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Development of novel solid lipid nanoparticles-loaded dual-reverse thermosensitive nanomicelle for intramuscular administration with sustained release and reduced toxicity

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Abstract

To develop novel solid lipid nanoparticles (SLNs)-loaded dual-reverse thermosensitive nanomicelle (DRTN) for intramuscular administration of flurbiprofen with sustained release and reduced toxicity, the DRTN was prepared with flurbiprofen-loaded SLNs, poloxamer 407 (P 407), poloxamer 188 (P 188) and water. Its rheological characterization, release, stability, pharmacokinetics and morphology were evaluated after intramuscular administration to rats. These SLNs were solid at 25°C and transformed into liquid form at physiological temperature due to their melting point of about 32°C. Furthermore, the DRTN retained a liquid state at 25°C and gelled inside the body owing to its gelation temperature of about 34.7°C, leading to an opposite reversible property of SLN. Compared to the hydrogel, it significantly decreased the drug release, respectively, exhibiting reduced initial fast release. It sustained high plasma concentration for 60 h, which was significantly higher compared to the suspension, indicating enhanced bioavailability. However, it showed lower plasma concentration, AUC, and C_{max} values than did the hydrogel, suggesting a retarded release and decreased side effects of the drug. Unlike the hydrogel, it induced no injury to the rat muscle resulting from no direct contact of the drug. It was stable for four months. Therefore, this novel DRTN system would be a strong candidate for the intramuscular administration of flurbiprofen.

Keywords: Flurbiprofen; Solid Lipid Nanoparticles; Dual-reverse Thermosensitive Nanomicelle; Stability; Bioavailability; Morphological Analysis.

1. Introduction

Hydrogel systems are the cross-linked networks composed of various polymers in aqueous solutions by means of intermolecular interactions, hydrogen, ionic bonding or hydrophobic forces.[1,2] In general, the conventional thermosensitive hydrogels were successfully developed using poloxamer solutions as a potential bases.[3,4] They were easy to administer to the body and gelled quickly, because they underwent a sol-to-gel conversion in return to temperature deviations. They were used as controlled release drug delivery systems for injectable,[5,6] ocular,[7] and rectal administration.[8-10] Moreover, they showed the enhanced bioavailability of the drug with appropriate safety in rats and human subjects.[2,8,11] However, in these conventional hydrogels, the drug was directly soluble or dispersed in aqueous medium, and thus may cause direct contact of the toxic drug to body tissue, leading to induced irritation or damage to the body tissue.[12] Furthermore, this may gave the initial fast release and higher C_{max} value of the drug, resulting in increased intrinsic side effects of the drug.[8-10] Therefore, a product without initial burst effect and direct contact of the toxic drug to body tissue must be developed.

In this study, to solve these serious problems, we have developed a novel dual-reverse thermosensitive nanomicelle (DRTN) system for intramuscular administration. The DRTN system is the dispersion of thermosensitive drug-loaded solid lipid nanoparticles (SLNs) in the thermosensitive poloxamer-based nanomicelle. The SLNs and nanomicelle in this DRTN system exist in a solid and liquid state at room temperature, and reversely transform to a liquid and gel state at body temperature, respectively. Thus, the SLNs and nanomicelle in the DRTN system may prevent the contact of the drug with body tissue, leading to reduced toxicity of the drug. Moreover, they regulate the release of the drug without burst effect,

which can reduce the initial fast release and C_{max} value of the drug, leading to decreased side effects. Additionally, this system is administered easily to the body like a conventional injectable gel, as it exists in a liquid state at room temperature and changes to a gel state at body temperature. The SLN system was increasingly interested as colloidal drug carriers.[13,14] The nanoparticles were in the submicron size range (50-1000 nm) composed of biocompatible lipids. They were stabilised with non-toxic surfactants such as poloxamer and lecithin.[15] The main advantages of SLN's include high absorption, appropriate tissue distribution, suitable pharmacokinetics, and biocompatibility.[14,16] Flurbiprofen, a widely used non-steroidal anti-inflammatory drug, was chosen as a model drug in this study. Because of its low aqueous solubility, flurbiprofen gave relatively weakened bioavailability and induced an irritation to the mucous tissues in the human body.[17,18]

2. Experimental Section

Materials

Flurbiprofen was kindly donated by Kolon Life Science, (Gwacheon, South Korea). Poloxamer 407 (P 407) and poloxamer 188 (P 188) were purchased from BASF (Ludwigshafen, Germany). Tricaprin and triethanolamine were obtained from Tokyo Chem. Inc. (Tokyo, Japan). Semipermeable membrane tubs were obtained from Spectrum Laboratories, Inc. (Los Angeles, CA, USA). All the other chemicals were of reagent grade and were used without extra purification.

