Injectable Thermosensitive Hydrogels for Intra-Articular Delivery of Methotrexate

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ABSTRACT: Intra-articular drug delivery is the preferred approach for targeting pharmacologic treatment directly at the joints to reduce undesirable side effects associated with systemic drug delivery. In this study, a controlled delivery system of methotrexate (MTX) based on injectable thermosensitive poly(ε-caprolactone)-poly (ethylene glycol)-poly(ε-caprolactone) (PCL-PEG-PCL, PEP) hydrogels was developed for the intra-articular drug delivery. The thermosensitive PEP copolymers were prepared by ring-opening polymerization. The synthesized PEP copolymers were characterized for their structure, composition, and the sol-to-gel transition. The *in vitro* MTX release from the PEP hydrogels was studied. MTX plasma concentration following intra-articular injection into healthy rats was determined by HPLC. Biocompatibility was confirmed by histology analysis after the intra-articular injection. The synthesized PEP copolymers aqueous solutions formed *in situ* gel rapidly after the injection. PEP hydrogels showed the ability to control the release of incorporated MTX. Following intra-articular injection, the PEP hydrogels decreased the clearance rate of MTX in the joint cavity. The maximum plasma concentrations of MTX in rats injected with free MTX were threefold higher than that of the groups injected with MTX hydrogels. These results suggest that the intra-articular delivery of the PEP hydrogels may be a viable strategy for the controlled release of drugs for treating arthritis diseases. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 122: 2139–2145, 2011

Key words: intra-articular delivery; injectable hydrogel; thermally responsive; methotrexate; controlled release

INTRODUCTION

Inflammatory arthritis diseases such as osteoarthritis (OA) and rheumatoid arthritis (RA) affect an enormous number of individuals. Patients afflicted with the disease may experience pain and loss of joint function with associated deleterious effects on patient activity level and lifestyle habits.[1] Treatment of arthritis disease is achieved through oral, parenteral, or intra-articular drugs. The direct drug delivery to an affected joint offers the possibility of reaching high drug concentrations at the action site with limited systemic toxicity.[2–4] However, the undeniable clinical efficacy of intra-articular injections is somehow restricted either by the rapid efflux of drugs from the joint cavity after injections or by the need of repeated injections, possibly causing joint instability and infections.

Retention of drugs in the joints using controlled release delivery system offers an exciting option for intra-articular drug delivery. Researchers thus have tried to encapsulate the drugs into different drug delivery systems such as liposomes, nanoparticles, and microparticles.[5–10] Although more promising than drug suspensions, these systems also faced a major drawback of short retention in the joint due to synovial capillary and lymphatic drainage, which takes place within a few days after injection.[11,12] To overcome these limitations, many researches have been carried out to find more controlled and prolonged drug delivery to the joint. For example, magnetic microparticles by encapsulating superparamagnetic iron oxide nanoparticles into polymer matrix are applied to enhance the retention of the microparticles in the joint with an external magnetic field.[13]

During the last decade, thermosensitive polymers-based injectable *in situ*-forming hydrogels received increasing attention as controlled drug carriers because of their many advantages such as the convenience of application, high drug loading, no organic solvents, sustained drug release behavior, and less systemic toxicity.[14,15] These drug delivery systems are flowable aqueous solution before administration, but once injected, they rapidly form gel under physiological conditions and hence an intra-articular "drug

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depot" forms. *In situ* gel formation after the intra-articular injection of aqueous solutions of such thermosensitive polymers (elastin-like polypeptides, ELPs) in rats resulted in a significantly prolonged intra-articular residence time when compared with that of the soluble ELPs.[16] Recently, we have constructed a biodegradable and injectable *in situ* gel-forming controlled drug delivery system based on thermosensitive PEP hydrogels.[17] PEP copolymer solution exhibited sol-to-gel transition at body temperature. *In vivo* gel formation and degradation studies indicated that PEP hydrogels sustained at least 45 days by subcutaneous injection, which was very useful for its application as *in situ* gel-forming drug delivery system.

