International Journal of Pharmaceutics 434 (2012) 243–250

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Fully biodegradable and cationic poly(amino oxalate) particles for the treatment of acetaminophen-induced acute liver failure

Hyungmin Kim[a], Yerang Kim[a], Kyeonghye Guk[b], Donghyuck Yoo[a], Hyungsuk Lim[a], Gilson Kang[b], Dongwon Lee[a,b,*]

[a] Department of BIN Fusion Technology, Chonbuk National University, Jeonju 561-756, Republic of Korea
[b] Polymer Fusion Research Center, Department of Polymer Nano Science and Technology, Chonbuk National University, Jeonju 561-756, Republic of Korea

ARTICLE INFO

Article history:
Received 26 April 2012
Received in revised form 23 May 2012
Accepted 27 May 2012
Available online 2 June 2012

Keywords:
Acute liver failure
Inflammation
Poly(amino oxalate)
Drug delivery
Microparticles

ABSTRACT

Acute inflammatory diseases are one of major causes of death in the world and there is great need for developing drug delivery systems that can target drugs to macrophages and enhance their therapeutic efficacy. Poly(amino oxalate) (PAOX) is a new family of fully biodegradable polymer that possesses tertiary amine groups in its backbone and has rapid hydrolytic degradation. In this study, we developed PAOX particles as drug delivery systems for treating acute liver failure (ALF) by taking the advantages of the natural propensity of particulate drug delivery systems to localize to the mononuclear phagocyte system, particularly to liver macrophages. PAOX particles showed a fast drug release kinetics and excellent biocompatibility *in vitro* and *in vivo*. A majority of PAOX particles were accumulated in liver, providing a rational strategy for effective treatment of ALF. A mouse model of acetaminophen (APAP)-induced ALF was used to evaluate the potential of PAOX particles using pentoxifylline (PTX) as a model drug. Treatment of PTX-loaded PAOX particles significantly reduced the activity of alanine transaminase (ALT) and inhibited hepatic cell damages in APAP-intoxicated mice. The high therapeutic efficacy of PTX-loaded PAOX particles for ALF treatment may be attributed to the unique properties of PAOX particles, which can target passively liver, stimulate cellular uptake and trigger a colloid osmotic disruption of the phagosome to release encapsulated PTX into the cytosol. Taken together, we believe that PAOX particles are a promising drug delivery candidate for the treatment of acute inflammatory diseases.

1. Introduction

Acute liver failure (ALF) is defined as the sudden loss of hepatocellular functions rapidly developed without preexisting liver injury and indicates that liver loses 80–90% functions of hepatic cells (Gill and Sterling, 2001; Ko et al., 2008; O'Grady, 2005). ALF is a complex multi-systemic illness and is more dramatic or more devastating than any other conditions in medicine, responsible for thousands of death each year in world (Lee et al., 2009; Lee, 1993). The common causes of ALF include viral hepatitis, drug- or toxin-induced liver diseases and metabolic disorders (Gill and Sterling, 2001; Ko et al., 2008). Among them, acetaminophen (APAP) overdose has recently been the most frequent cause of ALF in United States and most of European countries (Perkins, 2006). APAP intoxication accounts for more than 50% of all ALF cases in the United States, with a mortality of 30% (Larson et al., 2005; Ostapowicz et al., 2002; Williams et al., 2011). APAP is the most widely used analgesic and its overdose, intentional or unintentional, is known to cause massive hepatocellular apoptosis and hemorrhagic necrosis, leading to ALF (Stuart et al., 2011). Recognition of early stages of ALF is difficult and therefore initiation of treatment is frequently delayed (Ko et al., 2008; Larson et al., 2005). For these reasons, at the time of ALF diagnosis, significant tissue damages have already occurred and hepatic functions are rapidly deteriorating (Ogrady et al., 1993; Yang et al., 2008). Therefore, rapid and efficient therapeutics are critical in management of patients with ALF.

Macrophages resident in liver tissues are called Kupffer cells and represent an essential component of innate immunity (Bilzer et al., 2006). Kupffer cells are inflammation regulating liver cells, responding to potentially dangerous stimuli, such as bacteria, bacterial endotoxins and microbial debris derived from the gastrointestinal track and toxic chemical substances transported to liver. Macrophages play a central role in the establishment of APAP-induced ALF by producing various reactive oxygen and nitrogen species and pro-inflammatory cytokines, which regulate the phenotypes of macrophage themselves and neighboring cells and also mediate inflammatory responses (Bilzer et al., 2006;

* Corresponding author at: Polymer Fusion Research Center, Department of Polymer Nano Science and Technology, Chonbuk National University, Jeonju 561-756, Republic of Korea. Tel.: +82 63 270 2344; fax: +82 63 270 2341.
E-mail address: dlee@chonbuk.ac.kr (D. Lee).

0378-5173/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ijpharm.2012.05.067

Ishida et al., 2004; Ma et al., 2006). The tumor necrosis factor-alpha (TNF-α) produced by activated macrophages is a pleiotropic cytokine that plays a significant pathogenic role in APAP-induced hepatotoxicity and can even induce cell death in the setting of toxic insult (Das et al., 2010; Lee et al., 2009; Stuart et al., 2011). There are several studies reporting that the high level of TNF-α can induce the generation of a massive amount of nitric oxide that is one of the essential mediators in APAP-induced hepatotoxicity (Muriel, 2000; Waters et al., 2001). Reactive oxygen species produced by Kupffer cells are also known to mediate oxidative damage by attacking lipid membranes, protein and DNA, which in turn disrupts cellular functions and integrity (Das et al., 2010; Ma et al., 2006; Yen et al., 2009). Therefore, macrophages have great potential to serve as a target of therapeutics for the APAP-induced ALF.

There has been great interest in the use of antioxidant and anti-inflammatory drugs for the prevention and treatment of APAP-induced ALF (Das et al., 2010; Ferret et al., 2001; Lee et al., 2009; Ma et al., 2006; Waters et al., 2001; Yen et al., 2009). However, their therapeutic applications have been curtailed by inconstant effectiveness resulting from low bioavailability and lack of ability to target macrophages (Ferret et al., 2001; Hu et al., 2010; Khaja et al., 2007). Nano- or microparticles based on various biodegradable polymers have been widely used for controlled and sustained releases of therapeutics owing to their biocompatibility, safety profiles and easy manipulation of degradation rate (Heffernan and Murthy, 2005; Kim et al., 2010; Seong et al., 2011). In order to enhance the therapeutic efficacy in the treatment of ALF, it is greatly necessary for developing drug delivery systems that target drugs to macrophages and rapidly release them, allowing the rapid onset of therapeutic action (Lee et al., 2009). Polymers possessing ester linkages such as poly(lactic-co-glycolic acid) (PLGA) and poly(caprolactone) (PCL) have been extensively used as drug delivery systems, but their applications are problematic for treating inflammation-associated diseases such as ALF because of their slow hydrolytic kinetics. In addition, the acidic degradation products of PLGA are known to frequently cause inflammation (Seong et al., 2011; Yang et al., 2008). Consequently, great efforts have recently been made to develop new biodegradable polymer-based drug delivery systems that can target drugs to specific tissues and release them rapidly in damaged liver.

