

Inhalable Nitric Oxide Delivery Systems for Pulmonary Arterial Hypertension Treatment

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Pulmonary arterial hypertension (PAH) is a severe medical condition characterized by elevated blood pressure in the pulmonary arteries. Nitric oxide (NO) is a gaseous signaling molecule with potent vasodilator effects; however, inhaled NO is limited in clinical practice because of the need for tracheal intubation and the toxicity of high NO concentrations. In this study, inhalable NO-releasing microspheres (NO inhalers) are fabricated to deliver nanomolar NO through a nebulizer. Two NO inhalers with distinct porous structures are prepared depending on the molecular weights of NO donors. It is confirmed that pore formation can be controlled by regulating the migration of water molecules from the external aqueous phase to the internal aqueous phase. Notably, open porous NO inhalers (OPNIs) can deliver NO deep into the lungs through a nebulizer. Furthermore, OPNIs exhibit vasodilatory and anti-inflammatory effects via sustained NO release. In conclusion, the findings suggest that OPNIs with highly porous structures have the potential to serve as tools for PAH treatment.

Hence, oral drugs that dilate constricted and thickened blood vessels have been developed; however, they are limited because they can cause serious side effects such as systemic vasodilation and hypotension.^[2,4]

NO, which dilates blood vessels by activating the expression of cyclic guanosine monophosphate (cGMP) in smooth muscle cells (SMCs), has emerged as a potent therapeutic agent for PAH.^[5] NO can also regulate the inflammatory response by modulating the polarization of macrophages.^[6] Therefore, inhaled NO (iNO) is used to reduce the risk of pulmonary hypertensive crisis and improve oxygenation in the postoperative management of patients with congenital heart disease (CHD).^[7] A previous study demonstrated that routine administration of iNO after CHD surgery decreased the risk of pulmonary

hypertensive crisis.^[8] However, the use of iNO is limited in clinical practice because the exact NO concentration delivered to the lungs is unknown, raising concerns about the toxicity associated with high NO concentrations. Furthermore, iNO should be administered through endotracheal intubation using a complex delivery device.^[9]

Diazeniumdiolates (NONOates) are hydrophilic NO donors that comprise NO and nucleophiles, and they spontaneously release NO in an aqueous solution. NONOates, which depend on nucleophiles, have longer NO-release profiles than gaseous NO, which has a short half-life of 2–3 s.^[10] Previous studies have confirmed that the inhalation of various NONOates, such as DPTA/NONOate, DETA/NONOate, and Spermine NONOate, via a nebulizer can replace iNO in PAH treatment.^[9b,10c,11] However, nebulized NONOates are rapidly cleared by macrophages,

1. Introduction

Pulmonary arterial hypertension (PAH) is a rare and progressive disease that can ultimately lead to right heart failure and death. The common pathological hallmarks of PAH are pulmonary vasoconstriction and abnormal vascular remodeling.^[1] PAH lesions have reduced levels of vasodilatory mediators (nitric oxide [NO] and prostaglandin I₂) due to endothelial dysfunction.^[2] Furthermore, the overexpression of proinflammatory cytokines exacerbates the inflammatory response, causing structural changes in the pulmonary vessels.^[1a,3] These symptoms cause the pulmonary arteries to thicken and contract, thereby increasing pulmonary artery pressure and pulmonary vascular resistance.^[1a]

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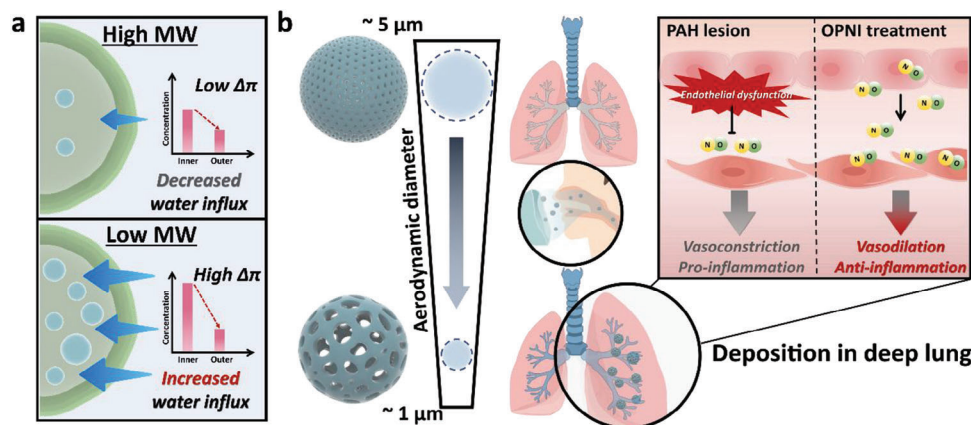


Figure 1. Schematic illustration of the therapeutic strategy for PAH a) Pore formation depending on the molecular weights of NO donors. b) Pulmonary delivery of NO inhalers depending on the porous structures.

attenuating NO bioavailability.^[12] NO also exhibits concentration-dependent effects on cells at a limited diffusion distance of <100 μm ; nanomolar NO has protective effects, whereas micromolar NO has harmful effects.^[13] Therefore, inhalable carriers that can locally deliver known NO concentrations into the deep lungs are required for easy and safe PAH treatment.