Animals

Male Sprague-Dawley rats (270±20 g, 6-7 weeks old) obtained from Nara Biotech (Seoul, South Korea) had easy access to a usual standard laboratory diet and tap water. The animals

were kept in a controlled environment of 23-26 ºC and 50-55% humidity throughout the research. The procedures for the animal studies were performed with the national institute of health (NIH) Policy and Animal Welfare Act under the endorsement by the Institutional Animal Care and Use Committee (IACUC) at Hanyang University.

Preparation of Flurbiprofen-loaded DRTN

Flurbiprofen (100 g) was added into 150 g of the lipid mixture of tricaprin and triethanolamine at the weight ratio of 8:2 at 45 °C. This drug loaded-lipid mixture was dispersed in 13 g of poloxamer solution (P 407, P 188, and water at the weight ratio of 15/25/60) at 45 °C using Ultra-Turrax (IKA, Guangzhou, China) and homogenized using a high pressure homogenizer (Emulsiflex B15, Avestin; Ottawa, ON, Canada) with the pressure drop of 500-1000 bar at 45 °C for three cycles.[19,20] Then, it was cooled down to room temperature to obtain the flurbiprofen-loaded SLNs dispersion.[21] Subsequently, 15g P 407 and 25 g P 188 were dissolved in 50 g of distilled water at 4 °C with mild stirring. Then, 10 g of the flurbiprofen-loaded SLNs dispersion was added to this poloxamer solution and kept overnight at 4 °C until a clear solution was obtained,[8] leading to the development of DRTN.

Measurement of Gelatin Tempertaure

Gelation temperature was determined as reported previously.[10] Briefly, 4 g of DRTN was placed in a 10 ml transparent glass vial with a magnetic bar (10×3 mm). The transparent glass vial was set in a low-temperature water bath and a digital thermometer (IKA ETS-D5; IKA , Guangzhou, China) was placed in the DRTN. The system was gradually heated at a constant rate of 1 °C min^{-1} with a constant stirring of 50-70 rpm. The point at which the magnetic bar stopped rotation was noted as the gelation temperature.

Rheological Behaviour of DRTN

Brookfield Viscometer LVDV-II+P (Middleborough, MA, USA) was employed to determine the rheological behavior of the DRTN at 25 °C and 36.5 °C. A revolving water bath (WC-05G, Daejeon, South Korea) was used to control the temperature. Software (RHEOCALC; Lorch, Geremany) was employed to determine the rheological parameters. The equipment was set up as explained previously.[9]

Transmission Electron Microscopy (TEM)

A transmission electron microscope (TEM) (Hitachi H-7600; Tokyo, Japan) was used at an accelerating voltage of 100 kV to observe the morphology of the DRTN. A drop of the DRTN was placed on a carbon-coated copper grid to adhere the particles to the carbon substrate. Negative staining was done with 2% phosphotungstic acid solution.

Release Test

A release test was performed using the paddle method. It was executed at 36.5 °C and 100 rpm with 500 ml of phosphate buffer (pH 7.4) containing 0.3% Tween 80 as a dissolution medium in the dissolution tester (VISION-6 Classic, Chatsworth, CA, USA).[8,9,22] Each DRTN, hydrogel, and suspension containing 40 mg of flurbiprofen was introduced into a semipermeable membrane tube. DRTN was comprised of [SLN/P 407/P 188/H_2O (10/15/25/50 %)], which enclosed 1% flurbiprofen. Hydrogel was composed of 1% drug, 15% P 407, 25% P 188, and 59% water. Suspension was a 1% drug-loaded sodium carboxymethylcellulose (sodium CMC) aqueous solution. Clamp was used to tie both sides of the tube to prevent leakage, and then the tube was positioned in the dissolution tester. Two ml of samples were withdrawn and each filtered at 1 h intervals. The filtrate was examined

by the HPLC technique as stated below.