Methotrexate (MTX) is one of the most frequently used drugs in the treatment of rheumatoid arthritis.[18,19] However, systemic toxicity effects such as liver fibrosis, pneumonitsi, and bone marrow depression limit the oral use of this drug.[20] MTX has also been administered intra-articularly to control the synovits in patients with the arthritic joints, but the results have generally been disappointing due to the rapid drug clearance from the joint.[21] The objective of this study was to develop a controlled release, intra-articular hydrogel delivery system for MTX with the goal of localizing MTX in the synovial joint. The *in vivo* pharmacokinetics of single intra-articular injections of either MTX solution or MTX-loaded PEP hydrogel was evaluated. In addition, biocompatibility of PEP hydrogels as intra-articular drug delivery system was also studied.

MATERIALS AND METHODS

Materials

ε-Caprolactone, poly (ethylene glycol) (PEG) (M_w = 1500), stannous octoate, were used as received from ς-Aldrich. Methotrexate (MTX, 99% purity) was supplied by Jiangsu Hengrui Pharmaceutical Co. Ltd. (Lianyungang, China). All other chemicals were AR grade and purchased from Tianjin No.1 Chemical Reagent Factory (Tianjin, China).

Synthesis of PEP triblock copolymers

The PEP triblock copolymer was prepared by ring-opening polymerization of caprolactone initiated by PEG and catalyzed by stannous octoate.[22] Briefly, PEG (15.0 g) was dissolved in anhydrous toluene (80 mL), and the solvent was distilled off to a final volume of 30 mL to remove the residual water adsorbed to the polymer. ε-Caprolactone (29.8 g) and stannous octoate (40 μL) were added to the reaction mixtures and stirred at 120°C for 24 h. The product was isolated by precipitation into diethyl ether. The copolymer was redissolved in methylene chloride and fractionally precipitated upon molecular weight by slowly adding diethyl ether. The residual solvent was removed under vacuum. The yield in copolymer after its purification was 86%.

^{1}H-NMR analysis and gel permeation chromatography (GPC)

A 500-MHz proton NMR spectrometer (DMX500 spectrometer, Bruker) was used for ^{1}H-NMR in $CDCl_3$ to study the molecular structure and composition of the triblock copolymers. Molecular weight and molecular weight distribution of copolymers were determined using a Waters 515 gel permeation chromatography (GPC) apparatus with a refractive index detector (Waters 410). Tetrahydrofuran was used as an eluting solvent at a flow rate of 1.0 mL/min at 35°C, and polystyrene standards were used as the calibration sample.

Sol-to-gel transition

The sol-to-gel transition was determined by the test tube inverting method.[23,24] The sample solution was prepared according to the literature[22] and briefly described as follows: the 10 mL vials containing 1 mL solution of the PEP triblock copolymers in distilled water were immersed in a water bath at 50°C, which is above the melting point of PCL; the solution was then quenched in an ice bath for 30 s. The phase transitions of the polymer solution were investigated by heating from 10 to 60°C in an increment of 2°C. The vials were kept at each temperature for 5 min and were removed from the water bath and then tilted. The sample was regarded as a "gel" in the case of no flow within 30 s by inverting the vial. The phase transition temperature was determined from the results of these inverting tests.

Gelation time

The time to form a gel was denoted as gelation time. The gelation time was also determined using the test tube inverting method.[25] Briefly, the test tube containing the solutions of the PEP triblock copolymers was immersed in a water bath at 37°C. The gel phase was determined if nonflowing solution was seen when the test tube was inverted.