Herein, we present a PAH treatment strategy that involves using inhalable NO-releasing microspheres (NO inhalers) to deliver NO into the deep lungs via nebulizers (Figure 1). Poly(lactic-co-glycolic acid) (PLGA), a biodegradable polymer, was used to prepare large porous microspheres for the pulmonary delivery of NO.^[14] We hypothesized that pore formation counts on water droplets that diffuse from the internal aqueous phase (W1) into the external aqueous phase (W2) in the double emulsion solvent–evaporation processes.^[15] Two distinct types of NO inhalers, referred to as closed porous NO inhalers (CPNIs) and open porous NO inhalers (OPNIs), respectively, were fabricated depending on the molecular weights of branched polyethyleneimine/NONOates (BPEI/NONOates). We confirmed that the porous structures determine the deposition of microspheres in a respiratory simulation model. Furthermore, we observed that the NO-release profile plays a crucial role in modulating the expression of cGMP, which is an essential mediator of vasodilation in SMCs, as well as the polarization of macrophages, which regulates the inflammatory response in PAH lesions. Therefore, we suggest that NO inhalers with highly porous structures could be a powerful tool for PAH treatment.

2. Results and Discussion

2.1. Synthesis and Characterization of NO Inhalers

NO inhalers with two different porous structures were fabricated using 0.8 and 25 kDa BPEI/NONOates, which are hereinafter abbreviated as BPEI0.8k/NO and BPEI25k/NO, respectively. BPEI, an amine-rich polymer, was used to generate NONOates in the polymer chain, offering several advantages. Notably, unlike other NO donors, such as DETA/NONOate and Spermine/NONOate, the adjacent amine groups stabilize the NONOates moieties through electrostatic interactions, re-

tarding NO release from BPEI/NONOates.^[16] Furthermore, the molecular weight of BPEI determines the stabilization of the NONOates, thereby controlling NO release.^[16c] This characteristic release behavior of BPEI/NONOates enables a sustained and controlled NO delivery, a crucial factor in PAH treatment. Therefore, we prepared BPEI0.8k/NO and BPEI25k/NO using the method employed in our previous studies (Figure S1, Supporting Information).^[17] The formation of NONOates was analyzed by UV–vis spectroscopy, Fourier-transform infrared (FT-IR), and H-NMR (Figures S2 and S3, Supporting Information). The characteristics of BPEI0.8k/NO and BPEI25k/NO are described in the Supporting Information. BPEI0.8k/NO released NO for an extended period of over 80 h, while BPEI25k/NO completed NO release within 67 h (Figure S4 and Table S1, Supporting Information). NO release tended to depend on the molecular weights of BPEI, which is similar to a previous finding.^[16c]

NO inhalers were prepared using the double emulsion–solvent evaporation method. Ammonium bicarbonate (ABC), which generates pores by producing ammonia and carbon dioxide, was added to the W1 phase to prepare the porous microspheres.^[18] Furthermore, we employed BPEI0.8k/NO for OPNI and BPEI25k/NO for CPNI producing two distinct types of NO inhalers. Porous PLGA microspheres without BPEI/NONOates (Blank) were also prepared (Figure S5, Supporting Information). To confirm the fabrication of NO inhalers, we first observed the morphologies of NO inhalers using scanning electron microscopy (SEM; Figure 2a,b). The SEM images revealed that porous microspheres of different pore sizes were successfully formed in both NO inhalers. The size distributions of NO inhalers were determined using laser diffraction analysis. Regardless of the molecular weight of BPEI, NO inhalers had geometric diameters of >20 μm . Figure 2c shows that after BPEI/NONOates were encapsulated into the PLGA microspheres, the surface charge of NO inhalers increased owing to the amine groups of the BPEI/NONOates. We performed UV–vis spectroscopy and X-ray photoelectron spectroscopy (XPS) analysis to confirm the presence of BPEI/NONOates in NO inhalers. The characteristic absorbance peak of NONOates ($\lambda_{\text{max}} = 253\text{--}257\text{ nm}$) was observed in NO inhalers (Figure 2d). The BPEI/NONOate amounts in NO inhalers were determined from

