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J Biomater Appl 2014 29: 524 originally published online 21 May 2014
DOI: 10.1177/0885328214535958

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Journal of Biomaterials Applications
2014, Vol. 29(4) 524–542
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DOI: 10.1177/0885328214535958
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In vitro evaluation of effects of sustained anti-TNF release from MPEG-PCL-MPEG and PCL microspheres on human rheumatoid arthritis synoviocytes

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Abstract

Anti-tumor necrosis factor α (TNFα) drugs such as etanercept (ETN) have been mostly used in systemic treatment of rheumatoid arthritis. To eliminate the side effects in long-term treatments and to achieve a local sustained anti-inflammatory effect, a controlled drug delivery system is needed for anti-TNFα drugs. This study aims to develop novel injectable microcarriers of ETN that can provide long-term controlled release of this protein drug upon intra-articular application. In this study, poly(ε-caprolactone) (PCL) and its copolymer with poly(ethylene glycol), methoxypoly(ethylene glycol)-poly(ε-caprolactone)-methoxypoly(ethylene glycol) microspheres (MPEG-PCL-MPEG) were compared for their prospective success in rheumatoid arthritis treatment. Microspheres with smooth surface of a mean particle diameter of approximately 5 μm were prepared with both polymers. MPEG-PCL-MPEG microspheres had higher encapsulation efficiency than PCL microspheres. The activity of encapsulated ETN within MPEG-PCL-MPEG microspheres also retained while 90% of the activity of ETN within PCL microspheres could retain during 90-day release. MPEG-PCL-MPEG microspheres showed faster ETN release compared to PCL microspheres in various release media. Cumulative amounts of ETN released from both types of microspheres were significantly lower in cell culture medium and in synovial fluids than in phosphate buffered saline. This was mainly due to protein adsorption onto microspheres. Hydrophilic MPEG segment enhanced ETN release while preventing protein adsorption on microspheres compared to PCL. Sustained ETN release from microspheres resulted with a significant decrease in pro-inflammatory cytokines (TNFα, IFNγ, IL-6, IL-17) and MMP levels (MMP-3, MMP-13), while conserving viability of fibroblast-like synoviocytes compared to the free drug. Results suggest that MPEG-PCL-MPEG is a potential copolymer of PCL that can be used in development of biomedical materials for effective local treatment purposes in chronic inflammatory arthritis owing to enhanced hydrophilicity. Yet, PCL microspheres are also promising systems having good compatibility to synoviocytes and would be especially the choice for treatment approach requiring longer term and slower release.

Keywords

Etanercept, methoxy poly(ethylene glycol)–poly(ε-caprolactone)–methoxy poly(ethylene glycol), microspheres, intra-articular delivery, rheumatoid arthritis, anti-tumor necrosis factor α drugs, amphiphilic triblock copolymer

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Introduction

Rheumatoid arthritis (RA) and its childhood counterpart juvenile idiopathic arthritis are common chronic inflammatory diseases of the joints characterized by synovial inflammation. RA prevalence ranges from 0.3% to 1% in the general population.[1] Although RA aetiology remains obscure, fibroblast-like synoviocytes (FLS) are the key players in the pathophysiological process by producing pro-inflammatory cytokines, chemokines, and degradative enzymes.[2] Tumor necrosis factor α (TNFα) plays a crucial role in the chronic inflammatory arthritis.[3] TNF enhances the inflammation of the synovial cells via stimulating FLS and activating a broad array of intracellular signaling mechanisms.[4] It has been shown that TNFα induces matrix metalloproteinases (MMPs), which are the mediators in destructive progression of chronic inflammatory arthritis.[5] Especially MMP-1, 3, and 13 are important for cartilage tissue.[6,7] Thus, the balance between MMPs and tissue inhibitors of MMPs (TIMPs) is thought to change in favor of MMPs leading to degradation of extracellular matrix and eventually cartilage.[8] The cellular infiltrate in chronic inflamed joints of RA/JIA patients mainly consists of T helper 1 (Th1) and Th17 cells.[9,10] The expression levels of IL-17, the signature cytokine of Th17, was significantly increased in RA synovium and Th17 cells were increased in peripheral blood mononuclear cells of RA patients when compared to healthy controls.[11–13] Levels of IFN-γ protein which is the principle cytokine product of Th1 were shown to be elevated in synovial tissue and fluid in RA patients as well.[14] On the other hand, IL-6 is crucial in enhancing the differentiation, survival, and stabilization of Th17 cells.[11] IL-17 induces the production of inflammatory cytokines such as IL-1, IL-6, IL-8, and TNFα.[15,16] Although TNFα does not directly induce these cytokines, all of the aforementioned are powerful mediators of inflammation in rheumatologic diseases and they have been shown in the synovial inflammation.[11,17]

Up to now, five anti-TNFα biologics have been approved for RA treatment and this therapy is effective in 70% of patients.[17] Etanercept (ETN) is a TNF-receptor-immunoglobulin-fusion protein functioning as a decoy receptor that binds to TNFα and β.[18] It is an effective drug; however, potential adverse effects are of concern after long-term systemic treatment.[19,20] For some patients, the inflammation in a few joints is the real cause of loss of function, highlighting the importance of local treatment instead of systemic treatment.[21] Thus, local application of these agents into chronically inflamed joints would be advantageous. A small number of preliminary clinical studies tested the efficacy of intra-articular use of ETN in RA patients.[22–24] These small-scale studies were conducted with drug doses as high as subcutaneous administration dose to increase duration of the drug activity. However, a sustained anti-TNF effect could not be achieved in synovial microenvironment.[22–24]

At this point, controlled drug delivery systems will provide the advantages as; suppressing the inflammation effectively via longer term drug supply, reducing the adverse effects arising from the use of high doses, and providing protection on protein based drug over the treatment period. Thereby, it will improve the potential for clinical use of ETN for intra-articular applications. An innovative way to maintain the therapeutic concentration of ETN over longer periods might be the use of microspheres, an ideal delivery system for intra-articular applications.

Biodegradable polymeric microspheres have been widely studied due to their biocompatibility and biodegradability.[25] Poly(ε-caprolactone) (PCL) has been frequently used in the design of controlled delivery systems in microsphere form due to its biocompatibility, high permeability to many drugs and the ability to be fully excreted from the body.[26,27] Additionally, drug delivery devices based on PCL had been approved by U.S. Food and Drug Administration (FDA).[26] Introduction of hydrophilic blocks into PCL chains enhances the hydrophilicity, biodegradability and mechanical properties compared to homopolymer, and thereby results with much wider application field for drug delivery.[28,29] Poly(ethylene glycol) (PEG), a hydrophilic, non-immunogenic, and FDA-approved polymer, has been used to form various block copolymers with PCL.[29,30] In literature, PEG-PCL and methoxy poly(ethylene glycol)-block-poly(ε-caprolactone) (MPEG-PCL) diblock and triblock copolymers have been used to prepare micelle, nanoparticle and microparticle forms of drug delivery systems.[29,31–34] Human serum albumin loaded MPEG-PCL microspheres,[31] fibroblast growth factor (bFGF) loaded PCL-PEG-PCL nanoparticles,[33,34] bovine serum albumin loaded PCL-PEG-PCL nanoparticles,[32] and immunoglobulin G (IgG) loaded MPEG-PCL-MPEG microspheres[35,36] have also been studied as peptide/protein delivery systems.

This study aims to develop novel intra-articularly injectable microcarrier systems for ETN that would provide long-term controlled drug release with sustained anti-inflammatory effect as a local treatment for chronic inflammatory arthritis. Even though ETN has been used in the RA treatment, this is the first report for the development of intra-articularly injectable microsphere formulation for ETN. ETN-loaded MPEG-PCL-MPEG and PCL microspheres were characterized for size, surface morphology, encapsulation efficiency, in vitro release properties in various media and compared. Additionally, protein adsorption on microspheres in synovial fluids of RA patients and healthy patients undergoing total knee replacement was also conducted to further study the effect of protein adsorption on release behavior and on preserving activity of the drug in microspheres. The present study was also designed to investigate the effects of ETN-loaded microspheres (PCL or MPEG-PCL-MPEG) on RA FLS and compare it to free ETN in an in vitro setting. Cytotoxic effects which might arise from the microsphere preparation method were evaluated by Alamar Blue Assay. We evaluated the changes at protein levels of critical pro-inflammatory cytokines of inflammation. (TNFα, IL-6, IFNγ, IL-17) and MMPs induced by TNF (MMP-3 and 13) to understand the effects of the ETN released from microspheres to the inflammatory milieu.

Materials and methods

Materials

Etanercept (ETN, Enbrel®) was obtained through the courtesy of Wyeth Pharmaceuticals, England. MPEG-PCL-MPEG triblock copolymer with [MPEG] / [ε-CL] monomer feed mole ratio of 1:200 (Mn 14,051 g/mol; Mw 47,038 g/mol; and PDI 3.35) was synthesized by ring-opening polymerization ε-caprolactone initiated by methoxy poly(ethylene glycol) (MPEG) in our previous study.[35,36] Poly(ε-caprolactone) (Mw 65,000), poly(vinyl alcohol) (PVA), chloroform, Pluronic F-68 (PLF-68), Tween 20, sodium azide, copper sulphate pentahydrate (CuSO4 5H2O), type IA collagenase, thiazolyl blue tetrazolium bromide, and bicinchoninic acid (BCA) were purchased from Sigma-Aldrich (Steinheim, Germany) and used as received. Hydrophilic polyvinylidene difluoride (PVDF) membrane was obtained from Millipore (Cork, Ireland). Dichloromethane (Merck, Darmstadt, Germany), dimethyl sulfoxide (DMSO; AppliChem, Darmstadt, Germany), sodium dodecyl sulfate (SDS, Bio-Rad Laboratories, Hercules, CA, USA), and Alamar Blue reagent (Invitrogen, Camarillo,CA, USA) were also used as received. Dulbecco's Modified Eagle Medium (DMEM), DMEM medium without phenol red, fetal bovine serum (FBS), penicillin/streptomycin, trypsin-EDTA were purchased from Biochrom AG (Berlin, Germany). Q-ETA ETN enzyme-linked immunosorbent assay (ELISA) was purchased from Matriks Biotek (Ankara, Turkey). Human TNFα, IL-6, IL-17, IFNγ, MMP-3, and MMP-13 ELISA kits were purchased from (eBioscience, San Diego, CA, USA). All other chemicals were also of analytical grade and were used without further purification.