We have recently developed cationic fully biodegradable drug delivery systems based on poly(amino oxalate) (PAOX) that contains peroxalate linkages and tertiary amine groups in its backbone (Seong et al., 2011). PAOX particles had excellent biocompatibility and exhibited fast drug release profiles due to its hydrophilic nature resulting from the presence of tertiary amine groups. In addition, PAOX particles had ability to disrupt endosomes via "proton sponge effects", which mediates the enhanced cytosolic delivery of membrane-impermeable drug payloads. It has been well known that macrophages readily phagocytose foreign matter in the range of 0.5–3.0 μm and particulate drug delivery systems have natural tendency to localize to mononuclear phagocyte system (MPS), particularly to liver and spleen macrophages (Khaja et al., 2007; Park et al., 2010; Yang et al., 2008). We therefore hypothesized that submicron PAOX particles may be suitable as a drug carrier for treating inflammation-associated diseases such as ALF.

The primary objectives of the present study are to evaluate the potential of PAOX particles as a drug delivery system for the treatment of ALF using pentoxifylline (PTX) as a model drug. PTX-loaded PAOX particles were prepared by a single emulsion method and their physicobiological properties were studied. Their biocompatibility and drug release profile were investigated and their therapeutic efficacy was studied using a mouse model of APAP-induced ALF.

2. Materials and methods

2.1. Polymer synthesis

PAOX was synthesized as previously reported (Seong et al., 2011). In brief, 1,4-cyclohexanediethanol and piperazinediethanol were dissolved in dry dichloromethane (DCM), under nitrogen, to which pyridine was added dropwise. The mixture was added oxalyl chloride in dry DCM at 4 °C. The reaction was allowed under nitrogen atmosphere at room temperature for 7 h and quenched with a brine solution. The resulting polymers were extracted with a large excess of DCM. The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated under vacuum. The obtained polymer was isolated by the precipitation in cold hexane (yield > 70%). The chemical structure of polymers was identified with a 400 MHz ^{1}H NMR spectrometer (JNM-EX400, JEOL, Japan).

2.2. Preparation and characterization of PTX-loaded PAOX particles

PTX-loaded PAOX particles were prepared by a single emulsion method. Twenty milligrams of pentoxifylline (Sigma–Aldrich, St. Louis, MO) and 100 mg of PAOX were dissolved in 1 mL of DCM. The solution was added into 10 mL of 5% (w/w) aqueous PVA (polyvinyl alcohol) solution and the mixture was sonicated using Sonic Dismembrator (Model 500, Pittsburgh, PA) for 40 s, followed by homogenization (PRO Scientific, PRO 200, Oxford, CT) for 1 min. The resulting o/w emulsion was stirred to evaporate the solvent for 15 min at room temperature. PTX-loaded PAOX particles were obtained by the centrifugation at 11,000 × *g* for 5 min at 4 °C followed by lyophilization of the recovered pellets. It was determined that 1 mg of PAOX particles encapsulate ~10 μg of PTX and the drug encapsulation efficiency is ~10%. Empty PAOX particles were prepared by a single emulsion method. The SEM images of PAOX particles were made using a scanning electron microscope (JSM-6400, JEOL, Japan). Their particle size was measured by dynamic light scattering using a particle analyzer (ELS-6000, Photal Otsuka Electronics, Japan).

2.3. Drug release kinetics of PAOX particles

A total of 50 mg of PTX-loaded PAOX particles was placed in a test tube containing 10 mL of phosphate buffered solution (PBS, pH 7.4). The tube was incubated at 37 °C with continuous shaking. At appropriate intervals, the tube was centrifuged at 1000 × *g* for 5 min. An 1 mL aliquot of supernatant was taken and replaced with an equal volume of fresh PBS. The concentration of PTX in the supernatant was measured using a liquid chromatography (Futecs, Korea) and the release kinetics was determined by comparing the concentrations of PTX standard solutions.

2.4. Cytotoxicity assay of PTX-loaded PAOX particles

The cytotoxicity of PTX-loaded PAOX particles was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. RAW 264.7 macrophage cells in a 24 well plate were with ~90% confluency were treated with various amounts of particles (10–1000 μg/mL) and incubated for 40 h. Each well was given 100 μL of MTT solution and was incubated for 4 h. Dimethyl sulfoxide (1 mL) was added to cells to dissolve the resulting formazan crystals. After 10 min of incubation, the absorbance at 570 nm was measured using a microplate reader (Synergy MX, BioTek Instruments, Inc., Winooski, VT). The cell viability was determined by comparing the absorbance of nanoparticles-treated cells to that of control cells.

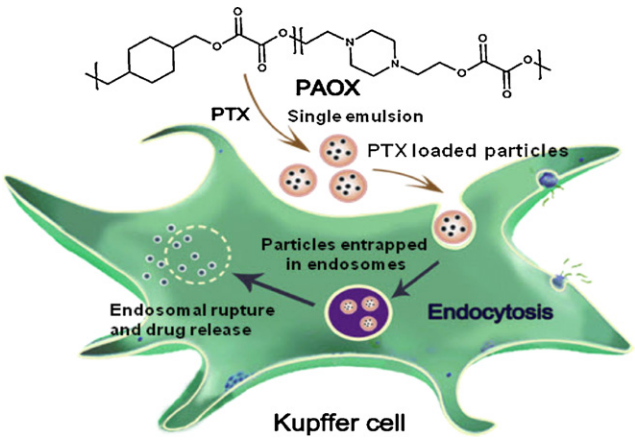

Fig. 1. A schematic diagram of enhanced cytosolic drug delivery by PAOX particles.

2.5. Confocal laser scanning microscopy

RAW 264.7 cells cultured on a glass bottom dish (MatTek Corp., Ashland, MA) were treated with 500 μL of calcein-loaded PAOX particle (1 mg/mL in PBS) suspension and LysoTracker Red (Molecular Probes, Eugene, OR) for 30 min. Cell culture media were removed and cells were washed with fresh media twice. Fluorescence images of cells were made at 2, 6 and 12 h post-incubation using a confocal scanning microscope (Carl Zeiss, Inc., Germany).