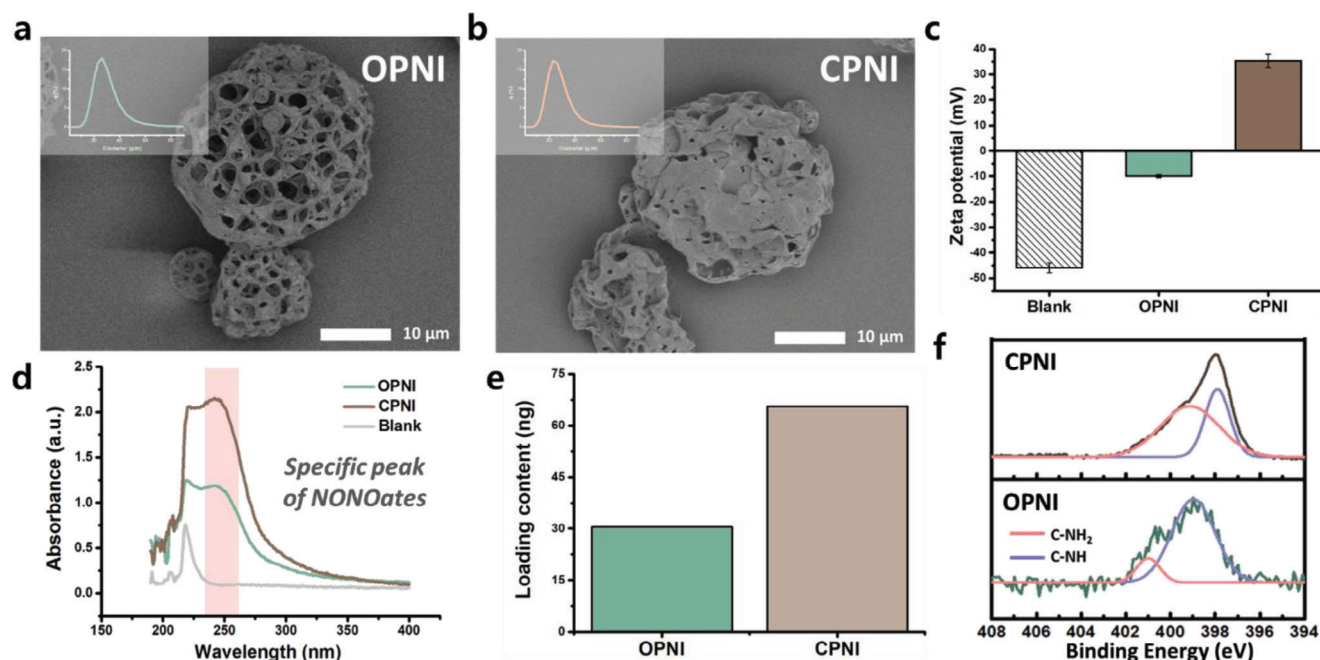


Figure 2. Analysis of NO inhalers with two different porous structures. a,b) SEM images and size distributions of NO inhalers. c) Surface charge of the NO inhalers measured in deionized water ($n = 5$). d) UV-vis spectra of NO inhalers incubated in an alkaline solution. e) Loading amount of BPEI/NONOates encapsulated into NO inhalers. f) XPS spectra of NO inhalers.

the UV-vis spectra. We confirmed that more BPEI25k/NO was loaded into NO inhalers than BPEI0.8k/NO (Figure 2e). The XPS spectra revealed that the atomic ratio of nitrogen increased in NO inhalers (Figure 2f and Table S2, Supporting Information). These results indicate that NO inhalers, micro-sized porous particles loaded with BPEI/NONOates, were successfully prepared.

2.2. Porous Structures of NO Inhalers

The formation of pores during the preparation process is attributed to the following two reasons. Tiny pores originated from evolved gas bubbles, and large pores were induced by water diffusion from W2 to W1.^[15b,18c,19] BPEI/NONOates can produce an osmotic pressure gradient, resulting in a water influx.^[15b,19b] The diffusion flux can be calculated as follows

$$\frac{dN_m}{dt} = \frac{C(\infty)A}{h} D(c_1 - c_2) \quad (1)$$

where N_m is the molar number of the diffused molecules (mol), t is the permeation time (s), $C(\infty)$ is the solubility of water in the oil phase (mol/mol), A is the mass-transfer area (m²), h is the oil membrane thickness (m), D is the diffusion coefficient (m² s⁻¹), and $c_1 - c_2$ is the molarity difference between the aqueous phases (mol mL⁻¹).^[1] The molarity in the W1 phase is inversely proportional to the molecular weights of BPEI/NONOates. Hence, more water influxes because BPEI0.8k/NO causes a larger molarity difference than BPEI25k/NO. The water molecules diffusing into the W1 phase form large aqueous droplets, which create pores in the particles after the water is removed by lyophilization.^[19b] Consequently, highly porous microspheres would be fabricated using BPEI0.8k/NO rather than BPEI25k/NO (Figure 3a).

We compare the water movement depending on the molecular weights of BPEI/NONOate using methylene blue (MB) as a tracer with a maximum UV-vis absorption at a wavelength of 663 nm.^[20] To determine the osmotic pressure difference during the preparation of NO inhalers, a Float-A-Lyzer G2 dialysis device containing MB solution was immersed in BPEI/NONOates solution (Figure 3b). Subsequently, we quantified the release of MB over time. As MB was released, the peak intensity at 663 nm in UV-vis spectra increased, indicating the migration of MB. Notably, more MBs were moved to the BPEI0.8k/NO solution compared to the BPEI25k/NO solution (Figure 3c). Furthermore, we introduced MB into the W2 phase and evaluated the amount of MB encapsulated within NO inhalers to confirm the entry of water molecules into the particles, driven by the osmotic pressure difference between the W1 and W2 phases (Figure 3d). We observed that the increased osmotic pressure generated by BPEI0.8k/NO allowed more MBs to enter OPNI (Figure 3e). These results suggest that the pore formation depends on the molecular weights of BPEI/NONOates.

To confirm the porous structures of NO inhalers, we investigated the apparent pore size on the NO inhalers' surfaces using SEM (Figure 3f,g). The OPNI exhibited open porous structures, whereas the CPNI showed very tiny pores. Nile red, a hydrophobic fluorescent dye, was incorporated into the PLGA solution during the preparation processes for confocal microscopic analysis (Figure S6, Supporting Information). Confocal Z-stack imaging was used to verify the internal porous structures (Figure S7, Supporting Information). The analysis of both surface and internal structures of NO inhalers confirmed that the porous structures of the particles are dependent on the molecular weight of BPEI/NONOates. The pore-size distribution was obtained using mercury intrusion porosimetry (Figure 3h).