In vitro MTX release

For the MTX release experiment, different amount of MTX was added to the 10 mL vials containing 1 mL solution of the PEP triblock copolymers at room temperature and was gently mixed till the copolymers

were completely dissolved. The vials were incubated at 37°C for 10 min to form a gel. Then, 5 mL of 0.01*M* phosphate buffer (PBS, pH 7.4) at 37°C was added to each vial, and the vials were shaken at 60 rpm at 37°C. At predetermined time, 5 mL of PBS was removed from the vial and the same volume of fresh phosphate buffer at 37°C was added to the vial. The amount of MTX released into the solution was determined by high-performance liquid chromatography (HPLC). The HPLC assay was performed on a reverse phase Diamond® C18 column (inner diameter 150 mm × 4.6 mm, pore size 5 μm). The mobile phase was a mixture of methanol and 10 m*M* sodium dihydrogen phosphate solution (25 : 75, v/v) at a flow rate of 1.0 mL/min. The column effluent was detected at 306 nm with a UV detector.

In vivo pharmacokinetic studies

Female Wistar rats (200 ± 20 g) were purchased from Tianjin Medical University Laboratory Animals Center (Tianjin, China). All the animal experiments were performed in compliance with the Guiding Principles for the Care and Use of Laboratory Animals, Peking Union Medical College, China. Twelve animals were randomly divided into two groups. Each animal was received an intra-articular injection into the right knee of either 100 μL of MTX solution in PBS (free MTX) or 100 μL of MTX-loaded PEP hydrogel (30 wt %) with a 30-gauge needle. The total dose of MTX injected was 250 μg. Serial blood were obtained by the retro-orbital sampling immediately before the intra-articular injection and at 5, 15, 30 min, 1, 2, 4, 6, 8, 24 h after the intra-articular injection. An equivalent volume of normal saline was administered into the animal after each blood draw. The blood samples were centrifuged at 3000 rpm for 10 min, and the plasma harvested and stored at −20°C for drug analysis.

The MTX in the plasma samples was extracted before analysis by HPLC. Briefly, 150 μL of rat plasma was extracted with 450 μL of ethyl acetated. Following the centrifugation at 3000 rpm for 10 min, the organic phase was transferred to a glass tube and the solvent was evaporated under nitrogen stream at 45°C. The dried sample was then dissolved in 100 μL mobile phase and 20 μL of the solution was injected into the column. Pharmacokinetic parameters were determined from MTX drug concentration-time data. Peak plasma concentration (C_{max}, μg/mL), and time to reach peak plasma concentration (t_{max}, h) were obtained from the MTX drug concentration-time curve. The area under the plasma concentration-time curve ($AUC_{0-\infty}$) was calculated by the trapezoidal rule for the observed values (AUC_{0-t}) and then extrapolated to infinity ($AUC_{t-\infty}$).[26]

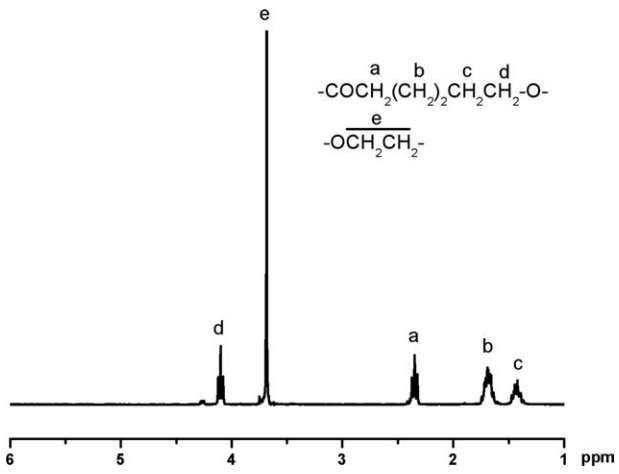

Figure 1 ^{1}H-NMR of PEP triblock copolymer.

Evaluation of biocompatibility

The biocompatibility of the PEP hydrogel to the synovium was evaluated by histopathological studies. Female Wistar rats (200 ± 20 g) were used. The PEP hydrogel (30 wt %) with a volume of 100 μL was injected into the right knee joint, and 100 μL of saline was injected into the left knee joint as the control. The swelling in all joints was monitored once a day for 7 days, and then the rats were sacrificed and the joints were isolated. Hematoxylin and eosin staining (H and E staining) was used to evaluate cell infiltration in the synovium.