Preparation of ETN-loaded microspheres

ETN-loaded PCL and MPEG-PCL-MPEG microspheres were prepared by the double emulsion-solvent evaporation method optimized in our previous study.[35] Briefly, 100 µL of the internal aqueous phase (phosphate buffered saline; PBS, 10 mM sodium phosphate, 145 mM NaCl, pH 7.2) containing 25 mg/mL ETN solution and 1% poly(vinyl alcohol) was added to 2 mL PCL in dichloromethane (5% w/v) or 4 mL MPEG-PCL-MPEG (2.5% w/v) solution in chloroform/DMSO mixture (1:1). The mixtures were sonicated for 60 s on ice using Sonorex sonicator (Bandelin, Germany). The resulting primary water-in-oil (w/o) emulsion was added to external 40 mL PVA solution (1% w/v) to prepare PCL microspheres or to external 40 mL PLF-68 solution (3% w/v) for MPEG-PCL-MPEG microspheres. Emulsions were stirred with a magnetic stirrer (Schott, Australia) at 1100 r/min for 15 min. The double emulsion (w/o/w) was poured into 180 mL PVA solution or 168 ml PLF-68 solution homogenized at 14,000 r/min for 3 min in an ice bath with a homogenizer (Ultraturrax T-25, IKA, Germany) to prepare PCL and MPEG-PCL-MPEG microspheres, respectively. The emulsion was stirred at 1100 r/min for 3 h to evaporate the organic solvents. Finally, formed microspheres were collected by filtration through a 0.45 µm hydrophilic PVDF membrane, washed with distilled water and then vacuum-dried overnight .The microspheres were stored at 4 °C until use.

ETN-loaded PCL and MPEG-PCL-MPEG microspheres were sterilized by exposure to γ-irradiation with a typical sterilization dose (25 kGy)[36] obtained from 60Co γ-source (Gamma-cell 220, MDS Nordion, Canada) at ambient temperature and at fixed dose rate (1.74 kGy/h) in Turkish Atomic Energy Authority.

Characterization of ETN-loaded microspheres

Surface morphology and particle size. The surface morphologies of ETN-loaded microspheres were examined by scanning electron microscopy (SEM, JSM – 6400 Electron Microscope, Japan). Microspheres were mounted onto metal stubs using carbon tape, vacuum-coated with gold (25 nm) by using Hummle VII sputter coating device (Anatech, USA) for SEM analysis.

The mean particle sizes and particle size distribution of ETN-loaded microspheres were determined from SEM images by measuring the diameters of 500 microspheres for each group using Image J analysis software (NIH, USA). The resulting size distribution of microspheres was plotted as a histogram with equal width intervals between the largest and smallest values and as a cumulative percent arithmetic curve. Measure of the width of the distribution of particle size (SPAN) values of microspheres was obtained from cumulative (% undersize) microsphere size distribution curves by using the following equation

$$SPAN = \frac{d[0.9] - d[0.1]}{d[0.5]} \quad (1)$$

where $d[0.9]$, $d[0.5]$, and $d[0.1]$ are the particle diameters determined respectively at the 90th, 50th, and 10th percentiles of undersized particles.

Protein loading and encapsulation efficiency. The protein loading and encapsulation efficiency of ETN-loaded microspheres were determined by modifying the method, which involves the hydrolysis of the microspheres by strong base and the extraction of the protein with SDS.[37] Briefly, microspheres were dissolved in 1 mL DMSO at 37°C for 1 h. 2150 µL of 0.25 N NaOH solution containing 0.5% SDS was added and gently mixed in water bath (Nüve, Turkey) at 37°C for 4 h. The mixture was then centrifuged (EBA-20, Hettich, Germany) at 3500 r/min for 5 min. After centrifugation, the protein amount in the supernatant was analyzed with bicinchoninic acid (µBCA) assay. In this assay, 500 µL of aliquots were incubated with 500 µL BCA working solution containing 50 parts BCA reagent and 1 part 4% copper sulphate pentahydrate solution 15 min at 60°C. After cooling to room temperature, the amount of encapsulated protein was determined by measuring the optical density at 562 nm with a microplate reader (µQuant, Biotek, USA) ($n = 6$). The calibration curve of µBCA assay was constructed with different concentrations of ETN (0–20 µg/mL) treated with DMSO and NaOH/SDS solution. Empty microspheres were also used as blank. The results of loading and encapsulation efficiency were obtained by the equations as given in our previous study.[36]

Protein adsorption test. Protein adsorption test was performed by placing 5 mg empty microspheres in 200 µL of healthy synovial fluid or in synovial fluid taken from patients with RA for 14 days. Synovial fluids were obtained from 5 patients with RA and healthy synovial fluid was obtained from 1 patient undergoing total knee replacements due to trauma. The study was approved by the Medical Ethics Committee of Hacettepe University, and informed consent was obtained from each patient. At defined time intervals, all synovial fluid was removed and microspheres were washed with d-H_2O three times to remove physically attached proteins on the surface of microspheres. Adsorbed protein was dissolved from the surface of microspheres by incubating microspheres in 3 mL 1% SDS solution in PBS (pH 7.4) in a shaking water bath at 37°C for 24 h.[38] Amounts of protein adsorbed on microspheres were determined by using µBCA assay ($n = 3$) at different time points. The calibration curve of µBCA assay was constructed with different concentrations of bovine serum albumin (BSA) (0–1.25 mg/mL) treated with 1% SDS solution in PBS (pH 7.4) in a shaking water bath at 37°C for 24 h. Additionally, total protein concentrations of RA and healthy synovial fluids were measured by using µBCA assay after dilutions with PBS and calibration curve for this measurement was constructed with different concentrations of bovine serum albumin (BSA) in PBS (0–1.25 mg/mL).

In vitro release of ETN from microspheres. In vitro ETN release profiles of microspheres were evaluated by incubating 5 mg microspheres in 2 mL of PBS (0.01M pH 7.4) containing 0.01% Tween 20 and 0.05% sodium azide in a shaking water bath at 37°C for 90 days.[36] At defined time intervals, all release medium was taken after centrifugation at 3500 r/min for 5 min and replaced by fresh medium. Total protein amount (active and inactive) in the removed sample was determined by BCA assay ($n = 3$) as described above. The calibration curve for BCA assay was constructed with different concentrations of ETN (0–15 µg/mL) in release medium to determine the released protein amount. Empty microspheres were used as blank. At the end of in vitro release study, the amount of ETN remaining in the microspheres was also determined by the extraction method described above. Additionally, Q-ETA Etanercept ELISA kit was also used to measure the amount of active ETN released from γ-irradiated microspheres ($n = 3$).

Release studies in cell culture medium (high glucose DMEM supplemented with 10% FBS and 100 units/mL penicillin/streptomycin) were also conducted for ETN-loaded microspheres during 60 days. At defined time intervals, cell culture medium was completely removed and replaced by fresh medium. ETN amount ($n = 3$) in sample aliquots were determined at defined time intervals with Q-ETA Etanercept ELISA.

For prediction of in vivo activity of free ETN and ETN released from microspheres, ETN-loaded microspheres (5 mg) and free ETN (5 µg/mL) were incubated in synovial fluids of healthy and RA patients at 37°C for 14 days. At defined time intervals, 20 µL samples were taken from the synovial fluids and changes in free ETN concentration and the amount of ETN released from microspheres were determined by Q-ETA Etanercept ELISA ($n = 3$).

Kinetics of ETN release. In order to investigate the mechanism of ETN release from microspheres in different media, the release data were analyzed with the following mathematical models given in equations (2) and (3) (more detailed information can be found in literature review[39])

$$Q_t = k_H\sqrt{t} \quad \text{(HiguchimodelbasedonFickiandiffusion)} \tag{2}$$

$$\frac{M_t}{M_\infty} = k_p t^n \quad \text{(Korsmeyer – Peppasmodel)} \tag{3}$$

where k_H and k_p are the rate constants of Higuchi and Korsmeyer–Peppas model, respectively. Q_t and M_t are the amounts of drug release in time t, M_∞ *is* the amount of drug release at time ∞, M_t/M_∞ is the fraction of drug released at time t and n is the diffusion exponent, which can be used to characterize both mechanism for both solvent penetration and drug release.

The following plots were evaluated to determine the rate constants and correlation coefficients for respective kinetic models: cumulative % drug release versus square root of time (Higuchi model) and log cumulative % drug release versus log time (Korsmeyer–Peppas model). Additionally, n values were also calculated from the graphs of Korsmeyer–Peppas model.

