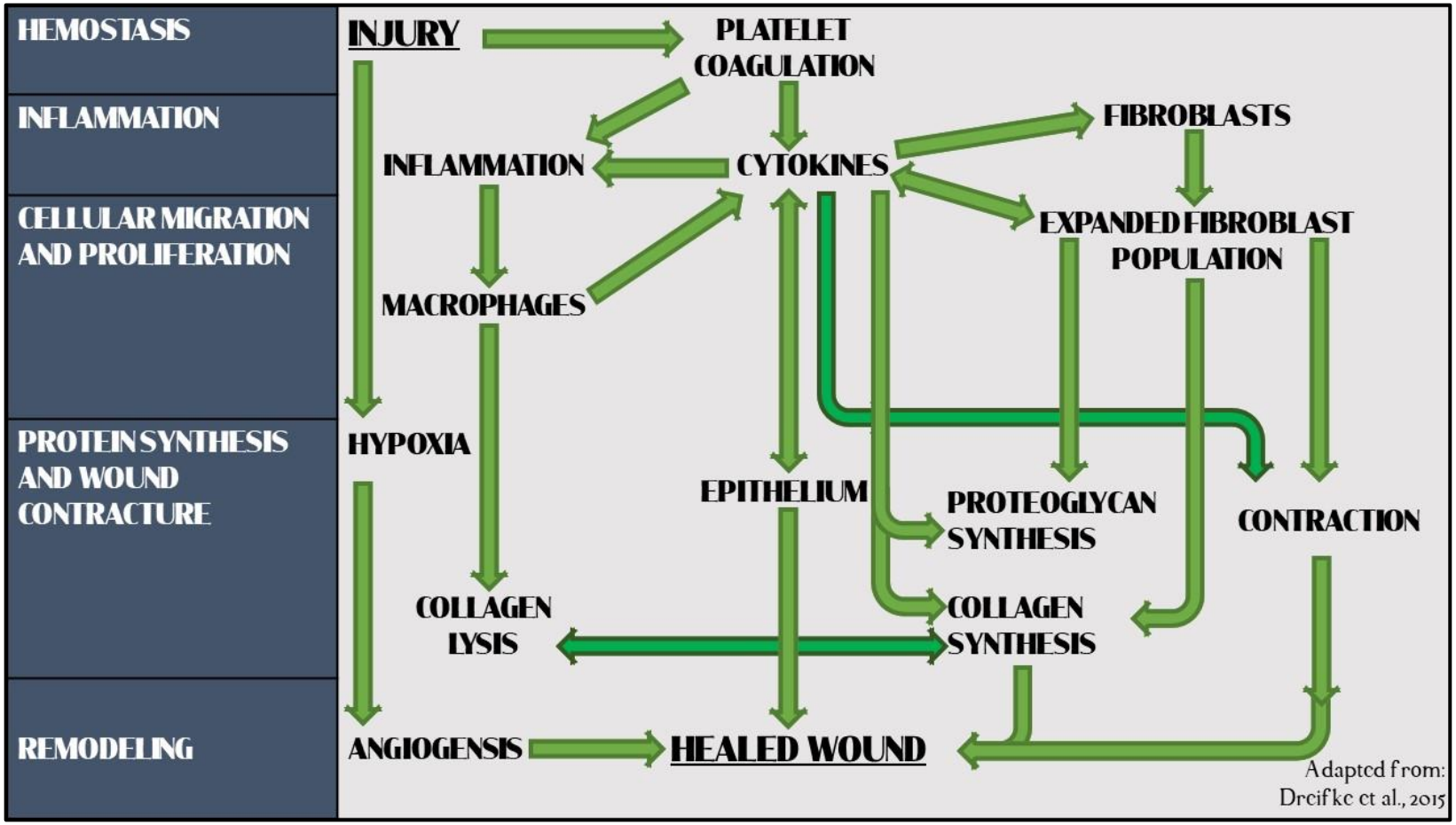


**Figure 2.1:** Pathway of Normal Wound Healing mechanism leading to a properly healed wound

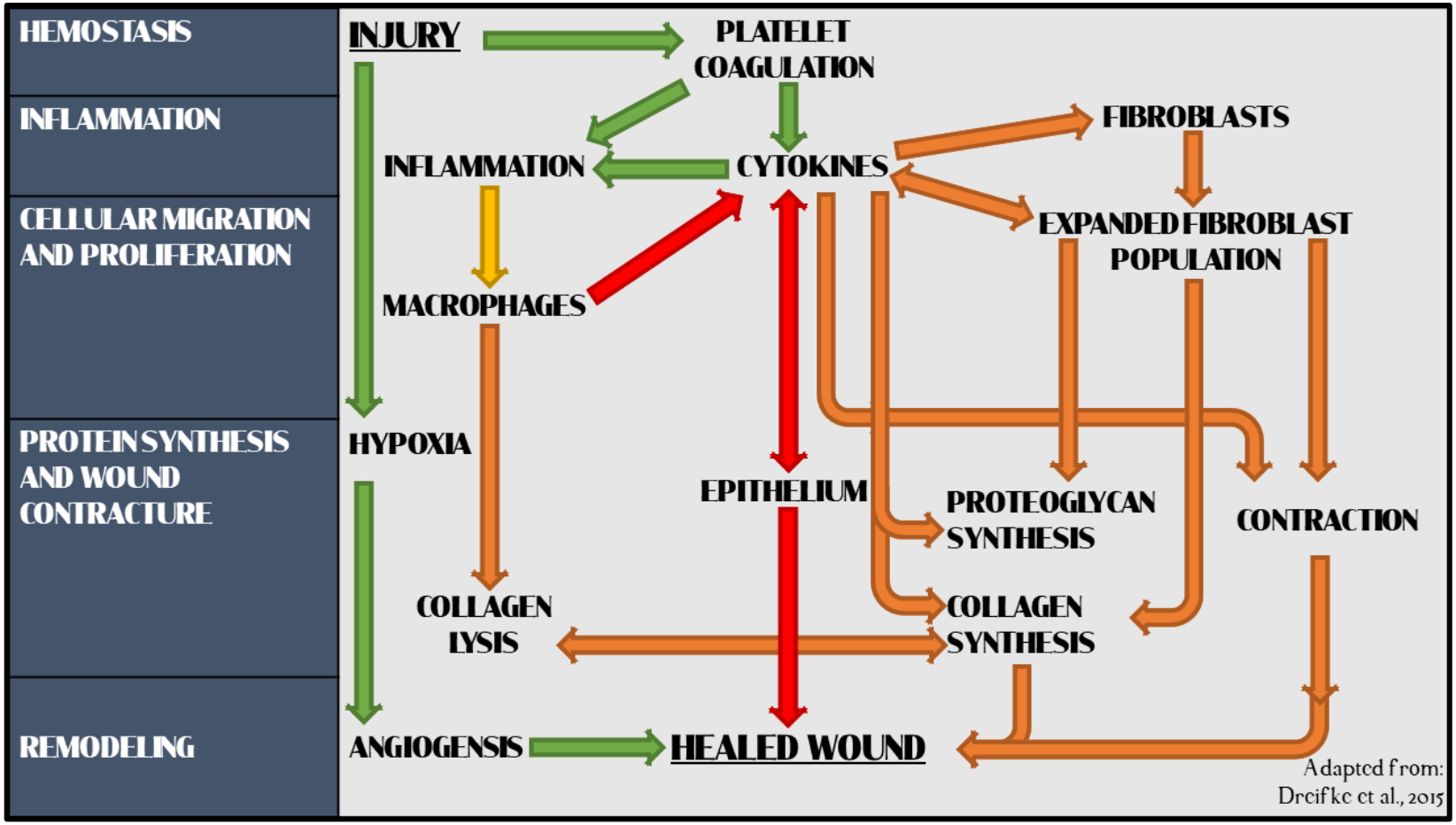


**Figure 2.2:** Pathway of an Impaired Wound Healing mechanism.

### 2.1.3 Healing process of wounds

All kinds of wounds be it burn or incision or even bone and tendon, follow the same mechanism and pathways for healing. Healing occurs in three main phases;

1. Inflammatory phase
2. Proliferative phase
3. Remodeling phase

Inflammatory phase was discussed above in the pathophysiology of burn wounds. After the inflammatory phase is over, the proliferative phase is mainly characterized by the fibroblast activity, angiogenesis and finally, the growth of new epithelial tissue on the surface of the wound. Proliferative phase of healing typically lasts from day 3 of the injury till 2-3 weeks depending on the extent of injury. During this phase, vitamin C is greatly required for collagen synthesis. The collagen synthesized at first is type-III collagen which is deposited in a random fashion with no alignment.

After the adequate deposition of collagen molecules and fibroblast proliferation, the wound enters the third and final phase of wound healing, the remodeling phase. Collagen type-III molecules are replaced by type-I and are realigned. Another process occurring throughout these three phases is the pH variability at the wound site with time. After the wound occurs, the pH at the site is alkaline, as the wound heals the pH progresses gradually from alkaline to neutral and neutral to acidic pH (Tsukada et al., 1992). It was indicated in a study by Leveen et al., 1973, wounds with a higher pH had lower rates of recovery.

### 2.1.4 Treatment of burn wounds

Management of burn wounds is the same regardless the degree or the total surface area of the burn. Burns are first cleaned, rid of dead or necrotic tissue, assessed for depth and then protected with an appropriate dressing to manage the wound. Superficial and partial thickness burns heal regardless of the dressing, however as the depth of the wound increases the chances of it being infected increases. Thus, with increasing depths, choice of treatment options and dressings can be the deciding factor between poor scarring and minimal scarring. Deep burn wounds are treated either with topical solutions or dressings incorporating those topical solutions. The most common topical solutions are mentioned in table 2.2 as follows;

| **Topical Agents** | **Effect** |
|---|---|
| Polyfax (Polymyxin B, Bacitracin) | Antibacterial |
| 1% Silver sulfadiazine cream | Broad-spectrum antibiotic, particularly effective against MRSA and Pseudomonas *aeruginosa* |
| 0.5% Silver nitrate solution | Less effective compared to Silver sulfadiazine, good antimicrobial activity against Pseudomonas *aeruginosa*, produces black staining |
| Silver sulfadiazine and cerium nitrate | Broad-spectrum antibiotic, boosts cell-mediated immunity. |

While the application of topical creams/agents and infused dressings is sufficient for superficial wounds and certain mixed depth wounds, they only serve to control the environment and protect against bacterial colonization in partial-thickness and full-thickness burn wounds. The full-scale treatment of 2nd and 3rd degree wounds at present requires grafting and surgery.

### 2.1.5 Scarring in wounds

In medical and surgical literature, it is stressed that the doctor must always inform the patient that a scar may be formed, either planned or accidental, regardless of how the procedure is done. Scars are almost certain after many human injuries such as burns, accidents, deep cuts, untidy wounds and even in the case of surgical incisions. Type of scar depends on the injury and the treatment option. If the wound is made to heal by primary intention, that is the wound is cleaned and closed earlier on, best scarring is achieved. Worst scar is achieved in healing secondary intention where the wound is left open and let to heal by itself. While this approach is preferred in small injuries like a small cut, this approach may potentially lead to disastrous scarring results.

Looking deep into healing by primary intention, it is observed that many a times the only scars left are those of sutures. Use of fine monofilament sutures reduces scarring as they produce less tension. Certain subcuticular suturing techniques have also been studied that minimize scars to being almost non-existent or non-observable.

But in the case of burns, multiple layers of the skin and a great amount of surface area are involved that make preventing scar formation a rather unachievable and unrealistic goal. Paul Martin in 1997, linked certain researches carried out by himself and others, regarding scarring. Eckes et al., 1988 observed that a week into wound healing, the initial clot is infiltrated completely by activated fibroblast which are

tasked with the deposition of collagen type 3 matrix. A ratio of these fibroblasts under the synergistic effect of transforming growth factor – beta1 (TGF-β1) (Desmoulière et al., 1993) and the mechanical stimulus provided by the contractile forces, transform into myofibroblasts, which lay down α-smooth muscle actin instead of type 3 collagen to generate stronger contractile forces (Desmoulière et al., 1993, Grinnell, 1994).

Martin et al., 1992 and Estes et al., 1994 observed that while activated fibroblasts transform into myofibroblasts in adults and is rendered normal process, same does not occur in the case of fetus. During the different fetal stages, the fetus receives wounds within the womb, but it is not until the later fetal stage that a scar remains. Fibroblasts do not convert into myofibroblasts and there is no response leading to scaring. Whitby and Ferguson, 1991, report that TGF-β1 expression is very low in embryos. Later on, research showed that in adults its amount was high during the healing process and even beyond the healing period (Roberts et al., 1996) and that high amounts of TGF-β1 were also implicated with pathogenic fibrotic conditions and scarring as well (Border and Noble, 1994). The increase in TGF-β1 was found to be directly related to the expression of mannose-6-phosphate IGFII receptor, and that application of mannose-6-phosphate could stop this pathway and prevent scarring (McCallion and Ferguson, 1997), as was proved in future researches along with the observed capability of mannose-6-phosphate in nerve regeneration (Harding et al., 2014).

