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Comparison of *in-situ* forming composite using PLGA-PEG-PLGA with *in-situ* forming implant using PLGA: *In-vitro*, *ex-vivo*, and *in-vivo* evaluation of naltrexone release

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ABSTRACT

An *in-situ* forming composite (ISFC) of naltrexone (NTX) was prepared using PLGA-PEG-PLGA (triblock) and N-methyl-2-pyrrolidone (NMP) for decreasing the initial burst release. The supercritical CO_2 method was used to achieve the ring-opening polymerization of the triblock. The Box-Behnken design was used to achieve the minimal initial burst release of NTX in the *in-vitro* release medium. *In-vitro*, *ex-vivo*, and *in-vivo* studies of the ISFC were compared with *an in-situ* forming implant (ISFI) composed of copolymer PLGA 504H. The equivalency of ISFC and Vivitrol® were compared by sampling the concentration of NTX in rabbit blood. The results of the *in-vitro* release evaluation showed that the initial burst release for the ISFC (5.69 ± 0.27%) was significantly lower than that for the ISFI (17.45 ± 1.07%). The C_{max} of NTX (15.16 ± 2.46 ng/mL) from the ISFC was significantly ($p < 0.05$) lower than that of the ISFI (24.46 ± 2.9 ng/mL) and Vivitrol® (21.11 ± 2.89 ng/mL). The bioavailability and the range of serum concentration of NTX for the ISFC formulation was similar to that of Vivitrol®. The results suggested that the ISFC could be used as a new type of sustained-release formulation with a smaller initial burst release than the ISFI.

1. Introduction

An *in-situ* forming implant (ISFI) can provide a controlled release of a drug while offering greater ease of administration than surgical implants. ISFIs include a water-insoluble biodegradable polymer, poly (DL-lactide-co-glycolide) (PLGA), dissolved in a water-miscible and physiologically compatible organic solvent, N-methyl-2-pyrrolidone (NMP). ISFIs are injected as a solution, and subsequent precipitation of the polymer creates a solid depot containing the drug (phase inversion mechanism). The NMP solvent, which is FDA approved, is used in manufacturing medical products such as Eligard® (leuprorelin acetate using PLGA), RBP-6000® (buprenorphine using PLGA), and Atridox® (doxycycline hyclate using PLA) [1,2]. However, ISFIs have a considerable tendency to burst (15–80% of the total drug), especially in the first 24 h [3]. The diffusion of NMP into the water upon injection causes high initial burst release rates because the drug emerges with the NMP. The initial burst release of some drugs causes tissue inflammation and systemic toxicity [4].

In our previous study, to control the initial burst release of naltrexone (NTX) from an ISFI, we used a combination of poly (lactic-co-glycolic acid) 75:25 copolymer (RG 756s) and 50:50 copolymer (RG 504H) along with additives. The initial burst release of the optimized formulation in the *in-vitro* release over the first 24 h, 6.18 ± 0.91%, was significantly ($p < 0.05$) lower than that of the formulation containing 100% of PLGA 504H (17.45 ± 1.07%) and 100% of PLGA 756s (11.82 ± 1.03%) [5]. In the current study, the use of PLGA-PEG-PLGA instead of PLGA was suggested to decrease the initial burst release. It seemed that the hydrogen bonding between the NMP molecules and PEG prevented the rapid diffusion of NMP into the release medium. Furthermore, the thermosensitive properties of PLGA-PEG-PLGA

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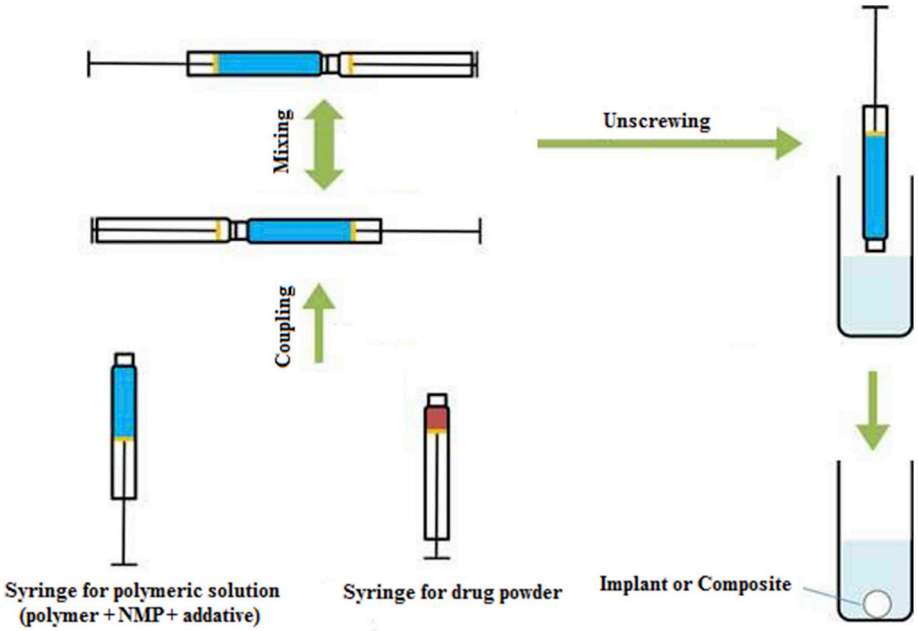

Fig. 1. Preparation of ISFI or ISFC using two coupled syringes.

triblock could be helpful in composite formation (*in-situ* forming composite (ISFC)). The matrix structure of the composite slowed the NMP escape rate, resulting in a slower initial burst release. This triblock is fluid below room temperature, but in the body (37 °C), it quickly converts to a highly viscous composite. As a result, the placement of injectable formulations based on triblock does not require surgery [6]. Since the triblock is degraded in its aqueous solution within a maximum of one month, the formulation should be prepared using three separate vials containing the triblock, water for injection (WFI), and the drug [7]. Vials should be combined only at the time of injection. However, the combination of triblock with an aqueous solution of the drug in clinical practice is impossible because the triblock dissolves slowly within 24 h in an aqueous solution using a stirrer at 4 °C. To solve these problems, in this study, NMP was used instead of water to dissolve the triblock. Formulations based on ISFIs were prepared using two coupled syringes of Eligard® injection purchased from a local pharmacy (Fig. 1). Briefly, the polymer was dissolved in NMP in a glass vial to prepare a polymeric solution. Two 1.2 mL syringes were then filled with this solution and the drug in powder form. The syringes were then coupled to each other and mixed for 60 cycles. After injection, the composite formed at the injection site through the mechanisms of phase inversion and the thermosensitive properties of the triblock.

In this investigation, supercritical carbon dioxide ($SCCO_2$) was used for the ring-opening polymerization (ROP) of PLGA-PEG-PLGA triblock. To achieve the minimum initial burst release of NTX (optimum formulation), the variables such as the lactide:glycolide (LA:GA) molar ratio of the triblock, % PEG of the triblock, % of the triblock in the formulation, and % of additive (ethyl heptanoate) in the formulation were evaluated using a Box-Behnken design (BBD). The *in-vitro*, *ex-vivo*, and *in-vivo* release and compatibility of the optimum formulation of the triblock were investigated. The surface and cross-section morphologies of the composite or implant were visualized using scanning electron microscopy (SEM). The optimum formulation was subcutaneously injected into rabbits to determine the pharmacokinetic profiles of the NTX and its main metabolite, 6β-naltrexol. Furthermore, the results of the *in-vitro, ex-vivo*, and *in-vivo* evaluations of the ISFC were compared with the ISFI prepared from PLGA 504H. To ensure their equivalency between ISFC and Vivitrol® (commercially produced naltrexone microspheres currently on the market), the rabbit-blood concentrations were compared.

2. Materials and methods

2.1. Materials

Glycolide, D,L-Lactide, polyethylene glycol (PEG 1500), and stannous octoate (Sn $(Oct)_2$) were obtained from Sigma-Aldrich for the ROP. Industrial carbon dioxide (≥ 99%) as the supercritical fluid was obtained from Zamzam Co. (Iran). PLGA RG 504H for the ISFI was obtained from Sigma-Aldrich (acid-terminated, lactide:glycolide 50:50, M_w 38,000–54,000). Vivitrol® was obtained from Alkermes (USA). NTX-HCl was purchased from Alhavi Pharmaceutical Co. (Iran) Naltrexone and 6β-naltrexol solution, as the standard materials, were purchased from Sigma-Aldrich for high-performance liquid chromatography (HPLC) analysis. N-methyl-2-pyrrolidone (NMP), 3-(4,5-dimethylthiazol-2-yl)-2, diphenyltetrazolium bromide (MTT), and other chemical reagents were purchased from Merck. Mouse fibroblast L929 cell lines were donated from the Pharmaceutical Technology Institute of Mashhad University of Medical Science (Iran). Roswell Park Memorial Institute (RPMI) 1640 culture medium, fetal bovine sera (FBS), trypsin, and penicillin-streptomycin were obtained from the Gibco (Germany).