2.6. In vivo host tissue responses

Six week old BALB/c mice (~20 g) obtained from Orient Bio, Seoul were anesthetized by an intraperitoneal injection of a mixture of Zoletil 50 and Domitor. Thighs were shaved and 200 μL of the suspension of PAOX or PLGA particles in PBS (10 mg/mL) was injected into the muscle. For histological examinations, mice were euthanized and muscle tissues injected with particles were removed at 5 days post-injection. The tissues were fixed with 4% formalin (Sigma–Aldrich, St. Louis, MO) and embedded into paraffin. Histological sections were made and stained with H&E (hematoxylin and eosin, YD Diagnostics, Korea) and anti CD 68 antibody (Abcam, Cambridge, MA). All experiment procedures were performed with the approval of Chonbuk National University Animal Care Committee.

2.7. In vivo biodistribution of PAOX particles

The biodistribution of PAOX particles was determined using rubrene-loaded PAOX particles. Rubrene-loaded PAOX particles were formulated with the same procedure for PTX-loaded PAOX particles as described above. Mice were injected 200 μL of rubrene-loaded PAOX (10 mg/mL in PBS) via tail vein injection and then sacrificed after 2 h. Immediately after the collection of organs, their fluorescence images were made using an IVIS imaging system (Xenogen, Alameda, CA). The organs were homogenized in a solution of 20% (v/v) Triton X-100 and 80% (v/v) PBS (pH 7.4) for 2 min and centrifuged at 5000 × *g* for 5 min. The fluorescence of rubrene in the supernatant was measured at 480 nm excitation and 554 nm excitation using a fluorospectrometer (Jasco, FP6500, Japan). The fluorescence from the organs removed from saline-injected mice was used as background and the relative fluorescence intensity of each organ was determined based on the organ weight.

2.8. Animal experiments

Mice were injected with 200 μL of PTX-loaded PAOX particles (10 mg/mL) and after 1 h ALF was achieved by intraperitoneal injection of 200 μL of APAP (25 mg/mL). Mice were sacrificed 10 h after APAP intoxication and whole blood and livers were collected. The activity of ALT in serum was determined using an ALT enzymatic assay kit (Bioo Scientific Corp., Austin, TX). The livers tissues were fixed with 4% formalin (Sigma–Aldrich, St. Louis, MO) and embedded into paraffin. Histological sections were made and stained with hematoxylin and eosin (H&E) and anti Terminal Deoxynucleotidyl Transferase, Recombinant (rTdT) (DeadEnd™ Fluorometric TUNEL System, Promega, Madison, WI).

3. Results and discussion

3.1. PTX-loaded PAOX particles

As illustrated in Fig. 1, PTX-loaded PAOX particles were formulated via a single emulsion method, which allowed PTX to be encapsulated in the interior of the particles. The prepared particles were freeze-dried and stored as a solid powder. The PTX-loaded PAOX particles were polydispersed round spheres with smooth surface, evidenced by SEM. Dynamic light scattering revealed that they have an average diameter of ~450 nm (Fig. 2). There was no remarkable difference from empty PAOX particles (data not shown). It is reasonable to expect that PAOX particles are useful for drug delivery involving phagocytosis because macrophages easily phagocytose particulate drug delivery systems with submicron size, up to 3 μm (Khaja et al., 2007; Moghimi et al., 2005; Park et al., 2010). We also found that the drug loading efficiency of the PTX-loaded PAOX particles was ~10%. The relatively low PTX loading efficiency of PAOX particles is due mainly to water solubility of PTX (Otsuka and Matsuda, 1994). It appears that during the particle formulation by a conventional single emulsion method using water and DCM, a small fraction of PTX in DCM was encapsulated in the PAOX particles and a majority of PTX was dissolved in water phase. It was also determined that 1 mg of PAOX particles encapsulate ~10 μg of PTX.

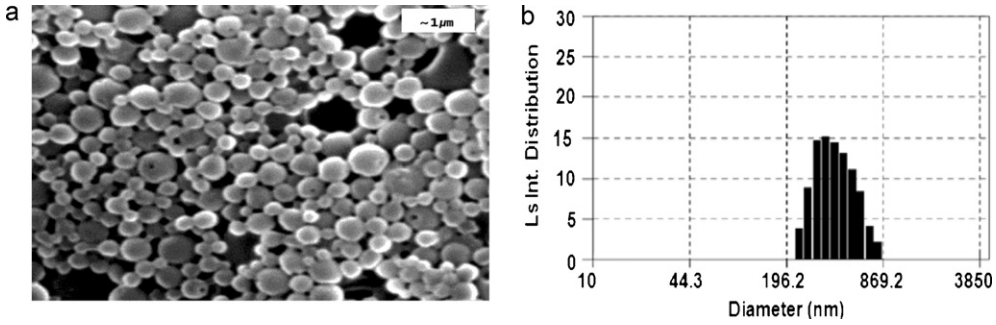

Fig. 2. Characterization of PTX-loaded PAOX particles. (a) A representative SEM image and (b) dynamic light scattering.

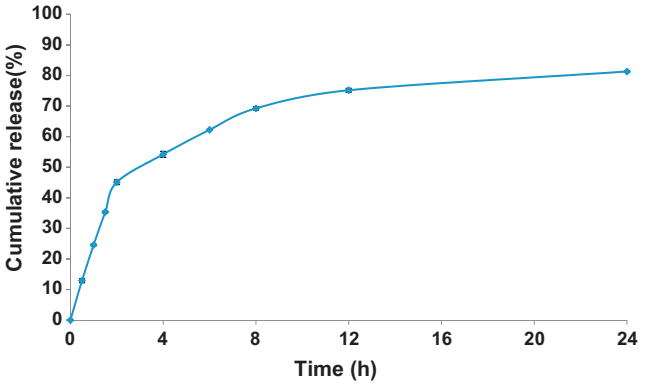

Fig. 3. Release rate of PTX from PAOX particles. Mean ± S.D., $n = 3$.

3.2. Drug release kinetics of PTX-loaded PAOX particles

In vitro drug release profiles of PAOX particles were investigated to determine whether PAOX particles are suitable as drug delivery systems for acute inflammatory diseases, which require fast onset of pharmaceutical action. Previously, it was reported that the tertiary amine groups in its back account for pH-dependent hydrolysis and enhance the hydrophilic nature, accelerating its hydrolytic degradation. PAOX exhibited a half-life of hydrolysis ~20 h at pH 5.5 and ~50 h at pH 7.4 (Seong et al., 2011). Fig. 3 shows that PTX was released rapidly at pH 7.4 from PAOX particles with an initial burst as high as 45% over the first 4 h. The initial burst was followed by slow release and 80% of PTX was released within 12 h. Thus, PAOX should be able to release drug payloads rapidly after phagocytosis, suggesting that PAOX particles have great potential as drug carriers for the treatment of acute inflammatory diseases such as ALF.