## 2.2 Current research trends in skin wound healing

Currently, plastics surgery is the go-to remedy to reduce scars, utilizing autologous skin grafts to cover wounds, it is however hindered by the limited availability of autologous skin. Even while sometimes having a lot of sites to choose from for

autologous grafts, the results do not qualify for being referred to as true tissue regeneration. Much work is currently being carried on developing alternatives to promote wound healing. The most promising avenue is tissue engineering scaffolds and regenerative medicine. 3-dimensional scaffolds are made from either synthetic or natural polymers or a combination of the two. When positioned over or embedded into the wound site, the scaffold enhances the wound healing process by preventing infection, dehydration, toxins, reinforces the wound site with essential components such as certain drugs or important chemical moieties. In some high-end researches being carried out, live cultured autologous cells are encapsulated within these scaffolds to enhance the process even further.

There is nonetheless still a lot of research to be carried out for optimal regeneration via tissue engineering techniques. Replicating the microenvironment to optimize cellular processes is one of the biggest challenges (Nicholas et al., 2016). There is a plethora of growth factors and cytokines associated with regeneration and the exact timing and pathways still need a lot of studies to be conducted. Although individual researches have been carried out for individual Interleukins (Akdis et al., 2011) and growth factors (Martin 1997, Werner 1998), there is yet to be a research conducted that could explain the synergistic effects of all the factors.

Most of the studies being done focus on the material components used to synthesize the scaffolds. These include synthetic and natural polymers, crosslinkers and other miscellaneous chemical and biological agents to help enhance the wound regeneration. Wide variety of these materials are being studied for the pore size they achieve, their mechanical and degradation properties as well as the biological characteristics. Polymers such as collagens, chitosan, elastin, gelatin, polyvinyl alcohol,

polylactic acid and polyethylene glycol, crosslinkers such as genepin and glutaraldehyde and chemical agents such as silver nanoparticles and ascorbic acid are amongst the most studied materials for scaffolds.

However, so far none of the researched scaffolds have reached the clinical stage (Tenenhaus and Rennekampff, 2016). To sum it all up, no optimized scaffold for skin has been found as of yet. Work is continuously being done on different materials, different combinations and different techniques to find a solution. With increasing knowledge about cellular interaction and cell signaling as well as the pathophysiological requirements of specific wound conditions, tissue engineering holds great perspectives for the future to enhance wound healing.

Apart from scaffolds, different dressings are now available in the commercial sector which improve the healing time and reduce scar formation. One such example is Tegaderm by 3M.

## 2.3 Hydrogels

There is no standard way of defining the term 'Hydrogels'. They are high-water content materials prepared from cross-linked polymers that are able to provide sustained, local delivery of a variety of therapeutic agents. Hydrogels are a kind of three-dimensional scaffolds with the ability to retain 90-99% water (Gibas et al., 2010). Their ability to retain large amounts of water and mimic the extra cellular matrix (ECM) makes them a good substitution for soft tissues as it cues the much necessary cell proliferation and differentiation (Mohan and Nair, 2005).

### 2.3.1 Hydrogel Types

Hydrogels can be differentiated into types on two bases; the method of cross-linking, and the type of material used.

**i. Types based on method of cross-linking**

Hydrogels can be divided into Chemical (Stable, Permanent) and Physical (Reversible, Degradable) on this basis. Chemical hydrogels, as apparent by the name, are formed due to formation of covalent bonds between the polymer and the cross-linking molecule. Physical gels, are formed by weak physical interactions such as molecular entanglement, hydrogen bonding and hydrophobic interactions (Hennink & Nostrum, 2002).

**ii. Types based on material**

Based on material, hydrogels can either be synthetic or natural.

**a. Synthetic Hydrogels**

Hydrogels constituting of Polyesters, Polyethers and similar structures are classified as synthetic hydrogels. Synthetic hydrogels are more controllable and reproducible as compared to natural hydrogels but lack the capacity to bind to the matrix forming cells. They are preferred over natural hydrogels for encapsulation and drug deliver purposes (Lee and Mooney, 2001).

**b. Natural Hydrogels**

Natural Hydrogels consist of substances found in nature such as peptides, proteins and polymeric compounds such as polysaccharides and their derivatives. These are water soluble in most cases and are capable of having a higher degree of intermolecular interactions (Malafaya et al., 2007). This higher degree of

intermolecular interactions gives natural hydrogels the ability to assemble into three-dimensional structures. Also, these polymers have the capacity to bind to matrix forming cells and are hence useful as scaffolds (Lee and Mooney, 2001).

## 2.4 Hydrogels as Scaffolds

Hydrogels are an attractive scaffolding material because their characteristics can be tailored according to the need. For the ability to be substituted for a natural tissue, hydrogels need to be compatible in terms of both, mechanical and physical properties. These properties are greatly influenced by the properties of the materials that make up the gel, their type and the concentration of the cross-linker molecules and physiological environment as well. Based on these properties, hydrogels can display excellent solid-like and liquid-like characteristics (Li, 2010).

## 2.5 Chitosan

Chitosan, a semi-synthetic polymer, is the deacetylated product of chitin, a compound found in fungal cell walls and crustacean exoskeletons. In the area of biomedicine, chitosan has proved to be a hemostatic agent, allowing it to rapidly clot blood (Neuffer et al., 2004). It has been approved in 2010 in the United States and Europe to be used in bandages and other hemostatic agents. Early trials had shown that chitosan bandages in swine models reduced blood loss and produced a 100% survival rate in lethal arterial wounds (Pusateri et al., 2003; Kheirabadi et al., 2005; Ward et al 2007).

### 2.5.1 Chitosan-based Hydrogels

Use of chitosan for hydrogels is being highly focused upon due to the polymer's biocompatibility, low toxicity, and biodegradability (Bhattarai et al., 2010) and its promising antimicrobial characteristics (Ming et al., 2010). Various formulations of

Chitosan-based hydrogels have been employed for their wound healing applications. Chitosan-based hydrogels induce wound-healing properties and reduces scarring (Azad et al., 2004). The presence of chito-oligomers at the wound site induce better collagen fibril incorporation into the extracellular matrix (Shigemasa et al., 1996).

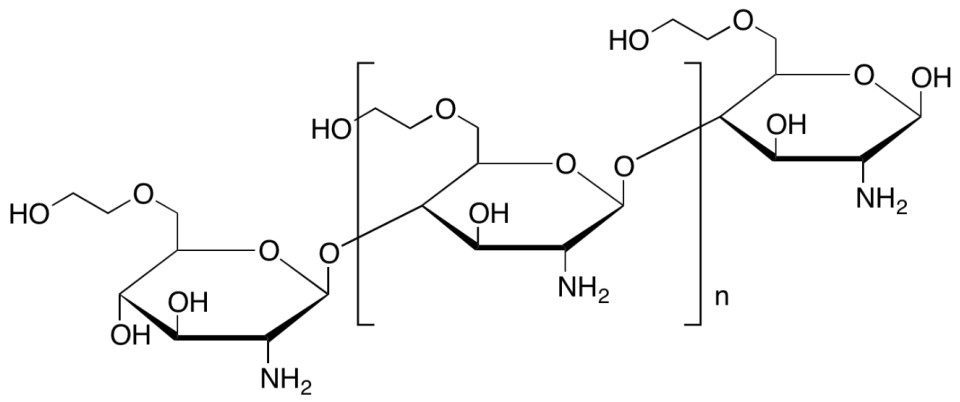


**Figure 2.3: Chemical structure of Chitosan**. *The amino groups at the bottom of every ring are responsible for the covalent bonds in hydrogels.*

## 2.6 Gelatin

Gelatin is a multifunctional ingredient used as a gelling agent, stabilizer, thickener, emulsifier in food, pharmaceuticals, cosmetics, and a film forming agent. Gelatin offers distinctive benefits over carbohydrate-based gelling agents as a thermoreversible hydrocolloid with a tiny gap between its melting and gelling temperatures. Gelatin is made primarily from the skin of porcine and hides and bones of cattle. Some alternative raw materials have lately attracted scientists and industry attention. A category of material that has been studied extensively as a source for gelatin manufacturing is fish skins from a range of fish species. Fish skins have noticeable potential to produce high-quality gelatin with distinct melting and gelling temperatures over a much broader spectrum than mammalian gelatins, but they have significantly high gel strength and viscosity levels (Boran and Regenstein, 2010). The

FDA usually recognizes that gelatin is a secure food or food ingredient (GRAS). Interestingly, horse gelatin has been researched and it has been discovered that gelatin affects the homeostasis of the amino acids needed for cartilage synthesis. A growing amount of novel collagen and gelatin apps have been discovered (Coenen et al., 2006; Liu et al., 2015).

In drug delivery and tissue engineering, gelatin-based hydrogels are used because they can support cell adhesion and proliferation. Furthermore, due to their appealing fluid absorbance characteristics, these hydrogels can be used as wound dressings (Jaipan et al., 2017). Gelatin is a denatured form of collagen, one of the cartilage and bone components and also acts as a sort of hemostatic agent (Palm and Altman, 2008). In relation to their well-established significance as a source of dietary protein, products derived from gelatin display multiple biological activities on cells and the extracellular matrix via the respective post-ingestion peptides derived from food (de Almeida et al., 2010; Liu et al., 2015).

Gelatin or collagen chains kept in solution can be cross-linked covalently to generate structures that can expand in the presence of aqueous solutions, creating what are called gelatin hydrogels. Hydrogels, defined by their hydrophilicity and water insolubility, can swell into an equilibrium volume while preserving their shape. The chemical crosslinkers used can be either tiny bifunctional molecules or polyfunctional macromolecules, such as glutaraldehyde (Gómez-Guillén et al., 2011).

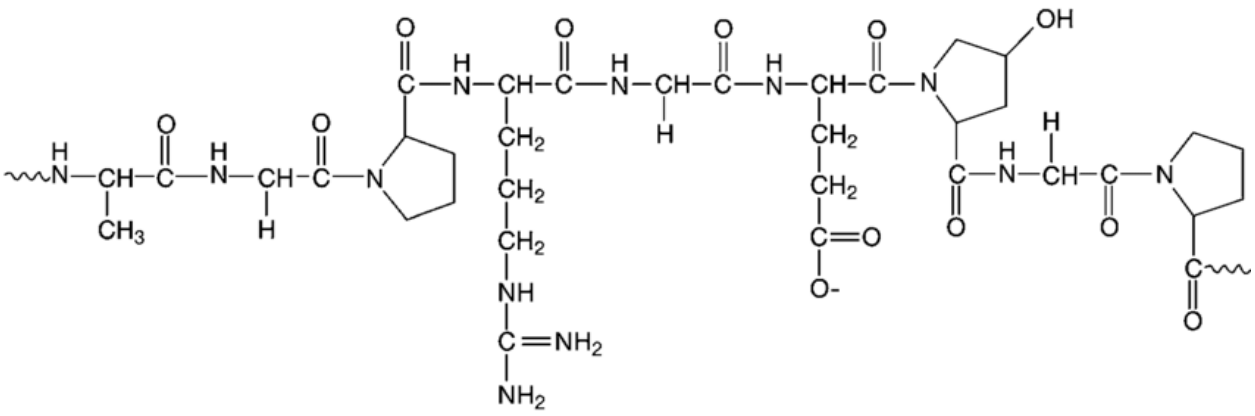

**Figure 2.4: Chemical Structure of Gelatin.** *The amide and carboxylic groups at the end along with hydrogen bonding in between the molecules are responsible for natural gelation.*

## 2.7 Acetic Acid

Acetic acid is a result of ethanol oxidation and destructive wood distillation. It is used as a counterirritant locally, sometimes internally, and also as a reagent. Acetic acid has been commonly used in 1% and 5% solutions in an effort to decrease wound pH. However, it has been noted that acetic acid reduces pH for only one hour after it returns to pretreatment concentrations (Leveen et al., 1978). Reducing the pH to a more acidic setting decreases the toxicity of bacterial end products such as ammonia, increases deterioration of defective collagen in the wound area, promotes angiogenesis, increases macrophage and fibroblast activity, and regulates enzyme activity (Thomas, 1990).

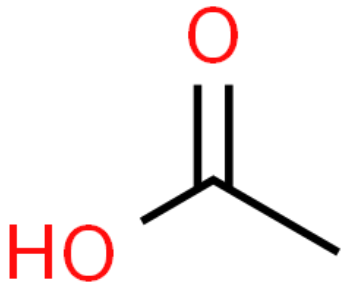


**Figure 2.5: Chemical Structure of Acetic Acid.**

## 2.8 L-Ascorbic Acid in Wound Healing

Many vitamins have been speculated to have effects on wound healing (Sinno et al., 2011). One such vitamin is Ascorbic acid (Vitamin C) (Mohammed et al., 2015). The body's rapid pace of production of collagen associated structures is owed to Vitamin C (Carr and Frei, 1999). Vitamin C is known to have an antioxidant nature (MacKay and Miller, 2003), and play essential roles in the synthesis of carnitine which is involved in fat metabolism, facilitation of iron absorption and collagen formation (Bendich et al., 1986). The wound healing effects of Vitamin C are primarily due the assistance it provides as a co-factor molecule in the synthesis of collagen. Vitamin C supplementation has been proved to enhance collagen synthesis in subjects suffering from Ehlers-Danlos syndrome (Ringsdorf and Cheraskin, 1982). Vitamin C has also been reported to have a pivotal role in the synthesis of mucopoly-saccharides that consist the extracellular matrix of the cell and help in proliferation, deficiency of which leads to impaired healing (Penney and Balfour, 1949).