Effects of ETN-loaded microspheres on fibroblast-like synoviocytes

Fibroblast-like synoviocyte (FLS) cell culture and study design. Synovial tissues were obtained from 5 patients with RA (5 women, aged 24–67 years) who were undergoing total joint replacement surgery. RA was diagnosed according to the 2010 ACR/EULAR (American College of Rheumatology/European League Against Rheumatism) classification criteria. The study was approved by the Medical Ethics Committee of Hacettepe University, and informed consent was obtained from each patient. The work described in this study has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans (http://www.wma. net/e/policy/b3.htm). Briefly, synovial membranes were minced and incubated with 0.4% (g/mL) Type IA collagenase in DMEM for 1 h at 5% CO_2/37°C.[40] FLS were cultured in DMEM supplemented with 10% FBS and 100 units/mL penicillin/streptomycin in a carbon dioxide incubator. 4th passage FLS were used in the experiments. The experimental groups were as follows:

(1) RA FLS (RA Control)
(2) RA FLS + free ETN
(3) RA FLS + ETN-loaded PCL microspheres
(4) RA FLS + ETN-loaded MPEG-PCL-MPEG microspheres

For groups 1, RA FLS were cultivated with cell culture medium containing 5% FBS, which was partially refreshed at pre-defined time periods. For group 2, FLS were incubated for 4 days only with cell culture medium containing free ETN equal to the loaded amount in 10 mg microsphere (10 µg/mL). After 4 days, fresh medium was added to existing medium. At day 7, half of the medium was refreshed and this was repeated at defined time periods. For groups 3 and 4, ETN-loaded microspheres (10 mg/per used flask) were incubated in cell culture medium containing 5% FBS. After day 3, medium of cells was replaced with 2.5 mL fresh medium of microspheres. After every 3 days, half of the culture medium was replaced with fresh medium and ETN released was determined.

Quantitation of ETN concentration. Changes in ETN concentrations in free ETN group and microsphere groups were monitored during 4 weeks using with Q-ETA Etanercept ELISA kit.

Determination of cell viability and cell numbers. At the end of each week, FLS in experimental groups were trypsinized with 0.1% trypsin-EDTA. After trypsinization, cell numbers and viability of FLS were determined with NucleoCounter (Chemometec, Allerød, Denmark) and by Alamar Blue Assay, respectively for 4 weeks. For cell number determination, 200 µL cell suspensions were taken and were first mixed with equal volumes of lysis/disaggregation buffer and then stabilizing buffer of NucleoCounter device. The NucleoCassette was loaded with the lysate solution by immersing the tip of the cassette into the solution and cells were counted in the instrument. For Alamar Blue assay, the culture medium was removed and fresh high glucose DMEM medium without phenol red containing 10% Alamar Blue reagent was added to each well at predetermined time intervals. After 4 h of incubation at 37°C in dark, media of cells were collected and their absorbance was read at 570 nm (reduced) and 600 nm (oxidized) with a microplate reader (µQuant, BioTek®, Winooski, VT, USA). Percent difference in the viability of FLS incubated with ETN-loaded microspheres compared to the RA control was evaluated according to the absorbance equation in which the ratio between the absorbance of microsphere groups and RA control group at 570 nm and 600 nm was used.[35]

Monitoring of changes in protein levels of pro-inflammatory cytokines and MMPs. Levels of TNFα, IL-6, IL-17, IFNγ, MMP-3, and MMP-13 released by FLS were determined by using ELISA kits. Levels of TNFα not bound by ETN were determined with a bioassay using WEHI 164 clone-13 murine fibrosarcoma cell line (American Type Culture Collection, CRL-1751).

In this bioassay, WEHI 164 cells were firstly sensitized to TNFα with exposure to actinomycin and only TNFα not bound by ETN was measured. Wehi cells were incubated for 20 h with culture media of RA FLS control and FLS exposed to free ETN or ETN released from microspheres. After incubation for 20 h, the TNFα not bound by ETN was determined indirectly by measuring the viability of WEHI cells. A calibration curve was obtained by measuring the viability of Wehi-164 incubated with human TNFα standard solutions using MTT [3-(4,5-dimetiltiazol-2-yl)-2,5-difeniltetrazolyum bromide] test. In MTT assay, the culture medium containing human TNFα standard solutions were removed and Wehi-164 cells were incubated with 500 μL of fresh MTT working solution (1:10 dilution of 5 mg/ml thiazolyl blue tetrazolium bromide in PBS with serum free DMEM medium) for 4 h at 37°C. After MTT working solution was removed, 500 μL of DMSO was added to solubilize the formazan complex and the mixture was shaken at room temperature for 15 min. The optical density was measured at 570 nm with microplate reader (μQuant, BioTek®, Winooski, VT, USA). The levels of TNFα IL-6, IL-17, IFNγ, MMP-3, and MMP-13 released by RA control group were also compared.

Statistical analysis

Data were statistically analyzed with Mann–Whitney nonparametric test for pair-wise comparisons of the groups using SPSS-9 Software (SPSS Inc., USA). Differences were considered significant at $p \leq 0.05$ and in some cases $p \leq 0.01$.

Results

Characterization of ETN-loaded microspheres

Surface morphology and particle size. The surface morphologies of ETN-loaded PCL and MPEG-PCL-MPEG microspheres were examined by SEM after γ-sterilization (Figure 1). Both types of microspheres possessed spherical shape with smooth surface and without observable pores.

The particle size distributions of microspheres were plotted as histogram (data not presented). As given in Table 1, no significant difference was found between the mean particle sizes (diameters) of microsphere groups. PCL microspheres had higher SPAN value compared to that of MPEG-PCL-MPEG microspheres, which indicated broader (more heterogeneous) size distribution for PCL microspheres.

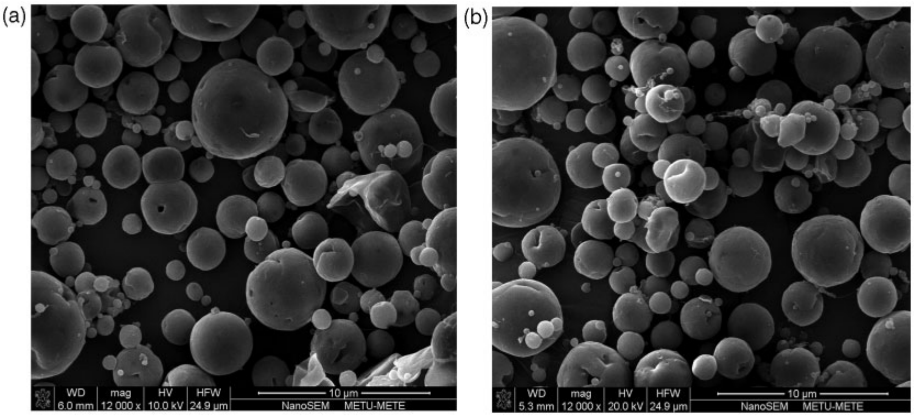
(a)
(b)
WD 6.0 mm | mag 12 000 x | HV 10.0 kV | HFW 24.9 μm | 10 μm | NanoSEM METU-METE
WD 5.3 mm | mag 12 000 x | HV 20.0 kV | HFW 24.9 μm | 10 μm | NanoSEM METU-METE

Figure 1. SEM images of γ-irradiated ETN-loaded: (a) PCL and (b) MPEG-PCL-MPEG microspheres.

Table 1. Mean particle sizes, particle sizes at 10%, 50%, and 90% and SPAN values of ETN-loaded PCL and MPEG-PCL-MPEG microspheres.

	Particle size (μm)	*d*[0.1] (μm)	*d*[0.5] (μm)	*d*[0.9] (μm)	Span
PCL microspheres	5.24 ± 0.17	2.15	4.24	10.12	1.88
MPEG-PCL-MPEG microspheres	4.98 ± 0.09	2.88	4.74	7.26	0.92

Data are given as mean ± SE.
PCL: **poly(ε-caprolactone);** MPEG-PCL-MPEG: methoxypoly(ethylene glycol)-poly(ε-caprolactone)-methoxypoly(ethylene glycol).

Protein loading and encapsulation efficiency. MPEG-PCL-MPEG microspheres showed higher ETN loading and encapsulation efficiency outcomes compared to PCL microspheres (Table 2).

Protein adsorption test. Total protein concentration of RA synovial fluid was observed to be higher (30.34 ± 1.25 mg/mL) than that of healthy synovial fluid (19.76 ± 1.44 mg/mL). No significant difference in the amounts of protein adsorbed onto microspheres was found between MPEG-PCL-MPEG and PCL microspheres upon incubation in same type of synovial fluid at 1st day (Figure 2). However, a significant increase in the adsorbed amount of protein on PCL microspheres was evident compared to the MPEG-PCL-MPEG microspheres after 1st and 2nd days for RA and healthy synovial fluid incubations, respectively.

For PCL microspheres, protein adsorption was significantly higher upon incubation in RA synovial fluid than in healthy synovial fluid for first two days whereas no significant difference was observed between these groups after 2nd day. On the other hand, no significant difference was observed in amounts of adsorbed protein for MPEG-PCL-MPEG microspheres after 14-day incubations in healthy or RA synovial fluids.

Table 2. ETN loading and encapsulation efficiency of γ-irradiated ETN-loaded PCL and MPEG-PCL-MPEG microspheres.

	Encapsulation efficiency (%)	Loading (%)
PCL microspheres	65.06 ± 1.85*	1.62 ± 0.03**
MPEG-PCL-MPEG microspheres	75.64 ± 1.78*	1.84 ± 0.04**

*,**Statistical significances between groups ($p \leq 0.01$).
Data are given as mean ± SD.
PCL: **poly(ε-caprolactone);** MPEG-PCL-MPEG: methoxypoly(ethylene glycol)-poly(ε-caprolactone)-methoxypoly(ethylene glycol).