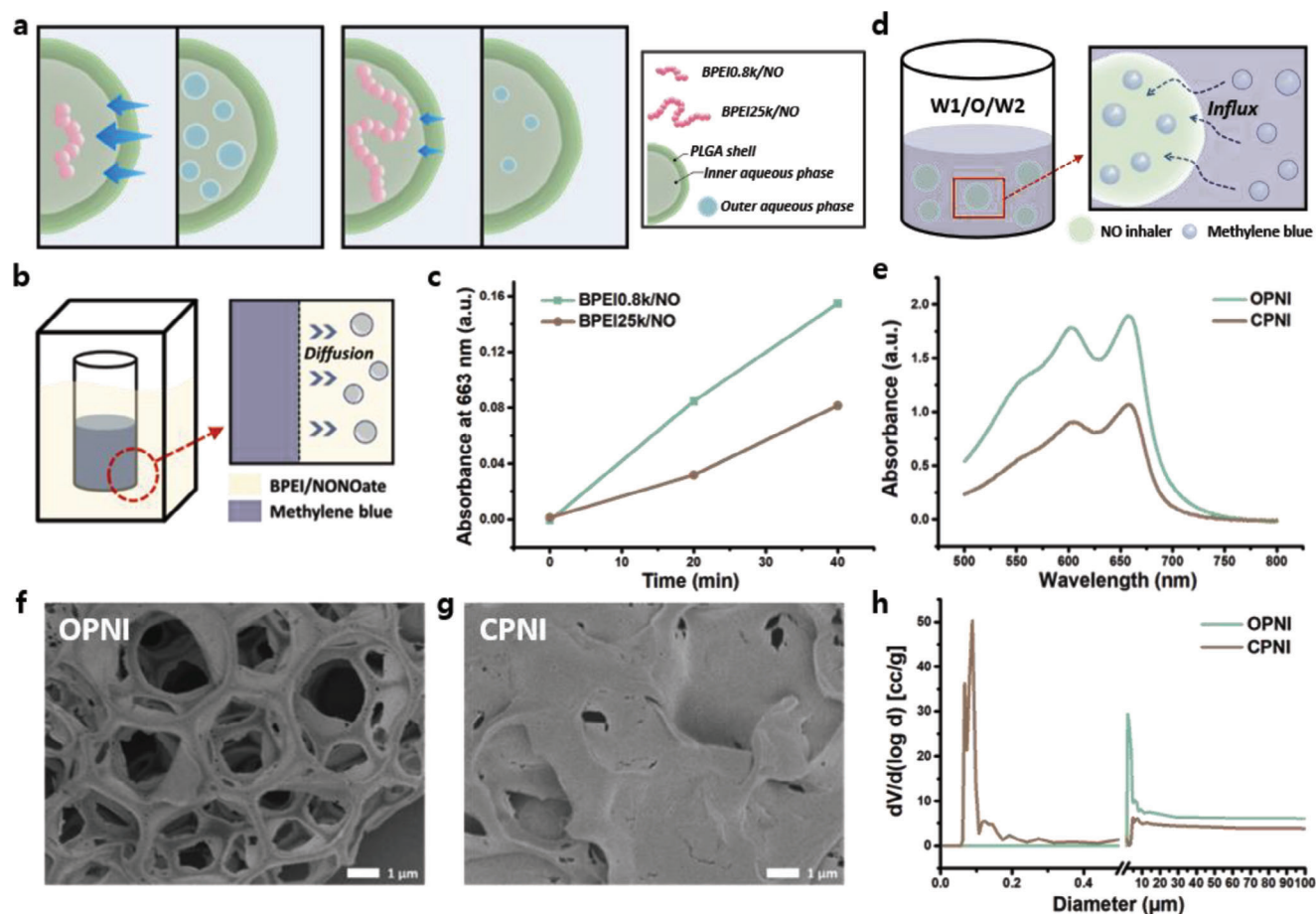


Figure 3. Porous structures of NO inhalers a) Pore formation depending on the molecular weights of BPEI/NONOates. b) Schematic illustration of the experiment to compare the osmotic pressure formed by BPEI/NONOates. c) Time-dependent absorbance spectra at 663 nm for diffused MB. d) Schematic illustration of the experiment to compare the movement of water molecules during NO inhaler preparation processes. e) UV-vis spectra of encapsulated MB into NO inhalers. f, g) Pore on the surface of NO inhalers. h) Pore-size distribution of NO inhalers obtained via mercury intrusion porosimetry.

Table 1. Porosity of NO inhalers obtained using mercury intrusion porosimetry.

	OPNI	CPNI
Pore size [μm]	5.383	0.203
Density [g/cc]	0.028	0.039
Aerodynamic diameter [μm]	3.54	4.56

Consistent with the SEM images, the pore-size distribution results revealed that OPNI had micro-sized pores, whereas CPNI had nano-sized pores. We calculated the aerodynamic diameter of NO inhalers using the following equation

$$D_a = D_v \sqrt{\frac{\rho}{\rho_0 x}} \quad (2)$$

where D_v is the geometric diameter of particles, ρ and ρ_0 are the particle and unit density (1 g cm^{-3}), respectively, and x is the dynamic shape factor (1 for spheres).^[21] Table 1 summarizes the av-

erage pore size, density, and aerodynamic diameter. Despite large geometric diameters ($>5 \mu\text{m}$), NO inhalers exhibited small aerodynamic diameters due to their low density ($<0.4 \text{ g cm}^{-3}$). As a result, our findings demonstrate that OPNI with highly porous structures is an ideal candidate for pulmonary delivery.^[14c,18a]

2.3. Nebulization Efficiency of NO Inhalers

iNO requires a gas cylinder and complex delivery devices, limiting its use in clinical practice. Previous studies have developed NO generators that produce NO electrically or electrochemically to overcome the limitations. However, these NO generators need specially designed devices, so additional costs and device malfunction are still concerns.^[22] Nebulizer therapy is easy and versatile, allowing outpatient use and rapid application to most patients even during pandemics. Therefore, we employed a nebulizer was used to deliver NO into the deep lungs using NO inhalers in this study.

A customized respiratory system with a nebulizer was prepared to evaluate the nebulization efficiency of NO inhalers

