

Nitric Oxide Gas in Carbon Nanohorn/Fluorinated Dendrimer/Fluorinated Poly(ethylene glycol)-Based Hierarchical Nanocomposites as Therapeutic Nanocarriers

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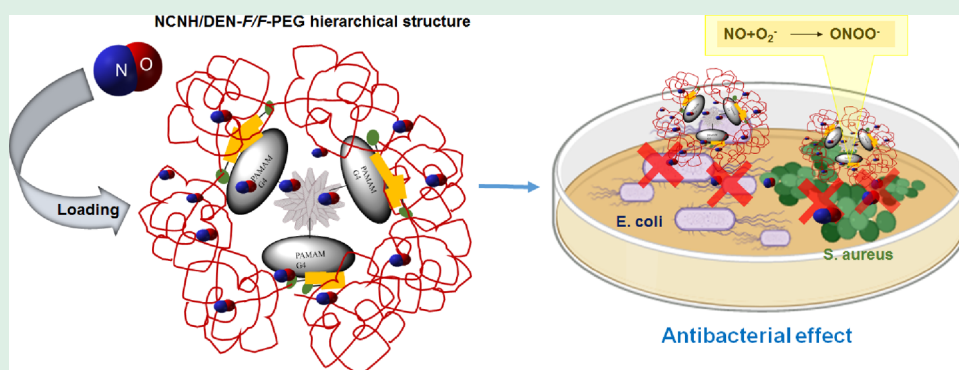
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ABSTRACT: Nitric oxide (NO) gas nanocarrier materials were prepared via a hierarchical assembly of poly(amido amine) dendrimers with fluorocarbon binding sites (DEN-F) and fluorinated poly(ethylene glycol) (F-PEG) on nitrogen-doped carbon nanohorns (NCNHs). The loading abilities of NO gas in these nanocarrier materials increased with the nitrogen doping of CNH and hierarchies formed by DEN-F and F-PEG. Especially, the ability of CNH-based nanocomposite materials was better than that of graphene-based materials. The loading of NO gas arose an infrared absorption band at 1387 cm^{-1} and increased the intensity ratio of D and G bands in Raman spectra, although these phenomena diminished after the degas treatment. The antimicrobial effects on bacteria (*Escherichia coli* and *Staphylococcus aureus*) increased depending on the loading amount of NO gas. It was confirmed from these results that NO gas weakly interacts with nitrogen-doped CNH and is trapped in the void volumes of DEN-F and F-PEG hierarchies. Thus, the concentric hierarchy is preferable for slow release of NO gas due to the void volumes in DEN-F, F-PEG, and CNH hierarchical organization. This sustained release of NO gas is advantageous with regards to the potential biomedical gas therapy against bacteria and other parasites.

KEYWORDS: nitric oxide gas, carbon nanohorn, poly(amido amine) dendrimer, fluorinated poly(ethylene glycol), hierarchical nanocomposite, therapeutic nanocarrier, antimicrobial effect

1. INTRODUCTION

Nitric oxide (NO) is the smallest endogenous molecule among the biological free-radical gases, can function as a chemical messenger in cells,¹ and has important roles in mammalian life, such as vasodilation and compression in arteries and improvement of blood flow in all organs.² NO provides some direct benefits to human organs, such as maintaining the normalized blood pressure, improving the memory and learning capacity, as well as increasing the quality of sleep.³ Additionally, the inhalation of NO gas⁴ has been employed as the main medical therapy in the treatment of neonatal pulmonary hypertension⁵ and it has received emergency approval for treating hypoxemia in COVID-19.⁶ In mammal tissues, NO is produced by four NO synthase isoforms and their presence correlate with the increased blood flow and the

reduced blood pressure.⁷ However, the biological production of NO gas in human body systems is limited because of the instability of gas supply and its extremely short efficacy (less than 1 s in circulating blood).^{8,9} Meanwhile, the potential of gaseous NO as an antimicrobial is also known.¹⁰ The role of NO in physiological function is revealed: Nevertheless, the deficiency of the adequate delivery systems is impeding the clinical use of gaseous NO. Another impact report is related to

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the therapy by inhaling NO for COVID-19,¹¹ although the additional verification should be necessary.

Recently, pharmaceutical researchers and industries have begun to focus on the research and development of NO gas carrier systems with effective sustainability,¹² instead of NO donor materials.¹³ By the highly porous materials as delivery systems, NO gas can be transported into cells, allowing reduction in the dose of NO gas to achieve therapeutic levels. The noncarbon-based porous materials most comprehensively studied include zeolites,¹⁴ metal–organic frameworks,¹⁵ silica nanoparticles,¹⁶ etc. The present limitation of noncarbon materials for NO loading/release is that they exhibit too rapid NO release, which results in an incremental toxicity risk.¹⁷ Carbon-based materials are preferable candidates and have been studied and reported for drug delivery due to their high surface area, porosity, compatibility with biological systems, and good encapsulation properties.¹⁸ They consist of graphene oxide (GO), carbon nanohorn (CNH), carbon nanotube, activated carbon, activated carbon nanofiber, and fullerene. Among them, CNH is a promising candidate, and the horn-shaped spherical structure of CNH has potential not only for gas loading/storage in biomedical treatments but also for imaging and energy systems^{19–23} because it possesses distinctive features, allowing interactions with organic molecules through van der Waals attraction, electrostatic attraction, hydrogen bonding, covalent bonding, and hydrophobic interaction forces, when it is functionalized by charged and oxidized moieties.²⁴

Dendrimers are macromolecules with a spherical structure and multiple functions in the interior of their structure and at its periphery. Dendrimers have been widely used as biomedical, electro-optical, catalytic, and carrier materials.^{25,26} Poly(amido amine) (PAMAM) dendrimers are employed for biomedical purposes due to their unique properties, such as biocompatibility, strong adsorption ability, hyperbranched structures, easy functionalization of terminal groups, and efficient antibacterial activity.²⁷ The amine-terminated dendrimers have been actively studied, but they were found toxic to eukaryotic (e.g., mammalian) cells.²⁸ By contrast, hydroxyl-terminated dendrimers were found less toxic than their amine-terminated analogues.²⁹ Thus, modifying the amine end groups of dendrimers by biocompatible molecules or polymers such as poly(ethylene glycol) (PEG) is commonly achieved. Moreover, the free volume in a dendrimer is propitious for encapsulation of small molecules like water and oxygen.^{30,31} Fluorinated PEGs (F-PEGs), which were considered as stabilizers of fluorocarbon-based emulsions for *in vivo* oxygen carriers,³² may provide an interesting moiety to promote hydro/oleophobic pathways for the transfer of gases.