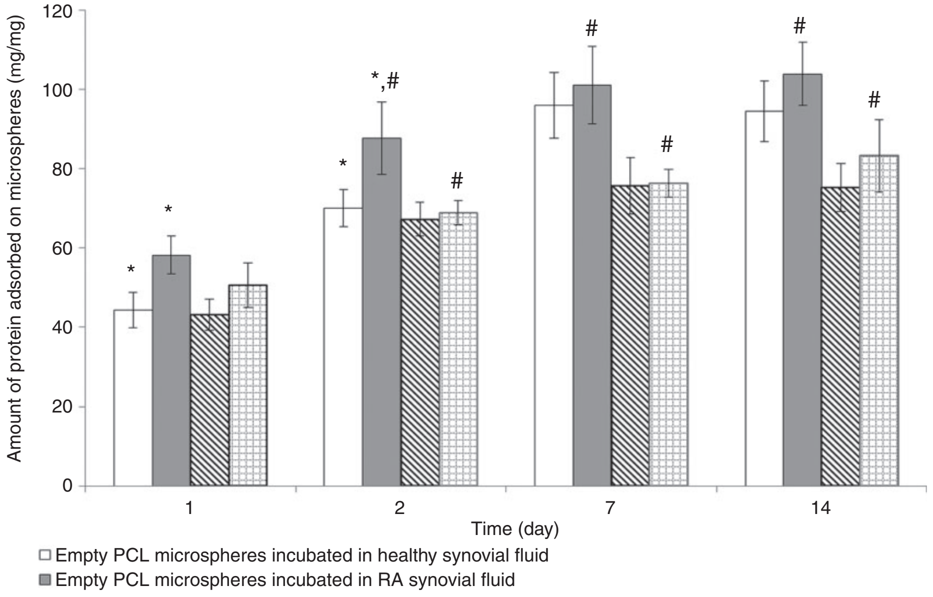

Figure 2. Amount of protein absorbed on empty PCL or MPEG-PCL-MPEG microspheres incubated in synovial fluids of healthy and RA patients for 14 days. Data are given as mean ± SD.
*Statistically significant differences between PCL microspheres incubated in synovial fluids of healthy and RA patients at the same time period.
,#Statistically significant differences between different types of microspheres incubated in synovial fluids of healthy and RA, respectively ($p \leq 0.01$).
PCL: poly(ethylene glycol); MPEG-PCL-MPEG: methoxypoly(ethylene glycol)-poly(ε-caprolactone)-methoxypoly(ethylene glycol); RA: rheumatoid arthritis.

In vitro release of ETN from microspheres. ETN release (in PBS) showed a slower release profile for PCL microspheres than from MPEG-PCL-MPEG microspheres (Figure 3). At the end of in vitro release study, the amounts of ETN remaining in the microspheres after the extraction (29.07 ± 3.95% and 22.97 ± 1.44% for PCL and MPEG-PCL-MPEG microspheres, respectively) was found lower than the amounts of ETN remaining in the microspheres obtained from the cumulative release profiles (39.71 ± 1.09% and 31.29 ± 1.59% for PCL and MPEG-PCL-MPEG microspheres, respectively), which could be due to the experimental errors. When the BCA and ELISA results were compared, the amount of active ETN released from PCL microspheres was numerically lower than the total amount released from these microspheres during 90 days (Figure 3).

To examine the activity loss, the ratio of the amount of active ETN released from microspheres (determined by ELISA) to the amount of total ETN released from microspheres (determined by BCA) was calculated (Table 3). Activity of ETN released from both type of

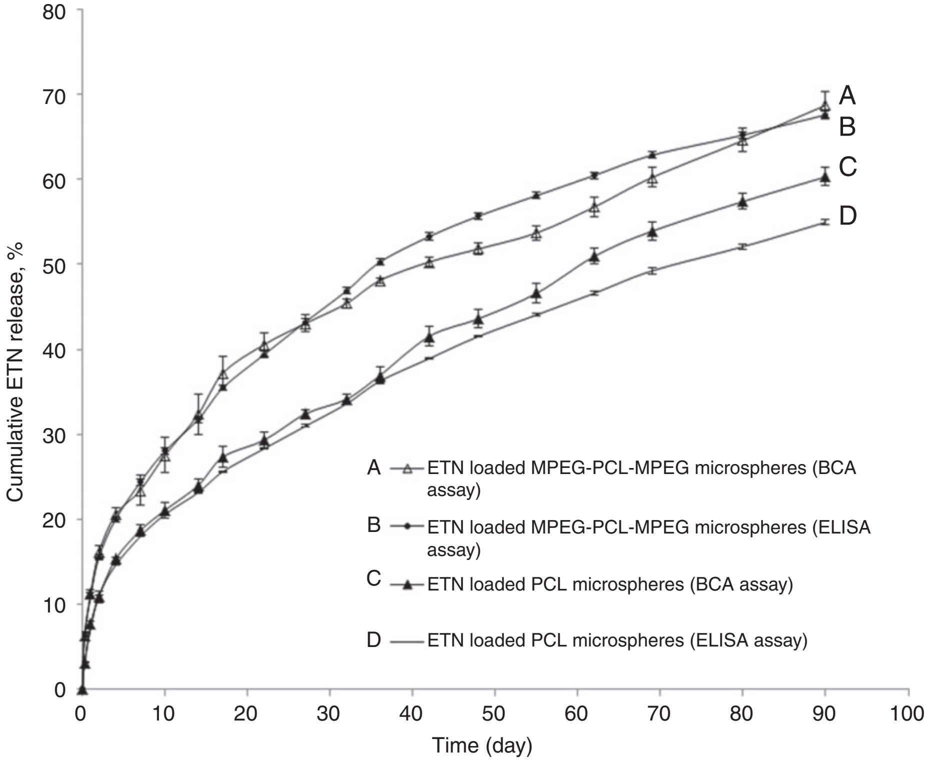

Figure 3. Cumulative release profile of ETN-loaded PCL and MPEG-PCL-MPEG microspheres in PBS for 90 days determined by: (a) BCA and (b) ELISA methods (data are given as mean ± SD, $n = 3$).
ETN: etanercept; MPEG-PCL-MPEG: methoxypoly(ethylene glycol)-poly(ε-caprolactone)-methoxypoly(ethylene glycol).

Table 3. The ratio of the amount of active ETN released to the total amount of ETN released from γ-irradiated PCL and MPEG-PCL-MPEG microspheres in PBS.

	Ratio of the amount of biologically active released protein to the amount of total released protein		
	6 h	20 days	90 days
PCL microspheres	1.00 ± 0.08	0.97 ± 0.03	0.91 ± 0.01*
MPEG-PCL-MPEG microspheres	1.00 ± 0.004	0.97 ± 0.03	0.98 ± 0.02*

*Statistically significant difference between groups at 90 days ($p \leq 0.05$).
Data are given as mean ± SD.
PCL: **poly(ε-caprolactone);** MPEG-PCL-MPEG: methoxypoly(ethylene glycol)-poly(ε-caprolactone)-methoxypoly(ethylene glycol).

microspheres was retained completely during 20 days. At the end of 90-day release study, 91.16 ± 1.22% activity of ETN released from PCL microspheres and 98.38 ± 2.11% ETN released from MPEG-PCL-MPEG microspheres were retained. Retention of ETN activity in MPEG-PCL-MPEG microspheres was significantly higher than that in PCL microspheres at the end of 90 days.

In vitro ETN release from PCL and MPEG-PCL-MPEG microspheres were also evaluated by incubating microspheres in cell culture medium for 60 days (Figure 4). As observed in PBS release, a slower release from PCL microspheres was observed for ETN compared to that from MPEG-PCL-MPEG microspheres.

Similarly, slower ETN release was observed from both PCL and MPEG-PCL-MPEG microspheres in healthy synovial fluid compared to their counterparts incubated in RA synovial fluid (Figure 5). For each synovial fluid type, percent cumulative amount of ETN released from MPEG-PCL-MPEG microspheres was found significantly higher than that from PCL microspheres at each time point. Additionally, free ETN was incubated in healthy and RA synovial fluids for 14 days. No significant change was observed in the concentration of free ETN in synovial fluids (data not shown) and activity of ETN was retained during 2-week incubation.

In all release media, percent cumulative release of ETN from MPEG-PCL-MPEG microspheres was significantly higher than that from PCL microspheres. For both microsphere groups, percent cumulative release observed in PBS was significantly higher than all other release media studied.

Kinetics of ETN release. In vitro release data of microspheres in different release media were fitted to Higuchi and Korsmeyer–Peppas release models to find out the mechanism of ETN release from microspheres. The coefficient of determination (R^2), rate constants (k_H and k_P) and n values obtained after linear regression on mathematical models are presented in Table 4. R^2 values showed that the best-fits were obtained with both models. For in vitro release studies evaluated in PBS and cell culture medium, n values for both microsphere formulations were above 0.43 indicating that ETN release was governed by an anomalous transport. However, release studies in healthy and RA synovial fluids showed n values (for both microsphere formulations) near 0.43 suggesting that ETN release was mainly diffusion controlled.

Effects of ETN-loaded microspheres on fibroblast-like synoviocytes

To investigate the effects of free ETN and ETN released from microspheres, in an in vitro setting, ETN released from microspheres in medium and free ETN were incubated with RA FLS during 4 weeks.

