



Pharmaceutical nanotechnology

Antioxidant polymeric nanoparticles as novel therapeutics for airway inflammatory diseases



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ABSTRACT

Successful pulmonary drug delivery requires polymeric drug delivery systems which have excellent biocompatibility and fast degradation rates, when frequent administration is necessary. Here, we report a new family of fully biodegradable hydroxybenzyl alcohol (HBA)-incorporated polyoxalate (HPOX) as a novel therapeutics of airway inflammatory diseases. HPOX was designed to incorporate antioxidant and anti-inflammatory HBA and peroxalate ester linkages capable of reacting with hydrogen peroxide (H_2O_2) in its backbone. HPOX nanoparticles exhibited highly potent antioxidant and anti-inflammatory effects by scavenging H_2O_2 , reducing the generation of intracellular oxidative stress and suppressing the expression of pro-inflammatory mediators such as inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and interleukin (IL)-1 β in stimulated macrophages. The potential of HPOX nanoparticles as an anti-asthmatic agent was evaluated using a murine model of asthma. Intratracheal administration of HPOX nanoparticles remarkably reduced the recruitment of inflammatory cells and expression of pro-inflammatory mediators such as IL-4 and iNOS. Based on their excellent antioxidant, anti-inflammatory and anti-asthmatic activities, we believe that HPOX nanoparticles have great potential as therapeutics and drug carriers for the treatment of airway inflammatory diseases such as asthma.

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1. Introduction

Pulmonary drug delivery using particulate drug carriers is an attractive approach for the treatment of airway disorders such as asthma, chronic obstructive pulmonary disease, pulmonary infections, pulmonary hypertension, lung cancer and cystic fibrosis (Kim et al., 2011a; Yang et al., 2009). Pulmonary drug delivery has great advantages over intravenous administration including a non-invasive method of delivery, a large surface area for absorption, rapid drug absorption, absence of first-pass metabolism, rapid onset of action, highly vascularized epithelium and high bioavailability (Cryan, 2005; Shoyele and Cawthorne, 2006). Targeted pulmonary drug delivery to the affected lung tissues or cells may result in the enhanced therapeutic efficiency and minimized adverse side effects (Dames et al., 2007). These advantages of pulmonary drug delivery are attributed to the unique physiological characteristics of lung. Lung is a complex organ with a primary function of gas exchange and consists of two functional

parts, the upper airways and the lower alveoli. It has been reported that human lung has about 2300 km of airway and 500 million of alveoli and its surface area is approximately 70–140 m² in adults (Sy et al., 2008). Lung provides substantially greater bioavailability for macromolecules and particulate matter than any other port of entry into the body (Jones et al., 2002; Sy et al., 2008). Therefore, lung has served as a means of delivering drugs systemically or locally and pulmonary drug delivery has gained increasing interest (Labiris and Dolovich, 2003).

Efficient pulmonary drug delivery necessitates particulate drug formulation at the micro- or nanometer scale, which effectively delivers therapeutic agents to the site of action for lung diseases. Recent and rapid advances in nanotechnology and materials science have resulted in the development of numerous polymers that can be formulated into particulate drug carriers for efficient drug delivery (Sy et al., 2008). Polymer particulates based on biodegradable polyesters such as poly(lactic-co-glycolic acid) (PLGA) and polycaprolactone have been widely used as delivery vehicles for various drugs and biopharmaceuticals (Kim et al., 2010; Park et al., 2010). PLGA has been approved by United States-Food and Drug Administration (US-FDA) for biomedical and pharmaceutical applications due to its excellent physicochemical properties, relatively inert composition, stable rate of degradation and well-defined degradation products and is one of the most commonly

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used biodegradable polymers (Dailey et al., 2003). However, the extremely slow degradation of PLGA limits its application for pulmonary drug delivery, especially when frequent dosing and rapid therapeutic action are necessary (Dailey et al., 2006, 2003). Consequently, there has been great interest in developing new strategies for the synthesis of fast degrading polymers such as poly(ether-anhydride) and amine-modified branched polyesters (Fiegel et al., 2004; Grenha et al., 2008, 2005). In addition to degradation rate, inflammation is also one of critical factors involving the design of pulmonary drug delivery systems. Non-biodegradable polystyrene nanospheres have shown to induce pulmonary inflammation after intratracheal instillation (Dailey et al., 2006, 2003). PLGA hydrolytically degrades into acid components, which are known to induce inflammatory responses. Previously, Fiore et al. (2010) reported that an intratracheal dose of 1 mg of PLGA microparticles provoked inflammatory responses, evidenced by a large recruitment of white blood cells to the lung. Therefore, there is great need for the development of new biodegradable polymers for pulmonary drug delivery, which have a fast degradation rate and do not produce inflammatory acidic degradation products.

We have recently developed HPOX as a polymeric prodrug of HBA and a drug carrier, which incorporates HBA and peroxalate ester linkage in its backbone (Park et al., 2010). HBA is one of components in a *Gastrodia elata*, which is a widely used herbal agent for the treatment of headache and tetanus. There are several studies reporting that HBA has antioxidant and anti-inflammatory activities and exerts therapeutic effects in the treatment of ischemic brain injury and coronary heart diseases (Descamps et al., 2009; Kim et al., 2007; Lim et al., 2007; Yu et al., 2005). By taking the therapeutic advantages of HBA, we developed HPOX, which incorporates HBA chemically in its backbone and releases HBA during its hydrolytic degradation. HPOX also possesses peroxalate ester bonds which are known to rapidly react with H_2O_2 and degrades hydrolytically, having a half-life of ~ 12 h (Park et al., 2010). Therefore, we reasoned that HPOX scavenges H_2O_2 and degrades into small molecules, one of which is antioxidant and anti-inflammatory HBA (Kim et al., 2011b). Previously, we reported that HPOX nanoparticles exert highly potent antioxidant activity by

reducing the generation of reactive oxygen and nitrogen species in LPS (lipopolysaccharide)-stimulated RAW 264.7 macrophage cells (Kim et al., 2011a,b). In addition, HPOX undergoes rapid hydrolytic degradation under physiological conditions due to unstable peroxalate ester linkages in its backbone. Therefore, it was hypothesized that HPOX has great potential as therapeutics for treating airway inflammatory diseases because of excellent antioxidant and anti-inflammatory activity of HBA generated during its hydrolytic degradation, H_2O_2 removal ability and rapid degradation as shown in Fig. 1.

