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

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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 the 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, ROS 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 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.¹² 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 is 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.²⁰ 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.²¹⁻³⁶ Magnetic Fe₃O₄ NPs can provide both T₁-weighted and T₂-weighted imaging contrast. Unlike gadolinium, magnetic iron oxide NPs exhibiting good biocompatibility, long blood retention and low cytotoxicity are fit for *in vitro* and *in vivo* diagnostics.³⁷

Recently, multifunctional nanocomposites combining drug delivery with imaging probe have provided promising potentials due to simultaneously imaging the cells and deliver drugs.³⁸⁻⁴¹ Layered double hydroxides (LDHs), a type of two dimension (2D) layered nanomaterials have been employed as a carrier for drug delivery and bioimaging.⁴²⁻⁴⁵ Moreover, as a unit block, LDHs have been used to construct multifunctional nanocomposites with polymers, NPs and proteins.^{44,46,47} LDHs consist of cationic brucite-like layers and anionic interlayers with the general chemical formula of $[M^{II}_{1-x}M^{III}_x(OH)_2]^{x+} [(X^{n-})_{x/n} \cdot yH_2O]^{x-}$ where M^{II} is divalent metal cation, M^{III} is trivalent metal cation and Xⁿ⁻ is the intercalating anion.⁴⁸ LDHs possess positive charge as M^{II} is partially replaced by M^{III} 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.^{49,50} They further modified SiO₂-LDH nanocomposites with mannose to achieve targeted siRNA delivery.⁴⁴ 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.⁵¹

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**, poly acrylic acid (PAA)-stabilized CeO₂ and Fe₃O₄ NPs were firstly 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 body⁵², 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.

Scheme 1. Schematic showing the theranostic function of $\text{CeO}_2\text{-Fe}_3\text{O}_4\text{@LDH}$ nanocomposites and potential applications in ROS-related diseases.

Materials and Methods

Materials

Cerium nitrate hexahydrate, poly (acrylic acid) (PAA, Mw =1800), iron (II) ammonium sulphate, hydrochloric acid (reagent grade, 37%), ammonium hydroxide solution (30% NH₃ in H₂O), magnesium chloride, aluminium chloride, hydrogen peroxide, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), horseradish peroxidase (HRP), 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]⁺Cl⁻) were prepared by co-precipitation with hydrothermal method.⁵³ A total of 40 mL of 0.15 M NaOH 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 polyacrylic acid (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 one 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 cut-off 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 MilliQ 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 (NH₄)₂Fe(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 refluxing the solution for 2 h. The resulting solution was concentrated using a 50K molecular weight cut-off Amicon filter (Millipore, Inc.). The concentrated solution was then dialyzed against 5 L of water at pH 10 for one day and pH 7 for 3 days. The PAA-coated Fe₃O₄ NPs were collected and stored at 4°C. The concentration of PAA-coated Fe₃O₄ NPs was measured using ICP-MS.

Synthesis of CeO₂-Fe₃O₄ @LDH nanocomposites

The prepared LDHs, CeO₂ and Fe₃O₄ NP suspensions were adjusted to the concentration of 1 mg/mL. A certain volume of LDH suspension was dropwise added into a mixture of CeO₂ and Fe₃O₄ suspension under continuous stirring for 24 h. The resulting solution was centrifuged at 14000 rpm for 15 min and washed with water twice. Finally, the pellets were resuspended in 2 mL of MilliQ water.

Dynamic light scattering

The hydrodynamic particle size, size distribution and zeta 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 follow with water as dispersant and a measurement angle of 173° backscatter. For LDHs and LDH nanocomposites, 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 (Zhongjingkeyi Technology Co., Japan).

