RESEARCH ARTICLE

Formulation and Evaluation of Metronidazole Loaded Biopolymer Hydrogel Films for Wound Healing Applications

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ABSTRACT
This study is based on creating and assessing citric acid (CA) cross-linked biopolymer composite Metronidazole (MTZ) loaded hydrogel films for wound healing applications. Different concentrations of sodium carboxymethyl cellulose (NaCMC), hydroxypropylmethyl cellulose (HPMC), and polyvinyl alcohol (PVA) were used to create hydrogel films using the solvent cast method. FTIR verified the chemical cross-linking (ester bond) between NaCMC and HPMC. WVTR for hydrogel films ranged between 167.10 ± 33 to 341.41 ± 18 g/m^2/day.S0 (Control) hydrogel film shows lowest tensile strength which indicates poor mechanical properties of this hydrogel film.Hydrogel films with more concentration of HPMC show a high swelling index initially but get dissolved completely within one hour, as the swelling index is inversely proportional to cross-linking density thus these results indicate poor cross-linking in S4 to S8 batches of film. Scanning Electron Microscopy (SEM), results shows drug particles on surface of control (S0) hydrogel film reveal absence of crosslinker. CA crosslinked hydrogel film show smooth and uniform morphology.X-ray diffraction (XRD) observed the crystalline nature of drug and amorphous nature of hydrogel film. *In vitro* studies reveal sustained release of MTZ from hydrogel films. Antibacterial study and Antimicrobial activity were investigated against *Bacteriod fragilis* and *Escherichia coli*. The hemocompatibility test reveals the biocompatibility of cross-linked hydrogel films. Overall, outcomes strongly recommended the prospective use of prepared biopolymer composite hydrogel films in wound healing applications.

Keywords: Biopolymer,Sodium carboxymethyl cellulose, Hydroxypropyl methylcellulose, Polyvinyl alcohol, Citric acid, Cross-linking, Metronidazole.

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INTRODUCTION

Wounds are rapid lacerations involving punctured skin or contusion, affecting organ tissue, skin, and mucous membranes. They can be open (surgical or non-surgical) or close, resulting from blunt force trauma or accidents. Based on the trauma to the skin, wounds are classified under four categories; clean, clean-contaminated, contaminated, and dirty or infected.[1] The establishment of microorganisms in open wounds may be offensively unpleasant also there is an accumulation of pus, the wound healing process becomes challenging for these kinds of infections.[2] U.S. Department of Health suggested the application of antibiotic ointment as a preventive measurement for wound infections. A sustained concentration of antimicrobial medication can be provided topically at the infected site.[3] Metronidazole(MTZ) is used in wound healing due to its cost-effectiveness, tissue penetration properties, and mild side effects.[4]Oral administration of MTZ has drawbacks as increased dosage required for bacterial infection treatment that leads to potential neutropenia, leukopenia, and CNS toxicity.[5] To resolve this problem drug incorporated hydrogel patches with minimal side effects have been widely developed. Hydrogels are cross-linked networks of hydrophilic polymers with excellent biochemical and mechanical properties, particularly beneficial in wound healing. Hydrogel structure is maintained through physical and chemical cross-linking, several chemical crosslinker are used to formulated hydrogel films that may be toxic however, recently Citric Acid (CA)is approved by the FDA for pharmaceutical formulation due to its low toxicity and good biocompatibility.[6,7]CA can stabilize polysaccharide structure by developing covalent intermolecular interaction by the formation of ester bonds with hydroxyl groups of polysaccharides.[8] Chang et al. (2022) reported the citric acid crosslinked hydrogel films that showed prolonged antibacterial activity with sustained release of the drug. These hydrogels showed good results for wound healing.[9] The majority of hydrogels for drug delivery and other biological applications have lately been created using natural polymers and their derivatives.[10] Out of all the polymers, cellulose is the most beneficial, non-

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Table 1: Composition table of hydrogel films

Film Code	NaCMC (wt %)	HPMC (wt %)	PVA (wt %)	Drug (wt %)	Citric Acid (wt %)
S0 (control)	5	1	1	0.5	-
S1	5	1	1	-	5
S2	5	1	-	-	5
S3	5	1	1	0.5	5
S4	5	1	-	0.5	5
S5	1	5	1	-	5
S6	1	5	-	-	5
S7	1	5	1	0.5	5
S8	1	5	-	0.5	5

toxic, and renewable one which is utilized extensively in the creation of hydrogels. Sodium carboxymethylcellulose (NaCMC),has high hydrophilicity because it contains carboxyl (-COOH) and hydroxyl (-OH) groups, and shows its outstanding properties among all other derivatives of cellulose, although electronic repulsion forces hinder the ability of NaCMC hydrogels to heal wounds when used alone.[11] To overcome this problem biopolymer composite-based hydrogels were formulated by using NaCMC, Polyvinyl Alcohol (PVA), and Hydroxypropyl methylcellulose (HPMC). PVA used with NaCMC increases to increase its tensile strength, further in the swollen state it helps to release drugs in controlled manner.[12]Ghorpade et al. (2019) prepared Citric acid cross-linked carboxymethyl cellulose (CMC) polyvinyl alcohol (PVA) hydrogel films for the extended release of water-soluble basic drugs.[13] Further mechanical strength, adhesive properties, water retention, and drug release from this composite matrix can be enhanced through use of HPMC. According to FDA these are harmless to the human body and can be used as, film former, and binders.[14,15]Based on group substitution, HPMC reveals a distinct tendency toward drug and aqueous phase.[16,17]Cellulose-based hydrogel does not impart any antibacterial activities; however, it can develop antibacterial activities when combined with antibacterial agents.[18,19] also, microbial contamination has been proven less for cellulose ether derivatives as compared to other gelling agents, thus manifested as an interesting choice for biomedical applications. Several studies have been done on cellulose-based hydrogel formulations,[11]although MTZ incorporated citric acid cross-linked hydrogel films of NaCMC, HPMC along with PVA for wound healing applications are not yet designed. Purpose of this study is to fabricate biocompatible, antibacterial, and mechanically stable citric acid cross-linked, cellulose ether derivatives (NaCMC, HPMC) and PVA-based hydrogel films. Further Metronidazole as an antimicrobial drug is incorporated in this biopolymeric composite hydrogel films.

