

Integration ferrihydrite prepared by an innovative method with oxidized activated carbon to construct a novel cascade nanozyme platform for inflammation therapy

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Materials and methods

Chemicals and materials

Ferric nitrate nonahydrate ($\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$, cell culture grade, 98 ~ 101%), activated carbon (pharmaceutical grade) were purchased from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Copper oxide (CuO , 99.9% metals basis, 1 ~ 2 μm) was purchased from Macklin Biochemical Technology Co., Ltd. (Shanghai, China). Hydrogen peroxide (H_2O_2 , 30%), Xylene were purchased from Fuyu Fine Chemical Co., Ltd. (Tianjin, China). Ferrous chloride tetrahydrate ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, analytical grade) was purchased from Beichen Fangzheng Reagent Factory (Tianjin, China). Salicylic acid (SA, analytical grade) was purchased from Hengxing Chemical Reagent Manufacturing Co., Ltd. (Tianjin, China). Concentrated nitric acid (GR grade, 65.0% ~ 68.0%) was purchased from Beijing Chemical Works (Beijing, China). Concentrated sulfuric acid (analytical reagent, 95.0% ~ 98.0%), diethyl ether (analytical reagent) were purchased from Lianlong Bohua Pharmaceutical & Chemical Co., Ltd. (Tianjin, China). Sodium bicarbonate (NaHCO_3 , analytical reagent grade, AR) was purchased from Damao Chemical Reagent Factory (Tianjin, China). Total SOD Activity Assay Kit (WST-8 Method) was purchased from Beyotime Biotechnology Co., Ltd. (Shanghai, China). Normal saline was purchased from Harbin Sanlian Pharmaceutical Co., Ltd. (Heilongjiang, China). 75% medical alcohol disinfectant was purchased from Jiacheng Fine

Chemicals Co., Ltd. (Liaoning, China). 4% PFA fixative was purchased from Lanjeke Technology Co., Ltd. (Beijing, China).

Instrumentation

The transmission electron microscopy (TEM) imaging was performed using a JEM 1200EX instrument from JEOL, Japan. The X-ray diffraction (XRD) patterns were measured using a D8 ADVANCE instrument from Bruker, Germany. The Fourier transform infrared spectroscopy (FT-IR) was obtained using an IFS55 infrared spectrometer manufactured by the German company BRUKER, with a detection wavenumber range of 4000 ~ 500 cm^{-1} and a resolution of 2.0 cm^{-1} . The measurement of Zeta potential was conducted using a Zetasizer Nano ZS90 laser particle size analyzer manufactured by Malvern Instruments Ltd., UK. The multifunctional microplate reader was from Thermo Fisher Scientific (China) Co., Ltd. The UV-Vis spectrophotometer used was an INESA model instrument produced by Shanghai INESA Analytical Instrument Co., Ltd. The dissolved oxygen meter was manufactured by Jiyi Technology Co., Ltd. (Shenzhen, China).

Preparation of nanozymes

Preparation of Fh

This study employed an innovative CuO-promoted iron salt hydrolysis method to prepare Fh nanozymes.¹ An aqueous solution of $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ (0.05 mol/L, 20 mL) was first heated to boiling at room temperature. After boiling, 0.16 g of CuO

powder with a particle size of about 1 ~ 2 μm was quickly added to the boiling $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ aqueous solution, which was continuously boiled and maintained for 60 min, with constant stirring during heating. After heating was stopped, the suspension was allowed to cool to room temperature and purified by dialysis for about 3 days using a dialysis bag with a molecular weight cut-off of 8000 ~ 14000 Da. The purified suspension was centrifuged at 3500 rpm for 15 min, and the supernatant was taken and preserved to obtain Fh nanozymes, which were stored at room temperature.

By examining the activity of Fh under various formulations, the optimal preparation strategy could be determined. Following the above reaction steps, the concentration of $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ was kept at 0.05 mol/L, and the molar ratios of $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ to CuO were varied as 2:1, 1:1, and 1:2 to explore the effect of the amount of CuO on the activity of the product. The molar ratio of $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ to CuO was fixed at 1:2, and the concentration of $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ was varied to 0.05, 0.15, and 0.25 mol/L to investigate the effect of the concentration of the reactants on the activity of the products.