In the present work, we sought to investigate the mechanistic aspects underlying the antioxidant and anti-inflammatory activities of HPOX nanoparticles and evaluate their potency as therapeutic agents for airway inflammatory diseases using a mouse model of allergic asthma. *In vitro* antioxidant and anti-inflammatory activities of HPOX nanoparticles in LPS-stimulated macrophages were evaluated by measuring the level of reactive oxygen species (ROS) and inflammatory mediators. HPOX nanoparticles were given to mice via intratracheal injection after induction of allergic asthma through OVA (ovalbumin)-sensitization and challenge. The effects of HPOX nanoparticles on cellular infiltration in bronchoalveolar lavage (BAL) fluid, expression of pro-inflammatory cytokines and histopathology of lung tissues were studied to determine their anti-asthmatic activity. The results demonstrate that intratracheal administration of HPOX nanoparticles remarkably reduced OVA-induced airway inflammation, suggesting the great potential of HPOX as therapeutic agents as well as pulmonary drug delivery systems.

2. Experimental

2.1. Materials

1,4-Cyclohexanedimethanol, oxalyl chloride, hydroxybenzyl alcohol and polyvinyl alcohol were obtained from Sigma-Aldrich (St. Louis, MO). PLGA (MW 90 kDa, 75/25 Resomer® RG755) was obtained from Boehringer Ingelheim Pharma KG (Ingelheim, Germany). Tetrahydrofuran (THF), dichloromethane and

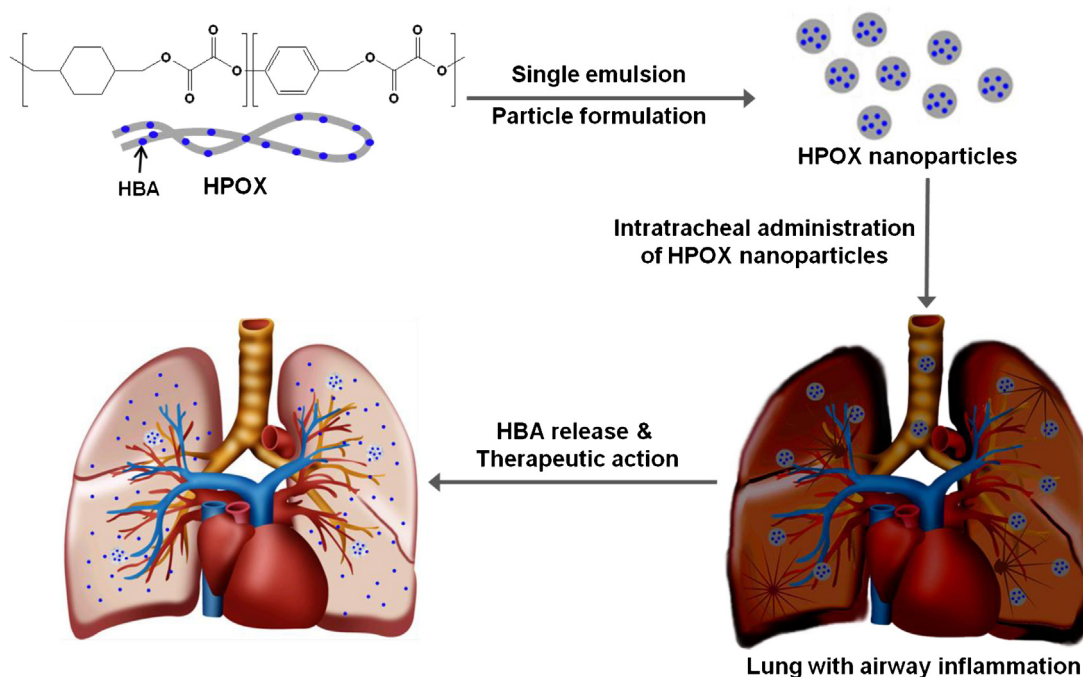


Fig. 1. A novel antioxidant biopolymer for treating airway inflammatory diseases. HPOX is a novel prodrug polymer which incorporates antioxidant and anti-inflammatory HBA in its backbone. HPOX is designed to release HBA during its degradation and exerts therapeutic effects.

triethylamine were obtained from Showa (Japan). All reagents were used without further purification except for THF, which was distilled over calcium hydride.

2.2. HPOX synthesis

HPOX was synthesized as reported previously (Kim et al., 2011a,b; Park et al., 2010). 1,4-Cyclohexanedimethanol (21.96 mmol) and hydroxybenzyl alcohol (5.49 mmol) were dissolved in 20 mL of dry THF under nitrogen. Triethylamine (60 mmol) was added dropwise to the mixture at 4 °C and then oxalyl chloride (27.45 mmol) in 25 mL of dry THF was added to the mixture dropwise at 4 °C. The reaction was allowed for 6 h under nitrogen atmosphere at room temperature and polymers were obtained through the extraction using dichloromethane and isolation by precipitating in cold hexane.