Investigation of the NO loading capacity of carbon-based materials has been performed for GO and F-PEG-coated GO nanocomposites, but the loaded amounts of gas were limited.³³ Thus, improved NO-loading systems with time-dependent self-sustained release properties are demanded. In this objective, we report here that another carbon-based material, CNH, is a hopeful candidate, which can be bound to a fluorinated dendrimer and hierarchically coated by F-PEG. Next, the loading capacity of NO gas in the nanomaterials were studied depending on the hierarchical structure and compared to the case of GO-based carrier materials. Moreover, the antimicrobial effect of NO-loading nanomaterials was investigated to confirm the potential antimicrobial ability of our carriers and compare the effect with the previously reported carriers.¹⁰ The

present results concerning the construction of nanocomposites with a hierarchical structure will demonstrate an improved efficiency as a NO carrier with sustained release character as well as their potential in the gas therapy.

2. EXPERIMENTAL SECTION

2.1. Materials and Reagents. Graphite flake (4 μm in size) was obtained from ITO Company, Japan. Hydrogen peroxide (H_2O_2) was purchased from Yakuri Pure Chemical, Japan. Oxidized CNH (CNHox) was purchased from NEC, Japan. Pentafluoropropionic acid (PFPrA) was purchased from Fluorochem, UK. Potassium permanganate (KMnO_4), 4-dimethylaminopyridine (DMAP), and *N,N*-dicyclohexylcarbodiimide (DCC) were purchased from Acros Organics, Belgium. OH-terminated PAMAM dendrimer (DEN-OH, generation 4.0, 10 wt % in methanol) was purchased from Sigma-Aldrich, USA. $\text{C}_8\text{F}_{17}(\text{CH}_2)_2\text{COOPEG}_{2000}$ ($\text{F}_8\text{H}_2\text{COOPEG}_{2000}$, F-PEG) was synthesized by chloration of $\text{C}_8\text{F}_{17}(\text{CH}_2)_2\text{COOH}$ by SOCl_2 followed by esterification with $\text{CH}_3(\text{OCH}_2\text{CH}_2)_{2000}\text{OH}$ ($\text{PEG}_{2000}\text{OH}$). Other reagents were of commercial grades and were used without further purification. The bacteria, *Staphylococcus aureus* ATCC6538P and *Escherichia coli* BL21, were obtained from Novagen, USA. Ultrapure water (a resistivity of 18.2 $\text{M}\Omega\text{ cm}$) was prepared on a Yamato Millipore WT100 system, Japan.

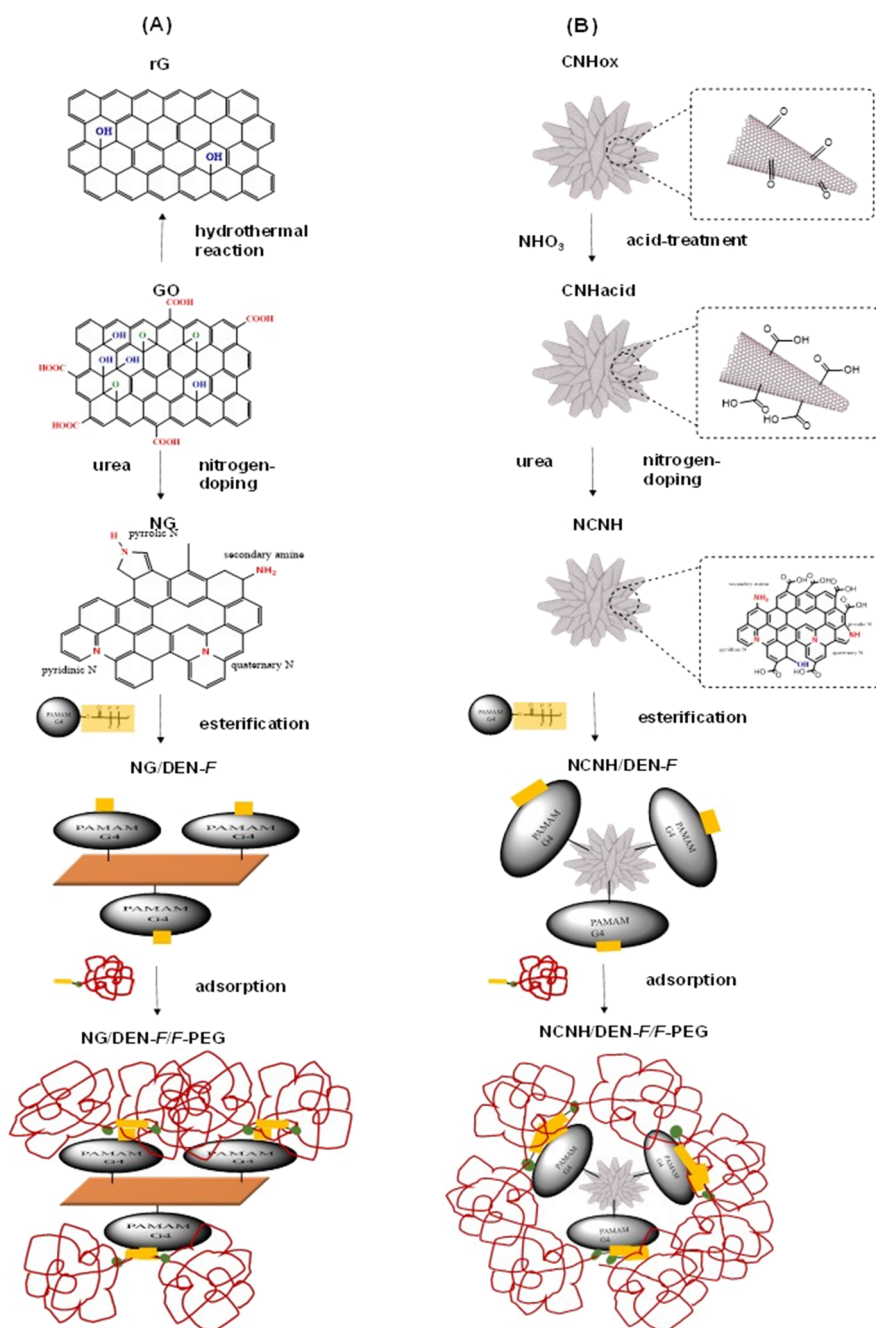
2.2. Instruments. Ultraviolet (UV)–visible absorption spectra were measured using a UV–visible spectrophotometer (V-670, JASCO, Japan) in a 1 cm path cell. The infrared absorption spectra were obtained on an FTIR spectrometer (Thermo Nicolet, Nexus 6700, Japan) with 500 scans for KBr pellets. Raman scattering measurements were performed using a Raman spectrometer (HORIBA Jobin Yvon iHR550, Japan) equipped with a Nd:YAG laser excitation source at 633 nm. The X-ray diffraction (XRD) was recorded at 10–80° and a step of 4 min^{-1} on a Rigaku D/Max-kA diffractometer with Cu $\text{K}\alpha$ radiation (1.54 Å) at 40 kV and 30 mA. Dynamic light scattering (DLS) and zeta potential were measured on a HORIBA SZ-100 (Japan) instrument. A transmission electron microscope (TEM, JEOL, Japan) was operated at 120 kV. The scanning electron microscope and energy-dispersive X-ray spectroscopy (SEM and EDS, JSM-6390, Japan) were operated at 10 kV. The N_2 adsorption–desorption isotherms were measured on a BELSORP-max (Bel, Japan) and analyzed by the Brunauer–Emmett–Teller (BET) method. All samples were degassed at 120 °C for 3 h before the measurement.

