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Original article

# Targeted drug delivery of Methotrexate *in situ* gels for the treatment of Rheumatoid Arthritis



Madhugiri Prakash Venkatesh\*, Tegginmat Pramod Kumar, Deeksha Ramananda Pai

Department of Pharmaceutics, JSS College of Pharmacy, JSS Academy of Higher Education and Research, Sri Shivarathreshwara Nagara, Mysuru 570015, Karnataka, India

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## ABSTRACT

Rheumatoid arthritis (RA) is considered a debilitating disease that increases the risk of significant morbidity and premature mortality. To circumvent drug-related toxicity and ineffectiveness of anti-inflammatory drugs, there is a significant need for an advanced delivery system that increases bioavailability. The feasibility of *in situ* gel of methotrexate sodium (MTS) as an effective management for Rheumatoid arthritis was investigated. It was formulated with pluronic F-127 (PLF-127) as primary polymer, hydroxypropyl methylcellulose K4M (HK4M), and polycarboxophil (PCL) as a copolymer and characterized by various parameters. The efficacy evaluation by Freund's complete adjuvant (FCA) model, biocompatibility assessment by histopathological studies conducted. The optimized *in situ* gel (M4) was thermoresponsive, released  $93.26 \pm 2.39\%$  MTS at 96 hours. In addition, distribution of MTS was even in the optimized sterile and syringeable *in situ* gel. *In vivo* studies on wistar rats demonstrated a substantial reduction in paw oedema during the 28-day study period and were biocompatible with the tissues at the injection site. The study was successful in formulating, optimizing MTS *in situ* gel for effective management of RA.

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## 1. Introduction

Rheumatoid arthritis (RA) is an “immune mediated disease characterized by joint pain and inflammation, with progressive erosion of the cartilage and bone, accompanied by synovial proliferation” (Chopra, 2000; Lee and Kavanaugh, 2003). The cause of RA is unknown, 1% of the population suffers from RA, usually beginning at the age of 30–50 (Pai et al., 2019). An estimated 2.5 million people in US is affected by RA and has economic impact in billions (Lipsky and Kavanaugh, 1999). About 0.5–0.75% Indian population suffer from the disease, with a woman to man ratio 3:1 (Misra et al., 2008). RA also promotes death rate, with continued existence of patients with ruthless ischemic heart disease or lymphoma which has great impact on monetary cost (Pincus, 1998; Feldmann et al., 1996).

Based on the pathogenic criteria, RA is an autoimmune disease against unidentified self-antigens. In RA, the damage to group of cells is caused by synovium inflammation (Magne et al., 2006). As a consequence of extra-articular immunologic defects, other organs are prone to get damaged (Abdel-Nasser et al., 1997; Sokka et al., 2008). RA leads to disability, joint deformation, and mortality as reported by various studies (Leigh and Fries, 1994; Pincus et al., 1984; Wolfe et al., 1994). Multiple studies have reported higher death rates along with RA patients with other comorbidities. The death is caused due to heart related disease, carcinomas and infections (Avina-Zubieta et al., 2008; Meune et al., 2009; Sacks et al., 2004; Larsen et al., 2008).

Despite of advancements in basic science and therapeutics, the present treatment of RA remains partly efficient with several disadvantages which include dose, time of administration or other related toxicities (Butoescu et al., 2009). The ideal system must be administered easily, delivering the drug in a sustained manner and retain optimum drug concentration without disagreeable adverse events. Various therapeutic agents available for the treatment of RA comprises of (a) NSAIDs (non-steroidal anti-inflammatory drugs)/selective cyclooxygenase-2 (COX-2) inhibitors, (b) Disease-modifying anti-rheumatic drugs (DMARDs), (c) Glucocorticoids, (d) Natural origin compounds and (e) Biological agents (Pincus, 1998).

\* Corresponding author.

E-mail addresses: [venkateshmpv@jssuni.edu.in](mailto:venkateshmpv@jssuni.edu.in) (M.P. Venkatesh), [pramodkumar@jssuni.edu.in](mailto:pramodkumar@jssuni.edu.in) (T.P. Kumar), [deeksharpai@jssuni.edu.in](mailto:deeksharpai@jssuni.edu.in) (D.R. Pai).

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Methotrexate (MTS), a folic acid antagonist that inhibits DNA and RNA synthesis, is the most potent, and commonly prescribed synthetic DMARD, and can retard joint destruction. It acts by reversibly inhibiting dihydrofolate reductase which reduces dihydrofolate to tetrahydrofolate. Inhibition of this enzyme interferes with purine and pyrimidine synthesis. MTX is used in low doses for the treatment of RA and is termed as the “Gold Standard Treatment for Rheumatoid Arthritis”. Also, MTS has been very commonly used as the “anchor” agent for combination therapy with other DMARDs and biological agents for the effective treatment of RA. But the precise mechanism of action of MTX in RA is understood only to a limited extent; however, MTS acts as both an antiproliferative as well as an anti-inflammatory agent against the cells responsible for the joint inflammation in RA. The studies showed that MTS induce immunosuppression by inhibiting both dihydrofolate reductase and other folate-dependent enzymes, leading to adenosine overproduction (Cronstein, 1997).

To avoid various toxic effects of anti-arthritis drugs, the requirement for developing customized formulation which delivers drug to reach effective concentration at the desired site is warranted. Novel injectable systems have gained considerable attention in this arena. Targeted delivery has numerous benefits such as site specific delivery, maximum drug concentration at the site and limiting systemic-drug related toxicities (Albert et al., 2006). The rapid clearance of drug from target site is a significant problem which can be overcome by giving multiple injections. However, multiple injections may cause infection or joint disability (Yang et al., 2011). A depot system which can retain the drug concentration for the desired time interval at injection site may help resolve the issue.

Polymeric *in-situ* gels have been used as drug delivery vehicles because of their ability to undergo transformation from solution to gels at body temperature. The formulated system can be sterilized easily, deliver the active ingredient at desired location and have prolonged action due to gel formation. The gels are modified to decrease the drug amount required for beneficial effects, thus provides better patient comfort and compliance. Pluronic F-127 (PLF-127) is listed in the FDA's inactive substance database in “GRAS” (generally considered safe) ingredients. These polymers are non-toxic, non-irritant, biocompatible and biodegradable (Vohra, T et al., 2013).