2.2. Ring-opening polymerization of PLGA-PEG-PLGA using $SCCO_2$

The PLGA-PEG-PLGA copolymer was synthesized under supercritical carbon dioxide as previously reported [8]. The ROP scheme and supercritical apparatus are shown in Fig. 2A and B [9]. The prepared mixture of PEG, Sn $(Oct)_2$ (10 μL), LA, and GA were loaded into the 10 mL cylindrical stainless-steel cell. Then the cell was placed in the $SCCO_2$ apparatus. To reach the optimum temperature (144 °C) and pressure (367 bars), the CO_2 gas was first liquefied using a cooler circulator (adjusted below −12 °C) and charged by an HPLC pump (Azura P 4.1S, APG20EA, Knauer). The CO_2 was heated using a coil preheater placed in an oven before it was allowed to enter the column. After the reaction was complete (13 h), the triblock copolymer was purified by dissolving it in water (5–8 °C) and precipitating at 60 °C. The supernatant, water-soluble, low-molecular-weight polymer and the unreacted monomer were discarded, and the precipitate was collected. The process of heating, precipitation, and discarding were repeated once more to obtain the purified polymer. Residual water was removed

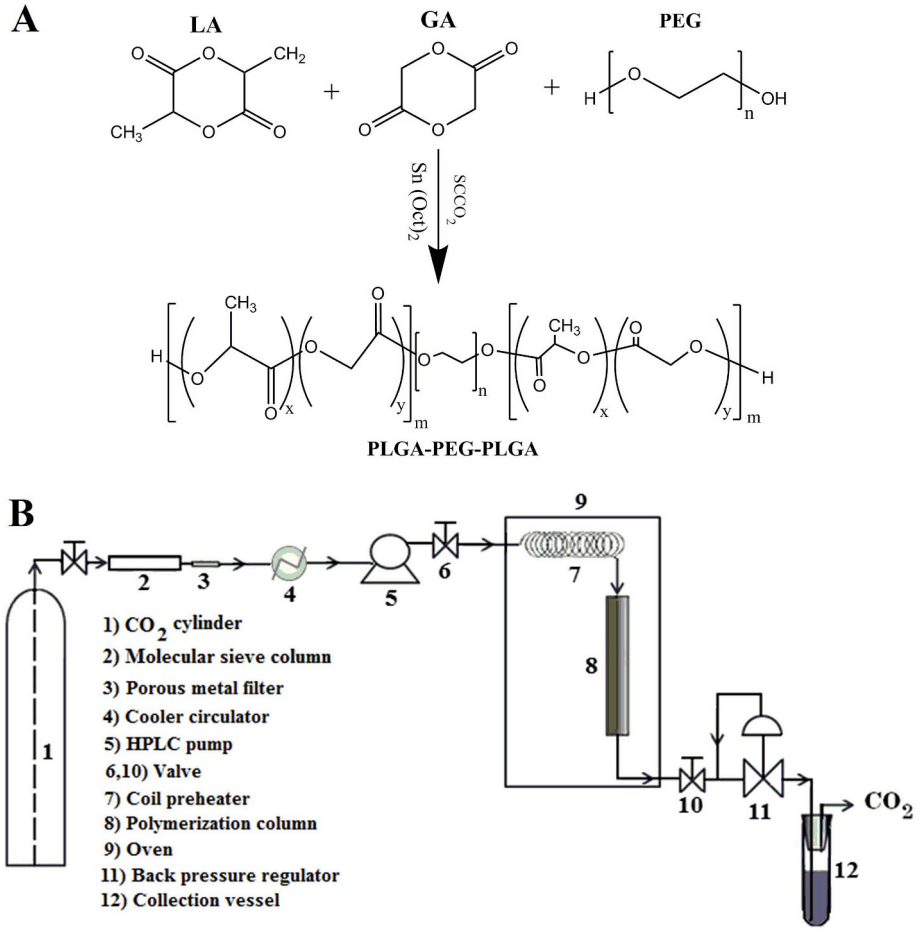

Fig. 2. (A) $Sn(Oct)_2$-Catalyzed ROP of PLGA-PEG-PLGA in $SCCO_2$. (B) Schematic diagram of $SCCO_2$ polymerization/purification system.

by freeze-drying (T = −20 °C, P = 103.2446 Pa) to make the triblock copolymer (PLGA-PEG-PLGA) as a viscous semisolid. Because freeze-drying is a change in state from the solid phase to the gaseous phase, material to be freeze-dried must first be adequately prefrozen (−20 °C). The product was stored at −20 °C until use.

The ^1H NMR (Bruker Avance 300 MHz NMR spectrometer, Germany) spectra of the PLGA-PEG-PLGA were measured in $CDCl_3$ at room temperature to confirm the structure and the LA:GA molar ratio [10,11]. The number average molecular weight (M_n), weight average molecular weight (M_w), and polydispersity index (PDI, M_w/M_n) of the synthesized triblock were determined using an Agilent GPC-Addon apparatus with Plgel® columns. Polystyrene standards as a calibration and tetrahydrofuran as an eluent with a flow rate of 1 mL/min were used [12]. The sol-gel transition temperature of PLGA-PEG-PLGA was determined using a refrigerated bath circulator instrument (WISD P-22, South Korea). The temperature was increased from 0 °C to 60 °C at a rate of 0.5 °C/min. Triblock concentrations of 10–50 (w/w %) in phosphate buffer saline (PBS) (pH 7.4) were prepared. The temperature at which the stirrer magnet was stopped inside the copolymer solution was considered as the sol-gel transition temperature [7].

2.3. Experimental design for in-vitro release evaluation

A response surface method (RSM) based on a Box-Behnken design (BBD) was modeled using the Design-Expert software (version 10.0.4) [13,14]. The % of initial burst release of NTX (Y) was measured for the LA:GA molar ratio (x_1) and the % PEG of the triblock (x_2), the % of triblock in the formulation (x_3), and the % of additive (ethyl heptanoate) (x_4) in the formulation. The quadratic model was predicted based on experimental data (Eq. (1)). The range of independent parameters is listed in Table 1.

$$Y = \beta_0 + \Sigma\, \beta_j X_i + \Sigma\, \beta_{jj} X_j^2 + \Sigma\, \beta_{jk} X_j X_k \quad (1)$$

where Y = % of the initial burst release, β_0 = the intercept, β_j = the linear coefficients, β_{jj} = the squared coefficients, β_{jk} = the interaction coefficients, and X_i, X_j^2, X_j. X_k = the levels of the respective independent parameters.

2.4. Preparation of the formulations

To prepare the formulations containing the triblock, the mixtures of the triblock, NMP, and ethyl heptanoate, according to Table 2, were incubated at room temperature for 24 h. Furthermore, the formulation containing PLGA 504H (ISFI) was prepared by mixing 33% PLGA 504H, 5% ethyl heptanoate, and 62% NMP (% w/w) [15]. After preparation, the formulations were sterilized using thermal sterilization via

Table 1
Range of four independent variables in the *in-vitro* release using the Box-Behnken method.

Independent variables	Levels		
	−1	0	+1
LA:GA molar ratio	3:1	5:1	7:1
% PEG (w/w %)	20	30	40
% triblock in formulation[a] (w/w %)	30	40	50
% ethyl heptanoate in formulation (w/w %)	1	4	7

[a] Formulation (Triblock + NMP + Ethyl heptanoate).

Table 2
Responses of dependent variables using three levels-four factors via BB method to independent variables (X_1, X_2, X_3, and X_4) of *in-vitro* initial burst release.

Run	X_1 LA:GA molar ratio	X_2% PEG (w/w %)	X_3% triblock in formulation (w/w %)	X_4% ethyl heptanoate in formulation (w/w %)	initial burst release of naltrexone %
1	5:1	20	30	4	11.55 ± 1.13
2	5:1	40	40	1	12.49 ± 1.22
3	5:1	30	40	4	6.69 ± 0.65
4	7:1	30	40	7	6.44 ± 0.63
5	3:1	30	30	4	10.63 ± 1.04
6	3:1	30	50	4	8.15 ± 0.79
7	5:1	30	50	7	6.70 ± 0.65
8	5:1	40	50	4	9.65 ± 0.94
9	3:1	20	40	4	10.63 ± 1.04
10	7:1	40	40	4	9.36 ± 0.92
11	7:1	30	30	4	8.79 ± 0.86
12	5:1	30	40	4	6.73 ± 0.71
13	5:1	40	30	4	12.13 ± 1.27
14	7:1	30	40	1	6.74 ± 0.71
15	3:1	40	40	4	11.21 ± 1.18
16	3:1	30	40	7	8.28 ± 0.87
17	5:1	30	40	4	6.73 ± 0.71
18	5:1	40	40	7	9.79 ± 1.03
19	5:1	30	40	4	6.75 ± 0.65
20	5:1	30	50	1	7.02 ± 0.74
21	5:1	30	30	1	9.50 ± 1.00
22	3:1	30	40	1	8.58 ± 0.94
23	5:1	20	40	1	9.51 ± 0.89
24	5:1	30	30	7	9.23 ± 1.02
25	5:1	30	40	4	6.72 ± 0.57
26	5:1	20	40	7	9.20 ± 0.69
27	7:1	20	40	4	8.79 ± 0.97
28	7:1	30	50	4	6.31 ± 0.49
29	5:1	20	50	4	9.07 ± 1.18

saturated steam at 121 °C and 3 bars for 15 min [16]. After sterilization, 100 mg of NTX was added and sonicated under the sterile conditions. To ensure that this sterilization method did not affect the copolymers' stability, the *in-vitro* release evaluations were checked before and after sterilization.

