Development of multifunctional hydrogel loaded with aminoacids as activity enhancers

Manoj Kumar K.A*, Hemananthan E, Renuka Devi P, Vignesh Kumar S

Abstract— In this present work of developing a multifunctional hydrogel material with aminoacids as activity enhancers, a gelatin-based hydrogel using glycerol as a plasticizer was developed in different ratio combinations. Among the combination, two different ratios of Gelatin:glycerol (26:4 & 27:3) which has good physical ability was chosen for further study. Different compositions of amino acids in different concentration were immobilized in the films which show that there was no impact of aminoacid in the morphology of the film. Films were characterized for FTIR and XRD. The surface morphology of the films was analyzed using SEM and AFM. The thermal degradation behavior of the gelatin-hydrogel composite was characterized using thermogravimetric analysis. Our gelatin film has good elongation and stretchability with the water vapour permeability rate in the range of normal human skin. The amino acid release study shows that amino acid release in the water was higher than in the PBS for aminoacid immobilized film at 5 hours of duration. *In-vitro* drug release study results show that there was a gradual drug release from the gelatin film. The films were processed for further swelling and dissolution studies. Swelling study results show that the swelling ratio was higher in water compared to PBS. The dissolution ratio for the films was observed in the following increasing order (blank gelatin film > Amino acid film devoid of glycerol > film devoid of amino acids > Amino acid film using glycerol). Water content evaporation rate was higher for amino acid immobilized film compared to blank gelatin film. Gel fraction percent was higher for blank gelatin film after the particular interval of time (blank gelatin film > Amino acid film devoid of glycerol > Gelatin film devoid of aminoacids > Amino acid film using glycerol). Gelatin film was impermeable to microbes and has antimicrobial activity. The hemolysis rate percent of gelatin film was negligible compared to control. Gelatin film also shows higher radical scavenging activity compared with that of control and has good compatibility with human NIH/3T3 fibroblast cells. This suggests that film can be used as human wound dressing material and study can be further developed to test and use this hydrogel as food packaging material and for seedling growth of food crops.

Index Terms— Hydrogel, gelatin, glycerol, characterization, morphology

1 INTRODUCTION

Hydrogels are vastly hydrophilic macromolecular networks, which are produced by chemical or physical cross-linking of soluble polymers. Peculiar properties of hydrogels such as high-sensitive to physiological environments, hydrophilic nature, soft tissue-like water content, and adequate flexibility, make them excellent candidates for biomedical applications. Hydrogels can swell and de-swell water in a reversible direction, showing specific environmental stimuli-responsive e.g. temperature, pH, and ionic strength [1]. Some hydrogels have antibacterial and antifungal activities and these properties could be useful for wound dressings and accelerating the wound healing process [2].

Hydrogels can be divided into two groups based on the sources from which they are obtained. Natural polymer-based hydrogels and synthetic polymer-based available hydrogel-based products are derived from petroleum-based vinyl monomers, which are very poor in degradability and environment friendliness. Using the concept of green chemistry, environmental protection has been made much stronger with the utilization of biodegradable and renewable biopolymers. The natural polymer has different applications in various fields such nano-drug delivery, Pharmaceutical industry, cosmetics, and gene delivery. In the past few years, research has focused on the use of natural polymers, such as collagen, peptide, and polysaccharides, in the synthesis of hydrogel for targeted applications like tissue engineering, drug delivery, metal ion adsorption, dye removal, and conductive materials [3].

Currently, researches are carried out in natural polymer-based hydrogel materials, which is focused on developing the hydrogel for a particular biological problem. So there was a need and huge space to develop a single polymer-based hydrogel with specific compositions to function for problems arising in different sectors such as agriculture, food industry, biomedical, wound healing, beverage industry, etc.

Drug delivery systems (DDS) are new technologies developed to control the release of therapeutic agents or the delivery location. Polymers have been widely used in drug delivery technology to control drug release. For a drug delivery application, hydrogels and other polymer-based carriers have an important application because of their physicochemical properties and structural characteristics that allow loading drugs and controlling their release [4]. Also, the modern concept of interactive wound dressings envisages a wound healing process in which the bioactive components incorporated in the dressing influence the physiological processes and direct the healing cascade. It has been proved that healing under a wet environment is augmented through easy migration of the extracellular matrix proteins and enhanced activity and availability of growth factors.

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Hydrogels and hydrocolloids are being widely used in this respect to maintain a moist environment at the wound site and aid the healing process. Due to their high water contents, skin-like consistency, and biocompatibility, hydrogels are expected to promote healing and enhance re-epithelialization [5]. Furthermore, such hydrophilic wound dressings would preclude trauma to the patient during ambulation or dressing removal. A hydrogel material that can influence the healing cascade and provide sustained antimicrobial activity is expected to lead to accelerated wound healing [6][7][8]. Apart from the control drug delivery, the hydrogel has been also used as the hydro film in agriculture for the seedling growth of food crops.

Agriculture is an environmental control technology. Although the environments of the aerial part such as temperature, light intensity, and humidity can be controlled, the environment control technology of the underground part has not been developed. This is because the soil which is a central player in the underground part has an unknown background. For example, soil varies dependent upon places, seasons, etc. Furthermore, the fatal problems of soil that salt is seriously accumulated by the lack of water resulting from the current global warming, makes farming deadly difficult. ''Hydrofilm'' is composed of hydrogel which absorbs only water and nutrients such as various ions, amino acids, and sugar but excludes viruses and microbes. Therefore, even if the culture medium is contaminated or decayed, the plants are not diseased. ''Hydrofilm'' minimizes the use of chemicals and also eliminates the need for circulation and sterilization of the culture medium in current hydroponics, leading to a significant reduction in the facilities and the running costs [9]. Despite in both medical and agricultural sectors, there is also a huge space in food packaging industries, where there is a need for bio-packaging material.

Various attempts had been studied to develop food packaging wrapping materials using biopolymers like starch, gum, gelatin, whey protein, chitosan, alginate, pectin, and others as promising biopolymers for many fields of manufacturing hydrogel applications. Packaging hydrogels based on gelatin have been suggested as replaceable materials to petroleum-based plastics. Biopolymer-based packaging is defined as packaging that contains raw materials originating from agricultural and marine sources. There are three such categories of biopolymers: (a) extracted directly from natural raw materials, such as starch, cellulose, protein, and marine prokaryotes; (b) produced by chemical synthesis from bioderived monomers; (c) produced by microorganisms such as hydroxy-butyrate and hydroxy-valerate. The main cause of spoilage of many foods is microbial growth on the product surface. The application of antimicrobial agents to packaging can create an environment inside the package that may delay or even prevent the growth of microorganisms on the product surface anhence, leading to an extension of the shelf life and/or the improved safety of the product. To control undesirable microorganisms in foods during storage and distribution, antimicrobial substances can be either incorporated into food packaging materials or coated onto the surface of food [10].

We aim to develop a multifunctional hydrogel using natural polymer materials with added amino acids as activity enhancers. Our hydrogel is developed using natural polymer gelatin and glycerol as the crosslinker. Gelatin is a natural origin biocompatible and non-cytotoxic material and it shows low immunogenicity compared to the native collagen. This polymer is considered generally recognized as safe (GRAS) by the Food and Drug Administration (FDA). Regarding the physicochemical properties of gelatin, one of the most important properties is the potential to form a thermally reversible network in water. Gelatin is a natural origin biocompatible and non-cytotoxic material and it shows low immunogenicity compared to the native collagen. This polymer is considered generally recognized as safe (GRAS) by the Food and Drug Administration (FDA). Regarding the physicochemical properties of gelatin, one of the most important properties is the potential to form a thermally reversible network in water [11]. Glycerol (1, 2, 3-propanetriol or glycerin) is a transparent, odorless, non-toxic liquid. Glycerol can reduce the freezing point of water, so the glycerol/ water solution is usually used in non-toxic antifreeze. A glycerol/water mixed system was used to prepare antifreeze hydrogel, and the hydrogel can maintain good elasticity and flexibility under a low-temperature environment. Glycerin is a humectant by definition and has been recognized by the U.S. Food and Drug Administration (FDA) [12].

Our single multifunctional hydrogel is to be designed on the fact that they can be utilized in the different fields of industry such as agriculture, biomedical, in the process of wound healing, food industry, and beverage industry. To enhance the functioning of hydrogel, a few of the amino acids are to be loaded into the hydrogel. Overall, this multifunctioning hydrogel saves the usage of more energy resources and reduces the release of toxic waste in the hydrogel manufacturing industry with the capability of solving problems in different biological sectors.

2 Materials and Methods

2.1 Materials

Gelatin, glycerol, and all other amino acids such as L – Lysine, L - Tryptophan, L - Glycine, L - Arginine, L - Proline, L – Glutamine, L - Methionine were also purchased from Himedia Laboratories Pvt. Ltd., India.

2.2 Preparation of Gelatin/Glycerol based Film

Gelatin and the glycerol-based film were formed by the solvent casting method as mentioned below. 10% w/v gelatin was pre-

pared using distilled water. For the complete dissolution of gelatin, the solution was homogenized using a magnetic stirrer at 40°C for 40 minutes. 50% v/v Glycerol was prepared using distilled water. For degassing, gelatin was centrifuged at 5000 rpm for 5 minutes. After centrifugation, gelatin solution was used for film-forming. 30mL total volume of gelatin and glycerol solution mixture was used for film-forming. Gelatin and glycerol proportion was mentioned in table 1. After degassing of gelatin, 30mL of the solution was poured into the Petri plates and kept in a hot air oven at 40°C for 8 hours until the film gets dried completely [13]. The following combination of gelatin and glycerol was used for film-forming by solvent casting method (Table 1).

TABLE 1
GELATIN and GLYCEROL PROPORTION

Proportion	Volume of sample (mL)	
	10% w/v gelatin	50% v/v glycerol
P 1	26	4
P 2	27	3

2.3 Immobilization of Aminoacids and Drug

Different combinations of amino acids were immobilized in the gelatin/glycerol hydrogel prepared as mentioned in table 2. 10 mL of amino acid and drug sample was prepared with distilled water in different combinations. 10 mL of the amino acid mixture was then added to 90 mL of 10% W/V gelatin solution drop by drop using the syringe. The resultant mixture was then left with continuous stirring for 30 minutes [14]. The hydrogel was formed by solvent casting technique as mentioned above.

2.4 Physicochemical Evaluation

The physicochemical evaluation was done by visual examination of the aminoacid immobilized gelatin films. The color, homogeneity, existence of lumps were analyzed visually for the films [15].