Various DMARDs such as methotrexate sodium (MTS) are used in therapy; however, the treatment is still unsatisfactory due to its undesired pharmacokinetic parameters such as low bioavailability, long half life and narrow therapeutic window limit its therapeutic benefits via conventional delivery. Treatment using MTS is discontinued in 8–19% of patients due to diverse drug-related toxicities, including suppression of the formation of new blood cells leading to a severe form of anaemia, liver damage, pulmonary disturbances, renal disturbances, gastrointestinal disturbances (stomatitis, enteritis, ulcerations, and bleeding of the mucous membranes of the mouth or other portions of the gastrointestinal (GI) tract and abdominal distress) and potential effects on the central nervous system. Therefore, its use in normal routine therapy is limited. In the present scenario, MTS is the preferred drug of choice as it has dual anti-inflammatory and immunosuppressant effects, which can be useful in RA (Wenham et al., 2013; Venkatesh et al., 2013; Schmolka and Artificial skin, 1972).

The ultimate goal in the treatment of RA is to prevent joint damage and restore normal life. To reduce the drug related toxic effects on the body, there is a need of dosage form which can ensure the availability of MTX at right amounts with minimum exposure to body tissues and still be effective in the treatment of RA.

The main objective of the research work was to prepare an injectable MTS *in-situ* gel for sustained release of MTS for the successful management of RA.

## 2. Material and methods

### 2.1. Materials

PLF-127 was sourced from Sigma-Aldrich, St. Louis, United States. MTS was gifted by Samarth Life Sciences, Bangalore. Hydroxy Propyl Methyl Cellulose K4M (HK4M) was sourced from Loba Chemie Pvt. Ltd., Mumbai; Polycarbophil (PCL) procured from Arihant Trading Co., Mumbai, India. All formulations were prepared with analytical grade chemicals and double distilled water.

## 3. Methods

### 3.1. Fourier transform infrared (FTIR) spectroscopy

FT-IR studies were carried out to assess the compatibility between drug and excipients using FT-IR spectrophotometer (8400S, Shimadzu, Japan). Compatibility was ascertained by matching the IR spectra of the pure drug, total lipid and its physical mixture to identify any shift, appearance or disappearance of peaks.

### 3.2. Differential scanning calorimeter (DSC) studies

The thermal behavior of the sample was analyzed using DSC (DSC-60, Shimadzu, Japan). The required amounts of samples (pure drug, total lipid and its physical mixture) were placed in aluminum pans which were accurately weighed and then crimped. The samples were heated at a rate of 20 °C/min from 40 °C to 300 °C under constant nitrogen purging at a rate of 40 ml/min.

### 3.3. MTS *in-situ* gels formulation

Traditional “cold method” was employed for MTS *in-situ* gels preparation (Yang et al., 2009). With reference to formulation chart (Table 1) weighed amounts of PLF-127, other copolymers (HK4M / PCL) were gradually added in varying concentrations and dispersed in cold water (5–10 °C) under magnetic stirring. As per the cold method, the mixture was kept at 4 °C for overnight to aid into the gel formation. Dissolved MTS in 0.1 N NaOH and added to the preformed gel to obtain final concentration. While triethanolamine was added to adjust the pH to neutral, 0.99%w/v benzyl alcohol added to polymeric gel as antibacterial and benzalkonium chloride (0.001%w/v) as preservative.

### 3.4. MTS *in-situ* gels characterization

#### 3.4.1. Gelation temperature and time

Heated, MTS *in-situ* gelling solution in specially designed thin walled (0.6 mm) borosilicate glass tube (65 mm length & 10 mm

**Table 1**  
Composition of MTS *in situ* gels.

Formulation Code	MTS	PLF-127	HK4M	PCL
M1	0.5	20	–	–
M2	0.5	20	0.5	–
M3	0.5	20	1.0	–
M4	0.5	20	1.5	–
M5	0.5	20	–	0.5
M6	0.5	20	–	1.0
M7	0.5	20	–	1.5

\*Hydroxy Propyl Methyl Cellulose K4M (HK4M).  
Polycarbophil (PCL).  
Methotrexate Sodium (MTS).  
Pluronic F-127 (PLF-127).

internal diameter) by placing in a thermostatically controlled water bath with incremental rise in temperature (every 2 °C for 5 min) till it converted to gel. The absence of flow when the tube was overturned indicated the gel formation and was rendered 'gelation temperature' (Schuetz et al., 2008).

Heated 2 ml of MTS *in-situ* gelling solution at respective gelation temperature (determined earlier) in specially designed thin-walled borosilicate tube (as described above) in a thermostatically controlled water bath. The tube was inspected at periodic intervals, overturned to observe the flow of gelling solution. The time lag, when the gelling solution didn't flow was considered as 'gelation time' (Schmolka and Artificial skin, 1972).

### 3.4.2. Syringeability test

The syringeability test apparatus consisted of MTS *in-situ* gelling solution (5 ml, maintained at  $5 \pm 1$  °C) loaded 5 ml glass syringe and 18G needle held by vertical support. The time taken for syringeability of gelling solution (expel completely from syringe) under constant pressure (0.5 kg placed on pan) was recorded (Ravani et al., 2013).

### 3.4.3. Viscosity study

The viscosity of MTS *in-situ* gels was measured ( $n = 3$ ) at  $5 \pm 1$  °C and at  $37 \pm 1$  °C using viscometer (Brookfield DV Pro-II, United States). The samples were equilibrated in thermo stated water jacket for 10 min; viscosity read at 50 rpm with spindle no. 5 (Thakkar et al., 2007).

### 3.4.4. Drug content

The drug content in MTS *in-situ* gels equivalent to 1 ml was extracted in phosphate buffer solution (PBS) pH 7.4, diluted upto 100 ml. The drug content was determined spectrophotometrically at 303.5 nm (Florey, 2001).

### 3.4.5. Sterilization

Gamma irradiation is simple, efficient, and convenient for terminal sterilization of pharmaceuticals and is recommended by European Pharmacopoeia (Dorpema, 1990; European Pharmacopoeia, 2005; Helena Madruga Lima-Ribeiro et al., 2012). The optimized formulation purged with nitrogen, sealed in borosilicate glass vials was gamma irradiated (Microtrol, Bangalore, India -Cobalt-60 source) for 15.76 kGy dose at 25 °C for sterilization. Compendial sterility testing was carried out on irradiated samples to ascertain the effectiveness of sterilization (Indian Pharmacopoeia, 2014).