2.5. In-vitro release evaluation

One mL of the formulation was passed through a 20-gauge needle and added into the 13 mL of release medium (PBS, pH 7.4, 37 °C, and 35 rpm). The composite or implant was formed as soon as the formulation was injected. To maintain a sink condition, 3 mL of the release environment was replaced with fresh PBS at specified times (2, 6, 8, 10, 12, 18, and 24 h, then 2, 3, 4, 5, 7, 10, 14, 16, 18, 21, 28, and 35 days). The concentrations of NTX from the samples were analyzed using HPLC compared with the calibration curve (0.0067–100 μg/mL). The HPLC apparatus was a diode array detector (SPDm) with λ 205 nm connected to an LC-6AD pump; HPLC column (C18, Brisa LC2, 4.6 × 250 mm, 5 μm); mobile phase of an isocratic mixture of 80% (v/v) HPLC-grade water (pH = 3.2 via phosphoric acid) and 20% (v/v) methanol at ambient temperature; flow rate 1.2 mL/min; and injection volume 20 μL [5,17].

The concentration of the released NMP was analyzed at specified time intervals. The NMP concentrations were calculated based on the calibration curves of various concentrations (4–22 μg/mL). The HPLC conditions were λ 220; column (C18, Brisa LC2, 4.6 × 250 mm, 5 μm); mobile phase of an isocratic mixture of 68% (v/v) trifluoroacetic acid (0.1% v/v) and 32% (v/v) acetonitrile at ambient temperature; flow rate 0.5 mL/min; and injection volume 20 μL [15,18]. The experiments were conducted independently in triplicate. (*Note:* NTX and NMP analyses were done differently using two different HPLC conditions).

The direct *in-vitro* degradation evaluation (a decrease in the molecular weight of the copolymer) of the optimum formulation of the triblock and PLGA 504H without NTX in the release medium at time intervals (1, 2, 3, 5, 7, 14, 21, 28, and 35 days) at 37 °C were investigated based on the variations in molecular weight. To maintain the pH of the degradation medium at 7.4, the PBS was replaced every 2 days. The samples (composites or implants) were freeze-dried for 48 h and stored at −20 °C until analysis using GPC, as described in section 2.2 [19]. The experiments were done in triplicate.

The surface and cross-section morphologies of the composite or implant were visualized after 3 days using SEM on a Tescan, model Vega II, Czech Republic. The freeze-dried samples were placed on the specimen holder using double-sided tape and coated with gold to render them electrically conductive [20].

2.6. Ex-vivo release evaluation

To evaluate the *ex-vivo* release and visually observe the formed composite or implant, the formulations were subcutaneously injected into a hen drummet using a 20-gauge needle. After injection, the hen drummet was immersed into a beaker containing PBS (pH 7.4) with sodium azide (0.1% w/v) at 37 °C. To confirm the composite formation using an ISFC formulation or the implant using an ISFI formulation, the drummet injection site was incised after 24 h. To maintain a sink condition, three mL of the release environment were replaced with fresh PBS at specified time (2, 6, 8, 10, 12, 18, 24, 48, and 72 h). The concentrations of NTX from the samples were analyzed using HPLC compared with the calibration curve (0.0067–100 μg/mL), as described in section 2.5 [5,21].

2.7. In-vivo release evaluation

Male New Zealand rabbits (2 ± 0.1 kg) housed individually in stainless-steel cages were supplied with food and water at ambient temperature (15–22 ± 1 °C). They were treated in accordance with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978). The animal studies were approved by the Institutional Animal Care and Use Committee of Mashhad University of Medical Sciences (No. 930788).

The animals were divided into 7 groups (n = 5 per group): Group I: Control; Group II: NMP (670 mg, subcutaneous injection); Group III: NTX solution in NMP (subcutaneous injection); Group IV: ISFI (subcutaneous injection); Group V: ISFC (subcutaneous injection); Group VI: Vivitrol® (intramuscular injection); and Group VII, which received 111 mg of naltrexone hydrochloride (equivalent to 100 mg NTX free base) in 1.1 mL of WFI solvent administered intravenously. (Note: all formulations contained 100 mg of NTX, and the subcutaneous groups were injected using a 20-gauge needle). NTX is extensively metabolized to 6β-naltrexol, 2-hydroxy-3-methoxy-6β-naltrexol, and 2-hydroxy-3-methoxynaltrexone. Blood samples of 1 mL were collected from the heart using a 2 mL syringe to analysis the NTX and 6β-naltrexol in the serum for pharmacokinetic evaluation at specified times (2, 4, 8, 10, 12, 18, and 24 h, then 2, 3, 4, 7, 8, 10, 14, 21, and 28 days). The blood serum was separated using a centrifuge (5000 rpm, 15 min) and stored frozen (−80 °C) until analyzed by HPLC [5].

NTX and 6β-naltrexol were extracted from the serum by adding 60 μL of perchloric acid (1.0 M) into 400 μL of serum in a 4 mL microtube. To liberate free NTX and 6β-naltrexol from the conjugates, the microtube was incubated at 37 °C for 24 h [22]. Extraction was accomplished by mixing (6000 rpm, 15 min) ethyl acetate and cyclohexane (9:1) (organic layer). The organic layer was transferred to a microtube and eliminated using a small flow of nitrogen. One mL of methanol was used to dissolve the residues of the organic layer. Finally, the NTX and 6β-naltrexol were detected using HPLC and compared with the calibration curve (0.0067–100 μg/mL), as described in section 2.5 [17,23].

The pharmacokinetics parameters, such as the C_{max} (maximum

serum NTX and 6β-naltrexol concentration after injection), T_{max} (time to reach maximum serum concentration), and AUC $_{0\text{-}t}$ (area under the serum NTX and 6β-naltrexol concentration vs. time curve in the finite time), were computed by PKSolver software [24]. The absolute bioavailability (F) of NTX and 6β-naltrexol from the formulations and subcutaneous solutions in comparison to the reference (intravenous injection) was calculated by dividing their AUC $_{0\text{-}t}$ by that of IV solution [5,25].

2.8. In-vitro compatibility evaluation

A culture medium containing the RPMI 1640 medium (100 mg/ml of streptomycin, 100 IU/ml of penicillin, 10% (v/v) heat-inactivated FBS) was used to grow the mouse L929 fibroblast cell lines in a humidified incubator in a 5% CO_2 atmosphere at 37 °C for 1 week. Twelve-well plates at a density of 5×10^4 cells/well were applied to seed the cells. Sterilized formulations (10 µL) and their components (PLGA 504H, triblock, $Sn(Oct)_2$, NMP, and ethyl heptanoate) were placed directly in the center of each well, and the plate was incubated for 24 h. The negative control was a wall containing cells and growth medium with no sample. Then, the culture medium was discarded, and 500 µL of MTT solution (0.5 mg/mL in PBS) was added to each well to determine the cell viability %. The culture medium was replaced with 100 µL of DMSO, and the plate was shaken slowly for 1 h. Finally, the microplate reader (Tecan Group Ltd., Switzerland) was applied to measure the absorbance at 570 nm (sample) and 630 nm (reference wavelength) [18]. The reported values are the means of five trials.

2.9. In-vivo compatibility evaluation

The rabbits were sacrificed by suffocation with CO_2 after the end of the *in-vivo* release study. The injection sites on the rabbits were shaved and surgically removed for histopathology study. They were excised and immersed in formalin 10% to fix them. Then, the samples were added to solutions of alcohol with increasing strength to dehydrate them. Toluene was added to clarify the solution. The samples were transferred to a vessel of molten paraffin. The samples were removed from the vessel and, after cooling to ambient temperature, 10 µm-thick sections were cut using a rotary microtome. Sections were mounted on glass slides using glycerogelatin, stained with chrysoidine and hematoxylin, and counterstained with eosin. Finally, they were dehydrated, cleared, and cover-slipped. To investigate the presence of signs of chronic inflammation, granulated tissue, foreign-body giant cells, and fibrous capsule formation, the histopathological changes of the samples were observed using a light microscope [25,26].

2.10. Statistical analysis

The data were assessed by one-way analysis of variance (ANOVA) followed by the Tukey–Kramer post-test. Data are expressed as mean ± SD (standard deviation). P-values of less than 0.05 were considered statistically significant.

3. Results and discussion

3.1. Synthesis and characterization of the PLGA-PEG-PLGA triblock copolymers

Ring-opening polymerization (ROP) of the triblock was successfully accomplished using the $SCCO_2$ method. During the polymerization process, the viscosity decreased due to the $SCCO_2$ condition overcoming the influence of the increased viscosity as polymerization progressed. Subsequently, the molecular weight of the copolymer increased more than has been found with the conventional method of synthesis [13,27,28]. A typical ^{1}H NMR spectrum of the PLGA-PEG-PLGA copolymer using $SCCO_2$ is shown in Fig. 3A. The characteristic signals appearing at 1.5, 3.63, 4.30, 4.76, and 5.15 are from the CH_3 of LA, the CH_2 of PEG, a terminal CH_2 of PEG, the CH_2 of GA, and the CH of LA, respectively. Fig. 3B shows a typical GPC chromatogram of triblock, which is a nearly symmetric peak. The low polydispersity and unimodal GPC trace of the triblock copolymers shows that the synthesis and purification methods were successful.

The phase-transition temperature of the triblock copolymers as a function of temperature and concentration is shown in Fig. 4. A decrease in the phase-transition temperature from sol to gel and an increase in the precipitation temperature occurred when the copolymer concentration was increased from 10 to 50% (w/v) and the molar ratio of LA:GA from 3:1 to 7:1. When the concentration and molar ratio of LA:GA were increased (Fig. 4A), the enhancement of micelle concentrations due to the accelerated aggregation of the micelles was observed, and the sol-gel transition happened at a lower temperature. With an increase in the % of PEG from 20 to 40% (Fig. 4B), the sol-gel transition temperature increased, and the precipitation temperature decreased. Since the H- and OH- of PEG can hydrogen bond with water, the amount of hydrogen bonding also increased when the % of PEG increased. As a result, the formation of hydrophobic bonds between the PLGA segments and the breaking of those bonds occurred at higher temperatures.