2.3. Preparation and characterization of HPOX nanoparticles

HPOX nanoparticles were formulated using a conventional single emulsion method. In brief, 100 mg of HPOX dissolved in 1000 μ L of dichloromethane was added to 10 mL of 5% (w/v) poly(vinyl alcohol) solution. The mixture was sonicated using a sonicator (Fisher Scientific, Sonic Dismembrator 500) for 40 s and homogenized (PRO Scientific, PRO 200) for 1 min to form a fine oil/water emulsion. The emulsion was added into 20 mL poly(vinyl alcohol) (1%, w/w) solution and homogenized for 1 min. The organic solvent was removed using a rotary evaporator for 30 min. HPOX nanoparticles were obtained by centrifuging at $11,000 \times g$ for 5 min at 4 °C, washing with deionized water twice and lyophilizing the recovered pellet. The morphology and size of HPOX nanoparticles were observed by a scanning electron microscopy (SEM, Hitachi, Japan).

2.4. Antioxidant activity of HPOX nanoparticles

HPOX nanoparticles and HBA were added into 1 mL of H₂O₂ solution (10 μ M). The H₂O₂ solutions were incubated at 37 °C with mechanical stirring for 3, 6, 12 and 24 h. After short centrifugation, the H₂O₂ concentration of the supernatant was determined using the Amplex Red assay (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

2.5. Measurement of intracellular reactive oxygen species

RAW 264.7 cells (1×10^6 cells) were seeded in 24-well plate and incubated for 24 h. Cells were treated with HBA or HPOX nanoparticles for 4 h and incubated with 0.5 mg of PMA (phorbol-12-myristate-13-acetate, Sigma–Aldrich, St. Louis, MO) for 4 h. DCFH-DA (dichlorofluorescein–diacetate, Sigma–Aldrich, St. Louis, MO) was added to each well and after 20 min flow cytometry was performed to quantify the fluorescent cells using the Flow Cytometry Caliber (Becton Dickinson, San Jose, CA).

2.6. Reverse transcription-polymerase chain reaction

RAW 264.7 cells seeded at a density of 4×10^5 in a 24-well culture plate were pretreated with 0.5 mM of HBA and HPOX nanoparticles for 20 h and then treated with 1 μ L of LPS (1 mg/mL) for 10 h. Total cellular RNA was isolated using 1 mL of Trizol (Invitrogen, Life Technologies Co., Groningen, Netherlands) according to the manufacturer's instructions. Three microgram of total RNA was reverse-transcribed into cDNA using oligo (dT) primer (Invitrogen), 5X First Strand buffer (Invitrogen), dNTP (dGTP, dATP, dTTP, dCTP, Gibco), RNase inhibitor (Invitrogen), SuperScript II (Invitrogen), RNase H reverse transcriptase (Invitrogen) and DNase/RNase free water (Gibco). PCR was performed on aliquots of the cDNA

preparations to detect iNOS, IL-1 β , COX-2 and GAPDH (the internal standard) gene expressions by Authorized Thermal Cycler (TP 600, Takara Bio Inc., Japan). After amplification, portions of the PCR reactions were subjected to electrophoresis using 2% agarose gel and visualized under UV after ethidium bromide staining.

2.7. Lung biocompatibility

Nanoparticles (PLGA and HPOX) of equivalent physical characteristics were suspended in sterile saline at a concentration of 20 mg/mL. Balb/C mice (~20 g, Orient Bio, Korea) were anesthetized according to IACUC standards using isoflurane (Hana Pharma, Korea). A small incision was made to expose the trachea. The trachea was punctuated using a sterile syringe and 50 μ L of particle suspension was injected. After injection, mice were sutured and allowed to recover. After mice were sacrificed on day 5, lung tissues were collected.

2.8. A murine model of asthma

BALB/c mice ($n=4$) anesthetized under isoflurane were sensitized intraperitoneally with 75 μ g ovalbumin (OVA, Sigma–Aldrich, St. Louis, MO) and 2 mg of aluminum hydroxide (Sigma–Aldrich, St. Louis, MO) dissolved in 200 μ L phosphate buffered saline (PBS) on days 1 and 8. On days 15, 16 and 17, mice were challenged intranasally with 50 μ g of OVA. HPOX nanoparticles (50 or 100 μ g) were injected mice intratracheally on days 16, 17 and 18. Control mice were given PBS only. On day 19, the mice were sacrificed and lung lobes were isolated along with the trachea.

2.9. Isolation of BAL fluid

Mice were euthanized on day 19 and lungs were lavaged with 500 μ L of PBS introduced through the trachea. BAL fluid was centrifuged at $300 \times g$ for 5 min, and cells were rinsed and resuspended with PBS. Aliquots of the cell suspension were applied to slides by Cytospin (Hanil, Korea) at $500 \times g$ for 5 min allowed to air dry and stained with modified Wright's stain (Hema-3, Fisher Scientific). The numbers of macrophages, eosinophils, neutrophils and lymphocytes were determined morphologically and a minimum of 200 cells per slide were counted under the microscope (Eclipse, Nikon, Japan). The level of IL-4 in the supernatant of BAL fluid was measured with an ELISA kit as advised by manufacturer (eBioScience, San Diego, CA).

2.10. Lung histology

Mice were euthanized and their lungs were perfused with PBS, removed and fixed in 4% buffered formalin. Lungs were embedded in paraffin and serial paraffin sections (3 μ m) were cut. The sections were stained with hematoxylin (Yeongdong Pharma, Korea) and eosin (Showa Chemicals, Japan). A semi-quantitative microscopic evaluation of inflammatory cells in the entire lung section was done by persons blinded to the type of treatment. Inflammatory infiltrates were assessed morphologically for numbers and cell types. For the detection of iNOS, the sections were also subjected to immunofluorescence staining using a monoclonal iNOS antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The sections were blocked with protein block, serum-free for 1 h and treated with a primary antibody iNOS for 14 h and a secondary antibody, mouse IgG labeled with FITC (Santa Cruz Biotechnology Inc., Santa Cruz, CA) for 3 h.

