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Citric Acid Crosslinked Hydrogel Dressings for Delivery of Metronidazole

Rajashri Badadare[1*], Kailas Mali[2], Remeth Dias[3]

[1]Department of Pharmaceutics, College of Pharmacy, Jawalwadi, Medha
[2]Department of Pharmaceutics, Adarsh Institute of Pharmacy, Vita
[3]Department of Pharmacy, Government Polytechnic, Jalgaon, Maharashtra, India
Email ID- rajbadadare[at]gmail.com

Abstract: ***The objective of this study was to synthesize and characterize citric acid crosslinked hydrogel films of carboxymethyl tamarind gum for topical drug delivery system. The hydrogel films were characterized by attenuated total reflectance- Fourier-transform infrared spectroscopy, solid state ^{13}C- nuclear magnetic resonance spectroscopy and differential scanning calorimeter. The prepared hydrogel films were evaluated for the carboxyl content and equilibrium swelling ratio. Metronidazole was loaded into these hydrogel films and drug release was monitored in different PH medium. Hemolysis assay was used to study Biocompatibility of hydrogel films. Results of attenuated total reflectance- Fourier- transform infrared spectroscopy solid state ^{13}C- nuclear magnetic resonance spectroscopy and differential scanning calorimeter confirmed the formation of citric acid crosslinked hydrogel films. Total carboxyl content of hydrogel film was found to be increased when polymer ratio and amount of citric acid was increased. On the other hand swelling of hydrogel film was found to be decreased with increase in polymer ratio and amount of citric acid. Results of haemolysis assay indicated that the citric acid crosslinked hydrogels were safe to be used in drug delivery. In vivo wound healing study showed that metronidazole loaded hydrogel film has significant higher wound healing rate than control group, group 1, and group 3. This indicated that prepared hydrogel films can be successfully used as dressings for wound healing.***

Keywords: Carboxymethyl Tamarind Gum, Citric acid, Crosslinking, hydrogel, Metronidazole

1. Introduction

Hydrogels are three-dimensional cross-linked polymer network that can respond to the fluctuations of the environmental stimuli. These biomaterials can incorporate large quantum of biological fluids and swell. When swelled, they are soft and rubbery and resemble the living tissue, exhibiting excellent biocompatibility. Their distinct physical properties make them suitable for drug delivery applications.(1) Hydrogels can be tailored to be sensitive to different environmental conditions, such as temperature, pH etc.(2)

Many crosslinking agents are widely used for the crosslinking of polymers but these crosslinking agents are either toxic or costlier and also crosslinking reactions are carried out in presence of organic solvent. Citric acid (CA) is a new crosslinking agent has low toxicity and costs compared to other crosslinkers.(3) It is inexpensive and non-toxic that have been used to improve the performance properties of cellulose and proteins in textile applications.(4)

Polysaccharides are one class of polymers that are able to form hydrogels that are highly hydrated and porous, being similar to living tissue and have low toxicity.(5) Many polysaccharides reported to be non-carcinogenic, biocompatible and bioadhesive in nature. They are widely used as a stabilizer, thickener, gelling agent, and binder in food and pharmaceutical industries. So, use polysaccharides in preparation of hydrogels holds a great promise in developing novel drug delivery systems. The preparation of hydrogels with citric acid as a cross-linker can overcome toxicity and costs when compared with other crosslinking agents. (6)

Polysaccharides are generally obtained from plant sources and are usually biocompatible. Due to their inherent biocompatibility, polysaccharides have been explored to design polymeric constructs of biomedical importance (pharmaceutical, cosmetic, and tissue engineering applications). The mechanical properties of the polysaccharide-based polymeric constructs are usually poor. Scientists have applied various methodologies to improve the mechanical properties of the polysaccharide constructs.

TG has been reported to be non-carcinogenic and non-toxic (biocompatible) in nature. The addition of TG improves the mucoadhesive property of the pharmaceutical formulations. The main disadvantages of TG include unpleasant odor and quick microbial degradation.

To overcome these disadvantages, the derivatization of TG by chemical treatment has been explored. Carboxymethylation is one such chemical modification. Introduction of carboxymethyl group in TG makes the polymer anionic (CMTG). This improves the hydration of the polysaccharide, thereby, resulting in the higher viscosity of the carboxymethylated product. It has been reported that the increase in the viscosity lowers the biodegradation of the polysaccharide.(7) CMTG has been reorted to have enhanced stability against degradability and therefore increases its material suitability for diverse potential biomedical application.

Topical antibiotics have broadspectrum antibacterial coverage which lasts for 12 h and are less toxic.(8) Metronidazole is a synthetic antibiotic and belongs to the 5-nitroimidazole group, which is highly effective against anaerobic bacteria and protozoa. Its mode of action is *via* the reduction of its nitro group which leads to the production of short-lived cytotoxic intermediates. The toxicity of the intermediates is due to their interaction with deoxyribonucleic acid and possibly with other macromolecules which results in and inhibition of nucleic

acid synthesis. It is the only antimicrobial agent which can be used systemically and/or topically to treat wound infections as it reduces the malodor of anaerobically colonized wounds. Metronidazole has good anaerobic coverage and helps in maintaining a moist wound healing environment. (9)

Metronidazole is a drug of choice for variety of infections including bacterial vaginosis, pericarditis as well as for wound dressings. But, when given orally, it has metallic taste, produces nausea, epigastric pain(10). So, an attempt will be made to design and develop citric acid cross-linked polysaccharide based hydrogel dressing loaded with metronidazole.

Intrinsic properties of membranes as wound dressings to endorse the skin healing and to protect the skin defect zone from infection have been progressively investigated and applied in the clinical sectors since early eighties. The mechanism of hydrogels as wound dressings can be described as follows.