### 3.4.6. Sterility testing of formulations

Sterility test was performed as per the compendial method. The effectiveness of sterilization was determined by direct inoculation of MTS *in-situ* gels in fluid thioglycolate (FT) media and soyabean casein digest (SD) media. 2 ml of the formulation was transferred aseptically (and mixed) to 20 ml of FT media and 20 ml of SD media separately. The inoculated media were incubated for 14 days at 30 °C to 35 °C in the case of FT medium and 20 °C to 25 °C in the case of SD medium. Sterile liquid media was used as negative control and unsterilized gel was used as positive control.

Clouding of the liquid media implied contamination and ineffective sterilization, while a clear and uncontaminated liquid media indicates efficient sterilization (Snehalatha et al., 2012). Effect of sterilization on all the characterization parameters was ascertained.

### 3.4.7. In vitro drug release studies

In vitro release of MTS from *in-situ* gels was performed using a dialysis bag diffusion method. Dialysis membrane 110 (Hi-media Labs, India) with an average flat width of 32.34 mm (21.5 mm average diameter, approximate capacity of  $3.63 \text{ ml cm}^{-1}$ ) was used for diffusion. 1 ml of *in-situ* gel was placed in hydrated dialysis membrane (soaked in freshly prepared 7.4 PBS), and tied from both ends. The dialysis tube was positioned (lower end touching the surface of buffer) in a glass beaker with 30 ml of pH 7.4 PBS maintained at  $37 \pm 0.5$  °C. Aliquots of 3 ml were pipetted out at predetermined intervals and the same was replenished with fresh, warm diffusion medium to maintain constant volume. Subsequent to appropriate dilutions, samples were evaluated spectrophotometrically at 303.5 nm for MTS (Florey, 2001). The *in vitro* drug release profile was plotted and mechanism of drug release from gels was ascertained by fitting release data into various mathematical models.

### 3.4.8. In vivo study

Approval from Institutional Animal Ethics Committee for the conduct of preclinical study was obtained (IAEC No: JSSCPM/249/2017). Twelve wistar rats of either sex weighing 150–200 g were used for the study. The rats were randomly grouped into two with six rats in each group. The grouping and treatment schedule of experimental animals are shown in Table 2. On day "0", induction of arthritis was initiated by injecting 0.1 ml of Freund's complete adjuvant (FCA) into the sub-planter area of the right hind paw for all rats after anaesthesia. Animals were cautiously and carefully examined on daily basis by examining the induced paw and their general well-being. The sub-planter injection of FCA produces local oedema after few hours with a step by step elevation and reaching its peak level by 28th day. On day 14, the animals were randomized into control and test groups. The same day, test group was administered with MTS *in-situ* gel subcutaneously (SC), with second administration on day 21. The control group was administered saline SC weekly once, till day 28. Paw volume was measured on alternate days till day 28. The paw inflammation was measured (read as paw volume) using Digital Plethysmometer (7141 UGO Basile, Italy) on alternative days till day 14 to assess the development of arthritis in animals and its subsequent reduction till day 28 (Nagai et al., 2012; Miao et al., 2011).

### 3.4.9. Assessment of biocompatibility

Biocompatibility of optimized MTS *in-situ* gels was assessed in male wistar rats (150–200 g weight, aged 6–8 weeks). The right and left knee joint received 100 µL of optimized formulation and 100 µL saline solution respectively by IA. Both joints were monitored for swelling till 7th day; both joints were separated by euthanizing the animals on 8th day. The dissected joints were soaked in formalin (10%) for 48 h, dehydrated serially, embedded in paraffin,

**Table 2**  
Grouping of animals and treatment schedule for Freund's complete adjuvant (FCA) induced chronic arthritis in rat model.

Group	Induction of arthritis	Treatment
Control	Arthritis was induced by injecting 0.1 ml of FCA into the sub planter region of the right hind paw on day 0.	Received saline solution subcutaneously every week.
MTS <i>in situ</i> gel	Arthritis was induced by injecting 0.1 ml of FCA into the sub planter region of the right hind paw on day 0.	Received a single dose of MTS <i>in situ</i> gel subcutaneously per week

stained with haematoxylin and eosin. Histopathology of these samples were studied using light microscope (Olympus BX 51: Olympus, Japan) by a qualified pathologist for tissue reactions and cell infiltration in the synovium (Klouda and Mikos, 2008).

3.4.10. Stability studies

During storage conditions, the degradation of formulation may occur due to chemical modification or lack of formulation stability. The objective of stability studies was to ascertain the effect of the temperature and relative humidity (RH) conditions on stability of formulation by increasing rate of degradation. The optimized MTS *in-situ* gel were stored at  $5 \pm 3$  °C and  $25 \pm 2$  °C,  $60 \pm 5\%$ RH for 6 months (as per ICH Q1A (R2) guidelines) in amber coloured glass vials sealed secured with rubber bungs (ICH harmonized tripartite guidelines, 2003). Samples were analyzed at 3 and 6 months for changes in physical characteristics and drug content.

4. Results and discussion

The FT-IR spectra of the pure drug (MTS) and formulation M4 & M7 showed that the characteristics peaks of MTS were not altered without any change in their position, thereby indicating no chemical interactions between the drug and polymers used (Fig. 1 and Table 3). FT-IR study demonstrated the compatibility of MTS with PLF-127, HK4M and PCL.

MTS showed three endotherms; the first two endotherms that emerge at about 95 °C and 120 °C are correlated with the loss of free and bound water, respectively (Fig. 2). The third endotherm at 178.11 °C represents the melting point of MTS. The DSC analysis of MTS indicated that the obtained sample was a hydrate form (see Fig. 3).

The MTS *in-situ* gels were prepared by cold method technique in which the concentrations of HK4M and PCL were varied; whereas PLF-127 concentration was kept constant to our pre-optimized

