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Antioxidant polymeric prodrug microparticles as a therapeutic system for acute liver failure

Eunbyul Ko [a], Dahee Jeong [a], Jihye Kim [a], Sanga Park [a], Gilson Khang [a,b], Dongwon Lee [a,b,*]

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

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ABSTRACT

Acetaminophen (APAP) is the most widely used analgesic and its overdose, intentional or unintentional, is known to cause massive oxidative stress and liver tissue damages characterized by hepatocellular apoptosis and hemorrhagic necrosis, leading to acute liver failure (ALF). There has been great interest in the use of antioxidant and anti-inflammatory drugs for the effective treatment of ALF. Manganese porphyrin (MnP), a nonpeptidyl mimic of superoxide dismutase is a promising compound with antioxidant activity, but its application is curtailed by a short half-life in blood. We have recently developed a new family of biodegradable and antioxidant polymeric prodrug, poly(vanillyl alcohol-co-oxalate) (PVAX), which is able to scavenge H_2O_2 and release antioxidant and anti-inflammatory vanillyl alcohol. In this work, we developed MnP-loaded PVAX particles and evaluated their potential as antioxidant and anti-inflammatory therapeutic agents for APAP-induced ALF. PVAX particles and MnP showed synergistic antioxidant and anti-inflammatory activities in macrophages stimulated with LPS (lipopolysaccharide). Animal studies using a mouse model of APAP-induced ALF revealed that MnP-loaded PVAX particles significantly reduced the serum ALT level and protected liver damages. We anticipated that MnP-loaded PVAX particles have great potential as a therapeutic agent for oxidative stress-associated diseases such as APAP-induced ALF.

1. Introduction

Reactive oxygen species (ROS) are a collective term of chemically reactive oxygen-containing molecules and include oxygen radicals (superoxide anion, nitrite and hydroxyl radical) and oxygen derivatives such as hydrogen peroxide (H_2O_2) and peroxynitrite [1,2]. ROS are generated as a natural byproduct of cellular metabolism of oxygen. Under the normal conditions, cells produce a basal level of ROS which have essential roles in cell signaling, defense mechanisms and homeostasis [3]. However, the overproduction of ROS leads to oxidative stress, causing significant damages to cell structures, decline of cellular function and cell death. Therefore, it is critical for cells to maintain ROS homeostasis to regulate ROS level and overcome the cytotoxicity of ROS through molecular mechanisms including antioxidant metalloenzymes (such as superoxide dismutase and catalase) and an array of thiol-based redox couples [4]. In particular, H_2O_2 converted from superoxide is an increasingly recognized molecule and is one of major contributors to oxidative damages [5]. Because H_2O_2 is most stable and abundant ROS and is considered to generate more toxic hydroxyl radicals from the Fenton reaction, elimination of H_2O_2 is important for complete protection of oxidative stress [6]. High level of H_2O_2 has been implicated in numerous pathophysiological conditions including atherosclerosis, Alzheimer's disease, cancer, arthritis, ischemia/reperfusion injury and acute liver failure (ALF) [7,8].

ALF is life-threatening complications characterized by the sudden loss of hepatocellular functions rapidly developed without preexisting liver injury, massive death of hepatocytes and only minor liver regeneration [9]. ALF is responsible for thousands of death worldwide and is devastating more than any other conditions in medicine [10]. Common causes of ALF include hepatitis and toxin- or drug poisoning. Among them, APAP overdose is the most common cause of ALF, accounting for more than 50% of all ALF cases in the United States [11]. APAP is a most frequently used effective analgesic and anti-pyretic agent, which is sold under many brand names including Tyrenol. APAP-induced hepatotoxicity is physiologically characterized by apoptotic and/or necrotic cell death with a massive leukocyte infiltration [9,12]. Toxicity of APAP is initiated

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

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by P450-mediated metabolism that converts APAP to reactive *N*-acetyl benzoquinone imine (NAPQI) which can deplete glutathione, a key cellular antioxidant. NAPQI also binds to cysteine residues on proteins, resulting in the formation of APAP adducts, leading to liver injury [13]. Recent studies suggest that massive production of ROS and lipid peroxidation are involved in APAP-induced hepatotoxicity [13,14]. Antioxidant *N*-acetyl-L-cystein (NAC) is known to restore the glutathione pool depleted by NAPQI during APAP metabolism and therefore can be used in the clinical treatment of APAP-induced ALF. However, NAC serves as an effective therapeutic agent only when administrated before a significant elevation in serum transaminase activities [15]. Therefore, it is critical to develop a new therapeutic agent that would rapidly reduce oxidative stress and give maximum protection of the liver during APAP-induced hepatic damage [8].