Hydrogels can absorb and retain the wound exudates, which promote fibroblast proliferation and keratinocyte migration. The last two processes are very necessary for complete epithelialization and healing of the wound. In addition the tight mesh size of hydrogels structure protects the wound from infection and prevents microorganism and bacteria to reach the wound area. However, hydrogels structure allows transporting bioactive molecules e.g. antibiotics, and pharmaceuticals to wound centre. Such molecules can be entrapped into hydrogel networks during gelling process, while these molecules can be exchanged with absorbing the wound exudates during the sustainable release process after contacting hydrogels with the wound surface. The significant tissue-like water content of hydrogels provides the needed flexibility and elasticity to adapt wounds located in different body sites.

The objective of the present work was to investigate the potential of citric acid crosslinked hydrogel dressings in the treatment of wound healing.(11)

In the present work we have prepared citric acid crosslinked hydrogel films. The effect of concentration of CMTG as well as effect of concentration of Citric acid on properties of hydrogel films was investigated. Metronidazole was used as model drug. The hydrogel films were characterized for the formation of crosslinks and evaluation was done for swelling index, drug loading, drug release. The biocompatibility of the hydrogel films was done by using haemolytic assay. In vivo wound healing activity of drug loaded hydrogel was performed. For this purpose Control gr- no treatment, Group 1- Blank hydrogel film, Group 2- Metronidazole loaded hydrogel film, Group 3- Standard marketed formulation (Metro gel) was used. From the above study it was concluded that the wound size of metronidazole loaded hydrogel film was significantly higher than any other group. From this it is concluded that prepared hydrogels can be used as a dressings in topical drug delivery system.

2. Materials and Methods

Carboxymethyl Tamarind gum (CMTG) and Metronidazole was kindly gifted by Chhaya industries, Barshi, Maharashtra, India. Citric acid (CA) and all other chemicals used of analytical grade were purchased from Loba Chemie, Mumbai, India.

2.1 Preparation of citric acid crosslinked CMTG based hydrogel films

The citric acid crosslinked hydrogel films of CMTG were prepared by esterification crosslinking mechanism, as reported elsewhwere.(12) The formula of Metronidazole hydrogel was developed by using CMTG as a polymer with the use of citric acid as a cross linking agent. Initially, citric acid and CMTG were weighed properly. Then the measured volume of water was added in the beaker mounted on mechanical stirrer. CA was dissolved in the beaker then polymer was dissolved in the solution with pinch by pinch addition until the viscous clear slurry gets prepared.

This slurry is then transferred in to the Petri dish cleaned properly and labeled well. Finally obtained hydrogel films were washed with distilled water and isopropyl alcohol for 1hr in order to remove untreated entities. Then the hydrogel films were dried in hot air oven at 50^0C for 24 hrs and stored in desiccator until use. The parameters such as concentrations of CMTG, Citric acid, curing time and curing temperatures were varied individually to study their effect on hydrogel properties. (13)

Table 1: CMTG Hydrogel Films

Parameter	*Batch code*								
	M1	M2	M3	M4	M5	M6	M7	M8	M9
CMTG (mg)	500	1000	1500	1000	1000	1000	1000	1000	1000
Citric Acid (mg)	200	200	200	200	200	200	200	150	250
Curing Temperature (^{0}C)	140	140	140	130	150	140	140	140	140
Curing Time (min)	10	10	10	10	10	15	20	10	10

CMTG- Carboxy Methyl Tamarind Gum

2.2 Determination of carboxyl content

Carboxyl content of the hydrogel films was estimated conducting acid-base titration (Doane, Wing, Farag, Reinhardt, &Farag, 1996; Salam & Brothers, 2011). An absolute quantity of hydrogel film was dissolved in enough quantity of 0.1N NaOH and stirred under a magnetic stirrer for 2h. Sodium hydroxide splits the ester linkages and reacts with the free carboxyl groups to form sodium carboxylate (citrate). The excess amount of 0.1N NaOH was titrated with 0.1N HCl using phenolphthalein as an indicator. The carboxyl content in milliequivalents per 100g of hydrogel films was determined as given below.(14)

$$\text{Carboxyl content} = \frac{(Vb\text{-}Va) \times N \times 100}{W} \quad (1)$$

where, N is the normality of HCl (eq/L), Vb and Va are the volumes of HCl in absence and presence of sample, and W is the weight of sample (g).

2.3 Attenuated total reflectance – Fourier transform infrared (ATR-FTIR) spectroscopy

ATR-FTIR spectroscopy was used for the study. The infrared spectra of CMTG, citric acid, and hydrogel films were obtained using ATR-FTIR spectrophotometer (Shimadzu, IR Affinity, Japan). The samples to be analyzed were transferred to the ATR compartment. The spectra were obtained for the range of 600–4000 cm−1 at an average of 25 scans and resolution of 4 cm^{-1}(15)

2.4 Solid state NMR spectroscopy

Solid state 13C cross-polarization-magic angle spinning (13C CPMAS) NMR spectra of CMTG, hydrogel film was measured using JEOL-ECX400 spectrometer operating at 400 MHz (contact time of 3.5 ms, relaxation delay of 5s, sweep width of 35 kHz and spinning speed of 10KHz). The chemical shifts were calibrated with the external hexamethylbenzene standard methyl resonance at 17.3 ppm**.** (15)

2.5 Thermal analysis

Thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) was performed on (batch M2, M7) hydrogel films using Mettler-Toledo TGA/DSC 1 thermogravimetric analyzer (Mettler-Toledo, Switzerland). Samples were heated from 30 ◦C–300 ◦C at the rate of 10 ◦C/min, under nitrogen atmosphere (flow rate: 10 ml/min)(16)(17)

2.6 Swelling index

The swelling index of hydrogel was determined by placing the weighed hydrogel in the beaker containing 20ml medium. Study was performed in 0.1N HCl and phosphate buffer pH 7.4 at 37ºC ± 0.5ºC. Hydrogel samples were withdrawn at a time interval of 30 min, blotted with tissue paper to remove the excess water and weighed on the analytical balance (Shimadzu, AX 120). Swelling index was calculated by using the following formula(18):

$$\text{Equilibrium Swelling (\%)} = \frac{\text{Weight of swollen hydrogel} - \text{weight of dry hydrogel}}{\text{weight of dry hydrogel}} \times 100 \quad (2)$$

All measurements were done in triplicate.

