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Research paper

# Myotoxicity studies of O/W-in situ forming microparticle systems

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**Abstract**

The objective of this study was to investigate the myotoxicity potential of the solvents used in the preparation of polymer solutions and O/W-in situ forming microparticle (ISM) systems. The acute myotoxicity studies of the tested solvents, emulsions of the solvents, polymer solutions as well as the O/W-ISM formulations with varying phase ratios were investigated using the in vitro isolated rodent skeletal muscle model by measuring the cumulative creatine kinase (CK) efflux. Phenytoin and isotonic sodium chloride solution served as positive and negative controls, respectively. Results from the in vitro myotoxicity studies suggested that the investigated five partially water miscible solvents caused muscle damage in the following rank order: benzyl alcohol > triethyl citrate > triacetin > propylene carbonate > ethyl acetate. Myotoxicity of ethyl acetate was found to be comparable to that of the isotonic sodium chloride solution. Emulsions of the undiluted solvents and an aqueous 0.5% Pluronic F 68 solution (ratio 1:4) could dramatically reduce the myotoxicities to 24–65%. The myotoxicity of O/W-ISM was less than those of the polymer solutions and the undiluted solvents. The cumulative CK level from the muscle treated with the O/W-ISM with phase ratio 1:4 was comparable to those from the negative controls. Area under the CK plasma curve from Sprague–Dawley rats was used to evaluate the in vivo myotoxicity following an intramuscular injection of the formulations. The in vivo myotoxicity data was well correlated with the in vitro myotoxicity data and confirmed the good muscle compatibility of the O/W-ISM formulations.
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*Keywords:* Myotoxicity; Creatine kinase; In situ forming; Microparticles; Solvents

## 1. Introduction

During the past decade, biodegradable polymer solutions that could be injected as liquid and form implants upon injection have been investigated extensively [1–21]. These systems allow for easier manufacturing compared to microparticles and avoid the need for surgical incision as required in solid implants [2,9–11]. Although these formulations have demonstrated some success in certain applications [14–16], several disadvantages are often encountered. The injection of these liquid implants exhibited variable consistency and geometry, which leads to the inconsistent and unpredictable drug release profile [22,23]. Initial drug burst releases of the systems were found to be 30–80%, which may associate with tissue irritation and systemic toxicity [8]. High viscosity of the polymer solutions may also cause an injectability problem and require the use of a large needle size [23,24]. Furthermore, the use of a large amount of undiluted toxic solvents is questionable [22].

In order to overcome the problems from the large, single-unit in situ implants, a novel injectable biodegradable in situ forming (ISM) microspheres method has been developed [23–30]. This novel ISM formulation can be simply prepared by a two-syringe system containing internal and external emulsion phases as described previously [24]. In this system, drug and polymer are dissolved in a biocompatible solvent. The solution is then emulsified into an external phase (oil or aqueous) containing a stabilizer to

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form emulsion droplets prior to injection into the body. Upon contact with the aqueous physiologic surroundings, solvent diffuses from the site and water permeates into the emulsion droplets causing the polymer emulsion droplets to solidify and harden to form microparticle walls. These multiple-unit ISM systems demonstrated regular and reproducible particle size and shape, therefore the risk of uncontrollable drug release patterns is minimized. Moreover, the presence of external oil phase in O/O-ISM systems has been reported to be useful in the reduction of initial drug burst release and toxicity of the solvent [23].

The ideal parenteral formulation is one which minimizes the extent of tissue damage, while at the same time providing the desired physicochemical and biopharmaceutical properties. Previously, our group has reported the myotoxicity of solvents used for the preparation of O/O-ISM systems and 2-pyrrolidone was the least myotoxic one compared with *N*-methyl-2-pyrrolidone and dimethyl sulfoxide [30]. Later we found that benzyl alcohol, ethyl acetate, propylene carbonate, triacetin and triethyl citrate were solvents which could be used to form microparticles. Ethyl acetate, triacetin [31] and propylene carbonate [32] have been reported as potential solvents used in the formation of in situ implants and microparticles for drugs such as doxycycline hyclate. Benzyl alcohol and triethyl citrate have been also investigated for the preparation of microparticles for diazepam [33] and leuprolide acetate [34], respectively. Metabolism as well as some toxicological data of these solvents have been reported earlier [35–37]. Although some toxicological data of the solvents have been reported, there is limited information available on the myotoxicity of these solvents used for the polymeric drug delivery systems administered intramuscularly.