MATERIALS AND METHODS

Materials

Sodium carboxymethyl cellulose (NaCMC) and hydroxypropyl methylcellulose (HPMC E10), were purchased from Sigma Aldrich, Bangalore, India. Polyvinyl Alcohol (PVA) and citric acid (CA) were procured from Loba Chemical Pvt. Ltd Mumbai, India. Metronidazole was collected as a endowment from Unichem Laboratory Ltd, Baddi, India. Every chemical and reagent employed in this investigation was of analytical grade.

Formulation of drug-loaded cross-linked hydrogel film

Hydrogel films were formulated using solvent casting method by taking varied amounts of NaCMC, HPMC, and PVA in distilled water and CA was added as a crosslinker. NaCMC acts as a thickening and stabilizing agent, HPMC acts as a film-forming agent and stimulate the intermolecular interactions and reduces intramolecular interactions between NaCMC macromolecules. PVA provides hydrophilicity to the film. Metronidazole (MTZ) is used as an antibacterial, anti-inflammatory drug for wound healing. Experimentally different formulations of transdermal patches were formulated in ratio as depicted in Table 1. To prepare transdermal hydrogel films, dissolved different concentrations of NaCMC and HPMC in water under continuous stirring conditions for 25-30 mins until a clear solution is obtained, then slowly add 1% PVA to this solution. Metronidazole (MTZ)solution was prepared in water and added to above solution. Finally, CA was introduced as a croslinker to this solution, followed by cooling at room temperature. Also one batch was prepared by the same procedure without crosslinker as control. 15 mL portion of all samples werespilled on a petri plate(10cm),left for dry at room temperature. Finally peeled out these transdermal films from petri plates and stored in vacuum desiccator for more evaluation.

Evaluation of drug-loaded cross-linked film

Appearance, thickness and weight variation

Stretchability, transparency and color of film were observed visually. A digital caliper (Digimatic Micrometer

Table 2:Evaluation of drug loaded cross-linked hydrogel films

Film Code	Thickness (mm)	Weight variation (g)	Moisture content (%)	Moisture uptake (%)	Moisture loss (%)	Drug content(%)	Folding endurance
S0	0.037± 0.02	479 ± 0.09	14.22 ± 0.77	19.89 ± 0.72	3.16 ± 2.65	70 ± 0.49	220 ± 1.19
S1	0.041 ± 0.06	480 ± 0.05	12.12 ± 0.78	14.89 ± 0.78	5.82 ± 3.86	-	312 ± 1.22
S2	0.039 ± 0.05	472 ± 0.04	11.43 ± 0.84	13.78 ± 0.77	3.66 ± 2.72	-	296 ± 1.11
S3	0.042 ± 0.05	481 ± 0.04	12.10 ± 0.82	15.87 ± 0.82	4.87 ± 4.33	97± 0.76	315 ± 1.90
S4	0.040 ± 0.07	472 ± 0.06	11.56 ± 0.86	13.98 ± 0.88	6.77 ± 3.82	87 ± 0.73	302 ± 1.18
S5	0.039 ± 0.06	482 ± 0.05	13.80 ± 0.72	18.90 ± 0.79	5.87 ±3.44	93 ± 0.66	306 ± 1.20
S6	0.038 ± 0.04	475 ± 0.07	12.12 ± 0.69	16.87 ± 0.81	4.74 ± 1.92	82 ± 0.63	299 ± 1.19
S7	0.040 ± 0.06	482 ± 0.07	14.17 ± 0.78	18.91 ± 0.82	6.94 ± 2.43	-	307 ± 1.17
S8	0.039 ± 0.05	475 ± 0.06	12.19 ±0. 93	17.99 ± 0.88	4.72 ± 2.34	-	300 ± 1.22

Table 3: Mechanical properties, WVTR, Swelling Index of cross-linked hydrogel films

Film Code	Swellingindex (%)	WVTR ($g/m^2/day$)	Tensile strength (N)	Elongation at Break (mm)
S0	25.57	169.23 ± 23	10.11± 0.43	30.51 ± 0.08
S1	69.78	339.11 ± 28	38.36 ± 5.39	25.51 ± 0.08
S2	62.24	335.83 ± 32	28.52 ± 1.45	28.10 ± 0.09
S3	70.46	341.41 ± 18	39.12 ± 2.32	27.23 ± 0.02
S4	65.14	336.22 ± 31	28.61 ± 2.12	29.11 ± 0.01
S5	33.51	232.62 ± 29	22.15 ± 2.66	28.98 ± 0.10
S6	31.27	167.10 ± 33	10.74 ± 0.83	29.58 ± 0.16
S7	34.79	234.34 ± 21	21.24 ± 1.31	30.12 ± 0.15
S8	30.57	170.23 ± 23	11.12 ± 1.22	28.22 ± 0.02

MDC-S, Mitutoyo Co, Japan) was employed to evaluate the thickness of film at five sides. For weight variation films were weighed independentlyand average weight of twelve films were calculated using average standard deviation (± SD).[20]

Surface pH measurement

Hydrogel films were placedin distilled water for half hr in test tube. Calculation of pH was done by calibrating pH meter in a buffered solution (pH varied from 6.8, 7.2, 7.4) then bringing the glass electrode near the surface of the film and allowing to equilibrate for 1-2 mins.[20]

Drug content uniformity (Metronidazole assay)

The quantification of MTZ was done by using UV spectrophotometer (Shimadzu-1800) at 220 nm. Stock solution of MTZ was prepared by taking 0.01 g drug in 10 ml phosphate buffer (pH 7.4) Concentration of MTZ was calculated by using calibration curve.