Pharmacokinetics

Eighteen rats were divided into three groups and were fasted for 24 h. These rats were intramuscularly administered with DRTN, hydrogel, and suspension, respectively, at a dose of 12 mg kg^{-1} in the gluteus maximus muscle. Sequential blood samples (300 µl) were obatined from the vein of right or left ear at specific time peroids and centrifuged at 9,000 g for 10 min to obtain plasma using a centrifuge (Smart 15 micro centrifuge, Hanil Science Industrial Co.; Incheon, South Korea). Plasma was reserved at -18 ℃ prior to the HPLC analysis. Plasma (150 µl) was mixed with acetonitrile (250 µl) and 10 µl of acetonitrile solution containing naproxen (40 µg ml^{-1}) as an internal standard. The mixture was centrifuged at 9,000 g for 10 min, and the supernatant (10 µl) was injected into the column inertsil ODS-4 C18 (GL Science, 5µm, 4.6 x 250 mm). The chromatograph was comprised of a high-performance liquid chromatograph (Agilent 1260 Infinity; Santa Clara, CA, USA) consisting of a variable wavelength detector (G1314B 1260) and a Quat Pump (G1311C 1260). The mobile phase was a mixture of 40% double distilled water adjusted to pH 2.6 with acetic acid and 60% acetonitrile. The effluent was observed at 245 nm with a flow rate of 1.0 ml min^{-1}.[23]

Morphology Characterization

The rats were intramuscularly administered DRTN and hydrogel for 96 h. After 96 h of dosing, the muscle was entirely isolated, cleaned with a saline solution, fixed in 10% neutral carbonate-buffered formaldehyde, and then embedded in paraffin, serially sectioned (3~4 µm), and stained with hematoxylin and eosin for overall characterization[24] or Masson's trichrome for collagen fibers.[25,26] The muscular tissue without dose administration was used

as a control. Next, morphological evaluation of each sample was performed under a light microscope (E400, Nikkon, Tokyo, Japan). Any damages to muscle tissues were observed, based on the degenerative lesions including muscle fiber necrosis, lysis and atrophy, inflammatory cell infiltrations, and fibrosis-collagen depositions.[24,27] To observe more detailed changes, mean muscle fiber thicknesses (μm), mean numbers of muscle fiber (fibers mm^{-2}), mononuclear cells (cells mm^{-2}), and mean collagen-occupied regions (% mm^{-2}) in muscle bundles around hydrogel-applied regions were determined using a computer-based automated image analyzer (*i*Solution FL ver 9.1, IMT *i*-solution Inc.; Quebec, Canada).[28,29] Multiple comparison tests for different dose groups were conducted. The Levene test was performed to determine the variance of homogeneity.[30] If the Levene test failed to show significant differences from variance homogeneity, the data were assessed by one-way ANOVA test and the least significant differences (LSD) multi-comparison test. In case of significant deviations from the variance, homogeneity was observed in the Levene test, a non-parametric comparison test and Kruskal-Wallis H test were performed. Statistical analysis was executed using SPSS for Windows (Release 14.0, SPSS Inc., USA).[31]

Stability

The 1% flurbiprofen-loaded DRTN was stored at 25 °C and 30 °C over a period of four months and analyzed for mean particle size and drug content.

3. Results and discussion

3.1. Preparation of DRTN

Generally, the SLN system was an aqueous colloidal dispersion containing solid lipids and surfactant.[32] Similarly, the flurbiprofen-loaded SLN prepared in this study was an aqueous dispersion, in which the drug-loaded lipid mixture was dispersed in a poloxamer solution, resulting in a composition of the drug, lipid mixture, and surfactant in the weight ratio of 10/15/1.3. This SLN had the excellent entrapment efficiency of 91.4±7.2 % and nano size of 188.4±4.0 nm (raw data not shown). In the preliminary study, this lipid mixture of tricaprin-triethanolamine at the weight ratio of 8:2 gave the melting point of 32.3 °C, suggesting that it was in solid form at 25 °C and changed to liquid form at body temperature. On the other hand, the DRTN system composed of 10% flurbiprofen-loaded SLNs, 15% P 407, 25% P 188, and 50% water was also an aqueous dispersion. This DRTN gave a gelation temperature, the temperature at which the liquid phase was changed to a gel phase,[8,9] of 34.7±0.6 °C. It showed free-flow characteristic at room temperature, but became a gel at 36.5 °C, suggesting that the hydrogel (poloxamer solution) in this DRTN gave the opposite thermosensitive property compared to SLN. Thus, as shown in Fig. 1, in this DRTN system, the thermosensitive flurbiprofen-loaded SLNs and hydrogel retained a solid and liquid form at room temperature, respectively. Upon injection into muscle, they transformed oppositely to a liquid and gel form, respectively.