From our previous investigation, five solvents: benzyl alcohol, ethyl acetate, propylene carbonate, triacetin and triethyl citrate were found as solvents with potential to be used in the preparation of O/W-ISM systems. Although these solvents have been widely investigated by many researchers and appeared in series of patents as solvents useful for the preparation of in situ implants (liquid gel) for several drug types, there is currently no commercial product using these solvents available because their toxicological data as well as myotoxicity are not known. The objective of this study was to evaluate benzyl alcohol, ethyl acetate, propylene carbonate, triacetin and triethyl citrate for their potential to cause acute skeletal muscle damage by using in vitro and in vivo myotoxicity protocols.

The myotoxicity was assessed by measuring the cumulative release of intracellular creatine kinase (CK) enzyme following an injection of the tested vehicles into rat extensor digitorum longus (EDL) muscle. The methods were well established and had been used to screen many pharmaceutical formulations for their potential to cause muscle tissue damage [38–46]. In this study, myotoxicity of pure solvent, emulsions, PLGA polymer solutions (in situ forming implants) was also compared with that of the O/W-ISM systems with varying internal to external phase ratios.

## 2. Materials and methods

### 2.1. Materials

The following chemicals were obtained from commercial suppliers and used as received: poly(D,L-lactic-co-glycolic acid) (75:25), Resomer® RG 752 (PLGA 752) $M_w$ 17,000; poly(D,L-lactic-co-glycolic acid) (50:50), Resomer® RG 503H (PLGA 503H), $M_w$ 33,400; poly(D,L-lactic-co-glycolic acid) (50:50), Resomer® RG 502H (PLGA 502H), $M_w$ 11,600, i.v. 0.2 were purchased from Boehringer Ingelheim, Ingelheim, Germany. Lutrol® F 68 (Pluronic F 68) (PF 68) was given by BASF, Ludwigshafen, Germany. Solvents: triacetin was from Eastman chemical, USA; triethyl citrate from Morflex, USA; propylene carbonate and benzyl alcohol were products from Merck, Germany; phenytoin, Dilantin®, Parke Davis, Berlin, Germany; isotonic normal saline (0.9% NaCl), Abott Laboratories, Chicago, USA. All chemicals were of at least reagent grades.

### 2.2. Methods

#### 2.2.1. Preparation of the tested formulations

Undiluted solvents, solvent mixture, polymer solution and O/W-ISM formulations were used to evaluate the myotoxic potential. The diluted solvents (benzyl alcohol, ethyl acetate, propylene carbonate, triacetin and triethyl citrate) were injected without any pretreatment. To further observe the possibility to reduce myotoxicity of the undiluted solvents, solvent mixtures were prepared by mixing each solvent using 0.5% PF 68 at a 1:4 ratio. Polymer solutions were prepared by dissolving 40% w/w of PLGA 752 in ethyl acetate (if not otherwise specified). These polymer solutions subsequently formed in situ implants after injection into muscles. The O/W-ISM formulations were prepared as described previously [24]. Briefly, a polymer solution was emulsified into an aqueous external phase of 0.5% PF 68 at the ratio of 1:1 and 1:4 in a two-syringe system. The emulsion droplets were achieved at 25 mixing cycles (approximately 1 cycle/s).