2.7 Drug loading

The loading of Metronidazole in the hydrogel films was carried out by placing the preweighed hydrogel films (~100) in 10ml mix containing metronidazole (5 mg/ml). After achieving the equilibrium swelling, hydrogel films were removed carefully and dried in hot air oven at 40°C for 24h.

2.8 Drug content

40-50 mg hydrogel was weighed and transferred to a beaker containing 0.1N NaOH which was previously mounted on a mechanical stirrer. Continued the stirring for 15-20 min. to dissolve the hydrogel completely in to solvent. Then obtained solution was filtered and made the dilutions in a proper manner. The resultant solutions were subjected to UV spectrophotometric analysis.(15)

Drug content can be determined by using formula:

$$\text{Drug content (\%)} = \frac{\text{Practical value}}{\text{Theoretical value}} \times 100$$

2.9 Drug release

Metronidazole loaded dry hydrogel films (≈ 50mg) were immersed in 10ml 0.1N HCl and PBS 7.4 at 25˚C by maintaining sink condition. Samples were removed periodically and replaced with fresh medium in order to maintain constant volume of the liquid (dissolution medium).The amount of Metronidazole released within the samples was determined spectrophotometrically at a wavelength λ = 276 nm and 320nm respectively. The experiments were conducted in triplicate.

2.10 Hemolysis assay

Hemolysis assay was performed according to the reported method with slight modification. Hydrogel films (2 cm^2) were equilibrated in PBS for 60 min at 37 ◦C and human CPD blood (0.5 ml) was added on films. After 20 min, 4.0 ml of 0.9% sodium chloride (NaC1) saline was added to each sample to stop hemolysis and the samples were incubated for 60 min at 37 ◦C. Positive and negative controls were obtained by adding 0.5 ml of human CPD blood and 0.9% NaCl saline, respectively, to 4.0 ml of double distilled water. The incubated samples were centrifuged for 10 min at 3500 rpm, the supernatant was taken, and its absorbance was measured on a UV–vis spectrophotometer (Shimadzu, Japan) at 545 nm.(19)(20)

The percent of hemolysis was calculated using the following relationship:(21)

$$\text{Hemolysis (\%)} = A_{\text{Test sample}} - A_{\text{–ve control}} \left(A_{\text{+ve control}} - A_{\text{–ve control}}\right) \times 100 \quad (4)$$

In vivo wound healing activity of optimized drug loaded hydrogel

After getting ethical clearance from Institutional Animal Ethics Committee; mice were procured from National institute of bioscience, Pune seven days before the commencement of the study. Animals were placed in polypropylene cages in a controlled room temperature 22±1°C and relative humidity of 60-70% in registered animal house (1915/PO/ReBi/CPCSEA dated 04/11/2016). They were maintained with standard pellet diet (Amrut, Sangali, India) and water *ad libitum*.

Wound healing studies of hydrogel wound dressings on rat model

Rats were anesthetized with diethyl ether, the surgical area will be shaved and a wound, approximately 1cm^2, will be created on the dorsal side of the rat, using surgical scissors.

Rats will be randomly divided into three groups:

a) Control group in which wound will be left to heal spontaneously,
b) Group I in which wound will be treated with blank hydrogel film,
c) Group II in which wound will be treated with metronidazole loaded hydrogel films.
d) Group III in which wound will be treated with standard marketed formulation of Metronidazole. (Metrogel).

Tissues of the wounded area will be taken on 4^{th}, 8^{th} and 12^{th} day. Tissue sections 5mm thick will be cut using microtome, stained with haematoxylin-eosin, and photographed with Motic microscope, to study the changes in wounded skin. Wound healing of open wound, wound covered with drug loaded and unloaded hydrogel films will be compared.(22)

3. Results and Discussion

The various preformulation batches were prepared in order to optimize concentration of citric acid, curing temperature and curing time to obtain hydrogel films. The prepared hydrogel films were washed with distilled water to removeany traces of unreacted citreic acid and polymer. The total polymer concentration, Citric acid concentration, curing time and curing temperature was varied in different batches.

At very low concentrations of citric acid, the CMTG based hydrogel films were not formed because the concentration required for proper crosslinkig was not adequate, which may lead to loss of integrity of hydrogel film. At high concentrations of citric acid, the hydrogel films formed were rigid, which are having low water absorbing capacity.(23) The hydrogel films were became rigid and they were having reduced ability to absorb water due to increase in crosslinking density which in turn may have lead to the reduction in the mobility of polymer chains and reduced the free volume of hydrogel network.(24)

The curing temperature was varied from 130^0C to 150^0C. At lower temperature poor crosslinked gel was formed, whereas when the curing temperature was increased at that time firm and tough hydrogel film was formed. This might be due the formation of strong hydrogen bond interactions between polysaccharide CMTG and citric acid leading to reduction in expansion and relaxation in polymer chains.(23) The optimal swellin.2g was observed at a curing temperature 140^0C.

The curing time was varied from 10 to 20 minutes. The 10 min curing time was found to be sufficient to form the citric acid crosslinked hydrogel film while higher curing time lead to formation of brown coloured films with insufficient swelling. This could be exhibited due to interaction between –OH gropus of polysachharides and dehydrated cityric acid leading to formation of strong crosslinking.

