Aloe vera Gel Incorporated Chitosan/PVA/Gelatin Hydrogel Film for Potential Biomedical Applications

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Acknowledgment

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Date: **Koyel Ghosal**

Declaration

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgment has been made in the text. All information in this document has been obtained and presented in accordance with academic rules and ethical conduct.

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This is to certify that the thesis entitled "***Aloe vera* Gel Incorporated Chitosan/PVA/Gelatin Hydrogel Film for Potential Biomedical Applications**" is being submitted by **Koyel Ghosal**, in partial fulfillment for the award of the degree of Master in Biomedical Engineering of Jadavpur University is a record of bona fide work carried out by her under my guidance and supervision. The project, in our opinion, is worthy for its acceptance.

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Certificate of Approval

The forgoing thesis is hereby approved as a creditable study of an engineering subject carried out and presented satisfactorily to warrant its acceptance as a pre-requisite to the degree for which it has been submitted. It is understood that by this approval the undersigned do not necessarily endorse or approve any statement made, opinion expressed or conclusion drawn therein but approve the thesis only for the purpose for which it is submitted.

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Abstract

The development of hydrogel film with multifunctional properties is highly desirable in the field of biomedical sciences for various pre-clinical and clinical applications. *Aloe vera* is renowned for its medicinal properties such as anti-inflammation, wound healing, anti-septic, skin protection, and many more. The medical efficacy of *Aloe vera* is well documented in ancient Indian scriptures. The study aims to integrate ancient Ayurvedic knowledge with modern therapy by developing a hydrogel film majorly composed of natural components and biodegradable polymer like PVA which is recommended and approved by Food and Drug Administration (FDA). In this paper, chitosan, gelatin and polyvinyl alcohol (PVA) were used in the ratio of (2:1:1), respectively, for preparing the hydrogel film, with *Aloe vera* integrated into the film at concentrations of 25%, 50%, and 75% relative to the total polymer solution. The films are chemically crosslinked using glutaraldehyde at 0.5% and 1% concentration, with glycerol serving as a plasticizer. The final film was fabricated utilising the solvent casting method. The hydrogels were investigated for physico-chemical properties such as swelling, biodegradation, water vapour transmission rate (WVTR), anti-oxidant assay, scanning electron microscopy (SEM), contact angle measurement, and Fourier transform infrared spectroscopy (FTIR). *In-vitro* biological assays including hemocompatibility and antibacterial assay as well as cell viability assays (namely MTT and FDA assay) were also performed. Hydrogels with 0.5% cross-linking exhibited superior swelling, biodegradation, WVTR, and antioxidant activity. All the samples exhibited smooth surface morphology, hydrophilicity, hemocompatibility, possessed antibacterial properties, cell viability, and proliferation. A consistent trend was observed among samples with the same concentration of *Aloe vera* gel. Further *in-vivo* investigation is required among 0.5% cross-linking samples to select the best film as potential wound dressing material.

Keywords: Hydrogel, *Aloe vera*, Chitosan, PVA, Gelatin, Wound healing

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CHAPTER 1

Introduction

The skin, being the largest organ in the human body, functions as a protective barrier against external dangers like radiation, chemicals, bacterial and fungal infections, and thermo-mechanical shock (1). Any damage to the skin or mucosa caused by heat or physical force is referred to as wound (2). Wound healing consists of 4 distinct stages: hemostasis, inflammation, proliferation, and remodeling. Hemostasis is the initial stage where the blood vessel undergoes constriction to prevent any further bleeding. The next stage of inflammation is marked by the release of cytokines and the accumulation of macrophages and neutrophils that use proteolytic enzymes to ingest the bacteria to clean the wound debris. The growth of granulation tissue, angiogenesis, fibrogenesis, wound contraction, and re-epithelialization are all included in the proliferation phase. The last stage is the remodeling stage in which the formation of new epithelium and scar tissue takes place along with collagen deposition which plays an important role in strengthening the tissue (3)(4). Traditional wound dressings, which include gauze and bandages, are applied to dry, well-cleaned wounds as part of the passive dressing category. The primary function of gauge dressing is to absorb fluid and other secretions from the wound region due to its fibrous structure. As supplemental wound dressings, bandages are applied. Cotton bandages shed fibers and stick to the surface of wounds, making them unsuitable for cleaning wounds(5). An ideal dressing for a wound should effectively absorb any formed exudates, keep the surrounding tissue moist, and promote healing(6). Modern dressings, with a highly absorbent layer and semi-permeability, have replaced conventional wound dressings. In addition, contemporary wound dressings expedite the development of granular tissue and promote the movement of epithelial cells from the wound edge towards the middle. (7). Tissue engineering research focuses on the use of polymeric biomaterials as matrices or scaffolds for regeneration when combined with natural extracts. Hydrogels are increasingly recognized as highly efficient wound dressing material because of their potential to expedite wound healing, maintain moisture,

biocompatibility, spontaneous degradation, and absorb exudates from wounds (8)(9). As hydrogel film is a cross-linked hydrophilic polymer, its tissue like characteristics i.e. adheres to neighbouring tissue and permits nutrient permeability (10).

Since ancient times, medicinal plants have been utilized to speed up the wound healing process. According to estimates, around 80% of people worldwide receive their primary medical care from traditional herbal medicine (11). *Aloe vera* (AV) is highly medically recognized plant and a member of the Liliaceae family, with *Aloe barbadensis* Miller being the most well-known species. It has a rich history of use in traditional cures spanning thousands of years. The demand for AV products in the global market has been consistently rising owing to its diverse medicinal property. AV extracts are commonly utilized for treating skin lesions because of their therapeutic, pain-relieving, anti-inflammatory, antibacterial, and vasodilatory characteristics (12)(13)(14)(15). The AV plant comprised of approximately 75 possible components, which encompass minerals and phenolics. AV has been extensively researched, with substances such as aloe-emodin, aloesin, aloin, and acemannan being the main focus of many studies. Among these compounds, acemannan has been found to have the ability to stimulate tissue healing (16). Its soothing and hydrating characteristics also render it a prevalent component in skincare and cosmetics, including creams, lotions, and face masks. AV extracts are utilized in the pharmaceutical industry to treat burns, wounds, psoriasis, and gastrointestinal ailments (17). Furthermore, these polysaccharides have emerged as valuable asset in biomedical research because of their inherent capacity to form gels, wide accessibility, and non-toxicity/biocompatibility.

Chitosan (CS) is the only cationic (positively charged) polysaccharide that occurs naturally and is a partly acetylated form of chitin polymer with the presence of amino groups (NH_2) that have the capability of reacting within its structure. CS, as a hydrogel for wound dressing, is one of the most explored natural derivative polymers because of its exceptional qualities,

which include biocompatibility, biodegradability, antibacterial activity, adhesion, non-allergenicity, and reactivity (18). Positively charged groups present in CS can react with negatively charged components present in the microbial cell membrane that are responsible for CS's antibacterial activity (19). As CS contains hydrophilic amino groups, it could promote fibrinogen adsorption, enhancing platelet adhesion and thrombosis. Thus, it is often used in hemostatic dressings(20). Furthermore, CS applied to the wound surfaces may stimulate the growth of new cells as well as the synthesis and deposition of collagen and hyaluronic acid (HA) (21). CS, although abundant in advantages, has limited applications because of its poor mechanical qualities and low water solubility. Several approaches have been made to overcome these challenges, such as developing a synthetic version of CS or changing its functional groups (22). Furthermore, combining CS with synthetic polymers like polyvinyl alcohol (PVA) improves the mechanical strength and stability of hydrogel films utilized in biomedical fields, especially in processes such as wound repairing (23). PVA is a vinyl polymer that has C-C backbone. It is soluble in water and is biodegradable. Additionally, it exhibits excellent biocompatibility and can self-crosslink due to the presence of hydroxyl (-OH) groups on its side chains. Gelatin is a highly versatile protein with significant biomedical applications and is obtained from the hydrolysis of collagen undergoing acidic treatment for the production of type A gelatin or basic treatment resulting in the production of type B gelatin. (24). Significantly, gelatin includes sequences that originate from collagen, such as RGD peptides and matrix metalloproteinase-sensitive breakdown sites. The arginine–glycine–aspartate (RGD) sequence present in gelatin serves as an adhesive site for cell proliferation and migration.

In addition, gelatin has various functional groups like primary carboxyl, amine, & hydroxyl groups, which can be changed with other crosslinkers or therapeutic agents. This enhances the versatility of gelatin for use in wound healing and regeneration of tissue regeneration as a

biomaterial (25)(26)(27). Apart from this, gelatin possesses exceptional properties such as excellent film-forming ability (28), biocompatibility, biodegradability, and hemostatic characteristics (29)(30). As a result, gelatin has been extensively used in various wound dressing materials.

As far as the author's understanding and knowledge, currently no existing research on the concurrent incorporation of AV gel into the CS/PVA/gelatin hydrogel film to serve as a wound healing material. Objective of this research is to investigate the impact of incorporating AV at three different concentrations into the blend of CS/PVA/gelatin at two different concentrations of cross-linking agent (i.e. glutaraldehyde) on several aspects of the hydrogel. These features include water adsorption capacity, biodegradation, water vapor permeability, chemical composition, surface morphology, surface wettability, hemocompatibility, antibacterial activity, and cytotoxicity.

CHAPTER 2

Literature review

Hydrogels are gaining remarkable attention in biomedical sciences and their utilization is increasing due to their fascinating physico-chemical characteristics, such as the ability to hold water, flexibility, softness, capacity to degrade naturally, and compatibility with living tissues (31). Many natural & synthetic polymer materials that are extensively investigated for developing hydrogels with specific uses (32). Patel et al. in their review on hydrogels based on biopolymers found that hydrogels have been utilized in multiple fields. Hydrogels are useful in wound healing as they operate as effective dressings by retaining moisture, preventing infections, and facilitating the healing process. Additionally, they possess antibacterial properties that aid in supporting the cellular activities required for tissue repair. They provide an ideal environment for cell proliferation, adhesion, migration, and differentiation, that are crucial for the development of new bone tissue during bone regeneration. Due to their significant swelling and smart reactivity, they are ideal for drug delivery applications. This allows the controlled release of therapeutic molecules in response to specific stimuli, leading to enhanced therapeutic efficacy and reduced side effects. Biopolymer-based hydrogels are used in bio-imaging as contrast agents or carriers for fluorescent dyes. They help to visualize biological processes and monitor the progression of diseases or the response to treatment. Hydrogels can be designed for therapy, especially in cancer treatment, to release drugs at targeted sites and alter the tumor microenvironment to improve the effectiveness of the treatment. In tissue engineering, hydrogels serve as scaffolds that can replicate the extra-cellular matrix (ECM) to facilitate growth and differentiation of the cells into functional tissues. The various uses of biopolymer-based hydrogels are advantageous because of their physico-chemical qualities, including biocompatibility, biodegradability, and the capacity to replicate the microenvironments of natural tissues(32).