#### 2.2.2. Viscosity measurement

Viscosity was measured using a controlled stress mode of the plate and cone device connected with a computer interface (Rheostress RS 100, Haake Meβ-TechnikGmbH, Karlsruhe, Germany) at 20 °C ± 0.2. The viscosity of concentrated polymer solutions (30% w/w of PLGA 503H) was measured by the plate and cone of 20 mm diameter/4° angle with a fixed shear stress (10 Pa) for a constant time period (120 s). Whereas the viscosity of undiluted solvents was determined by using the plate and cone of 60 mm diameter/1° angle which is a more sensitive method for less viscous liquid. Data values were expressed as

means ± SD from three measurements of new sample of the same batch.

*2.2.3. Observation of droplet formations*

O/W-ISM formulations were prepared as described above. After emulsification of a polymer solution (30% w/w PLGA 502H in ethyl acetate or ethyl acetate–PEG 400) into an external aqueous phase containing a stabilizer (0.5% PF 68), emulsions were obtained. The emulsion of the ISM systems was placed on a glass slide and droplet formation and particle size was observed under an optical microscope (Axioskop, Carl Zeiss Jena GmbH, Jena, Germany).

*2.2.4. Creatine kinase activity in vitro interference assay*

All of the tested solvents were evaluated to determine whether they stimulated or inhibited CK activity. Briefly, as described previously [38–46], rabbit muscle CK Type I (Lyophilized form, Sigma Chemical Company, St. Louis, MO.) was prepared by dissolving approximately 1 mg of the enzyme in 10 mL of balanced salt solution (BSS) at pH 7.4. A given aliquot of this solution was spiked into incubation vessels containing the BSS at 37 °C and bubbled with 95% $O_2$/5% $CO_2$. The tested solution was added to the test incubation vessel, while the same volume of 0.9% sodium chloride injection served as control. The amount of CK, approximately 500 U/L, was the same in both the test and control incubation vessels. All studies were conducted for 30 min. CK was determined spectrophotometrically at 340 nm using a commercially available kit (Sigma Chemical Company, No. 47-UV), which is based upon the change in the absorbance caused by a reduction of $NAD^+$ to NADH.

*2.2.5. Animal procedures*

Myotoxicity studies were conducted using male Sprague–Dawley rats (3-months-old, 225–250 g). Myotoxicity protocols were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Florida in accordance with National Institute of Health Guidelines.

*2.2.5.1. In vitro myotoxicity studies.* Extensor digitorum longus (EDL) muscles (approximately 150 mg) were isolated from male Sprague–Dawley rats as previously described [38–47]. Briefly, rodents were administered an anesthetic dose of sodium pentobarbital and sacrificed via cervical dislocation. The EDL muscles were injected with the test solution or emulsion formulation (15 μL) using a 100 μL Hamilton syringe equipped with a needle guard to control the depth and angle of injection. The injected muscles were placed into a Teflon coated plastic basket and immersed in 9 mL of a carbogenated (95% $O_2$/5% $CO_2$) BSS. The solutions were drained and fresh BSS was added at 30-min intervals. The drained solutions at 30, 60, 90 and 120 min were analyzed for CK using a commercially available spectrophotometric kinetic assay (Sigma Chemical No. 47-20 UV). Myotoxicity was calculated from the cumulative sum of the CK values (U/L) over a 120-min period. Phenytoin (50 mg/mL in normal saline) and 0.9% normal saline served as positive and negative controls, respectively.

*2.2.5.2. In vivo myotoxicity studies.* In vivo myotoxicity studies were conducted using male rats as described previously [22,28,38]. Briefly, rats were catheterized and allowed to recover for 3 days prior to the study to allow CK-levels to stabilize at baseline. Following intramuscular injection (0.3 mL) in the right thigh muscle, blood samples (0.5 mL) were collected via the carotid artery at 0, 0.5, 1, 2, 4, 6, 8 and 12 h. The blood samples were centrifuged immediately and plasma was stored at −20 °C for analysis of CK level, while blood cells were reconstituted in heparinized (40 U/mL) normal saline solution (0.25 mL) and reinjected into the rat following the next sample to maintain blood volume. Myotoxicity was assessed by the area under the plasma CK curve.