Quantitation of ETN concentration. Changes in ETN concentration for free ETN and microsphere groups

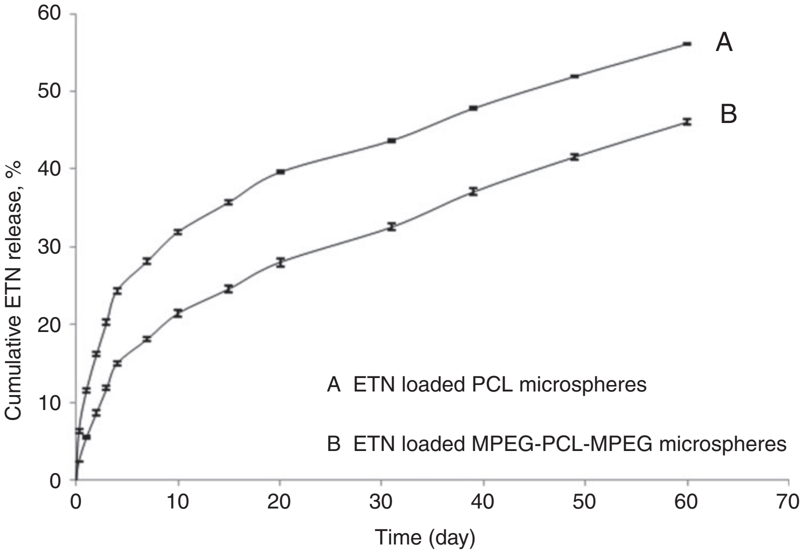

Figure 4. Cumulative release profiles of ETN-loaded PCL and MPEG-PCL-MPEG microspheres in cell culture medium for 60 days determined by ELISA (data are given as mean ± SD, $n = 3$).
ETN: etanercept; MPEG-PCL-MPEG: methoxypoly(ethylene glycol)-poly(ε-caprolactone)-methoxypoly(ethylene glycol).

were also compared during 4 weeks (Figure 6). ETN concentration of the free drug group decreased shortly whereas it increased with sustained release in microsphere groups.

Determination of cell viability and cell numbers. No significant differences were observed in cell numbers among RA groups during 4 weeks (Figure 7(a)).

Viability of FLS incubated with free drug was significantly lower than that incubated with microspheres at first week ($p \leq 0.05$) (Figure 7(b)). This indicates that high levels of the free drug caused a decrease in cell viability in group 3 at first week. However, after changing half of the medium, with a reduction in free drug level, cell viability increased. No decrease in cell viability was observed in groups 4 and 5, probably due to slow ETN release from microspheres.

Monitoring of changes at protein levels of pro-inflammatory cytokines and MMPs. No significant differences were observed for TNFα among groups because both ETN bound and unbound TNFα are quantitated by ELISA (data not presented). Thus, a bioassay was used to measure the amount of TNFα not bound by ETN (Figure 8(a)). After 2 weeks, percentage of biologically active TNFα decreased by 35% in microsphere groups while it decreased only by 10–25% in free drug group. IL-6 levels decreased by 25–30% in microsphere groups, whereas 10–15% decrease was observed in the free drug group (Figure 8(b)). At week 2, IL-17 levels were significantly lower only in MPEG-PCL-MPEG microsphere group when compared to the free drug group (Figure 8(c)). However, at the end of 4th week, IL-17 levels in both microsphere groups were significantly lower than that of free drug group. Furthermore, IFNγ levels were also significantly lower in both microsphere groups after 4 weeks compared to free drug application (Figure 8(d)).

In parallel with above outcomes at the end of 1st week, MMP-3 and MMP-13 levels were found significantly lower in the microsphere groups than the free drug group after 2nd and 1st weeks, respectively, (Figure 9(a) and (b)). These results demonstrated that sustained ETN release from microspheres significantly decreased the levels of pro-inflammatory cytokines and MMPs.

Discussion

For the treatment of chronic diseases, local delivery of therapeutic agents with the advantages in efficacy, prolonged duration, and convenience reduces the systemic exposure of anti-arthritic drugs; however, clinical improvement is often transient since the drugs quickly leave the joint. Moreover, intra-articular drug administration can cause serious side effects such as risk of infection due to numerous injections along with high financial burden and impaction to patient's quality of life.[41] Therefore, a number of micro- and nanocarrier

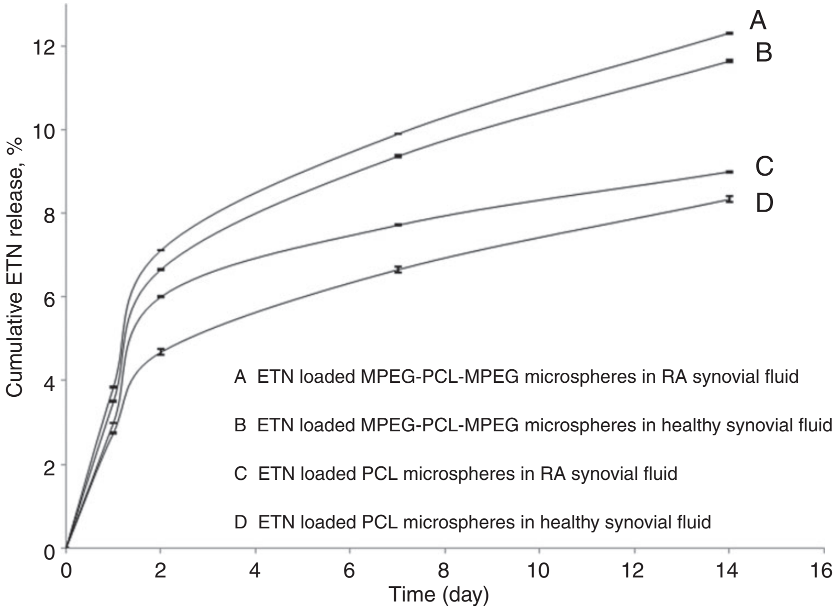

Figure 5. In vitro cumulative release profiles of ETN-loaded PCL and MPEG-PCL-MPEG microspheres in synovial fluids of healthy and RA patients for 14 days (data are given as mean ± SD, $n = 3$).
ETN: etanercept; PCL: **poly(ε-caprolactone);** MPEG-PCL-MPEG: methoxypoly(ethylene glycol)-poly(ε-caprolactone)-methoxypoly(ethylene glycol); RA: rheumatoid arthritis.

mediated drug delivery systems have been investigated for sustained release in joints.[41–43] Results showed that intra-articular drug delivery approaches may well be efficient alternatives in our arthritis treatment armamentarium. This is the first study investigating the beneficial effect of local ETN administration using microsphere delivery systems.

In active RA, intra-articular administration of a carrier includes its injection into a pathological environment; therefore, size, shape and type of the carrier are

Table 4. In vitro release kinetic parameters of ETN-loaded PCL and MPEG-PCL-MPEG microspheres after γ-sterilization incubated in different release media.

	Higuchi		Korsmeyer–Peppas		
	R^2	k_H ($h^{-1/2}$)	R^2	k_p (h^{-n})	n
PBS (BCA method)					
PCL	0.9967	1.27	0.9909	1.28	0.51
MPEG-PCL-MPEG	0.9872	1.39	0.9590	1.92	0.48
PBS (ELISA method)					
PCL	0.9975	1.16	0.9904	1.30	0.50
MPEG-PCL-MPEG	0.9891	1.45	0.9643	1.86	0.49
Cell culture medium					
PCL	0.9927	1.20	0.9908	1.06	0.53
MPEG-PCL-MPEG	0.9640	1.40	0.9392	1.94	0.49
RA synovial fluid					
PCL	0.9095	0.49	0.9677	1.01	0.39
MPEG-PCL-MPEG	0.9553	0.67	0.9838	1.03	0.44
Healthy synovial fluid					
PCL	0.9625	0.45	0.9866	0.98	0.37
MPEG-PCL-MPEG	0.9570	0.63	0.9827	1.01	0.43

PBS: phosphate buffered saline; PCL: **poly(ε-caprolactone);** MPEG-PCL-MPEG: methoxypoly(ethylene glycol)-poly(ε-caprolactone)-methoxypoly(ethylene glycol); RA: rheumatoid arthritis; ELISA: enzyme-linked immunosorbent assay; BCA: bicinchoninic acid.

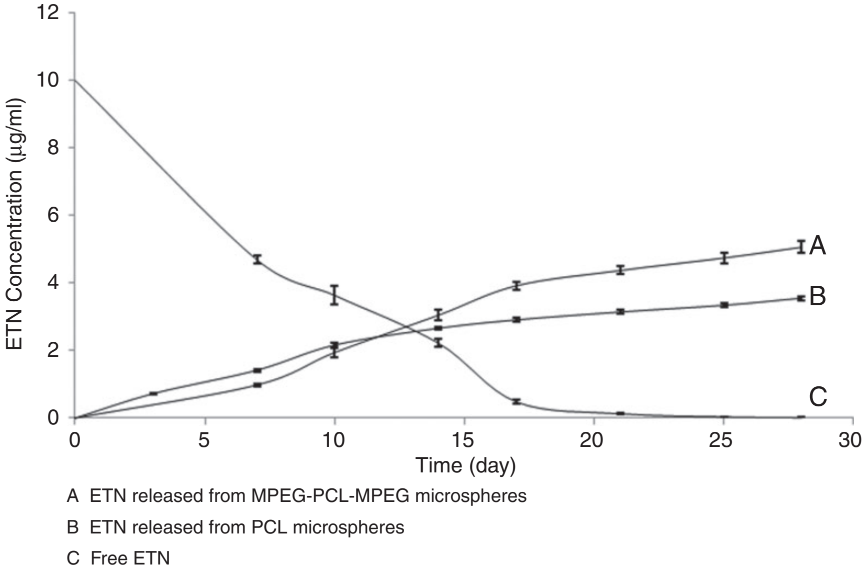

Figure 6. Changes in ETN concentration in free and PCL or MPEG-PCL-MPEG microsphere groups during 4-week cell culture studies (values are shown as mean ± SD, $n = 3$).