Hydrogels in wound healing

Zhang et al. (2021) synthesized hydrogels using sodium alginate, CS oligosaccharides, and zinc oxide. These hydrogels were designed to accelerate wound healing. Fabricated hydrogels showed high porosity and capacity for swelling. Hydrogels exhibited biocompatibility as they did not cause any damage to blood cells, 293 T cells, (epithelial-like cells isolated from the kidney) and 3T3 cells (cell lines of mouse embryonic fibroblasts). The rats treated with hydrogels during pre-clinical trials had superior wound healing responses in comparison to the control group, indicating higher wound healing capability attributed to increased cellular activity. Hydrogels showed antibacterial activity against both gram negative (*E. coli)* and gram positive *(C. albicans, S. aureus,* and, *B. subtilis)* and provided defence against these microorganisms during the healing process of the wound (33).

Sadeghi et al. (2023) synthesized hydrogel scaffolds using a combination of gelatin and sulfated alginate to treat chronic diabetic wounds. The scaffolds had no detrimental effects on cells, rather viability of the cells increased with a higher concentration of alginate, indicating their biocompatibility. *In-vivo* experiments demonstrated that hydrogels facilitated more uniform distribution of collagen tissue, improved the formation of the epidermal layer, and reduced immunogenic response(34).

Cao et al. (2023) conducted a study where they developed hydrogels for wound healing using natural polymers. They choose natural polymers since they are readily accessible and compatible with living organisms. The CPT (chitin/polyethylene glycol diglycidyl ether/tannic acid) hydrogels were synthesized and studies showed that hydrogels exhibited remarkable porosity, interconnected networks, and improved mechanical strength. Furthermore, they exhibited no cytotoxic properties, thereby verifying their biocompatibility. The CPT hydrogels exhibited antibacterial, antioxidant, and hemostatic characteristics as well. The hydrogels showed superior efficacy in promoting wound healing in rats compared

to other groups, which can be attributed to their enhanced cellular activity. The *in-vivo* results revealed that the development of a thicker layer of epidermis and a homogenous arrangement of collagen tissues (35).

Song et al. (2022) synthesized bioactive hydrogels by incorporating CS-based metal-organic polyhedrons and enzymes for application in wound healing. The hydrogels were made by combining glucose oxidase, vanadium metal organic polyhedrons, CS, and cross-linking them with glutaraldehyde. The hydrogels synthesized exhibited notable antibacterial properties against *E. coli* and *S. aureus*, which can be attributed to their capacity to produce hydroxyl radicals when glucose is present. Moreover, these hydrogels exhibited biocompatibility, demonstrating no harmful effects on cells, and efficiently facilitated the process of wound healing (36).

Additional instances of hydrogels specifically engineered for wound healing purposes, demonstrate a range of advantageous characteristics designed to optimize the healing process, including Peng et al. (2023) who developed hydrogels using CS/polyacrylamide/poly (N-isopropyl acrylamide-co-acrylic acid) that possess advantageous properties such as temperature and pH stimulated drug release, enhanced mechanical properties and adhesion, and significant antibacterial properties(37). Similarly, Yu et al. (2022) developed hydrogels composed of silk fibroin, hyaluronic acid, and bioactive glass. These hydrogels exhibited improved mechanical properties, enhanced cell growth and proliferation, and accelerated cell migration(38). Patel et al., (2022) developed hydrogels made of carboxymethyl and nanocellulose. These hydrogels exhibited enhanced viscoelasticity, injectability, adhesion, biocompatibility, and antibacterial effects. As a result, they enhanced the expression of gene markers associated to skin(39). Pilipenko et al. (2023) synthesized gelatin/nanocellulose hydrogels enriched with dopamine and iron. Dopamine was used to augment the process of crosslinking and assist the binding of gentamicin whereas, iron ions were incorporated to

permit a dynamic exchange that would result in controlled release of the antibiotic and boost its antibacterial effectiveness. These hydrogels demonstrated both biocompatibility and antibacterial characteristics (40). Jiang et al. (2023) from their study presented that arginine/silver hydrogels exhibited biocompatibility, antibacterial properties, and anti-inflammatory effects. These hydrogels have been shown to stimulate blood vessel formation (angiogenesis) and deposition of collagen (41). Deng et al. (2023) synthesized bacterial cellulose hydrogels composed of silver loaded zeolitic imidazolate framework 8 (ZIP 8), and dopamine. These hydrogels exhibited biocompatibility, antibacterial properties, near-infrared (NIR) responsiveness, and improved angiogenesis (42). Li et al. (2023) developed hydrogels by combining sodium alginate and polydopamine. These hydrogels are capable of responding to changes in pH and near-infrared (NIR) light, allowing for controlled drug release. The hydrogels were found to enhance cell growth and movement, and they demonstrated effectiveness in suppressing melanoma and boosting wound healing (43). Jing et al. (2019) created hydrogels using tannic acid, silk fibroin, and iron oxide. These hydrogels exhibited rapid gelation, shape-adaptive characteristics, improved adhesion, antioxidation and anti-bacterial capabilities (44).

Hydrogels in bone regeneration

Pu et al. (2022) synthesized 3D-printed hydrogels using a combination of gelatin methacrylate and hydroxyapatite for bone tissue regeneration. The hydrogels demonstrated good mechanical strength, excellent swelling capabilities, and slow degrading properties. Osteogenesis and angiogenesis were detected within one week of implantation, indicating their ability to generate new bone and blood vessels. Approximately 73% of a cranial lesion was successfully repaired with newly formed bone tissue within a 15-week after implantation (28).

Jiang et al. (2023) synthesized injectable hydrogels made of chitin and CS that include cells, promoting bone regeneration. These hydrogels possess self-healing, adhesive, and biodegradable properties, which makes them compatible with bone marrow derived mesenchymal stem cells (BMSCs) without causing adverse effects. They effectively reproduced the microenvironments of the extracellular matrix (ECM) that are present in natural tissues. Following 8 weeks of implantation, the hydrogels filled with BMSCs showed a remarkable ability to induce osteogenesis and reconstructed bone tissue.

Hydrogels in drug delivery

Stimuli-responsive hydrogels have gained considerable attention for their capacity to transport bioactive compounds, due to their high loading capability and regulated release processes(37). Hoang et al. (2022) developed CS/alginate hydrogels that are cross-linked in two ways, with the purpose of facilitating drug delivery that responds to changes in pH. The hydrogels showed a drug loading capacity of 44% (w/w) for hydrophobic drugs and showed no toxicity on HEK 293 cells (cell line isolated from kidney of a human embryo), indicating their compatibility with living cells. The drug release profile exhibited pH-dependent characteristics, with a gradual release detected at a pH of 2.2 and a fast release at a pH 7.4.

This demonstrates the pH-sensitive nature of the drug release behaviour in the hydrogels. Differential release behaviour was seen due to the development of hydrogen bonds (H-bonds) between the carboxyl and hydroxyl groups present in the polymer chains at low pH. These interactions impede the penetration of liquid and hinder the release process. On the other hand, when the pH levels are higher, the alginate chains elongate because of the electrostatic repulsion between COO^- functional group. This results in improved liquid penetration and faster release of drugs (45).

Zong et al. designed smart hydrogels that are thermo-responsive and redox-responsive for drug delivery utilizing oxidized nanocellulose (46). They developed a hydrogel with enhanced compressive strength by adding 2,2,6,6-tetramethylpiperidine-1-oxyl oxidized cellulose nanofibers and incorporating N, N′-bis(acryloyl) cystamine as crosslinker and for redox responsiveness in temperature-sensitive N-isopropyl acrylamide hydrogel as it has low strength. The drug release rate of hydrogel is controlled by temperature and reducing environments, showing significant swelling and transparency changes between 30–40 °C and low cytotoxicity.

Zhou et al. (2023) developed a hydrogel composed of hyaluronic acid & polylysine. This hydrogel showed notable effects in reducing inflammation, promoting collagen deposition, and exhibiting antibacterial and hemostatic properties. These findings highlight the possibility of this hydrogel for utilization in drug delivery applications (47).

CHAPTER 3

Materials and methods

Materials

CS (Molecular weight: medium, degree of deacetylation: 90%) was sourced from Sigma-Aldrich Company (USA). PVA (average M_w= 115000 g/mol, 98-99% hydrolyzed) supplied by Loba Chemie Private Limited. Gelatin from porcine skin (type B, Bloom 120g) was sourced from Sisco Research Laboratories Private Limited. Glutaraldehyde (25%) as cross-linker, glycerol as plasticizer and Phosphate Buffer Saline (PBS) were all obtained from Merck Life Science Private Limited. Fresh AV (*Aloe barbadensis* Miller*)* leaves obtained from the garden of medicinal plants and facilitation center Ramakrishna Mission Vidyalaya, Narendrapur. L-929 is the mouse fibroblast cell line that was obtained from NCCS Pune and *S. aureus* and *E. coli* were cultured in the microbiology laboratory of the School of Biosciences and Engineering, Jadavpur University.

Aloe vera gel extraction

AV gel was obtained using fresh leaves. Leaves were washed rigorously under the tap water to remove the dirt present and also to remove aloin completely. It was washed again with the distilled water. After this, the outer skin (green) was peeled off. The inner parenchyma gel was extracted and homogenized using a blender to obtain a clear liquid gel and stored in refrigerator at 4 °C until further use.

Hydrogel film preparation

At first CS solution (2% w/v) was prepared by mixing CS powder into the solvent (acetic acid) (3% v/v)**,** and stirring for 4 h at 500 rpm to obtain a complete uniform solution. Concurrently, a (5% w/v) PVA solution was made by mixing PVA in solvent (distilled water) and stirring at 500 rpm using a magnetic stirrer, and maintaining a temperature of 85°C until a clear solution was achieved. Likewise, a gelatin solution (5% w/v) was prepared by mixing gelatin powder in solvent (distilled water) and stirring for 20 minutes to ensure thorough

homogenization. Freshly prepared AV gel was prepared and kept in the refrigerator. Subsequently, a blend of CS, gelatin and PVA in a ratio of 2:1:1 respectively was prepared (48). The composite solution was subjected to further homogenization in a magnetic stirrer for 15 minutes. At this stage, AV gel was added at varying concentrations of 25%, 50%, and 75% of the total polymer solution(49). Cross-linking of the hydrogel was facilitated through the incorporation of glutaraldehyde (0.5% and 1% v/v), which was an essential step in improving the hydrogel's structural integrity, and mixed it for another one h at 400 rpm. To overcome the brittleness of the hydrogel and increase its flexibility thereby increasing folding endurance, glycerol as plasticizer was added at a concentration of 25% of the total polymer weight. Finally, the mixture was cast into a petri plate and subjected to incubation until complete drying, using the solvent casting method.

Sample name	**CS/PVA/Gelatin**	**AV (% of total polymer soln.)**	**Cross-linker (Glutaraldehyde %)**
Alo25|G0.5	2:1:1	25%	0.5%
Alo50|G0.5		50%	
Alo75|G0.5		75%	
Alo25|G1		25%	1%
Alo50|G1		50%	
Alo75|G1		75%	

Table 1: Tabular representation of the component composition present in hydrogel film.