2.3. Preparation of Materials. GO was prepared by a modified Hummers method.³⁴ A mixture of graphite (500 mg), concentrated sulfuric acid (50 mL), potassium nitrate (500 mg), and potassium permanganate (1500 mg) was heated at 35 °C for 24 h and diluted with water (20 mL). Twenty milliliters of H_2O_2 (30%) were then added dropwise. The product (GO) was washed with a 0.1 M HCl solution and water. Commercial CNHox (100 mg) was dispersed in HNO_3 (1 M, 60 mL) and refluxed at 100 °C for 1 h. The produced acid-treated CNH (CNHacid) was filtered on a 0.2 μm filter membrane and washed with water.

N-doped carbon materials were synthesized through a hydrothermal process.²² Urea (45 g) and GO or CNHacid (150 mg) were dispersed in water (50 mL). After 3 h of sonication (bath type) at 27 °C, the dispersion was heated in an autoclave at 180 °C for 12 h. The N-doped compound (NG or NCNH) was obtained by filtration and dried at 80 °C for 8 h. Reduced graphene oxide (rGO) was obtained by the same procedure as described for NG but without urea.

The fluorinated dendrimer (DEN-F) was prepared by esterification of PFPrA with DEN-OH. A PFPrA solution in DMF (10 mM, 140 μL) was mixed with an excess of DCC in DMF (10 mM, 150 μL) and DMAP in DMF (10 mM, 30 μL). A solution of DEN-OH in DMF (0.2 wt %, 10 mL) was then added, and the reaction was proceeded for 24 h. The dendrimer solution in DMF was prepared by adding DMF (40.7 mL) to the DEN-OH solid after evaporating the solvent in a commercial DEN-OH solution (10 wt % in methanol, 0.81 g). Then, the calculated mole ratio of DEN-OH/PFPrA in DEN-F was 1:1.

Scheme 1. Synthesis of Gas Carrier Nanomaterials. (A) GO-Based Nanomaterials and (B) CNH-Based Nanomaterials. The Sequential Process Involves Nitrogen Doping with Urea, Esterification with Fluorinated Dendrimer, and Subsequent Adsorption of Fluorinated PEG



The preparation of the hierarchical composite of NG or NCNH (200 mg) and DEN-F was obtained by mixing the carbon materials dispersed in DMF with an excess of DCC/DMAP and with DEN-F in DMF (10 mL). After being reacted for 24 h, the product, NG/DEN-F or NCNH/DEN-F, was collected by centrifugation and washed with DMF and water.³⁴

The coating of the resulting material by F-PEG was accomplished by mixing NG/DEN-F or NCNH/DEN-F (0.3 mg mL⁻¹, 180 mg) with F-PEG (0.757 μM, 10 mg) in water and exposing the dispersions in an ultrasound bath (with a power of 50 W) for 4 h at 27 °C. The synthesis processes are illustrated in Scheme 1 for the two different nanomaterials GO and CNH.

2.4. Determination of NO Gas Loading. The NO gas was generated in a modified Kipp's gas generator by reacting a copper metal (3 g) with aqueous nitric acid solution (25 mL). The generated

gas was introduced into a vessel placing the gas carrier materials in a closed system, following the reaction of $3\text{Cu} + 8\text{HNO}_3 \rightarrow 3\text{Cu}(\text{NO}_3)_2 + 2\text{NO} + 4\text{H}_2\text{O}$. The quantitative determination of NO gas in the carrier materials at a given time after stopping the loading was achieved following the previously reported method³³ by a Griess assay method.³⁵ The obtained quantity indicates NO gas in the carrier at a certain time after being loaded.

2.5. Antimicrobial Test. Gram-negative *Escherichia coli* (*E. coli*) and Gram-positive *Staphylococcus aureus* (*S. aureus*) were the strains used for the antimicrobial test based on the standard agar disk-diffusion method.³³ The suspension (100 μL) of bacteria grown in the agar medium (Luria–Bertani for *E. coli* and tryptone soya broth for *S. aureus*) with an optical density of 0.05 was spread homogeneously on the surface of sterile agar. The gas carrier materials (0.5% w/v) were mixed with 2,2,6,6-tetramethyl-1-piperidinyloxy radical-oxidized

cellulose nanofiber (TOCNF, 1 wt %) in water (20 mL), and the films of nanocomposites were prepared. The nanofiber is the same sample previously prepared.^{25,36} For the nanocomposite films adsorbed NO gas on sterile agar, where bacteria (*E. coli* and *S. aureus*) were fully cultured by the incubation for 12–16 h, the inhibition zone was measured. The films without NO adsorption were also treated as references.