RESULTS AND DISCUSSION

Characterization of synthesized PEP copolymers

The molecular structure and molecular weight of PEP copolymer were characterized by ^{1}H-NMR and GPC, respectively. In ^{1}H-NMR spectra, the sharp peak at 3.64 ppm was attributed to methylene protons of —CH_2CH_2O— in PEG units in block copolymer. Peaks at 1.38, 1.64, 2.32, and 4.06 ppm were assigned to the a, b, c, and d methylene protons of oxycarbonyl-1, 5-pentamethylene unit, which was derived from ε-CL ring opening. The very weak peak at 4.23 and 3.78 ppm were attributed to methylene protons of —O—CH_2—CH_2— in PEG end block that was linked with PCL blocks (Fig. 1). The ethylene peak of the ethylene glycol (CH_2CH_2O) unit at 3.64 ppm, the methylene peak of the caprolactone ($COCH_2CH_2CH_2CH_2CH_2O$) unit at 4.06 ppm and the methylene peak of the ethylene glycol end unit at 4.23 ppm in the ^{1}H-NMR spectra were used to determine the average molecular weight (M_n) of the PEP triblock copolymer.[27] The molecular weight and molecular weight distribution determined by GPC were in a range of 4350 and 1.3, respectively. According to Table I, the M_n values for PEP

TABLE I
List of PCL-PEG-PCL Triblock Copolymers Studied

Triblock copolymer	PCL/PEG[a] (theoretical)	Total M_n[a] (theoretical)	PCL/PEG[b] (calculated)	Total M_n[b] (calculated)	Total M_n (GPC)[c]	PDI[c]
PCL_{1250}-PEG_{1500}-PCL_{1250}	1.67 : 1	4000	1.8 : 1	4200	4350	1.3

[a] Theoretical value, calculated according to the feed ratio.
[b] Calculated from ^{1}H-NMR results.
[c] Determined by GPC analysis.

copolymer and PCL/PEG block ratios estimated from ^{1}H-NMR spectrum were consistent with theoretical value calculated from feed ratio. ^{1}H-NMR and GPC results indicated that the PEP triblock copolymer designed by controlling the feed composition was synthesized successfully.

Thermosensitivity characterization of PEP copolymers

The synthesized PEP triblock copolymers in this study exhibited a temperature-dependent reversible sol-to-gel transition in water: sol-to-gel transition at the lower transition temperature and gel-to-precipitate transition at the upper transition temperature. The phase diagram of the PEP triblock copolymers in water was shown in Figure 2, in which we found copolymer concentration of the triblock copolymers showed significant influence on the sol-to-gel transitions. An increase in the copolymer concentration from 15 to 30 wt % dramatically shifted sol-to-gel transitions to the lower temperature. The sol-to-gel transition temperature of the synthesized PEP triblock copolymers in this study was between room temperature and physiological temperature and thus suitable for drug delivery as an injectable biomaterial.

In our previous study, a series of PEP triblock copolymers were synthesized and the compositions of PEP on the mean characteristics of the copolymer were studied.[17] The results showed that increasing the length of hydrophobic PCL block with the same PEG block length resulted in a lower sol-to-gel transition temperature at a given copolymer concentration, which might be attributed to the enhanced hydrophobicity of the copolymer macromolecular backbone. This indicated that the thermodynamic driving force of such a physical sol-to-gel transition was the hydrophobic interaction. Increasing the length of hydrophobic PCL block with the same PEG block length resulted in a higher precipitate temperature at a given copolymer concentration. PEP copolymer with the composition of 1250-1500-1250 is suitable for drug delivery as an injectable hydrogel, and thus chose as the MTX-loaded delivery in this article.