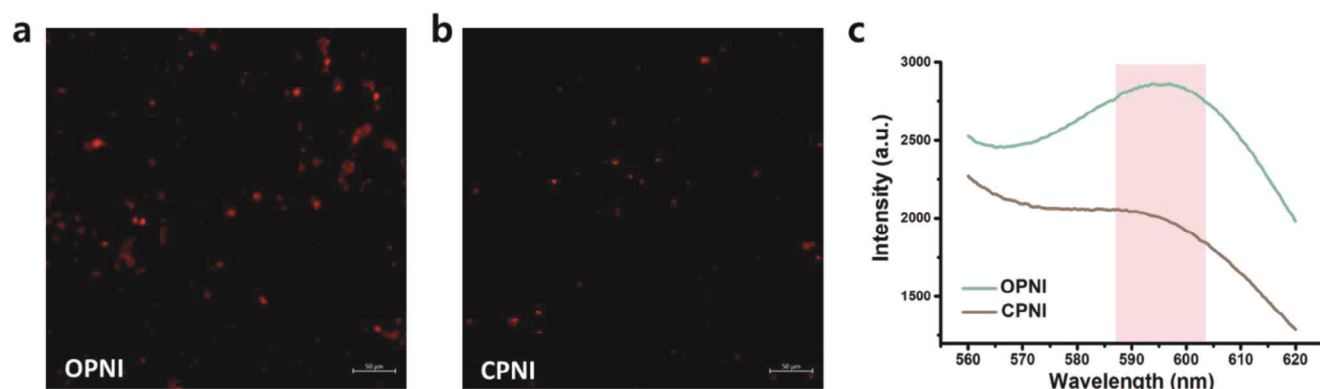


Figure 4. Nebulization efficiency of NO inhaler for pulmonary delivery a,b) Confocal images of collected NO inhalers after nebulization (scale: 50 μm). c) Photoluminescence spectra of nebulized NO inhalers (Nile red; emission maximum at 598 nm).

(Figure S8, Supporting Information). The movement of aerosols was monitored by observing the flask's opacity and changes in color on filter paper within a vial (Figure S9, Supporting Information). To quantify the delivered NO inhalers, we suspended Nile-red-loaded NO inhalers in a saline solution and placed a glass slide within a vial representing the deep lung. After nebulization, the deposited NO inhalers on the glass slide were analyzed. We found no morphological changes in NO inhalers after nebulization (Figure S10, Supporting Information). Confocal images clearly showed a greater deposition of OPNI compared to CPNI (Figure 4a,b). The fluorescence intensity of Nile red was analyzed using photoluminescence spectroscopy to confirm the amount of NO inhalers on the glass slide (Figure 4c). These results revealed that a larger amount of OPNI could arrive at the lower region than CPNI, similar to the confocal image results. These findings imply that the deposition of NO inhalers was determined by the porous structures. Consequently, we conclude that highly porous structures facilitated the delivery of OPNI to the deep lungs.

2.4. Vasodilatory Effects of NO Inhalers

Macrophage phagocytosis is a challenge that deteriorates the therapeutic effects of particles after administration. Large porous microspheres can escape uptake by macrophages because of their large geometric size, resulting in prolonged circulation time.^[23] We prepared 1 μm of PLGA microsphere that was likely to be consumed by macrophages as a control. After 4 h of coculture with the particles, confocal images were obtained to confirm the internalized particles in the macrophages. Both NO inhalers were rarely taken up by the macrophages even though they were close to the cells, whereas a cellular uptake of the 1 μm microspheres was observed (Figure S11, Supporting Information). Therefore, NO inhalers can deliver NO to cells and tissues within a prolonged circulation time without rapid clearance after being administered via a nebulizer.

NO produced by endothelial NO synthase (eNOS) induces vasodilation by increasing cGMP in vascular SMCs.^[1b] However, endothelial dysfunction reduces NO bioavailability in hypertension lesions, resulting in impaired endothelium-dependent vascular relaxation.^[5b,c,24] The concentrations of NO generated in normal endothelium are known to be 0.05 to 0.4 nmol

$\text{cm}^{-2} \text{min}^{-1}$.^[25] Therefore, NO inhalers should release less than 0.4 nmol $\text{cm}^{-2} \text{min}^{-1}$ of NO for PAH treatment. The NO-release profiles of NO inhalers were evaluated using a chemiluminescent method. Figure 5a shows the accumulated NO-release profiles, and Table 2 summarizes the detailed information. OPNI exhibited sustained NO release due to the stabilization effect derived from the electrostatic interaction between the NONOates and amine groups. Conversely, CPNI presented an initial burst release due to the decreased area where the amine groups stabilize NONOates.^[16c] Therefore, OPNI released 0.277 nmol $\text{cm}^{-2} \text{min}^{-1}$ of NO mimicking a healthy endothelial environment, while the CPNI released a higher NO concentration, 0.548 nmol $\text{cm}^{-2} \text{min}^{-1}$.

As endothelial dysfunction plays an important role in the progression of PAH, we first evaluated the toxicity of NO inhalers on human umbilical vein endothelial cells (HUVECs) before the evaluation of the vasodilatory effects. As shown in Figure S12, Supporting Information, the cell remained about 90% viable after being treated for 24 h, and then, a cytotoxicity test was conducted on NO inhalers using human umbilical artery SMCs (UASMCs), and it was confirmed that the NO inhalers exhibited good biocompatibility (Figure S13a, Supporting Information). To confirm that cGMP expression in the UASMCs was caused by NO released from NO inhalers, NO was delivered to the cells using the transwell insert method (Figure 5b). After 24 h of treatment, the concentration of cGMP produced in the cells was determined using ELISA. As shown in Figure 5c, OPNI increased the synthesis of cGMP in the cells, whereas CPNI presented adverse effects due to an initial burst release. Furthermore, CPNI delivered a higher NO concentration into the cells than OPNI (Figure 5d). These results demonstrate that the NO-release behavior of OPNI contributes to the vasodilatory effects by increasing the synthesis of cGMP.