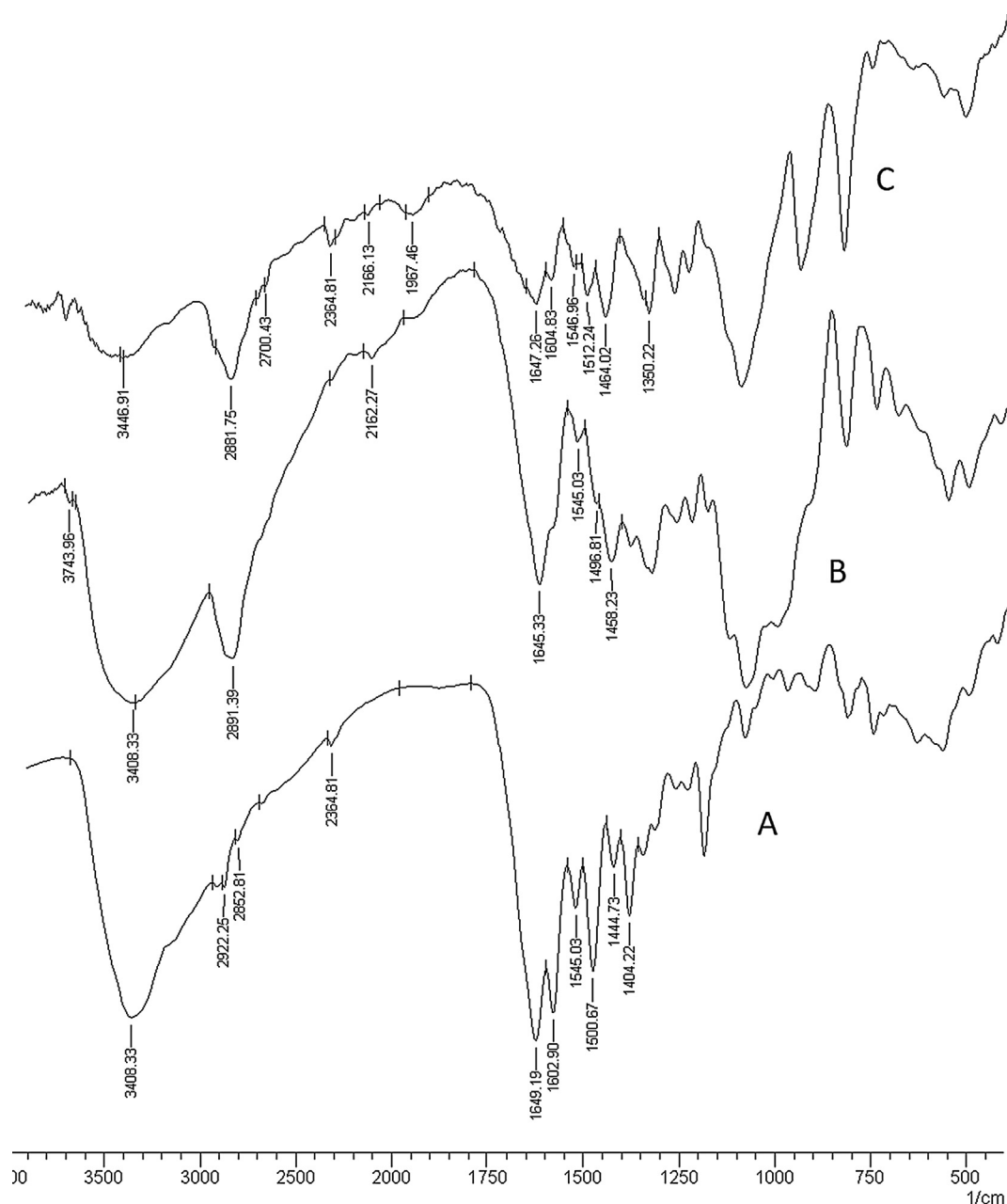
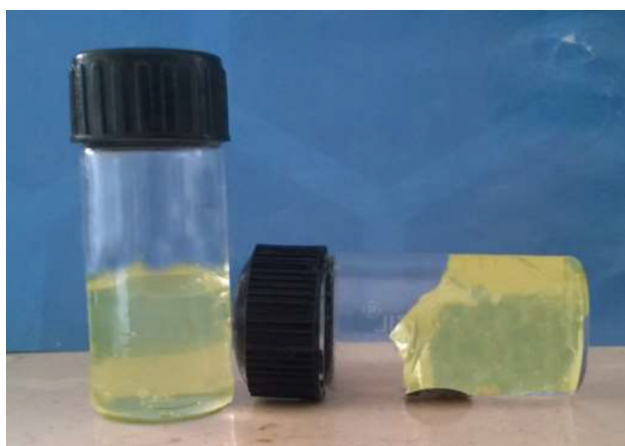


Fig. 1. FT-IR spectrum (A- Methotrexate, B- Formulation M4, C- Formulation M7).

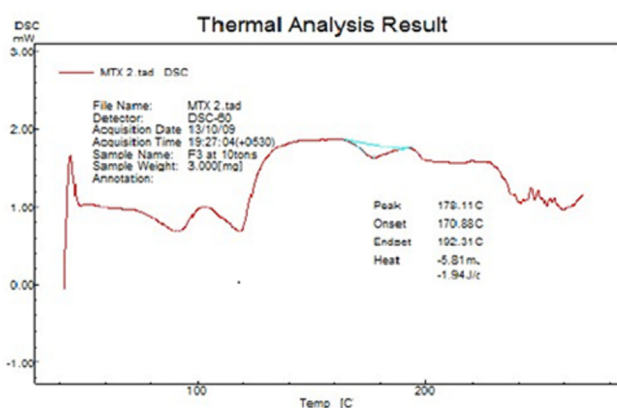


**Table 3**  
Interpretation of FT-IR spectra of Methotrexate and physical mixtures (M4 & M7).

Sl No.	Peak Observed ( $\text{cm}^{-1}$ )	Interpretation	Formulation M4	Formulation M7
1.	2960.70	O—H Stretch of COOH	2891.39	2881.75
2.	1642.74	C=O Stretch	1645.33	1647.26
3.	1546.72	C—C Stretch (aromatic)	1545.03	1546.96
4.	1448.35	C—H Deformation ( $\text{CH}_3$ )	1496.81	1464.02
5.	1404.22	C=C Stretch (aromatic)	1458.23	1350.22
6.	1207.72	C—O Stretch	1350.00	1300.00
7.	831.11	C—H Deformation (aromatic)	850.04	850.06
8.	3408.33	N—H Stretching	3408.33	3446.91



**Fig. 2.** Formulation of Methotrexate *in-situ* gels.



**Fig. 3.** DSC thermogram of Methotrexate.

level [20% (w/v)] (Yang et al., 2009). The preliminary study revealed that PLF-127 system alone at 20–22% w/v concentration was not quite satisfactory and exhibited prolonged gelation temperature and time. Therefore, the addition of co-polymer was initiated; HK4M and PCL were experimented in different concentration. The prepared *in-situ* gels were clear and transparent (Fig. 2). The overnight stay at cold temperature (2–8 °C) ensured the complete solubility of drug and polymers due to surfactant effect of PLF-127. Six formulations of MTS *in-situ* gels (M2 to M7) were prepared by altering two polymer constituents, the concentrations of HK4M and PCL (Table 1). The results of gelation temper-

ature (°C), gelation time (sec), syringeability test (sec), *in vitro* drug release studies (% cumulative drug release at 96 h) and viscosity (cps) are presented in Table 4. The final concentration of MTS in the *in-situ* gels was 0.5% (w/v). Table 5.