Our laboratory has developed several biodegradable antioxidant polymers using antioxidant and anti-inflammatory molecules naturally occurring in plants [2,7,16]. Vanillyl alcohol is one of major active pharmaceutical ingredients in *Gastrodia elata*, which is a herbal agent widely used in oriental medicine for the treatment of brain ischemic injury and coronary artery [17,18]. Vanillyl alcohol has been reported to have antioxidant, anti-inflammatory and anti-nociceptive activities. We have recently synthesized biodegradable polyoxalate incorporating vanillyl alcohol (PVAX) as an antioxidant polymeric prodrug, which has H_2O_2-responsive peroxalate ester linkages and therapeutic vanillyl alcohol in its backbone [7]. PVAX was designed to react with H_2O_2, leading to H_2O_2 scavenging and consequently reduction of oxidative stress. In addition, reaction of peroxalate ester linkages with H_2O_2 facilitates hydrolytic degradation, leading to the release of antioxidant and anti-inflammatory vanillyl alcohol. We have reported that H_2O_2-responsive molecularly engineered PVAX nanoparticles react with overproduced H_2O_2 and reduce cellular damages caused during ischemia/reperfusion injury by exerting highly potent anti-inflammatory and anti-apoptotic activities [7].

Manganese porphyrins (MnP) are a promising compound as a nonpeptidyl mimic of superoxide dismutase with catalase-like activity and chemical versatility [6]. Ferret et al. reported that manganese III tetrakis (5,10,15,20 benzoic acid) (MnTBAP) exerts beneficial effects in APAP-intoxicated mice [15]. However, MnP lacks the ability to circulate in blood stream for a long period of time and target cells that produce ROS including superoxide. There are therefore great needs to develop effective drug delivery systems to enhance the therapeutic efficacy of MnP [19]. In this regard, we utilized the biodegradable PVAX particles as a vehicle of MnP by taking the advantages of PVAX including rapid hydrolytic degradation and intrinsic antioxidant and anti-inflammatory activities. The underlying hypothesis is that MnP-loaded PVAX particles accumulate passively and preferentially at liver and the high level of H_2O_2 and superoxide generated during the APAP-induced ALF would be rapidly and effectively reduced by PVAX and MnP, respectively. In this study, we report the potential of antioxidant polymeric prodrug PVAX particles as therapeutics and drug delivery systems for the treatment of APAP-induced ALF.

2. Experimental details

2.1. Materials

Vanillyl alcohol, oxalyl chloride, cyclohexanedimethanol, 10,15,20-tetrakis (4-sulfonatophenyl)-21*H*,23*H*-porphine manganese (III) chloride (MnP), protoporphyrin IX and poly(vinyl alcohol) (PVA, MW 13,000–23,000) were obtained from Sigma–Aldrich (St. Louis, MO). Dichloromethane (DCM) and triethylamine were obtained from Showa (Japan). DCM was distilled over calcium hydride. RAW 264.7 cells were purchased from Korea Cell Line Bank (Seoul, Korea).

2.2. Synthesis of PVAX

1,4-Cyclohexanedimethanol (21.96 mmol) and 4-vanillyl alcohol (5.49 mmol) were dissolved in 10 mL of dry tetrahydrofuran (THF), under nitrogen, to which triethylamine (60 mmol) was added dropwise at 4 °C. Oxalyl chloride (27.45 mmol) in 20 mL of dry THF was added to the mixture dropwise at 4 °C. Polymerization reaction was continued for 6 h at room temperature under nitrogen atmosphere. The resulting polymers were obtained through the extraction using DCM and precipitation in cold hexane. PVAX was obtained as white solid and its chemical structure was identified with a 400 MHz ^{1}H NMR spectrometer. ^{1}H NMR in deuterated chloroform on a 400 MHz spectrometer: 7.0–7.3 (*m*, 3H, Ar), 5.3 (*m*, 2H OC**H**$_2$-PhO-CH$_3$), 4.1–4.2 (*m*, 4H, COOC**H**$_2$CH), 3,8 (m, 3H, OC**H**$_3$), 2.2 (*m*, 2H, C(C**H**$_2$)$_3$HO), 1.0–1.8 (*m*, 8H, Cyclic C**H**$_2$). The weight average molecular weight of PVAX was determined to be ~12,000 Da using a gel permeation chromatography (GPC).