3. RESULTS AND DISCUSSION

3.1. Loading of NO Gas on Gas Carrier Nanomaterials. Carbon material-based hierarchical composite nanocarriers were prepared to assess the ability for NO loading. Graphene-based nanocomposites were shown to consist of flat superposed graphene/dendrimer/fluorocarbon/PEG layers.³³ However, the morphology of CNH-based nanomaterials is assumed to be spherical, based on the shape of the core carbon material (CNH) (see Scheme 1).

When NO gas was loaded, the NO gas on nanomaterials self-decreases depending on time. Then, the contents of NO gas in various gas carrier materials were examined at different times after the NO gas was loaded for 10 min, as shown in Figure 1. In the GO-based series, although GO and rG showed a similar low loading ability, the loading ability increased in an order of magnitude for NG, NG/DEN-F, and NG/DEN-F/F-PEG. A similar tendency was observed for the CNH-based nanomaterial series. CNHox exhibited the lowest NO loading

ability, but the NO loading sequentially increased after DEN-F was combined and F-PEG was adsorbed. When the loading of NO was compared between GO-based and CNH-based composite materials, the loading ability of NO was higher for NG/DEN-F and NG/DEN-F/F-PEG than that for NCNH/DEN-F and NCNH/DEN-F/F-PEG, respectively.

However, the NO content at 180 min after loading was higher for NCNH/DEN-F and NCNH/DEN-F/F-PEG than that for NG/DEN-F and NG/DEN-F/F-PEG, respectively. The NO contents of GO, rG, and CNHox at 180 min after loading are not high because of the low affinity of NO to the graphitic structure. However, the substitution of carbon to nitrogen on GO or CNH increased the NO contents at 180 min after loading since nitrogen doping throughout the forms of quaternary N, pyridinic N, pyrrolic N, and secondary amine may provide nitric oxide affinities.³⁷ The binding of the dendrimer is also expected to increase the NO content because of its encapsulation ability of small molecules in its void volume.^{30,31,38} The adsorption of F-PEG on NG/DEN-F and NCNH/DEN-F resulted in the further increase of the NO content, originating from the hydrophobic and oleophobic fluorinated domain and the void volume created by the adsorption of F-PEG. Thus, DEN-F and F-PEG promoted the content of NO gas. By the way, the loading and the content of NO gas at 180 min after loading on GO/F-PEG nanomaterials³³ were lower than those on NG, NG/DEN-F, and NCNH/DEN-F. Then, these values should have a causal relationship with the nitrogen doping and the hierarchical structures in composites.

3.2. Characterization of Carrier Nanomaterials. The NO loading depended on the hierarchy of the carbon material-based composite nanocarriers. GO-based nanocomposites form the flat layer-by-layer hierarchy consisting of graphene/dendrimer/fluorocarbon/PEG layers, and CNH-based hierarchy may present a concentric hierarchy, depending on the shape of carbon materials, graphene and CNH (Scheme 1). Then, in this section, the characterization of CNH-based carrier nanomaterials was performed because the NO content at 180 min after loading was slightly better for CNH-based nanomaterials than that for GO-based nanomaterials.

The mean particle sizes and zeta potentials of CNH-based carrier nanomaterials dispersed in water at pH 4.9–6.3 are shown in Table 1. The results show that the mean hydrodynamic diameter increased by an order of magnitude for CNHox, CNHacid, NCNH, NCNH/DEN-F, and NCNH/DEN-F/F-PEG. The size increase of CNH after the acid treatment indicates an increase of the electric double layer by the charged functional groups such as carboxylic acids. The nitrogen doping results in a size increase through an increase of defects and an increase of the electric double layer by the addition of charged amine in the graphitic structure. The introduction of DEN-F and F-PEG induces a size increase by the practical increase of molecular weight of composites.

The zeta potentials reflected such structural variations. CNHox was more negatively charged due to the increase of negatively charged carboxylate groups after the acid treatment. The zeta potential of NCNH also decreased due to the reducing treatment of ionized functional groups and the increase of cationic amine groups. Since the dendrimers are positively charged by the tertiary amine groups, NCNH/DEN-F became positively charged and this cationic character was maintained even when F-PEG was bound to NCNH/DEN-F. These results confirmed that the capturing of NO gas depends

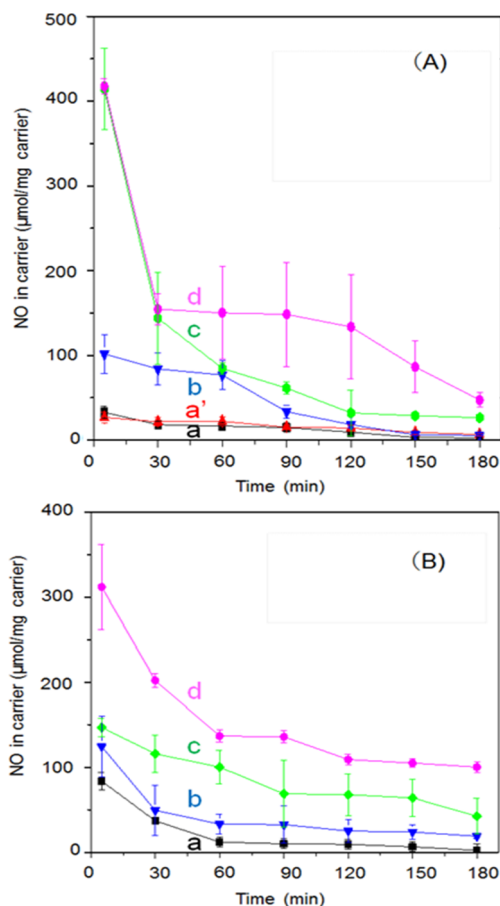


Figure 1. Time dependency of NO content in the carrier nanomaterials after loading NO gas for 10 min. (A) GO-based materials: (a) GO, (a') rG, (b) NG, (c) NG/DEN-F, and (d) NG/DEN-F/F-PEG. (B) CNH-based materials: (a) CNHox, (b) NCNH, (c) NCNH/DEN-F, and (d) NCNH/DEN-F/F-PEG.