Energy-dispersive X-ray spectroscopy

Energy-dispersive X-ray spectroscopy (EDS) images were obtained on an HT7700 Transmission Electron Microscope (Hitachi) and analyzed on an XFlash6Ti60 (Bruker). EDS sample was prepared in 100% ethanol and dried on the carbon film coated copper grid (Zhongjingkeyi 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 ($[\text{Mg}_2\text{Al}(\text{OH})_6]^+\text{Cl}^-$),⁵³ the molecular weight of LDH is identified to be 213.092 g/mol.

The concentration of CeO₂ NPs and Fe₃O₄ NPs was determined by Ce and Fe respectively. The estimation of CeO₂ or Fe₃O₄ NP percentage loading on the LDH was calculated using the following equation:

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

* 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 analysed 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 treated with 0.1 mM H₂O₂ in a 96-well plate and incubated for 1 h at 37°C with 200 rpm shaking in a total volume of 25 µL. 25 µL of HRP (0.01 U) was added into each reaction followed by 10 min incubation at room temperature. 50 µL of ABTS (10 mg/mL) was then added into the reaction, and the absorbance of samples at 405 nm was immediately measured by microplate reader Tecan X200. The absorbance of 0 µg/mL sample was determined as 0% ROS scavenging.

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% fetal bovine serum (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% fetal bovine serum 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 reactive oxygen species (ROS) was evaluated with 2',7' –dichlorofluorescein diacetate (DCFDA). When diffusing into the cell, DCFDA is deacetylated to a non-fluorescent compound and subsequently oxidized by ROS into the fluorescent 2', 7' –dichlorofluorescein (DCF).⁵⁴ 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.⁵⁵ 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 four-hour treatment with NPs to allow NP uptake by macrophages, the NP solution was removed, followed by adding 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 dissolved in PBS 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). 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:

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

Where $F_{\text{stimulation}}$ was the fluorescence intensity of the cells stimulated by H_2O_2 and F_{control} was the fluorescence intensity of the cells treated with DCFDA only without any additional stimulation. The fluorescence images were taken by Nikon ECLIPSE Ti microscopy system with Photometrics® CoolSNAP™ HQ² camera and Nikon INTENSILIGHT C-HGFIE fluorescent light source.

***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 10000 cells per well. After 24 h incubation, the cells were treated with $\text{CeO}_2\text{-Fe}_3\text{O}_4\text{@LDHs}$ at the concentration of CeO_2 ranging from 0.1 to 1 $\mu\text{g/mL}$. The concentration of Fe_3O_4 NPs, LDHs or $\text{CeO}_2\text{-Fe}_3\text{O}_4\text{@LDHs}$ was determined based on the content of CeO_2 attached on the surface of LDH. After 24 h treatment with NPs, the NP solution was removed followed by adding 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_{sample} was the fluorescence intensity of the cells treated with different conditions and F_{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 3×10^5 cells per well. After 24 h incubation, the cells were treated with $\text{CeO}_2\text{-Fe}_3\text{O}_4\text{@LDHs}$ at the concentration of 0, 0.5 and 1 $\mu\text{g/mL}$ based on the amount of CeO_2 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). T_2 -weighted images were captured under the following parameter settings: TR = 2630.0 ms, TE = 53.1 or 100 ms, FA = 90.0 deg, and TA = 0h11m13s261ms NEX 1.

Scheme 2. Schematic illustration of the preparation of phantom vessel for MRI.

Statistical analysis

Data are presented as mean $\pm$ SD from at least three independent experiments and analyzed with two-way ANOVA or one-way ANOVA. The level of statistical significance was defined as p-value < 0.05 . The graphs were plotted using GraphPad Prism 7 software (GraphPad Prism Inc. CA, USA).

Results and Discussion

Physicochemical features of CeO₂, Fe₃O₄, LDH, and CeO₂-Fe₃O₄@LDH nanocomposites.

