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NANOMEDICINE

Hydrogen peroxide-responsive copolyoxalate nanoparticles for detection and therapy of ischemia–reperfusion injury

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ABSTRACT

The main culprit in the pathogenesis of ischemia/reperfusion (I/R) injury is the generation of high level of hydrogen peroxide (H_2O_2). In this study, we report a novel diagnostic and therapeutic strategy for I/R injury based on H_2O_2-activatable copolyoxalate nanoparticles using a murine model of hind limb I/R injury. The nanoparticles are composed of hydroxybenzyl alcohol (HBA)-incorporating copolyoxalate (HPOX) that, in the presence of H_2O_2, degrades completely into three known and safe compounds, cyclohexanedimethanol, HBA and CO_2. HPOX effectively scavenges H_2O_2 in a dose-dependent manner and hydrolyzes to release HBA which exerts intrinsic antioxidant and anti-inflammatory activities both *in vitro* and *in vivo* models of hind limb I/R. HPOX nanoparticles loaded with fluorophore effectively and robustly image H_2O_2 generated in hind limb I/R injury, demonstrating their potential for bioimaging of H_2O_2-associated diseases. Furthermore, HPOX nanoparticles loaded with anti-apoptotic drug effectively release the drug payload after I/R injury, exhibiting their effectiveness for a targeted drug delivery system for I/R injury. We anticipate that multifunctional HPOX nanoparticles have great potential as H_2O_2 imaging agents, therapeutics and drug delivery systems for H_2O_2-associated diseases.

1. Introduction

I/R injury occurs in a variety of clinical conditions, such as coronary artery disease, peripheral arterial disease, and stroke [1–5]. Reperfusion of blood flow to previously ischemic tissues is accompanied by generation of large amounts of reactive oxygen species (ROS), which overwhelm cellular defenses and damage normal cellular functions. When oxygen is resupplied during reperfusion, NADPH oxidases are known to generate a large amount of toxic ROS which include H_2O_2, superoxide anions, hydroxyl radicals, hypochlorous acid and nitric oxide-derived peroxynitrite [6]. In particular, H_2O_2, the most abundant form of the ROS produced during I/R, induces oxidative stress and triggers apoptosis, further exacerbating initial tissue damages. Despite its essential role in cellular signaling in living organisms, overproduced H_2O_2 is known to be a major source of oxidative stress and serves as a precursor of highly reactive ROS such as hydroxyl radical, peroxinitrite and hydrochlorite [7]. Therefore, targeting H_2O_2 as a diagnostic marker as well as a therapeutic target for I/R injury has tremendous potential.

Over the past decades, tremendous efforts have been made in the development of biodegradable polymers for drug delivery systems which can enhance the therapeutic effectiveness of conventional drugs and improve patient compliance/convenience, while reducing their detrimental side effects and required dose. To date, the most thoroughly investigated biodegradable polymers have been members of polyesters, such as poly(lactic acid), poly(glycolic acid) and poly(lactic-co-glycolic acid) (PLGA) [8,9]. In general, therapeutic drugs are physically admixed within the matrix and released during the degradation of the polymer matrix. The polymeric drug carriers could provide considerable benefits such as enhanced therapeutic effects, prolonged bioactivity, controlled release rate and decreased administration frequency/dose. However, a drawback of these degradable polymers is that the high concentration of acidic degradation products at a localized site causes inflammatory responses. Moreover, nanoparticulate drug carriers have limited drug loading which prevents them from achieving the full drug delivery potential. In this regard, there has been increasing interest in the development of new polymeric drug carriers that have a high content of deliverable drugs and induce little to no inflammatory responses and oxidative

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stress. One of the approaches to fulfill these criteria involves the direct incorporation of bioactive molecules into the backbone of biodegradable polymers, pioneered by Uhrich [10,11].

Previously, we developed fully biodegradable polyoxalate copolymer (HPOX) which chemically incorporates naturally occurring bioactive hydroxybenzyl alcohol (HBA) in the backbone of polymers [12,13]. HBA is one of phenolic compounds found in diverse plants and is a major active pharmaceutical ingredient in *Gastrodia elata* Blume, which has been used as an herbal agent [14]. HPOX was designed to covalently incorporate antioxidant and anti-inflammatory HBA in its backbone, not attached to the side groups and release HBA during its hydrolytic degradation. Another unique property of HPOX is its ability to react with H_2O_2 to perform peroxalate chemiluminescence reaction in the presence of fluorescent compounds. Previously, nanoparticles based on polyoxalate were developed which could image H_2O_2 produced in a peritoneal cavity in mice during lipopolysaccharide-induced inflammation [15]. However, the polyoxalate was unsuitable for formulation into solid nanoparticles due to its instability under aqueous conditions, limiting its applications in both bioimaging and drug delivery.

In this paper, we report molecularly engineered solid HPOX nanoparticles with enhanced stability and high specificity for H_2O_2, thus allowing physiological bioimaging and therapy for I/R injury. We used a mouse model of hind limb I/R to evaluate the potential of multifunctional HPOX nanoparticles as H_2O_2 imaging agents and therapeutics for H_2O_2-associated inflammatory diseases. In addition, the potential of HPOX nanoparticles as site directed drug delivery systems for I/R injury was investigated using an anti-apoptotic agent, 4-amino-1,8-napthalimide (4-AN) as a model drug. Here, we present multifunctional H_2O_2-activatable nanoparticles that are able to image H_2O_2 *in vivo*, possess intrinsic antioxidant and anti-inflammatory properties, and capable of site directed drug delivery for the treatment of I/R injury (Fig. 1).