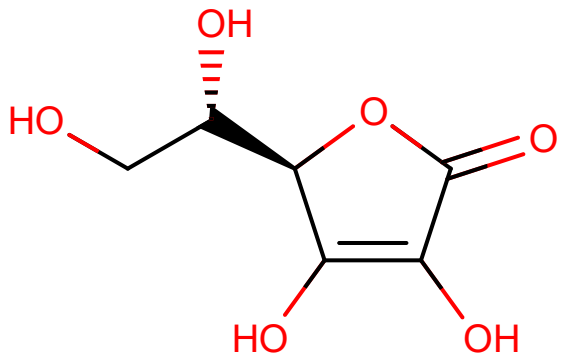


**Figure 2.6: Chemical Structure of Ascorbic Acid.**

## 2.9 Current Progress in Chitosan Gelatin Hydrogels

By means of electrostatic, hydrophobic and hydrogen bonding interactions, chitosan and gelatin polymer can produce hydrogels using appropriate polymer ratio, temperature, pH and ionic strength (Yin et al., 2005). Chang et al., manufactured chitosan / gelatin hydrogels by thermal gelation with a weak base like $NaHCO_3$ and increasing temperature at varying polymer concentrations. Due to the slight alkalinity of $NaHCO_3$ and the availability of gelatin, the system remained in solution between pH 6.6–7.3 and $4^0C$ but transformed into a hydrogel when heated at physiological temperature ($37^0C$) (Chang et al., 2009).

In order to enhance their effectiveness in biomedical apps, gelatin-chitosan hydrogels were altered or strengthened by certain physical cross-linking and chemical cross-linking techniques (Rivero et al., 2009).

Shen et al. have reported chitosan hydrogels fabricated by precipitation of the reported hydrogel via soaking in aqueous NaOH. The resultant structure was porous,

interconnected, robust and above all biocompatible and degradable. Gelatin was added in this system to improve their physiochemical and biological properties. Improvement in biological properties was due to the high absorptivity and very low or lack of antigenicity and therefore a lack of immune response to gelatin (Shen et al., 2015; Pei et al., 2015).

The hydrogels based on gelatin had a rough surface, lower pore size (100 μm), and higher compressive module (Young's module, 3.2 MPa), which favored chondrocyte adhesion, migration, and proliferation (Shen et al., 2015). Thein-Han et al., reported similar behavior using embryonic stem cells from buffalo (Thein-Han et al., 2009). Thein-Han stated that chitosan / gelatin scaffold degradation was higher than chitosan scaffolds. Chitosan / gelatin scaffolds degraded rapidly in lysozyme during the first seven days of incubation (30–50 percent), and their degradation accelerated with growing gelatin quantity.

This mechanism is ascribed in contrast with chitosan to the greater hydrophilic content of gelatin (Peter et al., 2010). If glutaraldehyde is used to crosslink chitosan / gelatin hydrogels, the strength and mechanical characteristics are enhanced and the rate of degradation can be modulated. In addition to this, it was also observed that Ascorbic Acid not only helps degrade Chitosan overtime but also increases its swelling capacity (Augustine et al., 2019; Kistriyani et al., 2018; Zoldners et al., 2005).

Huang et al., revealed pre-clinical studies using the implantation model and full-thickness cutaneous scar model of prior chitosan / gelatin hydrogels. The findings showed that the hydrogels had excellent biocompatibility with the colonization of host cells, a gentle inflammatory response, enhanced tissue regeneration with dense granulation tissue, and caused early re-epithelialization (Huang et al., 2013).

*CHAPTER*

# *3 Materials & Methodology*

## 3.1 Materials and Solutions

### 3.1.1 Chitosan

Chitosan used was analytical grade was purchased from Santa Cruz Biotechnology.

### 3.1.2 Ascorbic Acid

USP grade L-Ascorbic Acid was purchased from Phytotechnology laboratories, United States. Formula weight was noted to be 176.13 g/mol. 2 g of L-Ascorbic Acid were dissolved in 100 ml distilled water to make up 2% solution.

### 3.1.3 Gelatin

Gelatin used was procured from Daejung Chemical Co. (Siheung, Korea).

### 3.1.4 Glutaraldehyde

25% Glutaraldehyde solution (Sigma-Aldrich) was diluted with distilled water to make a 5% solution by the use of formula:

$$C_1V_1 = C_2V_2$$

### 3.1.5 Silver Sulfadiazine

1% w/v Silver Sulfadiazine solution was purchased from Novartis Pharma to be used as a positive control during microbial inhibition assays.

### 3.1.6 Solvent Used

Glacial acetic acid (MERCK, Germany) was utilized for dissolving Chitosan. Different concentrations of acetic acid were used in different variants of hydrogels to study the effects. The pH of 2% Acetic acid solution was checked with universal pH indicator from Merck Germany and observed to be 4.3.

### 3.1.7 Media Used

Sterilized Agar media for well diffusion assay was prepared by dissolving 28 g of Nutrient Agar (Oxoid) in 1 L of distilled water and autoclaving the solution at 121°C for 2 hrs.

Luria Bertani (LB) broth for bacterial cultivation was prepared by mixing 10 g of tryptone (BioWorld, USA), 5 g of yeast extract (MERCK, Germany), 10 g of NaCl (MERCK, Germany) and 1 L of distilled water followed by autoclaving at 121°C for 2 hrs.

Normal saline for reviving bacterial stocks was prepared by dissolving 0.9 g of NaCl (Oxoid) in 100 ml distilled water.

## 3.2 Methodology Followed

### 3.2.1 Acetic Acid (pH) Variant Hydrogels

100 ml distilled water was kept at constant stirring at 450rpm on a hotplate and heated to $60^0$C. 6g of Gelatin were added to the 100ml distilled water and kept under constant stirring until a homogenous solution was formed. 2g of Chitosan was then added to the solution and kept under stirring until equally dispersed.

The solution was divided into 4 equal parts of 25ml each and requisite amounts of acetic acid were added to make 1%, 2%, 5% and 10% acetic acid variants of the Chitosan/ Gelatin hydrogels. After homogenous mixing and complete dissolution of Chitosan in the solution, 0.5ml of 5% glutaraldehyde was added and gels were cast before gelation.

### 3.2.2 Polymer Ratio Variant Hydrogels

100 ml distilled water was kept at constant stirring at 450rpm on a hotplate and heated to $60^0$C. 6g of Gelatin were added to the 100ml distilled water and kept under constant stirring until a homogenous solution was formed.

The solution was divided into 4 equal parts of 25ml each and requisite amounts of Chitosan were added to make 0%, 1%, 2%, 3% and 4% Chitosan variants of the Chitosan/ Gelatin hydrogels, and kept under stirring until equally dispersed, after which requisite amount of acetic acid was added to each solution to keep acetic acid concentration at 5%. After homogenous mixing and complete dissolution of Chitosan in the solution, 0.5ml of 5% glutaraldehyde was added and gels were cast before gelation.

All the varying combinations synthesized can be easily summarized in the figure below:

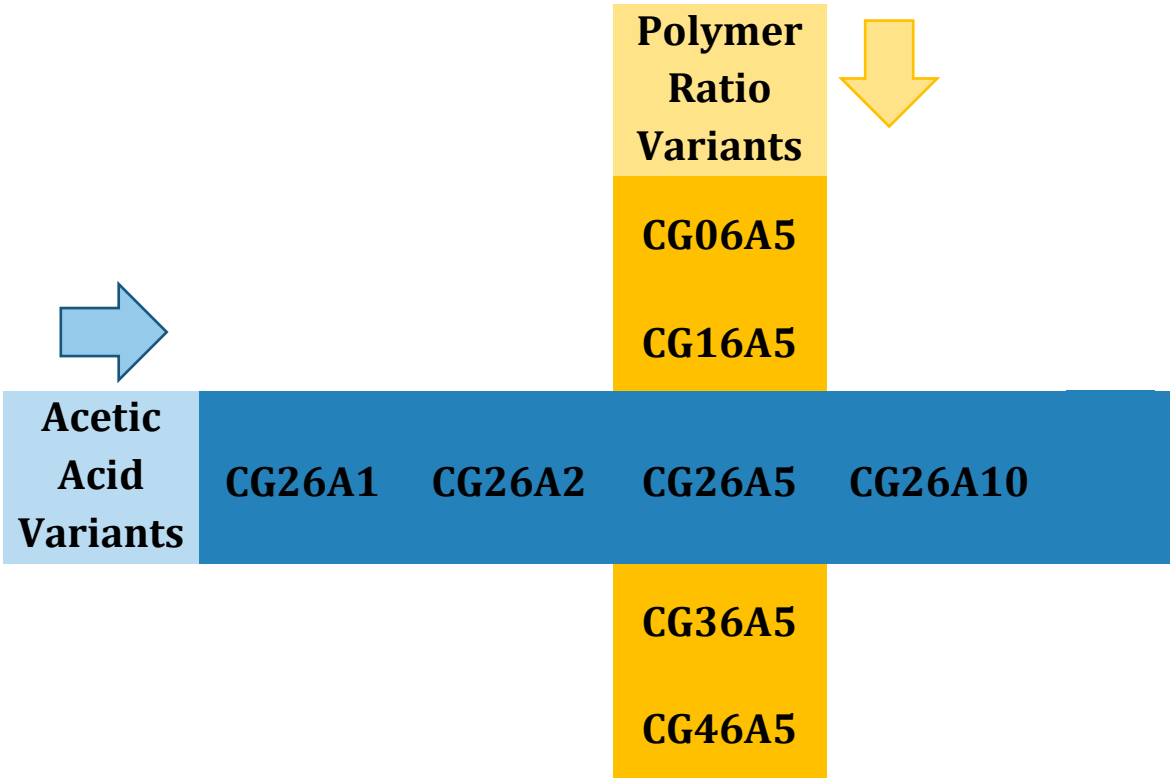


**Figure 3.1: Variants of Chitosan/Gelatin Hydrogels being studied.**

### 3.2.3 Gelation time

After casting of gels, time for the gels to achieve gelation was observed as an important parameter towards selection of the appropriate compositions. The gel formation was checked by the tilt-test (Mujeeb et al., 2013). Petri dishes containing hydrogels were tilted to confirm gelation.

### 3.2.4 Free Swell Absorptive Capacity

BS EN 13726-1 was modified and followed to observe the Free Swell Absorptive Capacity of the hydrogels. Test Solution was made with standardized amounts of Sodium Chloride and Calcium Chloride and the temperature was kept at the standardized levels as well. Modifications were made to the sample quantity and

sample size. Hydrogels were cut instead of 5cm x 5cm squares, into small cylinders of diameter and height 1cm each and dispersed in the test solution. However, due to the composition, water mass, and thickness of the gels, the samples weighed more than the amount mentioned in the standard (0.2g). Sample size was kept at 3 instead of 9 samples as mentioned in the standard and swelling was conducted in glass vials rather than petri dishes due to apparatus limitations. Apart from these, the standard was followed in every detail (British Standard Institute. 2002. 13726-1).

As suggested in the mentioned standard, readings were taken for the first 30 minutes, at 5min, 10min, 20min and 30min intervals. Free Swell Absorptive Capacity was observed as a function of the percentage increase in mass of the hydrogels. Values were calculated by the formula:

$$\frac{(\boldsymbol{Mass\ of\ hydrogel\ at\ t' - Mass\ of\ dry\ glass\ vial}) \times \mathbf{100}}{\boldsymbol{Initial\ mass\ of\ hydrogel}}$$

Where $\boldsymbol{t'}$ denotes the time intervals at which readings were taken.

### 3.2.5 Microbial Inhibition Assay

Well diffusion assay was performed to determine the antibacterial activity of the hydrogel. Nutrient agar was used as a medium to test antibacterial susceptibility. 28 g of nutrient agar was dissolved in 1 L of distilled water and was autoclaved at 121°C for 2 hrs. Upon cooling media was poured into petri dishes. Cultures of Pseudomonas *aeruginosa*, Methicillin resistant Staphylococcus *aureus*, and Escheria *coli* were spread over the dry agar plates. Wells of 6 mm diameter were dug into it and the gels were cut in to fit inside the wells. In all assays, commercially available silver sulfadiazine solution (Dermazin, Novartis Pharma) was used as a positive control.

### 3.2.6 Selection of Compositions

Compositions of hydrogels were shortlisted for further testing based on their swellability, gelation times and their potential for microbial inhibition.

### 3.2.7 Scanning Electron Microscopy

Scanning Electron Microscopy (SEM) is an effective technique for offering comprehensive surface topography data, morphology, sample composition by imaging with focused electron rays. For SEM, a thick section of the gel was cut, about 3cm x 3cm, and washed alternatively with dehydrating ethanol and 0.001M NaOH solution for 3-4 cycles for and left to airdry. A thin slice of hydrogel was then cut fixed on a 1cm×1cm slide with glutaraldehyde. This was followed by gold coating of sample to make the surface conductive by sputter coater device JFC-1500. Specimen after coating was placed on stubs by conductive tape and scanning was done by Scanning electron microscope (JEOL JSM-6490LA).