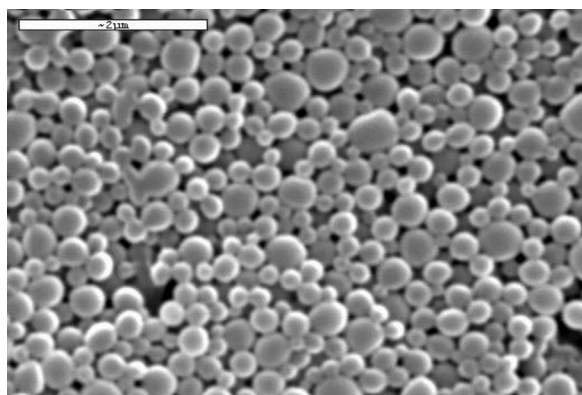


Fig. 2. A representative SEM image of HPOX nanoparticles.

2.11. Statistical analysis

All data are presented as the mean $\pm$ standard deviation. Statistical analysis was performed using a Student *t*-test. Values of $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ were considered statistically significant.

3. Results

3.1. Antioxidant activity of HPOX nanoparticles

A conventional single emulsion method generated HPOX nanoparticles with a mean diameter of ~ 500 nm. HPOX nanoparticles were solid spheres with smooth surface, as demonstrated by SEM (Fig. 2). Antioxidant activity of HPOX nanoparticles was evaluated by investigating their ability to scavenge H_2O_2 and prevent the generation of intracellular reactive oxygen species (ROS). HPOX nanoparticles were added into a $10 \mu M$ of H_2O_2 solution and the H_2O_2 concentration was monitored at an appropriate interval during a 24 h of observation. Fig. 3a shows the change of H_2O_2 concentration measured by Amplex Red assay. HBA showed no H_2O_2 removal ability, but HPOX nanoparticles gradually and continuously scavenged H_2O_2 in a concentration dependent manner. After 24 h, more than half of H_2O_2 was removed by HPOX nanoparticles at a concentration of 2 mg/mL . No further reduction in H_2O_2 concentration was observed thereafter.

The preventative effects of HPOX nanoparticles on ROS production were evaluated by measuring the level of ROS generation in macrophages stimulated with PMA, which was widely used as a

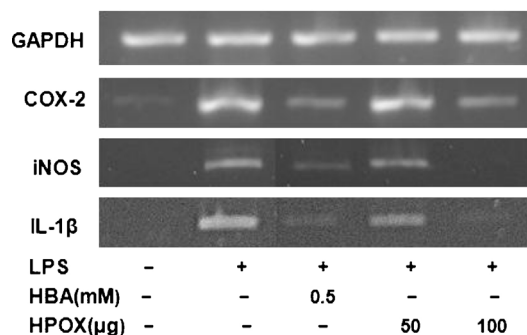


Fig. 4. The effects of HPOX nanoparticles on the production of pro-inflammatory mediators in LPS-stimulated macrophages.

stimulant (Moreno, 2003). DCFH-DA was used as a probe for intracellular ROS because it becomes fluorescent dichlorofluorescein (DCF) by intracellular ROS-mediated oxidation. PMA-treated cells showed a significant rightward shift of DCFH-DA fluorescence, suggesting a large generation of ROS including H_2O_2 (Fig. 3b). During the incubation with HPOX nanoparticles or free HBA, cells were stimulated with PMA and treated with DCFH-DA for 15 min. HBA (0.5 mM) showed a slight leftward shift in DCF fluorescence. In contrast, pretreatment of HPOX nanoparticles showed a remarkable leftward shift in DCF fluorescence in a dose dependent manner, suggesting that HPOX nanoparticles suppress the intracellular ROS generation.

3.2. Anti-inflammatory activity of HPOX nanoparticles

The anti-inflammatory activity of HPOX nanoparticles was assessed to determine their effects on the level of pro-inflammatory mediators. Fig. 4 shows the effect of HPOX nanoparticles on the expression of IL-1β, COX-2 and iNOS at the mRNA level in LPS-stimulated macrophages, detected by RT-PCR. LPS-stimulation caused a large expression of IL-1β, COX-2 and iNOS in macrophages. HBA at the concentration of 0.5 mM significantly suppressed the induction of IL-1β, COX-2 and iNOS, which is in good agreement with previous studies (Lim et al., 2007). HPOX nanoparticles also remarkably suppressed the expression of IL-1β, COX-2 and iNOS, in a concentration dependent manner. A dose of $100 \mu g/mL$ of HPOX nanoparticles almost completely inhibited the expression of IL-1β, COX-2 and iNOS, demonstrating the highly potent anti-inflammatory activity of HPOX nanoparticles.

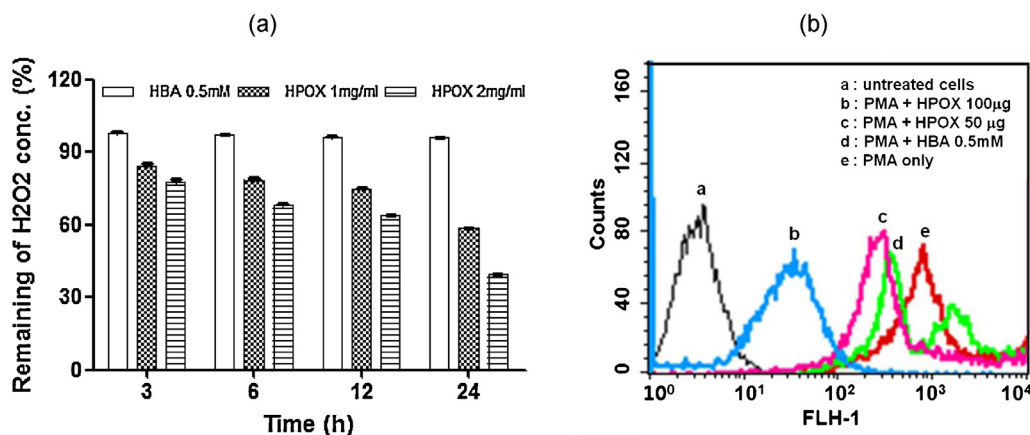


Fig. 3. Antioxidant activity of HPOX nanoparticles *in vitro*. (a) H_2O_2 scavenging activity of HPOX nanoparticles, (b) inhibitory effects of HPOX nanoparticles on ROS generation in PMA-stimulated macrophages.