ETN: etanercept; PCL: **poly(ε-caprolactone);** MPEG-PCL-MPEG: methoxypoly(ethylene glycol)-poly(ε-caprolactone)-methoxypoly(ethylene glycol).

important to avoid further formation of an inflammation or an immune response.[41] In a previous study, poly-L-lactic acid (PLA) microspheres containing neutron-activated ^{166}Ho were investigated as potential agents for radio nuclide synovectomy.[44] Biodistnbution analysis and gamma camera analysis showed that nearly 98% of ^{166}Ho-loaded PLA microspheres with the size range 2–13 µm retained in the joint space of rabbits after 120 h with no uptake by the lymph nodes. In the review article of Butoescu et al.,[41] various types of microspheres tested for intra-articular delivery of drugs were reported and it was reported that most suitable size for intra-articular administration is between 5 µm and 10 µm microspheres. This particle size provides a prolonged retention time for drugs in the joint cavity without inducing any inflammatory reactions. The particle size range between 1 µm and 250 µm is ideal for injectables.[45] In our study, 90% of particle size of ETN-loaded PCL and MPEG-PCL-MPEG microspheres were found smaller than 10 µm (Table 1) and only 0.2% of particle size of ETN-loaded PCL and MPEG-PCL-MPEG microspheres were found below 1.4 µm and 2 µm, respectively (data not represented). According to these results, both formulations were thought suitable for intra-articular applications.

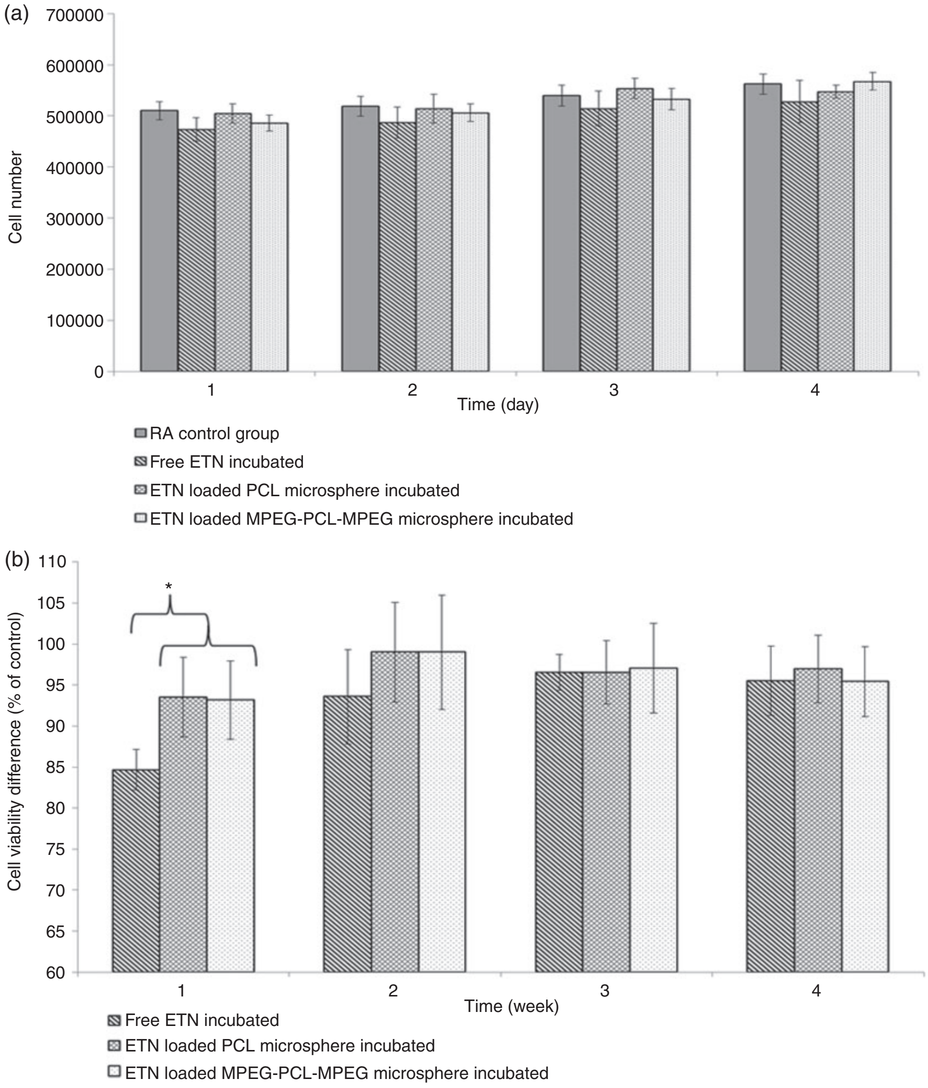

Figure 7. (a) Cell numbers determined with NucleoCounter and (b) relative viability of FLS determined by Alamar Blue Assay upon incubation with free ETN or ETN-loaded microspheres, for 4 weeks RA FLS without exposure to drug served as control for determination of cell viability (values are shown as mean ± SE of the mean, $n = 5$) ($p \leq 0.05$).
ETN: etanercept; PCL: **poly(ε-caprolactone);** MPEG-PCL-MPEG: methoxypoly(ethylene glycol)-poly(ε-caprolactone)-methoxypoly(ethylene glycol); RA: rheumatoid arthritis.

MPEG-PCL-MPEG microspheres showed higher ETN loading and encapsulation efficiency than PCL microspheres. This was thought to be caused by the presence of hydrophilic MPEG segments in polymer matrix, which improved the hydrophilicity of polymeric matrix and thereby enhancing the affinity of ETN for the polymeric core. This result is in agreement with the result observed in our previous study in which MPEG-PCL-MPEG microspheres showed higher IgG loading and encapsulation efficiency than PCL microspheres.[35,36] In a previous study, encapsulation efficiency of human serum albumin loaded PCL and PEG-PCL (diblock copolymer) microspheres were compared.[31] PEG-PCL microspheres with various PEG/PCL ratio showed higher encapsulation efficiencies (between 35.4% and 50.1%) compared to that of PCL microspheres (25.8%).[31] They concluded that PEG segment in polymer chains improved the affinity of polymer to protein molecules, resulting in higher loading efficiencies compared to PCL microspheres.

In general, hydrophobic surfaces adsorb more proteins than hydrophilic surfaces.[46] The adsorption of plasma proteins onto surface of carrier systems mediates cell adhesion, including platelet activation and leukocyte binding in the clotting and inflammation cascades, respectively. Additionally, it was previously shown that non-specific protein adsorption onto polymeric surface of microspheres limit the release of proteins and result in slow release profiles.[47] According to the results of protein adsorption test, protein adsorption on PCL microspheres was higher compared to the MPEG-PCL-MPEG microspheres for RA and healthy synovial fluid after 2-day incubations. Hydrophilic MPEG segment prevented protein adsorption on MPEG-PCL-MPEG microspheres. PEG is well known to minimize the adsorption of plasma proteins

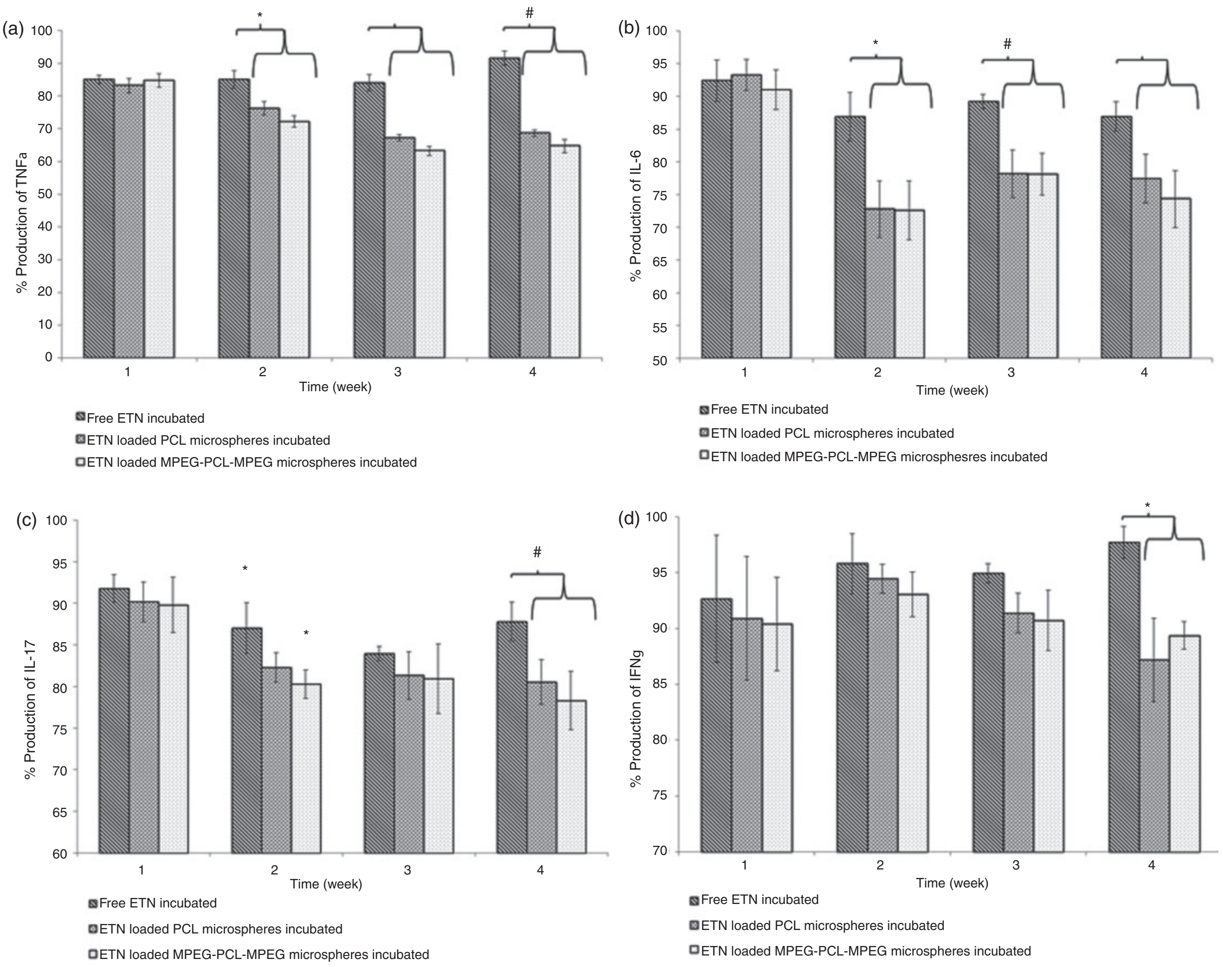

Figure 8. Comparison of production levels of TNFα (a), IL-6 (b), IL-17 (c), IFNγ (d) produced by FLS among treatment groups with respect to control group (values are shown as mean ± SE of the mean, $n = 5$) ($p \leq 0.05$).
ETN: etanercept; PCL: **poly(ε-caprolactone);** MPEG-PCL-MPEG: methoxypoly(ethylene glycol)-poly(ε-caprolactone)-methoxypoly(ethylene glycol).