Mono-dispersed 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 zeta 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.⁵⁶ 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).⁵⁷ Well-dispersed LDHs, possessed hexagonal shapes (**Figure 1e**), were obtained with the particle sizes of approximately 113 nm and the zeta potential of +42.5 mV (**Table 1** and **Figure 1a, b**), as reported previously.⁵⁸ 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.⁵³

To obtain the desired CeO₂-Fe₃O₄@LDH nanocomposites, the mass ratios of CeO₂ to LDH and Fe₃O₄ to LDH were firstly investigated. CeO₂@LDHs synthesized under the mass ratios of 4:1, 2:1, 1:1, 1:2 shared the similar particle sizes and zeta 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 nanoparticle suspension with the CeO₂ and Fe₃O₄ mixture drop-wisely 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 zeta 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 layered structure. Ce and Fe spectrum were also observed via energy-dispersive X-ray spectrometry (EDS) coupled to 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.⁵⁹

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 zeta 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-Fe₃O₄@LDH nanocomposites at the mass ratio of 1:4:4 for CeO₂ to Fe₃O₄ to LDH exhibited the highest yield of CeO₂ NPs (7.71 µg/ml) and Fe₃O₄ NPs (18.73 µg/ml). Thus, the optimized mass ratio of CeO₂ to Fe₃O₄ to LDH to make CeO₂-Fe₃O₄@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 CeO₂ and Fe₃O₄ NPs on the LDHs. As shown in **Figure 1d**, the characteristic bands of CeO₂ and Fe₃O₄ NPs at 1715, 1539, 1441 and 1440 cm⁻¹ are assigned to C=O stretch, C-H stretch, O-H stretch and N-O stretch, respectively.⁶⁰ These peaks were attributed to PAA polymer, suggesting CeO₂ and Fe₃O₄ NPs are stabilized by PAA coating.⁶¹ The absorption band at 758 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-M-O bending vibrations.⁶³ An additional peak at 1361 cm⁻¹ is found in LDH due to the carbonate formation.⁶⁴ After coating CeO₂ and Fe₃O₄ NPs the characteristic peaks of CeO₂ and Fe₃O₄ NPs at 758 and 582 cm⁻¹ were also observed in CeO₂-Fe₃O₄@LDH nanocomposites, suggesting that CeO₂ and Fe₃O₄ NPs were coated on the surface of LDHs.

Table 1. Characteristics of CeO₂, Fe₃O₄, LDHs and CeO₂-Fe₃O₄@LDH nanocomposites measured by DLS Zetasizer.

Sample	Polydispersity index	Z-average (d.nm)	Zeta potential (mV)
CeO ₂	0.379±0.071	11.1±0.8	-23.2±2.1
Fe ₃ O ₄	0.339±0.013	12.0±0.9	-15.2±2.4
LDH	0.132±0.016	113.1±1.1	40.1±0.3

Figure 1. Characteristics of CeO₂, Fe₃O₄, LDHs and CeO₂-Fe₃O₄@LDH nanocomposites. (a-c) The size distribution by intensity, number and Zeta potential of CeO₂, Fe₃O₄, LDHs and CeO₂-Fe₃O₄@LDH nanocomposites by DLS. (d) FTIR spectra (4000-400 cm⁻¹ region) of CeO₂, Fe₃O₄, LDHs and CeO₂-Fe₃O₄@LDH nanocomposites. (e) The TEM images of CeO₂, Fe₃O₄, LDHs and CeO₂-Fe₃O₄@LDH nanocomposites.

The stability of CeO₂-Fe₃O₄@LDH nanocomposites were further examined for 60 days. As shown in **Figure 2**, the size, size distribution and zeta potential of CeO₂-Fe₃O₄@LDHs remained stable after two months, indicating the favorable stability of the desired NPs.

Figure 2. Size distribution (a) and zeta potential (b) of CeO₂-Fe₃O₄@LDH nanocomposites synthesized at day 0 and day 60.

