Investigating the Use of Layered Double Hydroxide Nanoparticles as Carriers of Metal Oxides for Theranostics of ROS-Related Diseases

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Supporting Information

ABSTRACT: Overproduction of reactive oxygen species (ROS) is commonly known as a key factor in the progression of many chronic inflammation diseases such as atherosclerosis and rheumatoid arthritis. In this study, a metal oxide nanodot-coated layered double hydroxide (LDH) nanocomposite is constructed for theranostics of ROS-related diseases. This is the first time that both cerium oxide and iron oxide nanoparticles (NPs) were attached on the surface of LDH NPs through electrostatic interaction via a nanoengineering approach. LDHs served as nanocarriers, cerium oxide NPs served as therapeutic agents due to the antioxidant properties, and iron oxide NPs served as magnetic resonance imaging (MRI) contrast agents. In vitro studies have demonstrated that the constructed nanocomposites have good biocompatibility, good antioxidant capacity to reduce ROS level in the cells, as well as satisfying cell imaging effect in MRI. Functionalization of LDH surface with cerium oxide NPs and iron oxide NPs allows the simultaneous therapy and diagnosis of ROS-related diseases and may also allow biodistribution tracking of the therapeutic cerium oxide NPs.

KEYWORDS: atherosclerosis, macrophage, theranostics, layered double hydroxide, cerium oxide nanoparticle, iron oxide nanoparticle, reactive oxygen species scavenging, magnetic resonance imaging

INTRODUCTION

Reactive oxygen species (ROS) have been demonstrated to play an important role in human physiological and pathophysiological processes.1 ROS produced by phagocytic cells such as macrophages and neutrophils generally exert their physiological effects by killing invading pathogens.2 However, overproduction of ROS can cause dysfunction of tissues and cells and contribute to oxidative stress that accelerates the development of many diseases such as atherosclerosis, diabetes, rheumatoid arthritis, neurodegeneration, and cancer.3−9 ROS involves in the activation of cell signaling and immune responses. However, the excessive production of ROS can cause oxidative injury and lead to cell death eventually.10 The ROS family generally includes hydrogen peroxide, hydroxyl radicals, superoxide anions, and hypochlorous acid.11 They are reported to be linked to various chronic diseases such as cardiovascular diseases.10 Cellular enzyme oxidants and reductants enable cells to counteract the oxidative injury when excess ROS are produced. However, this cellular defense mechanism can be compromised by diseases or aging.12 Thus, an effective agent to decrease the cellular ROS levels is urgently needed.

The treatment for ROS-related diseases mainly focuses on therapeutic effect of natural antioxidants based on the assumption that they will reduce ROS levels in tissues and cells. In fact, the results from the clinical studies using antioxidants such as vitamin E, rottlerin, curcumin, and diclofenac were largely disappointing.13−17 In recent decades, novel therapeutic drugs like nanomedicine have been designed to inhibit specific ROS-producing systems. Cerium oxide (CeO2) NPs have received considerable attention in nanotherapeutics to scavenge mediators like ROS in chronic inflammation due to the excellent antioxidant properties.18 CeO2 NPs have shown great superoxide dismutase (SOD) mimetic activity, catalase mimetic activity, hydroxyl radical, and nitric oxide scavenging property.11 It appears that CeO2 NPs have the capability of scavenging most types of ROS, making it superior to the antioxidant enzymes that can only specifically inhibit the corresponding catalytic substrate. The excellent antioxidant properties of CeO2 NPs are attributed to the quick conversion of the oxidation state between Ce3+ and Ce4+ on their surfaces.19

Molecular imaging techniques are essential to investigate the specific cells and molecules involving in the lesion sites where
diseases occur and develop. Magnetic resonance imaging (MRI) has been widely used for disease diagnosis. Several imaging agents including gadolinium and superparamagnetic iron oxide NPs are developed for disease diagnosis. Superparamagnetic iron oxide (Fe₃O₄) NPs have been widely developed for MRI of diseases. Unlike gadolinium, magnetic iron oxide NPs exhibiting good biocompatibility, long blood retention, and low cytotoxicity are fit for in vitro and in vivo diagnostics.

Recently, multifunctional nanocomposites combining drug delivery with imaging probe have provided promising potential due to simultaneously imaging the cells and delivering drugs. Layered double hydroxides (LDHs), a type of two dimensional (2D) layered nanomaterials, have been employed as a carrier for drug delivery and bioimaging. Moreover, as a unit block, LDHs have been used to construct multifunctional nanocomposites with polymers, NPs, and proteins. LDHs consist of cationic brucite-like layers and anionic interlayers with the general chemical formula of \([\text{M}^{II}_{1-x}\text{M}^{III}_x\text{(OH)}_2]^{2+}\times (\text{X}^-)_{n}\text{H}_2\text{O}^{x-}\), where \(\text{M}^{II}\) is the divalent metal cation, \(\text{M}^{III}\) is the trivalent metal cation, and \(\text{X}^-\) is the intercalating anion. LDHs possess positive charge as MII is partially replaced by MIII in the brucite layers. This provides a possibility to conjugate LDHs with negatively charged biomolecules and nanoparticles (NPs). There have been a number of studies on the cooperation between different NPs and LDHs. Li et al. reported SiO₂ nanodot-coated LDH nanocomposites by attaching SiO₂ nanodots evenly on LDHs for drug delivery. They further modified SiO₂-LDH nanocomposites with mannose to achieve targeted siRNA delivery. LDHs were also functionalized with magnetic Fe₃O₄ NPs to act as photocatalysts for environmental remediation like water purification because they are capable of adsorption for organic dyes and heavy metal.

