Biomaterials 144 (2017) 30–41

Contents lists available at ScienceDirect

Biomaterials

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

Reactive oxygen species-responsive polymeric nanoparticles for alleviating sepsis-induced acute liver injury in mice

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ARTICLE INFO

Article history:
Received 10 May 2017
Received in revised form
7 August 2017
Accepted 8 August 2017
Available online 11 August 2017

Keywords:
Sepsis
Reactive oxygen species
Melatonin
Acute liver injury
Nanoparticles

ABSTRACT

Sepsis-associated acute liver injury contributes to the pathogenesis of multiple organ dysfunction syndrome and is associated with increased mortality. Currently, no specific therapeutics for sepsis-associated liver injury are available. With excess levels of reactive oxygen species (ROS) being implicated as key players in sepsis-induced liver injury, we hypothesize that ROS-responsive nanoparticles (NPs) formed via the self-assembly of diblock copolymers of poly(ethylene glycol) (PEG) and poly(propylene sulfide) (PPS) may function as an effective drug delivery system for alleviating sepsis-induced liver injury by preferentially releasing drug molecules at the disease site. However, there are no reports available on the biocompatibility and effect of PEG-b-PPS-NPs *in vivo*. Herein, this platform was tested for delivering the promising antioxidant therapeutic molecule melatonin (Mel), which currently has limited therapeutic efficacy because of its poor pharmacokinetic properties. The mPEG-b-PPS-NPs efficiently encapsulated Mel using the oil-in-water emulsion technique and provided sustained, on-demand release that was modulated *in vitro* by the hydrogen peroxide concentration. Animal studies using a mouse model of sepsis-induced acute liver injury revealed that Mel-loaded mPEG-b-PPS-NPs are biocompatible and much more efficacious than an equivalent amount of free drug in attenuating oxidative stress, the inflammatory response, and subsequent liver injury. Accordingly, this work indicates that mPEG-b-PPS-NPs show potential as an ROS-mediated on-demand drug delivery system for improving Mel bioavailability and treating oxidative stress-associated diseases such as sepsis-induced acute liver injury.

1. Introduction

Sepsis reflects a severe systemic inflammatory reaction triggered by overwhelming infection or trauma and is the leading cause of death in critically ill patients [1]. Sepsis can evolve to multiple organ dysfunction syndrome (MODS), the severity of which results in a high mortality rate [2]. During the process of sepsis, the liver is a potential target of a dysregulated inflammatory response. Sepsis-associated acute liver injury was reported to contribute to the pathogenesis of MODS and is usually associated with a poor prognosis [3,4]. Currently, there are no specific therapeutic interventions available for sepsis-associated liver injury [5,6]. Therefore, safe and efficacious therapeutic strategies against sepsis and sepsis-induced acute liver injury are needed urgently.

Excess production of reactive oxygen species (ROS) is believed to directly contribute to tissue injury sustained via membrane lipid peroxidation, inflammatory cascade initiations, and systemic disturbances [7]. Consequently, oxidative stress may be a critical factor in the pathogenesis of sepsis-induced acute liver injury, and thus, it

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http://dx.doi.org/10.1016/j.biomaterials.2017.08.008
0142-9612/

should be targeted by antioxidative therapies to prevent sepsis-induced acute liver injury [8]. However, for most antioxidative drugs delivered by systemic administration, only limited efficacy was observed in clinical trials, despite their success in animal studies [9,10]. This undesirable therapeutic effect has mainly been ascribed to rapid drug clearance, poor drug accumulation at the site of injury, inefficiency in maintaining the therapeutics at adequate levels for a prolonged period, and adverse effects due to supplementation overdose [8,11,12]. Stimuli-responsive drug delivery systems have shown a promising ability to circumvent these obstacles [13,14]. These smart drug delivery systems can stably package their cargo molecules and perform targeted release in response to specific microenvironments [15–17]. Because ROS overproduction has considerable implications in sepsis-induced acute liver injury, an on-demand delivery system that enables the targeted release of antioxidants or other drugs in regions of high ROS activity has the potential to improve drug efficacy for the prevention and treatment of sepsis-induced acute liver injury.

Melatonin (Mel) is a multifunctional hormone that can function as an endogenous free radical scavenger and broad spectrum antioxidant [18]. Previous studies have indicated that systemic administration of free Mel has a therapeutic benefit in alleviating sepsis-induced tissue injury by inhibiting NF-κB and NLRP3 inflammasome activation [19,20]. Specifically, Mel can exert a protective effect in mitochondria, which are the main intracellular source of ROS. However, the therapeutic use of Mel is limited by its short half-life ($t_{1/2}$ <30 min) and low bioavailability [21]. Previous approaches to improve Mel bioavailability and pharmacokinetics *in vivo* have involved polymeric nanoparticles (NPs) and encapsulation in liposomes [21,22]; however, limited control of the targeting and drug release profile hampered their therapeutic efficacy.