Drug content

It was calculated by using the UV spectroscopic method where samples of predetermined area were collected from various regions of films and immersed in 50 mL of phosphate buffer (pH 7.4) and drug distribution was calculated as drug content (%).

Moisture content

The weight of hydrogel films were taken (Wi), and was removed and left for a day at room temperature in a desiccators containing activated silica gel. Following 24 hours, each film was weighed, to take the final weight Wf.

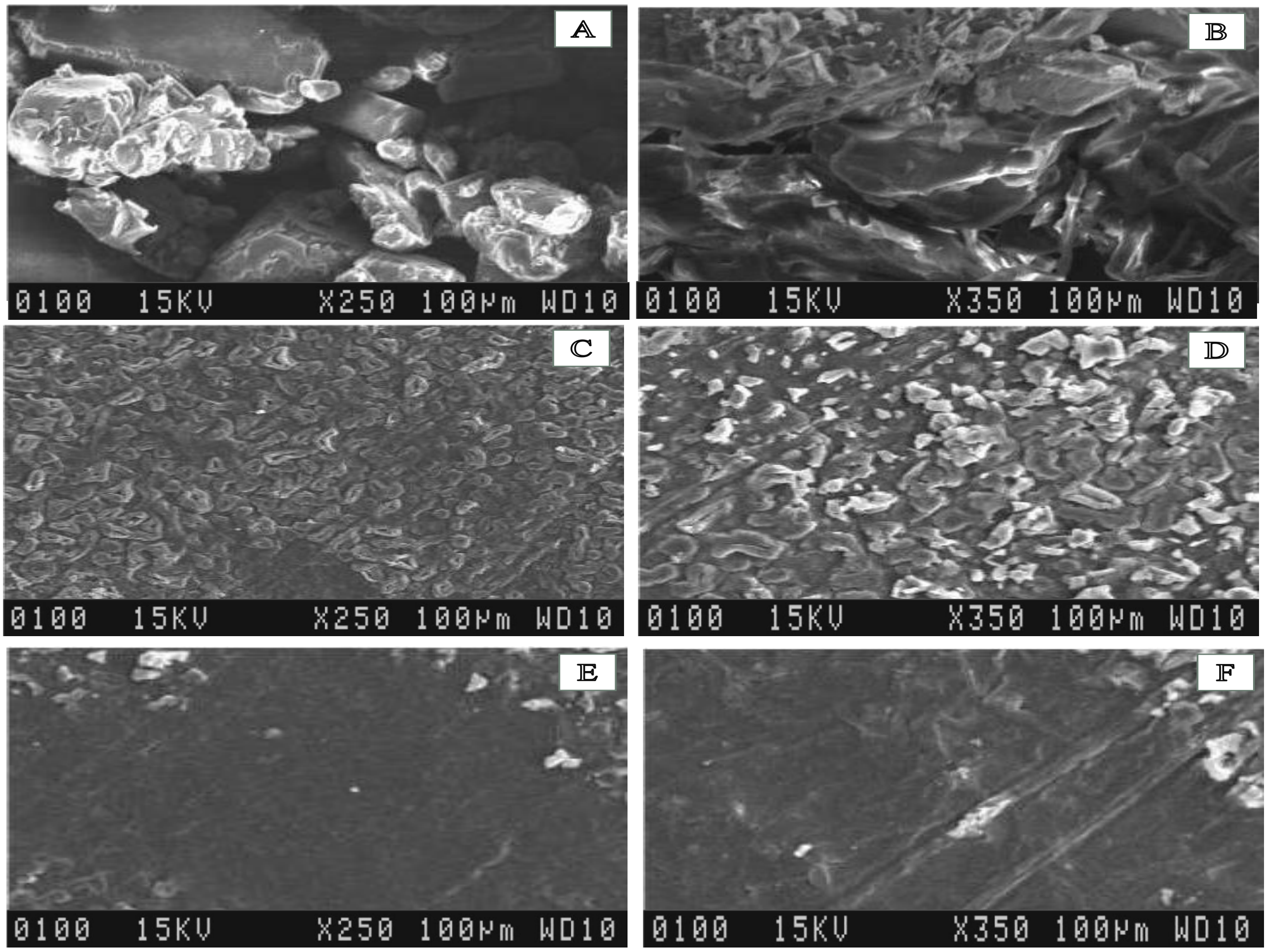

Figure 1: SEM images at magnification value (A) is 250×of drug; (B) is 350×of drug; (C) is 250×of S0 (Control) hydrogel film; (D) is 350×of S0 (Control) hydrogel film; (E) is 250×of hydrogel film (S3); (F) is 350×of hydrogel film (S3)

Moisture content was obtained by using equation 1.[20]

$$\text{Moisture Content \%} = \left[\frac{(\mathrm{Wi}-\mathrm{Wf})}{\mathrm{Wf}}\right] \times 100 \ldots\ldots.\ldots.(1)$$

Percent moisture uptake

Initial weight of hydrogel films were taken and put into a desiccator filled with saturated aluminum chloride solution (75% ± 25°C). Afterward films were removed and reweighed individually. The % moisture uptake capacity was calculated according to the following formula.[21]

$$\text{\% moisture uptake} = \frac{\text{Final wt.} - \text{Initial wt.}}{\text{Initial wt.}} \times 100 \ldots (2)$$

Percent moisture loss

Initial weight of hydrogel films were taken out and put into a desiccator with anhydrous calcium chloride and recalculate the weight after three days. The percentage of moisture loss was calculated by using following formula.[21]

$$\text{\% moisture loss} = \frac{\text{Final wt.} - \text{Initial wt.}}{\text{Initial wt.}} \times 100. \ldots. \quad (3)$$