2.5 Spreadability Measurements

Spreadability measurements of the hydrogel film were done by placing the hydrogel film between the two horizontal plates (20*20cm) and 5g of standard weight was placed on the plate. The whole setup was left for 5minutes where no more spreading was expected in the film. The diameter of the spread circle was measured and taken as the value of Spreadability [15].

2.6 Weight of the Films

The weight uniformity of the films was determined by measuring the weight of the different film specimens. Films were cut into 2*2 cm and all specimens were weighed using an electronic balance. The mean weight of the specimens was calculated [16].

TABLE 2
DIFFERENT PROPORTIONS OF AMINOACID AND DRUG

Film no	Amount of the sample (g)						
	L-lysine	L-Tryptophan	L-glycine	L-glutamine	L-arginine	L- proline	Amoxcillin
F1	0.3	0.3	0.3	0.3	0.3	0.3	250 mg
F2	0.6	0.3	0.3	0.3	0.3	0.3	250 mg
F3	0.3	0.6	0.3	0.3	0.3	0.3	250 mg
F4	0.3	0.3	0.6	0.3	0.3	0.3	250 mg
F5	0.3	0.3	0.3	0.6	0.3	0.3	250 mg
F6	0.3	0.3	0.3	0.3	0.6	0.3	250 mg
F7	0.3	0.3	0.3	0.3	0.3	0.6	250 mg

2.7 Folding Endurance

This property is used to find the flexibility of the film, which determines the flexibility of the film. Folding endurance can be found by folding the film at the same place or folded 300 times manually. The number of times, the film can withstand the folding capacity or fold at the same place without break gives the value of folding endurance [16].

2.8 Characterization Studies

Characterization studies were performed for gelatin film formed. Characterization such as FTIR, XRD, SEM, AFM, and thermogravimetric analysis was done for control and aminoacid immobilized film

2.8.1 Fourier transform infrared spectroscopy

FTIR was performed using a Bruker Vector 22 mid-IR spectroscope (Bruker, Germany). All the FTIR absorption spectra were recorded over the wavenumber range of 4000 cm^{-1} to 500 cm^{-1} at a resolution of 8 cm^{-1}, with 1024 scans, using a deuterated triglycine sulfate (DTGS) detector. A straight line between the two lowest points in the respective spectra region was selected as a baseline. The gelatin-based hydrogel film was cast onto glass slides before investigation.

2.8.2 X-ray diffraction

The gelatin films were subjected to XRD (XRD-PW 1700, Philips, Rockville, MD) using CuKα radiation generated at 40kV and 40 mA; the range of diffraction angle was 10.00 to 80.00- 2θ.

2.8.3 Field emission scanning electron microscopy

The morphological properties of the bacterial cellulose and gelatin-based hydrogel composites were investigated using a field-emission scanning electron microscope (FE-SEM, Hitachi, S-4800) at an acceleration voltage of 2 kV. Before the investigation,

the samples were stored in desiccators to avoid exposure to humidity. Each sample was placed on carbon tape and sputtered with gold particles before analysis.

2.8.4 Atomic force microscopy

AFM was performed using a Digital Instruments Nanoscope III Scanning Probe Microscope (Digital Instruments, CA, USA) under ambient conditions (22 °C, 45 - 55% relative humidity) over areas measuring 10 μm×10 μm. The gelatin-based-hydrogel composites were prepared in the form of a thin, flat sheet. The instrument was equipped with a silicon nitride tip and operated in the lateral contact mode. The measurements were repeated five times for comparable topological analysis.

2.8.5 Thermogravimetric analysis

The thermal degradation behavior of the gelatin-hydrogel composite was characterized using thermogravimetric analysis (TGA, TGA Q500, TA Instruments). To achieve this, 20 mg of the sample was heated from 35°C to 200°C under an N_2 atmosphere, using a heating rate and flow rate of 5 °C/min and 70 mL/min, respectively.

2.9 Thickness

The thickness of the film was measured by digital micrometer Mitutoyo Corporations, Japan (Model-UEN-2S2). The film is measured using a micrometer at five locations (center and four corners) by reading accurately and mean thickness is averaging it [16].

2.10 Strength and Elongation

The Strength and elongation of the gelatin film were measured using a universal testing machine test bench - 9000 series (DAK system inc, India) with a sample size of 5 x 2 cm, with a gauge length of 30 mm and 100 mm/min testing speed. The tear strength of the film was measured using MAG tearing tester Mec tear (MAG Solvics Pvt Ltd, India).

2.11 Film Transparency

Film samples of 10 x 35 mm were placed on a spectrophotometer cell and analyzed at 200 - 300 nm [17]. The transparency was determined by the following equation.

$$Transparency = Abs200-300 / \alpha \quad (1)$$

Where $Abs_{200-300}$ and α correspond to the value of absorbance at 200 - 300 nm and the film thickness (mm) respectively. Tests were performed in triplicate.

2.12 pH Measurement

the pH of the gelatin polymeric films was measured in distilled water, PBS, simulated wound fluid (SWF), ringers fluid [18]. For the pH measurements, ELICO LI 120 pH meter was used and all tested solutions were incubated at 37 °C under sterile conditions. For the tests, 1.0 g of dried hydrogel was used. The pH value of the tested solutions was measured after 0, 2, 4, 6, 8, and 10 hours. All reagents used for the preparation of 1 dm^3 of SWF and ringers fluid were listed in table 3 and table 4 respectively.

TABLE 3
SWF COMPOSITION

Chemical reagent	Amount (g)
NaCl	7.996
$NaHCO_3$	0.350
KCl	0.224
KH_2PO_4	0.228
$MgCl_2$	0.305
$CaCl_2$	0.278
$Na2SO_4$	0.071
HCl	Until pH = 7.25

TABLE 4
RINGER'S FLUID COMPOSITION

Chemical reagent	Amount (g)
KCl	8.60
NaCl	0.30
$CaCl_2 \cdot 6H2O$	0.48

2.13 Swelling Study

The swelling property of the Gelatin film was studied by the following method. The Film formed was cut into 2*2 cm length. The initial dry weight of the film was measured (W_0). The swelling property was studied with water and PBS. The whole setup was maintained at 37°C. The samples were removed at the time interval of 30 minutes and the weight of the film was measured (W_s) [19]. The swelling rate was calculated using the following equation.

$$Swelling\ rate\ (R) = (Ws*W0/W0)*100 \quad (2)$$

Where W_s and W_0 were the wet weight and dry weight of the film respectively.

2.14 Dissolution Behavior of Gelatin

The dissolution behavior study of the Gelatin film was carried out by the following method. The Film formed was cut into 2*2 cm length. The initial dry weight of the film was measured (W_0). Then the gelatin film was immersed in simulated wound fluid and heated at 50°C. The samples were removed at the time interval of 5 minutes and the weight of the film was measured (W_d) [20]. The dissolution ratio of the film was calculated according to the following equation.

$$Dissolution\ ratio = (Wd / WD)*100 \quad (3)$$

Where W_d and W_0 were the wet weight and dry weight of the film respectively.

2.15 Water Content

The water content of the hydrogel was determined by the weight of the hydrogel. Initially, the weight of the hydrogel film was measured with the electronic balance. The samples were placed in a hot air oven at a definite temperature of 37°C. The respective weight of the hydrogel was measured at different time intervals (12 hours to 72 hours) [21]. Water content (W_c) was calculated using the equation

$$Wc = Wi - Wd \quad (3)$$

Where W_i= initial weight of the film and W_d = dry weight of film at definite time intervals.

2.16 Gel Fraction

Gel fraction percentage indicates the degree of firmness of the film. Hydrogel films were dried at 40°C for 3 hours under a vacuum. Dried hydrogel film was weighed (W_0). The dried film was soaked in simulated wound fluid for 4 hours. They were further dried at the same temperature under the vacuum condition (W_e) [22]. The gel fraction percentage was calculated using the following equation.

$$Gel\ \ fraction = (Wa/Wi)*100 \quad (4)$$

2.17 Oxygen Permeability of Polymer Films

O_2 permeability of polymer samples was evaluated by using the following method [23]. The polymer film was placed on the top of the flask containing 200 mL of de-oxygenated water (boiled for 10 minutes) and was sealed properly to ensure the oxygen permeation only through polymer films. A completely closed flask was taken as negative control which did not allow oxygen permeation into the flask while the open flask was taken as positive control allowing complete permeation of oxygen into the water. These flasks were placed in an ambient environment for 24 h and then tested for dissolved oxygen.

2.18 Water Vapor Permeability of Polymer Films

Water vapor permeability of a series of polymer samples was determined using the desiccant method [23] and expressed as water vapour transmission rate (WVTR) (g/m²/day). The vials containing anhydrous CaCl2, with and without polymer films on the mouth, were placed in desiccators containing a saturated solution of NaCl, and their weight was determined after a specific interval. WVTR (g per m2 per day) was calculated using the equation

$$WVTR = [\Delta w/\Delta t * 24]/A] \quad (5)$$

where (Δw/Δt) was the slope of the plot 'w' vs. 't', where 'w' was the weight gain (g) along the specified time period, 't' (h) and 'A' is the effective transfer area (m²).

2.19 Porosity

The porosity rates of porous membranes were determined by dissolving the membranes in ethanol at room temperature. The cut-out porous membrane was placed in a scaled test tube with the media (ethanol) until the sample was fully immersed without an air bubble on the surface [24]. The porosity rate of porous membranes was calculated as the following equation

$$P = [V1 - V3/V2 - V3]*100 \quad (6)$$

Where, V - Initial volume of ethanol, V2 - Volume of ethanol after membrane immersed, V3 - Volume of ethanol after the membrane removed.

2.20 Evaluation of Integrity of Gelatin Films and Degradation *In-vitro*

The prepared films (12×12 mm) with a known weight were immersed into 1 mg/mL lysozyme/PBS at 37°Cfor 1 hr (Di) to swell and equilibrate before use. The equilibrated films were removed, washed with distilled water, wiped off the residual water, and weighed (Dt). The pre-equilibrated films were further immersed in 1 mg/mL lysozyme/PBS at 37°C for the 2, 4, 6, 8, 10 hr, and weighed at the designated time (Dt) [25]. The relative degradation ratio (RDR) was calculated using the following equation:

$$RDR\,(\%) = (Di - Dt)/Di*100 \quad (8)$$

Where, D_i is the initial weight after 1 hr equilibration and D_t is the weight after t-hours of incubation (t = 2, 4, 6, 8, 10).