3.3. Cellular uptake of PAOX particles

After phagocytosis of drug delivery vehicles, drug payloads should be rapidly released from the endosomal compartments (phagosomes) which are degradation machinery of cells with a pH as low as 4.5 (Hu et al., 2007; Seong et al., 2011), as illustrated in Fig. 1. In order to confirm whether PAOX particles are readily taken up by macrophages and facilitate the drug release from phagosomes, the internalization and cellular trafficking of PAOX particles were observed using a membrane impermeable calcein as a fluorescent probe. LysoTracker Red, a pH-sensitive fluorescent marker, was also used to label late endosomes and lysosomes during the PAOX particle uptake. RAW 264.7 cells were treated with LysoTracker Red for 30 min and then incubated with calcein-loaded PAOX particles for 2 and 6 h. For comparison purposes, cells were treated with free calcein only. Fig. 4 shows the confocal fluorescence micrographs of cells treated with calcein-loaded PAOX particles and LysoTracker. Cells treated with free calcein alone showed a punctuate distribution of green (calcein) and red (LysoTracker) fluorescence even after 12 h. The intensity of fluorescence of calcein and LysoTracker decreased with time. Strong yellow fluorescence was observed in the merged image because of colocalization of calcein and LysoTracker in late endosomal compartments in the periphery of cells, suggesting that endosomes retained calcein and LysoTracker inside and were not disrupted. In contrast, cells treated with calcein-loaded PAOX particles showed endosomal escape of calcein in a time dependent manner. At 2 h post-incubation, colocalization of calcein and LysoTracker was observed in endosomes, evidenced by yellow fluorescence. However, calcein released from PAOX particles diffused into cytosol and weak green fluorescence became observed in cytosol. The green fluorescence in cytosol became enhanced with time, indicating the more release of calcein to cytosol. At 6 h, majority of calcein was released into cytosol and the red fluorescence intensity of LysoTracker became very weak because LysoTracker that reached the cytosol lost its fluorescence under neutral conditions. Therefore, very weak yellow fluorescence was observed in the periphery of cells. However, cells treated with calcein-loaded PLGA particles show the colocalization of green and red fluorescence in the periphery of cells even at 6 h after incubation, suggesting no of less endosomal escapes of calcein. LysoTracker was also retained in the endosomes and its red fluorescence still remained high at 6 h, indicating the endosomes were not disrupted. These observations demonstrate that PAOX particles enhance the endosomal escape and diffusion of drug payloads into cytosol. As previously reported, the enhanced cytosolic delivery of PAOX particles can be explained by the rapid hydrolytic degradation and rapid endosome disruption via proton sponge effects and osmotic pressure buildup (Lee et al., 2009; Seong et al., 2011).

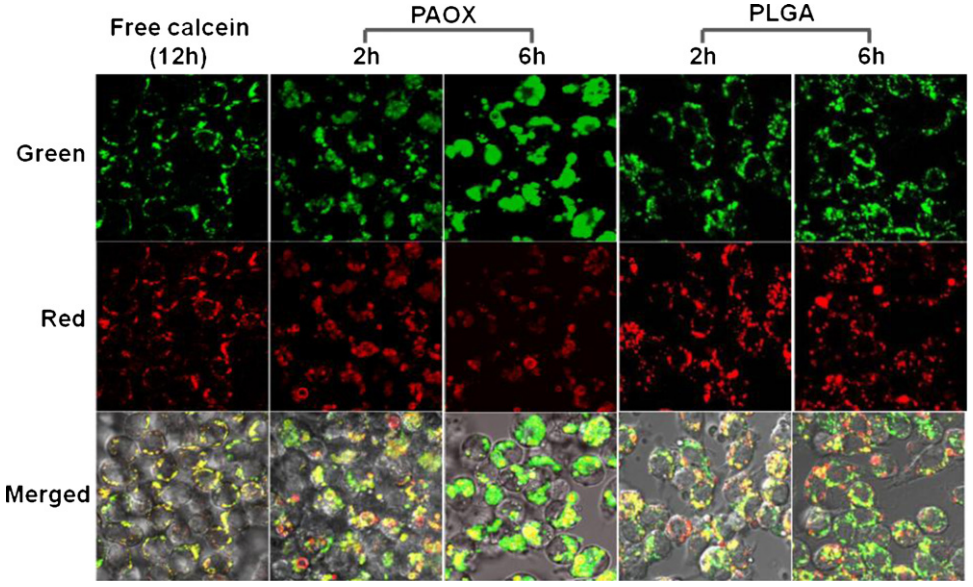

Fig. 4. Confocal fluorescence micrographs of cells treated with calcein-loaded PAOX particles in the presence of LysoTracker Red.

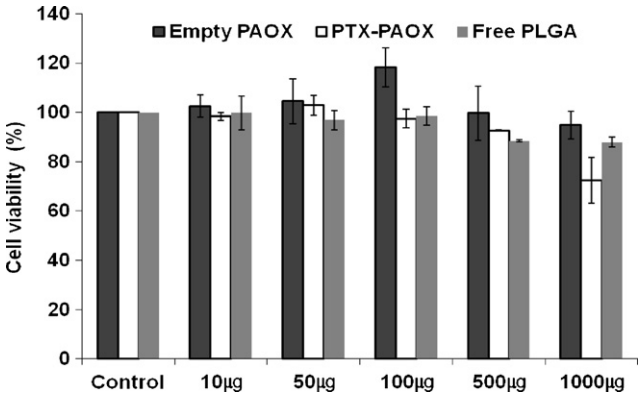

Fig. 5. Cell viability after incubation with PAOX particles determined by MTT assay. Mean ± S.D., $n = 4$.

3.4. Biocompatibility of PAOX particles

Cytotoxicity of PAOX particles was evaluated because cytotoxicity is one of critical concerns in development of drug delivery systems. RAW 264.7 cells were incubated with various amounts of PTX-loaded PAOX particles for 40 h and their viability was determined by MTT assay (Fig. 5). Both empty PAOX and PLGA particles showed negligible cytotoxicity at the concentration of up to 1 mg/mL. PTX-loaded PAOX particles also showed no or minimal cytotoxicity. The results demonstrate that PAOX has excellent biocompatibility *in vitro* and great potential as a drug delivery system.