Table 1. Mean Hydrodynamic Size, Zeta Potential, Specific Surface Area, Pore Volume, and Pore Diameter of the Various CNH-Based Materials

carrier material	solution pH	zeta potential (mV)	hydrodynamic particle diameter (nm)	specific surface area (m ² g ⁻¹)	pore volume (cm ³ g ⁻¹)	pore diameter (nm)
CNHox	6.25	-15.1 ± 0.5	150.2 ± 0.4	1202.6	1.99 ± 0.02	6.6 ± 2.2
CNHacid	5.25	-34.2 ± 0.2	234.4 ± 0.5	542.8	0.99 ± 0.06	7.3 ± 2.5
NCNH	5.64	-24.4 ± 0.1	273.3 ± 0.8	621.0	1.22 ± 0.01	7.9 ± 2.1
NCNH/DEN-F	6.01	+22.3 ± 0.9	286.1 ± 0.6	429.3	0.73 ± 0.02	11.7 ± 0.2
NCNH/DEN-F/F-PEG	4.88	+31.2 ± 0.8	318.0 ± 0.5	113.9	0.62 ± 0.01	21.8 ± 0.8

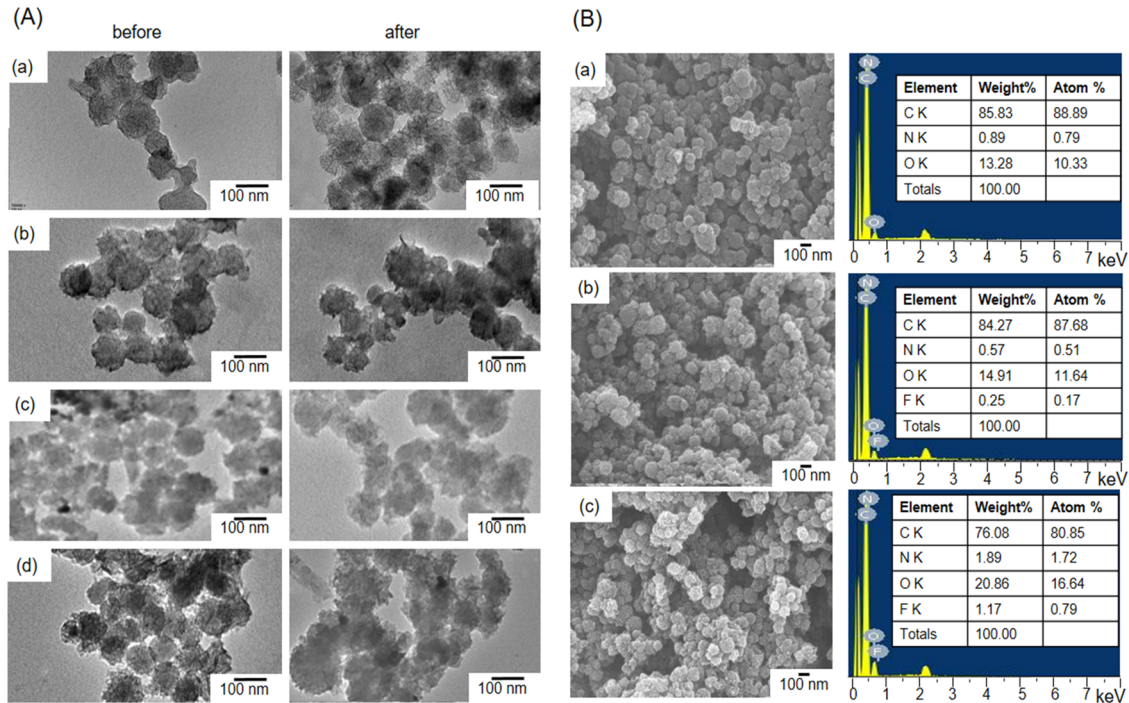


Figure 2. (A) TEM images of the carrier nanomaterials before and after NO loading (a) CNHox, (b) CNHacid, (c) NCNH/DEN-F, and (d) NCNH/DEN-F/F-PEG. (B) SEM images and EDX of (a) NCNH, (b) NCNH/DEN-F, and (c) NCNH/DEN-F/F-PEG.

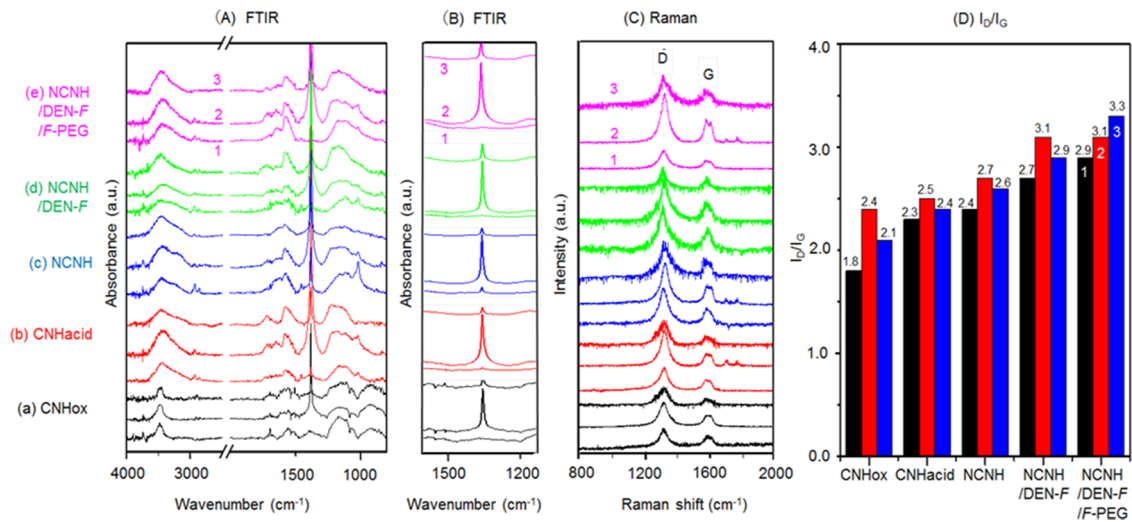


Figure 3. FTIR spectra (A) at 4000–800 nm and (B) at 1500–1200 nm, (C) Raman spectra, and (D) I_D/I_G values for CNH-based carrier materials (a–e). Measurements were achieved (1) before NO gas loading, (2) after NO gas loading, and (3) after degassing at 180 min after NO loading. Black, red, and blue bars in panel (D) denote (1)–(3), respectively.

on the size of carrier materials but is independent of their charge (anionic or cationic) because of the noncharge character of captured NO gas.