The physical gelation process of the PEP triblock copolymers in water was shown in Figure 3, which was in agreement with the earlier reports about PLGA-PEG-PLGA copolymers hydrogels. Yu et al.[14] summarized the hierarchy mechanism to interpret the physical gelation process of these kinds of amphiphilic triblock copolymers in water: First, amphiphilic block copolymers were self assembled into micelles [Fig. 3(A)]; Second, the micelles were aggregated into macroscopic gel with mesoscopically inhomogeneous micelle network (transparent gel) [Fig. 3(B)]; Finally, the micelle structure was destroyed due to the over hydrophobicity of the copolymers at higher temperature, eventually leading to macroscopic precipitate [Fig. 3(C)].

Gelation time

Besides the capability of gelation under physiological conditions, another prerequisite of PEP copolymers hydrogels as an injectable biomaterial drug

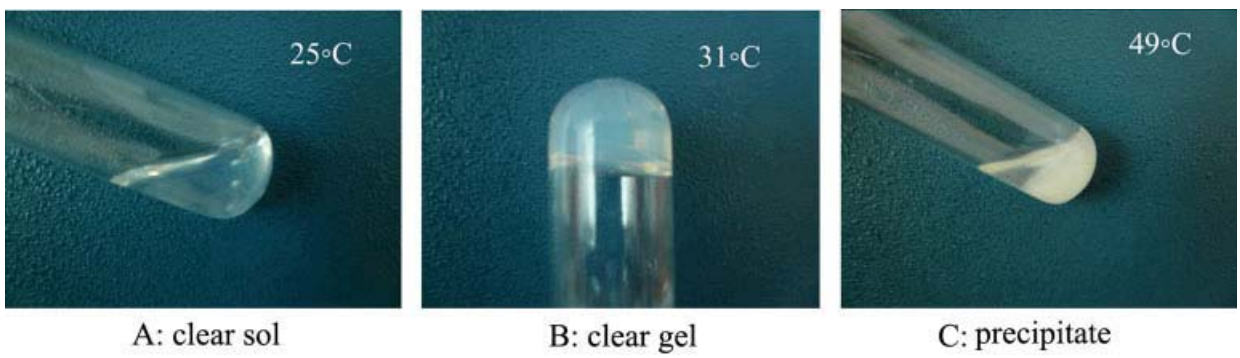

Figure 2 Phase diagrams of PEP aqueous solutions. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

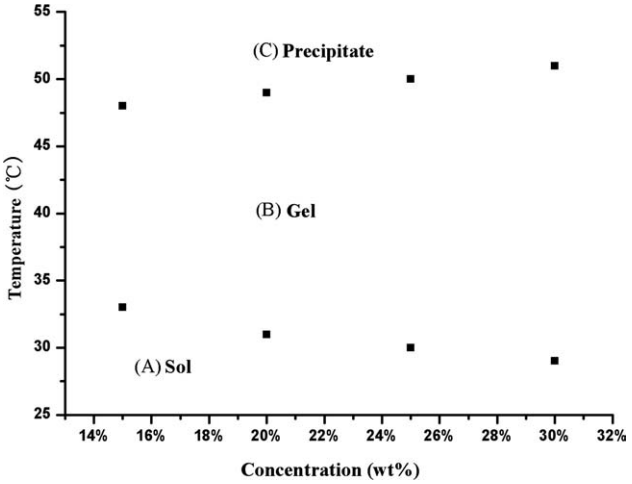

Figure 3 Optical images of PEP copolymer solutions (20 wt %) in the test tube at the indicated temperatures leading to the sol (A), transparent gel (B), and precipitate (C).

delivery system is to form gel within an appropriate gelation time. Fast *in situ* gelation is required to maintain drugs at the injection site. We previously showed the synthesized PEP copolymers aqueous solution could form *in situ* gel rapidly after injection under physiological conditions.[17] Figure 4 showed the effect of the PEP copolymer concentration on the gelation time. Increasing the copolymer concentration from 15 wt % to 30 wt % decreased gelation time from 2 min to 30 s.