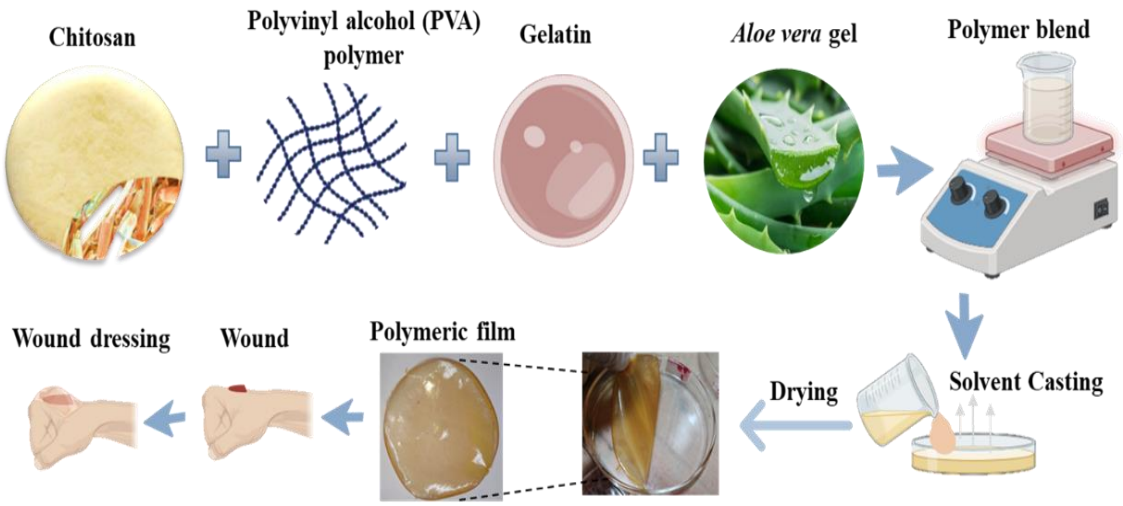


Figure 1: Schematic representation of the hydrogel preparation to its potential application.

Characterization

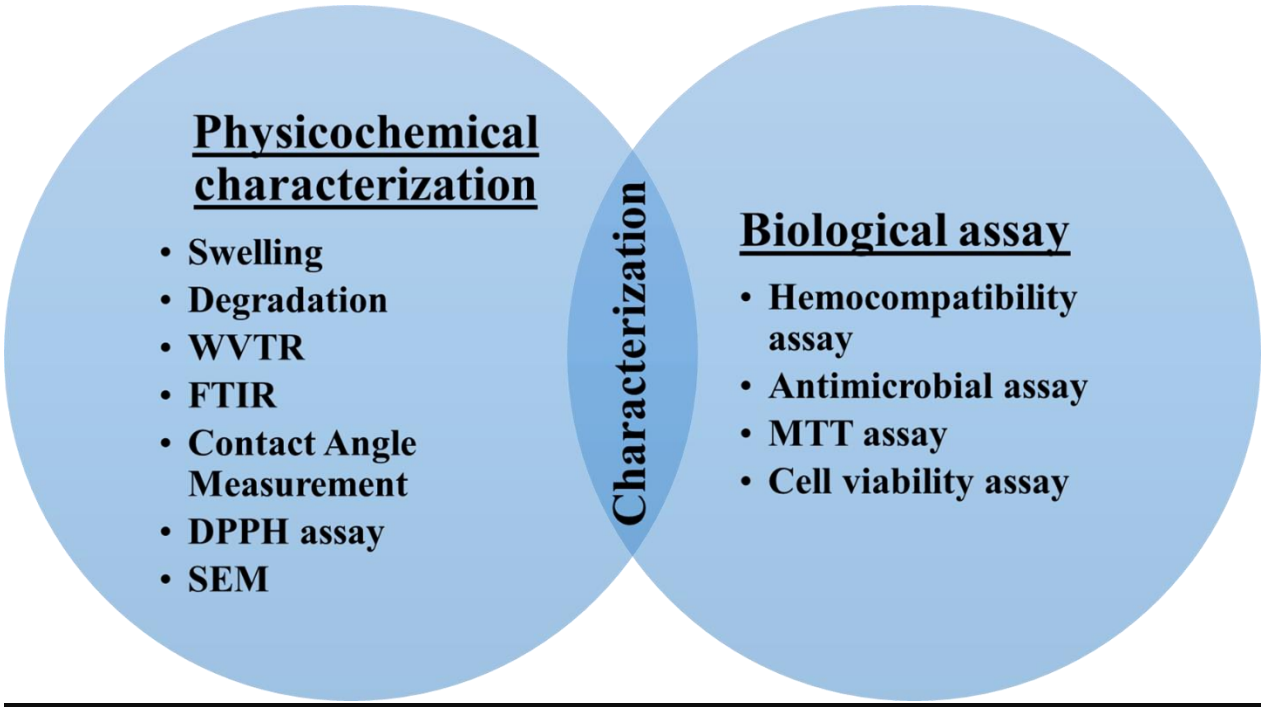


Figure 2: Different physico-chemical and biological assays

Swelling

The hydrogel samples were cut into square specimens ($1cm^2$) and dried entirely in the incubator at 60 °C to remove any moisture content if present to determine the swelling ratio of the hydrogel film samples. After that, the hydrogel samples were weighed and put in a 5 mL PBS at 37 °C with a pH of 7.4. At various intervals, the films were carefully taken out from the buffer solution and blotted to remove excess water using filter paper. The hydrogels were then weighed then put back in the same Petri plate. Until the weight of the samples reached a constant value, this process was repeated.

The swelling percentage (S) is calculated using the formula:

$$\text{Swelling (\%)} = \frac{\text{Initial weight (W0)} - \text{Final weight(W1)}}{\text{Initial weight (W0)}} \text{ X } 100$$

where, w_0 is for the initial weight of the sample and w_1 is for the final weight of the sample.

pH dependent swelling

Cut the hydrogel samples into uniform sizes of 1 cm^2 and measure the initial weight (W0) of each sample. Immerse each hydrogel sample in 5 mL of pH 4 buffer. Similarly immerse another group of samples in pH 7 buffer and pH 9 buffer solutions. Allow all samples to undergo swelling at room temperature for a predetermined duration until they reach a stable weight. Take the hydrogel samples from the buffer solutions, blot them to draw out any excess water content on the surface, and measure the weight of each swelled sample (W1). Subsequently, calculate using the above formula.

Degradation

Dry hydrogel samples were cut into square specimens of 1cm^2 and placed in PBS at 37°C. Then at different intervals of day 1, 3, 7, 14, 21 and 28th the hydrogel samples were removed from the PBS solution. The samples were dried in the incubator at 60°C until achieved a constant weight. The dried hydrogels were re-weighed. To calculate the degradation (%) of hydrogel, the following equation is used:

$$\text{Degradation (\%)} = \frac{\text{Initial weight (W0)} - \text{Final weight (W1)}}{\text{Initial weight (W0)}} \text{ X } 100$$

Where, W0 is the initial dry weight and W1 is the final weight of the sample.

Water vapor transmission rate (WVTR)

Using the European Pharmacopoeia (EP) standard, WVTR was computed (50)(51). Hydrogel samples were trimmed into a circular shape of 12 mm radius and attached to the aperture of a vial with an internal diameter of 20.3 mm, which contains 20 mL of distilled water, and secured with paraffin. Initial weight of the vials was recorded, after which they were stored in the incubator at a temperature of 37°C for 24 h. Subsequently, the final weights of the vials were recorded. WVTR was evaluated using the following equation:

$$\mathrm{WVTR}\left(\frac{\mathrm{g}}{\mathrm{m^2 day}}\right) = \frac{\text{Initial weight (W0)} - \text{Final weight (W1)}}{\text{Permeation area (A)}}$$

Where, W0 is the initial weight and W1 is the final weight while A is the permeation area of the vial.

Antioxidant assay

The hydrogel film's antioxidant activity was measured by measuring the reduction in absorbance of the free DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical. Thirty milligrams of each sample were added to 1 mL of DPPH solution in solvent (methanol) and incubated at room temperature in a place away from light as it is light sensitive for half an hour. After the incubation period the absorbance was measured at 517 nm with the utilization of the supernatant. This experimental procedure allowed for the evaluation of the antioxidant potential of the hydrogel formulations. Radical scavenging activity (%) can be calculated as follows:

$$\text{Radical scavenging activity}(\%) = \frac{\mathrm{OD(Control)} - \mathrm{OD\ (Sample)}}{\mathrm{OD(Control)}}\ \mathrm{X}\ 100$$

Contact angle measurement

The contact angle of the hydrogel was determined using a SURFECTENS 4.5 surface tensiometer from OEG GmbH, Germany. Hydrogel hydrophilicity or wettability was evaluated by depositing 10µL droplets (distilled water) onto the samples utilizing the sessile drop method. Images of the droplets on the hydrogels were captured with an image analyzer, and the mean values were calculated.

Fourier-Transform Infrared spectroscopy (FTIR)

FTIR spectroscopy was employed to examine the functional groups and interactions among all components of the hydrogel films that were synthesized using Thermo NICOLET -IS-50 instrument. The FTIR analysis was conducted with a resolution of 4 cm^{-1} at a scan rate of 60 scans with a wavenumber ranging from 400 to 4000 cm^{-1}.

SEM morphology

Using a scanning electron microscope (ZEISS EVO 50) and an accelerated voltage of 15 kV, morphological analysis of hydrogel films was carried out. Every hydrogel sample was placed onto the stage of metallic stub, secured with the help of double-sided tape, and then covered in a layer of gold which is known as gold sputtering.

Hemocompatibility

To assess the haemolytic activity of the hydrogel, hemocompatibility assay was performed. With a few minor modifications, this experiment was carried out in accordance with ISO 10993-4 criteria (35). 5 mL of fresh goat blood collected in an EDTA vial. 0.9% normal saline was prepared and it was treated as negative control. 0.1%(w/v) Na_2CO_3 was treated as positive control. Blood was mixed with saline in a ratio of 2:2.25. Samples were put in a tube

& 1 mL of normal saline was added and incubated for 30 minutes at 37°C. 0.02 mL of blood was added to each sample tube and centrifuged for 10 minutes at 3000 rpm. Haemolysis was measured by recording the absorbance of the supernatant collected at 540 nm including positive and negative control. Haemolysis (%) can be calculated using the formula:

$$\text{Hemolysis}(\%) = \frac{\text{OD(Sample)} - \text{OD (Negative control)}}{\text{OD(Positive control)} - \text{OD(Negative control)}} \text{ X } 100$$

Antibacterial assay

The antibacterial efficacy of the synthesized hydrogels was assessed utilising the direct colony count method with Luria broth (LB) and agar medium, against *S. aureus* and *E. coli*(52). Each specimen was initially cut into a square shape of 1 cm^2, positioned within a sterilized petri dish, and subsequently subjected to a 20-minute exposure to ultraviolet (UV) radiation. An aseptic LB culture of *E. coli* was grown overnight at 200 rpm and a temperature of 37°C. The bacterial suspension was diluted until its optical density reached 0.4-0.5 at 600 nm to standardize turbidity. The aseptic samples were put in 12-well polystyrene plate. Bacterial suspension of 200 µL was introduced to each sample. The plates were incubated at a temperature of 37 °C for two hours. Subsequently, the films underwent multiple washes with a 0.9% saline solution (sterile) to eliminate the non-adherent bacteria following the removal of the cell suspension. The films were transferred to a centrifuge tube containing 2 mL of saline solution. The tubes underwent bath sonication for ten minutes to eliminate the bacteria that had clung to the surface of the hydrogel film. The bacterial suspension underwent dilution and was thoroughly mixed for 10 seconds using a vortex mixer. Each diluted *E. coli* suspension was placed onto LB agar plates in 70 µL volumes, followed by incubation at 37 °C for 24 h. The *E. coli* colonies on agar plates were quantified and

documented using photography. Furthermore, the experiment was replicated for *S. aureus* as well. The data indicate the mean colony count obtained from three separate plates for each sample of hydrogel film.