An increase in the concentration of PLF-127 by 2% resulted in noticeable changes in the evaluation parameters of gel. PLF-127 gels with 22%w/w exhibited gelation at 35.1 °C within 59 sec and syringeability time of 6.18 sec. The complete satisfactory *in-situ* gel system could be achieved by increasing the concentration of PLF-127 or by incorporating co-polymers in the formulation. Hence, co-polymers (HK4M and PCL) were incorporated to investigate the feasibility of formulating thermoresponsive PLF *in-situ* gels.

Gelation of PLF-127 occurs due to dehydration of the polymer leading to an increased chain friction and entanglement, producing hydrophobic association. Micelles are formed in aqueous pluronic solutions. At higher temperature or at high concentrations, these micelles associate to form various lyotropic liquid crystalline phases. With the increase in temperature, micellar entanglement proceeds, leading to gel formation and an overall increase in bulk viscosity. PLF solutions forms gel at body temperature forming liquid crystalline phase due to increased intermicellar interactions. With increase in length of PEO chain the onset and temperature of gelation and thermal stability of the gel also increases.

The prepared MTS *in-situ* gels showed sol-to-gel transformation. The gelation temperatures were in the range of  $34.8 \pm 0.51$  °C to  $37.3 \pm 0.42$  °C (close to human body temperature). The formulation M2 and M5 exhibited highest gelation temperature whereas M4 and M7 showed lowest gelation temperature.

The results suggest concentration dependent gelation of the system; an upsurge in HK4M and PCL concentration, lowered the gelation temperature of the system. Although PLF-127 concentration had significant effects on the sol-gel transformation, HK4M and PCL concentrations also affected the gelation temperature. It was observed that the concentrations of the HK4M and PCL increased the viscosity of the system linearly; which lead to lowered gelation temperature (Fig. 4). Although both *in-situ* gels showed considerable impacts on the sol-gel transition, HK4M concentration had a greater impact on the gelation temperature. Klouda et al., addressed changes in PLF-127 molecule properties upon changes in temperature and concentration in the environment, which was a main reason for choosing PLF-127 (20%) for the study.

The gelation temperature lowering effect of co-polymer could be explained by two possibilities: (a) The gelation temperature lowering effect might be caused by increased viscosity after dissolution of co-polymer or (b) Due to their ability to bind to the polyoxyethylene chains present in the Pluronic molecules, they promote dehydration, causing an increase in entanglement of adjacent molecules and extensively increasing intermolecular hydrogen bonding which will lead to gelation at lower temperature.

The results of the gelation time revealed that the prepared MTS *in-situ* gels rapidly responded to gelation temperature, exhibiting quick gelation by a maximum of  $65 \pm 1$  sec. The gelation times were in the range of  $58 \pm 1$  to  $65 \pm 1$  sec. The formulation M4 and M7 showed lowest gelation time, whereas M2 and M5 showed highest gelation time.

The gelation time was significantly influenced by polymeric concentrations of HK4M and PCL. The change of gelation temperature with polymer concentration is depicted in the Fig. 4. The rise in the polymer concentration (HK4M and PCL) from zero to 1.5%w/w resulted in a decreased gelation time of about 7 sec, due to enhanced gel viscosity. Din et al. (2015) found similar results in designing novel solid-lipid nanoparticle hydrogels for the rectal administration.

**Table 4**  
Characterization of MTS *in situ* gels.

Formulation Code	Gelation temperature (°C)	Gelation time (Sec)	Syringeability (Sec)	Cumulative % drug release (at 96 h)	Viscosity	
					8 °C	37 °C
M1	37.3 ± 0.42	65 ± 2	5.33 ± 0.58	–	544 ± 12	26248 ± 223
M2	35.6 ± 0.46	65 ± 1	6.33 ± 0.58	98.80 ± 2.39	1053 ± 34	32021 ± 246
M3	35.2 ± 0.62	60 ± 2	6.66 ± 0.58	98.93 ± 3.04	1126 ± 42	39088 ± 258
M4	<b>34.8 ± 0.51</b>	<b>58 ± 1</b>	<b>7.00 ± 1.00</b>	<b>93.26 ± 2.39</b>	<b>1432 ± 61</b>	<b>43179 ± 269</b>
M5	37.2 ± 0.24	65 ± 1	5.33 ± 0.58	99.39 ± 4.93	583 ± 18	28356 ± 234
M6	36.7 ± 0.28	60 ± 2	6.00 ± 1.00	96.18 ± 4.83	721 ± 21	28924 ± 286
M7	36.0 ± 0.57	58 ± 2	6.66 ± 0.58	91.27 ± 4.48	915 ± 28	32623 ± 169
Post sterilization (M4)	<b>34.5 ± 0.58</b>	<b>57 ± 1</b>	<b>6.86 ± 1.00</b>	<b>94.28 ± 3.12</b>	<b>1394 ± 49</b>	<b>42981 ± 198</b>

\*Mean ± Standard deviation, n = 3.

**Table 5**  
*In-vitro* dissolution data and mathematical model fitting.

Formulation Code	T50% (Hrs)	Order of drug release	Mechanism of drug release	'n' value
M1	03.87	First-Order	Non-fickian	0.654
M2	44.42	First-Order	Non-fickian	0.760
M3	52.92	First-Order	Non-fickian	0.629
M4	55.02	First-Order	Non-fickian	0.585
M5	39.15	First-Order	Non-fickian	0.532
M6	43.58	First-Order	Non-fickian	0.613
M7	48.80	First-Order	Non-fickian	0.535

\*Mean ± Standard deviation, n = 3.