3.1 Mechanism of formation of hydrogel film-

In formation of hydrogel films of CMTG and CA, esterification reaction takes place therefore crosslinking occurs. When CA is heated at high temperatures, formation of intermediate cyclic anhydride takes place which is responsible for developments of crosslinks with CMTG. This formed intermediate anhydride opens under action of polysaccharide –OH functional groups via esterification. leading to a new carboxylic acid unit in carboxylic acid, which has the property of forming new intra-molecular anhydride moiety with neighboring carboxylic acid unit. In esterification process there may be involvement of primary –OH groups as it is more reactive than the secondary –OH from the structure of the polysaccharides.(25)

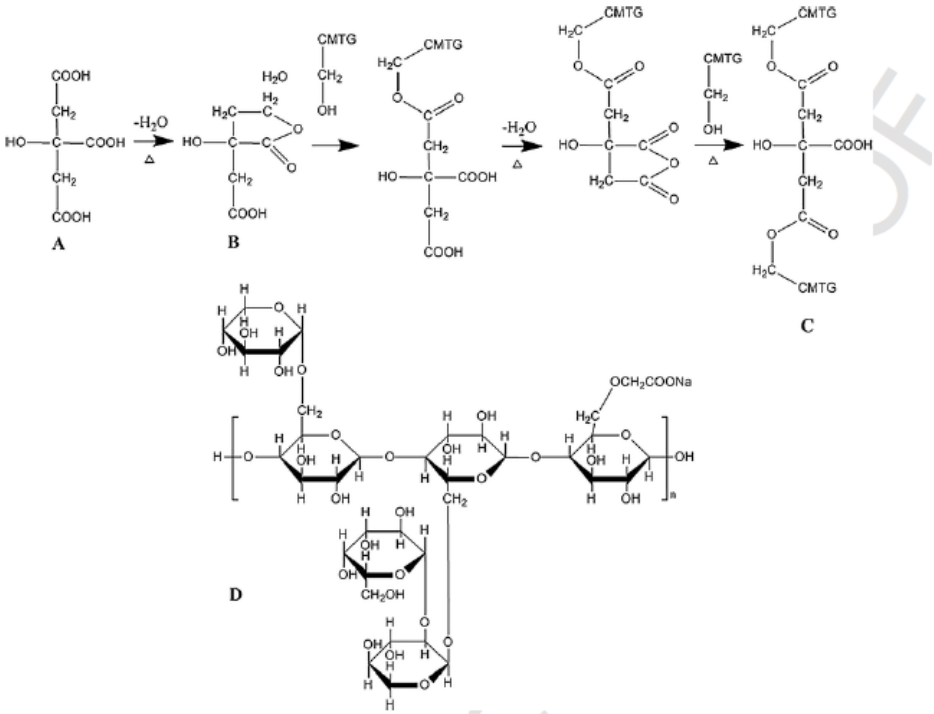

Figure 1: Possible crosslinking reaction between citric acid (A) and CMTG (D); citric acid anhydride (B); CMTG (C)

The total carboxyl content of citric acid cross linked CMTG hydrogel was found to be in range 2.285 to 2.857. The maximum total carboxyl content was observed in batch M7. An increase in the concentration of CMTG caused decrease in the carboxyl content of the hydrogel films. This indicates that the crosslinking density of the hydrogel films was reduced with increase in CMTG concentration. Also, an increase in the CMTG concentration may reduce the number of crosslinks per unit weight of the hydrogel films and eventually reduce the carboxyl content. Therefore, batch M1 has less carboxyl content than M2 and M3. When curing temperature and curing time was increased, carboxyl content was found to be increased indicating increased crosslinking.

Figure 2 shows Overlay ATR-FTIR spectra of Metronidazole, CMTG, Citric acid, Blank hydrogel film M6 and loaded hydrogel film M6 and NaOH treated M6 respectively.

The spectrum of Metronidazole shows broad peak at 3209.55 cm^{-1} due to intramolecular 'H' bonding of -OH stretching and peak at 3095.95cm^{-1} of aliphatic C-H stretching. The peak at 1531.48 cm^{-1} is due to (-NO_2, N-O) stretching & the peak at 1072.42 cm−1 is due to C-OH bending.The spectrum of citric acid shows broad peak at 3491.16 cm^{-1} (aromatic OH) then 3278cm^{-1} (O-H stretching) and sharp peak at 1693.50cm−1 (C=O stretch) and at 1138.0 (C-O) stretching. The FTIR spectrum of CMTG shows peak at 1010 cm^{-1} for C-O-C stretching. The peak at 1639 cm^{-1} is of C=O. Also, it shows peak at 1402 cm^{-1} of COO^- and confirms the formation of CMTG from TG. The peak at 3424 cm^{-1} represents the –OH group. Also peak at 1639 cm^{-1} and 1402 cm^{-1} corresponds to asymmetric and symmetric stretches of COO^- for pure CMTG.(26) The FTIR spectrum of blank hydrogel film M6 shows peak at 1631cm^{-1} of C=0 stretching, peak at 3452cm^{-1} corresponding to –OH group and peak at 1024.20 cm^{-1} of –C-O-C. Blank hydrogel film shows peak at 1631.78cm^{-1} corresponding to C=O stretching. It also shows peak at 3452.58cm^{-1} attributed to OH group and peak at 1024.20 relates to –C-O-C linkage. Loaded hydrogel film shows peak at 1265.30cm^{-1} attributed to C-O stretch, 2885.51cm^{-1} of aliphatic C-H stretching, it also shows peak at 1363.67cm^{-1} of C-N vibration. It shows peak of intramolecular 'H' bonding of O-H stretching at 3209.55cm^{-1}.The peak at 1531.48cm^{-1} represents C-NO_2 for symmetric stretching.(9) NaOH treated hydrogel film shows peak at 1587.42cm^{-1} and peak at - 1732 cm^{-1} of carboxylate band and ester carbonyl band respectively. Blank film shows peak at 1726.29 cm^{-1} which gets disappeared in NaOH treated film. NaOH treatment confirms the formation of ester linkages within the hydrogel films.(25)