This SLNs-loaded DRTN exhibited a well-sustained round shape with a clear boundary between each particle (Fig. 2A). The flurbiprofen-loaded thermosensitive SLNs were the solid form with a nano size of about 160 nm (Fig. 2B), which were surrounded by thermosensitive nanomicelle, poloxamer solution. The thermosensitive nanomicelle were formed owing to the gelling properties of hydrogel, which were liquid at 25 °C. Therefore, such formation of dually controlled thermosensitive system showed that the poloxamer

solution formed a nanomicelle surrounding the thermosensitive SLNs. [33]

In the preliminary study, the threshold of syringe-ability (viscosity at 25 °C) was determined by intramuscularly administering DRTN to rats using a syringe with a needle. It was easy to administer into muscle, beneath the threshold of syringe-ability, without separation of the needle from the syringe. However, the needle separated from the syringe, above the upper threshold value. In this study, the poloxamer-based DRTN had a viscosity of 275.9±3.2 mPa.s at 25 °C, which was beneath the threshold in syringe-ability, resulting in easy intramuscular administration of the DRTN to the body.[34] As shown in Fig. 3, at physiological temperature, the viscosity of the DRTN rapidly increased with the elapse of time, and then maintained a constant intrinsic viscosity, gel strength. This could be due to the gelling effect of nanomicelle, which was a liquid at room temperature; however, upon exposure to body temperature it promptly converted into gel.

From these findings, the DRTN, composed of [SLN/P 407/P 188/H_2O (10/15/25/50 %)], gave the suitable gelation temperature and good syringeability. Therefore, it was easy to administer intramuscularly and speedily formed a gel state in the body.

3.2. Release

The release profile of DRTN was compared with the suspension and hydrogel (Fig. 4A). DRTN was composed of [SLN/P 407/P 188/H_2O (10/15/25/50 %)], which contained 1% drug. The hydrogel was composed of 1% drug, 15% P 407, 25% P 188, and 59% water. The suspension was 1% drug-loaded sodium CMC aqueous solution. The hydrogel was clear because a poloxamer mixture played the role of a solubilising agent.[9-10] The DRTN significantly increased the release rate of the drug compared to the suspension. However, this

system significantly retarded the drug release compared to the hydrogel. The SLNs and nanomicelle in this DRTN system, which existed in a solid and liquid state at room temperature, were reversely transformed into a liquid and a gel state at body temperature, respectively. Thus, this DRTN system could dually control the release of the drug by SLNs and nanomicelle, resulting in retardation of the drug release.

To recognize the release mechanism of the drug from the DRTN and hydrogel, we designated the dissolution rate by the following equations: $Mt/M = Kt^n$, $Log [Mt/M] = log\ k + n\ log [t]$, where Mt/M is the portion of dissolved drug at time t. The n and k values are an indication of the release mechanism and a characteristic constant of the DRTN, respectively. From the plot of log (Mt/M) versus log (t) (Fig. 4B), the kinetic parameters n and k were determined. Release becomes rapid as the k value upsurges.[35, 36] The n value = 1 equals to zero-order dissolution kinetics, $0.5 < n < 1$ corresponds to a non-Fickian dissolution model, and the n value corresponds to 0.5 specifies Fickian diffusion (Higuchi model).[35] Table 1 shows the n values were close to 0.5 both in DRTN and hydrogel, suggesting that, like the hydrogel, the drug in this DRTN was released by Fickian diffusion through the extramicellar aqueous passages of the gel medium.[9,35] Their similar n values suggested that the presence of SLNs might hardly affect the release kinetics (mechanism) of the drug, because thermosensitive SLNs converted rapidly from solid to liquid form in 36.5 °C.[13,14] However, the lower k value indicated that flurbiprofen released more slowly from DRTN as compared to the hydrogel (Table 1). Thus, unlike the single control of hydrogel, the SLN and nanomicelle in the DRTN system could dually control the release of the drug, resulting in reduced initial fast release and no burst effect.

3.3. Pharmacokinetics

The pharmacokinetic study of DRTN after intramuscular administration at the dose of 12 mg kg^{-1} drug to rats was carried out and compared to the hydrogel and suspension. Their mean plasma concentration-time profiles and pharmacokinetic parameters are given in Fig. 5 and Table 2, respectively. The DRTN was observed to attain relatively high plasma concentration of about 18 μg ml^{-1} at 2 h, followed by maintained concentration in 18 μg ml^{-1} for 60 h. Likewise, the hydrogel gave a constant high plasma concentration of about 25 μg ml^{-1} from 1.75 to 60 h. In addition, the suspension showed the plasma concentration at about 4 μg ml^{-1} to 48 h, and then it gradually decreased to 0.87 μg ml^{-1} at 96 h.