Folding endurance

A specific section of film was cut and folded multiple time at the same site until it was interrupted in order to test folding endurance. The folding endurance of the film was determined by how many times it could be folded without breaking.[21]

Water vapor transmission rate

WVTR was determined by measuring weight loss of conical flasks (25 mL), which contains 10 mL of phosphate buffer (pH 7.4). Hydrogel films were mounted on the top of these conical flasks and took the weight (Wi), then placed in an oven at 35°C for 24 hrs. Following formula was employed to calculate the WVTR.

$$\mathrm{WVTR} = \frac{\mathrm{Wi} - \mathrm{Wt}}{\mathrm{A} \times 24} \times \frac{\frac{10^6 \mathrm{g}}{\mathrm{m}^2}}{\mathrm{day}} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \ (4)$$

Where, WVTR is expressed in10^6 g/m^2/day, A is area of top of conical flask (mm^2), Wi and Wt are the weight of conical flasks before and after being placed in the oven, respectively.[22]

Swelling index (%) of hydrogel films

Hydrogel films were dried and sliced into square shapes measuring about 1cm^2, and initial weights were recorded. Further, these films were dipped into PBS pH 7.4 and were removed after specific time intervals (30 to 1440 mins). The extra water from the film surface was removed by blotting films with tissue paper after each interval of time and the final weight was noted.[21] The % swelling index was calculated as following.

$$\text{Swelling Index (\%)} = \frac{\mathrm{Wt} - \mathrm{Wo}}{\mathrm{Wo}} \times 100 \ldots\ldots\ldots\ldots\ldots (5)$$

Where Wo is the initial weight of the film at time 0 and Wt is the weight of the swelled film at time t.

Characterization of drug loaded cross- linked hydrogel films

SEM, FTIR, XRD, DSC

Morphology of hydrogel film were observed by employing scanning electron microscopy (SEM) (S 4300 SE/N, Hitachi, CA, USA), 10kV (accelerating voltage).Fourier transform infrared spectroscopy (FTIR) (IFS66/S, Alpha Bruker, Ettlingen, Germany) was performed for all selected polymers and vacuum- desiccated hydrogel films. Investigation for these samples were done across the absorbance range of 4000-400 cm^{-1} with a resolution of 4 cm^{-1}. All of the selected samples were examined using an X-ray powder diffractometer (XRD) (Ragaku miniflex - 600 X - Ray diffractometer, Japan) to determine their crystalline phase, under specification as scanning with a counting time of 0.001s between 2 θ of 0-60°. For differential scanning calorimetry (DSC) (Mettler Toledo star 1, Switzerland) analysis, all samples were placed and sealed in aluminium pans and heated at rate of 10°C/min within a temperature range of 50-300°C.

In vitro drug release

The hydrogel films composed of 75mg equivalent weight of Metronidazole (MTZ) were attached to a mesh screen and put on a glass slide. The assembly was securely positioned at the bottom of the dissolution test apparatus (Paddle type- Lab India DS 8000, Mumbai, India). Phosphate buffer pH 7.4 used as dissolution media and set the temperature 37°C ± 0.5°C at 50 rpm. 5 mL sample were taken at regular time interval and replace by fresh buffer. The release of MTZ was determined by UV /Visible spectrophotometer (2202, Systronics, India) at 220 nm.

Antibacterial and antimicrobial study

MTZ-loaded cross-linked films (Control and S3) were left for drug release in PBS at 37 °C for one day. After the drug release in PBS for 24 hrs these films were used to observed the antibacterial activities. The Kirby-Bauer Agar diffusion method was employed for calculating antibacterial activity of MTZ-loaded cross-linked hydrogel films. Here, Bacteriodes fragilis *(B.fragilis*) (anaerobic gram -ve bacteria) and Escherichia *coli* (*E. coli*) (facultative anaerobic gram -ve bacteria) were used. Agar medium was prepared as a culture media for study. Both strains were cultured in nutrient broth and were kept in incubator shaker for 12 h at 37 °C. The bacterial culture suspension of E. coli and B. fragilis were prepared and modified in contrast with 0.5 McFarland standard (~1.5 × 10^8 bacteria per mL), then inoculated into different agar plates to induce steady growth. Clean filter paper with a diameter of 7mm was respectively immersed in the cross-linked hydrogel films S3 and without crosslinked hydrogel film S0 (- control), another uncontaminated filter paper was just immersed with MTZ for comparison study. Incubation was done for one day at 37°C and clear zone area was calculated. In order to evaluate antimicrobial activity seperatley the microbes were inoculated in yeast extract and cultured through the night at 37°C.About 200μL diluted microbial suspensions were mixed with drug (MTZ) control and sample film (1cm × 1cm) and incubated at 37°. A control group of microbes had no treatment and microbial viability was calculated as:

$$\text{Microbial viability} = \frac{\mathrm{N_{sample}}}{\mathrm{N_{control}}} \times 100 \ldots\ldots\ldots\ldots\ldots\ldots.(6)$$

Hemolysis assay

The cross-linked hydrogel films (2 cm^2) were standardize in phosphate buffer saline (PBS) for 1 hr at 37 °C. Afterward 0.5 mL citrate phosphate dextrose (CPD) blood was added. After 20 mins, 4.5 mL of 0.9% sodium