2.21 Aminoacids Release Study

An amino acid release study was performed by placing the hydrogel film in PBS and distilled water. 2mL of samples were taken from film immersed solution at different periods of time intervals (1hour). 2mL of the fresh solution was constantly replaced for the withdrawn samples. The amount of amino acid present in the solution was estimated by the ninhydrin method. Different volume (0.2 - 1mL) of standard amino acid solution (3mg/mL) was taken in respectively labeled test tubes. Now make up the volume of solution to 1 mL with distilled water. Add 1 mL of ninhydrin reagent to all test tubes both standard and unknown samples. Incubate the test tubes in the boiling water bath for 15 minutes. After 10 minutes, add 1 mL of ethanol to the test tubes and mix well. The absorbance of the samples was recorded using a UV – Spectrophotometer at 570 nm [26].

2.22 Amoxicillin Release Study

To determine the amount of released drug, the amoxicillin-loaded hydrogels were gently shaken in the release medium PBS and water at different periods of time intervals (2 hours). The hydrogels were taken out after a definite time period and the amount of drug remaining in the solution was quantified by measuring the absorbance of the solution at a definite wavelength of 234 nm (λmax) [27].

2.23 Thin Layer Chromatography

Pour the solvent mixture into the TLC chamber and close the chamber. The chamber should not be disturbed for about 30

minutes so that the atmosphere in the jar becomes saturated with the solvent. Using a pencil gently draw a straight line across the plate approximately 2 cm from the bottom. Using a capillary tube, a minute drop of amino acid is spotted on the line. Allow the spot to dry. Allow capillary action to draw the solvent up the plate until it is approximately 1 cm from the end. Under a hood, dry the plate with the aid of a blow dryer. Spray the dry plate with ninhydrin reagent. Dry the plates in a hot air oven at 105°C for 5 min [26].

2.24 DPPH Radical-Scavenging Assay

The test samples were compared to a known antioxidant, ascorbic acid (1000 ppm). Briefly, DPPH solution (0.2 mM, in ethanol) was mixed with the hydrogel samples. The reaction mixture sample was shaken for 30 min at 37 ◦C in the dark. The reaction of the DPPH radical was estimated by measuring the absorption at 517 nm against ethanol as a blank in the spectrophotometer [28]. The percentage of the DPPH scavenging inhibition capacity was calculated from the below equation.

$$\%inhibition = \{1 - (Absorbance of\ sample / Absorbance of\ control)\} * 100 \quad (9)$$

2.25 Microbial Penetration

The ability of membranes to prevent microbial penetration was tested by attaching the polymeric film to the top of a glass test tube containing 5mL sterile nutrient broth. Before the test, nutrient broth and glass test tubes were sterilized with an autoclave for 20 min at 121°C. The negative control was a sterile nutrient broth in a glass test tube closed with the cotton ball while the positive control was a sterile nutrient broth in a test tube open to the air. The cloudiness of the nutrient broth in test tubes after 1 week of incubation in an ambient environment was considered as microbial contamination [29].

2.26 Antimicrobial Studies

The antimicrobial nature of the hydrogel film was tested by the disc diffusion method [30]. Hydrogel film was tested on agar plates inoculated against *Escherichia coli, Staphylococcus aureus, Klebsiella pneumonia, Proteus mirabilis.* A lawn of bacteria was laid over the nutrient agar plate using a sterile cotton swab. Drug-loaded dressings (5 mm diameter discs) were placed on the agar plate using sterile forceps. Hydrogel film without amoxicillin was used as the control sample. The plates were incubated at 37°C for 24 h and the zone of inhibition was noted down.

2.27 Hemolysis Assay of Gelatin Films

8 mL fresh rabbit blood was diluted with 10 mL of physiological saline. 0.5 cm × 0.5 cm of hydrogel film was soaked in10 mL physiological saline and incubated at 37°C for 30 min, then 0.2 mL of diluted blood was added and incubated again at 37°C for 60 min, and centrifuged at 1500 r/min for 5 min. The supernatant was put to detect the absorbance at 545 nm. The positive control group was treated with distilled water and the negative control group was treated with physiological saline instead of the gelatin film soaked solution, respectively [31]. The hemolysis ratio was calculated according to the following formula,

$$HR\ (\%) = (ODsample - ODnegative) / (ODpositive - ODnegative) * 100\% \quad (10)$$

Here OD_{sample} is the absorbance value of the tested sample, and $OD_{positive}$ and $OD_{negative}$ are the absorbance values of positive (water) and negative (physiological saline) controls, respectively.

2.28 *In-vitro* Cytotoxicity Assay

The cytotoxicity/ biocompatibility of the prepared gelatin film was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [32]. This method is based on exposing any viable cell to test sample in presence of MTT. The solutions of gelatin film were diluted with media (0%, 20%, 40%, 60%, 80% and 100%). Medium without any CGH film sample i.e. 100% media was used as control. These dilutions were incubated with Dulbecco's Modified Eagles Medium (DMEM) media at 37ºC for 1h in an incubator. These dilutions were added to 96-well plates containing 3 × 10^5 cells/ml NIH/3T3 fibroblast cells (200 µl) in DMEM media incubated at 37ºC for 24h in a 5% CO2 humidified atmosphere. After this, DMEM media was discarded and cells were rinsed with phosphate-buffered saline. Then, 5 mg/ml MTT solution (10 µl) was added to each well and incubated for 4 h at 37°C. Hereafter, the medium was removed completely from each well, and then 200 µl of 40 mM DMSO lysis buffer was added to each well. One hundred µl of cell lysate was transferred into each well of a 96-well plate. Absorbance at 570 nm was measured with a microplate reader (SpecraMax M2e; Bucher Biotech, Basel, Switzerland) to determine cell viability.

3 Results and Discussion

3.1 Preparation of Gelatin/Glycerol based Film

Gelatin film (F1-26:4 & F2-27:3) was prepared with distilled water by casting technique (fig 1). Both the films formed were transparent and there were no precipitates formed over the hydrogel. Both the hydrogel formed were further evaluated for characterization studies.

3.2 Immobilization of Aminoacids and Drug

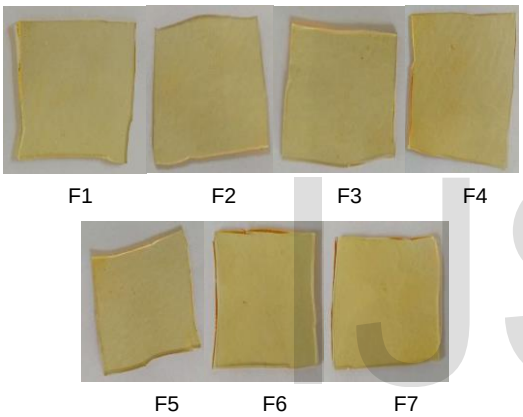
Different compositions of amino acids and drug were immobilized with the gel (fig 2). The immobilization of amino acids along with the drug was done to validate the influence of amino acids on the nature of the gel. For the immobilization of the amino acids, Film F1 was taken. The immobilization of aminoacids doesn't alter the nature of the hydrogel film and the film was consistent.

3.3 Physicochemical Evaluation

The obtained different hydrogel films were taken into observation which looks transparent golden color and homogenous.

F1 F2

Fig. 1. Gelatin hydrogel film. F1 - Gelatin:glycerol (26:4). F2 - Gelatin:glycerol (27:3).



F1 F2 F3 F4

F5 F6 F7

Fig. 2. Hydrogel comprising of amino acids and drug (lysine, tryptophan, glycine, glutamine, arginine, proline and amoxillin). Aminoacids were immobilized in different ratio. F1 - 1:1:1:1:1:1, F2 - 2:1:1:1:1:1, F3 - 1:2:1:1:1:1, F4 - 1:1:2:1:1:1, F5 - 1:1:1:2:1:1, F6 - 1:1:1:1:2:1, F7 - 1:1:1:1:1:1.

3.4 Spreadability Measurements

Spreadability was one of the important parameters which make hydrogel film a uniform application to the skin. It is very important in patience compliance. Spreadability measurements for blank gelatin film, glycerol plasticized film, and aminoacid immobilized film were listed in table 5. Aminoacids immobilized film was found to be highly spreadable compared to control gelatin film.

3.5 Weight of the Films

The weight of the films was measured for four different combinations (blank gelatin film, F1 - 26:4, F2 - 27:3, Aminoacid film devoid of glycerol, Aminoacid film using glycerol). Weight of the different film proportions such as blank gelatin film, F1 - 26:4, F2 - 27:3, Aminoacid film devoid of glycerol, Aminoacid film using glycerol were 0.786 g/m^2, 0.610 g/m^2, 0.645 g/m^2, 0.686 g/m^2, 0.657 g/m^2 respectively.

TABLE 5
SPREADABILITY MEASUREMENTS FOR DIFFERENT FILM TYPES

Film types	Spreadability measurements (cm)
Blank gelatin film	6.1±0.1
Gelatin: glycerol (26:4)	8.1±0.2
Gelatin: glycerol (27:3)	7.9±0.1
Aminoacids immobilized film (equal ratio)	8.1±.0.1

3.6 Folding Endurance

All the films tested for folding endurance have the same level of folding endurance that it can be folded to the maximum number (blank gelatin film, F1 - 26:4, F2 - 27:3, Aminoacid film devoid of glycerol, Aminoacid film using glycerol). It indicated that the addition of glycerol (plasticizer) significantly increases the folding endurance and improves the flexibility of the film. Flexibility is an important property of wound dressing as it influences the conformability of the dressing. So the film integrity with skin folding is considered to be very good when applied to the wound surface.

3.7 Characterization Studies

3.7.1 Fourier transform infrared spectroscopy

The FTIR spectral data were used to confirm the crosslinking of the gelatin chain, and to study the changes that occurred in the functional groups of the gelatin following the reaction with glycerol. It is important to note that only the amino acid mixture presented absorption peaks at 3281 cm^{-1} and 2943 cm^{-1} (fig 3a). These peaks were ascribed to N-H stretching and aliphatic C-H stretching, respectively. The FTIR spectrum of the glycerol was complex (fig 3b) showing peaks of several functional groups. O-H stretching frequency was observed at 3386.78 cm-^{1} while a strong peak at 1611.37 cm-^{1} was due to the C=O stretching of esters present in the crude glycerol. O-H bending was observed at 890.73 cm-^{1}. Moreover, the gelatin-based hydrogel composite exhibited broad peaks at 3312 cm^{-1} and 1627 cm^{-1} (fig 3c), which were ascribed to the stretching vibration of N-H, and C=O stretching, respectively. The characteristic peaks at 1421 cm^{-1} and 1036 cm^{-1} (fig 3a) were attributed to N-H deformation and C-N stretching, respectively. However, the existence of the peak at 3296 cm^{-1} (fig 3d) was ascribed to many reaction mechanisms, such as H-bond formation in the gelatin network. As the amount of gelatin increased, the characteristic peak position at 1631cm^{-1} (fig 3d) was shifted slightly.