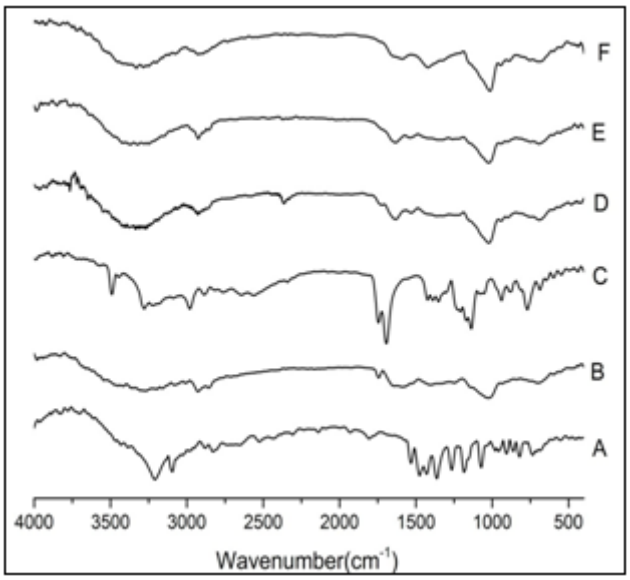

Figure 2: Overlay ATR-FTIR spectra of Metronidazole, CMTG, Citric acid, Blank hydrogel film M6 and loaded hydrogel film M6 and NaOH treated M6

Solid state ^{13}C NMR of hydrogel film is given in figure 3. The solid state ^{13}C NMR of hydrogel film shows the characteristics peaks of CMTG and CA. The broadening of the peak at ~174.34 ppm was observed due to cross liking of CMTG and CA. It confirms formation of hydrogel film.

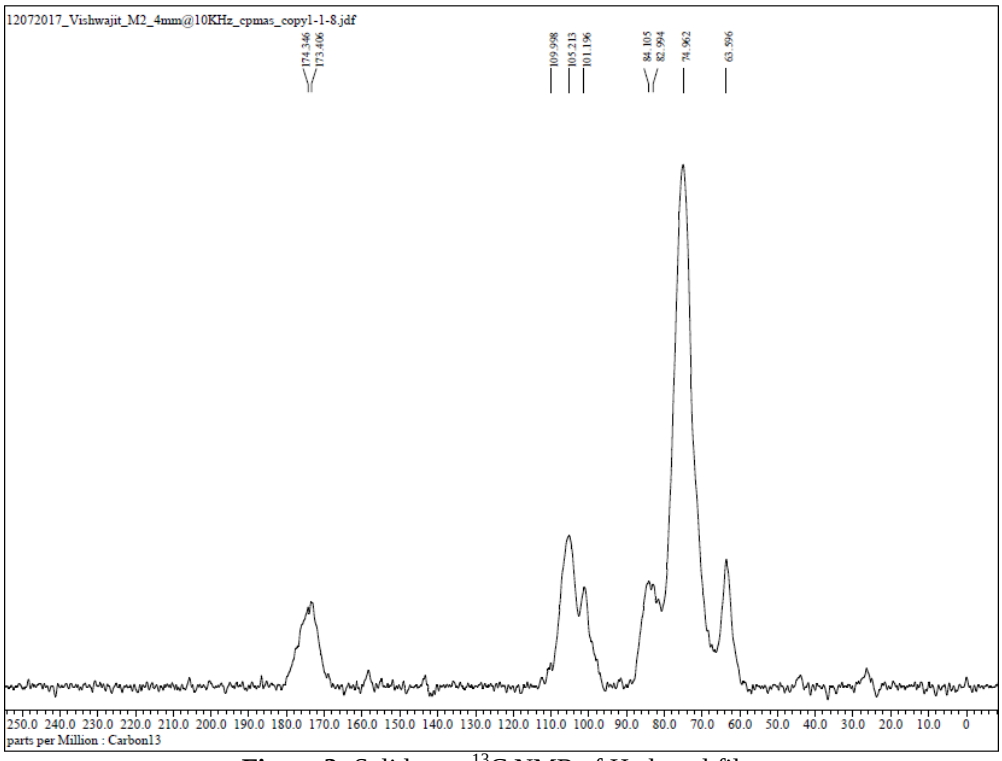

Figure 3: Solid state ^{13}C NMR of Hydrogel film

Figure 4 illustrates the TGA and DSC of hydrogel batch M2. Thermal decomposition curve of M2 showed two main stages of decomposition. The first stage begins at 28.29^{0}C and ends at 184.08^{0}C. This may be due to the removal of free and bound water from the polymer. The second stage of weight loss observed at around 286^{0}C and ends at 410.47^{0}C with 47.25 % loss of weight. In this stage loss in weight is attributed to decomposition of polymeric backbone. In third stage 27.05% weight loss occurs due to further decomposition of polymer. DSC shows exotherm at 504.90^{0}C