ROS scavenging capability of CeO₂-Fe₃O₄@LDH nanocomposites

Our previous studies showed that CeO₂ loaded hybrid NPs have anti-ROS properties in the ABTS-HRP system.²⁵ Briefly, CeO₂ 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 swift between Ce³⁺ and Ce⁴⁺. Here, we investigated the ROS scavenging capability of CeO₂-Fe₃O₄@LDH nanocomposites. As shown in **Figure 3**, CeO₂-Fe₃O₄@LDH nanocomposites could significantly reduce the ROS level with a quenching ability up to 40% when the concentration of Ce reached to 40 µg/mL.

Figure 3. ROS scavenging capability of CeO₂-Fe₃O₄@LDH nanocomposites. Different concentrations of CeO₂-Fe₃O₄@LDHs were tested in the ABTS-HRP system. The absorbance of the different samples was detected by Tecan X200. Control group is the one without nanocomposites determined as 0% of scavenging. Results were analyzed by one-way ANOVA. Data are presented as mean $\pm$ SD. *** $p < 0.001$ versus control, **** $p < 0.0001$ versus control, $n = 6$.

In vitro ROS scavenging by CeO₂-Fe₃O₄@LDH nanocomposites

Macrophages are used *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 CeO₂-Fe₃O₄@LDH nanocomposites in macrophages J774.1, it is imperative to confirm the efficacy of CeO₂ NPs on quenching the ROS as reported in the previous study.⁶⁶ Our study has proved that ROS was significantly scavenged at the CeO₂ concentrations of 0.5 and 1 µg/mL after stimulating cells with 0.5 mM H₂O₂ for 15-20 min (**Figure S5**). Notably, very high concentration of CeO₂ (20 µg/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 suggests that an appropriate dosing is essential to effectively quench the ROS, thus CeO₂-Fe₃O₄@LDHs (1:4:4) containing CeO₂ NPs at 0.5 and 1 µg/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 CeO₂-pretreated as well as in CeO₂-Fe₃O₄@LDH-pretreated macrophages with 0.5 mM H₂O₂ stimulating (see **Figure 4a and b**), suggesting a promising application for the desired CeO₂-Fe₃O₄@LDHs. It was noted that the ROS quenching effect did not increase when the nanocomposite concentration increased from 0.5 to 1 µg/mL (concentrations based on CeO₂).

Table 2. Composition of the combined nanocomposite and the concentration of each component.

Sample	Concentration ($\mu\text{g/mL}$)	Concentration ($\mu\text{g/mL}$)
CeO_2	0.5	1.0
Fe_3O_4	1.0*	2.0*
LDH	32 [†]	64 [†]
$\text{CeO}_2\text{:Fe}_3\text{O}_4\text{:LDH 1:4:4}$	33.5 (contain 0.5 $\mu\text{g/mL}$ of CeO_2)	67 (contain 1.0 $\mu\text{g/mL}$ of CeO_2)

* Concentration of Fe_3O_4 calculated based on the ratio between CeO_2 and Fe_3O_4 loading on LDH (1:2) determined by ICP-MS.

[†] Concentration of LDH calculated based on the ratio between CeO_2 and LDH (1:64) determined by ICP-MS.

The ROS scavenging capability of $\text{CeO}_2\text{-Fe}_3\text{O}_4\text{@LDH}$ nanocomposites was further confirmed by fluorescence images. ROS levels were directly measured by DCF-DA. As shown in **Figure 4c**, macrophages exhibited green fluorescence signal indicating they were stimulated by 0.5 mM H_2O_2 to produce excessive ROS. The fluorescence signal was much lower in the macrophages treated with $\text{CeO}_2\text{-Fe}_3\text{O}_4\text{@LDH}$ nanocomposites compared to the ones without NP treatment, implying that the $\text{CeO}_2\text{-Fe}_3\text{O}_4\text{@LDH}$ nanocomposites had ability to quench ROS.