All three formulations gave sustained property of plasma concentration, even if they gave significantly different plasma concentrations. At all times, the DRTN and hydrogel gave significantly higher plasma concentrations compared to the suspension ($P<0.05$). Furthermore, the DRTN and hydrogel showed significantly higher AUC and C_{max} values as compared to the suspension (Table 2). In particular, these preparations improved about 5.3- and 7-fold bioavailability, respectively. The DRTN gave lower plasma concentration, AUC, and C_{max} values of flurbiprofen than did the hydrogel, even if they were not significantly different. In general, high C_{max} of toxic drug may cause its intrinsic side effect as well as improve its therapeutic effect.[8-10] Thus, relatively low C_{max} of flurbiprofen in the DRTN reduced its intrinsic side effects such as nausea, stomach pain, low fever and loss of appetite.[37] The drug in the hydrogel was fast and directly released from it and then absorbed to the blood vessel of muscle, leading to the initial burst effect of drug.[8-10] After injection to the muscle, the SLNs and nanomicelle in this DRTN system speedily and reversely changed to a liquid and a gel state, respectively. In the DRTN system, the drug encapsulated in SLNs may move out of the liquid SLNs phase and sequentially move out of gelled nanomicelle phase, followed by absorption to the blood vessel of muscle. Thus, unlike hydrogel, the DRTN system composed of viscous SLNs and nanomicelle in the muscle could dually

regulate the release of the drug without burst effect, which could reduce the initial fast release and C_{max} value of the drug, leading to reduced intrinsic side effects of the drug.

3.4. Morphology Analysis

The morphology analysis was performed to check any irritation or injury to the muscular tissues in the rats after the administration of DRTN compared to the hydrogel. The normal muscular tissues without drug administration were used as the control. No abnormal damages or irritation signs were observed in the muscle tissues after intramuscular administration of DRTN, but noticeable focal muscle fiber necrosis and lysis were noticed in the hydrogel (Fig. 6). It was also demonstrated that no meaningful or significant changes in the histological damage scores, mean muscle fiber thicknesses and numbers, mononuclear cell numbers, and mean collagen occupied regions in the muscle bundles were demonstrated in the DRTN as compared with the control (Table 3). However, significantly ($p<0.01$) increased histological damage scores and significantly ($p<0.001$) decreased mean muscle fiber thicknesses and numbers were observed in the hydrogel as compared with the control (Table 3).

These findings are viewed as direct confirmations that the DRTN did not cause any local irritations or injuries to the applied muscle, but the hydrogel could cause noticeable local irritable signs, focal muscle fiber necrosis, and lysis. In general, flurbiprofen, a NSAID drug, caused local irritation or injury to body tissue.[17,18] Thus, the drug in the hydrogel directly contacted the muscle tissue, leading to irritation or injury. However, because the SLNs encapsulated the drug and dispersed in the nanomicelle, the DRTN system prevented direct contact of the drug to the body tissue, leading to no induced injury or damage to the muscle tissue.

3.5. Stability

The stability of the DRTN was evaluated by the drug content, mean particle size, and physical appearance over four months at 25 and 30 ℃ (Table 4). There was no noticeable change in the physical appearance of the DRTN during this period. Moreover, no significant and meaningful difference was found in the drug content. The contents of flurbiprofen decreased less than 3%, even at 30 ℃, suggesting that the DRTN was stable for at least four months.

4. Conclusion

The novel DRTN system composed of SLN and nanomicelle was easy to administer intramuscularly. It retarded the sustained release of the drug compared to the hydrogel, and improved the bioavailability compared to the suspension without burst effect. Furthermore, it caused no injury or irritation to the muscle, unlike the hydrogel, and remained physically and chemically stable over a period of four months. Thus, this DRTN system with sustained release, reduced toxicity, and excellent stability would be a strong candidate for the intramuscular administration of flurbiprofen.

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Table 1. Release kinetic parameters.

Formulations	Release exponent (n)	Kinetic constant (% h^{-n})	Correlation coefficient (r^2)
DRTN	0.53	1.17	0.98
Hydrogel	0.50	1.20	0.99

*Each value represents the mean ± SD (n=6).

Table 2. Pharmacokinetics parameters.

Parameters	Suspension	Hydrogel	DRTN
AUC (h.μg ml^{-1})	202.80±27.23	1678.87±575.90*	1196.37±245.21*
T_{max} (h)	1.33±0.57	1.75±1.67	2.17±1.47
C_{max} (μg ml^{-1})	3.54±0.72	24.73±6.91*	18.74±3.22*
$t_{½}$ (h)	0.03±0.02	9.43±1.70	10.09±0.67
K_{el} (h^{-1})	30.99±10.04	0.07±0.02	0.06±0.04

Each value represents the mean ± SD (n=6).