Preparation of C-dot

C-dot was prepared by oxidative etching of activated carbon using mixed strong acids.² Briefly, 0.5 g of powdered activated carbon was added to a mixture of 50 mL of HNO_3 and H_2SO_4 ($V_{\text{HNO}_3}:V_{\text{H}_2\text{SO}_4} = 1:1$) and refluxed for 1.5 h at 110 °C in an oil bath. At the end of the reaction the corresponding solution was neutralized with

NaHCO₃ and then purified containing C-dot by filtration through a 0.22 μm membrane and dialyzed for about one week. The purified C-dot solution was then centrifuged at 14000 rpm for 20 min, and the supernatant was separated to obtain the C-dot solution, which was stored at room temperature.

Preparation of Fh@C-dot

At room temperature, the Fh solution was placed in a 5 mL EP tube, quickly added the C-dot solution to the EP tube under vortex conditions, and continue vortexing for 30 s to obtain Fh@C-dot, which was stored at room temperature for later use.

Preparation was carried out with the same experimental steps but the mass ratio of Fh to C-dot was varied to prepare Fh@C-dot with mass ratios of 5:1, 3:1, 1:1, 1:3 and 1:5, which were stored at room temperature for spare use to investigate the effect of the ratio of Fh to C-dot on the activity of the cascade of nanozymes.

Characterization of nanozymes

TEM

TEM was used to analyze the microscopic morphology of Fh, C-dot and Fh@C-dot. A small amount of sample was ultrasonically dispersed in water, and 10 μL was dropped onto a copper mesh. After settling for 10 min, excess solution was absorbed with filter paper, and observation was performed using a JEM 1200EX transmission electron microscope.

FT-IR

FT-IR was used to analyze the molecular structure and chemical bonding of the nanozymes prepared in this paper and its analysis helped to corroborate the structure of the synthesized nanomaterials. An appropriate amount of the nanozyme sample was taken and mixed with approximately 200 mg of dried KBr, then ground uniformly in an agate mortar and pressed into a thin film using a mold. The resolution of FT-IR was set to 2.0 cm^{-1} , and the structure of the synthesized nanozymes was characterized within the range of $4000 \sim 500\text{ cm}^{-1}$, and then the structural features of the samples in transmission mode were studied.

XRD

The crystal structures of the prepared nanozymes were investigated and analyzed using XRD. Among them, the test material for Fh was cobalt, while the test materials for C-dot and Fh@C-dot were copper. The conditions were as follows: the scanning angle range for Fh and Fh@C-dot was $10 \sim 85^\circ$, and for C-dot, it was $15 \sim 50^\circ$, with a scanning speed of $5^\circ/\text{min}$ for all.

Zeta Potential

Zeta potential measurements were conducted on Fh, C-dot, and Fh@C-dot. Each nanozyme was tested 15 times at a concentration of 0.1 mg/mL .

Characterization of free radical scavenging activities

Scavenging $\cdot\text{OH}$ experiment

First, weighed out 17.5 mg of $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ solid and dissolved it in 35 mL of purified water to prepare a solution of $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ at a concentration of 0.5 mg/mL. Secondly, weighed 10 mg of SA solid and dissolved it in 10 mL of purified water to prepare an SA solution with a concentration of 1 mg/mL. At room temperature, 1 mL of 0.5 mg/mL $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ solution and 50 μL of 100 mmol/L H_2O_2 were sequentially added to 5 mL EP tubes, and then the EP tubes were incubated in a constant temperature oscillator at 37°C for 15 min to allow complete generation of $\cdot\text{OH}$. Then, different concentrations of nanozymes were added to the reaction solution to achieve final concentrations of 20, 40, 60, 80, and 100 $\mu\text{g}/\text{mL}$, with a total reaction volume of 3 mL. The mixture was incubated at 37°C for 30 min to scavenge $\cdot\text{OH}$. Finally, 500 μL of SA solution was added, and the mixture was shaken at 37°C for 30 min to capture the remaining $\cdot\text{OH}$ and develop color. The absorbance changes at 510 nm were measured, and the scavenging rate of Fh against $\cdot\text{OH}$ was calculated according to Equation (1) ($n = 3$).

$$\text{Scavenging rates of } \cdot\text{OH} (\%) = 1 - \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} \times 100\% \quad (1)$$

Scavenging $\text{O}_2^{\cdot-}$ experiment

The activity of nanozymes in scavenging $\text{O}_2^{\cdot-}$ was measured using the Total SOD Activity Assay Kit (WST-8 method). The assay was performed according to the manufacturer's instructions, with final concentrations of nanozymes at 5, 10, 20, 40, and 80 $\mu\text{g}/\text{mL}$, respectively. Absorbance was measured at 450 nm using a 96-well plate.