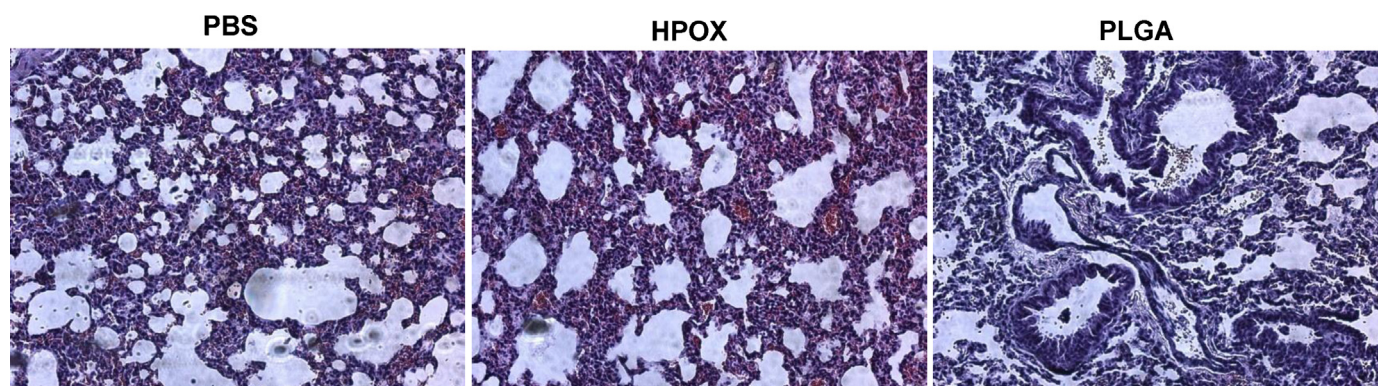


Fig. 5. H&E staining of lung tissues after intratracheal injection of HPOX nanoparticles. Magnification: 200 $\times$.

3.3. Inflammatory responses to HPOX nanoparticles in vivo

After intratracheal injection of HPOX nanoparticles (50 μ L of 20 mg/mL) mice were sacrificed on day 5 and lung histological study was performed to investigate the effect of HPOX nanoparticles on airway architecture and inflammation. Fig. 5 shows the histological analysis of lung inflammatory responses after intratracheal injection of HPOX and PLGA nanoparticles. PLGA nanoparticles with a mean diameter of $\sim$ 450 nm were used for comparison purposes. PLGA nanoparticles-treated lung displayed mild inflammatory

responses and increased alveolar wall thickening. In contrast, HPOX nanoparticles-treated group showed a phenotype similar to PBS-treated control group which has minimal inflammatory responses and no alteration of airway architecture.

3.4. Anti-asthmatic effects of HPOX nanoparticles in a murine model of allergic asthma

Allergic asthma was induced in mice using OVA-sensitization and challenge and the asthmatic mice were given HPOX