MTT assay

The hydrogel samples were at first sterilized using 70% ethanol and UV treatment in a sterile condition in a laminar flow hood for the *in-vitro* cell compatibility experiments. The compatibility of the cells was assessed using an extraction test in line with ISO standard 10993-5. Hydrogel samples labelled as Alo25|G0.5, Alo50|G0.5, Alo75|G0.5, Alo25|G1, Alo50G1 and Alo75|G1 were attached to cell crown and placed in a well plate. Five milliliters of DMEM medium was put in the well and kept at a temperature of 37 °C. 25,000 cells/well were placed in each well containing hydrogels and incubated for 24 h. Cell viability was evaluated using the MTT test, using the established protocol (53). Following a 24 h incubation period, the medium was extracted and 100 μL of MTT solution (5 mg/mL) was introduced into each well. The samples were then incubated at a temperature of 37 °C for 3 h. Following that, 100 μL DMSO was put into each well to dissolve the formazan crystals as they are not soluble. The measurement of absorbance (OD) was conducted at 570 nm. The cell viability percentage is computed as follows:

$$\text{Cell viability (\%)} = \frac{\text{OD value of sample}}{\text{OD value of negative control}}$$

Cell culture

L929 mouse fibroblast cell line was cultured in Dulbecco's Modified Eagle Media (DMEM) containing 10% Fetal Bovine Serum (FBS) and 1% antibiotics (Penicillin, Streptomycin, and amphotericin B), in a 5% CO_2 environment, at a temperature of 37 °C. Each well of a 96-well tissue culture plate (Nunc, Thermofisher) was seeded with 25,000 cells in 200 μL of the medium.

Cell Viability assay

The hydrogel samples were evaluated for cell viability using the FDA (Fluorescein Diacetate Assay) staining technique. At first, it is necessary to sterilize the hydrogel samples by subjecting them to UV radiation for 1 h on each side before conducting the FDA. L-929 mouse fibroblast cells (25,000 cells/well) were placed in a 24-well plate and they were let to adhere by incubating at 37 ℃ accompanied by 5% CO2 for 24 h. Sterilized samples were immersed in the wells containing cells, ensuring they were fully submerged in the DMEM medium. And was supplemented with 10% FBS and 1% antibiotics. Then the cells were cultured with the hydrogels for 1, 2, and 3 days. At each interval, the medium was extracted, cells were cleansed with PBS, and 100µL of FDA solution (10mg/ml) was introduced into each well. The plate was placed in the incubator at 37°C for 5 minutes, ensuring that it is kept in a dark environment. Cells were rinsed with PBS to eliminate any surplus FDA and visualized at 465 nm filter under the fluorescence microscope. The experiment was performed in triplicate to ensure statistical reliability.

Statistical analysis

The study was conducted in triplicates. The mean ± standard deviation and its statistical significance ($*p < 0.05$) of the mean comparison were assessed and examined using several approaches such as the student's t-test and Tukey's multiple comparison test. The graphs were prepared using the OriginPro software program.

CHAPTER 4

Results and Discussion

Physico-chemical characterization

Swelling study in PBS

The water retention ability of hydrogel films has a significant impact on their swelling capacity, which is essential for wound healing and other therapeutic applications (54). Hydrogels have the ability to absorb wound exudate, which accelerates the healing process by causing them to swell. The swelling properties of hydrogels are influenced by various factors, such as the presence of hydrophilic groups, the degree of cross-linking, the flexibility of the polymer structure, and the pH and temperature of the swelling medium. Consequently, any alterations in the hydrogel network structure will have a direct effect on its swelling properties (55). The results gathered on the swelling behavior of films with different concentrations of AV (25%, 50%, 75%) and varying degrees of glutaraldehyde cross-linking (0.5%, 1%) showed distinct trends. After 15 minutes, all samples exhibit significant swelling. However, the samples Alo25|G0.5 and Alo25|G1 containing 25% AV with 0.5% glutaraldehyde and 1% glutaraldehyde show more initial swelling because AV has a stronger affinity for water. Over time, the swelling of all samples rises with Alo25|G0.5 hydrogel film reaching a maximum after 180 minutes, which is the best among all samples as rapid absorption of exudates at the wound area will balance a moist environment that facilitates rapid healing. Moreover, its notable ability to swell implies exceptional fluid retention, which aids in maintaining a clean wound area and minimizing the possibility of infection. On the other hand, Alo75|G0.5 and Alo75|G1 hydrogels show the lowest swelling percentages. The decreased swelling may be a result of the increased crosslinking density within the hydrogel network as a higher concentration of AV engages more intermolecular interactions within itself and with the other components present, which forms a more constricted network, limiting water absorption. The AV concentration of 50% exhibits intermediate swelling behavior compared to the other two concentrations. Alo50|G0.5 show better swelling than

Alo50|G1 due to their reduced crosslink density. The observed behaviors can be justified by the fact of the presence of hydrophilic groups in AV, which promote water absorption, and the influence of glutaraldehyde concentration on the crosslink density, which affects the polymer network's capacity to expand and absorb water.

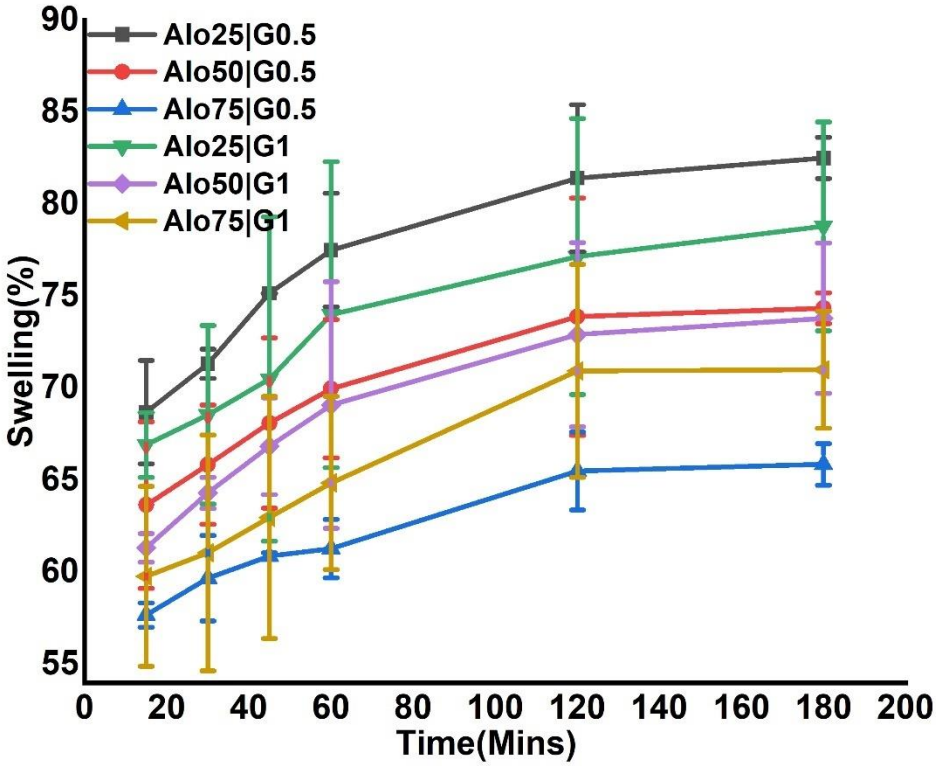


Figure 3: Swelling study of the hydrogel samples

pH-dependent swelling study

Investigating the swelling of films in acidic, neutral, and basic pH buffers is essential for wound dressing applications because it influences the hydrogel's ability to absorb exudate from the wound site, provide a moist environment, and promote healing. Wounds of different types may have different pH levels, and comprehending the swelling behavior of hydrogels in these conditions helps in optimizing their efficiency. Optimal swelling promotes the appropriate absorption of exudate, adhesion to the wound region, and the development of an ideal healing environment (56). The maximum swelling occurs at pH 4, because of the protonation of functional groups, such as amino groups in CS and gelatin, and O-H groups in PVA. Protonation of the hydrogel matrix enhances its hydrophilicity and promotes electrostatic repulsion within the polymer network, leading to increase of absorption. Alo25|G0.5 exhibits the highest percentage of swelling (158.452%) due to the optimal balance between hydrophilicity and network stability. Additionally, the lower cross-linking density (i.e. at 0.5% concentration of glutaraldehyde) permits greater swelling. At a pH of 7, the degree of swelling is moderate due to the partial ionization of the functional groups. This partial ionization preserves a state of equilibrium between the hydrophilicity and the structural integrity of the network. The range of swelling occurring from 96.324% to 133.5%, where Alo25|G0.5 shows the highest swelling, maybe because of increased hydrophilicity of the hydrogel with optimum cross-linking density. The good swelling (%) observed in Alo25|G1 is maybe because of achieving an ideal equilibrium between partial ionization and a stable network facilitated by a higher concentration of glutaraldehyde. At a pH of 9, the swelling of the hydrogel decreases because of the deprotonation of NH_2 groups. This leads to decreased swelling at basic or neutral pH. Maximum swelling was observed in the sample Alo25|G1 while the minimum percentage of swelling was observed in Alo75|G0.5 (57). May be because of poor cross-linking. The increased cross-linking in Alo75|G0.5 at pH 9

counterbalances the reduced hydrophilicity by preserving a more unified structure that is still capable of swelling, albeit to a smaller degree than in lower pH conditions. Figure 4 below compares the swelling percentage at the time when the samples have reached optimal swelling percentage.