The influence of drug loading and concentrations of PEP copolymers on *in vitro* MTX release

MTX release profiles were affected in some extent by initial drug loadings and concentrations of PEP copolymers hydrogels. *In vitro* release behavior of MTX from the PEP hydrogels in PBS was shown in

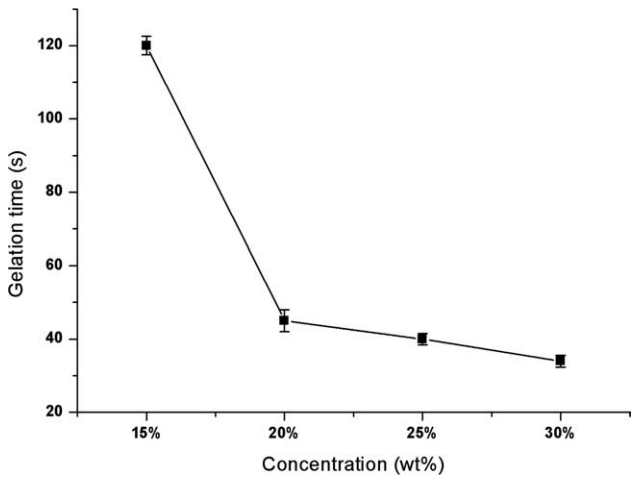

Figure 4 Gelation time of PEP aqueous solutions at different concentration (37°C). Each point represents the mean ± S.D., $n = 3$.

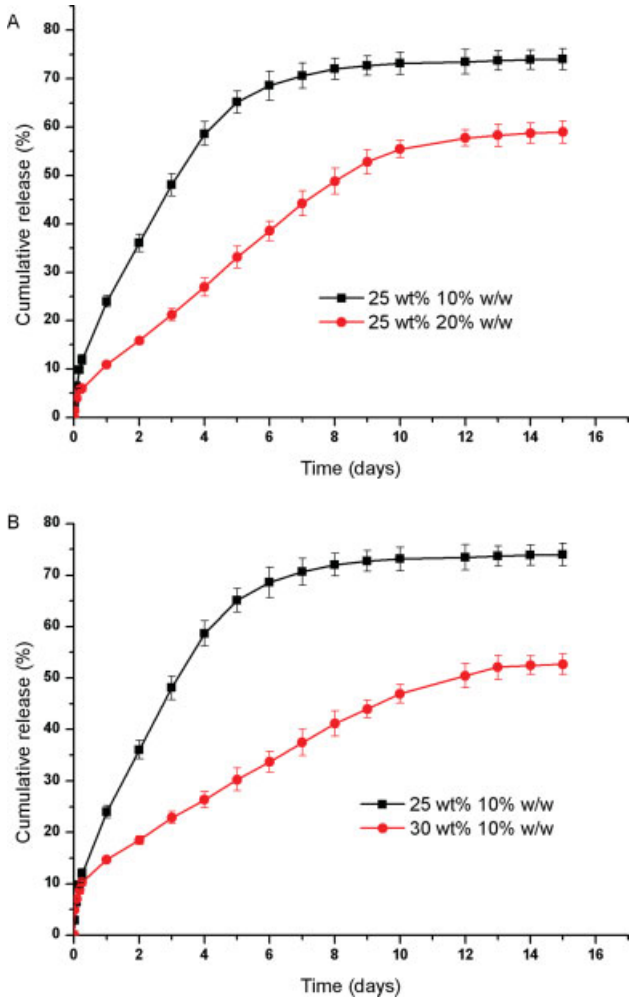

Figure 5 *In vitro* release of MTX from PEP hydrogels in PBS at 37°C with marked copolymer and drug loadings. A: Effect of initial MTX loading amount; B: Effect of hydrogel concentration. Each point represents the mean ± S.D., $n = 3$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 5, in which the higher initial drug loading amount resulted in slower release rate of MTX from the hydrogels. By increasing the initial loading of MTX from 10 to 20% (w/w), the cumulative release rate decreased dramatically from 74.0% to 58.9% in 15 days. Increasing the PEP hydrogel concentration significantly slowed down the release of MTX. As shown in Figure 5(B), 52.69% of MTX was released from the 30 wt % gel after 15 days, whereas 74.0% of MTX was released from the 25 wt % gel in the same time period. Therefore, it is quite flexible to select the copolymer concentration within a certain range as the release rate is concerned.