In this study, a novel LDH nanocomposite with both cerium oxide NP and magnetic iron oxide NP loading on the surface of LDH was synthesized for biological applications. As shown in Scheme 1, polyacrylic acid (PAA)-stabilized CeO₂ and Fe₃O₄ NPs were first prepared by precipitation approach and then were loaded on the surface of the LDHs to construct CeO₂-Fe₃O₄@LDH nanocomposites via electrostatic interaction. LDHs served as nanocarriers that can enhance the half-life of the CeO₂ and Fe₃O₄ NPs in the body. CeO₂ NPs served as therapeutic agents due to the antioxidant properties, and Fe₃O₄ NPs served as contrast agents in MRI to monitor NPs in the cells. Both ROS scavenging and cytotoxicity data showed promising results as well as MRI signal detection in the macrophages. The nanocomposites can be further modified to target ROS-related cells such as macrophages, smooth muscle cells, and endothelial cells. Functionalization of LDH surface with CeO₂ NPs and Fe₃O₄ NPs allows simultaneous therapy and detection of inflammatory diseases such as atherosclerosis and rheumatoid arthritis.

### MATERIALS AND METHODS

#### Materials

- Cerium nitrate hexahydrate, PAA (Mw = 1800), iron(II) ammonium sulfate, hydrochloric acid (reagent grade, 37%), ammonium hydroxide solution (30% NH₃ in H₂O), magnesium chloride, aluminum chloride, hydrogen peroxide, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), horseradish peroxidase (HRP), and 2,7′-dichlorodihydrofluorescein diacetate (DCF-DA) were purchased from Sigma-Aldrich. Phosphate-buffered saline (PBS), RPMI 1640 medium, fetal bovine serum (FBS), l-glutamine, penicillin/streptomycin solution, and TrypLE Express were purchased from ThermoFisher Scientific. PrestoBlue cell viability reagent was purchased from Invitrogen.

#### Synthesis of Layered Double Hydroxides

LDHs containing Mg²⁺ and Al³⁺ ([Mg₂Al(OH)₆]Cl₄) were prepared by co-precipitation with hydrothermal method. A total of 40 mL of 0.15 M Mg(NO₃)₂ was added to 10 mL of a mixed solution containing 0.3 M MgCl₂ and 0.1 M AlCl₃ and stirred at room temperature for 10 min. The resulting slurry precipitate was collected and washed twice by centrifugation, then dispersed in 40 mL of distilled water. The inhomogeneous suspension was transferred to an autoclave (Teflon-lined stainless steel) and heated at 100 °C for 16 h. The resulting homogeneous suspension was collected and used for subsequent investigations.

#### Synthesis of Cerium Oxide NPs

The PAA-stabilized CeO₂ NPs were prepared by a precipitation method. A total of 2 mL of a mixed solution containing cerium(III) nitrate (0.4 g) and PAA (0.3 g) was added dropwise to 6 mL of 30% ammonium hydroxide solution with continuous stirring for 24 h. The stirred mixture was then centrifuged at 4000 rpm for 30 min to settle down large agglomerates. The supernatant was collected and purified by dialyzing against 5 L of water at pH 10 for 1 day, followed by dialysis against 5 L of water at pH 7 for another day. The resulting solution was concentrated using a 10K molecular weight cutoff Amicon filter (Millipore, Inc.). The concentration of PAA-coated cerium oxide NPs was measured by inductively coupled plasma mass spectrometry (ICP-MS) analysis (Agilent 7900, Agilent Technologies, Japan).

#### Synthesis of Iron Oxide NPs

The PAA-coated Fe₃O₄ NPs were prepared by co-precipitation method. Typically, 0.2 g of PAA was dissolved in 50 mL of Milli-Q water. The PAA solution was purged with nitrogen for 30 min and then heated at 130 °C to reflux using an oil bath. A mixture of 0.51 mM FeCl₃·6H₂O (0.14 g) and 0.25 mM ACl₃·6H₂O (0.52 g) was added dropwise to the PAA solution with vigorous stirring. The resulting mixture was then refluxed at 130 °C for 40 h to form a highly magnetic powder. The obtained powder was then washed several times with ethanol and water and dried at 60 °C under vacuum.

#### Synthesis of CeO₂-Fe₃O₄@LDH Nanocomposites

Scheme 1. Schematic Showing the Theranostic Function of CeO₂-Fe₃O₄@LDH Nanocomposites and Potential Applications in ROS-Related Diseases

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(NH₄)$_2$Fe(SO₄)$_2$·6H₂O (0.10 g) was dissolved in 1 mL of 37% concentrated HCl. The mixed solution was then quickly added into the hot PAA solution. After stirring for 5 min, 15 mL of 30% concentrated ammonia solution was added into the mixture, followed by the addition of 15 mL of 30% concentrated HCl. The mixed solution was then quickly added into a mixture of CeO₂ and Fe₃O₄ suspension under continuous stirring for 24 h. The resulting solution was centrifuged at 14,000 rpm for 15 min and washed with water twice. Finally, the pellets were resuspended in 2 mL of Milli-Q water.

**Synthesis of CeO₂-Fe₃O₄@LDH Nanocomposites.** The prepared LDHs, CeO₂ NPs, and Fe₃O₄ NPs suspensions were adjusted to the desired concentration. CeO₂ NPs and Fe₃O₄ NPs percentages used in the synthesis were measured using ICP-MS.

**Dynamic Light Scattering.** The hydrodynamic particle size, size distribution, and ζ potential of LDHs, CeO₂ NPs, Fe₃O₄ NPs, and CeO₂-Fe₃O₄@LDH nanocomposites were determined by dynamic light scattering (DLS) using DTS1070 folded capillary cell (Malvern) on a Malvern Zetasizer. Each sample was measured at 25 °C in triplicate using the parameter settings as follows with water as dispersant and a measurement angle of 173° backscatter. For LDHs and CeO₂ NPs, refractive index = 1.550, absorption = 0.000; for cerium oxide NPs, refractive index = 2.2, absorption = 0.001; for iron oxide NPs, refractive index = 2.42, absorption = 0.001.

**Transmission Electron Microscopy.** Transmission electron microscopy (TEM) images were captured on an HT7700 Transmission Electron Microscope (Hitachi) at an accelerating voltage of 100 kV. TEM sample was prepared in 100% ethanol and dried on the carbon film-coated copper grid (Zholingkeyi Technology Co., Japan).