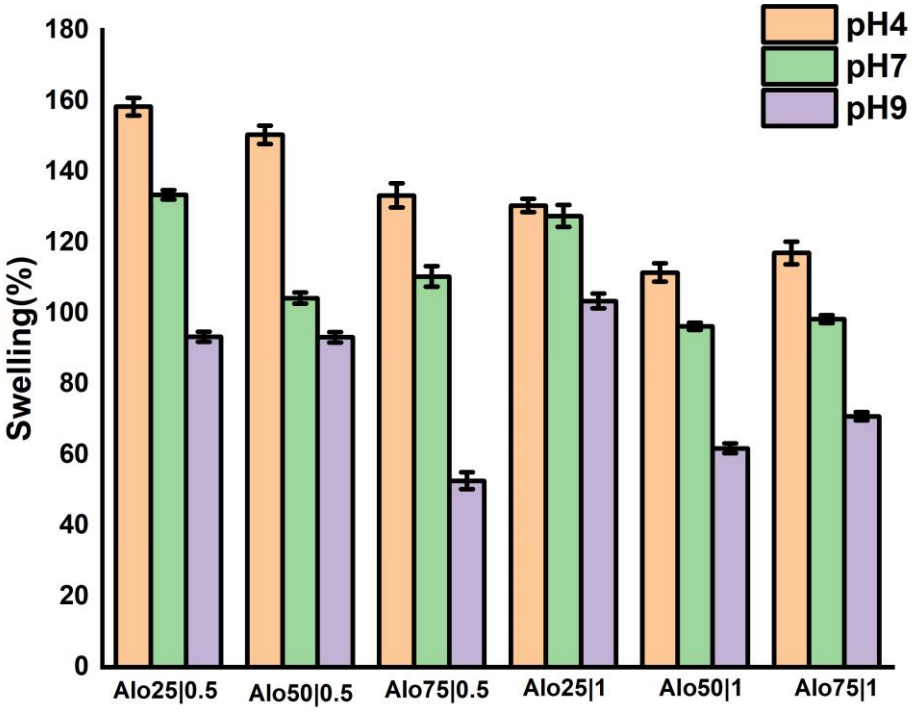


Figure 4: Swelling percentage of hydrogel samples at pH buffer 4, 7 and 9

Degradation study

The biodegradation of hydrogel films is crucial for the proper healing of wounds and ought to correlate with the process of skin regeneration (54). Modern hydrogels, unlike traditional wound dressings like gauze and bandages, necessitate a moist wound environment as upon contact with wound fluid, these hydrogels partially dissolve, leading to the generation of a soluble hydrophilic gel. This gel protects the wound region and promotes the growth of granulation tissue and epithelial cells. This environment helps to avoid cell mortality and expedite the healing process (58) (59). Figure 5 displays the weight loss trends for Alo25|G0.5, Alo50|G0.5, Alo75|G0.5, Alo25|G1, Alo50|G1, and Alo75|G1 hydrogels after a 28-day period of incubation in PBS at 37°C. All the samples demonstrated significant degradation over the initial week, namely from day 1 to day 7. The maximum amount of degradation is observed in Alo75|G0.5 and Alo75|G1 i.e. 61% and 58% respectively. And minimum amount of degradation observed is 42% in Alo25|G1.

The degradation of the hydrogels enhanced by a higher concentration of AV and a decreasing lower concentration of glutaraldehyde. On increasing the concentration of AV to 75% in the hydrogel matrix it results in the incorporation of a greater number of hydrophilic polysaccharides. This leads to improved water absorption and increased vulnerability of the hydrogel to hydrolytic degradation. In addition, the bioactive compounds present in AV accelerate the process of enzymatic breakdown. The enhanced hydrophilicity causes a disturbance in the regularity of the polymer network, resulting in the formation of amorphous areas that are susceptible to degradation. Increasing the glutaraldehyde concentration to 1% leads to the formation of a more compact and rigid network structure thereby making it less prone to degradation. A decrease in the concentration of glutaraldehyde (0.5%) leads to the depletion in the degree of crosslinks, thus making the samples more susceptible to degradation. This is due to an increase in the polymer chain mobility and fluid penetration.

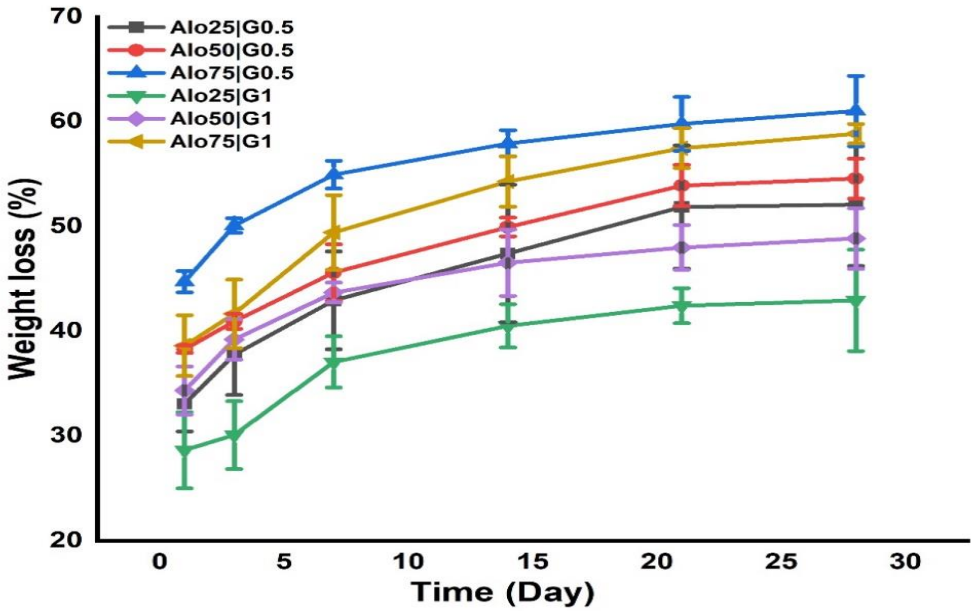


Figure 5: Weight loss (biodegradation) study of hydrogel films

WVTR analysis

In addition to maintaining elevated moisture content between the dressing and the damaged areas, successful wound dressings that utilize films must possess adjustable rates of transmission and absorption. Increased WVTR accelerate the drying of the injured area, perhaps resulting in the formation of scars. Nevertheless, wound dressings with lower WVTRs may render the wound region more vulnerable to excessive exudates, which may increase the infection risk and impede the healing process. An appropriate amount of moisture in the wound environment can be maintained by ensuring that the WVTR value falls within the range of 2000-2500 g/m^2/day (60). Figure 6 indicates the WVTR of the films that were prepared over 24 h. It is evident that the WVTR increases as the concentration of AV increases. In addition, previous studies have demonstrated that AV exhibits excellent water absorption capabilities and a favorable tendency for water transport(61).

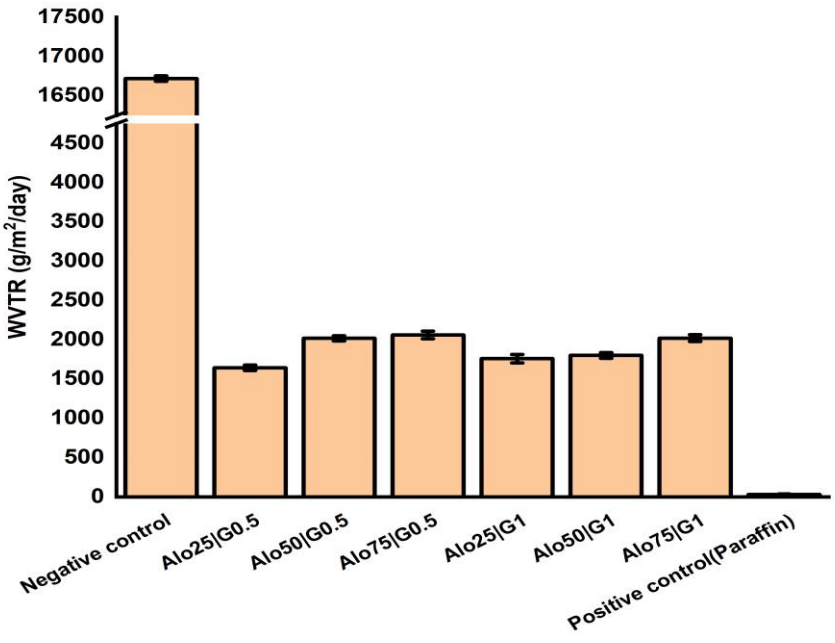


Figure 6: WVTR of the hydrogel films

At higher concentrations of AV, the hydrogel films show a significant increase in WVTR. This may be because of the enhanced hydrophilicity that allows for greater water vapor

diffusion. Both Alo75|G0.5 and Alo75|G1 have impressive WVTR values of 2060.379% and 2020.714%, respectively. These values fall within the optimal range of 2000-2500 g/m²/day, making them ideal for different applications one like wound dressings. On the other hand, when the concentrations of cross-linking are increased, the hydrogel structure becomes more compact, resulting in a slight decrease in the WVTR. This can be observed in the cases of Alo25|G1 and Alo50|G1. When using lower concentrations of 25% and 50% AV, such as in Alo25|G0.5, Alo25|G1, and Alo50|G1, the resulting WVTR values are lower. Specifically, the values are 1644.777%, 1759.365%, and 1801.23%, respectively. This decrease in values can be attributed to the reduced hydrophilicity and increased crosslink density. Therefore, by adjusting the levels of AV content and glutaraldehyde concentration, the WVTR of the hydrogel can be optimized, making it more suitable for medical applications.

SEM analysis

Figure 7 demonstrates the morphological properties of the hydrogel films. An analysis was conducted on the external surface of the material. The samples exhibited compact surfaces with a continuous structure of the films. Alo25|G0.5 exhibits a smooth surface, with some roughness and irregularities. Alo50|G0.5 has a relatively smoother surface compared to Alo25|G0.5. While the Alo75|G0.5 surface has a textured appearance with numerous small pits or dimples. Alo25|G1 has a smooth surface with few particles scattered across. On the other hand, Alo50|G1 shows minor roughness with some particulate matter, and Alo75|G1 has slight roughness and some linear features. The samples exhibit a variety of surface characteristics ranging from smooth to rough textures. Common features include the presence of small particles, cracks, pits, and surface deformations.

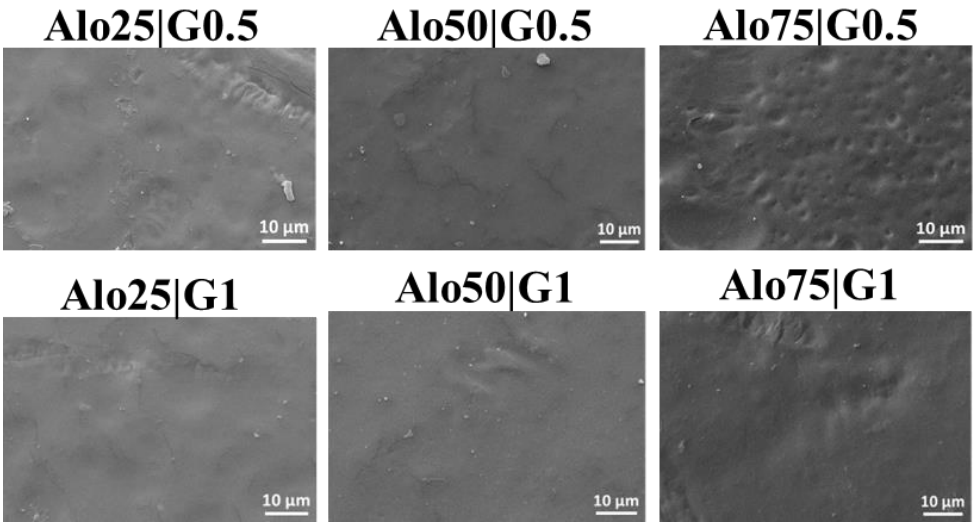


Figure 7: SEM images of hydrogels

Antioxidant assay

The figure 8 illustrates the antioxidant properties of hydrogel films. The determination of this property was accomplished using DPPH assay. Earlier researches has shown the presence of reactive oxygen species (ROS) disrupts the process of wound healing (62). Decreasing the level of ROS promotes the migration of cells and angiogenesis, which enhances the wound healing process (63). ROS accumulation in a wound is mostly caused by a persistent inflammatory response at the wound site, rather than the antioxidant potency of the wound. This inflammatory response hinders the transition from the inflammation phase to the proliferation phase (64).