TEM images in Figure 2A demonstrate that the morphology and size of the gas carrier materials do not change significantly after acid treatment, nitrogen doping, binding of DEN-F, and

adsorption of *F*-PEG. Likewise, the NO loading has no significant effect on either morphology or size of the nanocarriers. These morphological profiles differ from the DLS results, which were examined in aqueous solutions. This difference may arise from the fact that TEM observation is conducted on dried specimens. The morphology and particle size of dried CNH do not change significantly after the formation of the composite structures. It is likely that DEN-*F* and *F*-PEG bound on NCNH will be confined in the territory of horns of CNH, and so they cannot be distinguished at the periphery of NCNH on TEM images. Incidentally, although the layer thickness of GO increased by 0.21 nm after the adsorption of *F*-PEG and by 0.32 nm after the NO loading,³³ such small differences cannot be observed by TEM.

SEM images and EDX results are shown in Figure 2B. The SEM image of NCNH was not varied even after binding DEN-*F* and *F*-PEG, and they were similar with the TEM image. EDX indicated the appearance of fluorine besides carbon, nitrogen, and oxygen when DEN-*F* was bound on CNH, and the content of fluorine increased after further binding of *F*-PEG, as expected. Based on the elemental analysis results, the calculated mole ratio of the nanocomposite was CNH/DEN-*F*/*F*-PEG = 1:1.5:9.

Figure 3A compares FTIR absorption spectra of gas carrier materials before and after NO gas loading and materials degassing at 180 min after loading. CNHox was essentially characterized by a broad band at 3600–3400 cm⁻¹ (hydroxyl O–H), a band at ~1740 cm⁻¹ (carbonyl C=O), a band at 1545 cm⁻¹ (sp² C=C), and a band at 1150 cm⁻¹ (ether C–O–C).²² After the acid treatment, a broad band at 3600–3100 cm⁻¹ can be ascribed to the O–H stretching vibration mode of carboxyl groups in addition to hydroxyl groups. The absorption spectra of CNHacid also showed bands of carbonyl and carboxyl groups at 1740, 1625, and 1387 cm⁻¹.²² An N–H stretching band at 3250 cm⁻¹ was observed for nitrogen-doped CNH. DEN-*F* is characterized by 1649 and 1559 cm⁻¹ bands, which are assigned to amide I and amide II, respectively (see the Supporting Information, Figure S1). Thus, the spectrum is mainly contributed by DEN-OH because the weight content of PFPrA in DEN-*F* is less. For NCNH/DEN-*F* and NCNH/DEN-*F*/*F*-PEG, an absorption band at 1740 cm⁻¹ associated to a stretching mode of C=O in NCNH obviously decreased by the immobilization of DEN-*F*, and furthermore, absorption bands of amide I and amide II vibration modes of the dendrimer appeared at 1638 and 1548 cm⁻¹, respectively.³⁴ Incidentally, FTIR absorption bands of NG/DEN-*F* and NG/DEN-*F*/*F*-PEG similarly behaved to NCNH-based nanomaterials.

All carrier materials exhibited a new absorption band at 1387 cm⁻¹ after the loading of NO gas, whereas the desorption procedure of the NO gas from the NO gas-loading materials is accompanied by a decrease of the absorbance at 1387 cm⁻¹, indicating the removal of NO gas from the materials (see Figure 3B). Such a band at 1387 cm⁻¹ is also observed with the NO gas-loading GO and GO/*F*-PEG nanomaterials.³³ Thus, the appearance of a 1387 cm⁻¹ band after NO gas loading is a common phenomenon for both CNH-based and GO-based carrier nanomaterials.

Raman spectra were also analyzed to determine the composition in gas carrier materials. As seen in Figure 3C, Raman bands of NO carrier materials were observed at 1319 ± 3 cm⁻¹ (D band) and 1589 ± 3 cm⁻¹ (G band), but new bands derived from other moieties of materials were not observed.

The D and G bands are assigned to the sp³ bond deformation vibration of carbon in an amorphous structure and to the sp² bond deformation vibration originating from carbon in a hexagonal ring, respectively.³⁹ Thus, the *I*_D/*I*_G ratio of the integrated intensities of D and G bands reflects the graphitization degree, the particle size effect, or the lattice distortion of carbon-based materials.⁴⁰ The *I*_D/*I*_G values increased with acidification, nitrogen doping, and binding of DEN-*F* and *F*-PEG. Moreover, *I*_D/*I*_G increased when NO was loaded into the carrier material but mostly decreased slightly after the degassing procedure, as shown in Figure 3D. These results may indicate that the increase of *I*_D/*I*_G values after acidification and nitrogen doping results from a decrease in the graphitization degree and the formation of the composite increases *I*_D/*I*_G values by the lattice deformation of NCNH. Similarly, the adsorption of NO may result in a distortion of the lattice of carbon-based materials.

Figure 4 shows XRD patterns of the gas carrier materials. In the XRD of CNHox, peaks at 2θ = 26.34 and 43.51°,

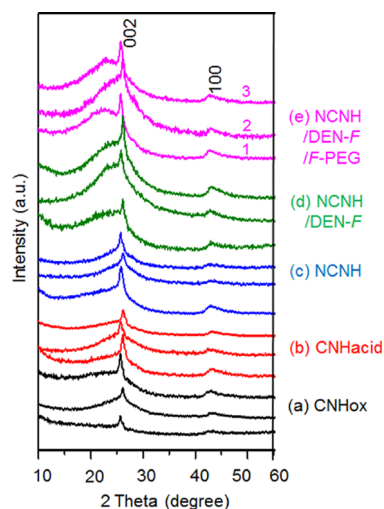


Figure 4. X-ray diffraction spectra of CNH-based carrier materials (a–e). Measurements were achieved (1) before NO gas loading, (2) after NO gas loading, and (3) after degassing at 180 min after NO loading.

respectively, are assigned to the (002) and (100) crystal planes of the graphitic structure.⁴¹ These Bragg peaks were observed on all carrier materials. Additional broad peaks at 2θ = ~23° were observed for NCNH/DEN-*F* and NCNH/DEN-*F*/*F*-PEG, which are attributed to amorphous domains of the dendrimer.⁴² However, the crystal structure was not affected by the loading of NO or its release from the carrier materials, as seen from XRD patterns. XRD patterns of NG/DEN-*F* and NG/DEN-*F*/*F*-PEG are shown in Figure S2. While Bragg peaks of NG was observed at 2θ = 25.3–25.6° (002),²² NG/DEN-*F* and NG/DEN-*F*/*F*-PEG had (002) peaks at 25.8 and 25.6°, respectively, indicating the coexistence of NG in carrier materials.