**Energy-Dispersive X-ray Spectroscopy.** Energy-dispersive X-ray spectrometry (EDS) images were obtained on an XFlash6TI60 (Bruker). EDS sample was prepared in 100% ethanol and dried on the carbon film-coated copper grid (Zholingkeyi Technology Co., Japan).

**Inductively Coupled Plasma Mass Spectrometry.** The elemental concentrations in each sample were determined using inductively coupled plasma-mass spectrometry using a ICP-MS apparatus (Agilent 7900, Agilent Technologies, Japan). Mg and Al levels in LDH-contained solution were used for determining the concentration of LDH. Based on the chemical formula of LDH ([Mg₆Al(OH)₁₆]⁺Cl⁻) [3,5] the molecular weight of LDH is identified to be 213.092 g/mol. The concentration of CeO₂NPs and Fe₃O₄NPs was determined by Ce and Fe, respectively. The estimation of CeO₂ or Fe₃O₄NP percentage loading on the LDH was calculated using the following equation:

\[
\text{NP loading efficiency (％) =} \frac{\text{concentration of NPs obtained by ICP-MS}}{\text{theoretical concentration of NP for synthesis}} \times 100\%
\]

where theoretical concentration of NPs is the concentration of the NPs used in the synthesis.

**Fourier-Transform Infrared Spectroscopy.** Fourier-transform infrared spectroscopy (FTIR) spectra were analyzed on a Nicolet 5700 FT-IR apparatus. Samples were prepared in the form of powder.

**Anti-ROS Capability of CeO₂-Fe₃O₄@LDH Nanocomposites.** CeO₂-Fe₃O₄@LDH nanocomposites with different concentrations of CeO₂ (0.5, 1, 5, 20, and 40 μg/mL) were prepared as per the procedure described above. The concentration of CeO₂-Fe₃O₄@LDHs was determined based on the content of CeO₂ attached on the surface of LDH. After 4 h of treatment with NPs to allow NP uptake by macrophages, the NP solution was removed, followed by addition of 100 μL ofDCFDA (25 μM final concentration) diluted in PBS. Macrophages were then stained with DCFDA for 45 min and washed with PBS once. The intracellular levels of ROS were stimulated by 0.05 mM or 0.5 mM H₂O₂ and determined by recording the fluorescence intensity at time points of 15 min after adding H₂O₂. Fluorescence intensity was measured (excitation/emission = 485/535 nm) using an EnSpire Alpha multimode plate reader (PerkinElmer, USA).

**In Vitro Cytotoxicity Assay.** Cell viability was determined using PrestoBlue cell viability reagent (Invitrogen). Macrophages were seeded in 96-well tissue-treated plates at a density of 10,000 cells per well. After 24 h incubation, the cells were treated with CeO₂-Fe₃O₄@LDHs at the concentration of CeO₂ ranging from 0.1 to 1 μg/mL. The concentration of CeO₂ NPs, LDHs, or CeO₂-Fe₃O₄@LDHs was determined based on the content of CeO₂ attached on the surface of LDH. After 24 h treatment with NPs, the NP solution was removed, followed by addition of 10 μL of PrestoBlue into the total volume of 100 μL of culture medium to each well. The cells were then incubated at 37 °C for 30 min. Fluorescence intensity was measured at the emission wavelength of 535 nm (25 nm bandwidth) and the excitation wavelength of 615 nm (10 nm bandwidth) after incubation using an EnSpire Alpha multimode plate reader (PerkinElmer, USA).

**Cell Culture.** Macrophage J774A.1 and Chinese hamster ovary (CHO) cell lines were attained from American Type Culture Collection (ATCC). Macrophages were cultured in RPMI1640 medium supplemented with 10% FBS, 1% l-glutamine and penicillin (10 U/mL)/streptomycin (10 μg/mL) in an incubator at 37 °C with 5% CO₂. CHO cells were cultured in DMEM medium with 10% FBS and penicillin (10 U/mL)/streptomycin (10 μg/mL). Cell numbers were counted using a Beckman Coulter Z. Series instrument.

**In Vitro ROS Scavenging Assay.** Cellular ROS was evaluated with 2′,7′-dichlorofluorescein diacetate (DCFDA). When diffusing into the cell, DCFDA is deacetylated to a nonfluorescent compound and subsequently oxidized by ROS into the fluorescent 2′,7′-dichlorofluorescein (DCF) [3,5]. Thus the fluorescent signal intensity can directly reflect and positively correlate with intracellular ROS level. To trigger the ROS production from macrophages, H₂O₂ as a stimulus can pass through the macrophage membranes and stimulate the generation of hydroxyl radicals (OH·), which induces oxidative stress and cellular damage [5,6]. Our aim is to detect the fluorescence intensity and determine whether the intracellular ROS can be quenched by the desired NPs. Macrophages were seeded in 96-well tissue-treated plates at a density of 10,000 cells per well. After 24 h incubation, macrophages were treated with CeO₂ NPs at the concentration ranging from 0.5 to 1 μg/mL. The concentration of CeO₂-Fe₃O₄@LDHs was determined based on the content of CeO₂ attached on the surface of LDH. After 4 h of treatment with NPs to allow NP uptake by macrophages, the NP solution was removed, followed by addition of 100 μL of DCFDA (25 μM final concentration) diluted in PBS. Macrophages were then stained with DCFDA for 45 min and washed with PBS once. The intracellular levels of ROS were stimulated by 0.05 mM or 0.5 mM H₂O₂ and determined by recording the fluorescence intensity at time points of 15 min after adding H₂O₂. Fluorescence intensity was measured (excitation/emission = 485/535 nm) using an EnSpire Alpha multimode plate reader (PerkinElmer, USA). The background fluorescence value containing cell-only control wells was subtracted from the fluorescence value of each experimental well. ROS levels were calculated using the following equation:

\[
\text{ROS level (fold change) stimulation} = \frac{F_{\text{stimulation}}}{F_{\text{control}}}
\]

where \(F_{\text{stimulation}}\) is the fluorescence intensity of the cells stimulated by H₂O₂ and \(F_{\text{control}}\) is the fluorescence intensity of the cells treated with DCFDA only without any additional stimulation. The fluorescence images were taken by Nikon ECLIPSE Ti microscope system with Photometrics CoolSNAP HQ² camera and Nikon INTENSILIGHT C-HGFIIE fluorescent light source.