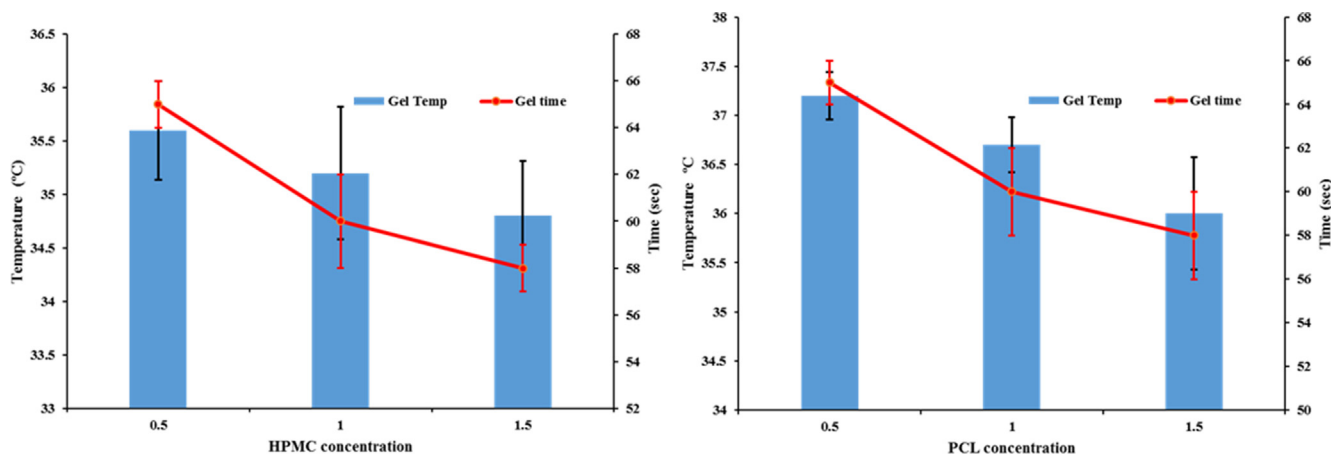
The duration to drive out the syringe contents by use of constant energy is called as syringeability time. The findings of syringeability testing revealed that the prepared MTS *in-situ* gels were conveniently syringeable via 18-gauge needle. It was observed that the syringeability times reported for gel formulations are within the range of 5.33 ± 0.58 sec to 7 ± 1.00 sec. Fig. 5 revealed a significant linear trend in syringeability time with proportional increase in polymer concentration (M2 to M7). This recognized the fact that, rise in the viscosity of the system resulted in improved opposition to flow (Cabana et al., 1997).

All the formulations exhibited sol state at refrigerated temperature (5 ± 3 °C) but transformed into clear stiff gels at body temperature (37 °C). The conversion from sol-to-gel is dependent on temperature, which is reversible when refrigerated. The viscosity of the MTS *in-situ* gels was measured at 5 ± 3 °C (storage temperature) and 37 ± 0.5 °C (human body temperature). A temperature dependent raise in viscosity was exhibited by the gel (Fig. 6). The

viscosity of the system was proportionally increased with increment in HK4M and PCL concentrations. Whereas Pluronic polymers, which are polypropylene oxide tri block copolymers and non-ionic in nature, got aggregated into micelles at 37 °C; this relates to dehydration of the polymer blocks with changes (rise) in temperature. It has been confirmed that the protective material and micellar concentration promotes gel formation; at larger PLF-127 concentrations, the gel is more tangled. Consequent to these, separation from each other becomes evidently difficult, causing inflexibility and higher gel viscosity at higher PLF concentrations (Jain et al., 1998; Dyondi et al., 2015). The viscosity values for optimized formulation was low at 5 ± 3 °C; although, a significant rise was reported at 37 ± 0.5 °C, owing to sol-gel conversion. The MTS *in-situ* gel (M4) viscosity was 1432cps (at 5 ± 3 °C), a notable increase was observed at 37 °C (43179cps). Taking into account all the results/findings of characterization, formulation M4 was considered for further studies.

The *in vitro* drug release studies help in providing important data regarding the performance of a dosage form under *in vivo* conditions. Sustained MTS release from formulations upto 96 h due to gradual degradation of the polymer matrix and chiefly influenced by the combination of polymers resulted in rapid gelation time and lowered gelation temperature. The formulations M4 and M7 prolonged the release compared to other formulations (Figs. 7 & 8). Preliminary burst discharge followed by a sustained release of MTS was observed with all *in-situ* gels. The burst release of MTS helps in immediate management of symptoms, prolonged release helps for maintaining the therapeutic concentration in the management of RA (Bozdog et al., 2005).

*In-vitro* drug release data was fit into various mathematical models to ascertain the best fit model and to determine the release mechanism. From the kinetics study, it was found that drug release



**Fig. 4.** Effect of polymer (HK4M and PCL) concentration on gelation time (Sec) and gelation temperature (°C).

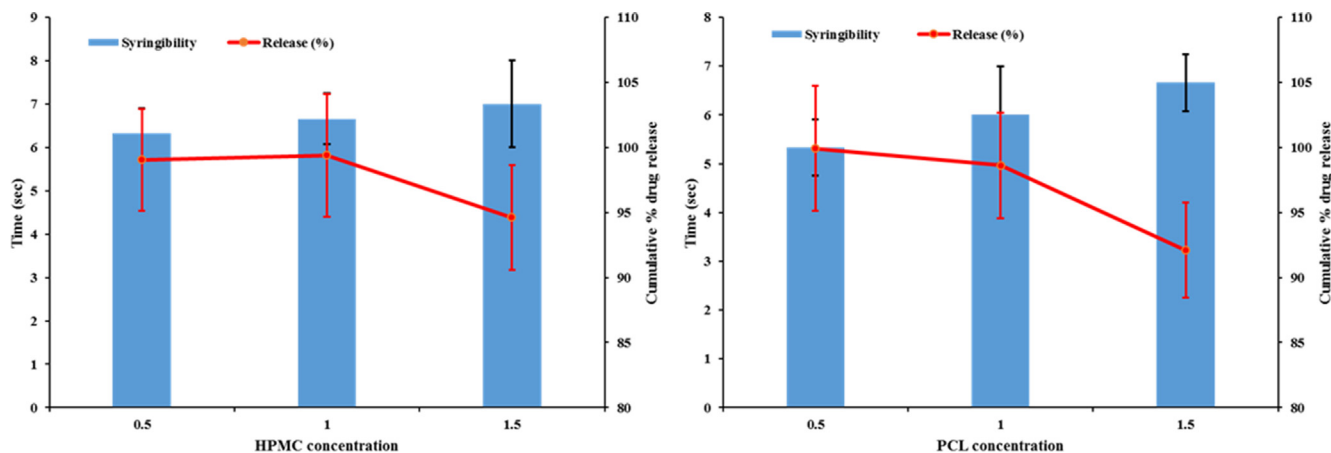


Fig. 5. Effect of polymers (HK4M and PCL) concentration on syringeability (Sec), *in vitro* drug release studies (cumulative % drug release).