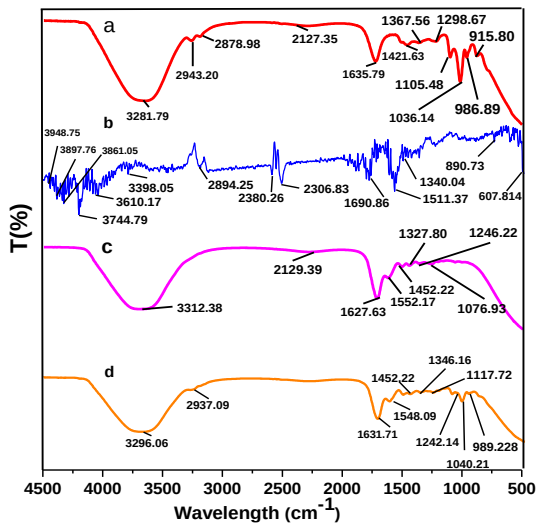


Fig. 3. FTIR spectrum of gelatin based film a.) Amino-acid mixture b.) glycerol c.) gelatin film (26:4) d.) ami-noacid immobilized gelatin film.

3.7.2 X-ray diffraction

X-ray diffraction studies also confirmed the amorphous na-ture of the drug in entrapped form. Gelatin film showed characteristic diffraction peaks at about 18.50°, 19.11°, 22.18°, 23.89°, 25.92° (2θ) with intense signal intensities (fig 4a & 4b). This further indicated that the drug was dispersed at the molecular level in the polymer matrix and hence, no crystals were found in the drug-loaded matrices [33].

3.7.3 Field emission scanning electron microscopy

The morphological properties of the gelatin hydrogel com-posites were evaluated using SEM. Fig 5a & 5c shows the closed-cell form of the porous network present throughout the hydrogel composite. The pores were of various sizes and considered to have a spherical shape. However, it is noteworthy that the glycerol in the hydrogel composite in-creased the size of the pores during swelling (fig 5b & 5d). This was owed to the reaction between the glycerol and wa-tery suspension as glycerol was hygroscopic. The gelatin presented as a network, amino acids, and drug were then inserted between the network. The properties of the hydro-gel composite depend on the amounts of gelatin present. The quantities of materials present, as well as the crosslink-ing agent, play an important role in the chemical reaction and the production of by-products [34].

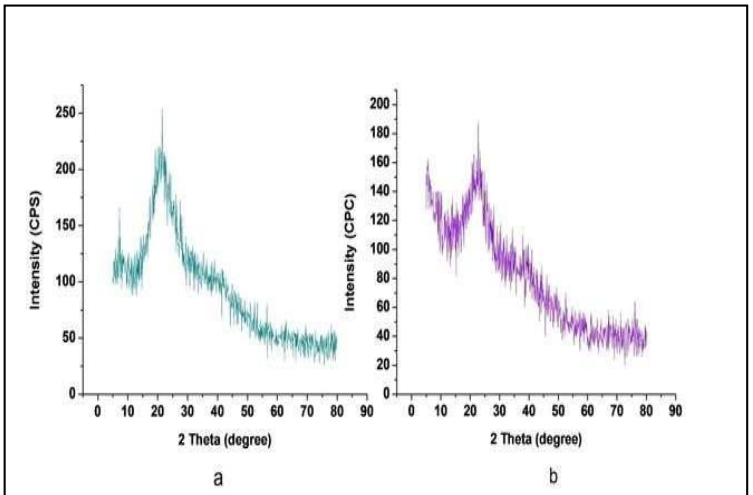


Fig. 4. XRD analysis of gelatin hydrogels a.) Gelatin film (26:4) b.) Aminoacid immobilized gelatin film.

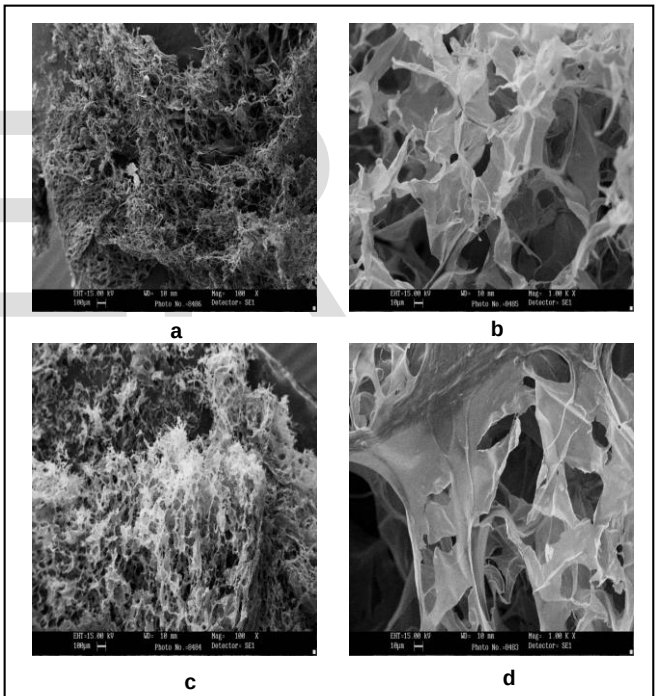


Fig. 5. SEM image of gelatin based hydrogel a.) Gel-atin film b.) Gelatin film after swelling (26:4) c.) Ami-noacid immobilized gelatin film d.) Aminoacid immo-bilized gelatin after swelling.

3.7.4 Atomic force microscopy

Concerning the utilization of the gelatin composite hydro-gel materials for drug-delivery systems, the surface rough-ness of the material is a key issue. The roughness of such hydrogels plays an important role with regard to biomedi-cal engineering applications, such as the controlled release

of water-soluble drugs, encapsulation of cells, tissue engineering, and adhesives. Concerning the controlled release of drugs from a hydrogel composite, the surface roughness of the material should be considered as well as its stability under acid and alkaline buffers. It is important to note that the stability of a hydrogel depends on its roughness. A hydrogel surface with a high degree of roughness can enable the dissociation of drug molecules. Fig 6 & fig 7 exhibits an AFM image captured in lateral contact mode. In this work, AFM images were captured at various areas of the gelatin composite, all of which revealed similar topologies. The AFM scan size (10 μm×10 μm) implies the uniformity of the surface roughness. The degree of roughness of the gelatin-based film was at the nano-scale level (fig 6 & 7). Moreover, it was obvious that the morphological properties of the composite determined from the AFM investigation were identical to those of the SEM investigation [34].

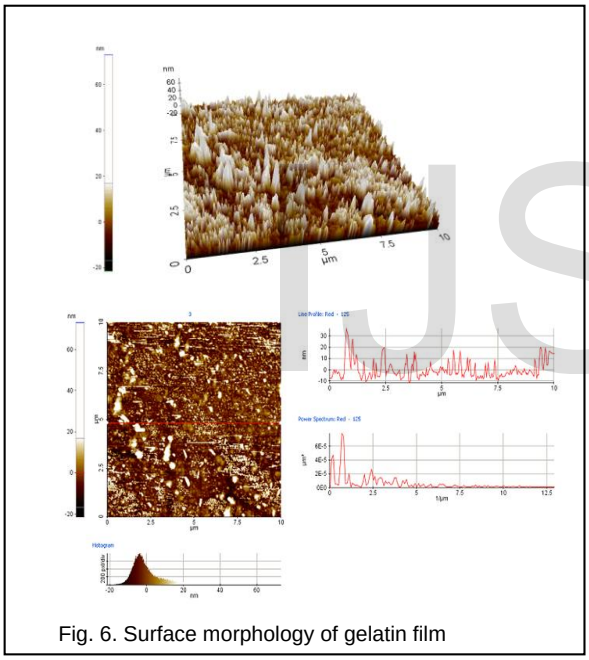


Fig. 6. Surface morphology of gelatin film

3.7.5 Thermogravimetric analysis

The thermal and kinetic behavior of the gelatin-based hydrogel composites is of importance concerning their stability and controlled release mechanism. The thermal and kinetic behavior of hydrogels depends on the thermo-responsive mechanism of the crosslinked hydrogel network. The TGA curves of the gelatin and bacterial cellulose hydrogel composite are presented in fig 8 & fig 9. The change in weight loss can be categorized into two different regions. From 37°C to 60°C, the weight loss was owed to water and solvent evaporation. From 60°C to 140°C, the change in the weight loss was due to organic decomposition. The polymer backbone can break over this temperature region. The pores within the material can be regarded as pathways for CO_2 and H_2O removal (fig 8). This phenomenon is associated with the results of the SEM observation. It is suggested that gelatin hydrogel composites for drug-delivery systems should be used at temperatures lower than 100 °C [34].

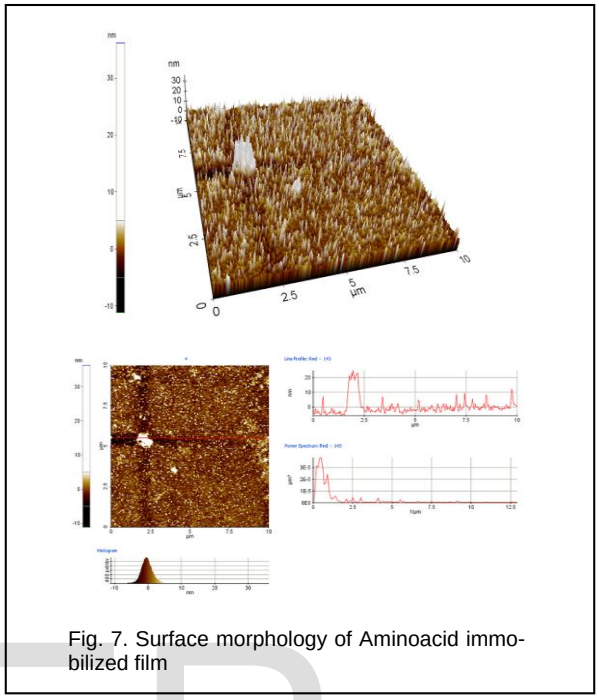


Fig. 7. Surface morphology of Aminoacid immobilized film

3.8 Thickness

The thickness of the gelatin-based films was measured using a digital micrometer. Moreover, the gelatin films possess nearly the same thickness. The thickness of the gelatin-based films were listed in table 6.