### 3.2.8 Fourier Transform – InfraRed Analysis

FTIR measurements were performed using Perkin-Elmer Spectrum-100 spectrometer by scanning wavelength range of 400-4000 $cm^{-1}$. The FT-IR spectra of the Chitosan/Gelatin hydrogels were analyzed by KBr disc technique. KBr being hygroscopic was heated to 110$^{o}$C to remove the traces of water. Pellet of KBr was prepared using hydraulic press. Sample was loaded in chamber where IR waves passed through sample to detect the functional groups in the hydrogels. The transmission spectra were recorded and interpreted to identify the bond stretching in functional groups. Essential FTIR software was used to analyze the FTIR spectra of the hydrogels.

- **Degradation Analysis via FT-IR**

Degradation rates of the selected hydrogels were observed in standard PBS solution and 2% L-Ascorbic Acid (Vitamin C) Solution. PBS degraded samples were studied at 24hrs while Vitamin C degraded samples were studied at 12hrs and 24hrs. The percentage transmittance of InfraRed through the sample at various wavelengths were compared to analyze the degradation of the samples and the effects of the medium on their degradation. John Coates's "Interpretation of infrared spectra, a practical approach" published in the 'Encyclopedia of analytical chemistry: applications, theory and instrumentation' was referred to as a guide while characterizing and analyzing the spectra (Coates, 2006).

*CHAPTER*

# *4 Results & Discussion*

## 4.1 Gelation Time

After casting of gels, time for the gels to achieve gelation was observed as an important parameter towards selection of the appropriate compositions. The gel formation was checked by the tilt-test (Mujeeb et al., 2013). Petri dishes containing hydrogels were tilted to confirm gelation.

It was observed that as the acetic acid concentration was increased, the gelation times increased noticeably. This can be explained by the masking of the domains crucial for gelation by the high concentration of $H^+$ ions, also known as the protonation. Hydrogels with Acetic Acid concentration at 1%, 2%, 5% and 10% achieved gelation at 1, 5, 50 & 110 mins respectively.

It was also observed that under a constant concentration of acetic acid concentration, as the concentration of Chitosan was increased, gelation times decreased significantly. This supports the explanation made earlier as an increase of Chitosan would see an increase in the number of domains crucial for gelation that are not protonated. Hydrogels with 2%, 3% and 4% Chitosan achieved gelation in 50, 20 and 2 mins respectively. 0% and 1% Chitosan containing solutions failed to achieve gelation in a desirable amount of time and achieved gelation only under lower temperatures of around $4^0$C-$8^0$C range over the span of 4 days and 2 days respectively. This data is compiled in Tables 4.1 and 4.2.

## 4.2 Free Swell Absorptive Capacity

BS EN 13726-1 was modified and followed to observe the Free Swell Absorptive Capacity of the hydrogels, also known as and mentioned in the standard as "Free Swell Absorptive Capacity (British Standard Institute. 2002. 13726-1).

As suggested in the mentioned standard, readings were taken for the first 30 minutes, at 5min, 10min, 20min and 30min intervals. Free Swell Absorptive Capacity was observed as a function of the percentage increase in mass of the hydrogels. Values were calculated by the formula:

$$\frac{(\boldsymbol{Mass\ of\ hydrogel\ at\ t' - Mass\ of\ dry\ glass\ vial}) \times \mathbf{100}}{\boldsymbol{Initial\ mass\ of\ hydrogel}}$$

Where $\boldsymbol{t'}$ denotes the time intervals at which readings were taken.

An increase in the mass of the gels was observed at intervals. The general trend amongst the acetic acid variants observed was that with the increase in acetic acid concentrations, the rate of uptake and the maximum capacity reachable within 30min was greatly increased. However, the 10% Acetic Acid variant lost its integrity overtime and assumed a soft, jelly-like consistency. However, 10% Acetic Acid variant showed an 80.22% increase in mass after 30min, whereas 5%, 2% and 1% Acetic Acid variant showed 64.07%, 39.34% and 30.92% increase in mass respectively. It is important to note that the bulk of medium uptake and consequently the bulk of hydrogel swelling and mass increase was observed within the first 5min of hydrogel dispersion.

It is also important to note from thee graph in Figure 4.1 that the effect of Acetic Acid content in increasing the swellability decreases as the Acetic Acid content is increased from 1% to 10%. Initially, an increase of 1%, from 1% to 2% of Acetic Acid content

increased the total free swell absorptive capacity by roughly 9% of the initial mass, whereas a 5% increase from 5% to 10% of Acetic Acid content increased the total free swell absorptive capacity by only about 14% of the initial mass.

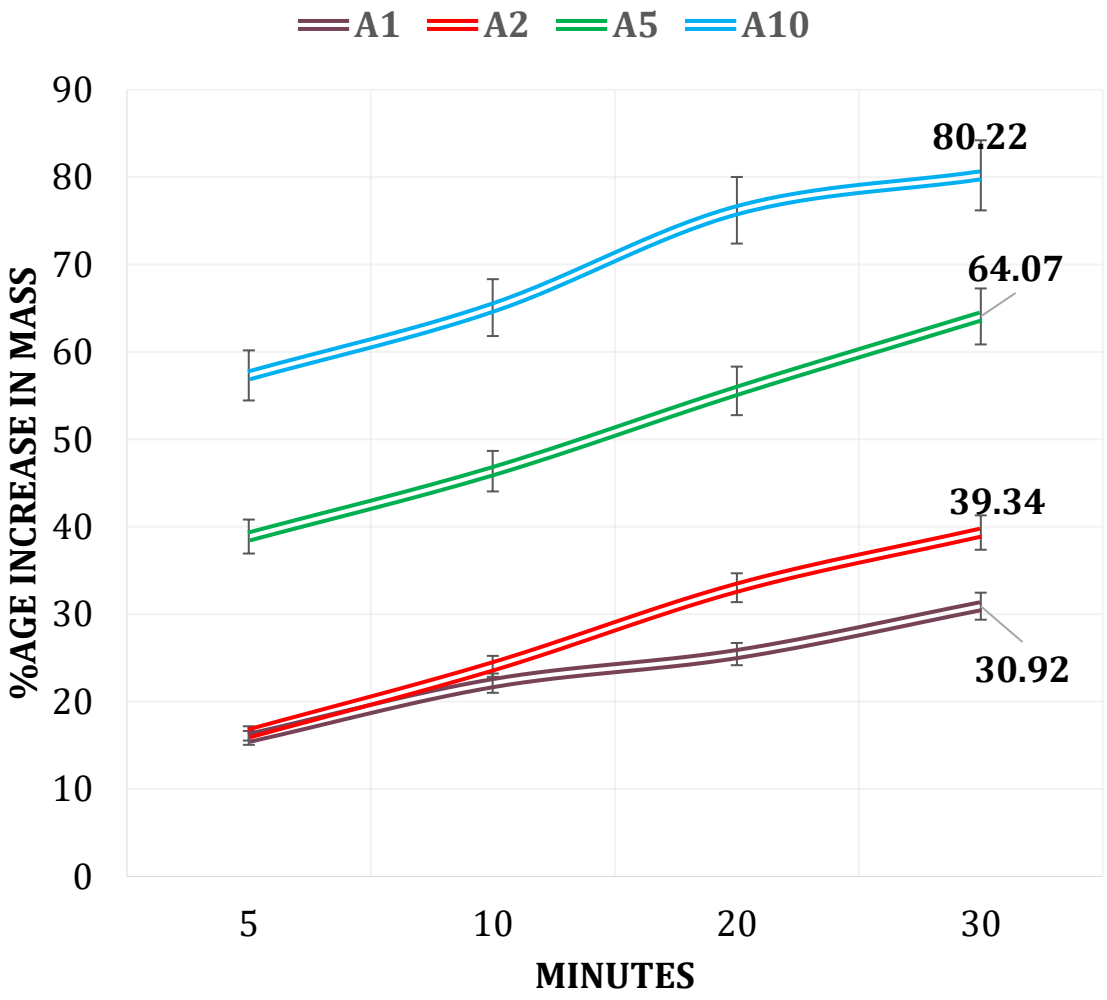


**Figure 4.1: Effect of Acetic Acid on Free Swell Absorptive Capacity.** *Here A1, A2, A5 and A10 denote the percentage by volume of Acetic Acid in the hydrogels.*

In a similar approach, the effect of increase of chitosan (to change the polymer ratio) on the Free Swell Absorptive Capacity was observed. In this case, it was observed that at a constant Acetic Acid content (5%), the increase in Chitosan decreased the swellability but in a very meagre amount. A 2% increase in Chitosan content decreased

the percentage increase in mass from 64.07% to 60.11% from 2% to 4% Chitosan content.

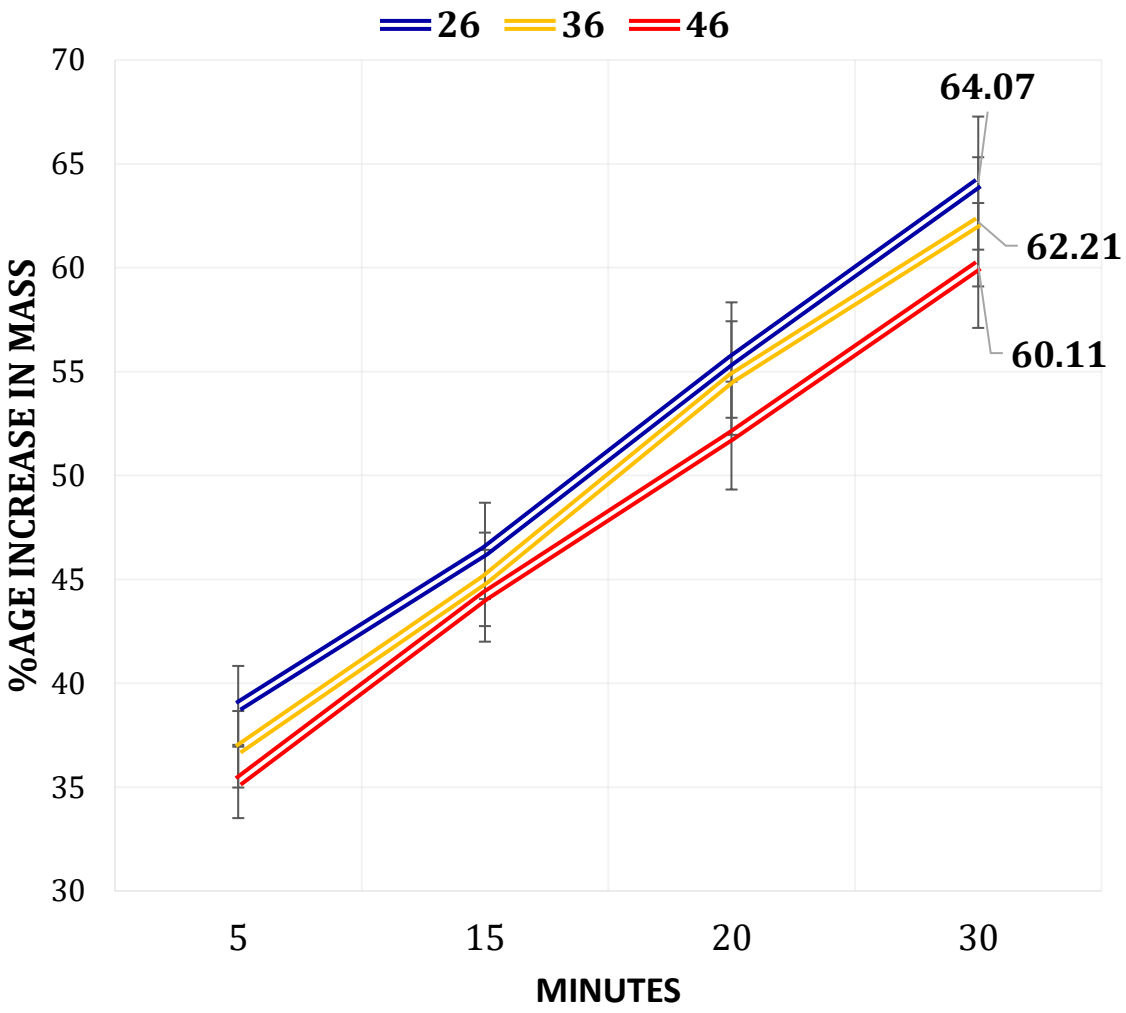


**Figure 4.2: Effect of Polymer Ration variation on the Free Swell Absorptive Capacity.** *The number 26, 36 and 46 denote the Chitosan/Gelatin Ratio, first digit denoting percentage by weight of Chitosan and the second digit denoting percentage by weight of Gelatin.*

## 4.3 Microbial Inhibition Assay

Well diffusion assay was performed to determine the antibacterial activity of the hydrogel. Cultures of Pseudomonas *aeruginosa*, Methicillin resistant Staphylococcus *aureus*, and Escheria *coli* were spread over the dry agar plates and the hydrogels were then tested against them. In all assays, commercially available silver sulfadiazine solution (Dermazin, Novartis Pharma) was used as a positive control. All analyses were performed in triplicate.