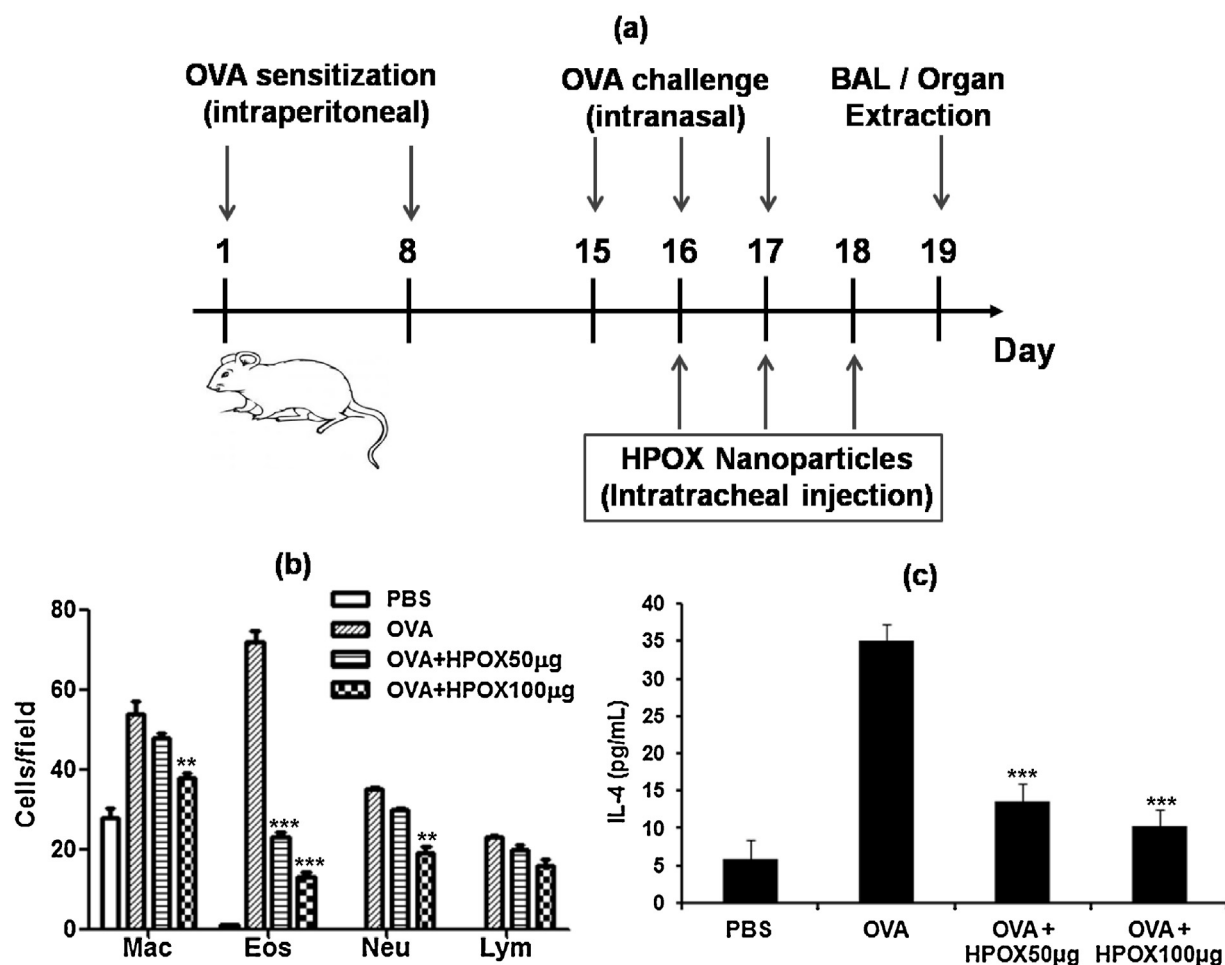


Fig. 6. HPOX nanoparticles for the treatment of OVA-induced allergic asthma. (a) An experimental protocol of a murine model of asthma. (b) Inflammatory cell populations in BAL fluid of asthmatic mice. (c) The effects of HPOX nanoparticles on the level of IL-4 in the BAL fluid of asthmatic mice. ** P < 0.01, *** P < 0.001 relative to OVA-sensitized and challenged group.

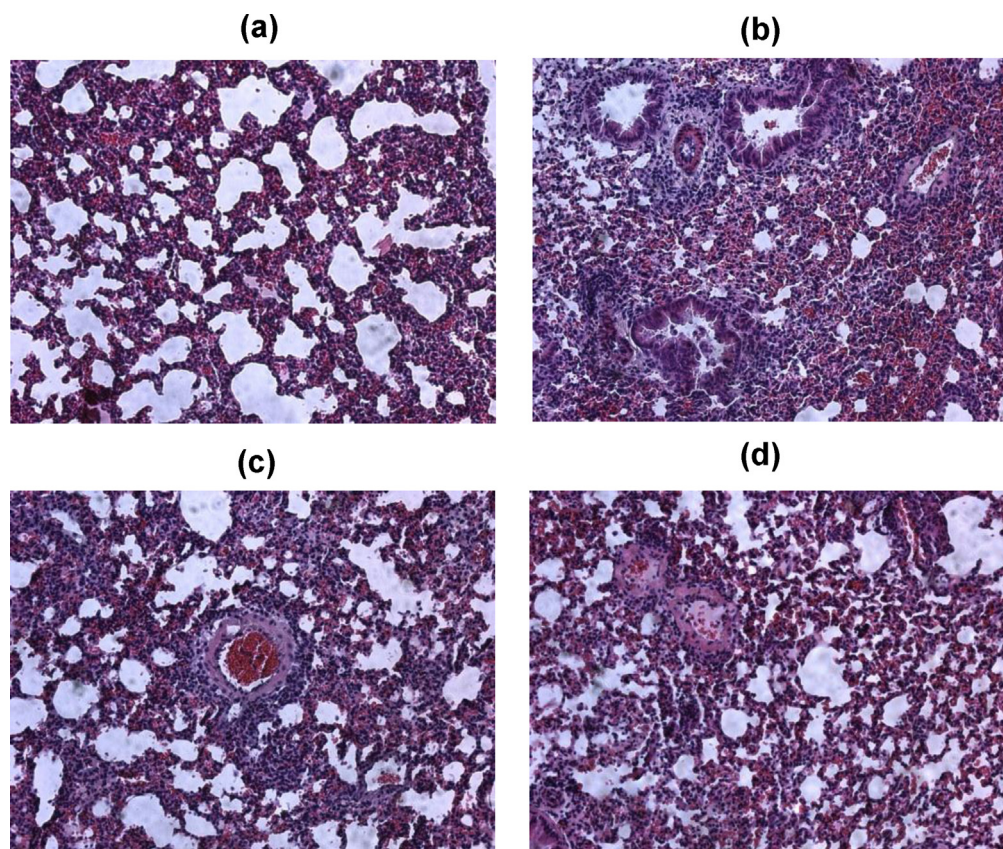


Fig. 7. H&E staining of lung tissues from asthmatic mice after intratracheal injection of HPOX nanoparticles. (a) PBS, (b) OVA, (c) OVA + 50 µg of HPOX nanoparticles, (d) OVA + 100 µg of HPOX nanoparticles. Magnification: 200×.

nanoparticles intratracheally for 3 days, as shown in Fig. 6a. At day 19, BAL fluid was collected and the population of cells in the BAL fluid was counted to investigate the therapeutic effects of HPOX nanoparticles. OVA-sensitization and challenge significantly increased the total number of cells and the populations of macrophages and eosinophils, which are the most representative inflammatory cells in asthma (Oh et al., 2011). Treatment of HPOX nanoparticles exerted therapeutic effects on OVA-induced allergic asthma, in a dose dependent manner. Reduction in populations of these inflammatory cells was observed with 50 µg of HPOX nanoparticles. However, treatment with 100 µg of HPOX nanoparticles reduced significantly the population of macrophages and eosinophils (Fig. 6b).

We also measured the level of IL-4 in BAL fluid, which is one of cytokines involving the pathogenesis of asthma and allergic airway diseases (Kandasamy et al., 2010; Wong et al., 2001). As shown in Fig. 6c, OVA-induced asthmatic mice showed a ~7-fold higher level of IL-4 compared to the control group. Treatment of HPOX nanoparticles significantly reduced the IL-4 level. No significant difference was observed between groups treated with 50 µg of HPOX nanoparticles and 100 µg of HPOX nanoparticles. These findings clearly demonstrate that HPOX nanoparticles exert strong anti-asthmatic effects on asthmatic mice.