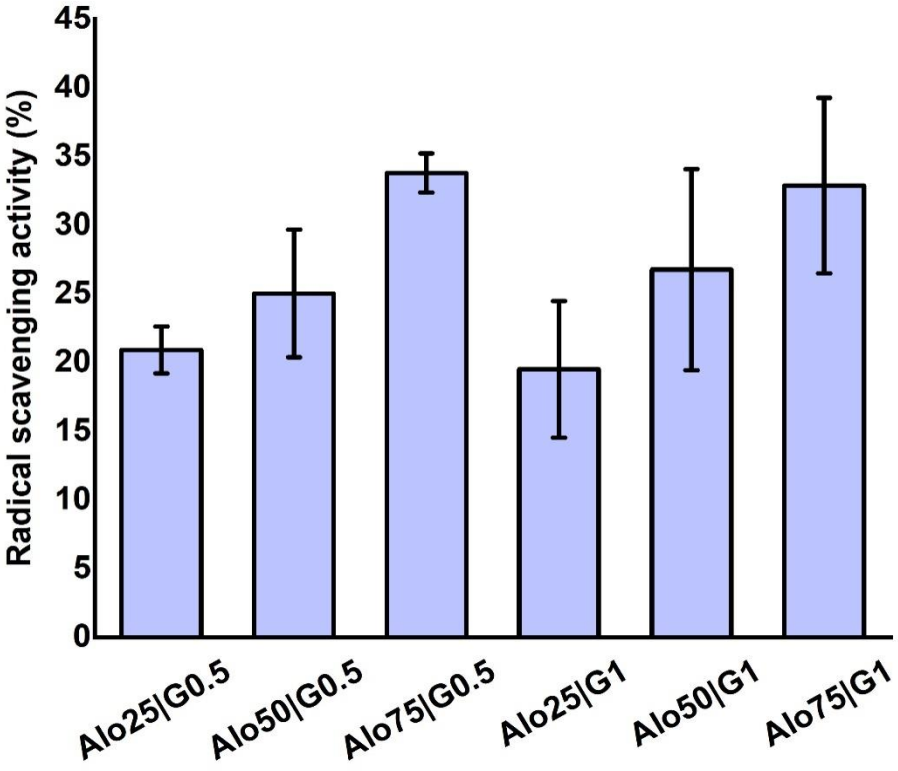


Figure 8: DPPH scavenging activity of the hydrogel samples.

Therefore, by incorporating an antioxidant agent into the wound dressing, it is possible to prevent the redox balance at the wound site. This would help prevent any interference with

the immune response and facilitate the healing process, specifically in chronic wound cases. According to the results obtained from the DPPH·-scavenging assay, the hydrogels Alo25|G0.5, Alo50|G0.5, Alo75|G0.5, Alo25|G1, Alo50|G1, and Alo75|G1 exhibited antioxidant activities of 20.93541±1.710, 25.03784±4.639, 33.81812±1.424, 19.52717±4.976, 26.77416±7.3117, and 32.89039±6.395, respectively. The maximum percentage of scavenging activity is seen in Alo75|G0.5 and Alo75|G1, this is maybe because AV has antioxidant characteristics due to the presence of alkaloids, polyphenols, and indoles (65). A consistent trend was observed in performance based on the concentration of AV.

FTIR Spectroscopy

The hydrogel incorporating AV, CS, PVA, and gelatin has been analyzed using FTIR spectroscopy to compare each of the components with the developed samples. From previous studies, it has been detected that CS exhibits a broad band region at 3441 cm^{-1} (overlapping -NH and -OH stretching) and peaks at 1598 cm^{-1} and 1680 cm^{-1} (amine and amide bending). PVA has broad bands at specific frequencies: 3450 cm^{-1} (O-H stretching), 1635 cm^{-1} for C-O stretching of the acetate group, and 2910 cm^{-1} for aliphatic C-H stretching, among others(54). Gelatin displayed characteristic peaks at 1603 cm^{-1} for C=O stretching and 1469 cm^{-1} (N-H bending) (66).

In contrast, AV exhibited broad bands at 3316cm^{-1} (indicating O-H stretching) due to the presence of carbohydrate monomers, including galacturonic acid, uronic acid, mannose, and, as well as phenolic compounds like aloe-emodin and aloin, in the AV sample (67). 2881 and 2922 cm^{-1} (representing symmetric and asymmetric stretching of -CH2 groups), 1657 cm^{-1} (indicating the presence of C=O bonds), and 1236 cm^{-1} (indicating stretching of C-O bonds in esters and phenols). The hydrogel (combination of CS/PVA/gelatin/AV) exhibited prominent peaks at 3251 cm^{-1}, 2955 cm^{-1}, 1651 cm^{-1}, and 1025 cm^{-1}. The broad peak at 3251 cm^{-1} corresponds to the stretching vibrations of O-H and N-H groups, which result from H-bonding interactions between PVA, CS, AV, and gelatin. Narrow peak observed at 2955 cm^{-1} indicates the presence of aliphatic C-H stretching. The presence of peaks at 1651cm^{-1} can be attributed to the formation of Schiff base. Chitosan undergoes a chemical crosslinking reaction with the glutaraldehyde, forming an imine bond through its primary amino groups. This reaction is known as Schiff's base reaction and requires a reduction step to convert it into a stable bond and enhance its mechanical properties (68).

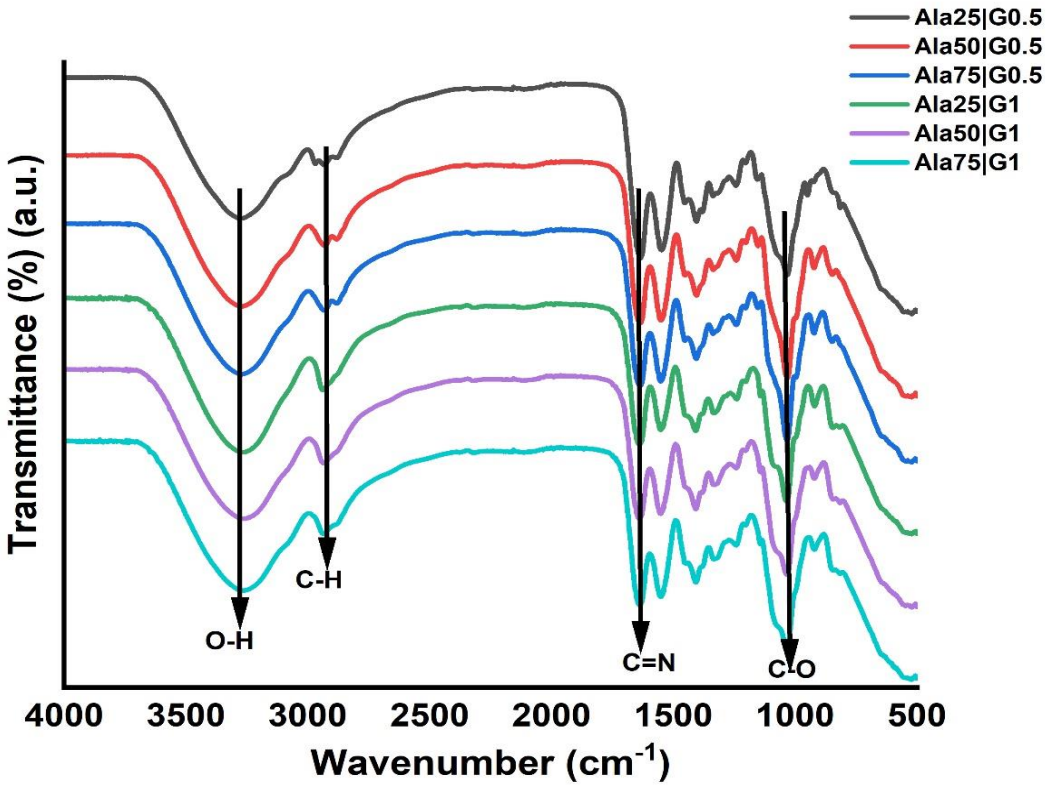


Figure 9: FTIR spectroscopy of AV/CS /PVA/gelatin hydrogel prepared

Conversely, the peak observed at 1025 cm^{-1} indicates the stretching of the C-O bond present in rhamnogalacturonan found in the side chain of pectin which in turn is a polysaccharide and is highly biocompatible and used as a coating material for biomedical applications (69). This outcome was comparable to the findings of prior research reported by Lim et al. (2015)(70). The results indicate robust connections and compatibility among the components, results in the creation of a stable hydrogel network with improved stability, and bioactivity.

Contact angle measurement

It is one of the crucial characterizations in identifying the hydrophilicity or hydrophobicity of hydrogels. This property is necessary for cell adhesion, cell proliferation, enhancing the clinical aesthetics, and speeding up wound closure (16). The wetting behaviour of hydrogel samples (Alo25|G0.5, Alo50|G0.5, Alo75|G0.5, Alo25|G1, Alo50|G1, and Alo75|G1) was investigated by evaluating the water contact angles of the hydrogels. The contact angle formed by Alo75|G0.5 and Alo75|G1 was found to be the lowest and then it gradually increased with decreasing AV concentration. This can be ascribed due to the rise in the density of hydrophilic groups at the surface of the film as the concentration of AV in the hydrogel increases. Therefore, there is a stronger interaction with water, which reduces the contact angle and increases the wettability of the hydrogel. All of the samples exhibited hydrophilic properties and had an angle below 90 degrees. The presence of polar groups in the substance will enhance its adsorption and the non-polar groups in AV will aid the incorporation of the films with proteins and growth factors in the wound. A correlation can be seen among the contact angle measurement and the adsorption of protein, which in turn enhances cell adherence on the material surface.