Characterization of CAT-like activity

Bubble generation experiment

The oxygen bubble generation experiment was conducted in a 2 mL EP tube. Fh was mixed with a 1000 mmol/L H₂O₂ solution to achieve final concentrations of 50 µg/mL for Fh and 500 mmol/L for H₂O₂ in the reaction system. The bubble formation was observed and photographed at 5 min, 10 min, and 30 min intervals at 37°C.

UV spectrophotometry

The absorbance changes of the reaction system at 240 nm were measured using a UV-visible spectrophotometer to evaluate the ability of nanozymes to decompose H₂O₂. Fh was mixed with 20 mmol/L H₂O₂ solution in a cuvette at 37°C, resulting in a final concentration of 15 µg/mL for Fh and 10 mmol/L for the H₂O₂ solution, with a total reaction volume of 3 mL. The absorbance changes of the reaction system at 240 nm over 10 min were measured to determine the ability of Fh to decompose H₂O₂.

Experiments to determine dissolved oxygen

The decomposition of H₂O₂ and the catalytic activity of the nanozyme were evaluated by monitoring the change in dissolved oxygen concentration using a dissolved oxygen meter. At 37°C, the nanozyme was uniformly mixed with a 200 mmol/L H₂O₂ solution to achieve a final H₂O₂ concentration of 100 mmol/L, with a

total mixture volume of 10 mL. The production of dissolved oxygen was recorded within 2 min.

Hemolysis test

The hemolysis test procedure was conducted according to the method described by Lv et al.³ First, fresh blood from healthy KM rats was extracted into heparinized centrifuge tubes and centrifuged at 10,000 rpm for 5 min to remove the supernatant and isolate the red blood cell pellet. Saline was added to the precipitate, shaken gently and then centrifuged at 3000 rpm for 5 min each time, and the above experimental steps were repeated until the supernatant was colorless and clear. Subsequently, the prepared Fh@C-dot was mixed with the red blood cell suspension, ensuring that the final concentration of red blood cell in the suspension was 2% (V/V), and the final concentration of Fh@C-dot nanozymes was 100, 200, 300, and 400 $\mu\text{g/mL}$. Deionized water and saline were used in the positive control and negative control groups, respectively. After thorough mixing, the groups of solutions were co-incubated at 37°C for 1 h. The mixture was then centrifuged to separate the supernatant. Collected the supernatant and measured its absorbance at 540 nm using a microplate reader ($n = 3$), and calculated the hemolysis rate using Equation (2).

$$\text{Hemolysis rate (\%)} = \frac{\text{OD}_s - \text{OD}_n}{\text{OD}_p - \text{OD}_n} \times 100\% \quad (2)$$

Where OD_s was the absorbance of the test sample, OD_n and OD_p were the

absorbance of the negative control and positive control, respectively.

Establishment and evaluation system of mouse irritant contact dermatitis model

Twelve KM mice were randomly divided into four groups: blank group (Saline), control group (Xylene + Saline), low-dose group (Xylene + Fh@C-dot 1 mg/kg), and high-dose group (Xylene + Fh@C-dot 2 mg/kg). The drug was administered by subcutaneous injection on the dorsal neck of mice. At 2.5 hours post-administration, 30 μ L of xylene was evenly applied to both surfaces of the right ear of mice in all groups except the blank group to establish a model of irritant contact dermatitis.⁴ The left ear of the mice remained untreated as a control. After 30 min, the mice were anesthetized with ether, and photographs were taken to document the degree of ear swelling in each group. Subsequently, the mice were euthanized by cervical dislocation, and both ears were cut off along the auricle. A circular ear pieces were punched in the same position in the left and right ears using a perforator with a diameter of 8 mm, weighed and recorded. The difference in weight between the two pieces of ear was regarded as the degree of swelling of the mouse ear, and the ear swelling rate was calculated according to Equation (3).

$$\text{Swelling rate (\%)} = \frac{M_{\text{right}} - M_{\text{left}}}{M_{\text{left}}} \times 100\% \quad (3)$$

Notes and references

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