*2.2.6. Data analysis*

Data values were presented as means and standard error of mean with $n = 6$ samples. Statistical analysis of cumulative CK activity in the different treatments was performed using unpaired $t$-test with $p < 0.05$ considered statistically significant difference.

## 3. Results and discussion

*3.1. Interference of assay*

The release of CK enzyme into buffer medium can be measured by a commercially available enzymatic kit. The spectrophotometric determination of CK activity was first described by Oliver in 1963 [43] and the method was subsequently optimized [44]. The assay was based on the principle that CK catalyzes the oxidation reactions and causes the changes in the absorbance at 340 nm. During this oxidation reaction, an equimolar amount of $NAD^+$ is reduced to NADH. The time dependent rate of absorbance change is directly proportional to CK activity.

Possible spectrometric and kinetic interference of the interested solvents was primarily tested to determine if they altered the activity of the CK. The degree of interference with CK activity was evaluated by measuring the CK activity in the presence of the tested solvent. The CK activity in the presence of 0.9% NaCl served as a control. The mean CK activity ratio was calculated from the CK activity value obtained in the presence of the tested solvent divided by the activity obtained from the control solution. If the CK activity ratio is not significantly different from 1, it is assumed there is no assay interference. Fig. 1 shows mean CK activity ratio of the tested solvents which was either one or slightly below. The interference assay indicated that none of the solvents significantly increased or decreased the activity of CK enzyme ($p > 0.05$).

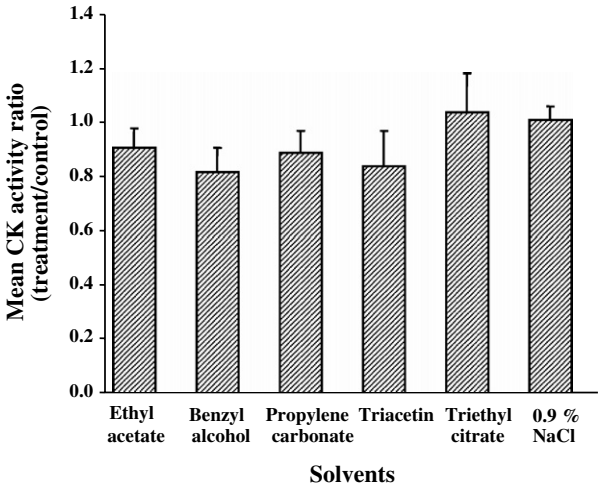


Fig. 1. In vitro interference assay of CK activity of tested solvents/control.

### 3.2. In vitro myotoxicity of pure solvent

In this study, the selected five solvents possible to be used in the formation of microparticles were tested for their potential to cause acute damage to muscles by using the in vitro myotoxicity protocol. Fig. 2 shows a linear CK release profile over 2 h from muscles treated with undiluted solvents. Muscles injected with isotonic normal saline solution were used as negative control to rule out muscle damage caused by needle puncture. The injection of phenytoin solution (positive control) was approximately 14 times more myotoxic than the negative control suggesting the potency to use the EDL muscle model to distinguish between the myotoxicity of different formulations by measuring the CK efflux. Cumulative release amount of the intracellular CK caused by benzyl alcohol, triethyl citrate and triacetin was even higher than that of the positive control indicating high potential damage of the solvents to the muscles when injected without any dilution. This result indicates the injection of in situ implant formulations composed of drug and polymer dissolved in the pure solvent such as triacetin as has been suggested by some researchers [3–21] is very myotoxic and can cause severe muscle damage at the injection site.

Benzyl alcohol noticeably caused the EDL muscles to turn pale immediately after injection and was ranked among the tested solvents as the most myotoxic one. Due to high toxicity, benzyl alcohol was not further investigated in this study. Propylene carbonate was slightly less myotoxic than the positive control. At 30 and 60 min, the cumulative CK release caused by propylene carbonate was statistically different from the positive control. However, after 90 and 120 min, the CK release from propylene carbonate was not different from the positive control with $p = 0.31$ and 0.85, respectively. Among all the tested solvents, only ethyl acetate showed low myotoxic potential. The cumulative CK release at 30, 60 and 90 min was not statistically different between the muscles treated with ethyl acetate and the negative control (0.9% NaCl). After 120 min, the cumulative CK following ethyl acetate treatment was slightly higher than the negative control ($p = 0.032$). This study suggested that there was a potential to develop the O/W-ISM systems using ethyl acetate due to relatively low toxicity of the solvent.