by steric repulsion and there is a direct correlation between the PEG chain-length and minimization of protein adsorption.[46] In a previous study, blend films based on PCL and PEG grafted PCL were prepared with different blend ratios and protein adhesion on these films was studied.[46] They concluded that the level of the protein adsorption decreased when PEG component was introduced. In another study, poly(L-lysine)-g-poly(ethylene glycol) (PLL-g-PEG) coated and uncoated poly(lactic-co-glycolic acid) (PLGA) microspheres were incubated in HEPES II buffer containing appropriate concentrations of human proteins such as human serum albumin, fibrinogen, fibronectin, γ-immunoglobulin G to investigate the efficiency of the protein repellent character of PLL-g-PEG.[48] PLL-g-PEG-coated PLGA microspheres showed a decrease in adsorption of proteins by two orders of magnitude in comparison to uncoated PLGA microspheres according to confocal laser scanning microscopy results.

The most abundant macromolecules in normal synovial fluid are sodium salt of hyaluronic acid (approx. 3 mg/mL) and blood plasma proteins such as albumin (approx. 11 mg/mL) and globulins (approx. 7 mg/mL).[49] Synovial fluid is very viscous because of its high concentration of hyaluronic acid.[50] Synovial fluids of RA patients have a higher albumin concentration compared to those of healthy individuals.[49] In agreement with literature, total protein concentration of RA synovial fluid was found to be significantly higher (30.34 ± 1.25 mg/mL) than that of healthy synovial fluid (19.76 ± 1.44 mg/mL). Thus, higher amount of protein adsorbed on PCL microspheres when incubated in RA synovial fluid compared to incubation in healthy synovial fluid.

In degenerative joint disease such as osteoarthritis and RA, the molecular weight and concentration of hyaluronic acid are reduced resulting in a decrease in viscosity of synovial fluids.[51,52] Due to lower viscosity in diseased state, the amount of ETN released from microspheres in RA synovial fluid was significantly higher than that of in healthy synovial fluid. Even though more protein adsorption on PCL microspheres was observed in RA synovial fluid compared to healthy synovial fluid, a slower release of ETN was obtained in healthy synovial fluid. Hence, it might be suggested that viscosity of synovial fluid is a more predominant factor than protein adsorption on microspheres for determining ETN release from microspheres. This finding can be supported with MPEG-PCL-MPEG results. No significant differences were observed between the amounts of protein adsorbed on MPEG-PCL-MPEG microspheres incubated in RA or healthy synovial fluids. However, ETN-loaded MPEG-PCL-MPEG microspheres showed slower release profiles in healthy synovial fluid compared to their counterparts incubated in RA synovial fluid during 14 days.

In all release media, presence of hydrophilic MPEG segment caused more ETN release from MPEG-PCL-MPEG microspheres owing to enhanced diffusion of water into microspheres. In a previous study, $(AB)_n$ type amphiphilic multiblock copolymers with various compositions were used to prepare bovine serum albumin (BSA) loaded PEG-PCL and PEG-PLLA microspheres.[53] They examined that the rate and amount of

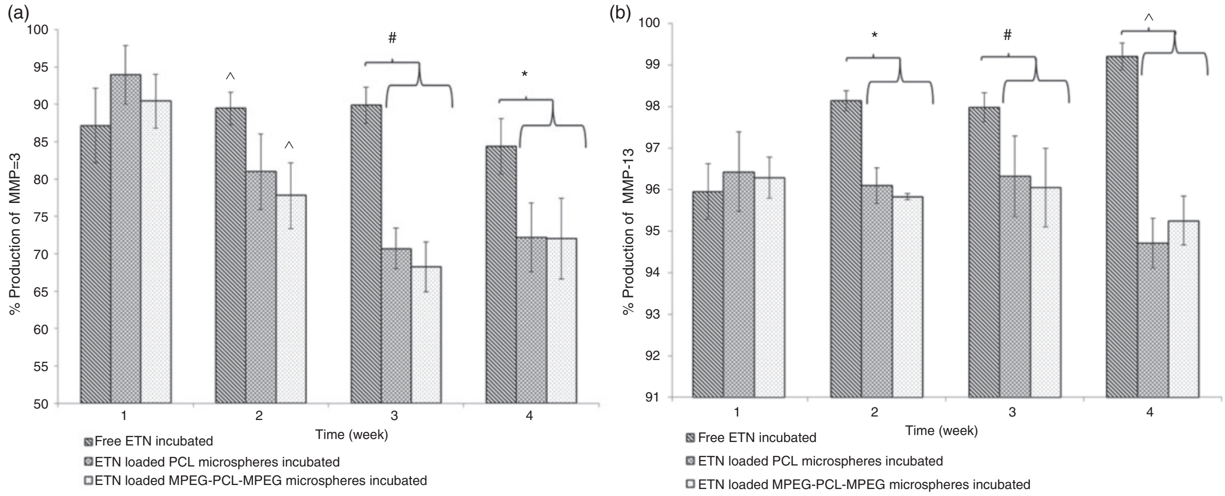

Figure 9. Comparison of production levels of MMP-3 (a), MMP-13 (b), produced by FLS among treatment groups with respect to control group (values are shown as mean ± SE of the mean, $n = 5$) ($p \leq 0.05$).
ETN: etanercept; PCL: **poly(ε-caprolactone);** MPEG-PCL-MPEG: methoxypoly(ethylene glycol)-poly(ε-caprolactone)-methoxypoly(ethylene glycol).

BSA release increased with increasing the ratio of PEG segment. In our previous study, γ-irradiated MPEG-PCL-MPEG microspheres also showed a much faster release compared to that of γ-irradiated PCL microspheres.[35,36] In another previous study, nanoparticles were prepared from di-block and tri-block copolymers of MPEG-PCL with different MPEG/PCL ratio for local delivery of tetradrine.[54] The nanoparticles prepared from tri-block copolymers exhibited a more sustained-release pattern compared to their diblock counterparts due to the differences between the "brush" structure for diblock copolymers and "mushroom" structure for triblock copolymers in aqueous solution. For obtaining more prolonged and sustained delivery of ETN, MPEG-PCL-MPEG tri-block copolymer was used to prepare ETN-loaded microspheres instead of their diblock counterparts in our study.

No significant difference was observed between the amounts of total and active ETN released from MPEG-PCL-MPEG whereas the amount of active ETN released from PCL microspheres was found lower than the total amount released from the PCL microspheres (Figure 3). This result showed that the stability of ETN during preparation step retained due to the presence of MPEG segment in microspheres. In the study of Pean et al.,[55] they encapsulated nerve growth factor with PEG 400 in PLGA microspheres to protect the stability of nerve growth factor. Co-encapsulated PEG protected nerve growth factor from denaturation by contact with the organic phase during the primary emulsification step.

Activity of ETN released from MPEG-PCL-MPEG and PCL microspheres was retained completely in PBS during 20 days (Table 3). In our previous study, IgG-loaded PCL and MPEG-PCL-MPEG microspheres were prepared with same method used for ETN-loaded microspheres, and only 33 ± 1% and 52 ± 1% of the activity of the IgG released from γ-irradiated PCL and MPEG-PCL-MPEG microspheres were protected for same time period, respectively.[35,36] For ETN-loaded microspheres, ETN drug formulation containing additives was used in addition to PVA in the inner phase during preparation. As described in Enbrel®, ETN US Prescribing Information of Wyeth also contains some additives such as mannitol, sucrose, and trometamol to protect the bioactivity of ETN. Therefore, activity of ETN released was retained completely for both formulations compared to the activity of IgG released from both microsphere groups during 20 days. In addition to additives in drug formulation, MPEG segment at polymer matrix improved the stability of ETN to denaturation by contact with the organic phase during the primary emulsification step as mentioned before.

As observed in PBS release, ETN released from PCL microspheres showed a slower release profile compared to that from MPEG-PCL-MPEG microspheres in cell culture medium and in healthy and RA synovial fluids. Here, presence of hydrophilic MPEG segment caused more release of ETN from MPEG-PCL-MPEG microspheres owing to enhanced diffusion of water into microspheres. Besides that, MPEG segments might have resulted in more ETN release from these microspheres by decreasing the adsorption of serum proteins in cell culture medium and synovial fluids. In a previous study, the effect of various additives (i.e. PEGs, bovine serum albumin (BSA) and poloxamer 188) on lysozyme release from PLGA microspheres in TRIS-HCL buffer medium containing 0.1% w/v BSA was studied.[47] They showed that BSA in release medium tended to be adsorbed onto PLGA microspheres when there is no additive and adsorption of BSA limited the release of lysozyme.