In this paper, ROS-responsive polymeric NPs formed via the self-assembly of diblock copolymers of poly(ethylene glycol) (PEG) and poly(propylene sulfide) (PPS) were explored as a drug delivery system for the encapsulation and on-demand delivery of Mel at sites of oxidative stress. The PPS block could undergo an oxidative conversion from hydrophobic to hydrophilic, which enabled on-demand delivery of the drug [23]. PEG-b-PPS has been explored for engineering polymersomes for vaccine delivery and NPs for encapsulating hydrophobic systems [24,25]. However, to the best of our knowledge, the biocompatibility and therapeutic effect of this system *in vivo* has still not been evaluated. The studies presented herein include the fabrication, characterization and assessment of on-demand release kinetics *in vitro* of the mPEG-b-PPS-NPs. Moreover, the *in vivo* biodistribution and therapeutic benefits of Mel-loaded mPEG-b-PPS-NPs in alleviating sepsis-induced acute hepatic injury were also investigated in a mouse sepsis model.

2. Materials and methods

2.1. Reagents and antibodies

Dichloromethane (DCM, CH_2Cl_2), poly(ethylene glycol) (mPEG, Mn = 2.0 × 10^3 g/mol), sodium hydride (NaH), methacryloyl chloride, trimethylamine, thioacetic acid, diethyl ether, propylene sulfide, sodium methylate, cholesterol, DiO (3,3′-dioctadecyloxacarbocyanine perchlorate), and hydrogen peroxide (H_2O_2) were purchased from GL Biochem Ltd. (Shanghai). DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) was purchased from Invitrogen (Carlsbad, CA, USA).

LPS (E. coli, serotype O127:B8; Sigma-Aldrich, MO, USA) was diluted in 0.9% saline; Melatonin (Sigma-Aldrich, MO, USA) was dissolved in saline containing 2.5% ethanol. Antibodies against iNOS, COX-2, and GAPDH were purchased from Proteintech (Chicago, IL, USA). Antibodies against phospho-NF-κB p65 (Ser536), NF-κB p65, NLRP3, and ASC were purchased from Cell Signaling Technology (Beverly, MA, USA). An antibody against caspase-1 p20 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Preparation of methoxy poly(ethylene glycol) methacrylate ($mPEG_{45}$ methacrylate)

mPEG (1.0 g, 0.5 mmol), trimethylamine (0.6 mL), and methacryloyl chloride (0.6 mL, 0.6 mmol) were added into dichloromethane under stirring. After 24 h, the solution was filtered, concentrated, and precipitated twice in cold diethyl ether.

2.3. Preparation of methoxy poly(ethylene glycol) thioacetate ($mPEG_{45}$ thioacetate)

mPEG methacrylate (1.0 g, 0.5 mmol) was introduced into a Schlenk tube and dissolved in THF (8 mL). AIBN (0.0312 g, 0.187 mmol) and thioacetic acid (0.54 mL, 7.6 mmol) were added under stirring. The reaction mixture was degassed by freezing the mixture under liquid nitrogen, evacuating under high vacuum, and filling the Schlenk tube with argon (Scheme 1) while warming it to room temperature. The degassing procedure was repeated three times. The reaction mixture was then stirred for 24 h at 60 °C. The solution was filtered, concentrated, and precipitated twice in cold diethyl ether.

2.4. Preparation of methoxy poly(ethylene glycol)-b-poly-(propylene sulfide) ($mPEG_{45}$-b-PPS_{60})

mPEG thioacetate (1.0 g, 0.5 mmol) was introduced in a Schlenk tube under an inert atmosphere and dissolved in THF. Sodium methylate (0.6 mmol, 0.5 M in methanol) was then added, and the mixture was stirred for 30 min at room temperature. Propylene sulfide (30 mmol) was added to the mixture, and the mixture was stirred overnight at 60 °C. The solvent was removed, and the resulting viscous liquid was twice extracted with methanol. The obtained material was characterized using ^{1}H nuclear magnetic resonance (NMR) spectroscopy (Agilent 500 MHz).

2.5. Mel-loaded NPs preparation and characterization

NPs loaded with Mel were prepared using an improved double emulsion (w/o/w) technique. Briefly, 8 mg of Mel was dissolved into 200 μL of saline/ethyl alcohol (1:1, v:v) and mixed with 1 mL of chloroform solution containing 16 mg of PEG-b-PPS. The mixture was emulsified by sonication for 3 min (70 W) in an ice bath. Then, the obtained primary emulsion was slowly added into 4 mL of saline with 0.5% (w/v) of polyvinyl alcohol (PVA) and further emulsified by sonication for 60 s (200 W) in an ice bath. The final emulsion was evaporated under reduced pressure with a rotary evaporator to remove the oil phase. Blank NPs and NPs containing DiO and DiI were fabricated using similar procedure.

Dynamic light scattering (DLS) measurements were performed to measure hydrodynamic diameter using a Malvern Zetasizer Nano-ZS (Malvern Instruments Ltd, Malvern, UK). The morphology of NPs was characterized using transmission electron microscopy (EM-200CX, JEOL Ltd., Tokyo, Japan).

2.6. Stability and ROS-responsive studies

Mel-loaded PEG-PPS-NPs were dispersed in phosphate-buffered saline (PBS) with or without 10 mM H_2O_2 and incubated under continuous stirring at 37 °C. At appropriate time points, the suspension was centrifuged at 1000 × g for 30 s. A 2 mL aliquot of the