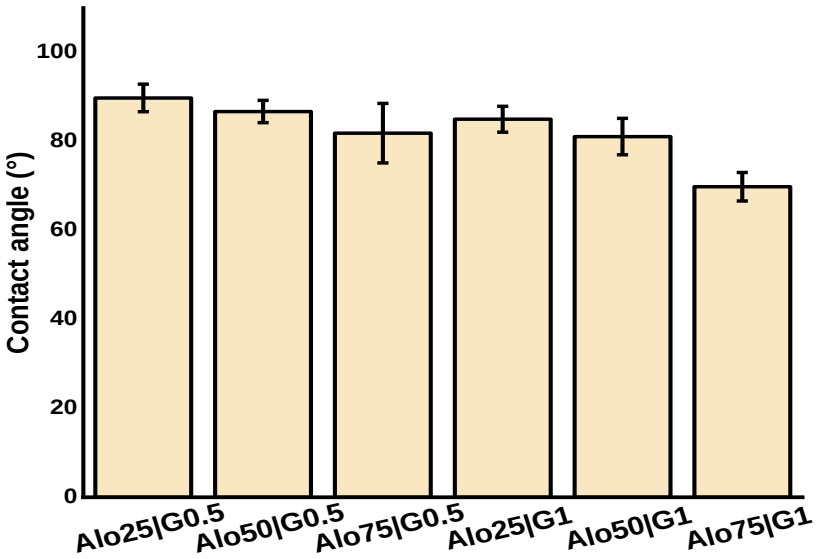


Figure 10A: Contact angle of the hydrogel samples.

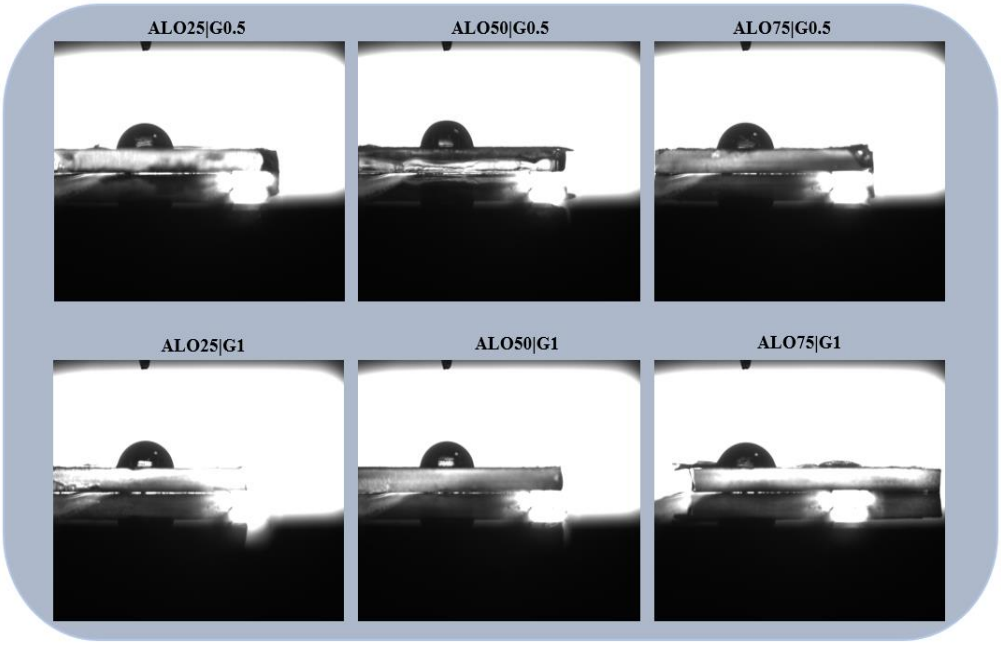


Figure 10B: Contact angle measurement Images obtained from tensiometer of hydrogel samples Alo25|G0.5, Alo50|G0.5, Alo75|G0.5, Alo25|G1, Alo50|G1, and Alo75|G1

Biological characterization

The CS/PVA/gelatin/AV hydrogels comprising three different concentrations of AV (25%, 50%, and 75%) were crosslinked with glutaraldehyde at two different concentrations of 0.5% and 1%. Specifically, the hydrogels designated Alo25|G0.5, Alo50|G0.5, and Alo75|G0.5 were chosen for further *in-vitro* cell culture assays The selection was based on comparative analysis, which indicated that increasing the concentration of glutaraldehyde to 1% had not much significant improvement in the performance of the hydrogels. In addition, the samples crosslinked with 0.5% glutaraldehyde showed slightly superior results in swelling, degradation, water vapor transmission rate (WVTR), and radical scavenging activity. Hence, it is preferable to employ a lower concentration of glutaraldehyde for this wound dressing application.

Hemocompatibility assay

The hemocompatibility of the hydrogel films were assessed using a haemolytic assay in human red blood cells (RBCs), as haemolysis is a simple and reliable method for evaluating the blood compatibility of samples (71)(72). The supernatant collected from Na_2CO_3 (positive control), NaOH (negative control), and hydrogel samples (Alo25|G0.5, Alo50|G0.5, Alo75|G0.5, Alo25|G1, Alo50|G1, and Alo75|G1) was analyzed using UV-Vis spectroscopy for absorbance. The absorbance values were then converted into percentages to represent the level of haemolysis, as shown in Figure 11A. All samples that were tested displayed haemolysis levels below 2%, indicating that there was no substantial release of haemoglobin resulting from the interaction between RBCs and the hydrogel samples.

Haemocompatibility (%)	Haemocompatibility grading criteria
0-2	Non - haemolytic
2-5	Slightly haemolytic
>5	haemolytic

Table 2: Based on the standard categorization system, haemocompatibility grading criteria (73).

The appearance of the supernatant for the hydrogels, saline solution, and Na_2CO_3 (Fig. 11B) supports these observations. All hydrogel samples exhibited a clear supernatant like that of the saline solution, but the Na_2CO_3 (positive control) had a vivid red hue. These results indicate that the hydrogels that were synthesized did not show any haemolytic activity against RBCs. The results indicate that the Alo25|G0.5, Alo50|G0.5, Alo75|G0.5, Alo25|G1,

Alo50|G1, and Alo75|G1 hydrogels exhibit favorable blood compatibility and are suitable for use in clinical applications.

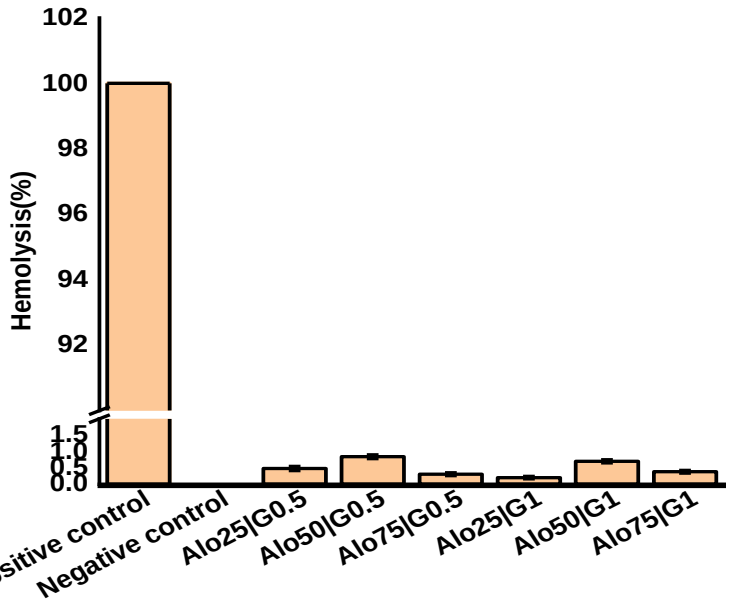


Figure 11A: Representation of the percentage of haemolysis observed following incubation with the positive and negative control and the prepared hydrogel samples

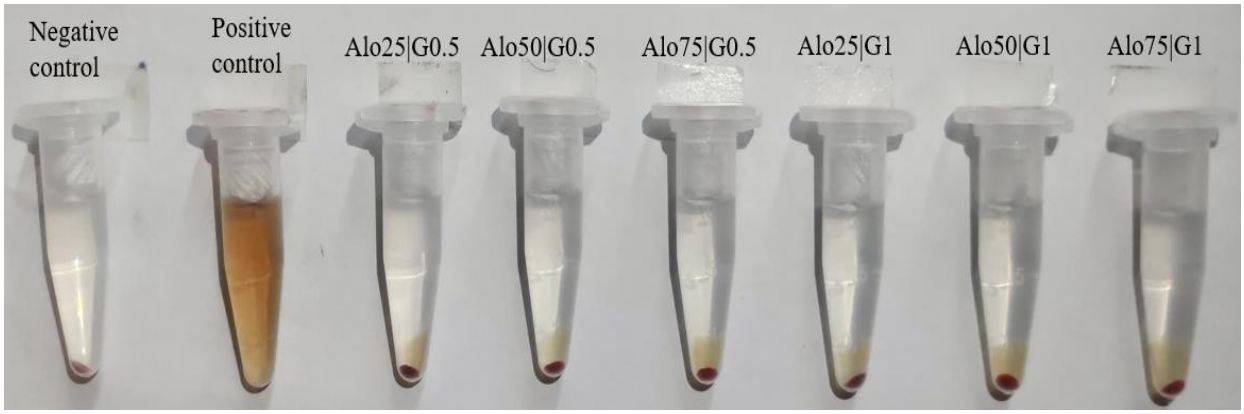


Figure 11B: Image depicting the change of color in the supernatant following centrifugation of the hydrogel films.

Antibacterial activity of hydrogels

Bacterial infections in biomedical applications constitute a major clinical problem as they often lead to implant rejection and the need for recurrent surgical procedures, which can result in consequences. Therefore, biomaterials that cause very little or no harm to human cells and can also reduce bacterial infection by preventing bacterial cell attachment or can kill bacteria, are quite desirable. The antibacterial properties of biomaterials are effected by the chemical and physical characteristics of their surface (74).

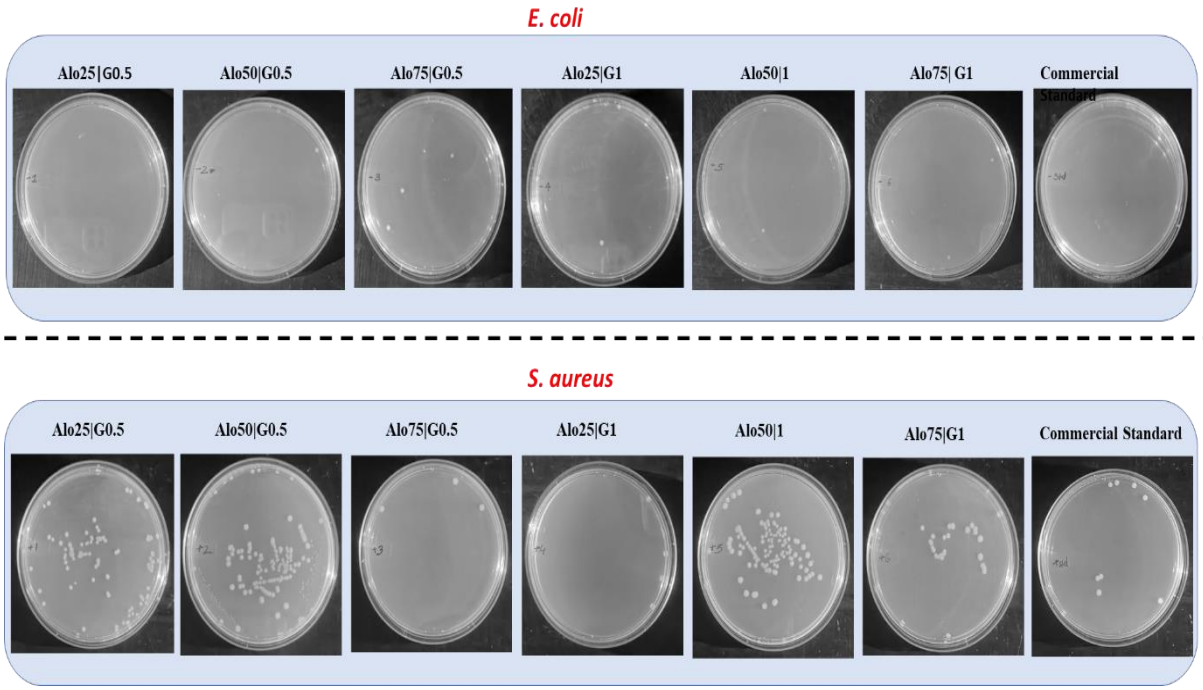


Figure 12: Digital images of viable E. coli and S. aureus colonies cultured on agar plates.