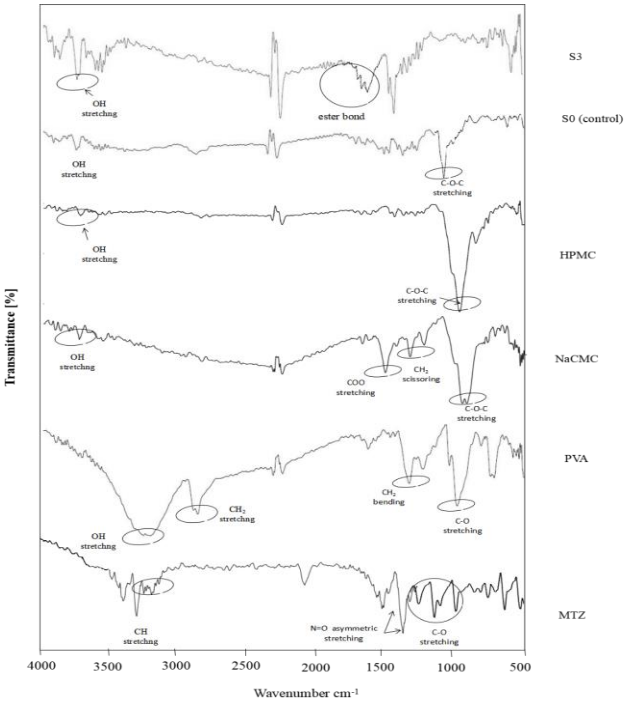

Figure 2: FTIR spectra of drug (MTZ), PVA, NaCMC, HPMC, Control film (S0) and crosslinked hydrogel film (S3).

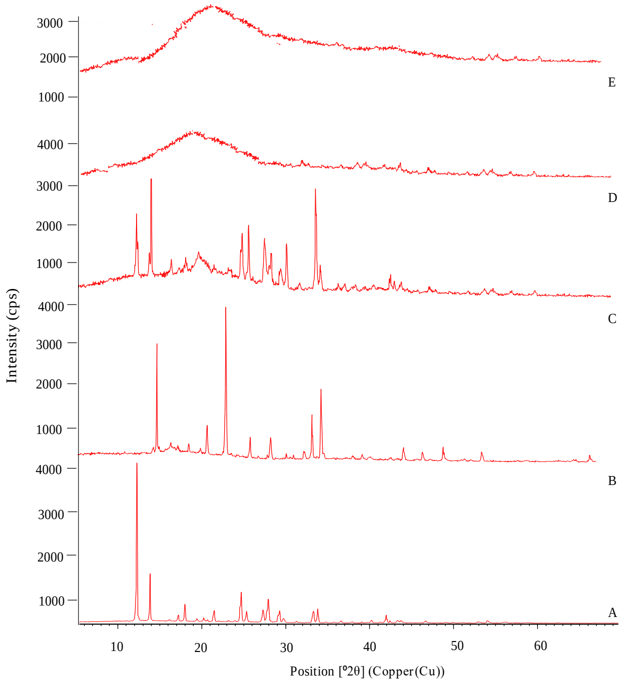

Figure 3: X-ray Diffraction of A MTZ; B mixture of NaCMC, HPMC, PVA; C mixture of NaCMC, HPMC, PVA, MTZ; D control hydrogel film (S0); E crosslinked hydrogel film (S3)

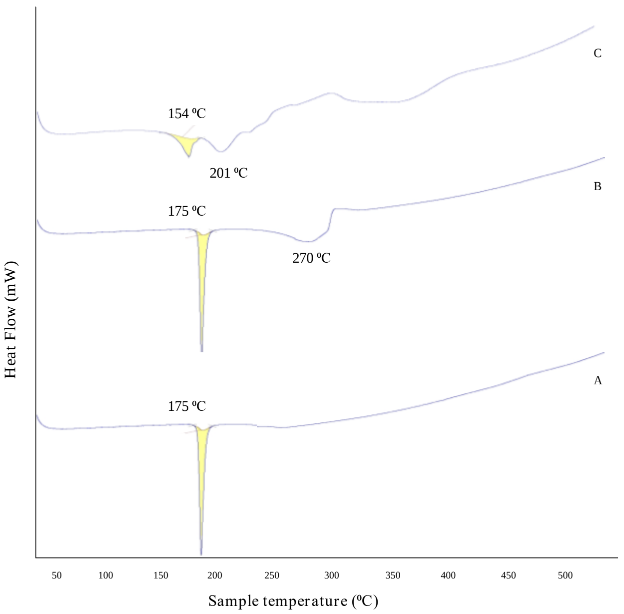

Figure 4: DSC thermograms of (MTZ);Control film (S0); crosslinked hydrogel film (S3)

chloride solution was introduced to each sample to stop hemolysis, and incubation was done for 1 hr at 37 °C. Positive and negative controls were prepared using 0.5 mL of human CPD blood and saline in 4 mL of distilled water. The incubated sample were Centrifuged at 1500 rpm for 15 mins and the supernatant was collected and measured spectrophotometrically at 220 nm. Following equation was used to determine % hemolysis rate.

$$\text{Hemolysis rate }\% = \frac{\text{At} - \text{An}}{\text{Ap} - \text{An}} \times 100 \ldots\ldots\ldots\ldots\ldots (7)$$

Where A is the absorbance values of experimental and control groups with CPD blood and Saline. At is test sample, An is negative control and Ap is positive control. Three readings were taken out by repeating the procedure three times.

RESULTS AND DISCUSSION

Evaluation of drug-loaded cross-linked hydrogel films

The outcomes of different physicochemical evaluations of hydrogel films are depicted in Table 2. These evaluation tests are standard of quality and reliability of the procedure used to prepare the transdermal films. Thickness and weight for hydrogel films varied between 0.037± 0.02 mm to 0.042 ± 0.05.mm and 472 ± 0.04 g to 479 ± 0.09 g, respectively which reveals the different concentrations of polymers in films with different viscosity. Moisture content ranged between 11.43 ± 0.84 % to 14.22 ± 0.77 %. Film with PVA have higher water content due to hydroxyl groups that absorb water. Moisture uptake and moisture loss ranged from13.78 ± 0.77 % to 19.89 ± 0.72 % and 3.16 ± 2.65 % to 6.94 ± 2.43 % respectively. Drug content in films were ranged from 70 ± 0.49 % to 97± 0.76 %. Low drug content in S0 (control) hydrogel film is due to lack of crosslinker that leads to inadequate distribution of drug in this batch. Uniform entrapment of MTZ was observed in batch S3 followed by S7 which indicated presence of PVA in these batches. Thus, using more than two polymers (biopolymer composite) signify the reliability of manufacturing process.Folding endurance assessment suggests ability of film to endure breakage. These values ranged from 220 ± 1.19 to 315 ± 1.90 for the hydrogel films. Control