2.5. Anti-Inflammatory Effects of NO Inhalers

Vasoconstriction can be intensified because of vascular remodeling induced by an inflammatory response.^[1b] In PAH lesions, NO acts as a multifunctional treatment agent with both vasodilatory and anti-inflammatory properties (Figure 6a). In our previous study, we confirmed that low NO concentrations can

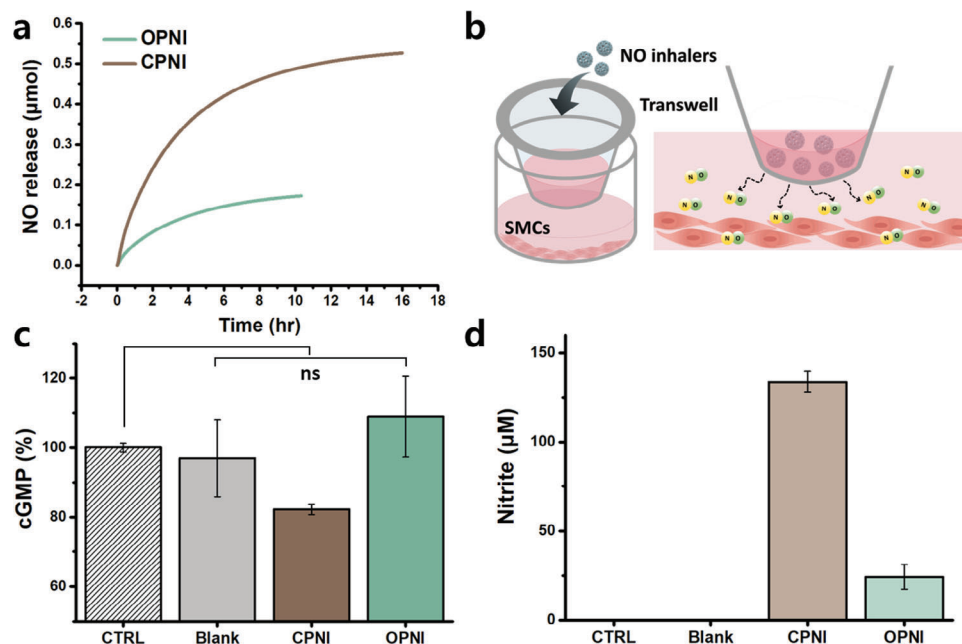


Figure 5. Vasodilation effects of NO inhalers a) NO release profiles of NO inhalers. b) NO was delivered into cells through transwell inserts. c) cGMP expression of SMCs after treatment with NO inhalers for 24 h. d) Nitrite concentrations in a culture medium determined using the Griess assay.

Table 2. NO-release profiles of NO inhalers.

	$t[\text{NO}]$ [$\mu\text{mol mg}^{-1}$]	$t_{1/2}$ [h]	$[\text{NO}]_m$ [pmol mg^{-1}]	t_m [min]	t_d [h]
BPEI0.8k/NO	0.17	2.13	23.33	4.83	10.35
BPEI25k/NO	0.53	2.33	63.82	6.75	16.0

$t_{1/2}$ (min): half-life of NO; $[\text{NO}]_m$ (pmol mg^{-1}): maximum flux of NO t_m (min): time until the maximum flux of NO; t_d (h): duration time $t[\text{NO}]$ ($\mu\text{mol mg}^{-1}$): the total amount of NO release Buffer: 1x PBS pH 7.4

serve as an anti-inflammatory agent by downregulating pro-inflammatory cytokines.^[16a] Hence, we hypothesized that ONPI with sustained NO release can alleviate PAH symptoms by regulating macrophage polarization.

We investigated the cytotoxicity of NO inhalers against RAW 264.7 macrophages and confirmed that NO inhalers exhibited no reduction in cell viability (Figure S13b, Supporting Information). Macrophages, as innate immune cells, can be classified into non-activated (M0), pro-inflammatory (M1), and anti-inflammatory (M2) phenotypes. Each phenotype has a distinct morphology and expresses specific markers that aid in identifying macrophage polarization. To evaluate the role of NO inhalers in regulating inflammatory responses, we examined macrophage polarization from M1 to M2 using flow cytometry, real-time quantitative PCR (RT-qPCR), and ELISA. Upon treatment with NO inhalers, we observed morphological changes in cells under an optical microscope. As shown in Figure S14, Supporting Information, LPS stimulation induced M1 polarization and resulted in a pancake-like shape, while the addition of IL-4 and IL-13 led to cellular elongation, indicating M2 polarization.^[26] Notably, treatment with OPNI resulted in a higher proportion of M2-induced cells compared to CPNI and blank particles.

To evaluate the polarized macrophage, we visualized the cell population using t-distributed stochastic neighbor embedding (t-

SNE) analysis (Figure S15, Supporting Information). Figure 6b–d shows that the OPNI treatment group had more overlapping areas with M2 cells than the CPNI and blank particle treatment groups. Quantification of the cell populations revealed that OPNI increased the number of M2-type cells while decreasing the number of M1-type cells (Figure 6e). Furthermore, protein and gene analysis demonstrated an increase in anti-inflammatory factors in the OPNI treatment group (Figure 6f–i). In conclusion, these results demonstrate that OPNI induced an anti-inflammatory response via sustained NO release.

3. Conclusion

We developed inhalable NO-releasing microspheres that deliver NO using a nebulizer to treat PAH. BPEI/NONOates with different molecular weights were chosen to control NO-release profiles based on the stabilization effects derived from the electrostatic interaction between amine groups and NONOates. Additionally, the porous structures of the microspheres, resulting from water diffusion from the outer to the inner aqueous phase, were influenced by the molecular weights of the BPEI/NONOates. Collectively, OPNIs exhibited excellent pulmonary delivery and sustained NO release that mimics NO production in the endothelium. As a result, we confirmed that OPNIs activated cGMP