Foreign-body responses by host immune systems is one of key issues of developing particulate drug delivery systems (Sy et al., 2008). The innate immune system plays an important role in determining the course of potential immune responses to foreign matter such as particulate drug delivery systems. Immune cells, especially macrophages, are recruited to the area of particulate injection and remain there until particulates are phagocytosed and metabolized, compromising the functions of particulates (Sy et al., 2008). We therefore assessed the tissue compatibility of PAOX particles. PAOX particles or PLGA particles were injected into thigh muscle of mice and mice were sacrificed after 5 days. Histological sections of the injection site were made and stained for H&E and ED1 which is an inflammatory cell marker (Ko et al., 2007; Meinel et al., 2005; Song et al., 2011). As shown in Fig. 6, H&E staining revealed that PLGA particles triggered acute inflammatory responses, evidenced by the recruitment of macrophages and multinucleated giant cells near the injection site. However, significantly less inflammatory responses were observed around PAOX particles. The bottom panel of Fig. 6 shows the histological sections stained with ED1 antibody. PAOX particles generated no or little recruitment of ED1 positive cells. In contrast, PLGA particles induced a large infiltration of ED1-positive cells, which is in agreement of a previous studies demonstrating *in vivo* inflammation induced by PLGA particles and PLGA films (Meinel et al., 2005; Song et al., 2011; Sy et al., 2008). Severe foreign-body responses were observed with PLGA particles that have acidic degradation products (Sy et al., 2008). We previously reported that PAOX has a hydrolysis half-life of ~50 h at pH 7.4 and therefore a majority of PAOX molecules formulating particles are expected to degrade into small molecules after 5 days of intramuscular injection. We found that minimal or no inflammatory responses were induced by PAOX particles. Therefore, our findings suggest that PAOX does not produce toxic degradation products and has excellent tissue biocompatibility, demonstrating the tremendous potential of PAOX as new family of biodegradable polymer.

3.5. Biodistribution of PAOX particles

The biodistribution of PAOX particles was investigated using rubrene as a fluorophore. Two hours after intravenous injection of rubrene-loaded PAOX particles, organs were collected and their relative fluorescence intensity was determined using a fluorospectrometer. As shown in Fig. 7, the majority of PAOX particles were accumulated in liver. PAOX particles were also accumulated in other organs such as lung, kidney, spleen and heart. However, the amount of PAOX particles accumulated in liver is more than the total of those accumulated in the other organs. It is not surprising because of the facts that the largest population of macrophages in contact with blood is located in liver sinuses (Kupffer cells) and particulates are localized to liver macrophages that have a primary scavenging role for foreign invaders. The ability of PAOX particles to target liver, passively but specifically, is similar to the natural tendency of particulate drug delivery systems.

In general, particulate drug delivery systems administrated intravenously are opsonized and rapidly and predominantly

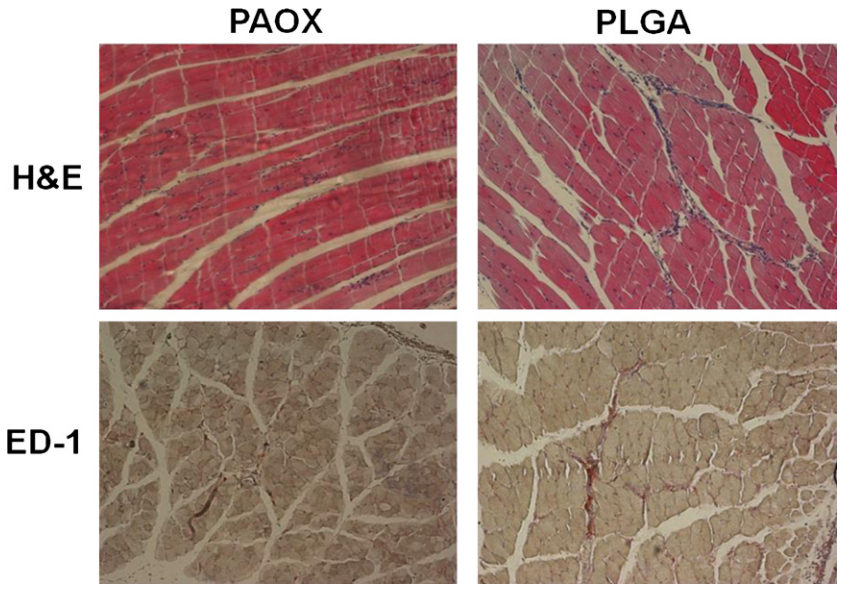

Fig. 6. Tissue compatibility of PAOX particles.

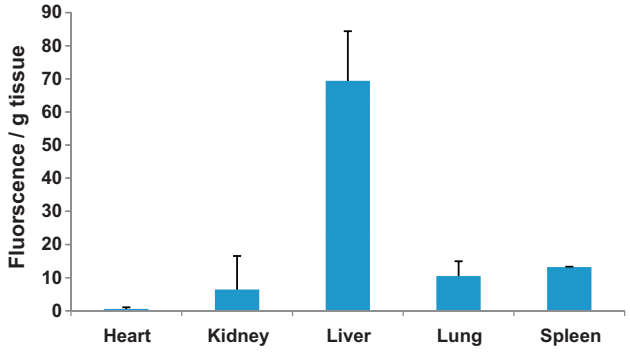

Fig. 7. Biodistribution of PAOX particles in mice. Mean ± S.D., $n = 3$.

intercepted by mononuclear phagocyte system (MPS), particularly to liver and spleen macrophages, leading to clearance from circulation. In addition, it was reported that macrophages from circulation are activated and entered the injured liver during ALF, which enhances macrophage-mediated phagocytosis in liver (Yen et al., 2009). Thus, passive liver targeting ability of PAOX particles and enhanced macrophage-mediated phagocytosis during ALF may provide a rational strategy for effective treatment of ALF using PAOX.

3.6. A mouse model of APAP-induced ALF

The ability of PAOX particles to target liver and enhanced macrophage-mediated phagocytosis during ALF prompted us to evaluate the efficacy of PAOX particles as a drug carrier for treating ALF. APAP is the most widely used analgesic and anti-pyretic agent for the relief of mild and moderate pain. APAP toxicity has been increasing since the 1980s and now is the most frequent cause of ALF in the United States (Larson et al., 2005). We used a mouse model of APAP-induced ALF because APAP is a dose-dependent hepatotoxicant and APAP-induced acute toxicity in mice is a clinically relevant model (Jaeschke et al., 2011). In addition, this model is one of the most popular experimental *in vivo* used today and is suitable to test the efficacy of various therapeutic compounds *in vivo* (Das et al., 2010; Wu et al., 2008).