It was observed that an increase in the Acetic Acid concentration increased the inhibitory activity of the Chitosan/ Gelatin hydrogels significantly. While significant antimicrobial activity was observed against Methicillin resistant Staphylococcus *aureus*, and Escheria *coli*, the highest inhibitory effects were observed against Pseudomonas *aeruginosa*. These results correlate with the work published by Leung et al, that demonstrated complete eradication of Pseudomonas *aeruginosa* in 92% (n=37) of cases with no relapse (Leung et al, 2001). These results are presented in figure 4.3, 4.4 and 4.5.

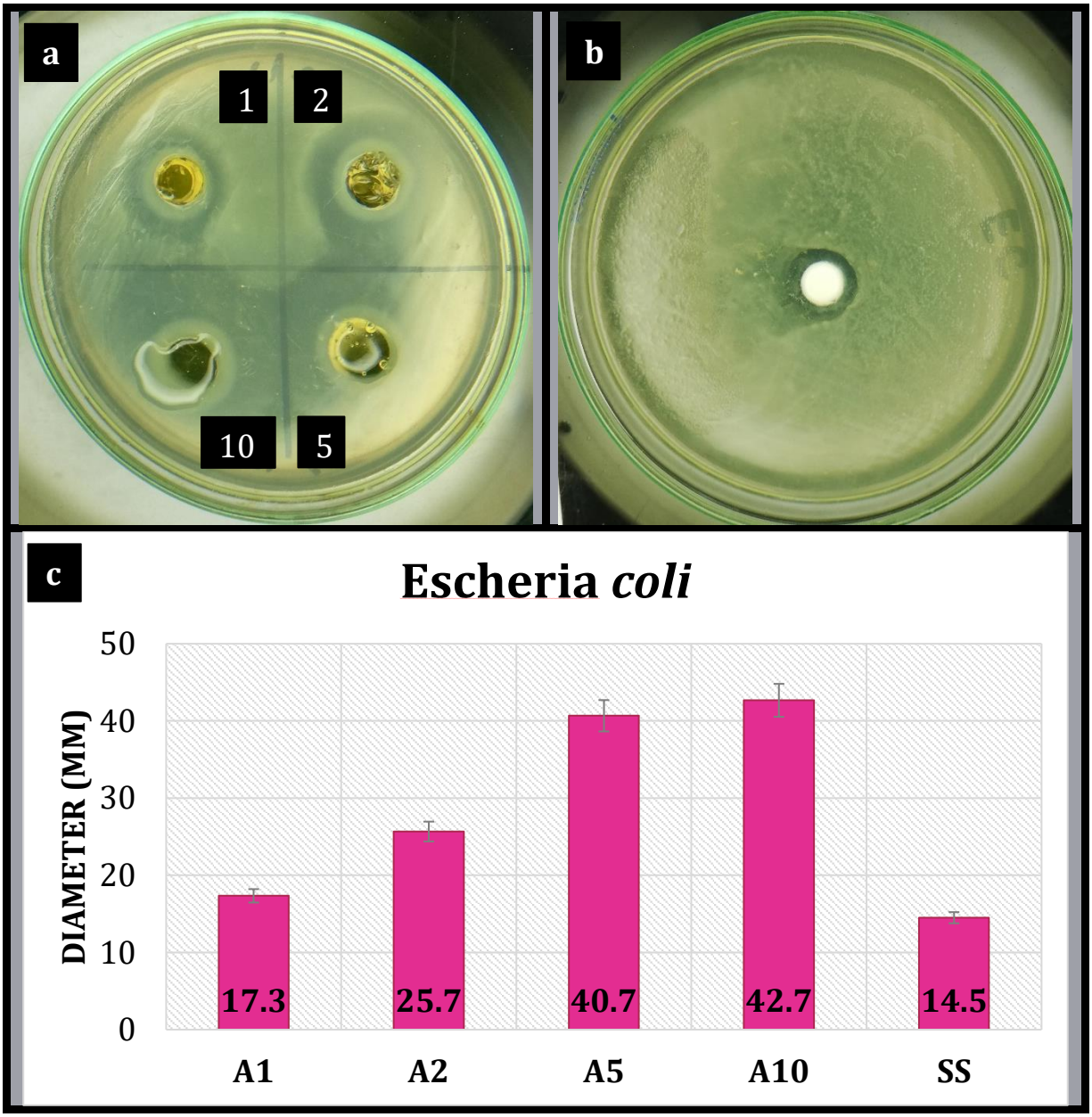


**Figure 4.3 Microbial Inhibition Assay against Escheria *coli* of Acetic Acid Variants.** (a) Hydrogels samples being tested, values denote percentage of Acetic Acid, (b) Silver Sulfadiazine tested as positive control, (c) Graphical representation of data obtained, values denote percentage of Acetic Acid, "SS" denotes Silver Sulfadiazine.

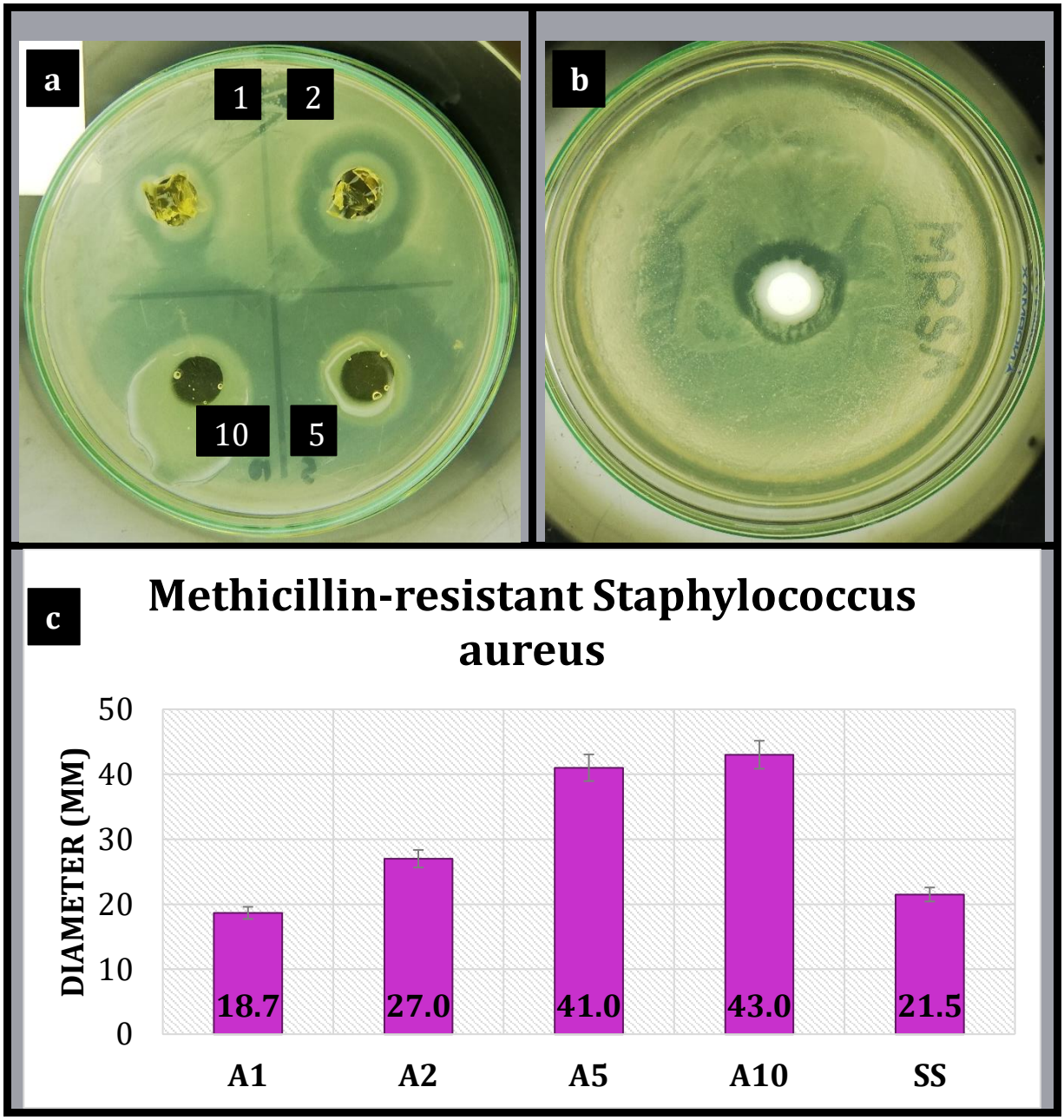


**Figure 4.4 Microbial Inhibition Assay against Methicillin-resistant Staphylococcus *aureus* of Acetic Acid Variants.** (a) Hydrogels samples being tested, values denote percentage of Acetic Acid, (b) Silver Sulfadiazine tested as positive control, (c) Graphical representation of data obtained, values denote percentage of Acetic Acid, "SS" denotes Silver Sulfadiazine.

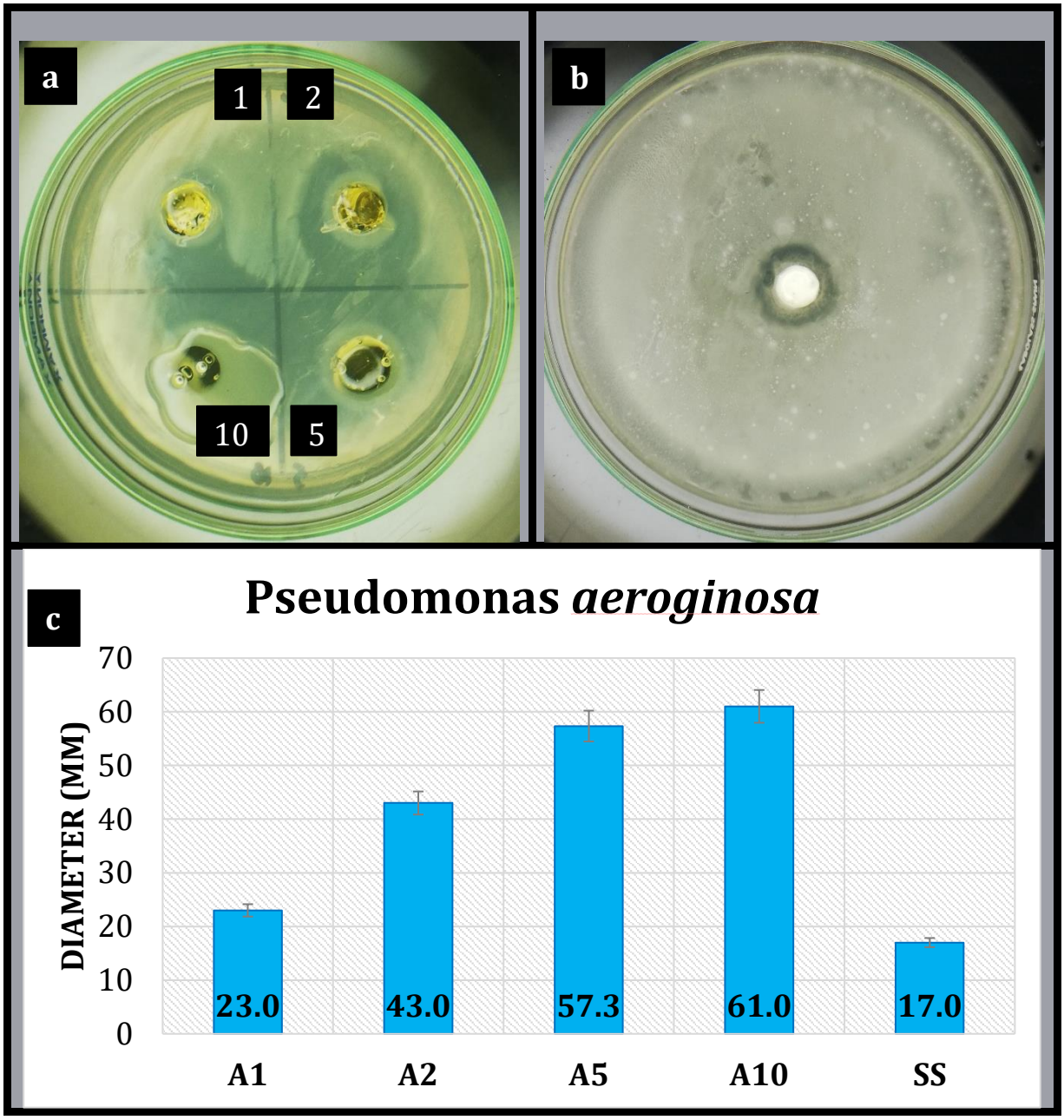


**Figure 4.5 Microbial Inhibition Assay against Pseudomonas *aeruginosa* of Acetic Acid Variants.** (a) Hydrogels samples being tested, values denote percentage of Acetic Acid, (b) Silver Sulfadiazine tested as positive control, (c) Graphical representation of data obtained, values denote percentage of Acetic Acid, "SS" denotes Silver Sulfadiazine.