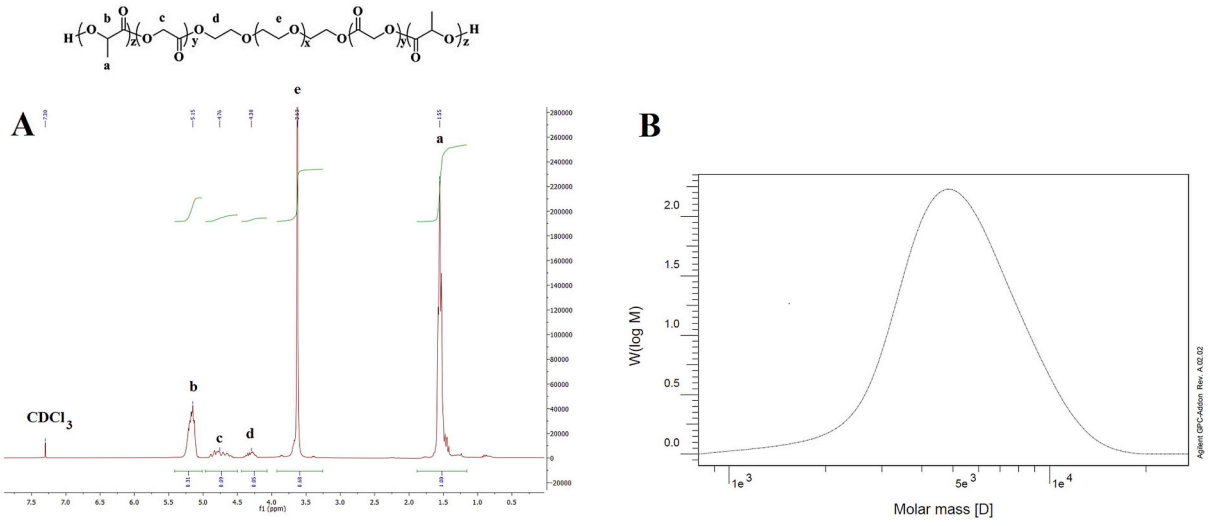

Fig. 3. ^{1}H NMR spectrum (A) and GPC chromatogram (B) of PLGA-PEG-PLGA using $SCCO_2$.

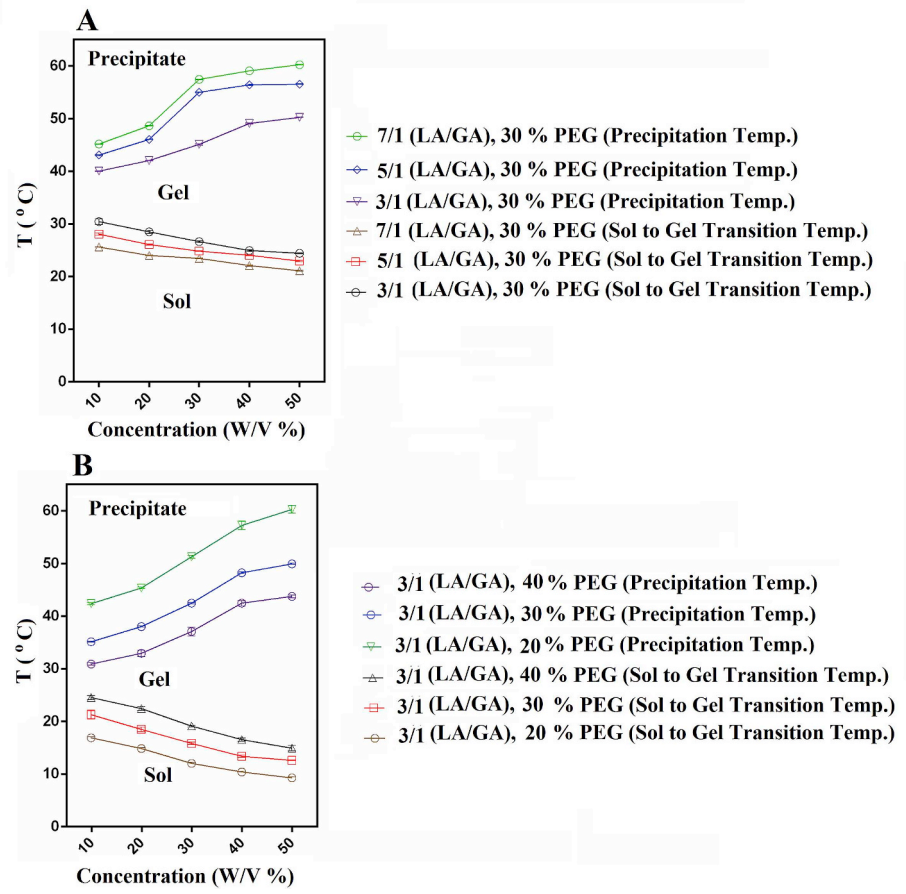

Fig. 4. Phase diagram of PLGA-PEG-PLGA; (A) Effect of LA to GA molar Ratio, (B) Effect of % PEG. (Mean ± SD, n = 3).

3.2. *In-vitro release evaluation*

The solubility of NTX-HCl and NTX in the NMP was 23 ± 2.09 mg/mL and 100 ± 3.14 mg/mL, respectively. Hence, to increase drug loading in the formulation, NTX-HCl was converted to NTX. The chromatogram of pure NTX and NMP in the *in-vitro* release medium (PBS) is shown in Fig. 5A and B, respectively. The HPLC chromatogram of NTX and NMP for the ISFC formulation into the *in-vitro* release medium after 24 h are shown in Fig. 5C and D, respectively. The retention times of NTX and NMP using HPLC analysis were observed at

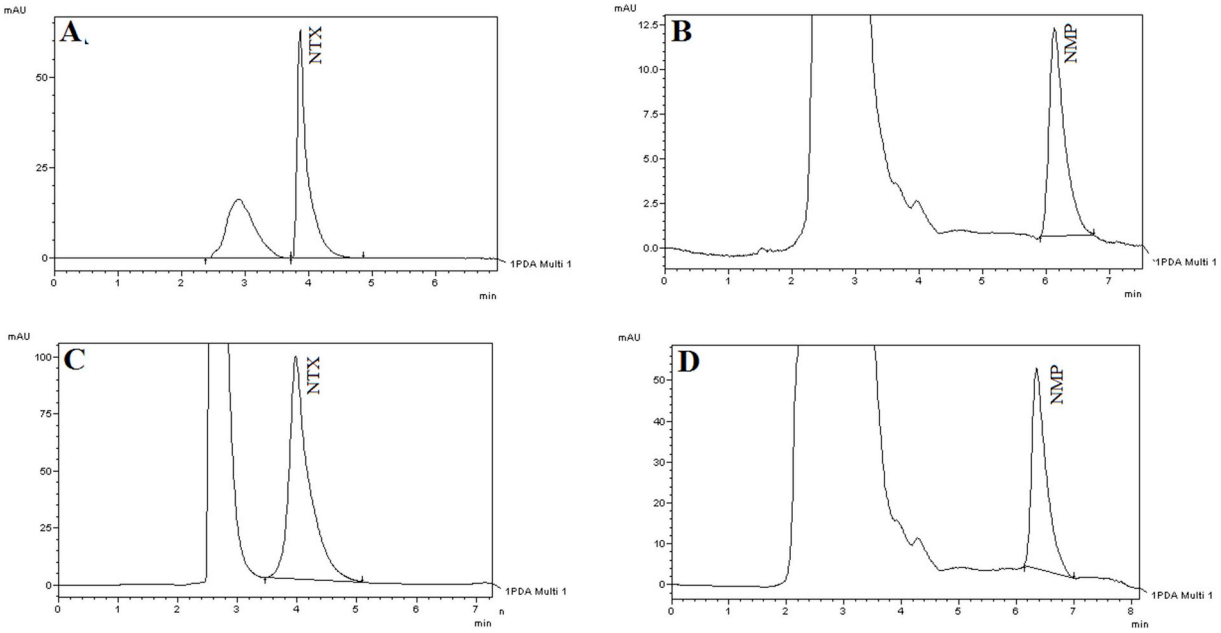

Fig. 5. Chromatogram of HPLC analysis for pure NTX (A) and NMP (B) in the PBS (50 µg/mL), sample of *in-vitro* release medium from ISFC formulation after 24 h for NTX (C) and NMP (D).

Table 3
Regression coefficients, significant *p*-values for the model estimated using Design Expert software.

	% Initial burst release of Naltrexone	
	Regression coefficients	*p*-values
Constant	+57.50639	–
X_1 [a]	−1.63708	< 0.0001 [e]
X_2 [b]	−1.61309	0.0011 [e]
X_3 [c]	−0.98023	< 0.0001 [e]
X_4 [d]	+1.12196	0.0111 [e]
X_1^2	+0.11804	0.0119 [e]
X_2^2	+0.029047	< 0.0001 [e]
X_3^2	+0.010709	< 0.0001 [e]
X_4^2	+0.045935	0.0241 [e]
$X_1 \times X_2$	0.0	0.9906
$X_1 \times X_3$	0.0	1.0000
$X_1 \times X_4$	0.0	1.0000
$X_2 \times X_3$	0.0	1.0000
$X_2 \times X_4$	−0.02	0.0121 [e]
$X_3 \times X_4$	0.0	0.9812

[a] LA/GA molar ratio.
[b] % PEG (w/w %).
[c] % triblock in formulation (w/w %).
[d] % ethyl heptanoate in formulation (w/w %).
[e] Significant factor.