**Cell Viability.** Cell viability was determined using PrestoBlue cell viability reagent (Invitrogen). Macrophages were seeded in 96-well tissue-treated plates at a density of 10,000 cells per well. After 24 h incubation, the cells were treated with CeO₂-Fe₃O₄@LDHs at the concentration of CeO₂ ranging from 0.1 to 1 μg/mL. The concentration of CeO₂ NPs, LDHs, or CeO₂-Fe₃O₄@LDHs was determined based on the content of CeO₂ attached on the surface of LDH. After 24 h treatment with NPs, the NP solution was removed, followed by addition of 10 μL of PrestoBlue into the total volume of 100 μL of culture medium to each well. The cells were then incubated at 37 °C for 30 min. Fluorescence intensity was measured at the excitation wavelength of 535 nm (25 nm bandwidth) and the emission wavelength of 615 nm (10 nm bandwidth) after incubation using an EnSpire Alpha multimode plate reader (PerkinElmer, USA).

The background fluorescence value containing no-cell control wells was subtracted from the fluorescence value of each experimental well. Cell viability was calculated using the following equation:

\[
\text{cell viability (％) =} \frac{F_{\text{sample}}}{F_{\text{control}}} \times 100\%
\]

where \(F_{\text{sample}}\) was the fluorescence intensity of the cells treated with different conditions and \(F_{\text{control}}\) was the fluorescence intensity of the cells cultured in the standard medium without any treatment.
Magnetic Resonance Imaging. Macrophages were seeded in 6-well tissue-treated plates at a density of 3 × 10⁵ cells per well. After 24 h incubation, the cells were treated with CeO₂·Fe₃O₄@LDHs at the concentration of 0, 0.5, and 1 μg/mL based on the amount of CeO₂ conjugated to the LDH. After an additional 8 h incubation, the NP solution was removed followed by washing cells with PBS once. The cells were then detached by TrypLE Express and centrifuged at 200 g for 5 min. TrypLE Express was removed after washing cells with PBS at 200 g for 5 min. The cells were subsequently resuspended with 20 μL of warm 1% low-gelling temperature agarose and quickly transferred into the prepared phantom vessel. To prepare the phantom vessel, the lower end of a straw with 3.2 cm long was sealed with hot glue, followed by adding around 100 μL of warm 1% agarose gel into the phantom vessel (see Scheme 2). After gel solidification for 10 min, the cell sample or NP sample was quickly added onto the agarose gel as mentioned before. Any bubble formed in the tubes was popped by a small needle, as it could influence the MRI signal. The cell pellet was set for 10 min, and then warm 1% agarose gel was added on the top of the cell pellet gelling for 10 min. Subsequently, the top end of the phantom vessel was sealed with hot glue. The prepared phantom vessel was embedded in a 1.5 mL Eppendorf tube containing 1% agarose gel. MRI was performed on a 9.4 T MRI scanner (Bruker).

![Scheme 2. Schematic Illustration of the Preparation of Phantom Vessel for MRI](Image)

Table 1. Characteristics of CeO₂, Fe₃O₄, LDH, and CeO₂·Fe₃O₄@LDH Nanocomposites Measured by DLS Zetasizer

<table>
<thead>
<tr>
<th>sample</th>
<th>polydispersity index</th>
<th>Z-average (d, nm)</th>
<th>ζ-potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CeO₂</td>
<td>0.379 ± 0.071</td>
<td>11.1 ± 0.8</td>
<td>−23.2 ± 2.1</td>
</tr>
<tr>
<td>Fe₃O₄</td>
<td>0.339 ± 0.013</td>
<td>12.0 ± 0.9</td>
<td>−15.2 ± 2.4</td>
</tr>
<tr>
<td>LDH</td>
<td>0.132 ± 0.016</td>
<td>113.1 ± 1.1</td>
<td>40.1 ± 0.3</td>
</tr>
<tr>
<td>CeO₂·Fe₃O₄@LDH 1:4:4</td>
<td>0.140 ± 0.017</td>
<td>170.9 ± 0.4</td>
<td>−18.7 ± 0.1</td>
</tr>
</tbody>
</table>

RESULTS AND DISCUSSION

Physicochemical Features of CeO₂, Fe₃O₄, LDH, and CeO₂·Fe₃O₄@LDH Nanocomposites. Monodispersed PAA-stabilized CeO₂ and PAA-stabilized Fe₃O₄ NPs were prepared via precipitation method. DLS data in Table 1 and Figure 1a,b indicated that CeO₂ and Fe₃O₄ NPs had narrow size distribution with the particle sizes in the range of 10–11 and 12–13 nm and ζ potentials of −21.0 and −17.0 mV, respectively. The TEM images in Figure 1e showed CeO₂ and Fe₃O₄ NPs exhibited irregular shapes. As shown in Figure S1a, the powder X-ray diffraction (XRD) patterns of the synthesized CeO₂ NPs exhibited very broad peaks at 28.4, 47.5, and 56.3, corresponding to (111), (220), and (311) planes of CeO₂ with a cubic fluorite structure. The broad diffraction peaks of CeO₂ NPs are ascribed to the small particle size of CeO₂ and PAA polymer coating. The XRD patterns of Fe₃O₄ NPs showed Fe₃O₄ NPs have inverse cubic spinel phase as displaying a series of characteristic peaks (220), (311), (400), (422), (511), and (440). Well-dispersed LDHs, possessing hexagonal shapes (Figure 1e), were obtained with the particle sizes of approximately 113 nm and the ζ potential of +42.5 mV (Table 1 and Figure 1a,b), as reported previously. As shown in Figure S1b, LDHs showed strong XRD peaks, indexing as typical (003) and (006) peaks of LDHs. LDHs exhibited the typical layered features with a rhombohedral symmetry in accordance with our previous report.