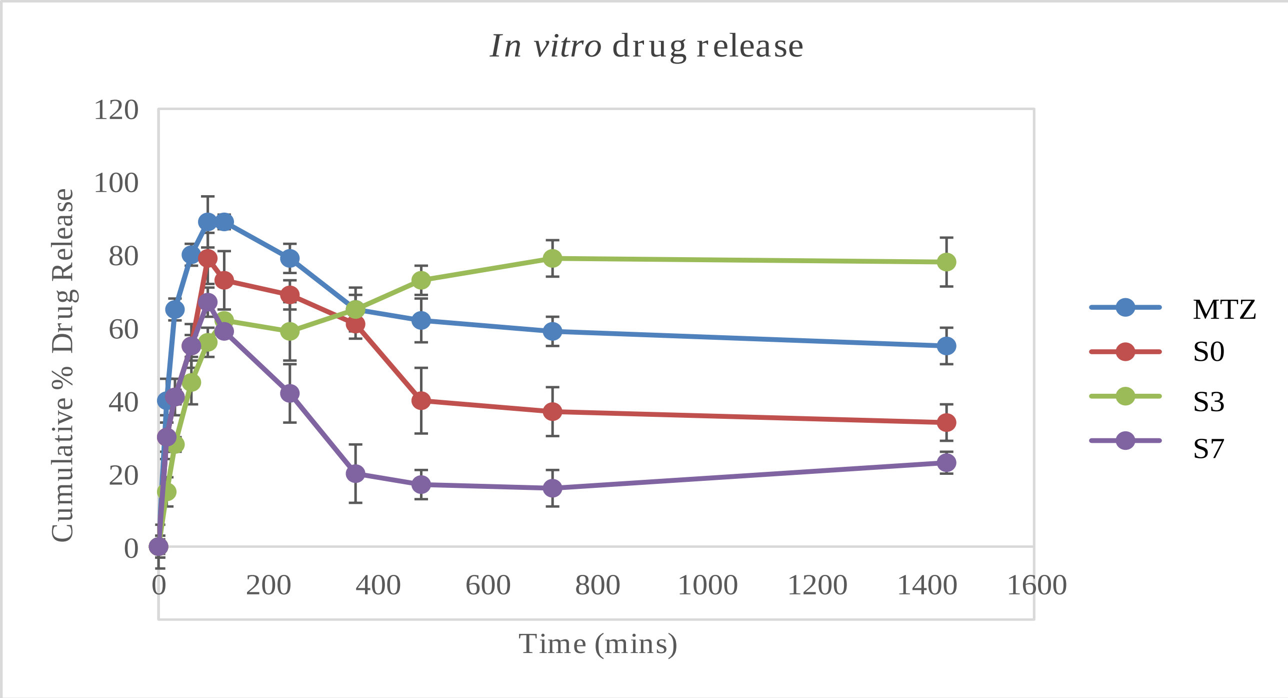

Figure 5:*In vitro* drug release of pure drug (MTZ), control (S0), S3 and S7 hydrogel films

hydrogel film without CA have lowest folding endurance which shows its poor strength.

Mechanical properties of drug-loaded cross-linked hydrogel films

Mechanical properties of hydrogel films were determined by evaluating tensile strength (N) and elongation at break (mm). Tensile strength for hydrogel films ranged between 10.11 ± 0.43 N to 39.12 ± 2.32 N as depicted in Table 3.S0 (Control) hydrogel film shows lowest tensile strength which indicates poor mechanical properties of this film. Hydrogel films S1 and S3 shows the good tensile strength due to more concentration of NaCMC in S1 & S3, also PVA is present in these films which make strong bond with NaCMC. Drug loaded S3 hydrogel film shows the highest value of tensile strength this is due to good cross-linking in film as OH group of PVA makes bond with carboxymethyl groups of NaCMC that leads to strong bonding. However, quantity of PVA should be minimum in order to prevent dilution effect of hydrogel film as more concentration of PVA may cause reduction of carboxyl content which further decrease the cross-linking density.[23]Elongation at break is inversely proportional to tensile strength as the tensile strength increases elongation at break decreases. These values ranged between 25.51 ± 0.08 mm to 30.51 ± 0.08 mm.

Water vapor transmission rate (WVTR)

The Water Vapor Transmission Rate (WVTR) studies assist in the determination of the moisture balance capacity of hydrogel films along with the films permeability to confirm its oxygen supply at the wound site.[1]WVTR studies have a strong impression on the evaluation of hydrogel films and ensure a moist environment around the wound site. In this analysis, WVTR for films ranged between 167.10 ± 33 to 341.41 ± 18 g/m^2/day, Table 3. Films with PVA shows more WVTR values due to its hydrophilic nature also increasing the concentration of NaCMC leads to an increase in WVTR.Increasing the concentration of HPMC leads to decrease in WVTR by making dense and less permeable films. HPMC in more concentration shows less porous structure due to entanglement of HPMC molecules and WVTR decreases, HPMC film without PVA shows the lowest WVTR (167.10 ± 33 g/m^2/day). If the WVTR value is too low, it will give rise to exudate accumulation at the wound site that may lead to microbial growth there.[24]

Swelling index (%)

The percent swelling index indicates the fluid intake potential of hydrogel films, that is an crucial variable in wound healing applications. It was found that control hydrogel film without any crosslinker have 25.57 % swelling index, which reveal that swelling % decrease with time. Hydrogel films (S4 to S8) that contains more concentration of HPMC show high swelling initially which decreases with time and films get dissolved completely in solvent. As the swelling index is inversely proportional to cross-linking density thus these results indicate poor cross-linking in these films. Drug-loaded (S3) NaCMC film shows more swelling as compare to control film (S1), this indicates more free -OH group of PVA which show O-H stretching vibration that increases the hydrophilic nature of this film.[25]Further HPMC in this film reduces the NaCMC intramolecular repulsion.