TABLE 6
THICKNESS OF THE DIFFERENT GELATIN BASED FILM

Film	Thickness (mm)
Gelatin	0.25±0.02
Gelatin (26:4)	0.23±0.01
Gelatin (27:3)	0.24±0.03
AA gelatin (devoid of glycerol)	0.25±0.04
AA gelatin (26:4)	0.26±0.02
AA gelatin (27:3)	0.26±0.03

3.9 Strength and Elongation

% elongation and elongation at break were calculated using the tensile tester. The data were given below in table 7. % elongation and elongation at break were found higher for aminoacid immobilized gelatin film (26:4). Tear strength was also found higher for the aminoacid immobilized gelatin film (26:4). This shows that aminoacids immobilized in the gelatin film maintain the strength of film compared to other films.

TABLE 7
ELONGATION (%), ELONGATION AT BREAK (%) AND TEAR STRENGTH OF GELATIN BASED FILM

Film	Elongation (%)	Elongation at break (%)	Tear strength (Kg force)
Gelatin	302.58	256.31	768
Gelatin (26:4)	568.16	490.15	782
Gelatin (27:3)	525.56	472.18	785.4
AA gelatin (devoid of glycerol)	301.96	240.87	815.65
AA gelatin (26:4)	558.34	486.70	832

3.10 Film Transparency

Light transmission works were performed to evaluate the transparency of the films and the influence of amino acids and glycerol in this property. The observed peaks of the gelatin film were given in fig 10. The results show that the transparency of the gelatin film was 23±1.4 and aminoacid immobilized film was 25±1.1. The developed films exhibit high transparency in both dry and wet states, which is of great importance for the proposed applications. The gelatin film posse's good transparency and it was possible to observe its ease application and removal, with good conformity, allowing the visualization of the skin.

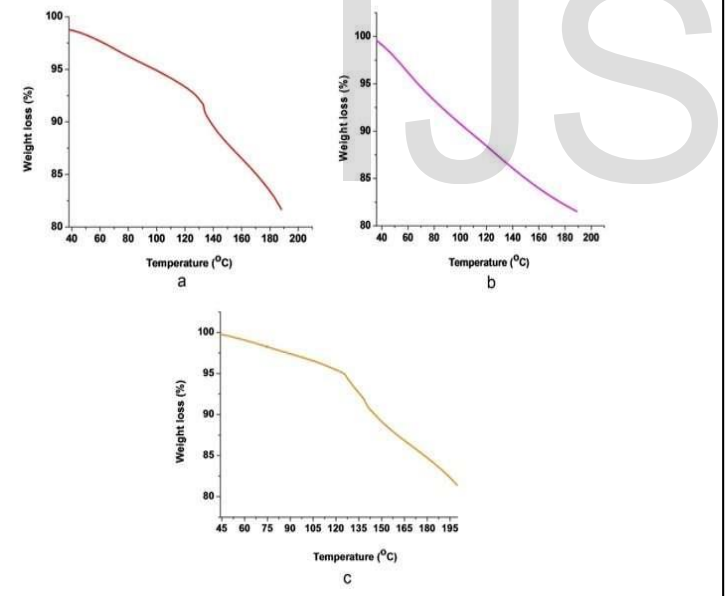


Figure. 8. Weight loss profile of gelatin based films. a.) Gelatin film b.) Gelatin film crosslinked with glycerol c.) Amino acid immobilized gelatin film.

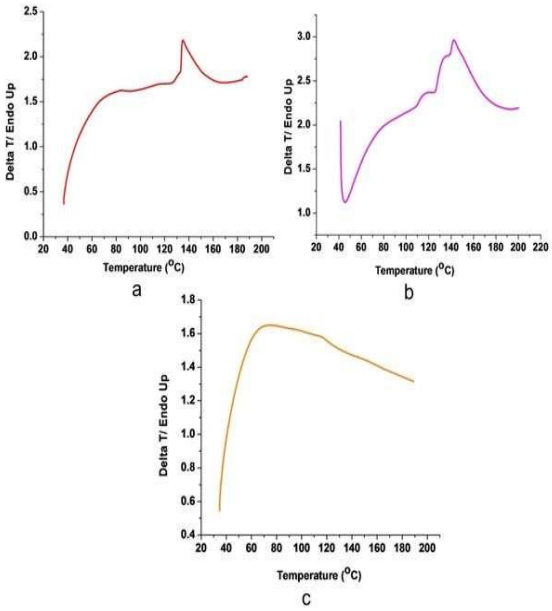


Fig. 9. Weight loss profile of gelatin based films. a.) Gelatin film b.) Gelatin film crosslinked with glycerol c.) Amino acid immobilized gelatin film.

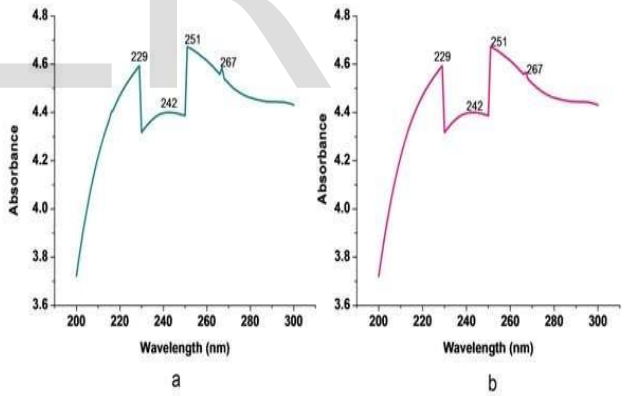


Fig. 10. Transparency of gelatin based film. a.) Gelatin film b.) Aminoacid immobilized gelatin film.

3.11 pH Measurement

The microenvironment of the wound site was determined by placing the gel in biological fluids such as simulated wound fluid and ringers' fluid. The results obtained showed that the pH of the fluids was in the range of 6.8 - 7.4 in the fluids. The results were shown in table 8 & 9

TABLE 8
pH VALUE OF GELATIN AND PLASTICIZED FILM

Fluids	pH value					
	Gelatin film		Gelatin film (26:4)		Gelatin film (27:3)	
	2nd hr	10th hr	2nd hr	10th hr	2nd hr	10th hr
Water	7.0±0.1	7.2±0.2	7.1±0.1	7.2±0.2	7.0±0.1	7.1±0.2
PBS	6.8±0.1	7.1±0.2	6.9±0.1	7.1±0.2	7.1±0.2	7.2±0.2
Simulated Wound fluid	6.9±0.1	7.1±0.2	6.8±0.2	7.1±0.3	6.9±0.2	7.1±0.1
Ringers' fluid	7.1±0.1	7.2±0.2	7.1±0.1	7.3±0.1	7.1±0.2	7.0±0.1

TABLE 9
pH VALUE OF AMINOACID IMMOBILIZED FILM

Fluids	pH value			
	Amino acid immobilized film (devoid of glycerol)		Amino acid immobilized gelatin film (26:4)	
	2nd hr	10th hr	2nd hr	10th hr
Water	7.0±0.1	7.2±0.2	6.8±0.2	7.1±0.1
PBS	6.8±0.1	7.1±0.2	6.8±0.3	7.2±0.2
Simulated Wound fluid	6.9±0.1	7.1±0.2	6.9±0.2	7.1±0.1
Ringers' fluid	7.1±0.1	7.2±0.2	7.0±0.2	7.1±0.2

3.12 Swelling Study

Swelling percent of the different films (gelatin film, gel: gly - 26:4, gel: gly - 27:3, AA film devoid of glycerol, AA film using glycerol) were performed in PBS and distilled water. The swelling percent of blank gelatin film increases gradually in PBS and water as time increases. The swelling ratio was higher for PBS than in water (fig 11a). The swelling percent of glycerol plasticized gelatin film (27:3) increases in PBS and water as time increases (fig 11b). The swelling capacity of the glycerol plasticized gelatin film was lesser than the blank gelatin film. The swelling ratio was higher for PBS than in water. Though glycerol increases the elasticity of the film, the film gets started dissolving in the water at a certain point of time may be due to the hydrophilicity nature of both gelatin and glycerol (fig 11c). The swelling percent of glycerol plasticized aminoacid immobilized increases in PBS and water as time increases. The swelling capacity of the glycerol plasticized gelatin film was lesser than the blank gelatin film. The swelling ratio was higher for water than in PBS. Though glycerol increases the elasticity of the film, the film gets started dissolving in the water at a certain point of time may be due to the hydrophilicity nature of both gelatin and glycerol in film (fig 11d).

3.13 Dissolution Behavior of Gelatin

The dissolution ratio of blank gelatin film was higher than the other films (gel: gly - 26:4, gel: gly - 27:3, AA film devoid of glycerol, AA film using glycerol). At a particular time interval, the dissolution ratio of blank gelatin film was higher than the other films (AA film devoid of glycerol > gel: gly - 26:4 > gel: gly - 27:3 > AA film using glycerol). Higher the dissolution ratio, lower the dissolution of gelatin in the solution. The dissolution ratio of the film decreases with respect to an increase in time. A decrease in the dissolution ratio is mainly due to the dissolving nature of the film components such as gelatin and glycerol in the solution. The dissolution ratio of the blank gelatin film and amino acid film decreases slowly and gradually compared to other plasticized films (fig 12a & fig 12b).