Figure 4. Comparison of ROS scavenging capability among different nanoparticles. (a-b) Macrophages were pretreated with CeO_2 , and $\text{CeO}_2\text{-Fe}_3\text{O}_4\text{@LDH}$ (1:4:4) or without NP treatment, followed by stimulating by H_2O_2 at the concentration of 0.05 mM and 0.5 mM for 15 min. (c) Brightfield and fluorescence images of macrophages were taken after NP treatment for 24h. Macrophages were pretreated with $\text{CeO}_2\text{-Fe}_3\text{O}_4\text{@LDH}$ s (with CeO_2 concentration of 0.5 $\mu\text{g/mL}$) and without the NPs, followed by H_2O_2

stimulating at the concentration of 0.5 mM for 15 min. Results were analysed by two-way ANOVA. Data are presented as mean $\pm$ SD. * $p < 0.05$ versus NP free control, ** $p < 0.01$ versus NP free control, *** $p < 0.001$ versus NP free control, **** $p < 0.0001$ versus NP free control, $n = 3$.

Although CeO₂ 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 CeO₂ NPs with sizes less than 6nm can be eliminated from the blood circulation quicker than the larger NPs.^{18,68} In addition, the combination of both imaging agent (Fe₃O₄ NPs) and therapeutic agent (CeO₂ NPs) in a single nanocomposite allows the tracking of the therapeutics.

Cytotoxicity of nanoparticles to macrophages and CHO cells

Cytotoxicity of the synthesized nanoparticles was preliminarily investigated by studying the effect of the nanoparticles 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 $\mu\text{g/mL}$ in CeO₂-Fe₃O₄@LDH nanocomposites (concentrations ranging from 0 to 33.5 $\mu\text{g/mL}$), but a higher CeO₂ concentration (1 $\mu\text{g/mL}$) led to a slight decrease of macrophage viability (approximately 10%). Compared with CHO cells at CeO₂ concentration of 1 $\mu\text{g/mL}$, 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 fusiform⁶⁹ as shown in the groups with CeO₂ concentration ranging from 0.1 to 0.5 $\mu\text{g/mL}$. However, the shape of macrophages started to change at the CeO₂ concentration of 1 $\mu\text{g/mL}$. These results indicate that CeO₂-Fe₃O₄@LDH nanocomposites have no

significant cytotoxicity on the cells at the CeO₂ concentration below 1 µg/mL. When the concentration of CeO₂ reached up to 1 µg/mL, the CeO₂-Fe₃O₄@LDH nanocomposites showed some cytotoxicity to macrophages but not to CHO cells. It implies that these nanoparticles 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.

Figure 5. Cytotoxicity of different nanoparticles to macrophages and CHO cells. (a) Cell viability of macrophages treated with the CeO₂-Fe₃O₄@LDH nanocomposites with

CeO₂ concentration ranging from 0 to 1 µg/mL (concentration of CeO₂-Fe₃O₄@LDH nanocomposites ranging from 0 to 67 µg/mL). (b) Comparison of cytotoxicity of different nanoparticles with the CeO₂ concentration of 1 µg/mL examined in macrophages and CHO cells. (c) Bright-field microscopy images for CeO₂-Fe₃O₄@LDH nanocomposites-treated macrophages with CeO₂ 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.

MRI of CeO₂-Fe₃O₄@LDH nanocomposites in macrophages

MR signals of CeO₂-Fe₃O₄@LDH nanocomposites at different concentrations were firstly investigated. The conversion for concentrations between Fe and Fe₃O₄ NPs was shown in **Table 3**. MRI images of the CeO₂-Fe₃O₄@LDH nanocomposites were shown in **Figure 6**. CeO₂-Fe₃O₄@LDH nanocomposites in 1% agarose gel or water exhibited the increasing T₂ contrast signals as the Fe concentration increased (**Figure 6a and c**). The T₂-weighted relaxivity determined in the 1% agarose gel (144.92 mM⁻¹s⁻¹) was lower than that in the water (243.46 mM⁻¹s⁻¹) in **Figure 6b and d**, indicating that the surrounding environment affects the nanomaterial relaxivity.