To obtain the desired CeO₂·Fe₃O₄@LDH nanocomposites, the mass ratios of CeO₂ to LDH and Fe₃O₄ to LDH were first investigated. CeO₂@LDHs synthesized under the mass ratios of 4:1, 2:1, 1:1, and 1:2 shared the similar particle sizes and ζ potentials, while the particles become much bigger at the mass ratio of 1:4 due to the particle aggregation (see Figure S2a,b). As for Fe₃O₄@LDHs, the particle sizes of the three tested particles at the mass ratios of 8:1, 4:1, and 2:1 were similar (see Figure S2c,d), while the particles were aggregated macroscopically at the mass ratio of 1:1, which was not being tested via DLS technique. Then the optimized mass ratios of CeO₂@LDHs and Fe₃O₄@LDHs were determined to 1:2 and 2:1, respectively. The TEM images in Figure S2e,f showed the successful loading of CeO₂ on the LDH with the mass ratio of 1:2 and Fe₃O₄ on the LDH with the mass ratio of 2:1 (or 4:2). Thus, we combined these two for the synthesis of CeO₂·Fe₃O₄@LDH nanocomposites, and the theoretical desired mass ratio of CeO₂ to Fe₃O₄ to LDH could be presumed as 1:4:4. Then, we properly mixed LDH NP suspension with the CeO₂ and Fe₃O₄ mixture dropwise at the mass ratio of 1:4:4 for CeO₂ to Fe₃O₄ to LDH. As shown in Figure 1c, the hydrodynamic size of CeO₂·Fe₃O₄@LDH increased to 170 nm due to the slight aggregation, and ζ potential was reversed to −18.7 mV. TEM images displayed that CeO₂ and Fe₃O₄ nanodots were evenly distributed on the surface of LDHs and CeO₂·Fe₃O₄@LDH nanocomposites retained the hexagonal and layered shapes. XRD results in Figure S1b also showed CeO₂·Fe₃O₄@LDH nanocomposites have similar diffraction peaks as LDHs in accordance with TEM results that CeO₂·Fe₃O₄@LDH nanocomposites retained a layered structure. Ce and Fe spectrum were also observed via energy-dispersive X-ray spectrometry (EDS) coupled with TEM (see Figure S3), indicating the existence of both CeO₂ and Fe₃O₄ NPs on LDHs. The even attachment of CeO₂·Fe₃O₄ on LDHs is attributed to the electrostatic interaction and space confinement.

Different mass ratios of CeO₂ to Fe₃O₄ to LDH (1:4:2, 1:8:4, and 1:8:2) were also investigated to further confirm whether the optimized ratio would be 1:4:4. As shown in Figure S4 and Table S1, CeO₂·Fe₃O₄@LDH nanocomposites at different mass ratios of CeO₂ to Fe₃O₄ to LDH displayed similar size distributions and ζ potentials. The TEM images in Figure S4 revealed that CeO₂ and Fe₃O₄ NPs were attached on LDHs. The loading amount of both CeO₂ NPs and Fe₃O₄ NPs was further tested using ICP-MS. Among the four NPs, CeO₂-
Fe$_3$O$_4$@LDH nanocomposites at the mass ratio of 1:4:4 for CeO$_2$ to Fe$_3$O$_4$ to LDH exhibited the highest yield of CeO$_2$ NPs (7.71 μg/mL) and Fe$_3$O$_4$ NPs (18.73 μg/mL). Thus, the optimized mass ratio of CeO$_2$ to Fe$_3$O$_4$ to LDH to make CeO$_2$-Fe$_3$O$_4$@LDH nanocomposites would be 1:4:4, used for further modification, characterization, and other tests unless stated.

FTIR spectrometry has further verified the loading of CeO$_2$ and Fe$_3$O$_4$ NPs on the LDHs. As shown in Figure 1d, the characteristic bands of CeO$_2$ and Fe$_3$O$_4$ NPs at 1715, 1539,
1441, and 1440 cm\(^{-1}\) are assigned to C\(=\)O stretch, C\(\cdots\)H stretch, O\(\cdots\)H stretch, and N\(\cdots\)O stretch, respectively. These peaks were attributed to PAA polymer, suggesting CeO\(_2\) and Fe\(_3\)O\(_4\) NPs are stabilized by PAA coating.\(^{50}\) The absorption stability of the desired NPs remained stable after two months, indicating the favorable Fe\(_3\)O\(_4\) NPs at 758 and 582 cm\(^{-1}\) corresponds to Ce\(=\)O stretching vibration.\(^{61}\) The specific band at 582 cm\(^{-1}\) attributes to Fe\(=\)O stretching vibration.\(^{62}\) The FTIR spectrum of LDH shows three strong peaks at 719, 667, and 549 cm\(^{-1}\) corresponding to O\(\cdots\)M\(\cdots\)O bending vibrations.\(^{63}\) An additional peak at 1361 cm\(^{-1}\) is found in LDH due to the carbonate formation.\(^{64}\) After coating CeO\(_2\) and Fe\(_3\)O\(_4\) NPs, the characteristic peaks of CeO\(_2\) and Fe\(_3\)O\(_4\)NPs were at 758 and 582 cm\(^{-1}\) were also observed in CeO\(_2\)-Fe\(_3\)O\(_4\)@LDH nanocomposites, suggesting that CeO\(_2\) and Fe\(_3\)O\(_4\)NPs were coated on the surface of LDHs.

The stability of CeO\(_2\)-Fe\(_3\)O\(_4\)@LDH nanocomposites were further examined for 60 days. As shown in Figure 2, the size, size distribution and \(\zeta\) potential of CeO\(_2\)-Fe\(_3\)O\(_4\)@LDHs remained stable after two months, indicating the favorable stability of the desired NPs.

**ROS Scavenging Capability of CeO\(_2\)-Fe\(_3\)O\(_4\)@LDH Nanocomposites.** Our previous studies showed that CeO\(_2\) loaded hybrid NPs have anti-ROS properties in the ABTS-HRP system.\(^{55}\) Briefly, CeO\(_2\) loaded hybrid NPs can scavenge and inhibit ROS such as hydrogen peroxide and superoxide radical in the solution system along with the cerium oxide NPs shift between Ce\(^{3+}\) and Ce\(^{4+}\). Here, we investigated the ROS scavenging capability of CeO\(_2\)-Fe\(_3\)O\(_4\)@LDH nanocomposites. As shown in Figure 3, CeO\(_2\)-Fe\(_3\)O\(_4\)@LDH nanocomposites could significantly reduce the ROS level with a quenching ability up to 40% when the concentration of Ce reached to 40 \(\mu\)g/mL.