3.97 ± 0.09 min and 6.25 ± 0.31 min, respectively. In this *in-vitro* release evaluation, the linear calibration curves of NTX and NMP in the release medium were fitted using a linear regression line with $R^2 \geq 0.98$. The limit of detection (LOD) and limit of quantification (LOQ) of NTX were 2 ng/mL and 6.7 ng/mL, respectively, and the LOD and LOQ of NMP were 1.2 μg/mL and 4.0 μg/mL, respectively.

The optimum formulation with a minimum initial burst release (5.69 ± 0.27%), the LA:GA molar ratio in triblock, the % of PEG in the triblock, the % of triblock in the formulation, and % of ethyl heptanoate were 6.5:1, 30%, 45.5%, and 5% respectively. A quadratic equation model predicted the initial burst release (Y) as follows:

$$Y = +57.50639 - 1.63708\,X_1 - 1.61309\,X_2 - 0.98023\,X_3 + 1.12196\,X_4 + 0.11804 \times {}_1{}^2 + 0.029047 \times {}_2{}^2 + 0.010709 \times {}_3{}^2 + 0.045935 \times {}_4{}^2 - 0.02\,X_2 \times X_4 \quad (2)$$

where X_1 is the molar ratio of LA:GA in the triblock, X_2 is the % of PEG in the triblock, X_3 is the % of triblock in the formulation, and X_4 is the % of ethyl heptanoate in the formulation. The response surface model obtained from the experimental design was evaluated using ANOVA and analysis of residuals. The results of the statistical analyses, including the p-values of the initial burst release, are shown in Table 3. The linear regression coefficients (R^2) for the initial burst release were 0.9715; this showed a good performance of the model based on the observed and predicted initial burst release. The effect of each term in the models could be significant provided that its p-value is smaller than 0.05 ($p < 0.05$) based on the statistical results (ANOVA) with a confidence level of 95% [29].

Fig. 6A shows the effect of different % of PEG and LA:GA molar ratios on the initial burst release of NTX. As the % of PEG in the PLGA-PEG-PLGA increased, two distinct trends were observed in the initial burst release. When the PEG content was in the range of 20–30%, the initial burst release decreased until it reached its minimum value and further increased up to 40%, which caused an increase in the initial burst release. Increasing the % of PEG in the triblock (20–30%) also increased the amount of hydrogen bonding between the NMP molecules and the PEG, preventing the initial burst release of NTX. Beyond 30% PEG, water diffusion into the composite increased; this phenomenon overcame the hydrogen bonding between the NMP molecules and the PEG. The initial burst release decreased with the increase in the molar ratio of lactide to glycolide to 6.5:1. With the enhancement of the LA:GA molar ratios of the triblock, the triblock became more hydrophobic and absorbed less water; this could have slowed the NMP diffusion [30].

Fig. 6B shows the effect of different % of the triblock and % of ethyl heptanoate on the initial burst release of NTX. Initial burst release decreased as % of the triblock was increased from 30 to 45.5%, at which point the minimum initial burst release was reached, and subsequent increases in the % of the triblock had no significant effect on the initial burst release. Increasing the copolymer concentration increased the cross-links between the copolymer molecules, the viscosity of the hydrogel, and the tortuosity; decreased the hydrogel network porosity; and lowered the drug diffusion rate [12,31]. Increasing the ethyl heptanoate (1–5%) decreased the initial burst release until a plateau was reached after 5% due to ethyl heptanoate's finite solubility in water. The ethyl heptanoate could have slowed NMP diffusion into the release medium and led to a slower drug release rate [31].

The implant of the ISFI into the copolymer PLGA 504H and the composite of the ISFC and the optimized formulation in the release media after 24 h are shown in Fig. 7A and B, respectively. The implant of ISFI in the *in-vitro* release medium had more stiffness in comparison to ISFC, which was in a gel state. This and other details (color and bilayer view) differed.

The cumulative release profile of NTX from the ISFC and ISFI formulations are shown in Fig. 8A and B, respectively. The results of the *in-vitro* release study of NTX from formulations before and after the

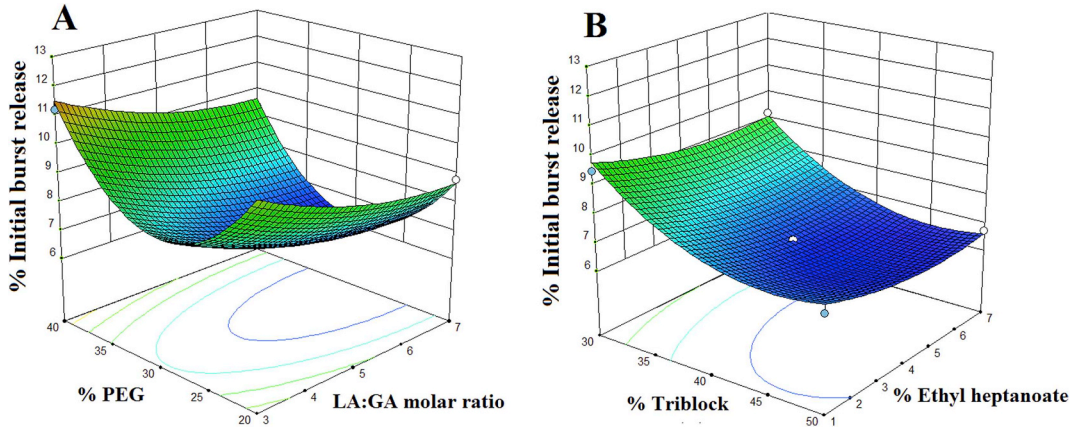

Fig. 6. (A) Surface and contour plot of % initial burst release of NTX as a function of % PEG and LA:GA molar ratio, (B) as a function of % triblock and % ethyl heptanoate.

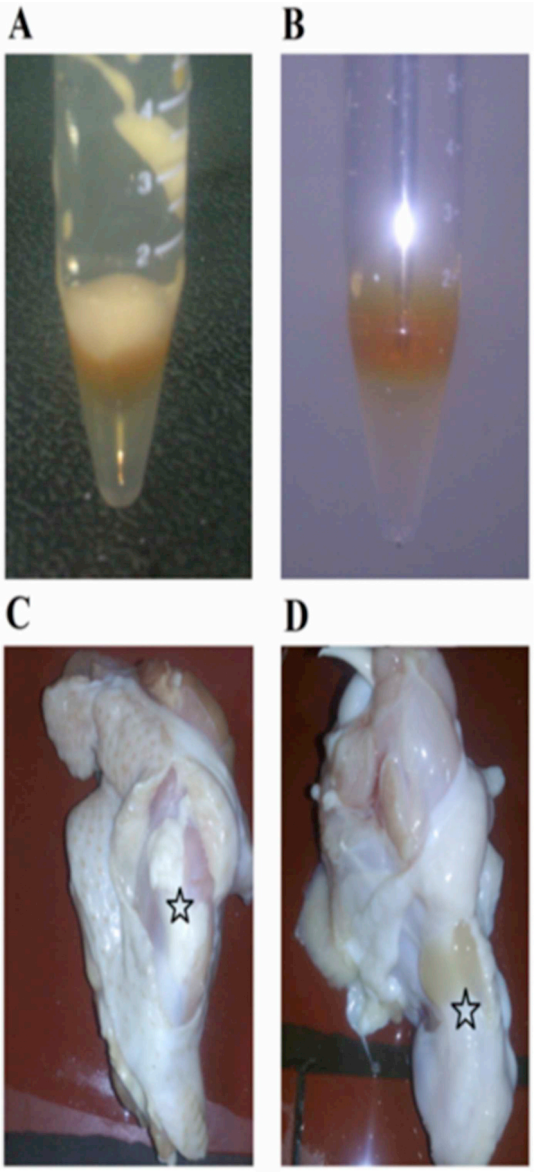

Fig. 7. (A) Formation of implant inside PBS using PLGA 504H after 24 h (ISFI), (B) Formation of composite inside PBS using triblock after 24 h (ISFC), (C) Formation of implant inside hen drummet using PLGA 504H after 24 h (ISFI), (D) Formation of composite inside hen drummet using triblock after 24 h (ISFC).