3.5. Histological studies

The effects of HPOX nanoparticles on OVA-induced histopathology in lung tissues are shown in Fig. 7. The lung tissues from PBS-treated mice showed normal structural architecture with no inflammatory cell infiltration around bronchiole and intra lumen. However, OVA-sensitized and challenged mice showed severe

peribronchial inflammation and airway remodeling, evidenced by airway thickening and a massive infiltration of inflammatory cells, predominantly eosinophils and mononuclear cells in perivascular and peribronchial regions. HPOX nanoparticles showed dose dependent therapeutic effects. Moderate anti-inflammatory effects were observed with 50 µg of HPOX nanoparticles. A dose of 100 µg of HPOX nanoparticles showed a considerable reduction in the established pulmonary inflammation and airway remodeling, evidenced by the less epithelial damage, the decreased number of infiltrating inflammatory cells in the interstitial and peribronchial regions and the decreased airway wall thickness.

3.6. Effects of HPOX nanoparticles on iNOS expression in lung

We measured the level of iNOS in lung of asthmatic mice to further determine whether the anti-asthmatic effects of HPOX nanoparticles are related with nitric oxide (NO). Fig. 8a shows the immuno-histological staining of lung tissues stained with iNOS. OVA-sensitized and challenged mice showed predominantly iNOS-stained cells in peribronchial and subepithelial regions. However, intratracheal administration of HPOX nanoparticles decreased the iNOS-stained cells in asthmatic lung tissues. To further quantify the inhibitory effects of HPOX nanoparticles on iNOS expression, we investigated the iNOS expression using Western blot analysis. As shown in Fig. 8b, the iNOS expression was significantly elevated in OVA-sensitized and challenged mice and the treatment of HPOX nanoparticles remarkably inhibited the iNOS expression. The level of β-actin was not affected by the treatment. These results demonstrate that intratracheal administration of HPOX nanoparticles exert anti-asthmatic activity by reducing the iNOS expression.

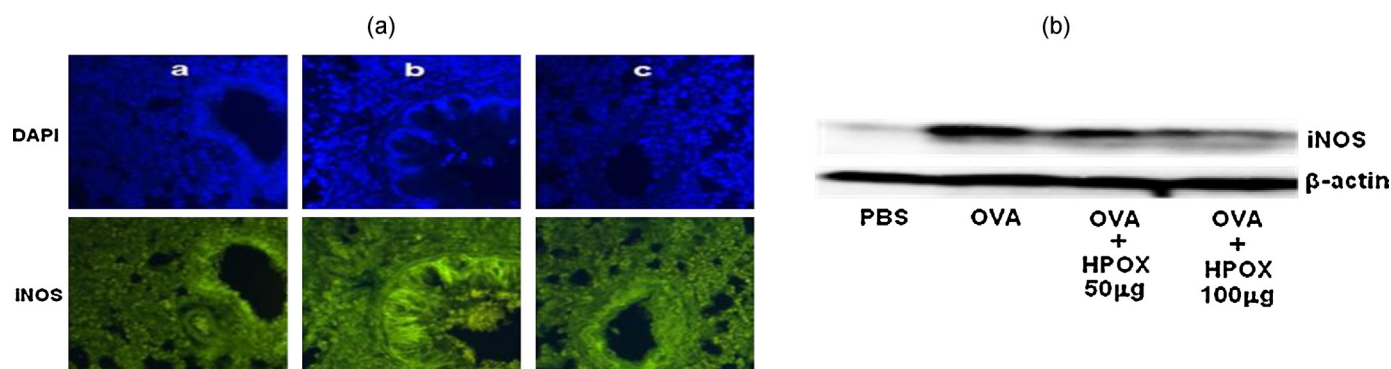


Fig. 8. The effects of HPOX nanoparticles on the expression of iNOS. (A) Immunofluorescence staining of lung sections. a: PBS, b: OVA, c: OVA + 100 µg of HPOX nanoparticles; (B) Western blotting analysis. Magnification: 200×.

4. Discussion

Asthma is characterized by airway hyperresponsiveness to a variety of allergens, airway edema, increased mucus secretion and infiltration of inflammatory cells such as eosinophils, neutrophils, macrophages and lymphocytes into the bronchial lumen and lung tissues (Lee et al., 2006a; Moon et al., 2008). These inflammatory cells are known to affect the structural cells of the airway such as bronchial epithelia, which orchestrate the airway inflammation (Mabalirajan et al., 2010). An elevated level of inflammatory cell populations is one of hallmarks of asthma and therefore, the most common treatment of asthma is the use of corticosteroids which have excellent anti-inflammatory effects and efficiently inhibit a variety of inflammatory responses that evoke airway hyperresponsiveness (Oh et al., 2011).

Allergic airway inflammation is associated with more than 50 inflammatory mediators, one of which is oxidative stress mediated by ROS and NO (Mabalirajan et al., 2010; Moon et al., 2008). H_2O_2 is one of ROS and its overproduction leads to oxidative stress, resulting in significant damages to cell structure and inflammatory responses. The overproduced H_2O_2 is highly correlated with the pathogenesis and progression of asthma and chronic obstructive pulmonary disease. NO is a highly reactive free radical and is also an important immunomodulatory and cytotoxic mediator of inflammatory responses in lung. NO is synthesized from the amino acid L-arginine, which is catalyzed by nitric oxide synthase (NOS). The excessive production of NO contributes to the pathogenesis of inflammation, cellular injury and tissue damages in lung. It was also reported that human asthmatics have a higher level of exhaled NO and iNOS expression in the airway of epithelium compared to non-asthmatic humans. Therefore, reduction of H_2O_2 - and NO-mediated oxidative stress provides a rational strategy for effective treatment of allergic asthma.