![Figure 3. ROS scavenging capability of CeO\(_2\)-Fe\(_3\)O\(_4\)@LDH nanocomposites.](image)

**In Vitro ROS Scavenging by CeO\(_2\)-Fe\(_3\)O\(_4\)@LDH Nanocomposites.** Macrophages are used in *in vitro* study as they produce excessive ROS when the immune system responds to the atherosclerotic lesions. Toxic ROS can further accelerate the development of atherosclerosis.\(^{65}\) Our aim is to decrease the ROS level in the macrophages and then inhibit the downstream regulation induced by toxic ROS. Before determining the ROS scavenging capability of the desired CeO\(_2\)-Fe\(_3\)O\(_4\)@LDH nanocomposites in macrophages J774.1, it is imperative to confirm the efficacy of CeO\(_2\) NPs on quenching the ROS as reported in the previous study.\(^{66}\) Our study has proved that ROS was significantly scavenged at the CeO\(_2\), concentrations of 0.5 and 1 \(\mu\)g/mL after stimulating cells with 0.5 mM H\(_2\)O\(_2\) for 15–20 min (Figure S5). Notably, very high concentration of CeO\(_2\)(20 \(\mu\)g/mL) did not provide a satisfactory scavenging effect, which might be due to the increasing toxicity to the macrophages. It was reported that at high concentrations, the NPs are toxic to the cells and trigger the elevation of ROS.\(^{67}\) The data suggest that an appropriate dosing is essential to effectively quench the ROS, thus CeO\(_2\)-Fe\(_3\)O\(_4\)@LDHs (1:4:4) containing CeO\(_2\) NPs at 0.5 and 1 \(\mu\)g/mL were chosen to evaluate the ROS quenching capability of the combined NPs in the macrophages. The composition of the combined NPs is shown in Table 2. ROS levels were significantly quenched in CeO\(_2\)-pretreated as well as in CeO\(_2\)-Fe\(_3\)O\(_4\)@LDH-pretreated macrophages with 0.5 mM H\(_2\)O\(_2\) stimulating (see Figure 4a,b), suggesting a promising application for the desired CeO\(_2\)-Fe\(_3\)O\(_4\)@LDHs. It was noted that the ROS quenching effect did not increase when the nanocomposite concentration increased from 0.5 to 1 \(\mu\)g/mL (concentrations based on CeO\(_2\)).

The ROS scavenging capability of CeO\(_2\)-Fe\(_3\)O\(_4\)@LDH nanocomposites was further confirmed by fluorescence images. ROS levels were directly measured by DCF-DA. As shown in Figure 4c, macrophages exhibited a green fluorescence signal indicating they were stimulated by 0.5 mM H\(_2\)O\(_2\) to produce excessive ROS. The fluorescence signal was much lower in the macrophages treated with CeO\(_2\)-Fe\(_3\)O\(_4\)@LDH nanocomposites compared to the ones without NP treatment, implying that the CeO\(_2\)-Fe\(_3\)O\(_4\)@LDH nanocomposites had ability to quench ROS.

Although CeO\(_2\) NPs were observed to perform similar to the nanocomposites when scavenging ROS, the advantages of the nanocomposite system lie in its multifunction and longer circulation time. It was reported that small nanodrugs like CeO\(_2\)NPs with sizes <6 nm can be eliminated from the blood circulation quicker than the larger NPs.\(^{18,68}\) In addition, the combination of both imaging agent (Fe\(_3\)O\(_4\) NPs) and therapeutic agent (CeO\(_2\) NPs) in a single nanocomposite allows the tracking of the therapeutics.

**Cytotoxicity of Nanoparticles to Macrophages and CHO Cells.** Cytotoxicity of the synthesized NPs was

Table 2. Composition of the Combined Nanocomposite and the Concentration of Each Component

<table>
<thead>
<tr>
<th>sample</th>
<th>concentration ((\mu)g/mL)</th>
<th>concentration ((\mu)g/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CeO(_2)</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Fe(_3)O(_4)</td>
<td>1.0*</td>
<td>2.0*</td>
</tr>
<tr>
<td>LDH</td>
<td>32*</td>
<td>64*</td>
</tr>
<tr>
<td>CeO(_2)-Fe(_3)O(_4)@LDH 1:4:4</td>
<td>33.5 (contain 0.5 (\mu)g/mL of CeO(_2))</td>
<td>67 (contain 1.0 (\mu)g/mL of CeO(_2))</td>
</tr>
</tbody>
</table>

\(a\) Concentration of Fe\(_3\)O\(_4\) calculated based on the ratio between CeO\(_2\) and Fe\(_3\)O\(_4\) loading on LDH (1:2) determined by ICP-MS.

\(b\) Concentration of LDH calculated based on the ratio between CeO\(_2\) and LDH (1:64) determined by ICP-MS.
preliminarily investigated by studying the effect of the NPs on cell viability. It was conducted with macrophage and CHO cells incubated with different concentrations of the NPs. Figure 5a showed that there was no significant cytotoxicity observed at CeO₂ concentrations from 0 to 0.5 μg/mL in CeO₂-Fe₃O₄@LDH nanocomposites (concentrations ranging from 0 to 33.5 μg/mL), but a higher CeO₂ concentration (1 μg/mL) led to a slight decrease of macrophage viability (approximately 10%).