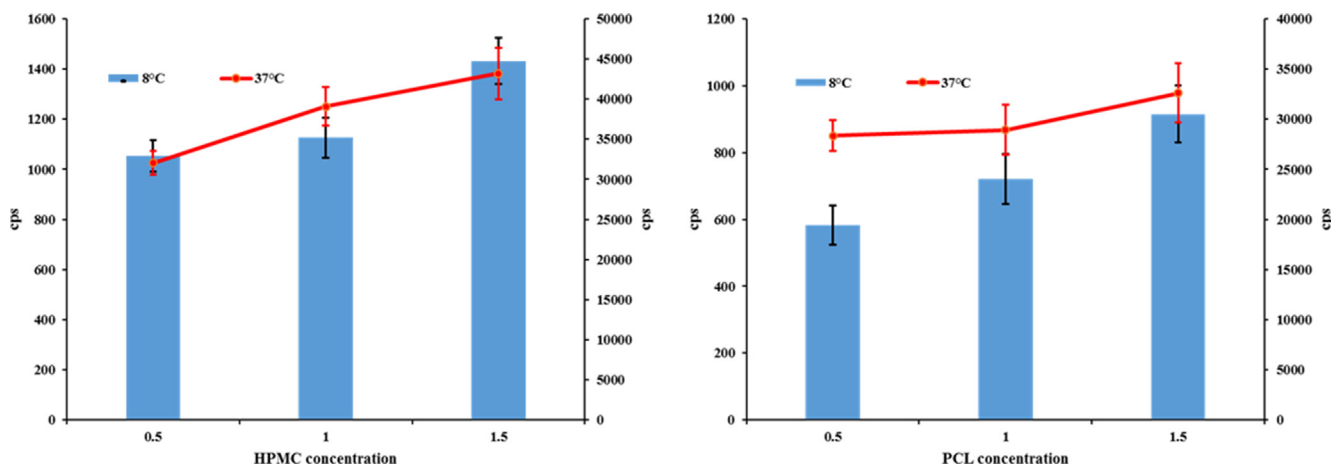


Fig. 6. Effect of polymers (HK4M and PCL) concentration on viscosity (cps) at two different temperature i.e. 8 °C & 37 °C.

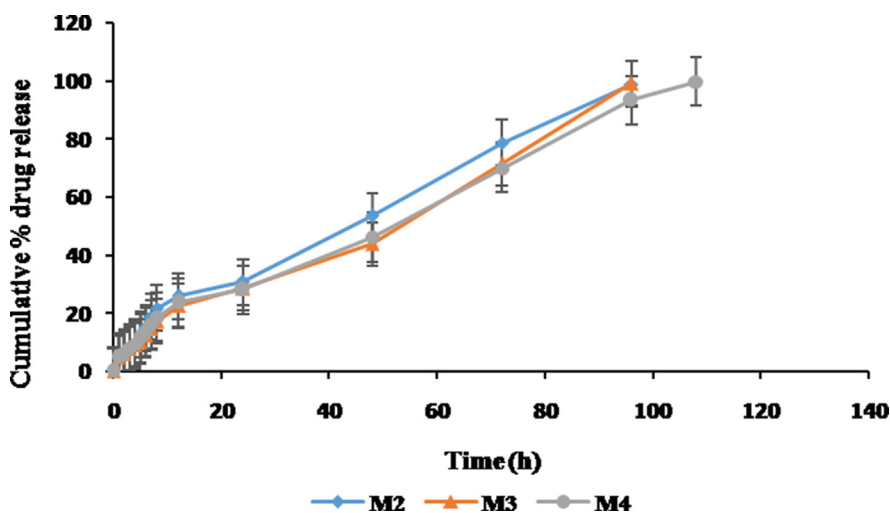


Fig. 7. Comparative *in vitro* release profile of MTS *in situ* gels (Formulation- M2, M3 & M4).

from all the formulations followed first-order kinetics (concentration dependant) and the best fit model was peppas model. Drug release data (<70%) was fitted during the peppas model. The value of 'n' determined from Korsmeyer-Peppas equation if found to be

<0.45 = Fickian diffusion, 'n' value is between 0.5 and 0.85 = non-fickian diffusion or anomalous mechanism (relaxation controlled) and if 'n' value is >0.89 = super case II transport. The calculated 'n' values were between 0.5 and 0.85, indicating that the release

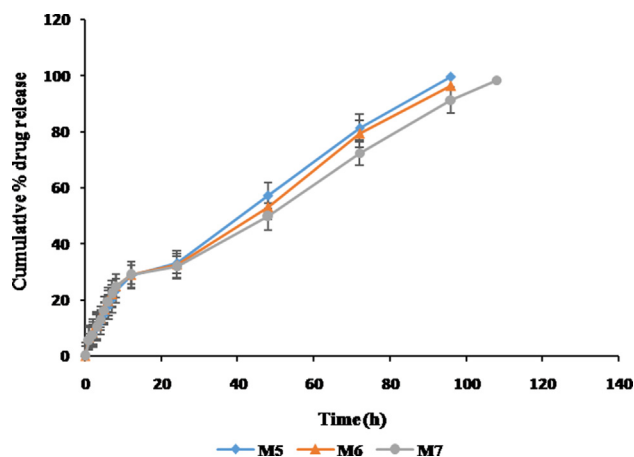


Fig. 8. Comparative *in vitro* release profile of MTS *in situ* gels (Formulation- M5, M6 & M7).

of MTS was by non-fickian diffusion. The statistical analysis of cumulative drug release data, duration of drug release (hours) along with T50% with reference to increase in concentration of co-polymers (HK4M and PCL) was found to be highly significant ( $p < 0.005$ ).

The MTS content in the M4 gel was  $98.08 \pm 0.12\%$ , which implied significant loading of *in-situ* gel formulation.

Sterility is very important requisite for a parenteral formulation. MTS *in-situ* gel is designed for parenteral administration to target site. The optimized formulation (M4) was sterilized by gamma irradiation and accessed for sterility after the exposure. The microbiological evaluation assures the product's sterility and effectiveness of the sterilization method. Post incubation period, no turbidity was observed both in the negative control as well as test sample; implying absence of microbial growth. Only in the non-irradiated formulation, used as a positive control, saw clouding of broth indicating microbial presence and its proliferation. With consideration of observations from the sterility test, the developed formulations passed the test for sterility.