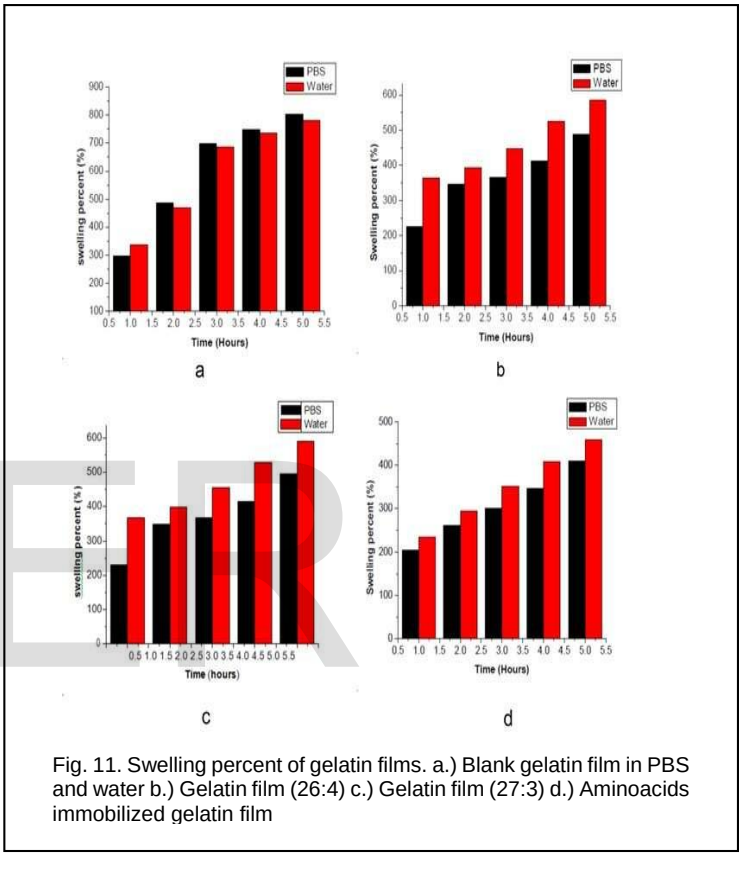


Fig. 11. Swelling percent of gelatin films. a.) Blank gelatin film in PBS and water b.) Gelatin film (26:4) c.) Gelatin film (27:3) d.) Aminoacids immobilized gelatin film

3.14 Water Content

The water content of the gelatin-based film (blank gelatin film, gel: gly - 26:4, gel: gly - 27:3, AA film devoid of glycerol, AA film using glycerol) was analyzed at different time intervals at 37°C (table 4.8). Water content (g) evaporated from the film was noted down at regular time intervals (12 hours to 72 hours). Water content evaporation rate was higher for the films prepared using glycerol compared to the films devoid of glycerol (AA film using glycerol > gel: gly - 27:3 > gel: gly - 26:4 > AA film devoid of glycerol > blank gelatin film). water content evaporation for gelatin and aminoacid film (devoid of glycerol) started at the same rate and increased gradually but for plasticized film, evaporation started at a higher rate but after 12 hours, there was a gradual release of water content (fig 13a & fig 13b). This indicates that as the time period increases, the water content of the film gets released from the film at the higher temperature.

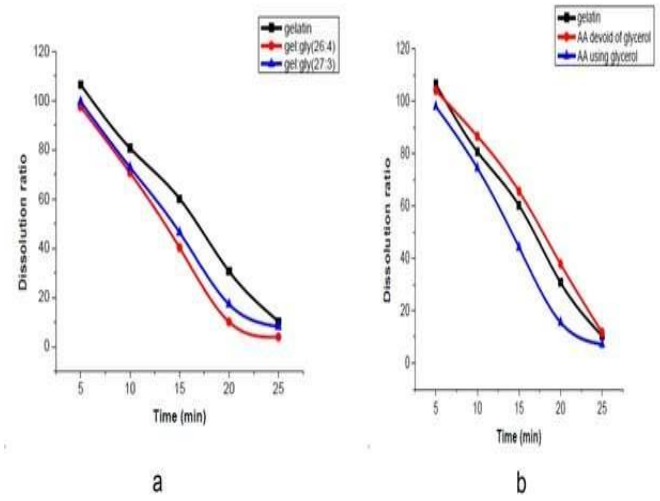


Fig. 12. Dissolution ratio a.) Gelatin film devoid of glycerol compared with Gelatin-glycerol films b.) Gelatin film devoid of glycerol compared with gelatin aminoacid films

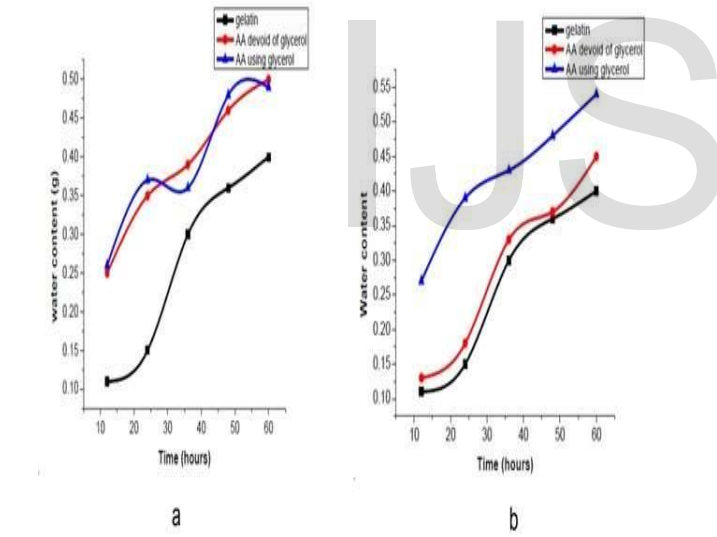


Fig. 13. Water content a.) Gelatin film devoid of glycerol compared with Gelatin-glycerol films b.) Gelatin film devoid of glycerol compared with gelatin aminoacid films

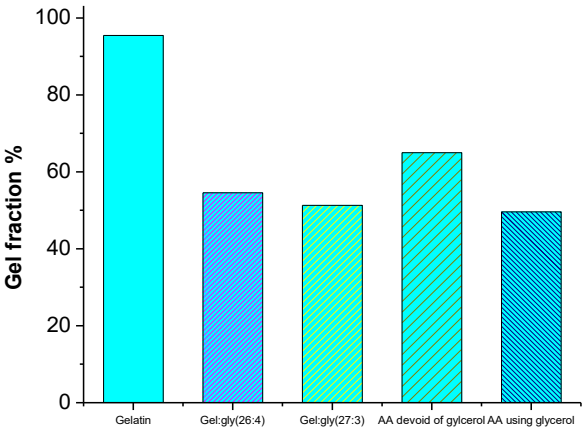


Fig. 14. Gel fraction percent of the gelatin, gel: gly (26:4), gel: gly (27:3), Aminoacid film devoid of glycerol, Aminoacid film using glycerol.

3.15 Gel Fraction

Gel fraction of the gelatin film was assessed by analyzing the film in simulated wound fluid for a period of time intervals. Gel fraction percent obtained for blank gelatin film, gel: gly - 26:4, gel: gly - 27:3, AA film devoid of glycerol, AA film using glycerol was 95.5%, 54.6%, 51.3%, 65%, and 49.6% respectively (fig 14). Gel fraction percent was higher for the blank gelatin film compared to other films (blank gelatin film > AA film devoid of glycerol > gel: gly - 26:4 > gel: gly - 27:3 > AA film using glycerol).

3.16 Oxygen Permeability of Polymer Films

The material used for wound dressing applications should have permeability towards oxygen, which can help in wound repair. Keeping in view that a proper supply of oxygen is required to accelerate the wound healing process. Results of various polymer samples prepared with gelatin content are given in table 10. The results from negative control (closed flask) and positive control (open flask) are 5.01 mg/L and 9.3 mg/L of oxygen respectively. Dissolved oxygen in the flask covered with gelatin (26:4) showed the highest value 6.72 ± 0.12 mg/L and the flask covered with gelatin allowed the least oxygen (4.98 ± 0.04) to pass through. Optimized dressing amino acid gelatin film (26:4) allowed 6.53 ± 0.07 mg/L of oxygen to pass across it. Oxygen plays an important role in increasing cell metabolism, increasing the rate of cell proliferation, epithelialization increasing collagen synthesis, increasing antibacterial activities, increasing angiogenesis, and promoting revascularization. Hence, hydrogel film permeable to oxygen can fasten the overall wound healing process.

3.17 Water Vapor Permeability of Polymer Films

Water vapor permeability was assessed as the amount of water evaporated, from a container sealed with polymer films (thickness 0.2mm) as a function of time, and results are shown in table 10. The highest vapor evaporation rate was around 1822.44 ±8.67 g/m2/day for AA gelatin (26:4) and the lowest water vapour transmission rate was 1420.70 ±4.06 g/m2/day for AA gelatin (devoid of glycerol) hydrogel films. Water permeation showed a clear dependence on the crosslinking agent irrespective of the amino acid content. Hydrogel wound dressings must control the water loss from the wound to maintain a moist environment and avoid excessive dehydration or edema.

TABLE 10
WATER VAPOUR TRANSMISSION RATE AND OXYGEN PERMEABILITY OF DIFFERENT GELATIN FILM

Film type	WVTR (g/m²/day)	Oxygen permeability (mg/L)
Gelatin	1475.70 ± 10.36	4.98 ±0.04
Gelatin (26:4)	1785.34 ±8.90	6.72 ±0.12
Gelatin (27:3)	1588.59 ±7.46	6.32 ±0.08
AA gelatin (devoid of glycerol)	1420.70 ±4.06	5.50 ±0.13
AA gelatin (26:4)	1822.44 ±8.67	6.53 ±0.07
AA gelatin (27:3)	1636.97 ±11.30	6.39 ±0.79

3.18 Porosity

The porosity percentage of films was observed from the results, porosity percentage of aminoacid immobilized film showed higher when compared to all other blended films (table 11), because glycerin content play a role for to develop and arrest the pore gap on to the film surface and core level, from the observation other proportion also give better porosity percentage compared to aminoacid immobilized films.

TABLE 11
POROSITY OF GELATIN BASED FILMS

Film type	Porosity (%)
Gelatin	66.7
Gelatin (26:4)	74.2
Gelatin (27:3)	76
AA gelatin (devoid of glycerol)	69
AA gelatin (26:4)	76.7
AA gelatin (27:3)	78.4

3.19 Evaluation of Integrity of Gelatin Films and Degradation *In-vitro*

The relative degradation ratio (RDR) of gelatin and blend films is summarized in fig 15. After soaking in lysozyme/PBS solution for 10 hours, the RDR of gelatin film, gel:gly (26:4), gel:gly (27:3), AA gelatin film (devoid of glycerol), AA gelatin film (26:4) was 32%, 47%, 42%, 38%, 46%, 43% respectively. So, the blend films presented lower degradation. The glycerol added into the blend films is a widely-used polymer additive and has a positive effect on the flexibility and mechanical properties of gelatin film. The improved flexibility and high mechanical endurance of the would-care dressing materials not only can endow it with a lasting function until the wound is completely healed, but also can contribute to being an appropriate substrate for adhesion, proliferation, and migrations of cells.