For both microsphere groups, percent cumulative release in cell culture medium was significantly higher than those observed in healthy and RA synovial fluids. However, percent cumulative releases in cell culture medium and synovial fluids were significantly lower than that observed in PBS. In the study of Verma and Maitra,[56] the release profiles of 5-fluorouracil-hexyl-carbamoyl fluorouracil (HCFU) loaded nanospheres of copolymers N-isopropylacrylamide and vinyl pyrrolidone were studied in PBS and diluted serum media. They also observed a slower release in serum due to the absorption of complement proteins present in serum. The percent cumulative release of ETN from microspheres in PBS and cell culture medium was significantly higher than that of in synovial fluids because of higher viscosity of synovial fluids compared to PBS and cell culture medium. This lower protein release from microspheres in synovial fluids can be attributed to adsorption of proteins on the surface of microspheres or to viscosity difference of the release environments, which affects diffusion process.

In vitro release studies in PBS and cell culture medium showed that ETN-loaded PCL and MPEG-PCL-MPEG microspheres can provide a sustained-prolonged release of active ETN for at least 90 days after intra-articular administration. Therefore, once weekly or twice weekly administration period of ETN in conventional treatment can be extended to longer periods with this microcarrier approach.

In vitro release data of microspheres in different release media were fitted in Higuchi and Korsmeyer–Peppas models. According to the release data fitted in Higuchi model, the cumulative amounts of released ETN from microspheres were diffusion controlled. This observation was also confirmed by fitting the release data to Korsmeyer–Peppas model. Korsmeyer–Peppas model is limited to the first 60% of the cumulative amount of drug release and the release exponent (n) is used to characterize release mechanism. If n is 0.43, it means that release mechanism follows pure Fickian diffusion, and higher values for mass transfer ($0.43 < n < 0.85$) shows that release follows a non-Fickian model which is denominated as anomalous transport.[39] In PBS and cell culture medium, release from all microsphere groups was governed by an anomalous transport ($n > 0.43$). This revealed that the water transport mechanism was mainly due to the combination of both diffusion through and surface erosion from PCL and MPEG-PCL-MPEG microspheres incubated in PBS and cell culture medium during 90 and 60 days, respectively. In agreement with these results, significant defects were observed at the surface of γ-irradiated model protein (IgG) loaded MPEG-PCL-MPEG microspheres at the end of 6 months PBS incubation in our previous study.[35,36] However, for in vitro release studies in healthy and RA synovial fluids, release from all microsphere groups was mainly diffusion controlled. Protein release from biodegradable polymeric microspheres can be controlled by two main mechanisms: diffusion of the protein out of microsphere and erosion of the polymer matrix surface. However, polymer hydrolysis and polymeric matrix erosion of PCL and MPEG-PCL-MEG are very slow. In our previous study, decrease in molecular weight (Mw) of γ-irradiated model protein (IgG) loaded PCL and MPEG-PCL-MPEG microspheres were found to be small at the end of 6-month PBS incubation.[35,36] Therefore, diffusion can be the more dominant factor affecting ETN release kinetics for the early stages of in vitro release as observed in 14-day synovial fluid release study. On the other hand, release of ETN from microspheres was controlled by diffusion and polymer erosion during the later stages as seen in the 60-day cell culture medium and 90-day PBS release studies. Rate constants for ETN release from PCL microspheres were found lower than that for ETN release from MPEG-PCL-MPEG microspheres (Table 4). This correlated well with the in vitro release profile of ETN from microspheres, in which ETN release from PCL microspheres showed a slower release profile compared to that from MPEG-PCL-MPEG microspheres.

The free drugs dissolved in the synovial fluid are rapidly eliminated from the joint ($t_{1/2}$ of about 0.1–6 h). Hence, maintenance of therapeutic concentration for an extended period of time necessitates the administration of the drug in the form of repeated intra-articular injections or an injectable depot formulation.[43] Microspheres are sustained drug release systems which offer an excellent alternative to multiple intra-articular injections. The free drug may also endanger the viability of local cells. In fact, in our study, FLS viability decreased in the free drug group at first week compared to microsphere groups. Therefore, microspheres also provided advantage in preserving viability by conservation of safe environment in the joint milieu. In addition, ETN-loaded microspheres have efficiently decreased the protein levels of pro-inflammatory cytokines and MMPs. These results show that MPEG-PCL-MPEG microspheres might be better candidates for sustained and efficient drug release.

A significant decrease in IL-17 and IFNγ levels of RA FLS in microsphere groups was not observed after 2 weeks whereas significant decreases were observed in TNFα and IL-6 levels of RA FLS in microsphere groups compared to those of RA FLS in free ETN group. Similarly, MMP-3 and MMP-13 levels of RA FLS were significantly lower in the microsphere groups after 3 and 2 weeks, respectively. Even though the mechanism of RA is not still fully understood, TNFα is the major cytokine responsible for the inflammatory cascade. Clinical therapy using ETN involves subcutaneous injections of 25 mg (twice a week) or 50 mg ETN (once a week) and this subcutaneously injected drug is released slowly from the site. It has been reported that the peak plasma concentrations are achieved 48 to 60 h after injections. It has been shown that serum and synovial levels of ETN were similar after 5 weeks of treatment (approx. 2.5 mg/mL) due to ease of penetration of the drug into joints. The therapeutic effect can only be observed 2 or more weeks after injections, therefore the neutralization effect of ETN is not also expected to be observed immediately in vitro in terms of decrease in the levels of pro-inflammatory cytokines and MMPs. TNFα is the key cytokine that induces the production of other pro-inflammatory cytokines with its mediator role in the positive and negative feedbacks in the intricate network of signaling pathway. With TNFα antagonist blockage of TNF receptor (TNFR) mediated signaling pathway, the production of these cytokines is lowered with time. It has been reported that when ETN concentration is low, it neutralizes TNF more effectively.[57] In addition, results of high dose intra-articular injections imply that inflammation suppression is not as successful as subcutaneous injections in which maintaining a low dose of approximately 2.5 µg/mL for long period achieves the required suppression. From these it can be suggested that sustained release from microspheres resulted gradual suppression and this prolonged suppression with steady lower doses instead of high dose triggered a decrease in other proinflammatory cytokines and MMPs after 2 weeks.

In patients with RA, the MMPs play a major role in the destruction of cartilage and other components of connective tissue in the joints. TNFα can induce the synthesis and secretion of MMPs, which in turn affects chemokine and cytokine action. A positive feedback loop exists between cytokines and metalloproteinase activities, creating a vicious cycle of destruction.[58] In parallel with the above outcomes, MMP-13 and MMP-3 levels in the microsphere groups were found significantly lower than the free drug group (Figure 9) at the end of 1st and 2nd weeks, respectively. Sustained release from microspheres provides prolonged suppression at MMP-13 levels in these microsphere groups and the levels of MMP-13 remained at same level for all groups. However, a significant increase in the MMP-13 level in free drug was observed at weeks 2 and 4, which can be explained with the ETN concentration decrease due to medium replacement at defined periods (Figure 9(b)). For free ETN and each microsphere group, there were no significant differences between their MMP-3 levels at weeks 3 and 4. However, the level of MMP-13 level at week 4 was significantly higher than that of observed at week 3 in free drug group whereas no significant differences were observed for each microsphere groups. Overall, results demonstrated that sustained ETN release from microspheres significantly decreased the levels of pro-inflammatory cytokines and MMPs.

Conclusion

In this study, ETN-loaded PCL and MPEG-PCL-MPEG microspheres as a novel potent long-term controlled ETN delivery system have been developed for the first time in literature. The microspheres have been successfully formulated with spherical morphology with a suitable size for intra-articular applications. Introducing MPEG segments into PCL chains to obtain triblock copolymer significantly improved the encapsulation efficiency of microspheres as well as ETN loading. It was also demonstrated that PCL and MPEG-PCL-MPEG microspheres were able to release active ETN for a prolonged period time, which will be superior to the conventional treatment applied at frequent intervals. MPEG-PCL-MPEG microspheres showed increased release rate of ETN and enhanced the stability of ETN within the microspheres compared to their PCL counterpart. Additionally, these microspheres have the advantage of lower serum protein adsorption due presence of outer MPEG segments. ETN in microspheres decreased effectively the pro-inflammatory cytokines and MMPs for a longer period of time, in a sustained manner, conserving the FLS viability. These results show that both PCL and MPEG-PCL-MPEG microspheres may be considered as a promising intra-articular ETN delivery system for treatment of RA, especially for cases of inflammation in few joints. On the other hand, enhanced hydrophilicity of MPEG-PCL-MPEG microspheres can be considered more advantageous compared to PCL microspheres. However, further in vivo studies are necessary to investigate the treatment efficacy of ETN-loaded PCL and MPEG-PCL-MPEG microspheres.

Funding

This work was financed and supported by The Scientific and Technological Research Council of Turkey (Project no: SBAG109S104).

Acknowledgements

WEHI 164 clone-13 murine fibrosarcoma cell line was a kind gift of Prof. Dr Atilla Işık from Department of Virology of Faculty of Veterinary Medicine of Selcuk University.

Declaration of conflicting interests

The authors of this manuscript have a patent pending (PCT/TR2012/000148) for the manufacture and use of PCL and MPEG-PCL-MPEG microspheres for intra-articular delivery of anti-TNFα drugs used in the treatment of rheumatic diseases.

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