2. Materials and methods

2.1. Synthesis of HPOX

All chemicals and solvents were of American Chemical Society grade or HPLC purity and were used as received. HPOX was synthesized using cyclohexanedimethanol, 4-hydroxybenzyl alcohol and oxalyl chloride. Briefly, 1,4-cyclohexanedimethanol (21.96 mmol) and 4-hydroxybenzyl alcohol (5.49 mmol) were dissolved in 20 mL of dry tetrahydrofuran (THF) and triethylamine (60 mmol) was added dropwise to the solution under nitrogen at 4 °C. Polymerization was initiated by adding oxalyl chloride (27.45 mmol) in 25 mL of dry THF to the reaction solution at 4 °C, and the reaction mixture was kept under nitrogen atmosphere at room temperature for 6 h. Polymers were obtained through extraction using dichloromethane (DCM), followed by precipitation in cold hexane. The chemical structure of polymers was identified with a 400 MHz ^{1}H NMR spectrometer (JNM-EX400 JEOL), and the molecular weight was determined by gel permeation chromatography (GPC, Futecs, Korea) to be approximately 20 kDa with a mean polydispersity of 1.8.

2.2. Nanoparticle preparation and characterization

HPOX nanoparticles were generated using an emulsion/solvent evaporation method. In brief, 100 mg of HPOX dissolved in 1 mL of DCM was added to 5 mL of 10 (w/v)% polyvinyl alcohol (PVA) solution and homogenized using a sonicator and homogenizer to form a fine oil/water emulsion. The emulsion was transferred to a 20 mL PVA (1 w/v%) solution and homogenized for 1 min. The remaining solvent was removed using a rotary evaporator. The particles were then centrifuged and washed with de-ionized water three times to remove residual PVA. The suspension was then frozen in liquid nitrogen and lyophilized to produce free-flowing particles. To develop HPOX nanoparticles loaded with rubrene or 4-AN, 5 mg of rubrene or 10 mg of 4-AN was dissolved in 100 μL of DCM or dimethylsulfoxide, respectively. The procedures for particle formulation were the same as for empty HPOX nanoparticle formulation. For comparison, PLGA (MW 30 kDa) was also formulated into nanoparticles using the same procedure for HPOX. It was determined that 1 mg of PLGA and HPOX nanoparticles contained ~95 μg and ~75 μg of 4-AN, respectively.

2.3. Release kinetics of 4-AN

HPOX or PLGA nanoparticles (5 mg) loaded with 4-AN were dispersed in 20 mL of phosphate-buffered saline (PBS) with or without 100 μM 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 supernatant was taken and replaced with an equal volume of fresh PBS. The concentration of 4-AN in the supernatant was measured using a UV-spectrometer (S-3100, Scinco, Korea) and the release kinetic was determined by comparing the concentrations of 4-AN standard solutions.

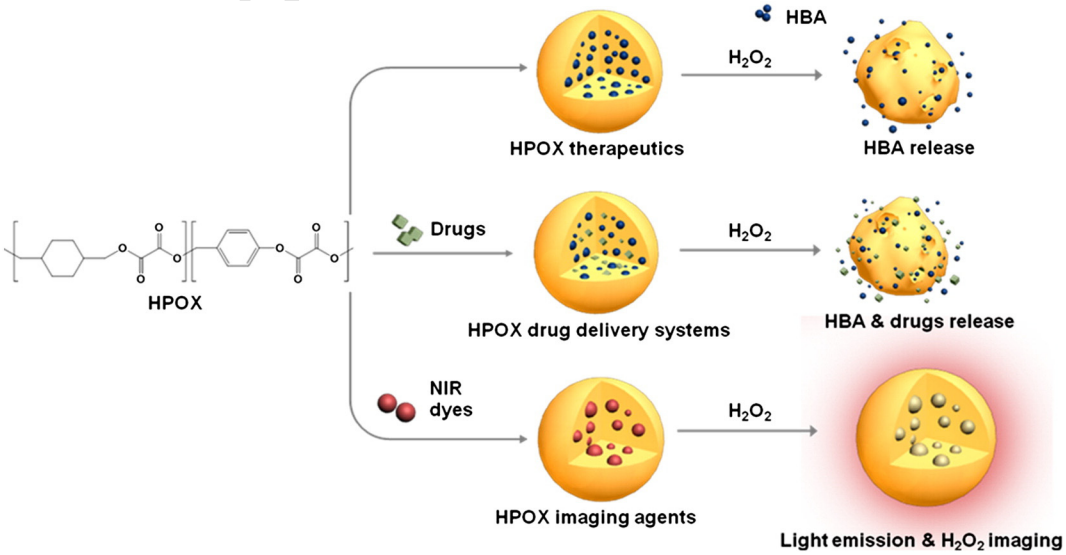

Fig. 1. Multifunctional H_2O_2-activatable nanoparticles as a novel strategy for bioimaging and therapy. HPOX nanoparticles are able to serve as H_2O_2 imaging agents, therapeutics and site-directed drug delivery systems for I/R injury.

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