Characterization of drug loaded hydrogel films

Scanning Electron Microscopy (SEM) images display the surface morphology that are depicted in Figure1. Here A and B shows morphology of pure drug at magnification of × 250 and × 350 respectively, The surface of S3 is smoother than S0, indicating good cross-linking density of the hydrogel film. Further crosslinking was confirmed by

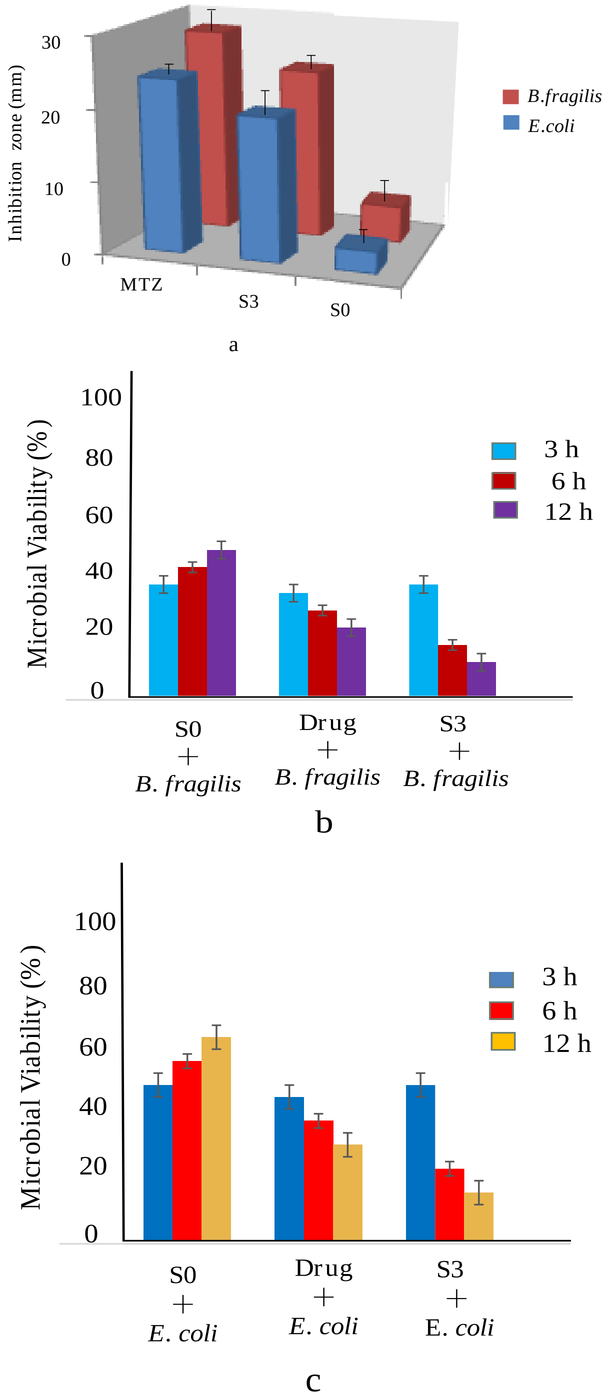

Figure 6:Antimicrobial andAntibacterial activities of drug (MTZ) and drug loaded control hydrogel film S0, crosslinked hydrogel film S3 against B.fragilis and E. *coli.*(a) zone of inhibition;(b) Microbial viability against *B. fragilis;* (c) Microbial viability against *E. coli*

the FTIR peak at 1795-1749 cm^{-1} due to the formation of ester bond between cyclic anhydride of CA and -OH groups of NaCMC, HPMC and PVA.[26]S0 (control) hydrogel film shows small rod-shaped structures on its surface which indicates the presence of the drug and drug is not properly distributed in the film according to SEM images. This indicates the lack of crosslinker in this film and absence of peak at 1795-1749 cm^{-1}.Fourier transform infrared (FTIR) spectra results are illustrated in Figure 2, that shows different peaks for different polymers.

These peaks helps to determine the structure and compatibility of polymer and drug used in formulation. NaCMC show peaks at 3734 cm^{-1} (-OH stretching),2874 cm^{-1} (C-H stretching), 1590 cm^{-1} (COO stretching),1318 cm^{-1} (OH bending), HPMC at 3725 cm^{-1} (-OH stretching),1057cm^{-1} (C-O-C stretching).[27] For PVA, OH stretching observed at 3242 cm^{-1} and C-O stretching at 1083cm^{-1}. For the drug (MTZ) FTIR shows peaks at 1466 cm^{-1}(N=O asymmetric stretching), 3011cm^{-1} (C-H stretching), 1275-1096 cm^{-1} (CO stretching), 1387 cm^{-1} (CH_3 bending), 1590 cm^{-1} (C=C stretching). Hydrogel film S3 show extra peak at 1795-1749 cm^{-1} due to crosslinking and in S0 (control) this peak is absent. XRD pattern of drug, mixture of polymers, mixture of polymers and drug, cross-linked hydrogel film (S3) are shown in Figure 3 (A-E). Drug (Metronidazole) shows several sharp diffraction peaks which reveal the crystalline nature of drug. All polmers and drug mixed in polymers shows several crystalline peaks. The diffraction pattern of control (S0) shows small crystalline peak which indicates lack of intermolecular hydrogen bonding between HPMC and NaCMC. However hydrogel film show amorphous nature even in absence of crosslinker. Cross-linked hydrogel film (S3) shows the absence of sharp peaks which indicate reduction in crystallinity also absence of HPMC peak reveal that there is proper bonding between polymers and crosslinker (citric acid). Dilaver et al. (2016) reported fumaric acid cross-linked hydrogel film of NaCMC and PVA that shows the broadening of hydrogel film.[28] Shi et al. (2008) reported a decrease in the height of the peak in citric acid cross-linked PVA/ Starch hydrogel film.[29] Peak broadening is directly related to crosslinking density, as the crosslinking increases in the film, the mobility of polymers within the film decreases and peaks become broaden.[30] DSC thermogram of pure drug Metronidazole, control hydrogel film without crosslinker (S0) and CA cross-linked hydrogel film (S3) batch are shown in figure 4. Characteristic endothermic peaks for MTZ, observed at 175 °C, which indicates the melting point of a drug. Control hydrogel film (S0) show same peak as pure drug i.e. 175 °C, indicates inadequate distribution of drug without crosslinker. Water evaporation peak is shown by CA crosslinked S3 hydrogel film at 154 °C. Endothermic peaks 201 and 270 indicates degrdation of NaCMC side chain.[31]