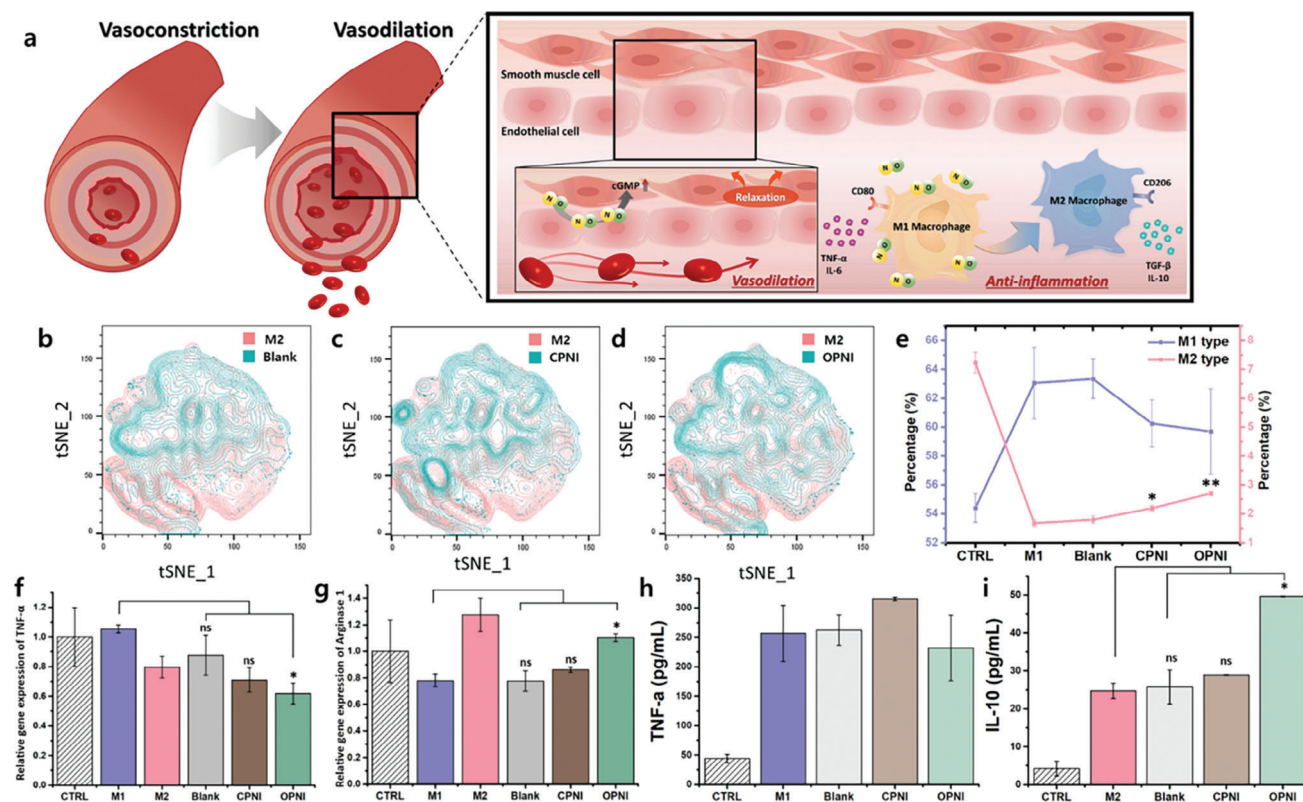


Figure 6. Anti-inflammatory effects of NO inhalers a) Schematic illustration representing the treatment of PAH by the NO inhalers. b–d) t-SNE plots showing the cellular distributions. e) Cell populations characterized as M1 and M2 phenotype cells. f,g) Expression levels of pro- and anti-inflammatory genes in the RAW 264.7 cells. h,i) Concentrations of pro- and anti-inflammatory cytokines produced by the RAW 264.7 cells.

synthesis in SMCs and polarized M1 phenotype macrophages into M2 phenotype macrophages, relieving inflammatory responses. This study demonstrates the potential to expand the versatility of NO in PAH treatment by overcoming the limitations of previous approaches that require an invasive procedure.

4. Experimental Section

Reagents and Materials: BPEI, PLGA (Resomer RG 504H), poly(vinyl alcohol) (PVA), ABC, dichloromethane (DCM), methanol, and the Griess reagent (modified) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Sodium methoxide (5.4 M, 30 wt% in methanol) was purchased from Thermo Scientific Chemicals (CA, USA). Diethyl ether was purchased from Daejung (Siheung-si, Republic of Korea). Phycoerythrin (PE) anti-mouse F4/80, Pacific Blue (PB) anti-mouse/human CD11b, fluorescein-5-isothiocyanate (FITC) anti-mouse CD80, Alexa Fluor 647 anti-mouse CD206, purified anti-mouse CD16/32, and 7-AAD viability staining solution were purchased from Biolegend (CA, USA).

Synthesis of NO Inhalers: The NO inhalers were prepared using a double (W1/O/W2) emulsion-solvent evaporation method with ABC serving as an effervescent porogen, as previously reported.^[18] PLGA was dissolved in DCM to create the oil phase. The BPEI/NONOates and ABC were dissolved in deionized water to create the W1 phase. The W1 solution was added to the PLGA solution, and the mixture solution was sonicated in an ice bath (VC-505; SONICS). Thereafter, the primary emulsion (W1/O) was obtained and added dropwise to the PVA solution (W2). The mixture was emulsified using a homogenizer (T18 digital ULTRA TURRAX, IKA, Staufen, Germany) at a rate of 10 000 rpm for 10 min. The obtained double emulsion was stirred for 5 h to harden the particles while DCM evap-

orated. The particles were collected via centrifugation, washed two times with deionized water, and lyophilized. To prepare the blank microspheres, the aforementioned process was employed without BPEI/NONOates in the W1 solution. Nile red was added to the PLGA solution for confocal imaging.

NO Inhalers Characterization: The morphologies of the NO inhalers were investigated using SEM (JSM-7610F-Plus). The size distribution was measured using a laser diffraction analyzer (LA-960, HORIBA, Japan). The surface charge was characterized using a nanoparticle analyzer (SZ-100, HORIBA). The NONOates were detected by a UV-vis spectrometer (Evolution 300, Thermo Fisher, CA, USA). The atomic composition of the NO inhalers was determined by XPS (K-alpha +; ThermoFisher Scientific). The porosity was detected using a mercury porosimeter (PM33GT, Quamtachrome, Graz, Austria). Z-stack images were obtained using confocal microscopy (LSM 900, Carl Zeiss, Oberkochen, Germany) to compare the porous structures of the NO inhalers based on the molecular weights of the BPEI/NONOates.