The drug delivery efficacy of PAOX particles was investigated with PTX as a model drug. PTX, [1-(5-oxohexyl)-3,7-demethylxanthine], has been approved in the United States for use in the treatment of patients with intermittent claudication due to chronic occlusive arterial disease (Lebrec et al., 2010). There are several lines of studies reporting that PTX exerts therapeutic effects of liver diseases such as cirrhosis and acute alcoholic hepatitis. However, PTX has low therapeutic effectiveness due to fast elimination resulting from high water solubility (Otsuka and Matsuda, 1994). We reasoned that the therapeutic efficacy of PTX can be enhanced by using PAOX particles as drug carriers, which are capable of targeting liver specifically and release drug payloads rapidly in damaged liver.

Mice were treated with various amounts of PTX-loaded PAOX particles 1 h prior to APAP injection. A group of mice treated with saline was used as a control. After mice were sacrificed, the activity of ALT (alanine transaminase) was determined to evaluate the liver injury. ALT is an enzyme which has been widely used as a surrogate clinical marker for liver injury such as ALF and hepatitis because the serum ALT level is correlated with the severity of liver injury (Hu et al., 2010; Yang et al., 2008). Fig. 8 shows the level of ALT of APAP-intoxicated mice. Mice treated with 2 mg of empty PAOX particles had the same ALT activity as the control group, suggesting no harmful effects of PAOX on liver. Treatment of 200 μL of APAP (25 mg/mL) significantly increased the ALT activity, indicating severe damage to hepatic tissue membranes (Das et al., 2010). However, PTX-loaded PAOX particles suppressed the elevation of ALT activity in a dose-dependent manner. A dose of 0.5 mg of PTX-loaded PAOX particles showed no effects on the ALT level, but approximately 50% reduction of the ALT level was observed with a dose of 1 mg of PTX-loaded PAOX particles. The elevation of ALT activity was almost completely suppressed by 2 mg of PTX-loaded PAOX particles. Free PTX at a dose of 20 μg, which is the same amount as those loaded in 2 mg of PAOX particles, showed no effect on the ALT activity. Therefore, the results demonstrate that PAOX particles significantly enhance the therapeutic efficacy of PTX. The high drug delivery efficiency of PAOX particles in ALF treatment can be explained by the facts that PAOX particles passively target liver and macrophage-mediated phagocytosis is enhanced during ALF. In addition, the drug delivery efficiency of PAOX particles is further enhanced by the effective endosomal escape of drug payload, as expected from Fig. 4. PAOX particles trigger escapes of PTX from phagosomes by proton sponge effects and colloid osmotic disruption of phagosomes (Lee et al., 2009; Seong et al., 2011).

We next performed the histological studies to further confirm the enhanced therapeutic efficacy of PTX delivered by PAOX particles (Fig. 9). In contrast to the control group of mice, APAP caused extensive liver damage and disruption of tissue architecture, evidenced by hemorrhagic necrosis, hepatic apoptosis, leukocyte infiltration and extensive destruction of parenchyma (Chandrasekaran et al., 2011; Hu et al., 2010; Ishida et al., 2004;

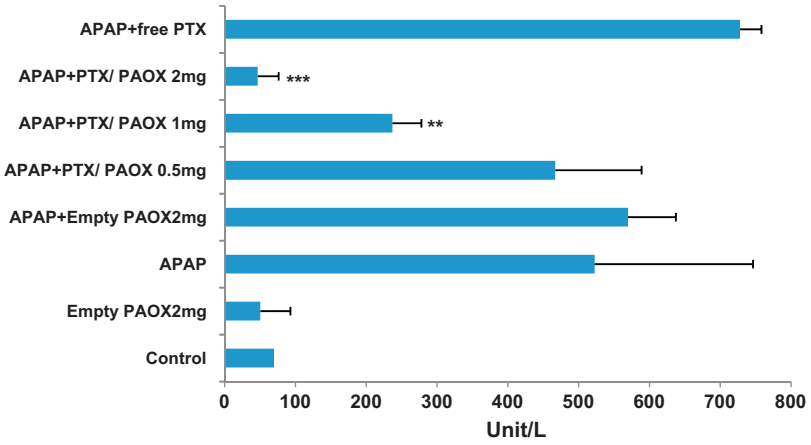

Fig. 8. Therapeutic effects of pentoxifylline on APAP-intoxicated mice. Mean ± S.D., $n = 4$. $^{**}P < 0.01$, $^{***}P < 0.001$ relative to APAP group.

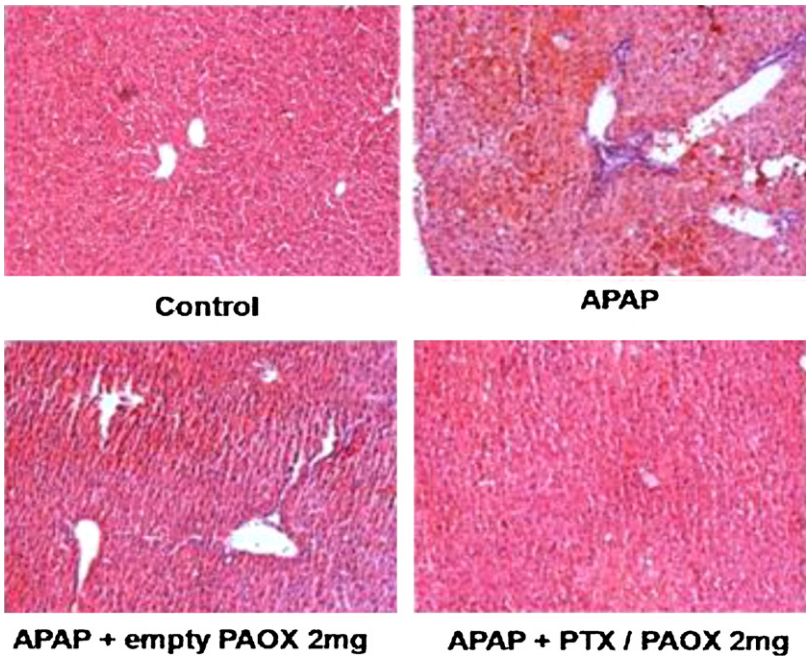

Fig. 9. H&E staining of liver tissues from APAP-intoxicated mice.