2.3. Preparation of MnP-loaded PVAX particles

One hundred milligrams of PVAX was dissolved in 1 mL of DCM, to which 5 mg of MnP dissolved in 300 μL of deionized water was added. The mixture was sonicated using a sonicator (Fisher Scientific, Sonic Dismembrator 500) for 30 s. The resulting emulsion was added 10 mL of PVA (5 wt%) solution and treated with a homogenizer (PRO Scientific, PRO 200) for 1 min to form w/o/w emulsion. DCM was evaluated at room temperature for 2 h and the MnP-loaded PVAX particles were obtained after centrifugation (1000× *g*) for 5 min. The obtained MnP-loaded PVAX particles were washed with deionized water twice and freeze dried at −80 °C. The morphology and size of MnP-PVAX particles were observed by a scanning electron microscopy (SEM, S-3000N, Hitachi, Japan) with accelerating voltage of 10 kV. Their hydrodynamic size was determined using a particle analyzer (Brookhaven Instruments Corporation, Holtsville, NY).

2.4. Release kinetics of MnP-PVAX particles

MnP-loaded PVAX particles (10 mg) were dispersed in 2 mL of phosphate-buffered saline (PBS) and incubated under continuous stirring at 37 °C. At appropriate time points, the suspension was centrifuged at 1000× *g* for 5 min and 200 μL of supernatant was taken and replaced with an equal volume of fresh PBS. The concentration of MnP in the supernatant was measured using a UV-spectrometer (S-3100, Scinco, Korea) at a wavelength of 466 nm and the release kinetic was determined by comparing the concentrations of MnP standard solutions.

2.5. Cytotoxicity of MnP-loaded PVAX particles

The cytotoxicity of MnP-loaded PVAX particles was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells (RAW 264.7) were cultured using Dulbecco's Modified Eagle Medium (Gibco, Gland Island, NY) containing 10% fetal bovine serum). Cells in a 24 well plate with ~90% confluency were treated with various amounts of particles for 24 h. The PVAX particles-containing medium was replaced with fresh medium. 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).

2.6. Measurement of nitric oxide (NO)

RAW 264.7 cells (4×10^5 cells/well in a 24 well plate) were pretreated with a various amount of PVAX particles for 4 h and then treated with 1 μg of lipopolysaccharide (LPS, 1 mg/mL) for 20 h. The concentration of NO in the medium was determined using a colorimetric assay based on the Griess reaction. Fifty microliters of cell culture medium was collected and given 50 μL of Griess reagent (6 mg/mL, Promega, Madison, WI) at room temperature for 10 min, and then the NO concentration was determined by measuring the absorbance at 570 nm using a microplate reader (Synergy MX, BioTek Instruments, Inc, Winooski, VT). The NO standard curve was constructed using known concentrations of sodium nitrite. Untreated cells were used as negative control.

2.7. Hydrogen peroxide scavenging

To 1 mL of H_2O_2 solutions (10 μM) was added 1 mg of PVAX or MnP-PVAX particles and the solution was left at 37 °C under gentle mechanical stirring. At appropriate time intervals, the H_2O_2 concentration of the supernatant was measured using the Amplex Red assay (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

2.8. Reduction of ROS

RAW 264.7 cells (4×10^5 cells) were treated with vanillyl alcohol, PVAX or MnP-PVAX particles for 24 h and incubated with 1 μg of LPS for 24 h. DCFH-DA of 5 μM was added to each dish and 0.5 h later the fluorescence images were made with a confocal laser scanning microscope (LSM 510 Meta, Carl Zeiss, Germany). To quantify the fluorescent cells, flow cytometry was also performed with a Flow Cytometry Caliber (Becton Dickinson, US). The percentage of cells in positive events was calculated as the events within the gate divided by total number of events then subtracting percentage of the control sample (untreated cells).