In an identical protocol, it was observed that with the increase in Chitosan (in order to change the polymer ratio), the antimicrobial activity of the gels decreased only by a couple of millimeters only. This supports the explanation made earlier in section '4.1 Gelation Time', that an increase of Chitosan would see an increase in the number of domains crucial for gelation that are not protonated. Hence a higher degree of crosslinking would occur. Higher degree of crosslinking means better structural integrity and capacity to withhold materials incorporated within. And while significant antimicrobial activity was observed against Methicillin resistant Staphylococcus *aureus*, and Escheria *coli*, the highest inhibitory effects were observed against Pseudomonas *aeruginosa*. These results correlate with the work published by Leung et al, that demonstrated complete eradication of Pseudomonas *aeruginosa* in 92% (n=37) of cases with no relapse (Leung et al, 2001). It should be noted however, that due to certain high readings during the triplicate testing of polymer ratio variants, some readings have means that are unusually high while their mode suggests the trend mentioned above. The results are therefore less reliable. These results are presented in figure 4.6, 4.7 and 4.8.

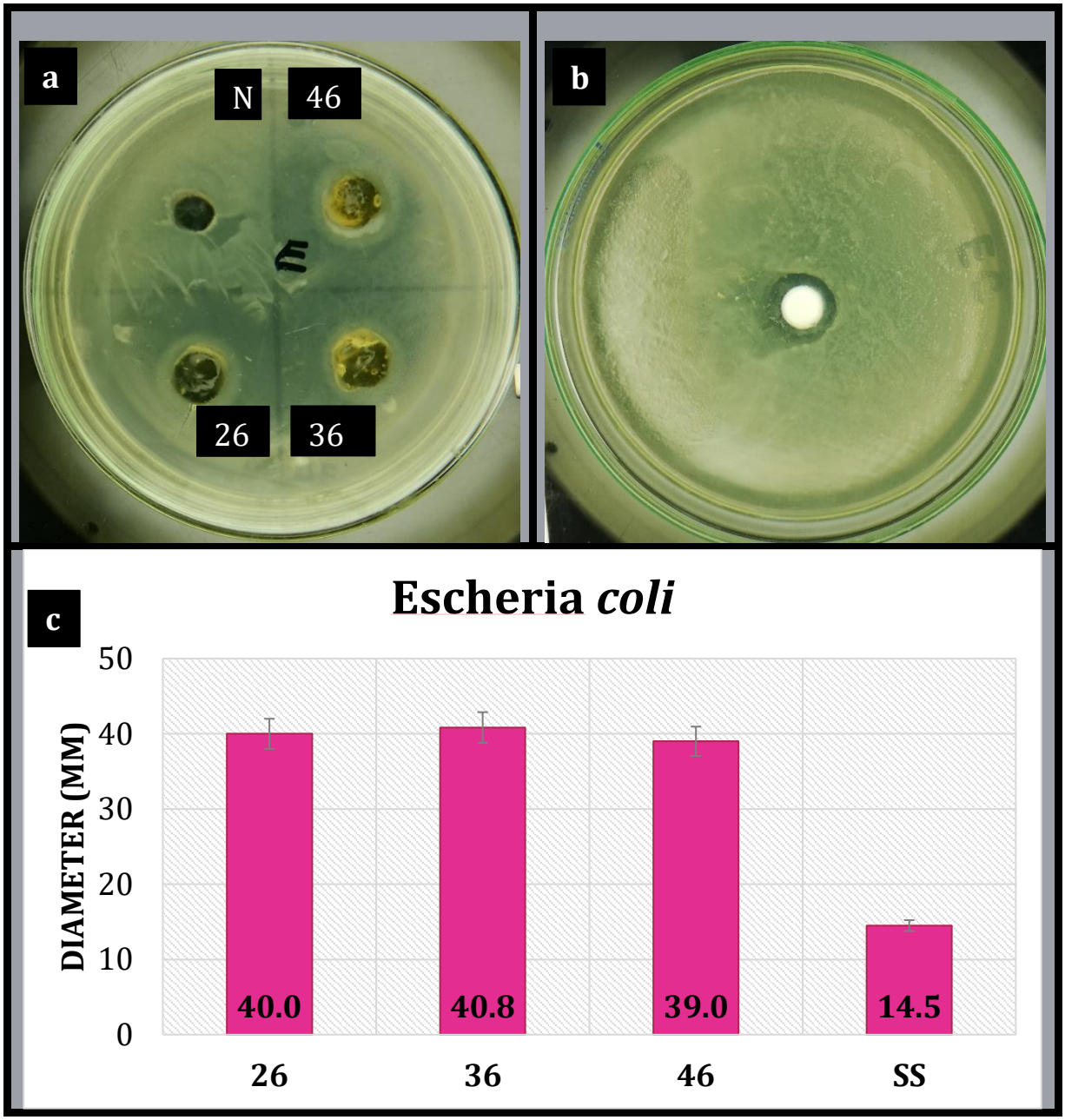


**Figure 4.6 Microbial Inhibition Assay against Escheria *coli* of Polymer Ratio Variants.** (a) Hydrogels samples being tested, values denote Chitosan/Gelatin ratio, "N" denotes negative control, (b) Silver Sulfadiazine tested as positive control, (c) Graphical representation of data obtained, values denote Chitosan/Gelatin ratio, "SS" denotes Silver Sulfadiazine.

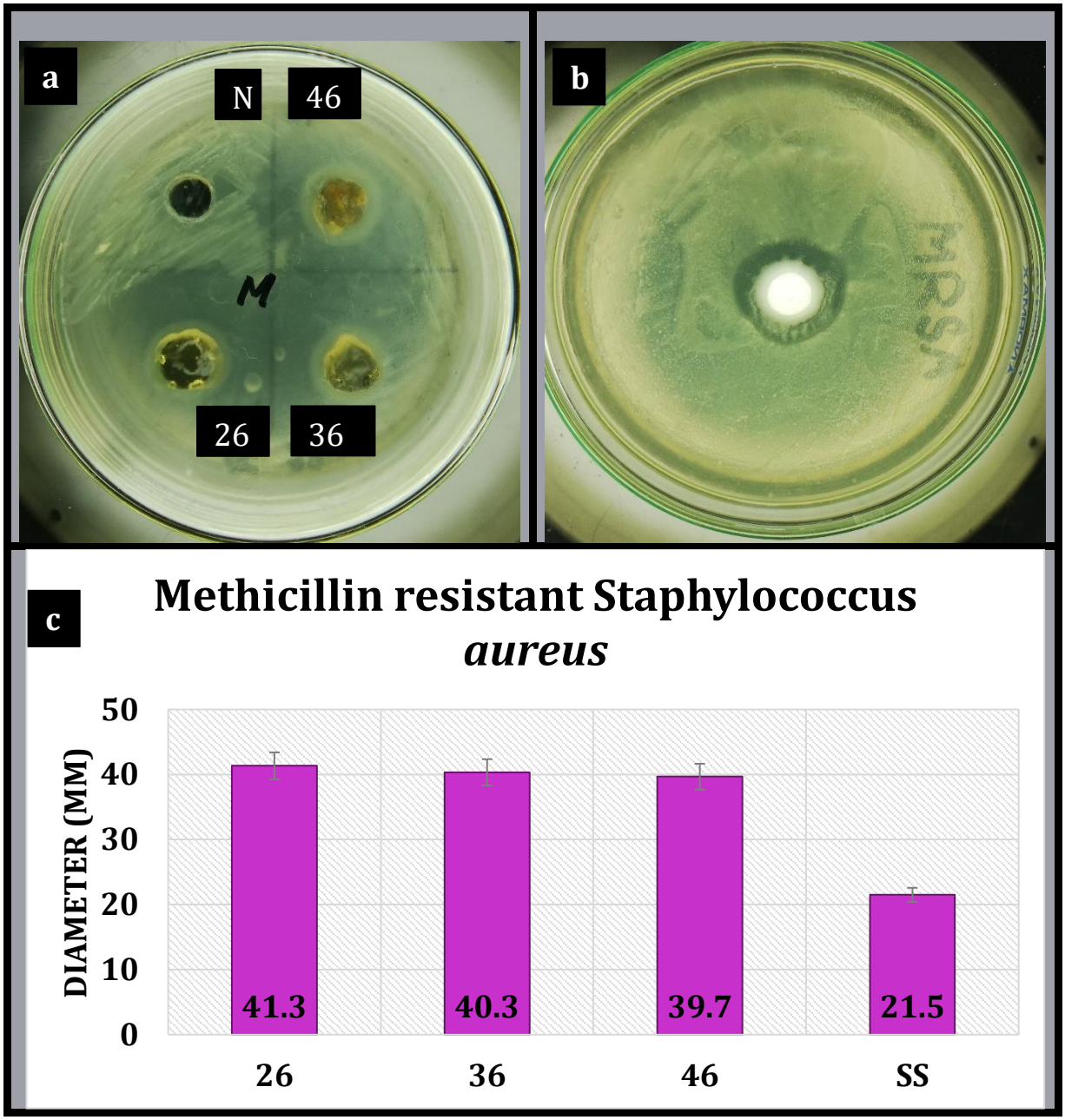


**Figure 4.7 Microbial Inhibition Assay against Methicillin-resistant Staphylococcus *aureus* of Polymer Ratio Variants.** (a) Hydrogels samples being tested, values denote Chitosan/Gelatin ratio, "N" denotes negative control, (b) Silver Sulfadiazine tested as positive control, (c) Graphical representation of data obtained, values denote Chitosan/Gelatin ratio, "SS" denotes Silver Sulfadiazine.

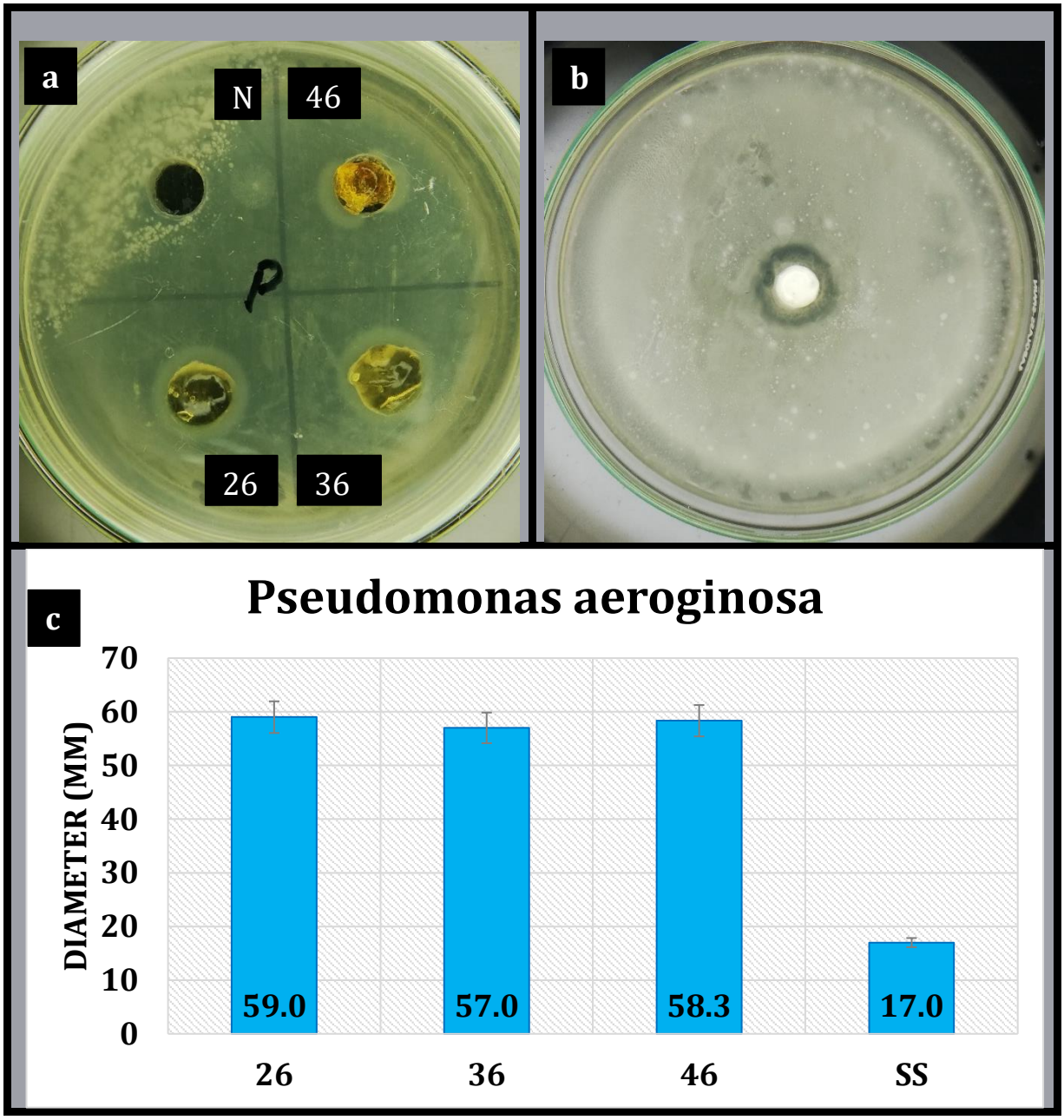


**Figure 4.8 Microbial Inhibition Assay against Pseudomonas *aeruginosa* of Polymer Ratio Variants.** (a) Hydrogels samples being tested, values denote Chitosan/Gelatin ratio, "N" denotes negative control, (b) Silver Sulfadiazine tested as positive control, (c) Graphical representation of data obtained, values denote Chitosan/Gelatin ratio, "SS" denotes Silver Sulfadiazine.

