Highly Biocompatible and Recyclable Biomimetic Nanoparticles for Antibiotics-Resistant Bacteria Infection

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Experimental Section

Materials

Tryptone and yeast extract were purchased from OXOID. Carboxymethylcellulose was purchased from Merck. Vancomycin was purchased from Biosharp. urethane and mucin（M2378）was purchased from Sigma-Aldrich (St Louis, MO, USA). Fe_3O_4－COOH was purchased from Nanoeast (Wuhan, China), Cy5-Streptavidin was obtained from Ruixi (Xian, China), Propidium iodide (PI) was obtained from Beyotime Biotechnology (Shanghai, China). SYTO 9 green fluorescent nucleic acid stain was ordered from Thermo Fisher Scientific (Waltham, MA). All aqueous solutions were prepared using deionized (DI) water, which was purified on an experimental water purification system (Direct-Q3, Millipore, USA). All other solvents used in the present study were purchased from Aladdin-Reagent (China) and Sinopharm Chemical Reagent (China).

Preparation and Characterization of Fe_3O_4 nanoparticle

Fe_3O_4 nanoparticles were synthesized by using a hydrothermal method. Firstly, 1.5 g $FeCl_3.6H_2O$ was dissolved in 40 mL ethylene glycol, and then added the 3.6 g NaAc into the ethylene glycol. The mixture was stirred for 1 h, and then sealed in a Teflon-lined stainless steel autoclave. The autoclave was heated to 200 °C and staying for 14 h and then pour out the solution from the reactor as the temperature drops to room temperature. The obtained solution was washed several times with ethanol and DI water. Finally, vacuum dry for 12 h to get the Fe_3O_4 nanoparticles. The preparation of Fe_3O_4 nanoparticles was monitored by measuring the hydrodynamic diameter and zeta potential, using dynamic light scatter (DLS; Nano-Zen 3600, Malvern Instruments, UK).

Preparation and Characterization of RBC

The blood collected from the BALB/c mice was centrifuged at 2000 rpm, 5 min at 4 °C to remove the plasma, and then the RBC were washed with cold 1×PBS for three times. Next, we used 0.25×PBS for hemolysis via a hypotonic medium treatment in an ice-bath for 30 min. Finally, the solution was centrifuged at 12000 rpm for 5 min, and the pellet with a light pink color was collected and washed twice with 1×PBS. Then, the obtained solution was sonicated in a capped glass vial for 5 min, and subsequently extruded sequentially through 400 nm and 200 nm polycarbonate porous membranes with a mini extruder (Avanti Polar Lipids, USA). And then the obtained RBC membrane was monitored by measuring the hydrodynamic diameter and zeta potential (DLS; Nano-Zen 3600, Malvern Instruments, UK).

Preparation and Characterization of RBC@Fe_3O_4

To coat RBC onto the surface of Fe_3O_4 nanoparticles, 1 mL PBS containing Fe_3O_4 at the Fe content of 25 μg mL^{-1} was mixed with RBC membrane-derived vesicles derived from 1 mL of mice blood. The mixture was subsequently extruded 11 times through a 200 nm polycarbonate porous membrane, and then excess RBC was removed using the external magnetic field. Finally, the obtained prepared RBC membranes coated Fe_3O_4 nanoparticles (RBC@Fe_3O_4) was left in 1×PBS at 4 °C for further use. The RBC@Fe_3O_4 was characterized with transmission electron microscope (TEM; JEM-

2010 ES500W, Japan). The TEM copper grids were contacted with RBC@Fe_3O_4 suspension droplets for 60 s, stained by uranium acetate for 30 s, dried under ambient conditions. The hydrodynamic diameter and zeta potential were measured by DLS (DLS; Nano-Zen 3600, Malvern Instruments, UK). RBC@Fe_3O_4 was suspended in 1 mL 1×PBS and measurements were performed at room temperature. The stability experiments of the RBC@Fe_3O_4 were carried out by measuring the samples of RBC@Fe_3O_4 in PBS or FBS for 6 days with DLS.