Compared with CHO cells at CeO₂ concentration of 1 μg/mL, all NPs showed no toxicity (Figure 5b). The cytotoxicity for macrophages was further confirmed by the bright-field cell imaging (Figure 5c). The morphology of macrophages was generally characteristically circular and fusiform, as shown in the groups with CeO₂ concentration ranging from 0.1 to 0.5 μg/mL. However, the shape of macrophages started to change at the CeO₂ concentration of 1 μg/mL. These results indicate...
that CeO$_2$-Fe$_3$O$_4$@LDH nanocomposites have no significant cytotoxicity on the cells at the CeO$_2$ concentration below 1 μg/mL. When the concentration of CeO$_2$ reached up to 1 μg/mL, the CeO$_2$-Fe$_3$O$_4$@LDH nanocomposites showed some cytotoxicity to macrophages but not to CHO cells. It implies that these NPs could have the potential ability to induce macrophage cell death without harming normal somatic cells, which may subsequently quench the inflammatory reactions at the site. However, more study is needed to investigate this aspect.

MRI of CeO$_2$-Fe$_3$O$_4$@LDH Nanocomposites in Macrophages. MR signals of CeO$_2$-Fe$_3$O$_4$@LDH nanocomposites at different concentrations were first investigated. The conversion for concentrations between Fe and Fe$_3$O$_4$ NPs is shown in Table 3. MRI images of the CeO$_2$-Fe$_3$O$_4$@LDH nanocomposites are shown in Figure 6. CeO$_2$-Fe$_3$O$_4$@LDH nanocomposites in 1% agarose gel or water exhibited the increasing $T_2$ contrast signals as the Fe concentration increased (Figure 6a,c). The $T_2$-weighted relaxivity determined in the 1% agarose gel (144.92 mM$^{-1}$ s$^{-1}$) was lower than that in the water (243.46 mM$^{-1}$ s$^{-1}$) in Figure 6b,d, indicating that the surrounding environment affects the nanomaterial relaxivity.

After determining the CeO$_2$-Fe$_3$O$_4$@LDH nanocomposites as the contrast agent in the macrophages was conducted. The cells were incubated with the nanocomposites, then collected and evenly embedded in agarose gel for MRI. As shown in Figure 6e,f, the MRI signal of cells incubated with NPs at Fe concentrations of 0.013 mM and 0.027 mM could be readily detected corresponding to the darker color in $T_2$-weighted MR images, implying the application of these nanocomposites for labeling and tracking macrophages in vivo.

### Table 3. Conversion for Concentrations between Fe and Fe$_3$O$_4$ NPs Based on the Effective Concentrations of CeO$_2$ NPs Determined by ROS Quenching Activity

<table>
<thead>
<tr>
<th>concentration of CeO$_2$ (μg/mL)</th>
<th>concentration of Fe$_3$O$_4$ (μg/mL)</th>
<th>[Fe]$_3$O$_4$</th>
<th>concentration of CeO$_2$ (μg/mL)</th>
<th>concentration of Fe$_3$O$_4$ (μg/mL)</th>
<th>[Fe]$_3$O$_4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>0.4</td>
<td>0.005</td>
<td>0.5</td>
<td>1</td>
<td>0.013</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>0.027</td>
<td>5</td>
<td>10</td>
<td>0.131</td>
</tr>
<tr>
<td>10</td>
<td>20</td>
<td>0.260</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

*Concentration of Fe$_3$O$_4$ calculated based on the ratio between CeO$_2$ and Fe$_3$O$_4$ loading on LDH (1:2) determined by ICP-MS.

Figure 5. Cytotoxicity of different NPs to macrophages and CHO cells. (a) Cell viability of macrophages treated with the CeO$_2$-Fe$_3$O$_4$@LDH nanocomposites with CeO$_2$ concentration ranging from 0 to 1 μg/mL (concentration of CeO$_2$-Fe$_3$O$_4$@LDH nanocomposites ranging from 0 to 67 μg/mL). (b) Comparison of cytotoxicity of different NPs with the CeO$_2$ concentration of 1 μg/mL examined in macrophages and CHO cells. (c) Bright-field microscopy images for CeO$_2$-Fe$_3$O$_4$@LDH nanocomposites-treated macrophages with CeO$_2$ concentration ranging from 0 to 1 μg/mL. Results were analyzed by one-way or two-way ANOVA. Data are presented as mean ± SD *$p < 0.05$ versus vehicle, **$p < 0.01$ versus vehicle, $n = 3$. 
CONCLUSIONS
The multifunctional CeO$_2$-Fe$_3$O$_4$@LDHs have been successfully developed for ROS scavenging and imaging in macrophages. The optimal mass ratio between CeO$_2$, Fe$_3$O$_4$, and LDHs should be 1:4:4 due to the highest loading mass of both CeO$_2$ and Fe$_3$O$_4$ NPs on the surface of LDHs compared to other NPs synthesized at different mass ratios. In in vitro studies, CeO$_2$-Fe$_3$O$_4$@LDH nanocomposites have proven to have antioxidant properties determined by ROS scavenging activity in the macrophages. The cytotoxicity assay of this nanohybrid shows there remains no cytotoxicity to macrophages and CHO cells at concentrations where ROS can be significantly scavenged. In MRI, CeO$_2$-Fe$_3$O$_4$@LDH nanocomposites exhibit good magnetic resonance signal in the macrophages, making it a potential contrast agent for cell imaging.

Current treatments for ROS-related diseases such as atherosclerosis and rheumatoid arthritis are performed systemically, leading to low therapeutic efficacy. With the development of the NP-mediated delivery system, therapeutic and diagnostic agents are allowed to be delivered locally and simultaneously. Theranostics approach or co-loading of therapeutic agents with imaging agents allows the monitoring and tracking of the drug location and distribution. In this study, the co-loading of Fe$_3$O$_4$ NPs (imaging agents) and CeO$_2$NPs (therapeutic agents) on LDH nanocarriers enables the tracking of CeO$_2$NP distribution. This approach also potentially increases the circulation retention time of the small CeO$_2$NPs, eventually enhancing the chance that the NPs get to the target areas. Our studies have demonstrated the applications of functionalized LDH nanocomposites in vitro. Further studies in animal models need to be performed to test the feasibility and efficacy of these LDH nanocomposites in vivo. A co-delivery strategy in combination with targeted molecules and therapeutic drugs can also be developed to enhance the therapeutic efficacy, stability, detection sensitivity, and pharmacokinetics of nanocomposites to the tissues we target. Future studies are expected to deliver NPs more specifically to the lesions where diseases form and develop. We are anticipating a broader application for the LDH nanocomposites.