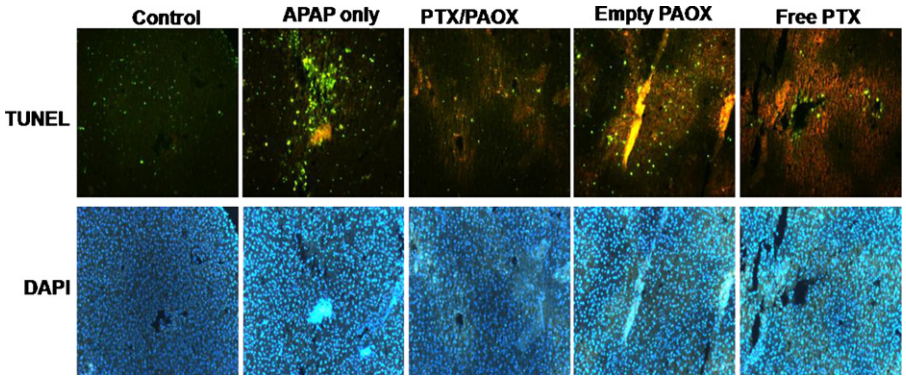

Fig. 10. TUNEL staining of liver tissues from APAP-intoxicated mice.

Williams et al., 2011). These observations are in good agreement with the previous study reporting that most of hepatocytes were destroyed, leaving esoinophilic bodies and macrophages (Ferret et al., 2001). These histopathological alterations were remarkably reduced in the liver sections of mice only treated with PTX-loaded PAOX particles.

APAP intoxication has been known to induce apoptotic cell death, characterized by nuclear condensation, cell shrinkage, DNA fragmentation and DNA fragments in cytosol (Jaeschke et al., 2011). TUNEL staining was also performed to study the effects of PTX on APAP-induced apoptosis. As shown in Fig. 10, liver sections from APAP-intoxicated mice showed extensive area of apoptosis. DNA fragmentation (TUNEL staining) was remarkably decreased in livers of mice treated with PTX-loaded PAOX particles. No or minimal reduction in DNA fragmentation was observed with free PTX and empty PAOX particles. The results demonstrate that PTX-loaded PAOX particles save hepatocytes from APAP-induced liver damage by inhibiting apoptosis.

4. Conclusions

We evaluated the potential of fully biodegradable and biocompatible PAOX particles as drug delivery systems for treating ALF. PAOX particles with a mean diameter of ~450 nm were accumulated in mainly liver after intravenous administration, demonstrating the great potential as drug delivery systems for treating ALF. PAOX particles showed no influence on cell viability and exhibited no inflammatory responses after intramuscular injection. The studies of confocal fluorescence imaging revealed that PAOX particles are readily phagocytozed and disrupted endosomes by proton sponge effects and colloid osmotic pressure without overt cytotoxicity. The ability of PAOX particles to deliver drug was evaluated using a mouse model of APAP-induced ALF and PTX as a model drug. PTX-loaded exhibited fast drug release profile because of fast hydrolytic degradation resulting from the presence of tertiary amine groups in the backbone of PAOX. PTX-delivered by PAOX particles remarkably reduced the hepatic cellular damages, evidenced by the ALF assay and histological studies. The excellent biocompatibility and rapid drug release profile make PAOX a promising drug delivery system for the treatment of acute inflammatory diseases.

Acknowledgements

This study was supported by the Basic Science Research Program through the National Research Foundation (2011-0003492,

2010-0021903) and the World Class University program (R31-20029) funded by the Ministry of Education, Science and Technology.

References

Bilzer, M., Roggel, F., Gerbes, A.L., 2006. Role of Kupffer cells in host defense and liver disease. Liver Int. 26, 1175–1186.

Chandrasekaran, V.R.M., Periasamy, S., Liu, L.L., Liu, M.Y., 2011. 17 Beta-estradiol protects against acetaminophen-overdose-induced acute oxidative hepatic damage and increases the survival rate in mice. Steroids 76, 118–124.

Das, J., Ghosh, J., Manna, P., Sil, P.C., 2010. Acetaminophen induced acute liver failure via oxidative stress and Jnk Activation: protective role of taurine by the suppression of cytochrome P450 2e1. Free Radic. Res. 44, 340–355.

Ferret, P.J., Hammoud, R., Tulliez, M., Tran, A., Trebeden, H., Jaffray, P., Malassagne, B., Calmus, Y., Weill, B., Batteux, F., 2001. Detoxification of reactive oxygen species by a nonpeptidyl mimic of superoxide dismutase cures acetaminophen-induced acute liver failure in the mouse. Hepatology 33, 1173–1180.

Gill, R.Q., Sterling, R.K., 2001. Acute liver failure. J. Clin. Gastroenterol. 33, 191–198.

Heffernan, M.J., Murthy, N., 2005. Polyketal nanoparticles: a new Ph-sensitive biodegradable drug delivery vehicle. Bioconjugate Chem. 16, 1340–1342.

Hu, J.J., Yan, D.J., Gao, J., Xu, C.Y., Yuan, Y.S., Zhu, R.Z., Xiang, D., Weng, S.Y., Han, W., Zang, G.Q., Yu, Y., 2010. Rhil-1ra reduces hepatocellular apoptosis in mice with acetaminophen-induced acute liver failure. Lab. Invest. 90, 1737–1746.

Hu, Y., Litwin, T., Nagaraja, A.R., Kwong, B., Katz, J., Watson, N., Irvine, D.J., 2007. Cytosolic delivery of membrane-impermeable molecules in dendritic cells using Ph-responsive core-shell nanoparticles. Nano Lett. 7, 3056–3064.

Ishida, Y., Kondo, T., Tsuneyama, K., Lu, P.R., Takayasu, T., Mukaida, N., 2004. The pathogenic roles of tumor necrosis factor receptor P55 in acetaminophen-induced liver injury in mice. J. Leukoc. Biol. 75, 59–67.

Jaeschke, H., McGill, M.R., Williams, C.D., Ramachandran, A., 2011. Current issues with acetaminophen hepatotoxicity—a clinically relevant model to test the efficacy of natural products. Life Sci. 88, 737–745.

Khaja, S.D., Lee, S., Murthy, N., 2007. Acid-degradable protein delivery vehicles based on metathesis chemistry. Biomacromolecules 8, 1391–1395.

Kim, S., Seong, K., Kim, O., Seo, H., Lee, M., Khang, G., Lee, D., 2010. Polyoxalate nanoparticles as a biodegradable and biocompatible drug delivery vehicle. Biomacromolecules 11, 555–560.

Ko, J., Park, K., Kim, Y.S., Kim, M.S., Han, J.K., Kim, K., Park, R.W., Kim, I.S., Song, H.K., Lee, D.S., Kwon, I.C., 2007. Tumoral acidic extracellular Ph targeting of Ph-responsive mpeg-poly (beta-amino ester) block copolymer micelles for cancer therapy. J. Control. Release 123, 109–115.