In this work, we evaluated the potential of HPOX nanoparticles as novel therapeutics for airway inflammatory diseases using a cell culture model and a murine model of allergic asthma. First, we investigated the antioxidant activity of HPOX nanoparticles *in vitro*. As shown in Fig. 3, free HBA at a concentration of 0.5 mM of showed negligible H_2O_2 removal ability, but HPOX nanoparticles continuously and gradually reduced H_2O_2 concentration for 24 h of observation time, demonstrating that peroxalate ester bonds in the backbone of HPOX react with H_2O_2 , removing H_2O_2 . HPOX nanoparticles also remarkably inhibited the generation of intracellular ROS including H_2O_2 in PMA-stimulated macrophages. HPOX nanoparticles showed a significantly higher inhibitory effect than free HBA. A dose of 0.5 mM of HBA suppressed the expression of iNOS, COX-2 and IL-1 β in LPS-stimulated macrophages, which are major pro-inflammatory mediators in allergic asthma. These observations are in good agreement with previous studies (Lee et al.,

2006b; Lim et al., 2007). HPOX nanoparticles exhibited remarkable suppressive effects on the expression of iNOS, COX-2 and IL-1 β in LPS-stimulated macrophages, demonstrating that HPOX nanoparticles have great potential as an anti-asthmatic agent. Theoretically, 100 µg of HPOX nanoparticles are supposed to release ~15 µg of HBA, which is ~0.083 mM in this experiment. However, 100 µg of HPOX nanoparticles exhibited remarkably higher antioxidant and anti-inflammatory activities than 0.5 mM of free HBA. These observations can be explained by the combined effects of peroxalate ester bonds and HBA, as aforementioned. HPOX scavenged H_2O_2 through the H_2O_2 -consuming oxidation of peroxalate esters and then released HBA which exerts antioxidant and anti-inflammatory activities to prohibit the generation of intracellular ROS and pro-inflammatory mediators (Kim et al., 2011a,b).

One of critical issues involving particulate drug delivery systems is the foreign-body responses by the host immune system. We compared the inflammatory responses of HPOX and PLGA nanoparticles in lung (Fig. 5). PLGA is one of the most commonly used biodegradable polymers for drug delivery applications. However, PLGA nanoparticles displayed a characteristic increase in inflammation, thickening of alveolar wall, distortion of lung architecture and significant macrophage infiltration. This is in a good agreement of previous studies reporting that degradation of PLGA into acidic byproducts could serve to aggravate the underlying inflammation (Dailey et al., 2006; Fiore et al., 2010). As opposed to PLGA, HPOX nanoparticles caused very little remodeling of lung and recruitment of inflammatory cells, suggesting their excellent biocompatibility in lung. The excellent biocompatibility of HPOX in lung may be due to its rapid degradation rate (half-life of ~12 h) and the reported anti-inflammatory activity of its degradation product, HBA (Park et al., 2010). Although further studies would be necessary to elucidate the mechanism of this HPOX action, HPOX displays a very attractive biocompatibility profile in lung and is a promising candidate as drug carriers.

In order to establish the clinical usefulness of HPOX nanoparticles as anti-asthmatic agents, we tested their therapeutic effects using a mouse model of OVA-induced asthma. As shown in Fig. 7, OVA-sensitization and challenge caused severe lung infiltration, pulmonary edema and inflammation, which is one of hallmarks of asthma (Oh et al., 2011). All these detrimental effects of OVA exposure were significantly reduced by the intratracheal administration of HPOX nanoparticles for 3 days. HPOX nanoparticles exerted anti-inflammatory effects by inhibiting the migration of leukocytes to the inflammation site and leakage from the blood vessels. This suppressive effect on OVA-induced inflammation may be due mainly to inhibited production of pro-inflammatory mediator such as IL-4, COX-2 and iNOS. NO is a highly reactive radical produced by iNOS and leads to the recruitment of inflammatory cells in airway, including eosinophils and neutrophils. The overproduction of

NO is known to contribute the pathogenesis of inflammation, airway narrowing and disease severity in lung (Ramesh et al., 2001). Importantly, we found that intratracheal administration of HPOX nanoparticles remarkably suppressed the generation of iNOS which are predominately expressed in inflammatory cells. Moreover, it was found that treatment of HPOX nanoparticles significantly reduced the populations of eosinophils which are an important effector cell in the inflammatory response to allergens. Increased numbers of eosinophils are detectable in bronchial biopsy specimens and sputum from asthma patients (Lee et al., 2006a,b). Taken together, HPOX nanoparticles exert potent anti-asthmatic activities by reducing oxidative stress and suppressing the pro-inflammatory cytokines and mediators.

5. Conclusions

We developed antioxidant HPOX nanoparticles and evaluated their potential as therapeutic agents for inflammatory airway diseases *in vitro* and *in vivo*. HPOX nanoparticles significantly reduced the generation of intracellular ROS and pro-inflammatory cytokines in stimulated macrophages. HPOX nanoparticles exhibited the excellent biocompatibility in lung, confirmed by the little or no inflammatory responses. The feasibility of HPOX nanoparticles for clinical applications was evaluated using a mouse model of allergic asthma. HPOX nanoparticles significantly reduced the recruitment of inflammatory cells, especially eosinophils and neutrophils and suppressed the expression of iNOS. The potent anti-asthmatic effects of HPOX nanoparticles may be due to the combined effects of antioxidant and anti-inflammatory HBA and H₂O₂ scavenging ability of peroxalate ester linkages. Given their excellent biocompatibility and highly potent antioxidant and anti-inflammatory effects, HPOX nanoparticles exhibit tremendous potential as therapeutics as well as drug delivery vehicles for the treatment of airway inflammatory diseases.

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