### 3.3. Viscosity of solvent and polymer solution

The relationship between the viscosity of the solvent and its myotoxicity was also determined. Fig. 3 depicts the viscosity of the tested solvents and 30% PLGA 503H solutions in each solvent type. The viscosity of the solvents was ranked from the least to most viscous one in the following order: ethyl acetate < propylene carbonate < benzyl alcohol < triacetin < triethyl citrate. The viscosity of the

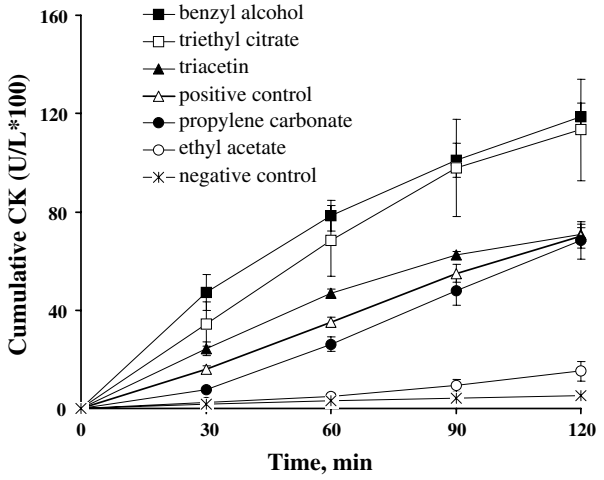


Fig. 2. Cumulative CK release after 120 min following the injection of undiluted solvents in comparison to the positive control (phenytoin) and the negative control (0.9% NaCl).

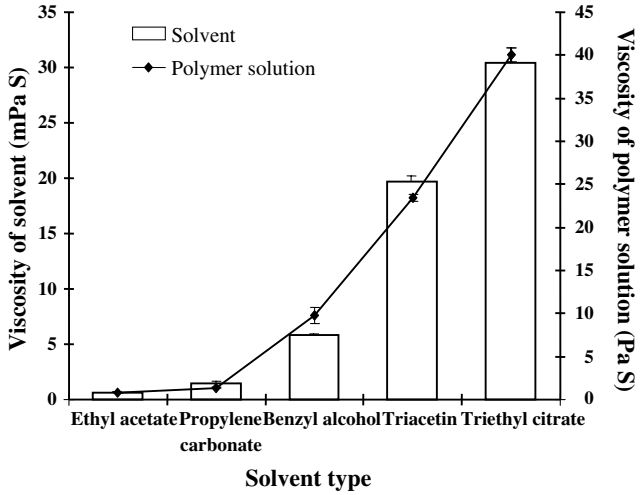


Fig. 3. Viscosity of solvents and 30% PLGA 503H solutions in each tested solvent at 20 °C.

polymer solutions was in the same rank order as the solvent alone. Surprisingly, the extent of myotoxicity has a tendency to follow the degree of viscosity of the solvent except for benzyl alcohol.

### 3.4. In vitro myotoxicity of formulations

In vitro myotoxicity of O/W-emulsions prepared from triethyl citrate, triacetin, propylene carbonate and ethyl acetate was investigated. The O/W-emulsions were prepared by emulsifying the solvent into 0.5% aqueous PF 68 solution at the internal to external phase ratio of 1:4. Fig. 4 shows emulsion formulations could reduce the myotoxicities of the pure solvent to approximately 24–65%. It is noteworthy to mention here that the CK release from muscles treated with the emulsions of ethyl acetate and aqueous pluronic solution (1:4) was not significantly different from that of the negative control ($p = 0.236$).