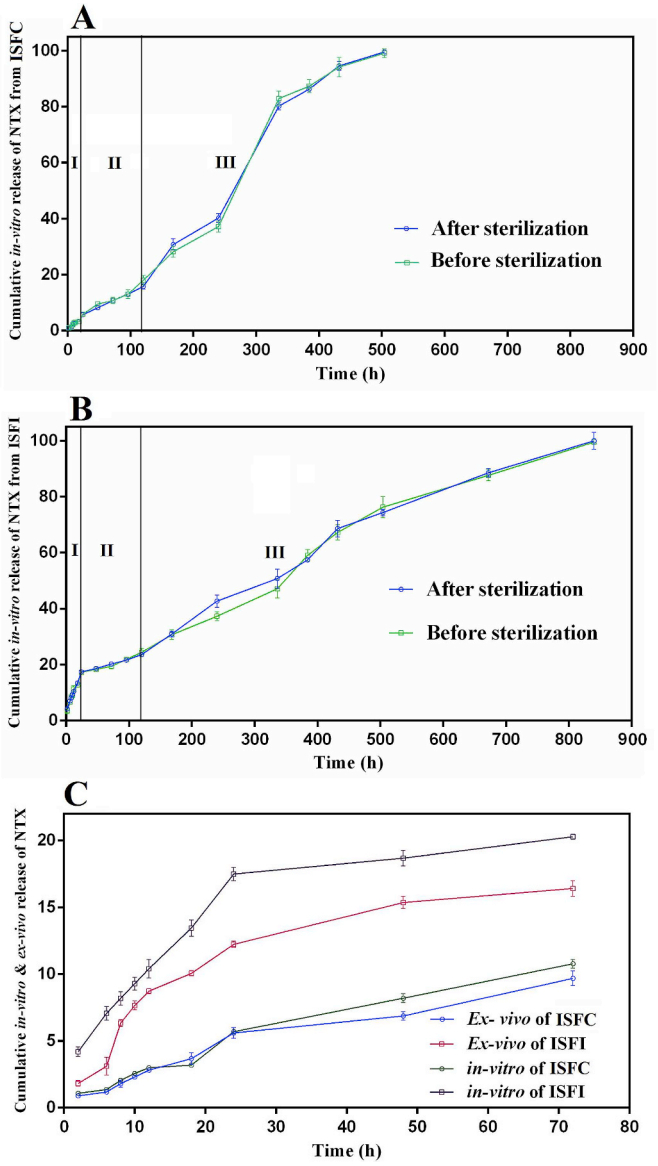

Fig. 8. Cumulative *in-vitro* release of NTX from triblock (ISFC) (A) and from 504 H (ISFI) (B), and cumulative *ex-vivo* and *in-vitro* release of NTX (C). (Mean ± SD, n = 3).

sterilization process are also shown. No significant changes in the *in-vitro* release profile of NTX from either the ISFC formulation or the ISFI formulation were observed during the 28 days ($p > 0.05$); this indicates that the sterilization method was appropriate.

The decrease in the molecular weight of the copolymer PLGA 504H and triblock (*in-vitro* degradation) using GPC analysis in the release environment [7,19] are shown in Fig. 9A and B, respectively.

The cumulative release and degradation profiles had three phases. The cumulative amount of drug released by the ISFI over the first 24 h, 17.45 ± 1.07%, was significantly ($p < 0.05$) higher than that released by the ISFC, 5.69 ± 0.27% (Phase I for both). NMP release can significantly influence the release rate of the studied formulations. The ISFI exhibited a higher initial NMP release (36.7 ± 2.89%) within the first day than did the ISFC (13.54 ± 2.08%) (Fig. 9C). These results confirmed that replacing PLGA with PLGA-PEG-PLGA in the ISFI was accomplished correctly, due to hydrogen bonding between the NMP molecules and PEG and the thermosensitive properties of the triblock. Phase II showed the completion of matrix formation; this happened over 5 days. In this phase, the mechanism of release was by diffusion from the polymeric system because the molecular weight of the copolymer in these two phases (I and II) was almost constant (Fig. 9A and B). Phase III (degradation) showed a much higher release rate (slope) than phase II (diffusion). The degradation rate of the ISFC was faster than that of the ISFI due to the presence of PEG in the triblock, which caused more water to penetrate the matrix [32]. NTX release from the ISFI was complete after 35 days, and from the ISFC after 21 days. This was due to

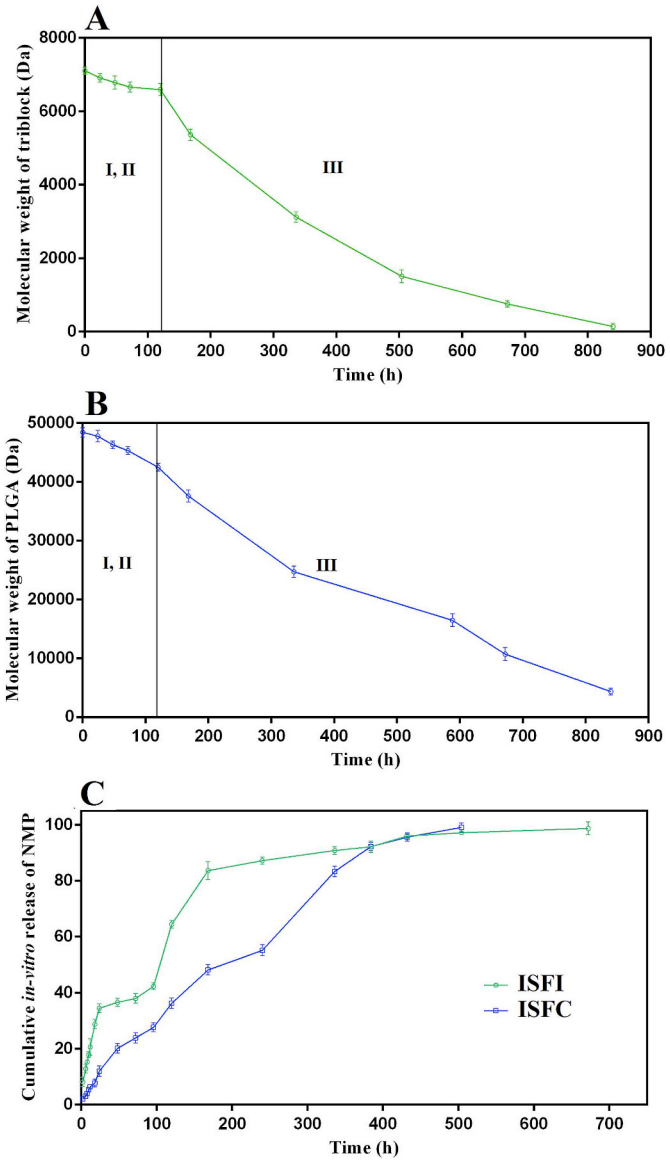

Fig. 9. *In-vitro* degradation of formulation without NTX containing triblock (A) and PLGA 504H (B), cumulative *in-vitro* release of NMP (C). (Mean ± SD, n = 3).

PLGA-PEG-PLGA degrading faster than PLGA 504H. The observed *in-vitro* drug-release profiles were in good agreement with the *in-vitro* degradation and NMP release.

The surface and cross-section morphologies from the implant using the formulation containing PLGA 504H and the composite from the optimized formulation of the triblock are shown via SEM after 3 days in the release medium, in Fig. 10. The ISFI using PLGA 504H caused a fast diffusion of NMP into the *in-vitro* release medium and, subsequently, a porous polymeric matrix [5]. The ISFI using PLGA 504H had a finger- and sponge-like structure with a large pore size (Fig. 10A). However, the optimized formulation containing the triblock decreased the rate of NMP exchange with water due to the hydrogen bonding between the NMP molecules and the PEG (Fig. 10B). This prevented the rapid diffusion of NMP into the release medium and created a more compact structure with a smooth surface, as it reduced the porosity of the surface. Also, it prevented the formation of the sponge- and finger-like structure with large pore sizes [33].

In previous studies, water was used to dissolve the PLGA-PEG-PLGA triblock [12,34,35]. The results for the release behavior found by Zhang et al. indicated that *in-situ* gels with PLGA-PEG-PLGA in water could serve as carriers for delay-released NTX delivery systems for 7 days [34]. Furthermore, the *in-vitro* NTX release study by Khodaverdi et al. indicated that NTX continuously delivers for 17 days [35,36]. In the current study, NMP was used instead of water to dissolve the PLGA-PEG-PLGA. The *in-vitro* release of NTX from the ISFC was complete after 21 days and from the hydrogel (without NMP) in less than 17 days. This was due to the PLGA-PEG-PLGA degrading faster in the hydrogel formulation.

3.3. Ex-vivo release evaluation

The asterisks in Fig. 7C and D shows the location of the composite of the ISFC formulation and the implant of the ISFI formulation, respectively, after 24 h. The ISFI formulation had more adhesion to tissue. This may be due to its higher hydrophobicity in comparison to the ISFC. The ISFI also had more stiffness than ISFC, which was in a gel state. This and other details (color and bilayer view) differed. Since the release media were corrupted after 3 days, the *ex-vivo* release study was impossible after this time. Fig. 8C indicates that there was no significant difference between the *ex-vivo* and *in-vitro* release ($p > 0.05$).

3.4. In-vivo release evaluation

The chromatogram of pure NTX, pure β-naltrexol, a mixture of the two in the rabbit serum, and a sample of an ISFG formulation administrated to the rabbits are shown in Fig. 11A, B, C, and D, respectively. The retention times of NTX and 6β-naltrexol using HPLC were observed at 4.12 min and 4.83 min, respectively. The calibration curve of NTX and 6β-naltrexol in the release medium were fitted using a linear regression line with $R^2 \geq 0.98$. The LOD and LOQ for NTX and 6β-naltrexol were 2 ng/mL and 6.7 ng/mL, respectively, in the rabbit serum; these results were later applied in the pharmacokinetic study.

The pharmacokinetic profile of an intravenous and a subcutaneous administration of the NTX solution were reported in our previous study (Fig. 12A and B) [5]. Constant serum levels of NTX were detected for nearly 4 weeks for the ISFI (Group IV), the ISFC (Group V), and the Vivitrol® (Group VI), indicating a constant rate of NTX release *in-vivo* upon a single injection after T_{max} (Fig. 12C and D). The AUC and absolute bioavailability (F) of NTX were enhanced by using the ISFC and ISFI compared to subcutaneous injection (Group III).