*P<0.05 compared to suspension.

Table 3. Morphological analysis of DRTN and hydrogel applied to muscle.

Morphology	Control	Hydrogel	DRTN
Muscle bundles	0.00±0.00	1.89±0.78$^{\#}$	0.00±0.00
Muscle fiber thickness (μm)	46.30±5.21	30.53±6.55*	46.36±5.05
Muscle numbers (fibers mm^{-2})	148.22±13.35	85.89±28.34*	148.89±18.20
Mononuclear cell numbers (cells mm^{-2})	46.78±14.31	58.67±18.81	47.22±11.17
Collagen percentage (% mm^{-2})	7.24±3.80	7.53±2.98	7.13±3.34

Each histological value represents the mean ± SD (n=9).

*$p < 0.01$ and $^{\#}p < 0.001$ as compared with control.

Table 4. Stability of DRTN.

	Particle size (nm)			Drug content (%)		
Period (months)	0	2	4	0	2	4
25 °C	168.7±7.7	169.9±8.5	169.9±6.9	100	98.03±0.2	97.30±0.4
30 °C	168.5±7.5	169.8±9.3	170.0±5.2	100	97.53±0.8	97.32±0.9

Each value represents the mean ± SD (n=3).

Fig. legends

Fig. 1. Scheme for dual-reverse thermosensitive nanomicelle (DRTN) system

Fig. 2. Transmission electron micrograph (10,000X) (A) and particle size (B) of DRTN.

Fig. 3. Rheological profile of DRTN at 36.5 °C. The arrow indicates the gel strength which means the constant intrinsic viscosity of the DRTH at 36.5 °C.

Fig. 4. Release profile (A) and release kinetics (B) of drug from DRTN, suspension and hydrogel.

Fig. 5. Plasma concentration-time profiles of flurbiprofen after intramuscular administration of DRTN, hydrogel, and suspension to rats. Each value represents the mean ±S.D. (n=6). All plasma concentrations in the DRTN and hydrogel at each time were significantly different from that in the suspension ($P<0.05$).

Fig. 6. Morphology of rat muscle: (A) control, (B) after the application of DRTN, (C) after the application of hydrogel. The muscles were isolated, rinsed with a saline solution, fixed in 10% neutral carbonate-buffered formaldehyde, entrenched in paraffin successively sectioned (3~4μm), and stained with hematoxylin and eosin. Each muscle was observed under a light microscope. MB = muscle bundle; MF = muscle fiber; IS = interstitial. Scale bars = 120 μm. The muscular tissues without administration were used as a control.