3.20 Aminoacids Release Study

Different concentrations of aminoacid immobilized film using glycerol and the film devoid of glycerol was subjected to aminoacid release study in the diffusion medium (PBS and water). AA release from the diffusion medium (PBS) was at a high rate compared to water. The concentration of AA in water gradually increases but the concentration of AA in PBS suddenly increases compared to water (fig 16a). This may be due to the pore size of gelatin in the PBS increasing at a higher rate compared to the pore size of the film. AA release from the diffusion medium (PBS and water) started at the same rate in the film but there was no gradual increase of AA from the film in both the medium. The concentration of amino acid in the diffusion medium (PBS and water) was non – linear and goes in a zig-zag way (fig 16b). At each time interval, there was the same level of amino acid concentration in the diffusion medium (PBS and water), which indicates that there was the same level of release rate in the medium. At 4^{th} hour, there was some concentration difference in the medium (fig 16c). However, at the final hour of study, both of the mediums reached the same concentration value. The concentration of aminoacid at the initial point of time (1^{st} hour and 2^{nd} hour) was comparatively at the same level in the diffusion medium (PBS and Water). At the final time of hour (5^{th} hour), both of the diffusion mediums reached the same concentration level of Amino acid. This graph (fig 16d) indicates that the swelling of film in the PBS and water was initially at the same rate. At 3^{rd} and 4^{th} hours, swelling was higher in water and amino acid concentration was high in water compared to water. However, finally both of the mediums reached the same level of concentration at the final time period. At the end of time (1hour), the concentration of aminoacid in water was high. The amino acid content in the water maintained a gradual increase till 3 hour, after 3^{rd} hour, aminoacid release maintained a steady-state in the medium (fig 16e). The amino acid in the PBS medium showed a gradual increase till the final hour of study. At an initial point of time in diffusion medium (PBS and Water) (fig 16f), there is a higher release of amino acid content in the PBS compared to water. The concentration of amino acid in the diffusion medium (PBS) at every time interval was comparatively high compared to water. This may be due to the swelling of film in PBS being higher than in water. As much there is a swelling of film, pores of the film enlarge such that there will be a higher content of amino acid.

3.21 Amoxicillin Release Study

In vitro drug release studies shows that there was a higher amount of drug release in PBS compared to water. For amoxicillin (fig 17), the drug release was in the zig-zag manner such that there was no gradual release of the drug.

3.22 Thin Layer Chromatography

Thin-layer chromatography was performed to analyze the release of amino acids at different time intervals. The sample solution from the releasing medium was taken at a different time interval and run in the TLC plate. There were thick bands of

colour obtained in the TLC plate for the sample obtained at 3hr interval of time interval (fig 18a& 18b) and 2hr interval of time (fig 18c). This confirms that the amino acids were released at a different time interval.

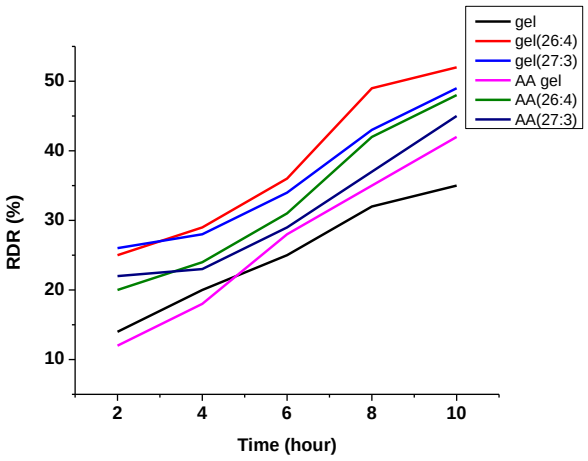


Fig. 15. Relative degradation rate (%) of different gelatin films.

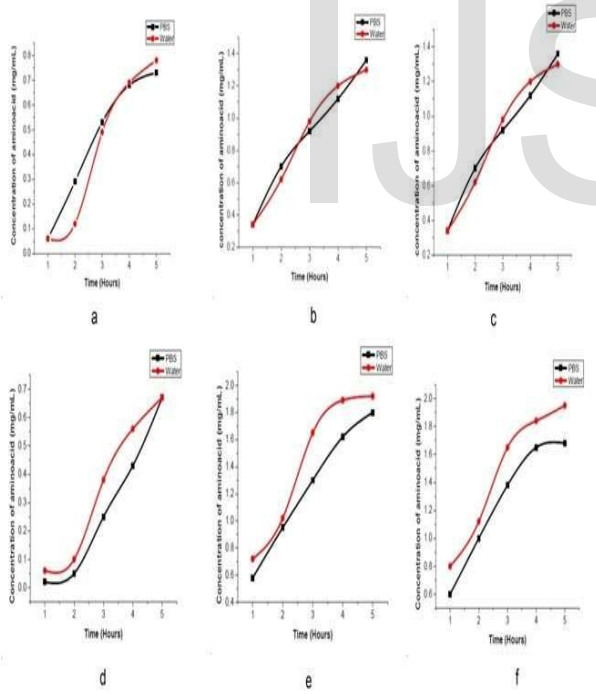


Fig. 16. Concentration of aminoacid release in the diffusion medium. a.) Aminoacid (2mg/mL) released from the gelatin film (devoid of glycerin) b.) Aminoacid (4mg/mL) released from the gelatin film (devoid of glycerol) c.) Aminoacid (2mg/mL) released from the glycerol plasticized gelatin film (26:4) d.) Aminoacid (2mg/mL) released from the glycerol plasticized gelatin film (27:3) e.) Aminoacid (4mg/mL) released from the glycerol plasticized gelatin film (26:4) f.) Aminoacid (4mg/mL) released from the glycerol plasticized gelatin film (27:3).

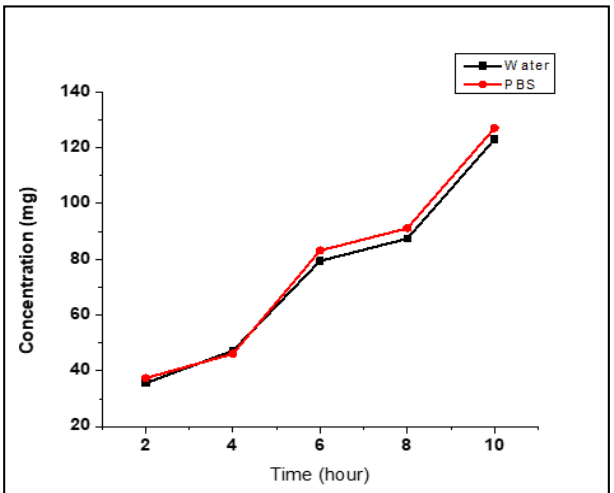


Fig.17. Amoxicillin release in different intervals of time

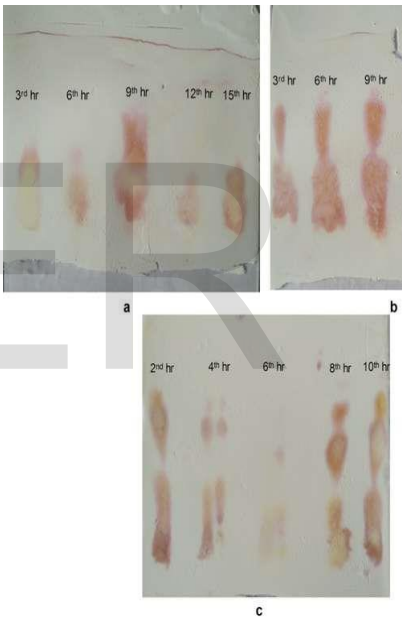


Fig. 18. TLC of aminoacid film medium in different time intervals. a.) Gelatin film medium b.) Aminoacid film medium at 3 hour time interval c.) Aminoacid film medium at 2 hour time interval.

3.23 DPPH Radical-Scavenging Assay

The results of the antioxidant activity of polymer samples were determined via Dpph assay. The antioxidant activity was higher for the aminoacid immobilized gelatin film (97%) as compared to the control ascorbic acid (fig 19 & fig 20). All the other films also showed a good antioxidant profile above 90% (fig 20). Hence, if sufficient antioxidant activity is present in wound dressing, it can provide a positive effect on the wound healing process due to their regulation of the overproduction of reactive oxygen species. Reactive oxygen species are generated by an antioxidant enzyme system of cells during the normal

wound healing process that can detoxify radicals, or repair oxidized molecules at the wounded site for effective defense against pathogens and especially for angiogenesis. But, during excessive oxidative stress, mammalian cells are not able to generate that much amount of antioxidant molecules to counteract, which results in oxidative stress, which leads to prolonged inflammation and delay in chronic wound healing.

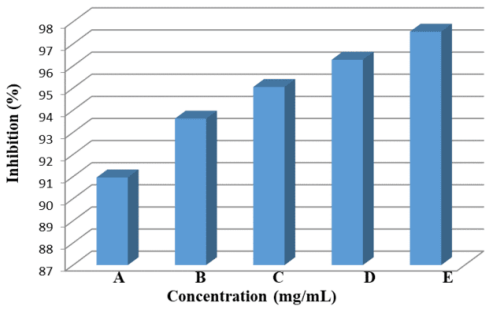


Fig. 19. Inhibition % of ascorbic acid.

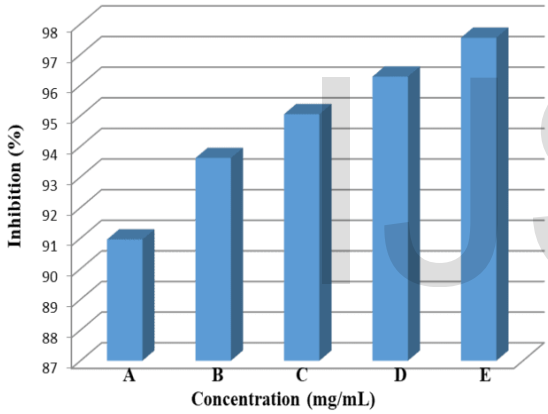


Fig. 20. Inhibition % of hydrogel film A - Control, B - Gelatin film (26:4), C - Gelatin film (27:3), D - Aminoacid film (26:4), E - Aminoacid film (27:3).