Tables 4 and 5 list the pharmacokinetic data for this study. The C_{max} of NTX (15.16 ± 2.46 ng/mL) from the ISFC was significantly ($p < 0.05$) lower than that from the ISFI (24.46 ± 2.9 ng/mL) and Vivitrol® (21.11 ± 2.89 ng/mL). Furthermore, the C_{max} of 6β-naltrexol (25.31 ± 3.98 ng/mL) from the ISFC was significantly ($p < 0.05$) lower than that from the ISFI (36.61 ± 3.98 ng/mL) and Vivitrol® (30.64 ± 4.67 ng/mL). The results of the C_{max} showed that the proposed formulation (ISFC) successfully reduced the initial burst release. Furthermore, the AUC, the absolute bioavailability (F), and the range of serum concentration of NTX for the ISFC formulation were like those for Vivitrol®.

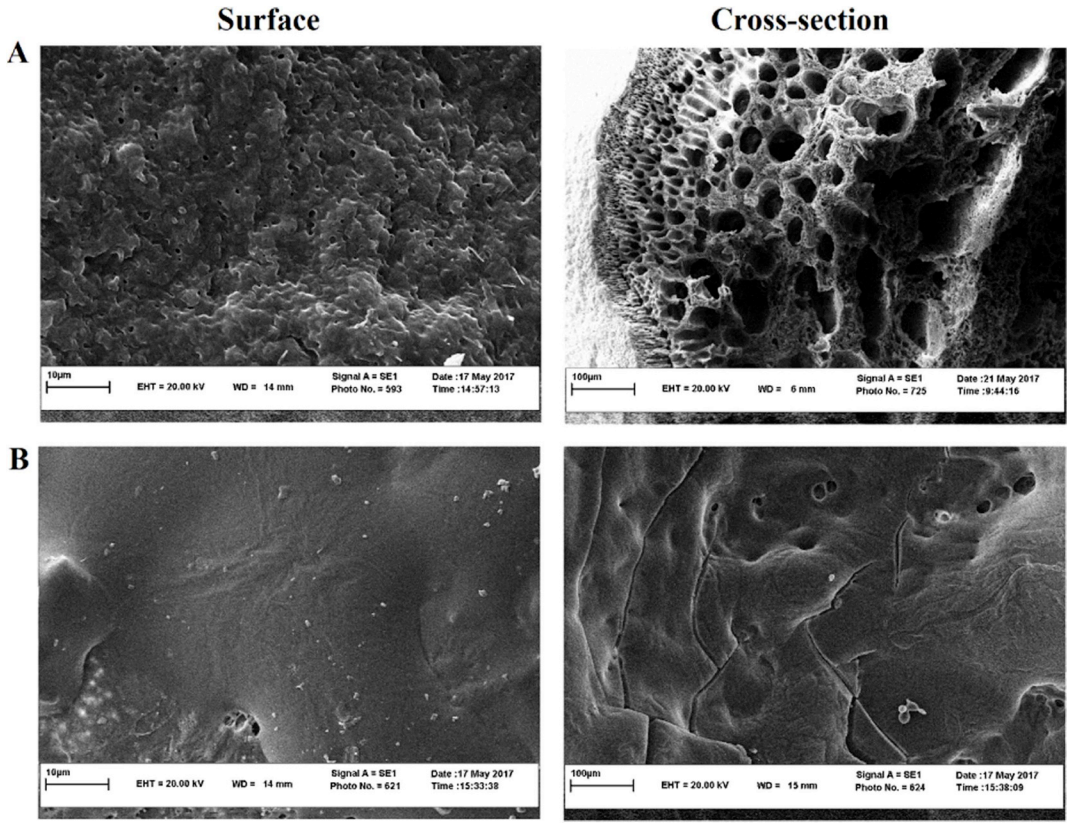

Fig. 10. Morphologies of the implant from the formulation containing PLGA 504H (A) [5] and composite from the optimized formulation containing triblock (B). Surface (scale bar: 10 µm) and cross-section (scale bar: 100 µm).

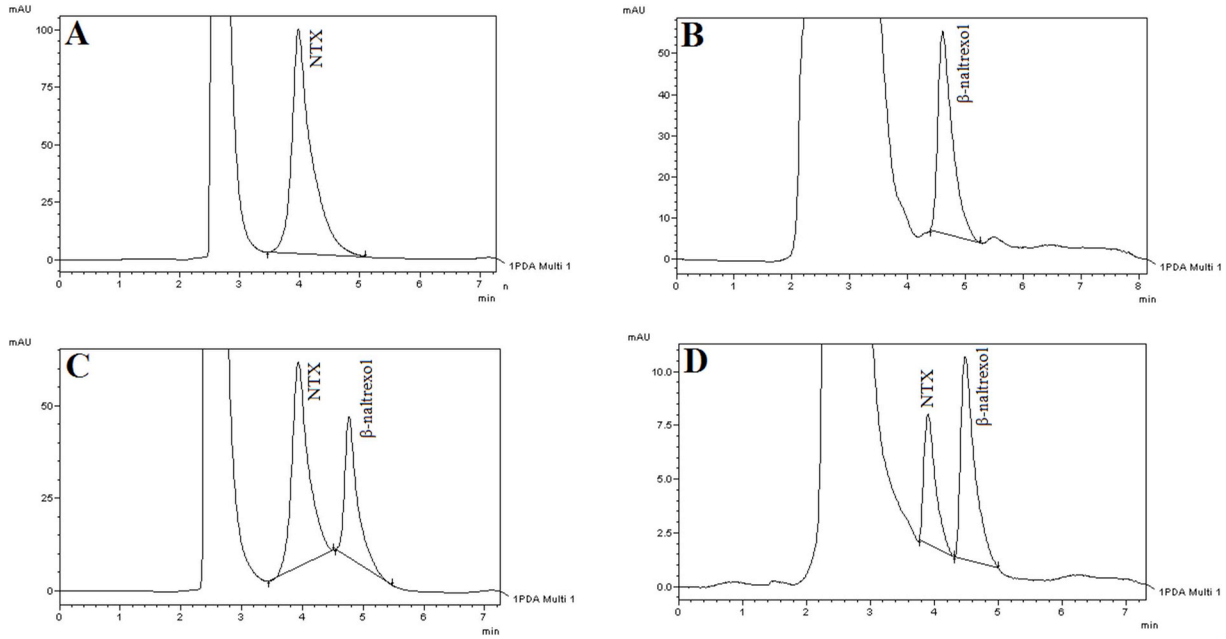

Fig. 11. Chromatogram of HPLC analysis for pure NTX (A) and 6β-naltrexol (B) and mixture of pure NTX and 6β-naltrexol in the serum of rabbit (C), and sample of serum from ISFC administration into the rabbit after 24 h (D).

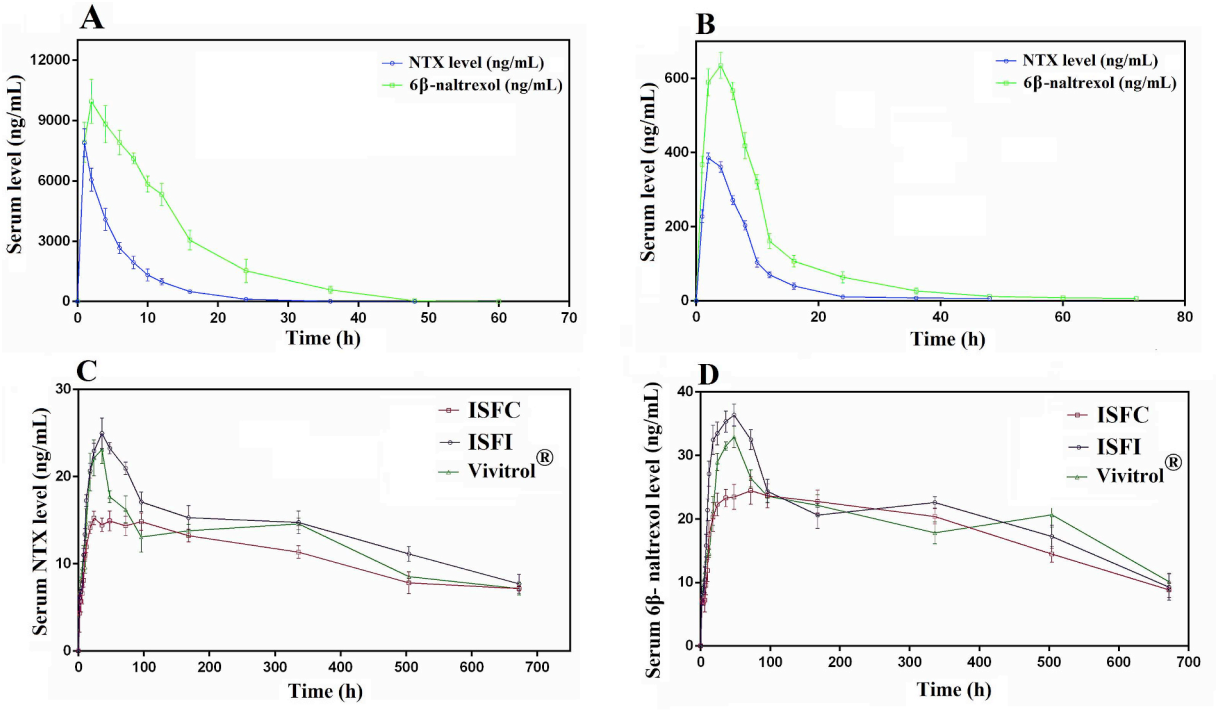

Fig. 12. *In-vivo* absorption of 100 mg NTX in rabbits after administration: (A) intravenous administration (111 mg naltrexone HCl in 1.1 mL WFI solvent) [5]; (B) subcutaneous administration of NTX solution in NMP [5]; (C) Serum NTX level of formulations as subcutaneously; (D) Serum 6β-naltrexol level formulations as subcutaneously. (Mean ± SD, n = 3).