In vitro drug release

The *in vitro* drug release was executed for different hydrogel films to evaluate the release of drug, for extended period of time that is essential parameter in wound healing application. As depicted in Figure 5, it was observed that Pure drug MTZ show higest drug release but this decrease with time due to immediate release effect. Formulation S3 shows highest drug release for 24 hrs, this is because strong linkage between PVA and cellulose derivatives (i.e. NaCMC5 %, HPMC 1%), S0 hydrogel film without crosslinker shows drug release lesser extent as compared to S3 but better than S7 due to low concentration of NaCMC in this batch. Here S7 shows highest drug release initially because of high swelling index and poor cross-linking density of this hydrogel film. Afterward there was decrease in drug release from these hydrogel films due to poor cross-linking and drug was not entrapped properly in this batch.[26] Value of n for drug loaded films were ranged between 0.301 to 0.671, n indicates anomalous transport, where drug release was affected by both diffusion and relaxation of drug from polymer matrix. Films S0 and S8 indicates Fickian diffusion where process is primarily controlled by diffusion, whereas hydrogel film S3 shows anomalous diffusion which is combination of both diffusion and swelling.

Antibacterial and antimicrobial study

To investigate the antibacterial and antimicrobial properties of MTZ and drug-released cross-linked hydrogel film S3 and control S0, against B. *fragilis* and *E. coli*, the filter paper method and colony count method was employed.(figure 6 a, b,c) Antibacterial experimental data were analyzed and the diameter of the inhibitory zone was applied as a benchmark of antimicrobial activity. The inhibitory zone around filter paper was observed higher for MTZ, while cross-linked film S3 shows a higher zone of inhibition for both strains as compared to S0 batch hydrogel film. S0 batch films have poor cross-linking and drug release occurs at a very fast rate initially and there may be a small quantity of drug available after 24 hrs. Although S3 batches hydrogel films release drug in a controlled manner and the highest amount of drug remained in this hydrogel film due to the ester bond formation.Also the viability of microbes decreases after coculturing with pure MTZ as well as MTZ-loaded hydrogel film for 3, 6, and 12 hours. After 12 hr viability of B. *fragilis* remains at 16 % in pure drug and 10 % in film-treated culture. However, *E. coli* shows viability up to 22% in pure drug and 15 % in film-treated culture. Although film S0 (without crosslinker) show reduction in microbial viability at 3 hour but there is increase in microbial viability with time. This indicates poor crosslinking and drug may not entrapped properly in this hydrogel film. This low bactericidal activity for *E. coli* is due to presence of an additional lipopolysaccharide-containing membrane inthese bacteria.[32,33]

Hemolysis assay

A hemocompatibility test was performed to determine the biocompatibility of cross-linked hydrogel films. This test involved the disintegration of red blood cells (RBCs) near the cross-linked hydrogel films. Liberated hemoglobin (Hb) dissolved in surrounding fluid giving it a yellowish color, that could be determined spectrophotometrically.% hemolysis for all hydrogel films was ranged between 1.16-2.68%. According to the ISO standard, a drug is considered as safe if its hemolysis index is <5%.[34] Lower percent hemolysis of hydrogel film accredits to good hydrophilicity of polymer matrix, which reduces the interaction of polymers with RBC, and also lower the destruction of RBC.[35] Observed % hemolysis was found to be with in permissible limit that indicate the hemocompatibility of cross-linked hydrogel films.

CONCLUSION

In this study biopolymer composite hydrogel films were successfully formulated and evaluated forl wound healing applications. Different concentrations of NaCMC, HPMC and PVA were taken to develop hydrogel films by solvent casting method. FTIR verified the ester bond formation at peak 1795-1749 cm^{-1} that confirms chemical cross-linking between cyclic anhydride of crosslinker and OH groups of polymers.WVTR for hydrogel films were ranged between 167.10 ± 33 to 341.41 ± 18 g/m^2/day. Drug-loaded S3 batch hydrogel film shows the highest WVTR (341.41 ± 18 g/m^2/day) value, due to more concentration of NaCMC and low concentration of HPMC which leads to water retention. S3 batch hydrogel films exhibit good moisture uptake, significant % swelling index and good mechanical strength. XRD result reveal drug loaded film S3 have broaden peak which indicates good cross-linking between polymers and drug. SEM, FTIR and DSC parameters were studied for surface morphology, compatibility and molecular interaction between biopolymers. The kinetic investigation was carried out and fitted into a number of kinetic models, where the n value showed that all of the created formulations behaved anomalously. Batch S3 hydrogel film shows non – Fickian transport. In vitro studies reveal, release of drug from batch S3 for 24 hrs. The prepared biopolymer composite hydrogel films can be employed as prospective wound healing applications.

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