Fig. 5 shows the in vitro myotoxicity of ethyl acetate, 40% polymer solution in ethyl acetate and O/W-ISM with internal to external phase ratio of 1:1 and 1:4. As previously mentioned, ethyl acetate was less myotoxic and comparable to the negative control. There was a trend of myotoxicity rank order as follows: ethyl acetate >40% polymer solution > O/W-ISM phase ratio 1:1 > O/W-ISM phase ratio 1:4. The cumulative CK release from the muscle treated with the O/W-ISM phase ratio 1:4 was not statistically different from that of the muscles treated with the negative control. This confirmed that O/W-ISM systems using ethyl acetate with the phase ratio of 1:4 gave low myotoxic potential. This finding showed the advantage of the ISM formulations over the in situ implants. In the ISM system, solvent is diluted to the external phase 1–10 times, thus irritation of the muscle cells at the site of injection is significantly reduced.

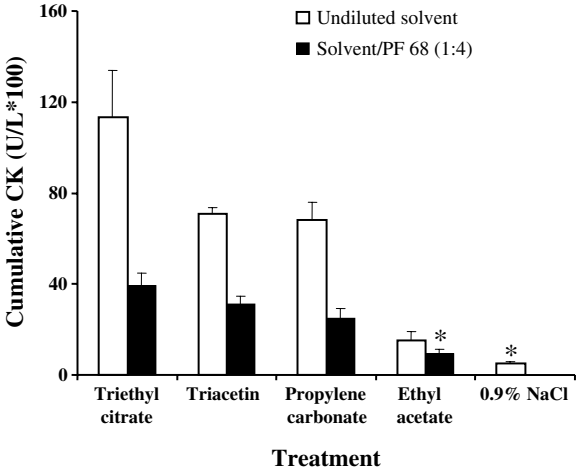


Fig. 4. Cumulative CK release after 120 min following the injection of dilution of pure solvent and emulsions of solvent/0.5% PF 68 solution (1:4) in comparison to the negative control (0.9% NaCl).

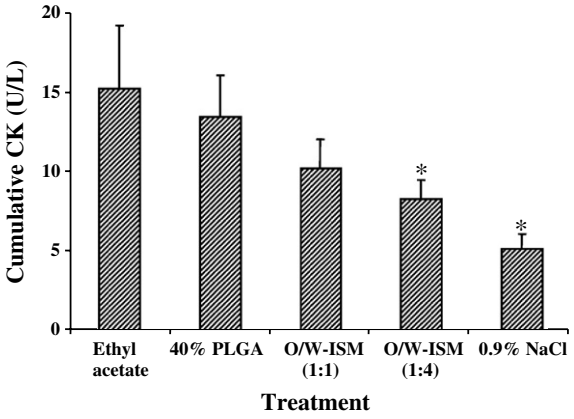


Fig. 5. Cumulative CK release after 120 min following the injection of pure solvent, polymer solution and O/W-ISM systems with phase ratio of 1:1 and 1:4 in comparison to the negative control (0.9% NaCl).

### 3.5. In vivo myotoxicity of in situ forming formulations

While the in vitro myotoxicity can be used to determine the acute effect of formulations on muscle tissue, these methods cannot be utilized to investigate long-acting or repeated injection formulations for their potential to cause tissue damage at an intramuscular site. Therefore, in some circumstances, it is necessary to employ the in vivo release of cytosolic enzymes as a marker of tissue damage caused by parenteral products. The major advantage of this type of experimental system is that the nervous, vascular, and immune systems associated with skeletal muscle remain intact and can contribute to parenteral product-induced tissue toxicity [35,39].