ASSOCIATED CONTENT
S Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsabm.9b00852.

Table S1: Comparison of particle size and $\zeta$ potential of CeO$_2$-Fe$_3$O$_4$@LDH nanocomposites between different mass ratios. Figure S1: XRD patterns of Fe$_3$O$_4$ and CeO$_2$, LDH, and CeO$_2$-Fe$_3$O$_4$@LDH nanocomposites at a mass ratio of 1:4:4. Figure S2: Characteristics of different mass ratios of CeO$_2$@LDHs and Fe$_3$O$_4$@LDH nanocomposites. Figure S3: EDS spectrum of CeO$_2$-Fe$_3$O$_4$@LDH nanocomposites at a mass ratio of 1:4:4. Figure S4: Characteristics of different mass ratios of CeO$_2$-Fe$_3$O$_4$@LDH NPs. Figure S5: ROS scavenging capability of CeO$_2$ NPs (PDF)
Our current understanding of the mechanisms that generate specificity in ROS homeostasis is limited. Further research is needed to fully understand the role of ROS in various biological processes and their implications for age-related disorders.

**ACKNOWLEDGMENTS**

This work received financial support from the Australian National Health and Medical Research Council (H.T.T.; APP1037310). The authors would like to acknowledge the Australian National Fabrication Facility (Queensland Node) and National Imaging Facility, Centre for Advanced Imaging for access to key items of equipment. The authors gratefully thank all of the colleagues at the Australian Institute for Bioengineering and Nanotechnology and School of Pharmacy for their comments and help.

**REFERENCES**


(57) Loh, K.-S.; Lee, Y.; Musa, A.; Salmah, A.; Zamri, I. Use of Fe3O4 nanoparticles for enhancement of biosensor response to the herbicide 2, 4-dichlorophenoxyacetic acid. Sensors 2008, 8, 5775–5791.
Supporting Information

Investigating the Use of Layered Double Hydroxide Nanoparticles as Carriers of Metal Oxides for Theranostics of ROS-related Diseases

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Table S1. Comparison of particle size and zeta potential of CeO$_2$-Fe$_3$O$_4$@LDH nanocomposites between different mass ratios.

<table>
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<tr>
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<tbody>
<tr>
<td>Polydispersity index</td>
<td>0.140±0.017</td>
<td>0.167±0.013</td>
<td>0.124±0.004</td>
<td>0.127±0.015</td>
</tr>
<tr>
<td>Z-average (d, nm)</td>
<td>170.9±0.4</td>
<td>166.4±1.1</td>
<td>150.9±2.0</td>
<td>138.7±0.1</td>
</tr>
<tr>
<td>Zeta potential (mV)</td>
<td>-18.7±0.1</td>
<td>-23.8±0.2</td>
<td>-24.1±0.2</td>
<td>-24.0±0.5</td>
</tr>
<tr>
<td>Concentration of CeO$_2$ on LDH (µg/ml)</td>
<td>7.71</td>
<td>2.97</td>
<td>5.58</td>
<td>2.87</td>
</tr>
<tr>
<td>Concentration of Fe$_3$O$_4$ on LDH (µg/ml)</td>
<td>18.73</td>
<td>9.01</td>
<td>9.36</td>
<td>7.15</td>
</tr>
</tbody>
</table>
Figure S1. The X-ray diffraction (XRD) patterns of Fe$_3$O$_4$ and CeO$_2$ (a), LDH and CeO$_2$-Fe$_3$O$_4$@LDH nanocomposites at a mass ratio of 1:4:4 (b).
Figure S2. Characteristics of different mass ratios of CeO$_2$@LDHs and Fe$_3$O$_4$@LDHs. (a-b) Size distribution by intensity and Zeta potential of CeO$_2$@LDHs at different mass ratios determined by DLS. (c-d) Size distribution by intensity and Zeta potential of Fe$_3$O$_4$@LDHs at different mass ratios determined by DLS. (e) The TEM image of CeO$_2$@LDHs at a mass ratio of 1:2. (f) The TEM image of Fe$_3$O$_4$@LDHs at mass ratio of 2:1. All the scale bars represent 100 nm.
**Figure S3.** EDS spectrum of CeO$_2$-Fe$_3$O$_4$@LDH nanocomposites at a mass ratio of 1:4:4. (a) TEM image obtained from CeO$_2$-Fe$_3$O$_4$@LDH nanocomposites at a mass ratio of 1:4:4. The scale bar is 200 nm. (b) An analyzing line derived from the individual particle was drawn in yellow to be performed by EDS. (c) EDS spectrum of the individual CeO$_2$-Fe$_3$O$_4$@LDH nanocomposite derived from the line analyzed in (b).
**Figure S4.** Characteristics of different mass ratios of CeO$_2$-Fe$_3$O$_4$@LDH nanocomposites. (a) Size distribution by intensity of CeO$_2$-Fe$_3$O$_4$@LDH nanocomposites at different mass ratios determined by DLS. (b) Zeta potential distribution of CeO$_2$-Fe$_3$O$_4$@LDH nanocomposites at different mass ratios determined by DLS. The TEM images of CeO$_2$-Fe$_3$O$_4$@LDH nanocomposites at a mass ratio of 1:4:4 (c), 1:4:2 (d), 1:8:4 (e) and 1:8:2 (f). All the scale bars represent 100 nm.
**Figure S5.** ROS scavenging capability of CeO$_2$ NPs. Graphs showing ROS levels of macrophages treated with CeO$_2$ NPs at different concentrations. Cells were stimulated with 0.05 and 0.5 mM H$_2$O$_2$. ROS was measured at different time points after stimulation. Results were analyzed by two-way ANOVA. Data are presented as mean ± SD. Significant was set as *p < 0.05 versus no NP control, **p < 0.01 versus no NP control, ***p < 0.001 versus no NP control, ****p < 0.0001 versus no NP control, n = 3.