Table 4
Pharmacokinetic parameters of NTX after administration of NTX in rabbits (n = 5) using PKSolver software.

Groups	NTX (mg)	C_{max} (ng/mL)	T_{max} (h)	AUC $_{0\text{-}t}$ (ng h/mL)	F%	Model
i.v. of NTX HCl solution in WFI [5]	100	9126.96 ± 300	0.25 ± 0.09	46829.64 ± 528	100	One compartment
s.c. of NTX solution in NMP [5]	100	382.98 ± 24	2.93 ± 1.08	3328.34 ± 472	7.11	Two compartment
s.c. of ISFI [5]	100	24.46 ± 2.9	38.2 ± 3.46	9021.53 ± 701	19.26	Two compartment
s.c. of ISFC	100	15.16 ± 2.46	35.82 ± 4.90	7214.19 ± 498	15.41	Two compartment
i.m. of Vivitrol® [5]	100	21.11 ± 2.89	31.85 ± 3.98	7882.96 ± 564	16.83	Two compartment

NTX: Naltrexone; C_{max}: maximum serum NTX concentration after dosing; T_{max}: time to reach C_{max} after dosing; AUC $_{0\text{-}t}$: area under the serum NTX concentration vs. time curve in the finite time; F: absolute bioavailability. *Note*—i.v.: intravenous injection (NTX solution), s.c.: subcutaneous injection, i.m.: intramuscular injection.

Table 5
Pharmacokinetic parameters of 6β-naltrexol after administration of NTX in rabbits (n = 5) using PKSolver software.

Groups	NTX (mg)	C_{max} (ng/mL)	T_{max} (h)	AUC $_{0\text{-}t}$ (ng h/mL)	Model
i.v. of NTX-HCl solution in WFI [5]	100	11703.50 ± 847.00	1.53 ± 0.64	154439.23 ± 1546	One compartment
s.c. of NTX solution in NMP [5]	100	652.31 ± 49.30	3.46 ± 0.78	7416.07 ± 683	Two compartment
s.c. of ISFI [5]	100	36.61 ± 6.27	37.77 ± 5.69	12950.68 ± 1035	Two compartment
s.c. of ISFC	100	25.31 ± 3.98	56.62 ± 6.84	11609.33 ± 809	Two compartment
i.m. of Vivitrol® [5]	100	30.64 ± 4.67	44.21 ± 7.08	12507.73 ± 911	Two compartment

NTX: Naltrexone; C_{max}: maximum serum 6β-naltrexol concentration after dosing; T_{max}: time to reach C_{max} after dosing; AUC $_{0\text{-}t}$: area under the serum NTX concentration vs. time curve in the finite time; F: absolute bioavailability. *Note*—i.v.: intravenous injection, s.c.: subcutaneous injection, i.m.: intramuscular injection.

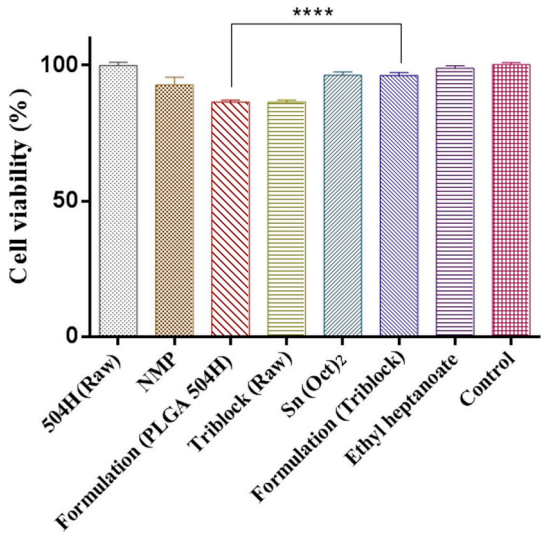

Fig. 13. Cell viability of L929 mouse fibroblast cells in contact with samples after 24 h using MTT assay. (Mean ± SD, n = 3). ****$p < 0.0001$.

3.5. In-vitro compatibility evaluation

The cell viability of samples after 24 h was investigated against mouse L929 fibroblast cell lines via an MTT assay (Fig. 13). The cell viability percent of the samples was in the range of 86.4–100%, which indicates that the samples were not cellular cytotoxic. The cell viability of the ISFC (96.2 ± 1.15%) was higher than that of the ISFI (86.4 ± 0.76%) due to the burst release of NMP, and the NTX from the ISFC over 24 h was lower than from the ISFI ($p < 0.0001$). Thus, the formulation containing the triblock (ISFC) was biocompatible, which indicates that it can be administered subcutaneously [37].

3.6. In-vivo compatibility evaluation

The histopathology section of groups I (Control), II (NMP), and III (NTX solution in NMP) (Fig. 14A and B) showed normal architecture. A marked granulomatous reaction characterized by the presence of multinucleated foreign-body giant cells and epithelioid histiocytes induced by the subcutaneous injection of the ISFI formulation was observed (group IV) (Fig. 14C). Also, lymphocytes were seen around the implant particles. The histologic changes in the dermal layer of the ISFC (group V) after 1 month of injections were increased collagen deposition and neovascularization with minimal inflammatory cells (Fig. 14D). The pathological responses of groups IV and V did not show a significant difference with groups I, II, and III. These responses were considered as a component of the normal tissue or cellular host reaction to injury [25,38].

4. Conclusion

In-situ forming implants (ISFIs) can provide a controlled release of a drug while offering greater ease of administration than surgical implants. ISFIs have a considerable tendency to burst (15–80% of the total drug), especially in the first 24 h. The diffusion of NMP into water upon injection causes the high initial burst release rates because the drug comes out with the NMP. In the current study, the use of PLGA-PEG-PLGA instead of PLGA was suggested to decrease the initial burst release. It seemed that the hydrogen bonding between the NMP molecules and the PEG prevented the rapid diffusion of NMP into the release medium. Furthermore, the thermosensitive properties of the PLGA-PEG-PLGA triblock could be helpful in composite formation (*in-situ* forming composite (ISFC)). The matrix structure of the composite slowed the NMP escape rate, resulting in a slower initial burst release. An ISFC formulation using PLGA-PEG-PLGA with NMP as a solvent that delivers medication continuously for 21 days *in-vitro* and 28 days *in-vivo* has not yet been reported in the literature. This study provides evidence that an ISFC can control the delivery of NTX both *in-vitro* and *in-vivo* for 28 days after a single subcutaneous injection, with a smaller initial burst release than with the ISFI using PLGA 504H and a combination of PLGA 504H and PLGA 756s, as previously studied [5]. The ISFC system was biocompatible and is a good formulation for sustained release for NTX with minimal initial burst release.

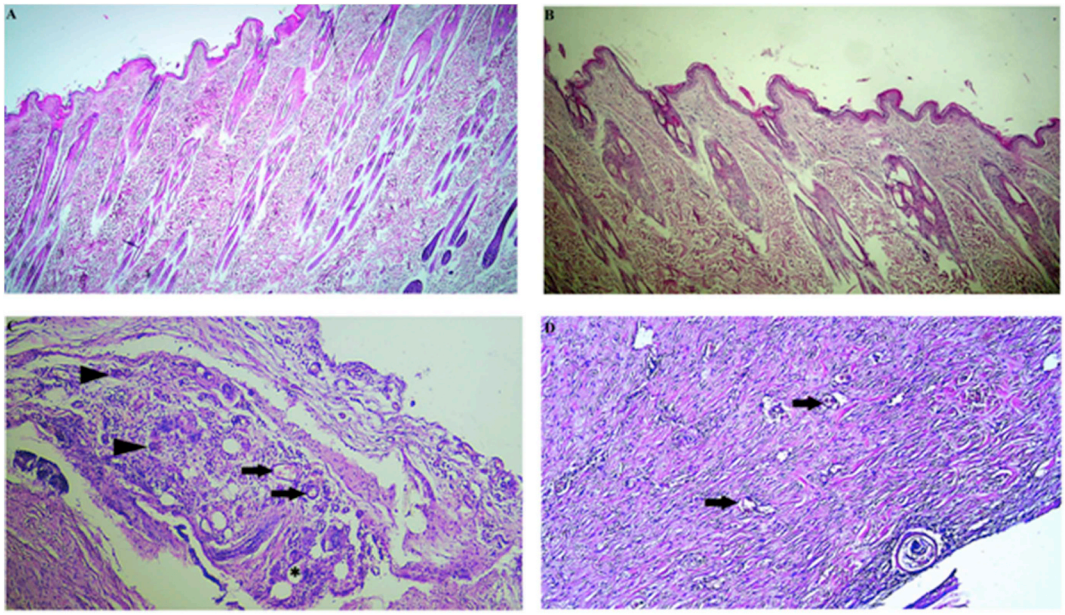

Fig. 14. Photomicrographs of hematoxylin and eosin stained skin tissues. A: Group I (Control), and Group II (NMP), 40× [5]; B: Group III (NTX solution in NMP), 40× [5]; C: Group IV (ISFI); the arrowheads show multinucleated foreign body giant cells surrounding implant particles, the blood vessels are indicated by arrows, and * indicates the implant of PLGA 504H, 100× [5]; D: Group V (ISFC), foreign body granulomatous reaction with numerous histiocytes, 100×.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jddst.2019.01.011.

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