Nebulization Efficiency: The Nile-red-loaded NO inhalers were suspended in a saline solution and then nebulized using a jet nebulizer (Philips Respironics InnoSpire Essence) in a respiratory model. The nebulized particles were collected on a glass slide in a vial and then quantified by photoluminescence spectroscopy (FP-8300, JASCO, Tokyo, Japan). Additionally, morphological changes were confirmed using confocal microscopy of nebulized particles present on the glass slide.

NO-Release Measurement: NO release from the NO inhalers was measured in phosphate-buffered saline (PBS; 0.01 M) at pH 7.4 and 37 °C using a chemiluminescence NO analyzer (Sievers NOA 280i; GE Analytical Instruments, Boulder, CO). The analyzer was calibrated with a zero filter (0 ppm of NO) and standard NO gas (45 ppm balanced with N₂; AIRKOREA Inc.) each time before measuring the samples. NO was delivered into the chemiluminescence detection chamber along with Ar gas

flow (70 mL min⁻¹). The NO amount was calculated per 1 mg of each sample.

HUVECs Culture and Viability Test: The HUVECs and cell culture media with supplements (EGM-2 Bulletkit) were purchased from Lonza (Basel, Switzerland). The cells were cultured using Lonza's protocol. To investigate the viability against the samples, 50 000 cells per well were seeded in 24-well plates and loaded samples in the hanging insert. After 24 h of treatment, the hanging insert was removed and washed with PBS. A cell counting kit-8 (CCK-8) reagent (Dongin Biotech Co., Korea) was added to the culture medium, which was then incubated for 2 h. The absorbance at a wavelength of 450 nm was detected using a plate reader (Spectra Max 340 PC; Molecular Devices, San Jose, CA, USA).

SMC Culture and cGMP ELISA Assay: The UASMCs and cell culture media with supplements (SmGM-2 Bulletkit) were purchased from Lonza (Basel, Switzerland). The cells were cultured using Lonza's protocol. The cell viability was evaluated as described in the section 'HUVECs Culture and Viability Test', Experimental Section. For the cGMP ELISA assay, cells were seeded in 6-well plates at a density of 120 000 cells per well and then incubated for 24 h. After 24 h of sample treatment, the cells were lysed through repeated freeze-thaw cycles to remove cGMP from the cells. Thereafter, the cGMP levels were measured using a human cGMP ELISA kit (MyBioSource, CA, USA).

RAW 264.7 Cell Culture and Cellular Uptake Analysis: Raw 264.7 mouse macrophage cells were obtained from the Korean Cell Line Bank (Seoul, Korea). The cells were cultured in low glucose-Dulbecco's modified eagle medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Welgene, Gyeongsan, Korea) and 1% penicillin-streptomycin-glutamine (Gibco). The cell viability was evaluated as described in the section 'HUVECs Culture and Viability Test', Experimental Section. To investigate the cellular uptake of the NO inhalers, the cells were seeded in a confocal dish (glass bottom dish; SPL Life Science, Pocheon, Korea). Subsequently, the cells were treated with the NO inhalers and 1 µm of the PLGA particles and then incubated for 4 h. After incubation, the cells were washed with PBS and stained with Alexa Fluor 488 phalloidin and DAPI. The intracellular uptake of the particles was observed using confocal microscopy.

Anti-Inflammatory Effects of NO Inhalers: Macrophages were plated in 6-well plates at a density of 250 000 cells per well and allowed to attach overnight. The cells were then stimulated with 50 ng mL⁻¹ of LPS (Sigma) and incubated for 12 h. Subsequently, the stimulated cells were exposed to NO inhalers for 24 h using hanging inserts. Following treatment, the cells were washed with PBS and cultured with fresh media for 48 h to allow for an immune response. M2 phenotype macrophages were prepared by treating the cells with IL-4 and IL-13 (20 ng mL⁻¹; Peprotech, USA) for 12 h.

ELISA: To investigate the production of cytokine by the macrophages, the culture supernatant was collected and analyzed using ELISA kits (R&D systems, Abingdon, U.K.) according to the manufacturer's protocols.

Flow Cytometry Analysis: Flow cytometry analysis was conducted to quantify the populations of M1 and M2 macrophages using CD80 (M1 marker) and CD206 (M2 marker) cell surface markers, respectively. The cells were treated with CD16/32 for 20 min to block nonspecific binding. Subsequently, the cells were stained with FITC anti-mouse CD80, Alexa Fluor 647 anti-mouse CD206, PE anti-mouse F4/80, and PB anti-mouse/human CD11b for 30 min. The cells were then washed with FACS buffer three times. Finally, the 7-AAD staining solution was added and incubated for 30 min. The data were acquired using a BD FACSVerse system (BD Bioscience, CA, USA) and analyzed with FlowJo X software (FlowJo, LLC, Ashland, OR, USA). The visualization of data was performed by t-distributed stochastic neighbor embedding (t-SNE) (Figure S15, Supporting Information).

RT-qPCR Analysis: RT-qPCRs were performed to evaluate the expression of inflammation-related genes. mRNA was extracted from the cell using a ReliaPrep RNA Cell Miniprep System (Promega, USA) according to the manufacturer's instructions. Afterward, cDNA was synthesized using a cDNA VILO kit (Invitrogen, MA, USA). The primers were purchased from Bioneer (Daejeon, Korea) and the primer sequences are listed in Table S3,

Supporting Information. RT-qPCR reactions were performed in Applied Biosystems StepOne Plus (Foster City, CA, USA).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords

nebulizer, nitric oxide, pulmonary arterial hypertension, pulmonary delivery

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