Sterilization by gamma rays helps in terminal sterilization of the dosage form before its administration into the body. Among other methods for sterilization, gamma irradiation was preferred for its high ability to penetrate the product that permits sterilization of even susceptible material without elevation of temperature. However, gamma irradiation may have affected characterization of *in-situ* gels (Memisoglu-Bilensoy and Hincal, 2006). Moreover, the effect of gamma sterilization on gelation temperature and time, syringeability time, drug release *-in vitro* and drug content was evaluated. The results demonstrated that there were no significant changes in the said parameters between non-irradiated and irradiated formulations (Table 3).

FCA is a denatured *Mycobacterium butyricum* suspended in mineral oil (paraffin oil). FCA induced arthritis model for chronic inflammation in rats is considered critical for the pathophysiological and pharmacological management of inflammatory processes. It helps in the assessment and evaluation of anti-inflammatory and anti-arthritis agents since it mimics the RA progression in humans.

Paw volume reports for the MTS *in-situ* gel treated rats (test) and control rats were compared to evaluate whether the site targeted injection of MTS *in-situ* gels may indicate positive course for arthritis. Fig. 9 demonstrates the impact of MTS *in-situ* gels on swelling of the right hind paw in different groups. The MTS *in-situ* gels showed a more beneficial result than control group during the study period. In comparison, the paw swelling with MTS *in-*

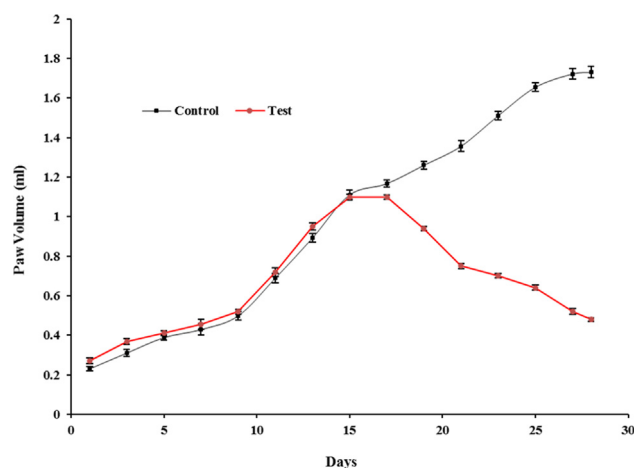


Fig. 9. The anti-inflammatory effect of MTS *in situ* gels as assessed by a reduction in paw volume in rats with adjuvant arthritis model. (One day after treatment).

*situ* gels treated rats ( $0.94 \pm 0.2\%$ ;  $P < 0.05$ ) was substantially less when compared to the rats in the control group ( $1.26 \pm 0.4\%$ ) after 14 days treatment period. This variation remained significantly throughout the duration of the study up to 28th day, where paw volume for the MTS *in-situ* gel treated rats was  $0.48 \pm 0.3\%$  ( $P < 0.05$ ). The effectiveness of MTS *in-situ* gel in FCA-induced arthritis model was aimed to retain MTS within the joint space. The findings from the study can be supported by the previous literature where lipid nano emulsion of MTS was assessed for anti-inflammatory activity in RA treatment (Maranhao et al., 2013). It was concluded that improvement in the RA inflammatory condition could be ultimately resulted from sustained MTS release and its concentration in the target site.

Drug delivery systems can enhance the drug retention at target site while reducing its toxicity. The findings of the current research verified these hypotheses by demonstrating gradual reduction in paw volume in MTS *in-situ* gels treated rats which progressed towards normal condition by the end of treatment period. These findings establish that incorporation of MTS into *in-situ* gels could desirably improve efficiency in suppressing inflammation in RA.

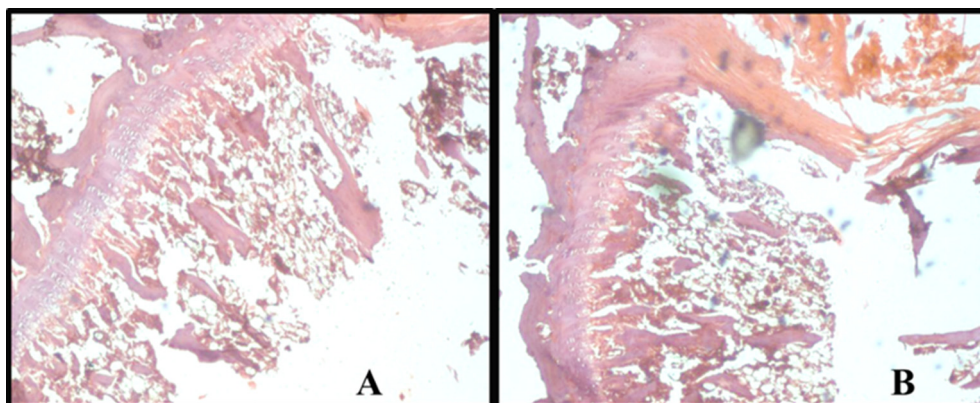
Significant paw swelling was not reported in test group; since macroscopic signs of paw stiffness, swelling or redness was not observed. The histological samples of haematoxylin and eosin stained joints of both test and control group are shown in Fig. 10. Inflammatory infiltration was not found in the joint tissue. The joints treated with MTS *in-situ* gels did not differ from the control joint tissue. These results inferred that MTS *in-situ* gel shows favourable biocompatibility with tissues at the target site. Thus, MTS *in-situ* gels could provide a non-toxic, biocompatible solution for the successful management of RA.

The stability studies for optimized formulation were conducted at  $5 \pm 3^\circ\text{C}$  (long term storage condition) and at  $25^\circ\text{C}/60\% \text{RH}$  (accelerated storage condition) for 6 months. There was no remarkable difference in the physical properties; gelation temperature and time, and drug content during the stability period, which indicated that the optimized formulation displayed superior stability during evaluation.

## 5. Conclusion

MTS *in-situ* gels were formulated to provide a novel injectable, biodegradable system for managing RA. The MTS *in-situ* gel remained stable, thermoresponsive and transformed to gel lower than the body temperature. The optimized *in-situ* gel exhibited rapid and sustained release of MTS enhancing the treatment





**Fig. 10.** Representative H and E (hematoxylin and eosin) stained histological slides of joint tissues from healthy rat knees after injection with (A) 100 µL of saline and (B) 100 µL of MTS *in situ* gels (magnification: 100×).

modality; it was non-toxic and biocompatible with joint tissue. A novel drug delivery system for prolonged release of MTS which can reduce dose, dosing frequency, associated side effects, cost of therapy and thereby improve patient compliance was developed. This research demonstrated advantages of MTS *in-situ* gel and may be suitable as an injectable drug delivery system for management of RA to overcome the limitations of currently available formulations.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jsps.2020.10.003>.

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