Drug release from the PEP hydrogel was a combined result of two processes: drug diffusion and hydrogel degradation.[28] As PCL is a highly hydrophobic crystalline polymer that degrades very slowly *in vitro* in the absence of enzymes[29,30]; the degradation of the PEP hydrogel in the entire release

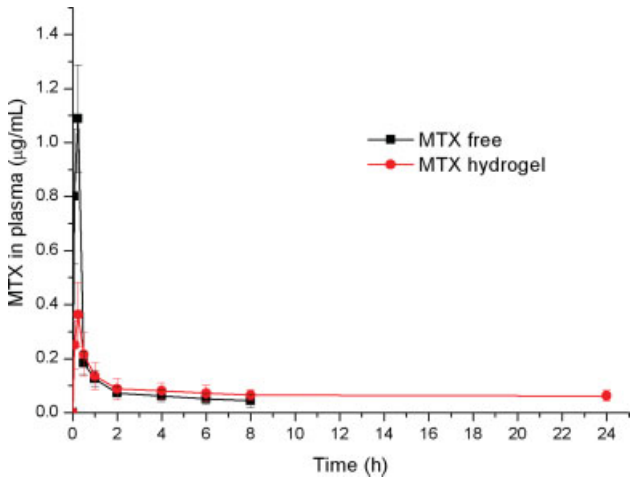

Figure 6 MTX concentrations in rat plasma after a single intra-articular injection of either free MTX or MTX-loaded hydrogel. The dose of MTX injected is 250 μg. Each point represents the mean ± S.D., $n = 6$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

experimental period (15 days) could be neglected. In other words, the drug diffusion process is the key factor to determine the drug release from the PEP hydrogel. MTX could be released with a higher cumulative release rate (74.0% in 14 days) from PEP hydrogel with drug loadings (10%, w/w) and hydrogel concentration (25% wt). Because MTX could diffuse through the pores of hydrogel and almost 75% of the drug in the hydrogel was released. In this study, higher drug loading resulted in slower release rate, which was reported previously.[31] At the constant diffusion surface of the drug-hydrogel system, the diffusion rate of the incorporated drugs might be limited to some extent at the higher drug-loading amount. As a result, the cumulative release rate decreased with increasing drug loading, although the cumulative amount of drug released increased. The otherwise, a higher hydrogel concentration resulted in a more condensed structure, therefore decrease the diffusion rate of the drugs, leading to a lower cumulative release rate.[32]

Pharmacokinetics of intra-articular MTX-loaded hydrogel

The plasma MTX concentration after a single intra-articular injection of either free MTX or MTX-loaded hydrogel was shown in Figure 6. Similar to previous data, rapid clearance of free MTX from rat joints into the systemic circulation was shown in Figure 6.[33] The plasma MTX peaked 15 min following the free MTX injection (t_{max} of 15 min and C_{max} of 1.09 μg/mL). The plasma concentration gradually declined and was below the limit of quantitation of the assay (0.03 μg/mL) 8 h after the injection. For rats injected with the MTX-loaded hydrogels, the plasma MTX was detectable 5 min following injection and peaked 15 min following the injection (t_{max} of 15 min and C_{max} of 0.36 μg/mL). The concentration gradually declined and was still detectable (0.06 μg/mL) 24 h after injection indicating that MTX was still being released from the hydrogels. In this study, the AUC of the group injected with free MTX (59.23 μg/mL h) was about threefold larger than that of the group injected with the MTX-loaded hydrogel (17.62 μg/mL h). Liang et al. also reported that the AUC of the group treated with free MTX was significantly higher than that of the group treated with MTX-loaded microspheres, indicating that more