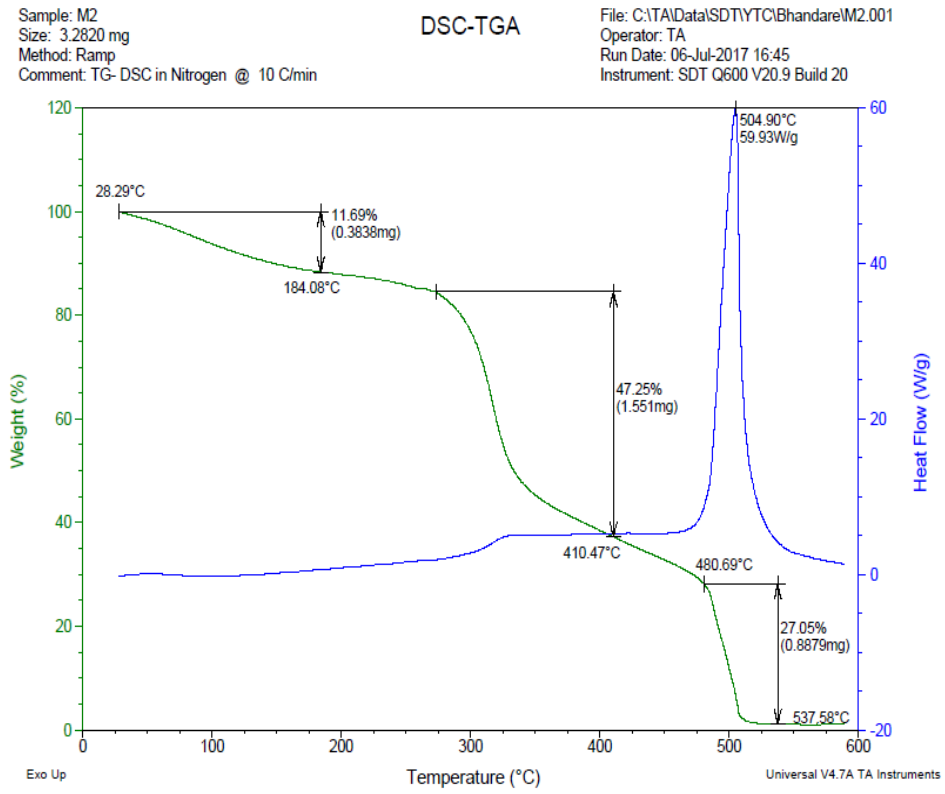

Figure 4: DSC AND TGA of HYDROGEL FILM M2

The swelling study was performed in 0.1N HCL, Phosphate buffer 7.4, and in water. The result of swelling study is presented in figure 5A.Batch M3 exhibited maximum swelling in PBS 7.4(14.15g/g). An increase in CMTG

concentration resulted in increase in swelling ratio. Therefore, batch M1 has less swelling ratio (10.06g/g) than M3 (14.15g/g).This may be due to high crosslinking density which caused decrease in the network space and mobility of polymer chains. Therefore, polymer network becomes more rigid, so entry of diffusion medium into the polymer network gets retarded. This means that CMTG concentration may affect swelling ratio. Curing time and curing temperature also affected swelling ratio. Increase in curing time resulted in decrease in swelling ratio due to increase in extent of crosslinking, resulting in formation of strong polymer network at higher curing time. Therefore, Batch M7 exhibited least swelling (6.99g/g) due to higher at higher curing time. Therefore, Batch M7 exhibited least swelling (6.99g/g) due to higher curing time.

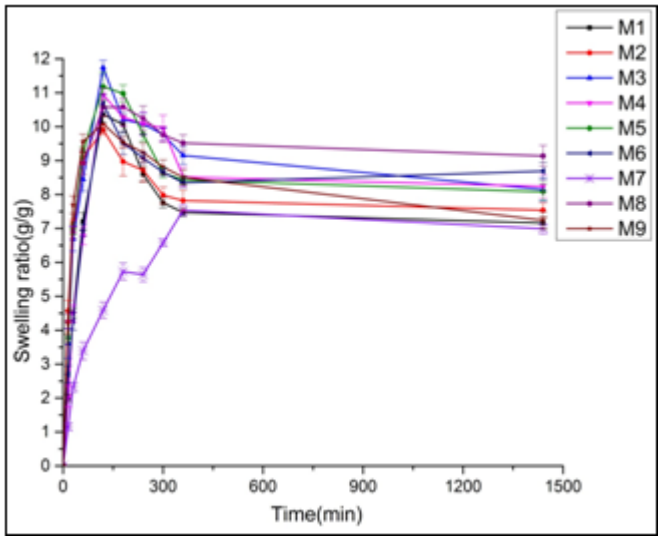

Figure 5 (A): Equilibrium swelling of hydrogel film in phosphate buffer 7.4

Effect of CMTG concentration on swelling is presented in the figure 5B. An increase in CMTG concentration resulted in increase in swelling ratio. Therefore, batch M1 has less swelling ratio than M3.

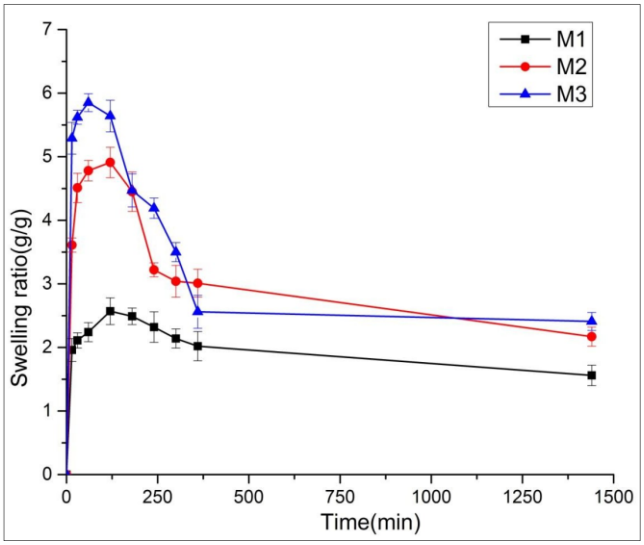

Figure 5 (B): Effect of CMTG concentration on swelling

It was noticed that concentration of citric acid was increased the swelling ratio of hydrogel film was found to be decreased significantly. This might be due to an increase in the degree of crosslinking, which altered the mobility of polymer chains. Also diffusion of swelling medium into the polymer chains was also decreased giving rise to more rigid structure of polymer network.(27)

The drug loading was done in aquous solution of Metronidazole through diffusion process in swollen networks. The results of drug loading studies are presented in Table-1.