Ko, Y.K., Choi, M.K., Kim, S.H., Kim, G.A., Lee, H.B., Rhee, J.M., Khang, G., 2008. Sustained release of proteins using small intestinal submucosa modified Plga scaffold. Polymer (Korea) 32, 199–205.

Larson, A.M., Polson, J., Fontana, R.J., Davern, T.J., Lalani, E., Hynan, L.S., Reisch, J.S., Schiodt, F.V., Ostapowicz, G., Shakil, A.O., Lee, W.M., Acute Liver Failure Study, G., 2005. Acetaminophen-induced acute liver failure: results of a United States Multicenter Prospective Study. Hepatology 42, 1364–1372.

Lebrec, D., Thabut, D., Oberti, F., Perarnau, J.M., Condat, B., Barraud, H., Saliba, F., Carbonell, N., Renard, P., Ramond, M.J., Moreau, R., Poynard, T., Pentocir, G., 2010. Pentoxifylline does not decrease short-term mortality but does reduce complications in patients with advanced cirrhosis. Gastroenterology 138, U1740–U1755.

Lee, S., Yang, S.C., Kao, C.Y., Pierce, R.H., Murthy, N., 2009. Solid polymeric microparticles enhance the delivery of Sirna to macrophages in vivo. Nucleic Acids Res. 37.

Lee, W.M., 1993. Medical progress – acute liver-failure. N. Engl. J. Med. 329, 1862–1872.

Ma, S.F., Nishikawa, M., Katsumi, H., Yamashita, F., Hashida, M., 2006. Liver targeting of catalase by cationization for prevention of acute liver failure in mice. J. Control. Release 110, 273–282.

Meinel, L., Hofmann, S., Karageorgiou, V., Kirker-Head, C., McCool, J., Gronowicz, G., Zichner, L., Langer, R., Vunjak-Novakovic, G., Kaplan, D.L., 2005. The inflammatory responses to silk films in vitro and in vivo. Biomaterials 26, 147–155.

Moghimi, S.M., Hunter, A.C., Murray, J.C., 2005. Nanomedicine: current status and future prospects. FASEB J. 19, 311–330.

Muriel, P., 2000. Regulation of nitric oxide synthesis in the liver. J. Appl. Toxicol. 20, 189–195.

O'Grady, J.G., 2005. Acute liver failure. Postgrad. Med. J. 81, 148–154.

Ogrady, J.G., Schalm, S.W., Williams, R., 1993. Acute liver-failure – redefining the syndromes. Lancet 342, 273–275.

Ostapowicz, G., Fontana, R.J., Schiodt, F.V., Larson, A., Davern, T.J., Han, S.H.B., McCashland, T.M., Shakil, A.O., Hay, J.E., Hynan, L., Crippin, J.S., Blei, A.T., Samuel, G., Reisch, J., Lee, W.M., Grp, U.S.A.L.F.S., 2002. Results of a prospective study of acute liver failure at 17 tertiary care centers in the United States. Ann. Intern. Med. 137, 947–954.

Otsuka, M., Matsuda, Y., 1994. Controlled drug-release of highly water-soluble pentoxifylline from time-limit disintegration-type wax matrix tablets. Pharm. Res. 11, 351–354.

Park, H., Kim, S., Song, Y., Seung, K., Hong, D., Khang, G., Lee, D., 2010. Antioxidant and anti-inflammatory activities of hydroxybenzyl alcohol releasing biodegradable polyoxalate nanoparticles. Biomacromolecules 11, 2103–2108.

Perkins, J.D., 2006. Acetaminophen Sets Records in the United States: Number 1 Analgesic and Number 1 Cause of Acute Liver Failure – Acetaminophen-Induced Acute Liver Failure: Results of a United States Multicenter, Prospective Study. Larson Am, Polson J, Fontana Rj, Davern Tj, Lalani E, Hynan Ls, Reisch Js, Schiodt Fv, Ostapowicz G, Shakil Ao, Lee Ww, Acute Liver Failure Study Group. Hepatology 2005;42: 1364–1372. Liver Transplantation 12, 682–683.

Seong, K., Seo, H., Ahn, W., Yoo, D., Cho, S., Khang, G., Lee, D., 2011. Enhanced cytosolic drug delivery using fully biodegradable poly(amino oxalate) particles. J. Control. Release 152, 257–263.

Song, Y., Kwon, J., Kim, B., Jeon, Y., Khang, G., Lee, D., 2011. Physicobiological properties and biocompatibility of biodegradable poly(oxalate-co-oxamide). J. Biomed. Mater. Res. Part A 98A, 517–526.

Stuart, W.D., Kulkarni, R.M., Gray, J.K., Vasiliauskas, J., Leonis, M.A., Waltz, S.E., 2011. Ron receptor regulates Kupffer cell-dependent cytokine production and hepatocyte survival following endotoxin exposure in mice. Hepatology 53, 1618–1628.

Sy, J.C., Seshadri, G., Yang, S.C., Brown, M., Oh, T., Dikalov, S., Murthy, N., Davis, M.E., 2008. Sustained release of a P38 inhibitor from non-inflammatory microspheres inhibits cardiac dysfunction. Nat. Mater. 7, 863–869.

Waters, E., Wang, J.H., Redmond, H.P., Wu, Q.D., Kay, E., Bouchier-Hayes, D., 2001. Role of taurine in preventing acetaminophen-induced hepatic injury in the rat. Am. J. Physiol. Gastrointestinal Liver Physiol. 280, G1274–G1279.

Williams, C.D., Antoine, D.J., Shaw, P.J., Benson, C., Farhood, A., Williams, D.P., Kanneganti, T.D., Park, B.K., Jaeschke, H., 2011. Role of the Nalp3 inflammasome in acetaminophen-induced sterile inflammation and liver injury. Toxicol. Appl. Pharmacol. 252, 289–297.

Wu, Y.L., Piao, D.M., Han, X.H., Nan, J.X., 2008. Protective effects of salidroside against acetaminophen-induced toxicity in mice. Biol. Pharm. Bull. 31, 1523–1529.

Yang, S.C., Bhide, M., Crispe, I.N., Pierce, R.H., Murthy, N., 2008. Polyketal copolymers: a new acid-sensitive delivery vehicle for treating acute inflammatory diseases. Bioconjugate Chem. 19, 1164–1169.

Yen, F.L., Wu, T.H., Lin, L.T., Cham, T.M., Lin, C.C., 2009. Naringenin-loaded nanoparticles improve the physicochemical properties and the hepatoprotective effects of naringenin in orally-administered rats with Ccl(4)-induced acute liver failure. Pharm. Res. 26, 893–902.