## 4.4 Selection of Compositions

Hydrogel variants were selected for further testing based on the properties they exhibited in terms of gelation rate, free swell absorptive capacity and microbial inhibition. Gelation time and free swell absorptive capacity of Acetic Acid variants and polymer ratio variants are given in table 4.1 and 4.2 below.

| GEL SAMPLE | GELATION AFTER | SWELLABILITY |
|---|---|---|
| CG26A1 | 1 min | 30.92% |
| CG26A2 | 5 min | 39.34% |
| CG26A5 | 50 min | 64.07% |
| CG26A10 | 110 min | 80.22% |

**Table 4.1: Acetic Acid variants**

| GEL SAMPLE | GELATION AFTER | SWELLABILITY |
|---|---|---|
| CG06A5 | - | - |
| CG16A5 | - | - |
| CG26A5 | 50 min | 64.07% |
| CG36A5 | 20 min | 62.21% |
| CG46A5 | 2 min | 60.11% |

**Table 4.2: Polymer Ratio variants**

During the microbial inhibition assay, 5% Acetic Acid variants delivered the best results while retaining structure and morphology, 1% and 2% Acetic Acid hydrogels

observed shrinking while 10% Acetic Acid hydrogels lost integrity into fluid state at incubation temperature. Gelation time was an important factor while selecting the gels as the hydrogels should achieve gelation in a reasonable time, too soon and it is not cast properly, too delayed and it is not feasible in terms of production economy.

On the basis of these factors, hydrogels CG26A2, CG26A5 and CG36A5 were selected.

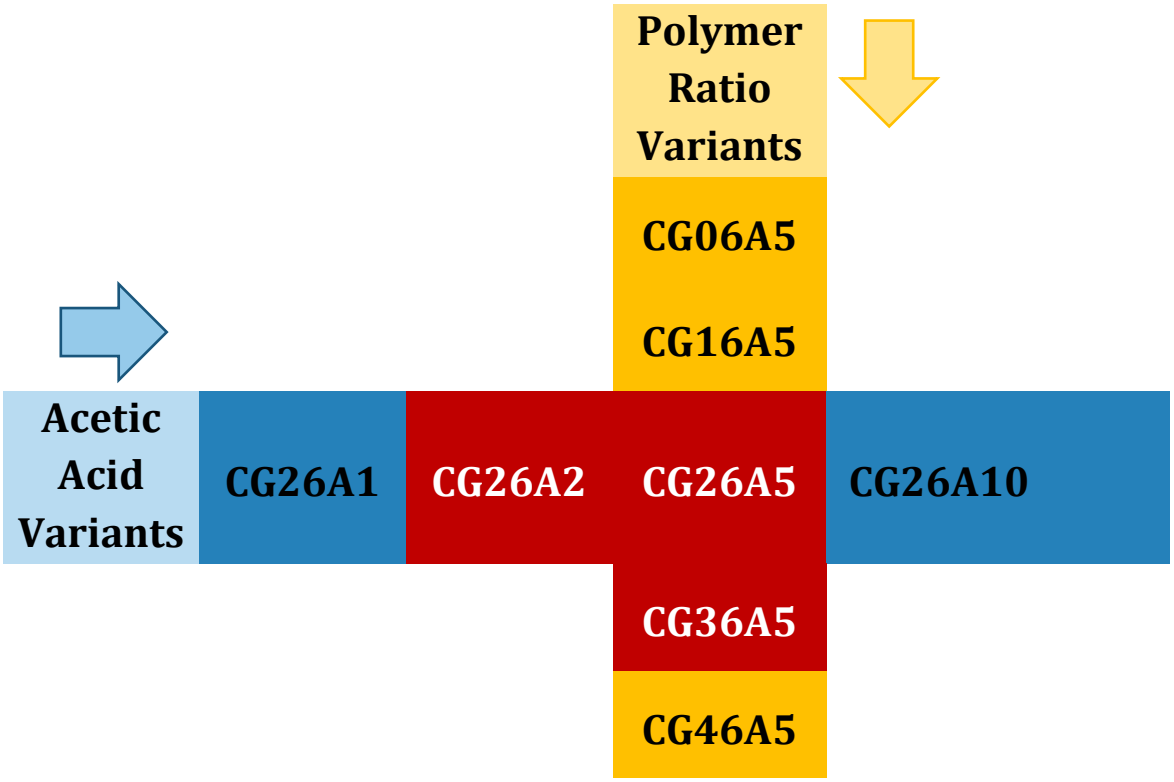


**Figure 4.9: Variants of Chitosan/Gelatin Hydrogels studied with selected Hydrogels highlighted in Red.**

## 4.5 Scanning Electron Microscopy

Scanning Electron Microscopy (SEM) was performed as it is an effective technique for offering comprehensive surface topography data, morphology, sample composition by imaging with focused electron rays. Pore sizes of the hydrogels were observed. Refer to figure 4.10 below.

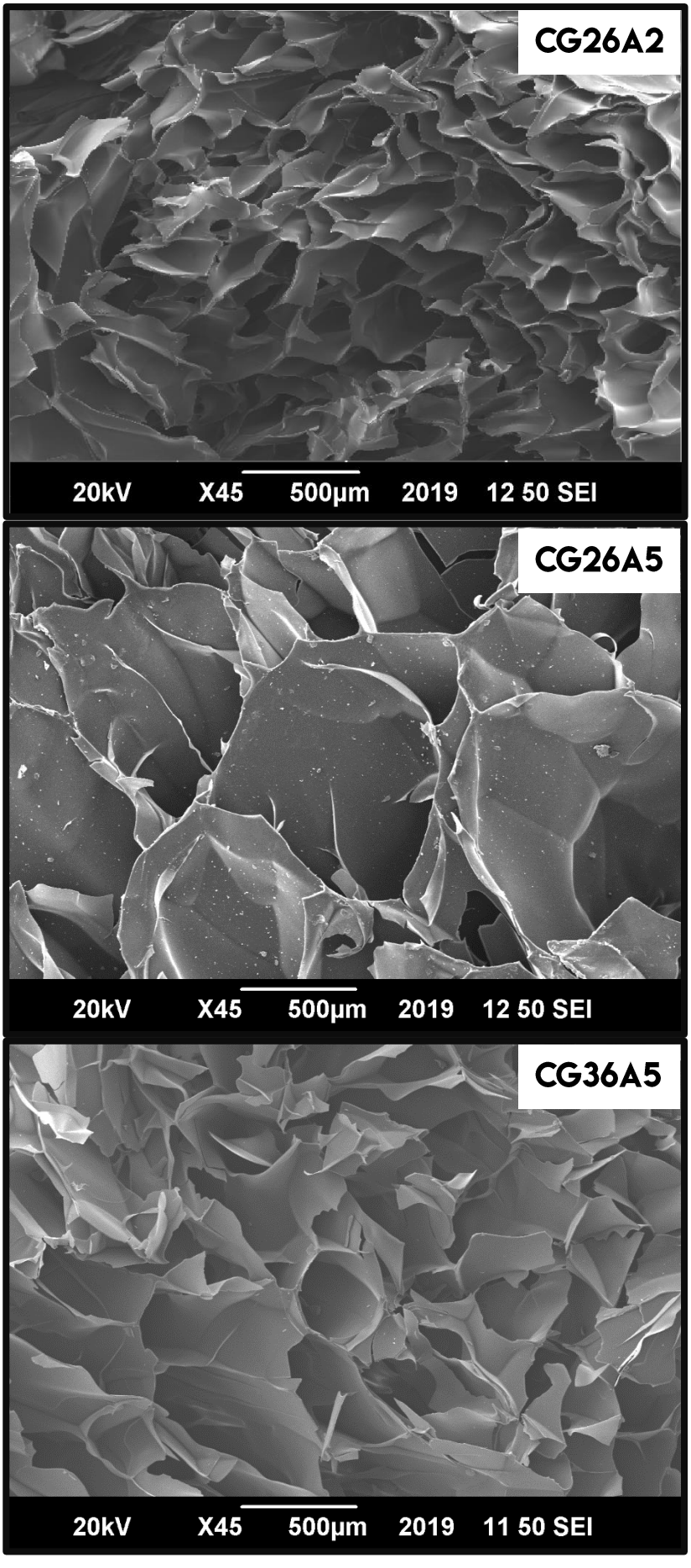


**Figure 4.10: SEM images of selected hydrogels.** As can be observed from by comparing the images to their provided scales, as the Acetic Acid content is increased from 2% to 5%, the pore size increase and after that when the Chitosan content is increased from 2% to 3%, the pore size decreases again. These results gather proof and support the argument of the effects of increasing and decreasing the amounts of Acetic Acid and Chitosan.

From the images obtained we gathered that as the Acetic Acid content is increased from 2% to 5%, the pore size increase and after that when the Chitosan content is increased from 2% to 3%, the pore size decreases again. These results are in line with the earlier results and prove the effects of increasing and decreasing the amounts of Acetic Acid and Chitosan. As the Acetic Acid content is increased from 2% to 5%, the pore size increase and hence a higher pore size allows a greater influx of an aqueous medium. Whereas when the Chitosan content is increased from 2% to 3%, the pore size decreases again and hence the swellability is affected as influx is decreased. In a similar fashion, efflux is decreased as well and consequently release of acetic acid within the gel is affected.

## 4.6 Fourier Transform – InfraRed Analysis

FTIR measurements were performed using Perkin-Elmer Spectrum-100 spectrometer by scanning wavelength range of 400-4000 $cm^{-1}$. The FT-IR spectra of the Chitosan/Gelatin hydrogels were analyzed by KBr disc technique. Essential FTIR software was used to analyze the FTIR spectra of the hydrogels. Degradation rates of the selected hydrogels were observed in standard PBS solution and 2% L-Ascorbic Acid (Vitamin C) Solution. PBS degraded samples were studied at 24hrs while Vitamin C degraded samples were studied at 12hrs and 24hrs. The percentage transmittance of InfraRed through the sample at various wavelengths were compared to analyze the degradation of the samples and the effects of the medium on their degradation. John Coates's "Interpretation of infrared spectra, a practical approach" published in the 'Encyclopedia of analytical chemistry: applications, theory and instrumentation' was referred to as a guide while characterizing and analyzing the spectra (Coates, 2006). FTIR Graphs are provided in figures 4.11, 4.12 and 4.13.