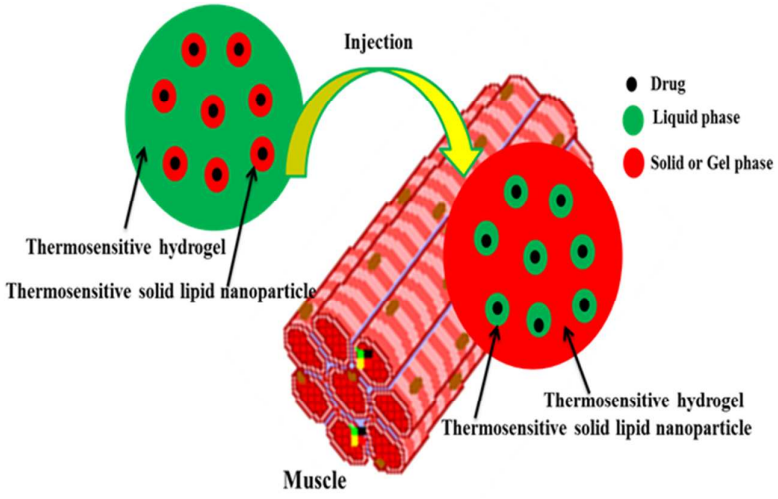

Fig. 1

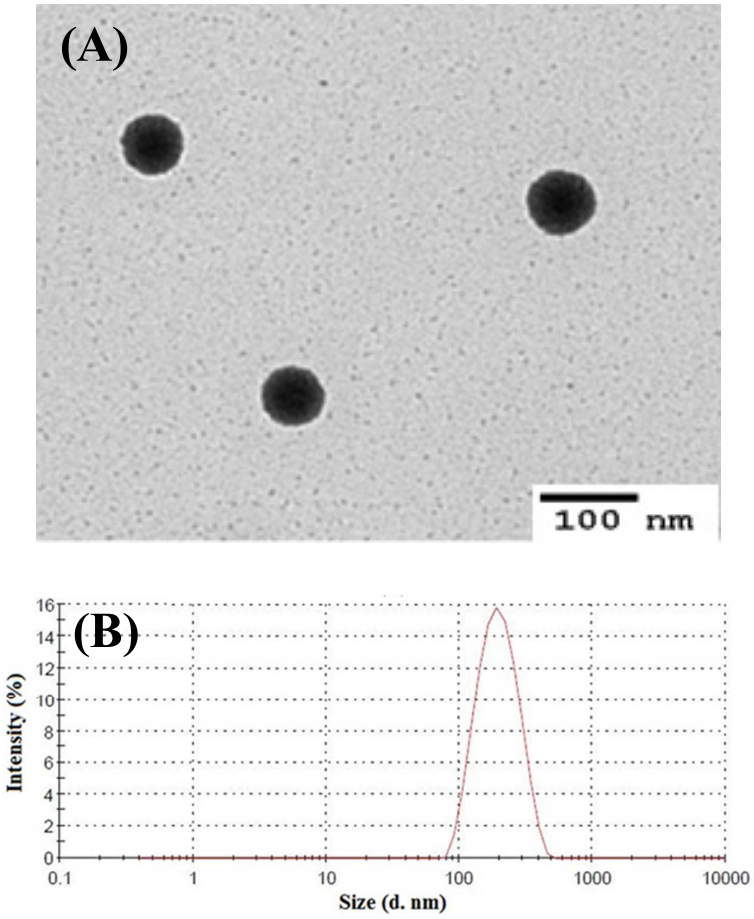

Fig. 2

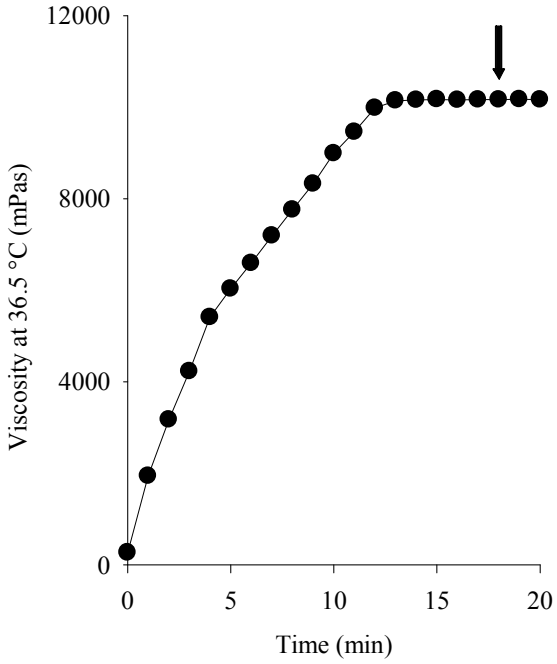

Fig. 3