SDS-PAGE Protein Analysis

The RBC lysate, RBC, and RBC@Fe_3O_4 were prepared in SDS sample buffer (Invitrogen, USA). And then heating the samples to 95 °C and staying for 5 min. And then each well that contained 10% SDS polyacrylamide gel (Beyotime, China) loaded 20 μL of the sample. Finally, we used Coomassie blue to stain the resulting polyacrylamide gel, and washed overnight.

Measurement of Photothermal Properties of Nanoparticles

The photothermal effect of nanoparticles in PBS was observed. The nanoparticles of RBC, RBC@Fe_3O_4, and Fe_3O_4 nanoparticles were dispersed 1×PBS. We use an 808 nm laser to irradiated at a power density of 2.5 W cm^{-2} for 6 min, meanwhile, used an FORTRIC225 IR thermographic camera (Shanghai Thermal Image Electromechanical Technology Co. Ltd., China) to measure the temperature changes.

Stability Studies of RBC@Fe_3O_4 Nanoparticles

To verify the stability of the synthesized nanoparticles, the size of the synthesized nanoparticles was first measured by DLS, and then stored in 1×PBS and FBS for 7 days. The diameter of the nanoparticles was measured by DLS (DLS; Nano-Zen 3600, Malvern Instruments, UK) and recorded every day.

Cell lines processing

The Raw264.7 cells were purchased from the China Center for Type Culture Collection. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), which was supplemented with 10% bovine serum (FBS), and 1% penicillin/streptomycin, at 37 °C in a humidified 5% CO_2 atmosphere. All the experiments were performed in compliance with the relevant laws and institutional guidelines of China and were

approved by the ethics committee at Wuhan University

Macrophage Uptake Study

Fe_3O_4-COOH nanoparticles was stirred with 100 mL EDC (4 mg/mL)、 NHS (6 mg/mL) solution at room temperature for 30 min, the surface carboxyl group was activated and washed with PBS for three times. Then, added Cy5-Streptavidin to Fe_3O_4-COOH solution for 10 h at 4℃, and wash three times with PBS. Next, we used the above method to obtain the RBC@Fe_3O_4 nanoparticles. Moreover, the nanoparticles' immune-evading ability has been studied by examining RBC@Fe_3O_4 nanoparticles ability to resist phagocytosis. First, RAW264.7 cells were cultured at 37℃， 5% CO_2. Then, Fe_3O_4 and RBC@Fe_3O_4 nanoparticles cy5-labeled were added to the RAW264.7 cell culture dish. The nanoparticles were co-cultured with the cells for 30 min, and then we used X81 to observe the fluorescence.

Quantification of Bacteria Hemolytic Activity

Firstly, we taken fresh blood from mice and centrifuged at 2000 rpm for 5 min to remove plasma, and then RBC were washed 3 times with 0.9% normal saline. The washed RBC was re-suspended with 0.9% normal saline into 2% (v/v) RBC suspension. The 10 μL MARS bacteria was removed from -80 ℃ and added to 10 mL of no-antibody medium and shaken for 24 h at 37 ℃, OD 630=1.5, and then centrifuging to obtain supernatant. The collected bacterial supernatant including bacterial toxin was then freeze-dried and concentrated at -80 ℃ for subsequent use. Then, the supernatant solutions were diluted with 0.9% normal saline at different ratio. Next, 100 μL RBC suspension and 100 μL supernatant solutions with different concentrations were added to the 96-well plates, with 3 parallel samples of each concentration, and 1% Triton X-100 and 0.9% normal saline were used as positive control and negative control, respectively. After mixing, the mixture was incubated at a low speed in a shaking bed at 37 ℃ for 1 h. Finally, the absorbance of supernatant at 490 nm was measured with a microplate reader (BioTek Instruments, USA), and the corresponding percent hemolysis was calculated by using the following formula.

Hemolysis = $(H_{sample}-H_{negative})/(H_{positive}-H_{negative}) \times 100\%$

Neutralization of Bacterial Toxin with RBC@Fe_3O_4 *in Vitro*

The 10 μL MARS bacteria was removed from -80 ℃ and added to 10 mL of no-antibody medium and shaken for 24 h at 37 ℃, OD 630=1.5, and then centrifuging to obtain supernatant. Freeze and dry bacterial supernatant at different concentration and mix it with 100 μL RBC@Fe3O4 (concentration of 100 μg Fe mL−1), at 37℃for 30 min. The mixture was then added to 2% of the red blood cell suspension and incubated together for 1h. The supernatant was obtained by centrifugation and measured the absorbance with a microplate reader (BioTek Instruments, USA), and the corresponding percent hemolysis was calculated by using the following formula.