Formulations tested by in vivo myotoxicity model were selected based on the in vitro myotoxicity data. The area under plasma CK for 12 h was used to assess muscle damage. The myotoxicity of injection of positive control (phenytoin) was approximately 6.9 times higher than the myotoxicity of an injection of the negative control

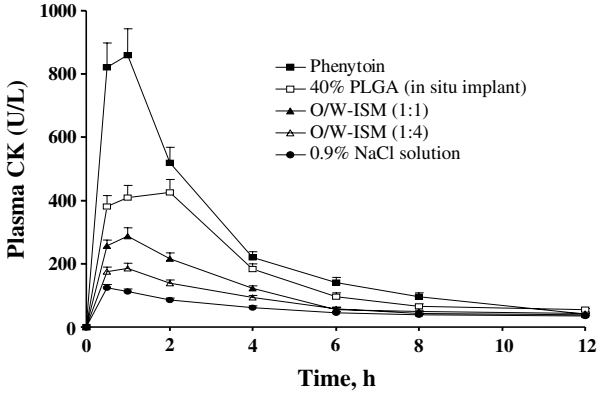


Fig. 6. Mean plasma CK-levels versus time following the intramuscular injection of 40% PLGA 752 in ethyl acetate (in situ implant), O/W-ISM systems (40% PLGA 752 in ethyl acetate, polymer solution to aqueous pluronic F68 phase ratio 1:1 and 1:4), phenytoin (positive control) and 0.9% NaCl (negative control).

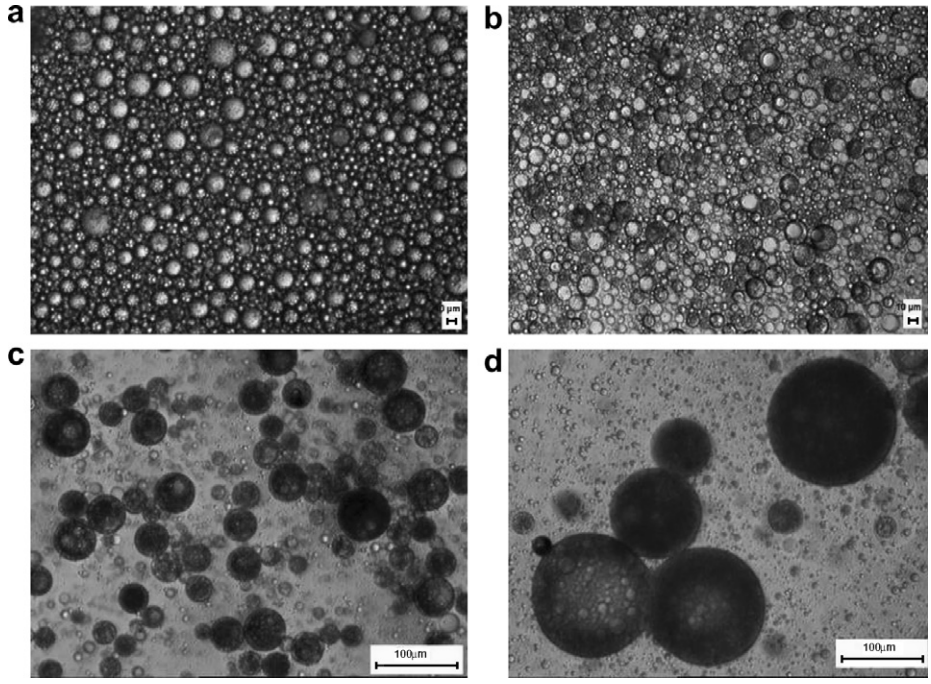


Fig. 7. Microscopic pictures of O/W-ISM formulation with varying solvent systems for polymer. Internal phase: 40% PLGA 502H in solvent; External phase: 0.5% Lutrol F 68; Phase ratio 1:1; (a) 60% ethyl acetate; (b) 40% ethyl acetate/20% PEG 400; (c) 30% ethyl acetate/30% PEG 400 and (d) 20% ethyl acetate/40% PEG 400. Magnification 20×.