Table 3. Conversion for concentrations between Fe and Fe₃O₄ NPs based on the effective concentrations of CeO₂ NPs determined by ROS quenching activity.

Concentration of CeO ₂ (µg/mL)	Concentration of Fe ₃ O ₄ (µg/mL)	[Fe], mM
0.2	0.4	0.005
0.5	1	0.013
1	2	0.027
5	10	0.131

* Concentration of Fe_3O_4 calculated based on the ratio between CeO_2 and Fe_3O_4 loading on LDH (1:2) determined by ICP-MS.

After determining the $\text{CeO}_2\text{-Fe}_3\text{O}_4\text{@LDH}$ nanocomposites as the contrast agent for MRI, further *in vitro* investigation on the underlying $\text{CeO}_2\text{-Fe}_3\text{O}_4\text{@LDH}$ 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 and 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 T2-weighted MR images, implying the application of these nanocomposites for labelling and tracking macrophages *in vivo*.

Figure 6. MRI of $\text{CeO}_2\text{-Fe}_3\text{O}_4\text{@LDHs}$. (a) T₂-weighted MR images of NP solution prepared in 1% agarose gel. (b) Plot of $1/T_2$ over Fe concentration of $\text{CeO}_2\text{-}$

Fe₃O₄@LDHs prepared in 1% agarose gel. (c) T₂-weighted MR images of NP solution prepared in water. (d) Plot of 1/T₂ over Fe concentration of CeO₂-Fe₃O₄@LDHs prepared in water. (e) T₂-weighted MR images of macrophages incubated with CeO₂-Fe₃O₄@ nanocomposites at different Fe concentrations. (f) Table of 1/T₂ over Fe concentration of CeO₂-Fe₃O₄@LDH nanocomposites in macrophages.

Conclusions

The multi-functional CeO₂-Fe₃O₄@LDHs have been successfully developed for ROS scavenging and imaging in macrophages. The optimal mass ratio between CeO₂, Fe₃O₄ and LDHs should be 1:4:4 due to the highest loading mass of both CeO₂ and Fe₃O₄ NPs on the surface of LDHs compared to other NPs synthesised at different mass ratios. *In vitro* studies, CeO₂-Fe₃O₄@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, CeO₂-Fe₃O₄@LDH nanocomposites exhibit good magnetic resonance signal in the macrophages, making it a potential contrast agent for cell imaging.

Current treatment for ROS-related diseases such as atherosclerosis and rheumatoid arthritis perform systemically leading to low therapeutic efficacy. With the development of the nanoparticle-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 Fe₃O₄ NPs (imaging agents) and CeO₂ NPs (therapeutic agents) on LDH nanocarriers enables the tracking of CeO₂ NP distribution. This approach also potentially increases the

circulation retention time of the small CeO₂ NPs, eventually enhancing the chance 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 nanoparticles more specifically to the lesions where diseases form and develop. We are anticipating a broader application for the LDH nanocomposites.

Supporting Information

Supporting information includes Table S1: Comparison of particle size and zeta potential of CeO₂-Fe₃O₄@LDH nanocomposites between different mass ratios; Figure S1: The X-ray diffraction (XRD) patterns of Fe₃O₄ and CeO₂, LDH and CeO₂-Fe₃O₄@LDH nanocomposites at a mass ratio of 1:4:4; Figure S2: Characteristics of different mass ratios of CeO₂@LDHs and Fe₃O₄@LDHs; Figure S3: EDS spectrum of CeO₂-Fe₃O₄@LDH nanocomposites at a mass ratio of 1:4:4; Figure S4: Characteristics of different mass ratios of CeO₂-Fe₃O₄@LDH nanoparticles; Figure S5: ROS scavenging capability of CeO₂ nanoparticles.

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