Studies have demonstrated that chemically altered biomaterial surfaces hinder the adherence of bacteria and the production of biofilms. Figure 12 displays the presence of live bacterial colonies cultured on agar containing plates for Alo25|G0.5, Alo50|G0.5, Alo75|G0.5, Alo25|G1, Alo50|G1, and Alo75|G1 in a direct contact test. The results indicate that the samples showed more potent antibacterial activity against *E. coli* in comparison to *S. aureus*.

The hydrogel samples Alo25|G0.5, Alo50|G0.5, Alo75|G0.5, Alo25|G1, Alo50|G1, and Alo75|G1 all exhibited similar degrees of effectiveness against *E. coli*, indicating significant antibacterial activity with minimal variation in the colony counts. The minor decrease in effectiveness, specifically in Alo50|G0.5 and Alo50|G1 in *S. aureus*, could be attributed to surface property of the material and the dispersion of active substances. However, the hydrogels were still able to successfully decrease the number of *S. aureus* colonies, highlighting their potential for use in a broad range of antimicrobial applications.

MTT assay

Figure 13 displays the results from the cytotoxicity study on hydrogels. The viability of fibroblasts (L929 cell lines) was assessed using MTT assay, following the ISO-10993-5-2009 Standard. As to the ISO criteria, a substance is classified as cytotoxic if the viability percentage of cells exposed to it for 1 day is below 70% (75). The hydrogel samples exhibit values exceeding 80%, surpassing the toxicity threshold. All of the investigated hydrogels exhibited viability values above the established threshold for toxicity.

Cell viability (%)	Cytotoxicity grading criteria
100	0 (non-toxic, qualifying)
99-75	1 (light-toxic, qualifying)
74-50	2 (moderate-toxic, disqualifying)
49-25	3 (severe-toxic, disqualifying)
24-1	4 (disqualifying)
0	5 (disqualifying)

Table 3: Cytotoxicity analysis in accordance with the cytotoxicity grading standards (76).

Therefore, the examined materials can be classified as non-toxic to the cell lines. Also, no significant difference was seen in cell viability between the control and the hydrogel samples. The results of the biocompatibility assay indicates that the wound dressing films (CS/PVA/Gelatin/AV) are non-cytotoxic.

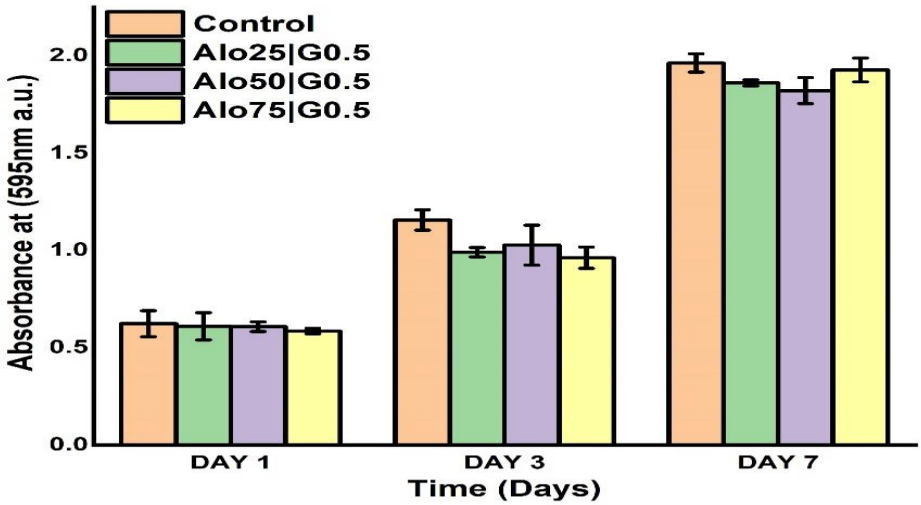


Figure 13: MTT assay of the hydrogel samples

Cell viability of hydrogels

Fluorescein diacetate (FDA) staining was conducted on the hydrogels on days 1, 2, and 3 of incubation to examine the viability and proliferation of live cells on the hydrogel film (Figure 14). The live cells were uniformly dispersed across the whole surface of all three samples, as shown by their green fluorescence. The data presented in the image demonstrates a progressive increase in viable cells count from day 1 to day 3 across all the samples. This indicates that there was an increase in the number of cells during the specified period.and the cells remained alive and functional in the samples. Additionally, it is evident that the cell populations in Alo75|G0.5 and Alo50|G0.5 were greater compared to Alo25|G0.5, hydrogel film. Thus, drawing conclusions from these findings, it may be shown that the CS/PVA/Gelatin/AV hydrogel films created a favourable environment for supporting cell growth. Further increasing the concentration of AV led to enhanced cell proliferation. The cells remained viable and were able to multiply for a longer length of time.

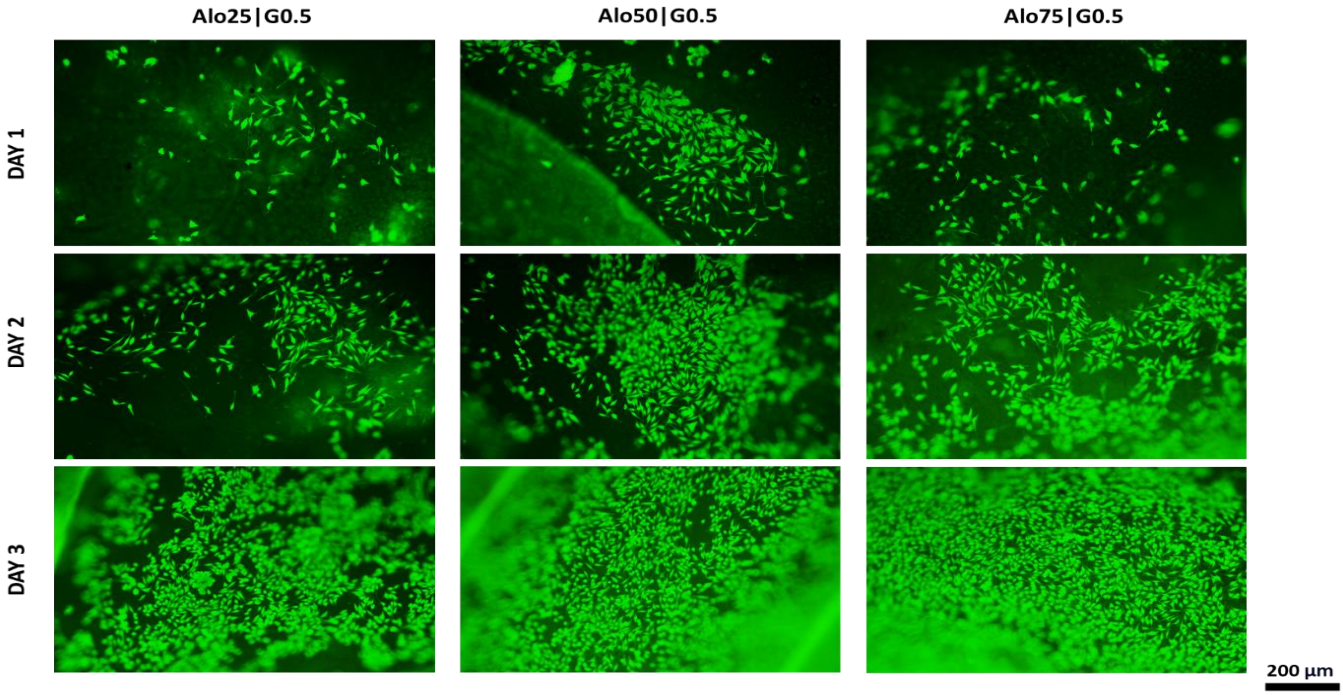


Figure 14: Cell viability assay, green fluorescence implies live cells.

CHAPTER 5

Conclusion

In this study, AV incorporating CS, PVA, gelatin hydrogel films for prospective application as wound dressings were successfully developed. The hydrogels were crosslinked using glutaraldehyde at concentrations of 0.5% and 1%. Among CS/PVA/Gelatin/AV hydrogels containing various concentrations of AV and cross-linkers, 0.5% glutaraldehyde was chosen for additional *in-vitro* cell culture assay as they showed better performance in terms of swelling, degradation, water vapor transmission rate (WVTR), and radical scavenging activity compared to 1% glutaraldehyde containing films.

The swelling studies demonstrated that hydrogels containing higher concentrations of AV displayed increased water absorption, with the Alo25|G0.5 hydrogel exhibiting the maximum swelling percentage. The degradation studies indicated that higher concentrations of AV and 0.5% of cross-linking resulted in enhanced biodegradation. The WVTR analysis showed that hydrogels containing a higher concentration of AV maintained an ideal level of wetness that is good for promoting wound healing. The antioxidant test confirmed the presence of radical scavenging activity, increasing with the increasing concentration of AV.

The use of SEM revealed smooth and continuous surface structure, indicating a consistent morphology. Additionally, FTIR spectroscopy confirmed that AV effectively integrated into the hydrogel matrix. Increasing the content of AV was found to promote hydrophilicity, leading to improved cell adhesion and proliferation, as determined by contact angle measurements. The hemocompatibility assay confirmed that the hydrogels showed no substantial haemolytic activity and antibacterial studies showed that the films were effective against gram-positive and gram-negative bacteria, hence, indicated good antibacterial properties.

Cytotoxicity evaluation of hydrogels assayed using cell viability assay, which showed that higher cell proliferation over time was supported by increased AV content. An extensive

assessment of the physico-chemical and biological characteristics of these hydrogels indicates their outstanding ability to be used as wound dressings. They create an environment that promotes wound healing by effectively retaining moisture, facilitating biodegradation, exhibiting antioxidant activity, and being compatible with living tissues.

CHAPTER 6

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