The nitrogen adsorption–desorption isotherms of the CNH-based materials are plotted in Figure 5, and the obtained numerical values of the surface area, pore volume, and pore size are listed in Table 1. All isotherms belonged to the type II profile with low hysteresis. The specific surface area and the pore volume decreased but the pore diameter increased after DEN-*F* was bound on NCNH and *F*-PEG was adsorbed on

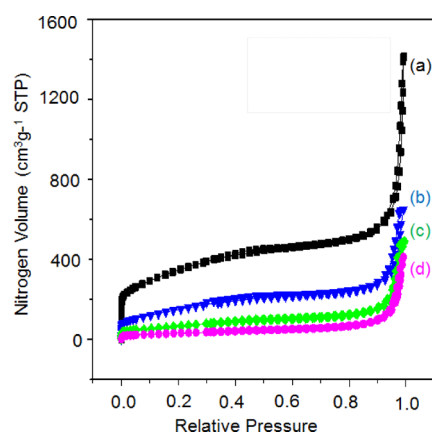


Figure 5. Nitrogen adsorption–desorption isotherms of (a) CNHox, (b) NCNH, (c) NCNH/DEN-F, and (d) NCNH/DEN-F/F-PEG.

NCNH/DEN-F. These results from the isotherms indicate that the decreasing tendency of the surface area and pore volume inverses the increase of loading amount of NO gas. Thus, another motive force like void volume as noticed later must be considered for loading of NO gas. Incidentally, the increase of pore diameter may suggest the enlarging channel of the gas passing to facilitate the passage of NO gas within carrier materials.

3.3. Assessment of the Antimicrobial Activity of the Nanomaterials. In this test, TOCNF films embedding NCNH, NCNH/DEN-F, and NCNH/DEN-F/F-PEG, which loaded NO gas, were prepared for the investigation of the antimicrobial activity of the nanomaterials toward *S. aureus* (Gram positive) and *E. coli* (Gram negative). Various films, including a pristine TOCNF film used as a reference that does not incorporate NO and was sealed in the agar plate, did not inhibit the growth of both bacteria. On the other hand, films of NO-captured nanomaterials were able to eradicate the bacteria, as proved by a clear increase of the pathogen inhibition zones of 0.5, 4, 5, and 7 mm for *S. aureus* and 2, 5, 5, and 5 mm for *E. coli*, respectively, for TOCNF, NCNH/TOCNF, NCNH/DEN-F/TOCNF, and NCNH/DEN-F/F-PEG/TOCNF, as shown in Figure 6. In the case of TOCNF, a small amount of loaded NO gas can be trapped near functional moieties such as carboxyl or hydroxyl groups. The results indicate that the presence of NO is efficient in killing both bacteria and the efficiency is higher for the nanomaterials encapsulating the larger amount of NO.⁴³ A possible process of the antimicrobial activity is that the NO-captured nanocomposite is carried in the bacterial cell and releases NO into the mitochondria. Then, NO reacts with superoxide anion O_2^- to form peroxynitrite ($ONOO^-$) and this active species may damage both the mitochondria and DNA of the microorganisms.⁴⁴

3.4. NO Gas Capture in Hierarchical Nanocomposites.

Our characterization study confirmed the sequential construction of the hierarchical nanocomposites, as illustrated in Scheme 1. DEN-F is chemically bound on NCNH, and thus, NCNH is surrounded by a layer of dendrimers. The fluorocarbon chain of F-PEG can interact with the fluorocarbon group present on the NCNH-nucleated DEN-F through the hydrophobic interactions, forming a hydrophobic/oleophobic domain between the hydrophobic dendrimer layer grafted on the NCNH core and the hydrophilic PEG outermost shell. NO may interact with nitrogen or oxygen present on the NCNH surface by London dispersion forces. It

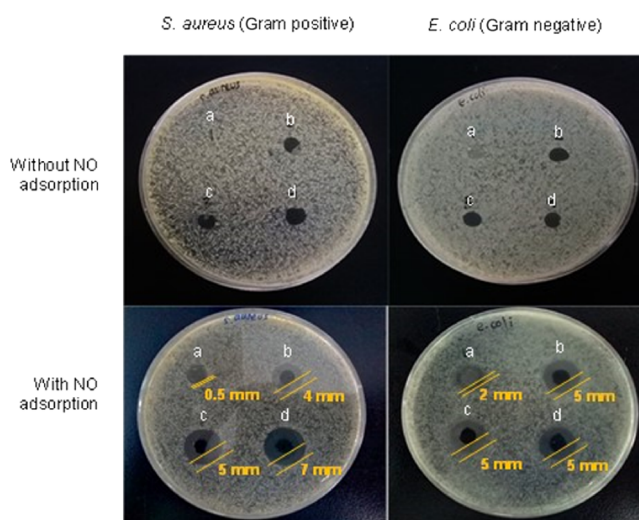


Figure 6. Antimicrobial activity of CNH-based nanocarriers without and with loaded NO toward *S. aureus* (Gram positive) and *E. coli* (Gram negative). (a) TOCNF, (b) NCNH/TOCNF, (c) NCNH/DEN-F/TOCNF, and (d) NCNH/DEN-F/F-PEG/TOCNF.

may also be located in the dendrimers that have significant encapsulation ability for small molecules and possibility of the interaction of tertiary amine with NO, and the PEG moiety containing ether groups can provide void volumes that are able to store small molecules and the interaction of ether with NO. The void volume could generate even at interlayers of NCNH, dendrimer, fluorocarbon, and PEG moieties owing to the steric hindrance between moieties. Thus, the loading of NO gas increases with the accumulation of the different moiety layers and the self-loss of NO gas as measured in Figure 1 happens through the void volume in parallel to the loading amount of NO gas. The herewith released NO gas attacked the cell of bacteria and deadened them, as illustrated in Scheme 2. As already explained in the previous paragraph, the carried NO generates peroxynitrate by binding with O_2^- and peroxynitrate plays an important role in the antimicrobial effect. This procedure is applicable to other bacteria and virus related to any diseases.