($860 \pm 82$ vs. $125 \pm 9.2$) (Fig. 6). All of the peak plasma CK-levels occurred at 0.5–2 h and fell off to normal level 6 h after the injection which was in close agreement with a previously published article [38]. The injection of polymer solution (40% PLGA 752 in ethyl acetate) resulted in the peak plasma CK-levels of 425 U/L after 2 h while that from the O/W-ISM with phase ratio 1:1 was 288 U/L after 1 h. Increasing the amount of the external aqueous phase of the O/W-ISM from 1:1 to 1:4 could reduce the myotoxicity to approximately half of the polymer solution. The plasma CK curve of the O/W-ISM with phase ratio 1:4 was only slightly higher than that of the negative control ($185.87 \pm 16.33$ for O/W-ISM vs. $125 \pm 9.2$ for the isotonic normal saline solution). The in vivo toxicity results correlated very well with the in vitro model, thus providing their values for rapid screening formulations.

### 3.6. Formation of ISM with ethyl acetate and cosolvent

Previous results suggested that the ISM systems prepared by ethyl acetate were well tolerated in the muscular tissues at the injection sites. Apart from the low myotoxic potential of ethyl acetate, its solubility in water at approximately 8–10% [45] allows spherical microparticles to form and solidify in situ. However, ethyl acetate cannot dissolve some hydrophilic active compounds, therefore a use of cosolvent in the ISM formulations may be necessary.

An example of cosolvent investigated for the formation of the O/W-ISM was PEG 400. The solvent has been approved to be used in the injectable formulation up to 50% w/w [46]. Cumulative CK released from muscles treated with 20 and 50% PEG 400 water mixture has been reported to be not significantly different from that treated with isotonic sodium chloride solution [36]. The use of regulatory authority approved solvent such as PEG 400 which was also reported as low myotoxic in the formation of O/W-ISM can also dilute the amount of ethyl acetate used.

Fig. 7a shows a microscopic picture of microparticles obtained from the O/W-ISM systems using ethyl acetate as the solvent. The formulation was prepared by emulsifying 40% PLGA 502H in ethyl acetate into 0.5% aqueous Pluronic F 68 with the internal to external phase ratio of 1:1. The obtained microparticle size was in the range of 5–20 μm. Replacing 20% of ethyl acetate with PEG 400 did not affect the resulted particle size, but was expected to modify the drug release profile due to a pore-forming property of the PEG 400 (Fig. 7b). This could be beneficial especially for active agents with large molecular size such as proteins, which diffuse through the polymer network very slowly. Increasing the amount of PEG 400 to 50% obviously resulted in larger microparticle size of approximately 20–50 μm (Fig. 7c). This was probably due to the higher viscosity of PEG 400 in comparison to ethyl acetate (105.95 mPa S vs. 0.59 mPa S) and thereby producing larger particle size. Microparticle size was the largest (100–200 μm) when 40% of PEG 400 was used to substitute ethyl acetate in the formulation (Fig. 7d).

## 4. Conclusions

The in vitro myotoxicity method with the use of isolated rodent skeletal muscle system can provide the pharmaceutical

formulator a means to quickly and directly investigate the effect of an injectable or its components to cause muscle damage using the release of CK into an isolated bath [38]. In this study, the in vitro myotoxicity was used to screen the potential of different solvents used in the preparation of polymer solution and the O/W-ISM systems to damage muscle tissues. Results showed that benzyl alcohol, triethyl citrate, and triacetin gave higher toxic potential than the positive control while propylene carbonate was slightly more toxic than the positive control. Among investigated solvents, ethyl acetate was found to be comparable to the isotonic sodium chloride solution. Further reduction of ethyl acetate toxicity can be achieved by the use of a lower internal to external phase ratio as well as substitution of the solvent amount by other regulatory authority approved solvents such as PEG 400. The in situ forming formulations were found to be much less myotoxic than the polymeric liquid implant because the solvent has been diluted with the external aqueous phase 1–10 times. The low myotoxicity of the ISM systems with the dilution of external phase was confirmed by the in vivo myotoxicity data. The results suggested that the ISM formulations were interesting drug delivery systems which were well tolerated in muscle tissues. For the commercial application of these solvents and the systems, long term and chronical administration should be further investigated.

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