3.24 Microbial Penetration

The results showed that the microbial contamination was not observed in all the formulations of gelatin films covered tubes (fig 21 & fig 22) and the negative control tubes. Only the positive control tubes (fig 21 & fig 22) had bacterial contamination. This indicates that the developed composite films have good potential to be used in wound dressings because of their ability to bind the negatively charged bacteria to the positively charged amino groups of the gelatin film by reducing the primary wound contamination. Hence, the protection of a wound from secondary bacterial infection can be achieved

3.25 Antimicrobial Studies

The main aim of the antibacterial activity of dressing is to cater a sterile atmosphere around the wound area, which promotes wound healing mechanism and improves cellular functionality. Gelatin-based films were tested against microorganisms to determine antibacterial activity. Fig 23 shows a typical antibacterial test of gelatin film 1 (gelatin film) and gelatin film 2 (aminoacid film) against four different microorganisms named *Proteus mirabilis, Streptococcus pneumoniae, Serratia sp., Staphylococcus aureus, and Klebsiella pneumoniae* determined by the zone inhibition method. The diameter of the growth inhibition zone was dependent on the antibiotics immobilized in the gelatin film. The gelatin film has the highest inhibition against *Staphylococcus aureus* with the zone of inhibition of 1.3cm (gelatin film 1), 1.6cm (gelatin film 2), and *Proteus mirabilis* with the zone of inhibition of 1.5cm (gelatin film 1), 1.6cm (gelatin film 2). Both the gelatin film exhibited better antibacterial activity against gram-positive bacteria than gram-negative bacteria. The difference in the cell membrane structure and chemical composition were responsible for the difference in the antibacterial activity.

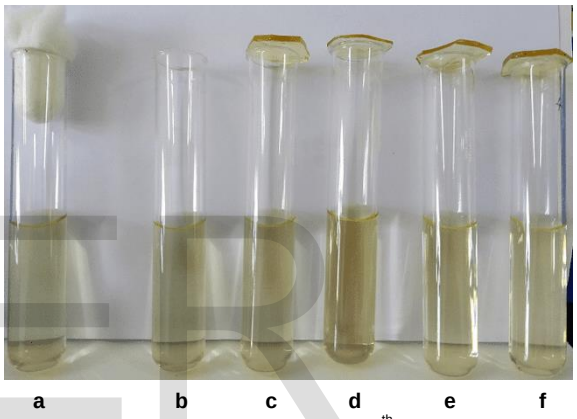


Fig. 21. Microbial penetration test (at 0th day). a - Negative control, b. Positive control, c - Gelatin film, d - gel:gly (26:4), e - gel:gly (27:3), f - AA gelatin film (26:4).

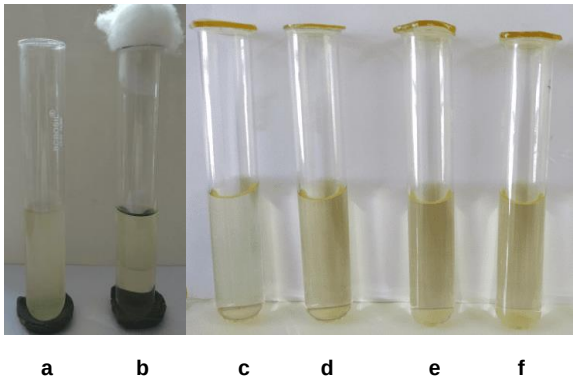


Fig. 22. Microbial penetration test (at 0th day). a - Negative control, b. Positive control, c - Gelatin film, d - gel:gly (26:4), e - gel:gly (27:3), f- - AA gelatin film (26:4).

3.26 Hemolysis Assay of Gelatin Films

When the materials were in contact with blood, they often

caused the activation of blood cells and plasma proteolytic enzyme systems, such as complement, coagulation, fibrinolysis, and hemolysis. The *in - vitro* blood compatibility of gelatin composite dressing was performed (fig 24). Absorbance at 545 nm of the positive control was 0.970±0.0015 and the absorbance of the aminoacid gelatin film was 0.014±0.0045. The hemolysis rate was 0.64%, and this result suggested that gelatin composite dressing should have a good hemocompatibility and it is a non-hemolysis medical dressing.

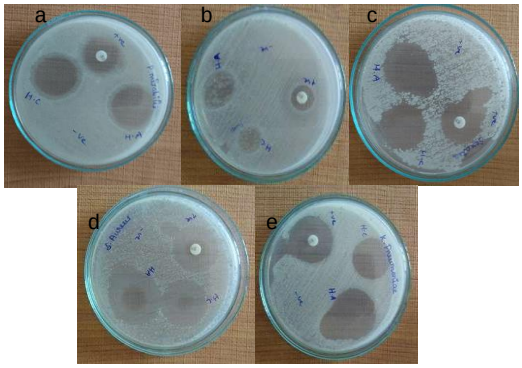


Fig. 23. Antibacterial activity of gelatin based films a. *Proteus mirabilis* b. *Streptococcus pneumoniae* c. *Serratia sp.* d. *Staphylococcus aureus* e. *Klebsiella pneumoniae*

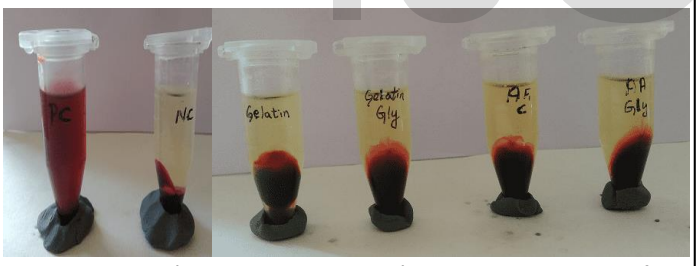


Fig. 24. Hemolysis activity of gelatin based films. a - positive control, b - negative control, c - gelatin, d - gelatin: glycerol (26:4), e - amino acid immobilized gelatin film.

3.27 *In-vitro* Cytotoxicity Assay

The effect of prepared amino acid immobilized gelatin film on cell viability (cytotoxicity) was examined using the MTT assay, which is a colorimetric method to determine the metabolic activity of viable cells. The result of MTT assay showed satisfactory cell viability on gelatin film dilutions. All the dilution of gelatin film extracts had shown no toxicity as more than 95% of cells were viable and proliferating (fig 25). The results clearly showed that gelatin film has the capacity to stimulate the cell viability to 128% which is 20% higher (fig 25) than that of the control value. The MTT assay is based on the conversion of insoluble purple formazon crystals from soluble yellow tetrazolinum salt by enzyme mitochondrial dehydrogenase of living cells that is measured in a spectrophotometer. The absorbance corresponds to the number of living cells. Thus amino acid immobilized film offered acceptable cell viability and non-toxicity.

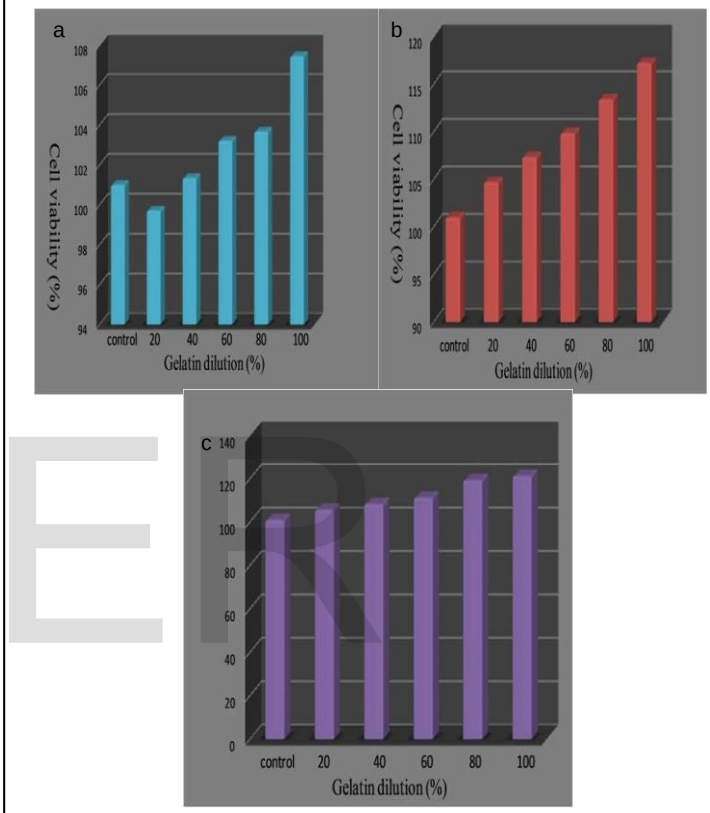


Fig. 25. Cytoxicity studies of gelatin based hydrogel. a.) Gelatin film b.) Aminoacid immobilized film devoid of glycerol c.) Aminoacid immobilized film using glycerol.

4 Conclusion

Gelatin-based hydrogel films were formed and those films were good in physical characteristics. In order to increase the elasticity of the film, glycerol was used as the plasticizer. Glycerol increased the plasticity of the gelatin. Immobilization of the amino acids and antimicrobial required for wound healing was also carried out in gelatin films. Hydrogel films immobilized with different concentrations of amino acids were prepared. All different ratio aminoacid films formed with gelatin were good in physical nature. Characterization results show that gelatin film has a good morphological structure and mechanical properties. The swelling ability of the films was also tested. Results show

that amino acids immobilized gelatin film has a good swelling ratio. The stability of the amino acids gelatin films was also high compared to other films. The dissolution ratio of the film shows the dissolution was steady throughout the time interval. The film has high flexibility, determined by repeated folding of the film. The film also possesses medium degree of firmness indicated by gel fraction percentage. Other gelatin-glycerin and gelatin- glycerin-aminoacids-based films were initiated to dissolve in water and PBS after a few hours. Amino acid release study shows that amino acids from the gelatin- glycerol-aminoacids film gradually increased at particular time intervals. Drug release study shows that the drug release was higher in PBS compared to water. Also, the aminoacid immobilized film maintained its water content and gel fraction percentage. Gelatin film has barrier properties against the microbes which act as an impermeable layer against microbes. Gelatin film inhibited the pathogenic organisms which form a major threat during the wound healing process. Gelatin film scavenges most of the free radicals comparably to that of control which can be used as dressing material scavenging the free radicals in the wound healing site. Gelatin film has a very low hemolysis rate percent and cytotoxicity result shows that gelatin film has good biocompatibility with human skin cells. Morphological, mechanical, biological properties of the gelatin film immobilized with amino acids show that film suits as a wound dressing material for skin injuries.

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