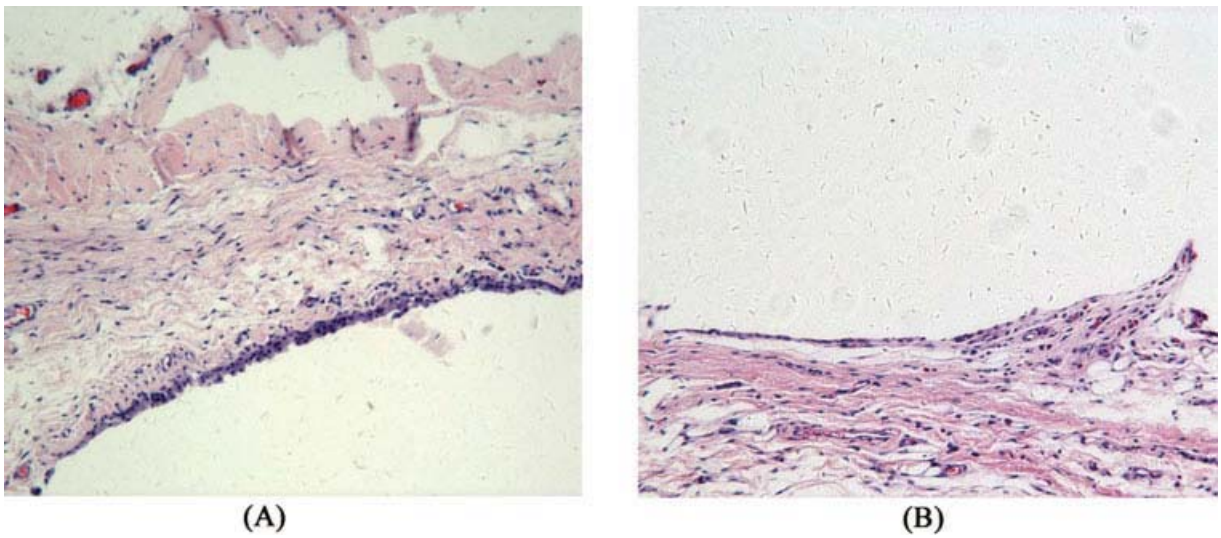

Figure 7 Representative H and E (hematoxylin and eosin) stained histological slides of synovial tissues from healthy rat knees after intra-articular injection with 100 μL of saline(A) and 100 μL of PEP hydrogels (B) (magnification: ×20). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

MTX entered the systemic circulation following the injection of MTX solution.[15] The plasma data suggested that the MTX-loaded hydrogel was able to slow down the clearance of MTX and control the MTX release in the joint cavity.

Evaluation of biocompatibility

The animals did not present any macroscopic signs of knee inflammation such as swelling or joint stiffness. Representative histology with H and E staining of healthy rat joints injected with the PEP hydrogels or saline was shown in Figure 7. The PEP hydrogels treated joints did not differ from saline-treated control joints. No obvious inflammatory infiltration in the synovial membrane was observed. Previous reports revealed size-dependent inflammation of polymeric microparticles administered intra-articularly.[34] However, our results demonstrate the biocompatibility of the PEP hydrogel and their suitability for intra-articular drug delivery. Hence, the PEP hydrogel offers a better and nontoxic alternative for the intra-articular drug delivery.

CONCLUSIONS

This study demonstrates that the biodegradable PEP hydrogel is a promising *in situ* gel-forming controlled drug delivery system for the intra-articular injection. Data for the pharmacokinetic study after the intra-articular injection demonstrate that MTX-loaded hydrogel is able to slow down the clearance of MTX and control the release of MTX in the joint cavity. Moreover, PEP hydrogel is not toxic to the synovium, an indication of their biocompatibility. In an ongoing work, the evaluation of therapeutic effects of MTX-loaded hydrogel has been done in an antigen-induced inflammatory arthritis model in rabbits.

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