The maximum drug loading was observed in batch M8 which may be due to maximum equilibrium swelling and lowest concentratiom of CA. The drug loading in hydrogel film was mainly dependent on the equilibrium swelling degree. When the concentration of CMTG and citric acid was increased the equilibrium swelling was found to be decreased. This may results in reduction in drug loading efficiency of hydrogel film.

Batch M1 showed less drug loading (45.126) as compared to M2 and M3.This may be attributed to low concentration of CMTG in M1 as compared to M2 and M3.

Table 1: Drug loading

Batch	Drug/gm of hydrogel (mg)	Standard Deviation
M1	45.126	2.52
M2	56.980	2.15
M3	73.020	2.16
M4	38.385	3.78
M5	34.872	2.87
M6	37.037	2.54
M7	31.108	3.12
M8	97.152	2.87
M9	28.671	2.14

As the prepared hydrogel films could be used to deliver the drug toically, in vitro drug release study was performed at pH7.4. The in- vitro drug release profile from Metronidazole loaded hydrogel films is given in Figure 6.

All CMTG hydrogel films exhibited ~10 to 60% of burst release of MTZ. This may be attributed to the surface associated drug. The free drug molecule back diffused from the bulk of the hydrogel matrix at the surface along with solvent during drying of drug loaded swollen hydrogel films. So, when the drug loaded hydrogel film comes in contact with the dissolution medium, the surface associated free drug is released at faster rate. The retardation of drug release is associated with the swelling of the drug loaded hydrogel film. The swelling of hydrogel film increased the thickness of film from which drug gets diffused in to bulk of dissolution medium.

When CMTG concentration was increased in the hydrogel film drug release was found to be increased. In case of M1 high degree of crosslinked network in which drug gets entrapped reduces the drug diffusion from the hydrogel matrix to the bulk of solution. In case of M3, loose crosslinked network structure of the hydrogel film contributes to increase the drug release. (Figure 6B)

When the concentration of crosslinking agent i.e citric acid was increased (figure 6C), drug release was found to be decreased in hydrogel films. This might be due to fact that as the concentration of citric acid was increased, the crosslinking density was also increased, which in turn resulted into decrease in swelling of the polymer matrix resulting into retardation of drug release.

The curing temperature and curing time also affects the drug release from hydrogel matrix. At low curing temperature (batch M4), the poorly crosslinked hydrogel matrix was formed which increased the drug release whereas at high curing temperature (M5) hydrogel matrix with high crosslinking density was obtained causing decrease in the drug release. Drug release was retarded with increase in the curing time.

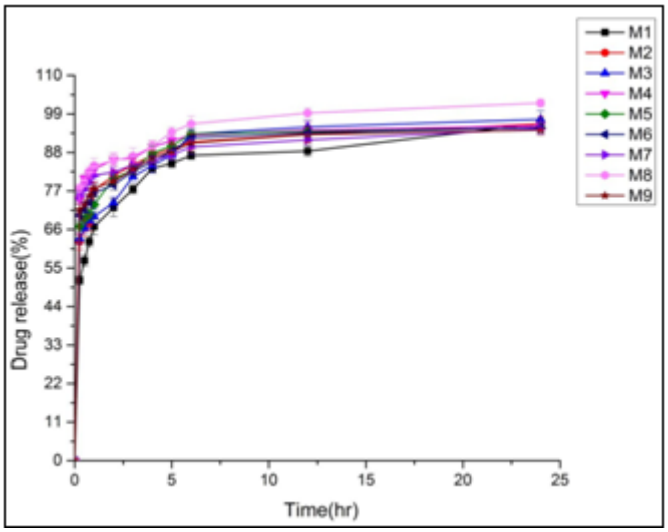

Figure 6: Drug release of hydrogel films M1 to M9 in phosphate buffer pH 7.4

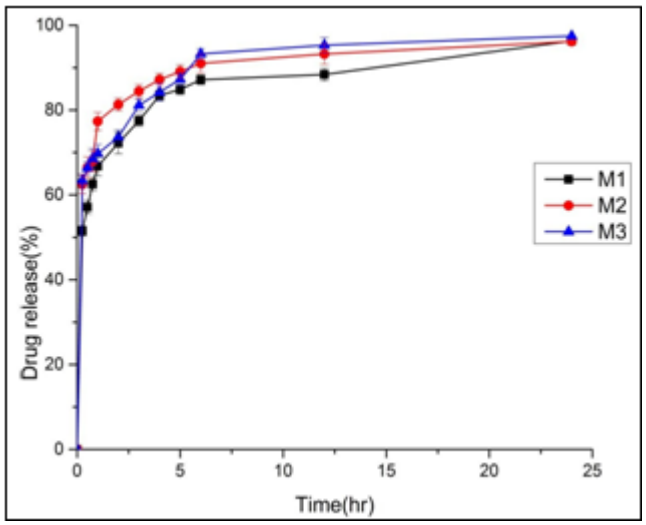

Figure 6 (B): Drug release of hydrogel film from M1 to M3 in phosphate buffer pH 7.4 Showing effect of CMTG concentration