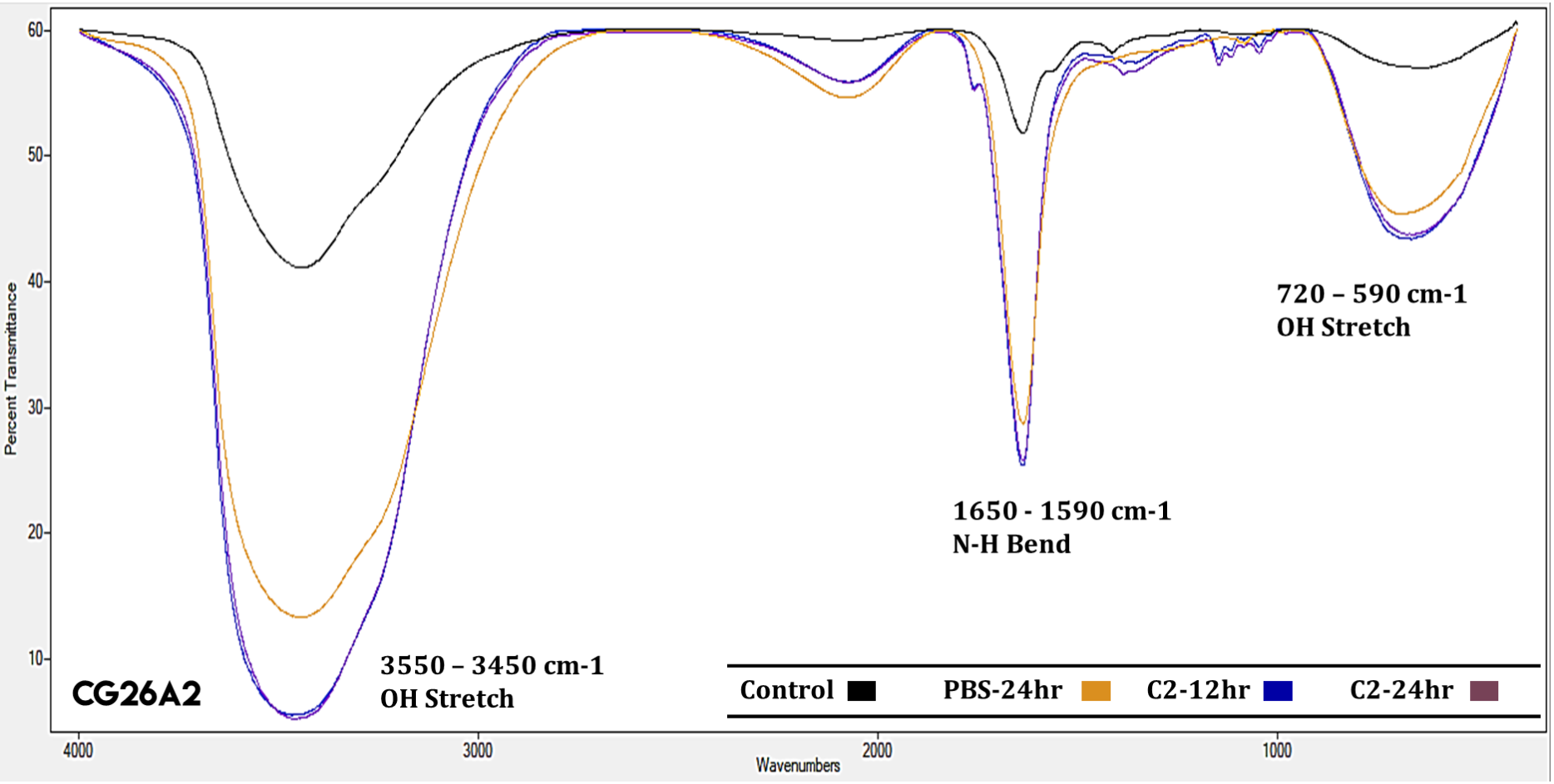


**Figure 4.11: FTIR Analysis and degradation profile comparison of CG26A2**

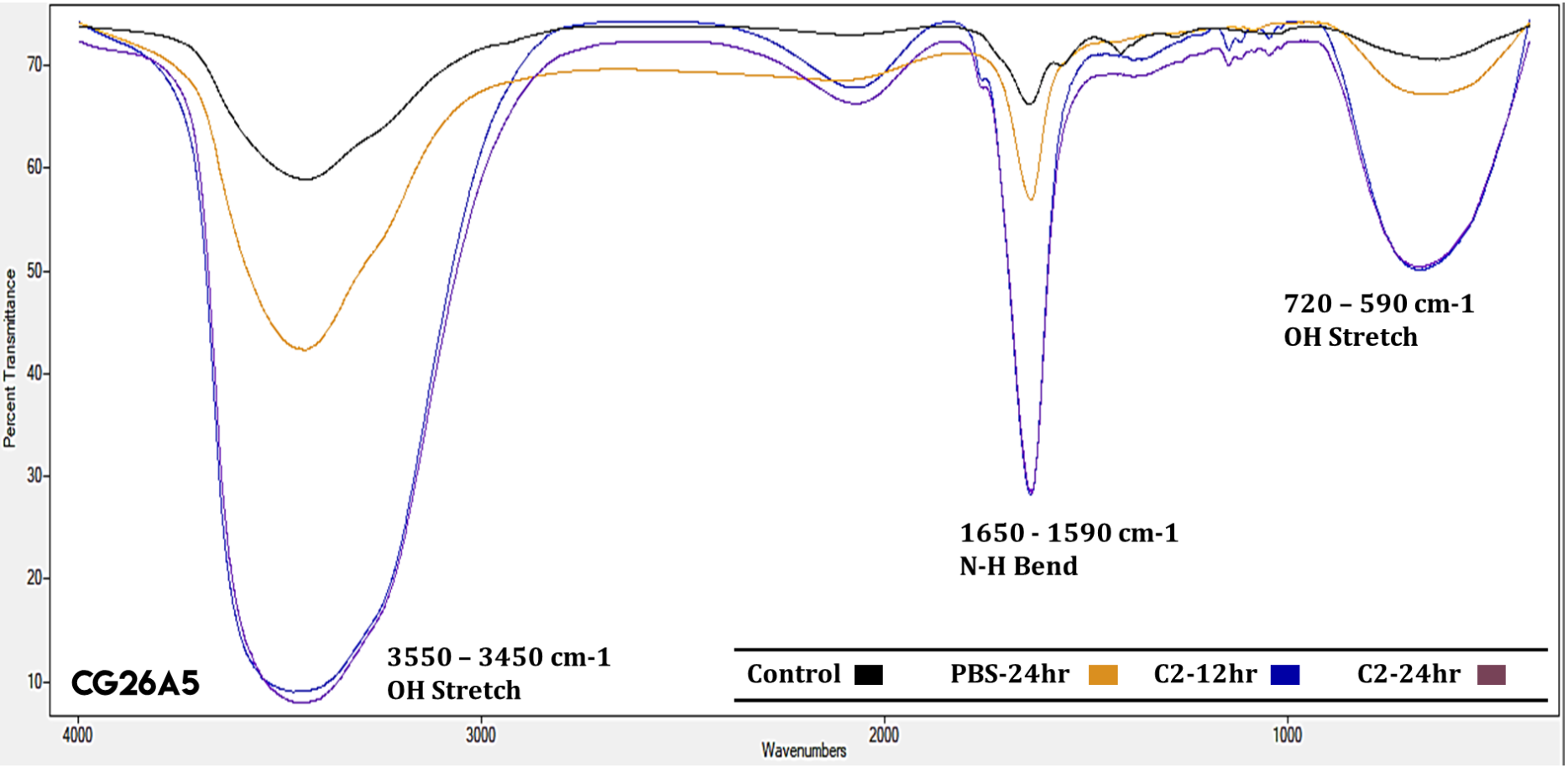


**Figure 4.12: FTIR Analysis and degradation profile comparison of CG26A5**

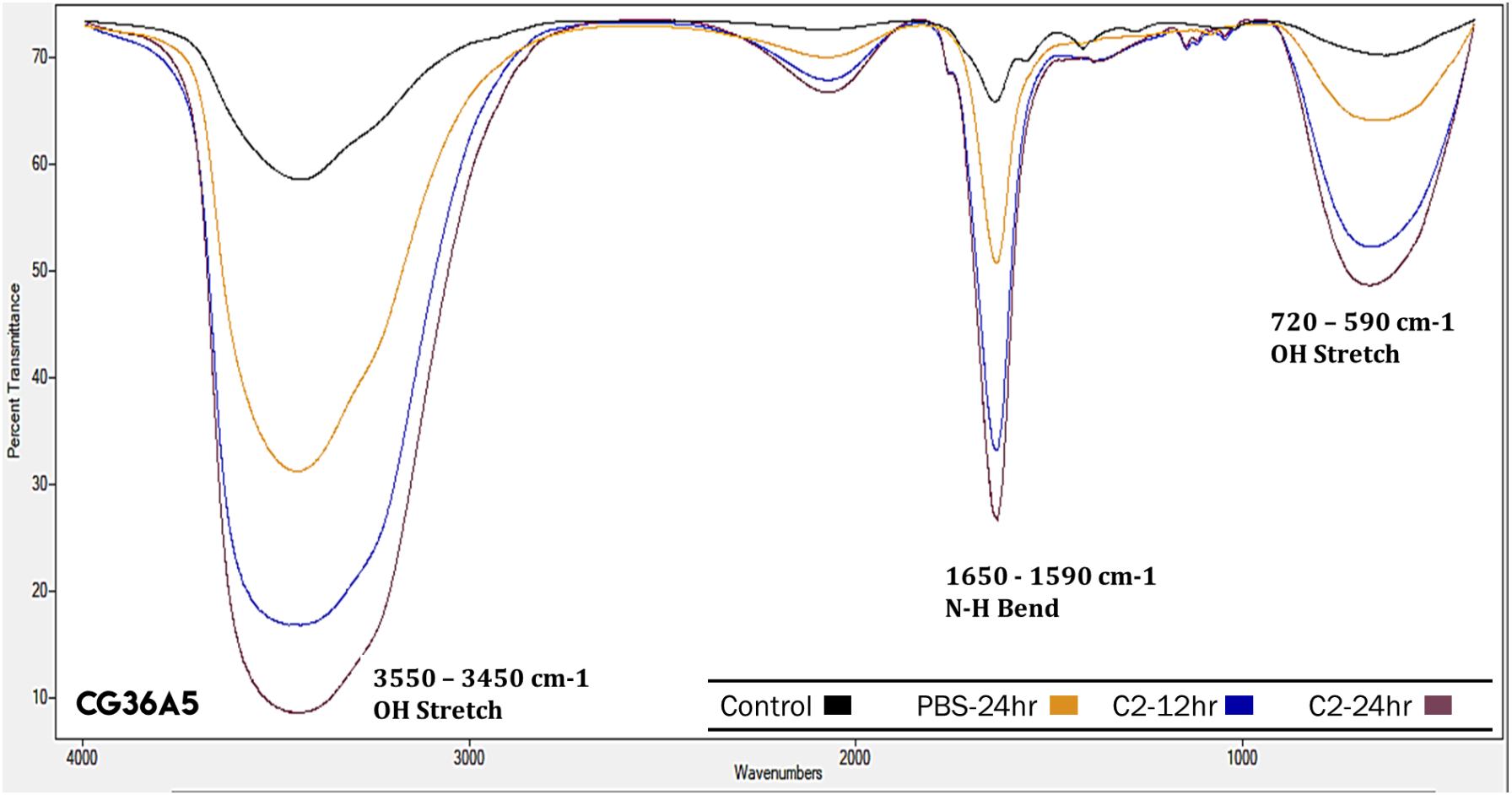


**Figure 4.11: FTIR Analysis and degradation profile comparison of CG26A2**

Results obtained from the FT-IR analysis were in line with the earlier results and prove the effects of increasing and decreasing the amounts of Acetic Acid and Chitosan. It was observed that as the Acetic Acid content is increased from 2% to 5%, the pore size increase and hence a higher pore size allows a greater influx of an aqueous medium and consequently a greater degree of degradation. The increase in absorbance in the O-H regions at 3550 – 3450 $cm^{-1}$ shows this. Hydrolysis of polymers yields hydroxyl groups which are the reason behind the increase in absorbance. The increase in N-H regions at 1650 - 1590 $cm^{-1}$ and O-H regions at 720 – 590 $cm^{-1}$ also proves the degradation of gelatin molecules as the peptide linkages within are broken yielding $NH_2$- and -COOH functional groups. However, the degradation profile of only Ascorbic Acid is increased while PBS is decreased. In all hydrogel samples, Ascorbic Acid mediated degradation was significantly more than PBS mediated degradation. Another important observation is that in CG26A2 and CG26A5, there is no difference in Ascorbic Acid mediated degradation between 12hr and 24hr observation.

However, when the Chitosan content is increased from 2% to 3%, with more substrate (Chitosan) to degrade, there is observed a profound difference between the Ascorbic Acid mediated degradation peaks at 12hr and 24hr.

*CHAPTER*

# *5 Conclusions*

Hydrogels synthesized exhibit excellent antimicrobial activity and swelling characteristics. It was observed that degradation under PBS affected under high Acetic Acid content and that gelation rate can be tweaked by Acetic Acid content. Higher Acetic Acid content not only provides tweakable gelation rates but also an increase in the antimicrobial activity. Similarly, Ascorbic Acid (Vitamin C) and Chitosan can be used to tweak the degradation rate.

Gels that were studied can provide excellent wound microenvironment modulation by providing simpler saccharides, amino acids, vitamins and moist acidic environment to the wound. 3:6 Chitosan-Gelatin Gels in 5% Acetic Acid solution provide the best overall results in terms of gelation time, antimicrobial activity, swelling and degradation.

Relationships were found on how the amount of Chitosan and Acetic Acid provide different porosity and packing density and can be studied extensively to obtain tailor-made porosities or hybrid and gradient porosities according to the desired applications. Structures with desired strengths can be created by influencing the packing density as well. Influx of solutions or media can be controlled via porosity control, hence helping synthesize time-dependent or timed-release drug delivery systems. For this aspect, tailored degradation can be achieved as well with these materials. Tailored degradation can also be achieved according to desired applications by tweaking the amounts of Chitosan and Ascorbic Acid. As Ascorbic Acid acts on

Chitosan this will approach will not only be tailored but also targeted degradation and would make it easier to predict and calculate the degradation rate. Another application of this would be to imbed Chitosan nanoparticles for controlled drug release in a scaffold non-degradable by Ascorbic Acid, so while the scaffold retains structural integrity, drugs can be released as required by degrading just the Chitosan. Additional advantages of this approach include the collagen synthesizing, inflammation reducing and antioxidant properties of Ascorbic Acid that could be received by the healing tissue.

Other applications include; superabsorbent gels, high strength patches, conformable bandages, gels with ointment-like consistency, bandages for prolonged usage medicated bandages.

Further, in vivo analysis of the hydrogels could be performed, study of wound sections could be conducted with HPLC analysis to quantify absorption of different components and the degradation end-products.

Mannose-6-phosphate and TGF-β3 have been studied extensively over the course of years and their roles in scarring have been well documented (Shah et al., 1992; Shah et al., 2000; Ferguson, 1994; Ferguson et al., 2004). Mannose-6-Phosphate can be incorporated into these gels to reduce scarring by downregulating the TGF-β3 pathway.

*CHAPTER*

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