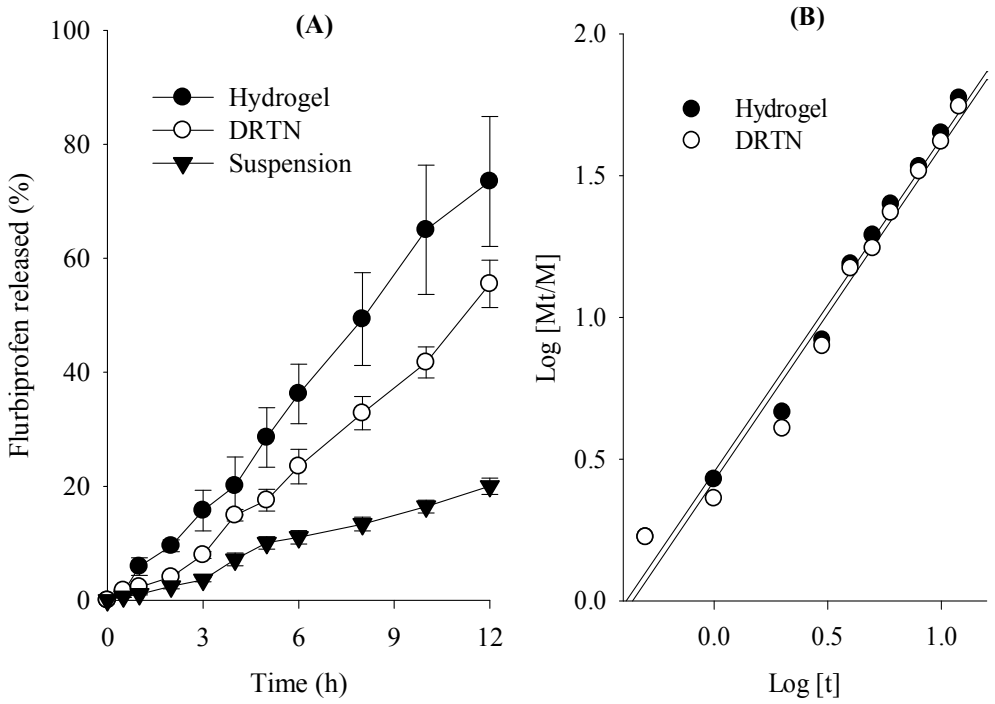

Fig. 4

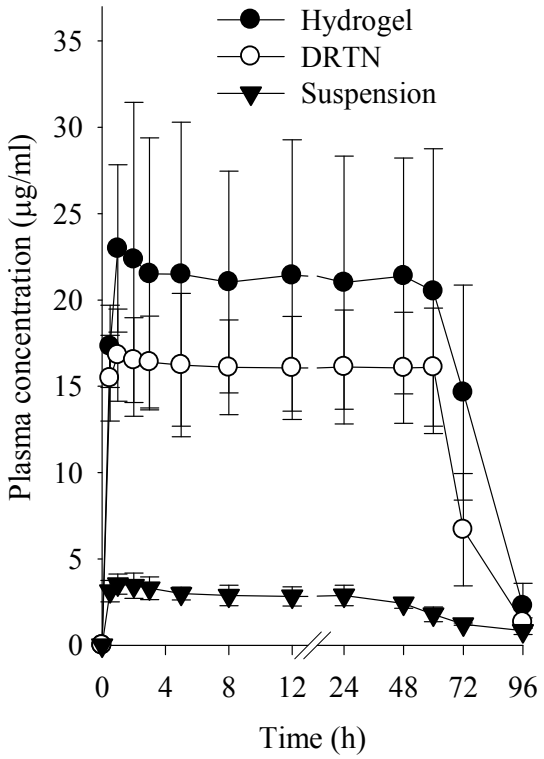

Fig. 5

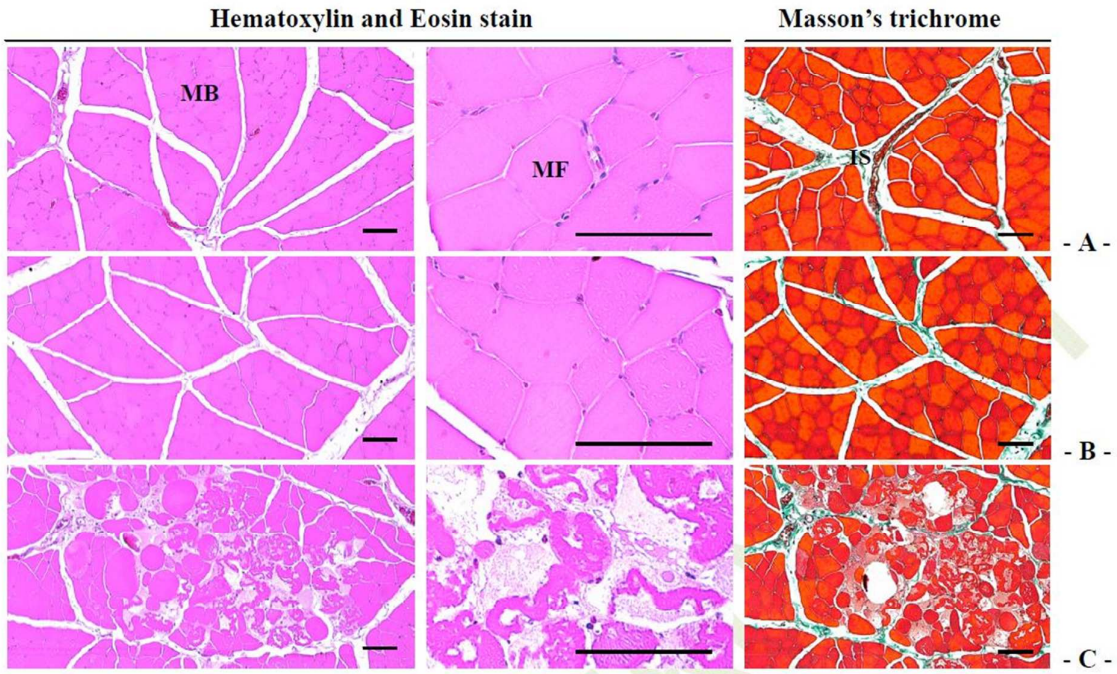

Fig. 6