Recently, the progress of NO-releasing platforms is impressive.⁴⁵ NO-releasing materials reported so far are compared in Table 2. The materials are composed of polymers, dendrimers, inorganic materials, or carbon-based composites. One notable feature of these materials is that most of them contain a NO-donor moiety based on *N*-diazoniumdiolate that functions as the NO-loading site,^{46–56} although zeolites and organoclays are functioned by complexation and the specific interaction, respectively.^{53,54} We present a different concept, both in our previous report³³ and in the present work, in which void volumes presented in 2D hierarchically organized layers and hierarchy and in 3D concentric hierarchies are the main sites for NO loading. As a consequence, the NO payload is high (e.g., reaching 417.4 and 311.9 $\mu\text{mol mg}^{-1}$ for NG/DEN-F/F-PEG and NCNH/DEN-F/F-PEG, respectively). These values are absolutely higher than those (less than 2 $\mu\text{mol mg}^{-1}$) of NO-donor moiety systems. Especially, the utilization of nitrogen-doped carbon materials and the introduction of DEN-F were strongly affected superior to GO/F-PEG (58.5 $\mu\text{mol mg}^{-1}$)³³ because the nitrogen unit in nitrogen-doped carbon materials and DEN-F attracts NO gas and the DEN-F contributes to the increase of void volume to encapsulate NO

Scheme 2. Schematic Illustration of Loading of NO in NCNH/DEN-F/F-PEG Nanocomposites Leading to Potent Antimicrobial Effects

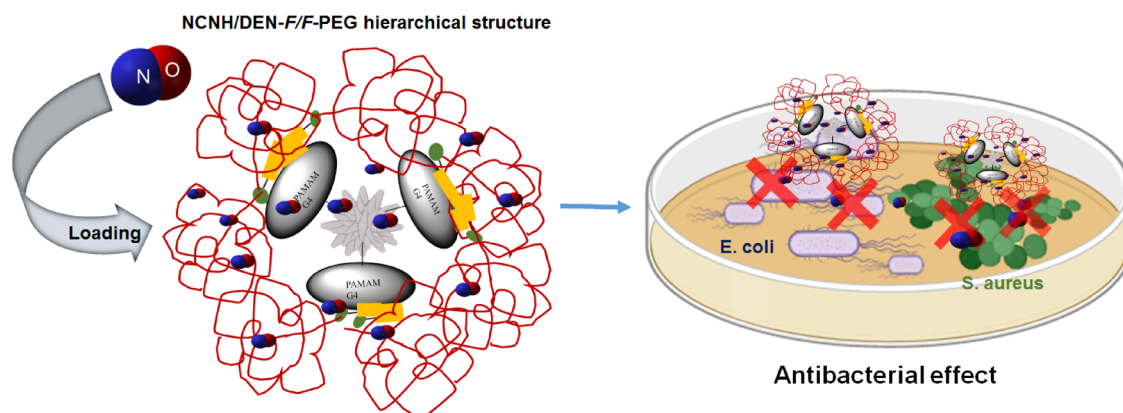


Table 2. Comparison of NO-Releasing Systems

material	NO-releasing mechanism	NO payload ($\mu\text{mol mg}^{-1}$)	half-life of NO (min)	ref.
polymer				
polymethacrylate-based copolymers	NO-donor moiety	0.94	N/A	46
polyesters		($0.81 \mu\text{mol cm}^{-2}$)	54	47
alginates	NO-donor moiety	0.4–0.6	18–780	48
dendrimer				
S-nitrosothiol-modified dendrimer	NO-donor site	2	N/A	49
nanoparticle (star polymer)	NO-donor moiety	($50 \mu\text{M}$)	N/A	50
alkyl-modified G4 PAMAM dendrimers	NO-donor moiety	0.91	20	51
alkyl-modified G1 PAMAM dendrimers	NO-donor moiety	1.06	21	52
inorganic material				
zeolites	complexation	1	5.6	53
silica	NO-donor moiety	1.78	54	54
organoclays	specific interaction	(1.06 nM)	N/A	55
carbon-based composite				
GO film assembly	NO-donor moiety	($0.2\text{--}0.3 \mu\text{mol cm}^{-2}$)	1020	56
GO/F-PEG 2D layer	void volume	58.5	62	33
NG/DEN-F/F-PEG 2D hierarchy	void volume	417.4	25	this work
NCNH/DEN-F/F-PEG concentric hierarchy	void volume	311.9	52	this work

gas as described above. On the utilization of NO gas for biomedical purposes, the release process as well as NO payload also must be taken into account. The half-life of NO is also compared in Table 2. The half-life time varied widely depending on the generation systems of NO, but the relevant time (0.5 to 1 h) by proposed NO-releasing systems in the current work is suitable for advancing the loading efficiency and the enhancing release on the medical treatment as with the previous reports.

4. CONCLUSIONS

In this work, the preparation and characterization of CNH-nucleated dendrimer/fluorocarbon/PEG hierarchical nanocarriers and their ability to capture NO gas were reported as well as their antibacterial effects for the most pertinent candidates. The NCNH/DEN-F/F-PEG nanomaterials were found to be the most efficient for NO loading because of NO-loading ability on each layer and were highest even for the NO content at 180 min after loading due to the weak interaction of NO with a component at each layer. Both behaviors likely result from the existence of the gas channel through the void volumes in nanocomposites, and the NO exercises the antibacterial effect. Thus, the NCNH/DEN-F/F-PEG hier-

archy can be considered as a promising system for NO delivery due to its easy preparation, ability to promote the sustained NO release, and antibacterial effect. Thus, the present hierarchical structure will be expected to be useful for any other gas therapies using not only NO but also other gases like oxygen.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsabm.0c01577>.

FTIR spectra of PFPrA, DEN-OH, and DEN-F and XRD spectra of NG/DEN-F and NG/DEN-F/F-PEG (PDF)

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Notes

The authors declare no competing financial interest.

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