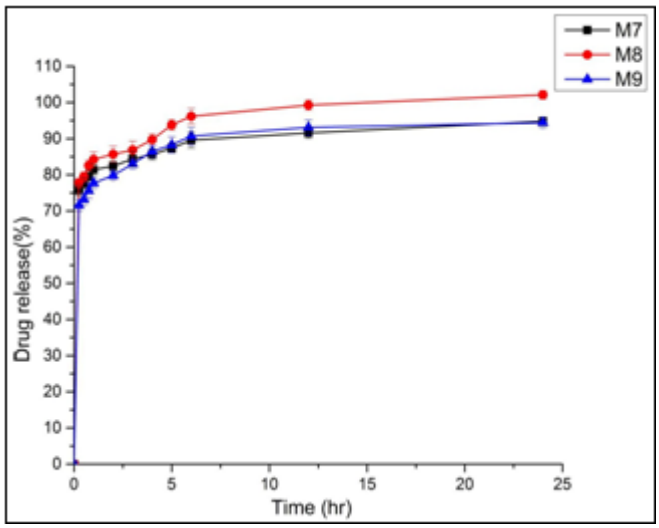

Figure 6 (C): Drug release of hydrogel films M7 to M9 showing effect of citric acid concentration in phosphate buffer pH 7.4

As the prepared hydrogel films could be used for topical drug delivery, hemocompatibility study was performed for determination of biocomapatibility. The test is based on the determination of lysis of red blood cells in presence of the hydrogel films. The releasaed hemoglobin is dissolved in the external fluid giving a yellowish colour, which could be measured spectrophotometrically. The higher the optical density of the supernatant, the greater the cell damage.(28) The results of hemolysis assay were given in Table -2

Table 2: Hemolysis assay of hydrogel film

Batch	Hemolysis (%)
M1	4.22
M2	2.04
M3	4.75
M4	3.70
M5	4.46
M6	4.90
M7	4.30
M8	3.76
M9	2.82

The percent hemolysis for all hydrogel films was found be 2.04 to 4.90 %.Lower percent hemolysis for hydrogel film could be attributed to higher hydrophilicity of polymer matrix, which decreased polymer RBC- interactions and lowered disruption of RBCs.(24) We found that the hemolytic activity of all batches was within the acceptable limit of 5%. This indicates that the Citric Acid Cross linked CMTG hydrogel films are hemocompatible.(15)

3.2 In vivo wound healing activity of drug loaded hydrogel

Control group-no treatment, **Group 1**- Blank hydrogel film, **Group 2**- Metronidazole loaded hydrogel film, **Group 3**- Standard marketed formulation (Metrogel)

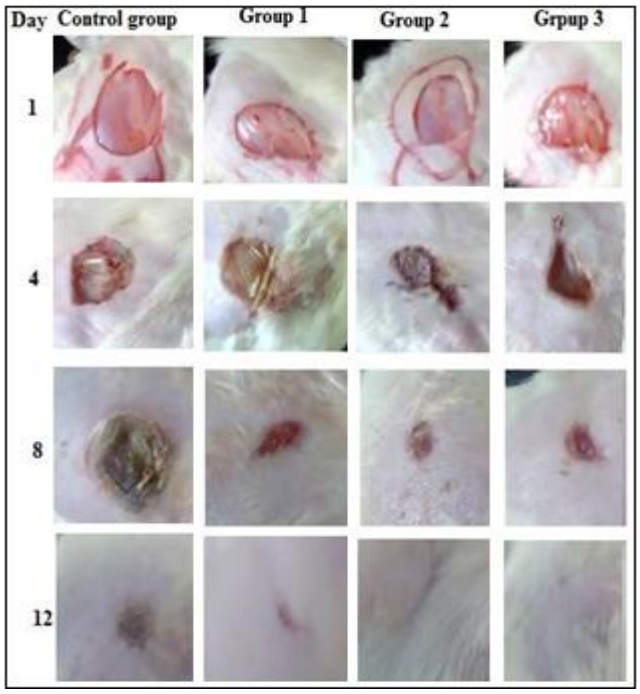

Figure 7: In vivo wound healing activity of drug loaded hydrogel

Figure 7 shows in vivo wound healing activity of drug loaded hydrogel and table 19 illustrates wound size (mm) of control group, group 1**(**Blank hydrogel film), Group 2-(Metronidazole loaded hydrogel film), Group 3- (Standard marketed formulation -Metrogel) in Rats. The wound contraction was faster in case of Metronidazole loaded hydrogel dressing than control group. Results revealed that the wound size of all groups of rats was decreased with time interval of 4^{th},8^{th} and 12^{th} day. The healing was significantly high in case of drug loaded hydrogel dressing as compared to other groups. This indicates that Metronidazole loaded hydrogel dressings exhibited good wound healing activity.(29)(22)

Table 3: In vivo wound size (mm)

Day	Control group	Wound size (mm)		
		Group 1	Group 2	Group 3
1	2.5	2.5	2.5	2.5
4	1.5	1.4	1.0	1.2
8	1.1	1.1	0.4	0.9
12	0.6	0.8	0.1	0.6

From the above table it is clear that wound size of Metronidazole loaded hydrogel significantly differs from all other groups. From this, prepared hydrogels can be used as dressings for topical drug delivery.

4. Conclusion

In the present study, hydrogel films were successfully prepared by using CMTG and CA as a crosslinking agent. Formation of ester linkage between CMTG and CA was confirmed by ATR-FTIR study, thermal analysis, ^{13}C NMR study. Different parameters such as concentration of CMTG, curing time, curing temperature showed significant effect on carboxyl content, swelling ratio, drug loading and drug release of prepared hydrogel films.

Hemolysis assay of hydrogel films revealed that hydrogel films are hemocompatible and can be successfully used as a drug delivery system. In vivo wound healing study showed that metronidazole loaded hydrogel film has significant higher wound healing rate than control group, group 1, and group 3.This indicated that prepared hydrogel films can be successfully used as dressings for wound healing.

From the study, it can be concluded that citric acid has ability to crosslink CMTG and forms hydrogel. The prepared CMTG hydrogel films can be used for the controlled release in topical drug delivery systems.

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