Hemolysis = $(H_{sample}-H_{negative})/(H_{positive}-H_{negative}) \times 100\%$.

Neutralization Capacity of Bacterial Toxins *in Vivo*

MRSA bacteria preserved in the laboratory was inoculated into the LB liquid culture medium with the bacteria under the ratio of 1:1000, and cultured overnight at the culture temperature of 37 ℃, 250 rpm. The bacteria cultured overnight was inoculated again in the LB liquid culture medium with the ratio of 1:100. The culture temperature was 37 ℃ and the shaking rate was 250 rpm. The bacteria in the logarithmic phase was washed and suspended with 0.9% normal saline of the sterilized bacteria, so that the concentration of bacteria was 10^8 CFU. 500 μL bacterial suspensions was taken and incubated with different materials (RBC, Fe_3O_4, and RBC@Fe_3O_4), In addition, the two groups of Fe_3O_4+bacteria and RBC@Fe_3O_4+bacteria were irradiated with 808 nm laser, and then 5% (w/v) mucin was added. Meanwhile, mice injected with only the bacteria served as a control group. And then the mice were observed 2-4 times a day for 5 days, and the survival rate was calculated. And each group contains six mice.

Photothermal Sterilization Experiment *in Vitro*

The bacteria preserved in the laboratory were inoculated into the LB liquid culture medium with a ratio of 1:1000, and cultured overnight at 37℃ and 250 rpm shaking rate. The bacteria cultured overnight were inoculated in the LB liquid culture medium

with the ratio of 1:100, and cultured at 37 ℃ at the shaking rate of 250 rpm until the logarithmic growth stage, OD_{630} = 0.25. 100 μL bacterial suspension containing RBC, Fe_3O_4, and RBC@Fe_3O_4 was added to the 96-well plate, and then irradiated with 808 nm laser for 5 min at 2.5 W cm^{-2}. Then added 100 μL culture medium to retrain for different times at 37 ℃, 250 rpm. The bacteria were collected by centrifugation at low speed, and the RBC membrane was removed by centrifugation at high speed. Then the bacteria were re-suspended with 150 μL PBS, and the light absorption value of the bacterial suspension at 630 nm was measured with the microplate reader within 36 h. In addition to, the bacteria with radiated are diluted 100 times, the diluted bacteria were inoculated on LB medium and cultured for 12 h at 37 ℃. Moreover, we used fluorescence live/dead staining to visualize the bacterial activity and distinguish the live bacteria (stained in green) and dead bacteria (stained in red), and the fluorescence images were observed under a fluorescence microscope (IX81, Olympus, Japan).

Morphology Observation of Bacteria

In order to further study the photothermal sterilization effect, we observed the morphological of bacteria after different treatment by SEM. First, we fixed the bacteria with 2.5% glutaraldehyde, and then dehydrated them with ethanol of different concentrations. Finally, the dried bacteria were sprayed with gold, and the morphological changes of the bacteria were observed under SEM (SEM, S-4800II, and Japan).

Photothermal Sterilization *in Vivo*

The laboratory preserved bacteria were inoculated into the sterilized LB liquid culture medium at a ratio of 1:1000, and cultured overnight at the shaking rate of 37 ℃ and 250 rpm. The bacteria cultured overnight were inoculated again in the LB liquid culture medium with the ratio of 1:100, and cultured at 37 ℃ at the shaking rate of 250 rpm. The bacteria were washed and suspended with sterile 0.9% normal saline. BALB/c mice at 6 weeks of age were anesthetized by intraperitoneal injection of uratan solution with a concentration of 20% (w/v) of 150 μL. Hair was removed from the back of the

anesthetized mice with a hair shaver and disinfected with iodine volts and 70% alcohol, respectively. Use a scissors to make a full-thickness skin cut on each mouse. Each wound was first added with the above treated bacterial suspension of 20 μL, the concentration of bacteria was 5×10^8 CFU mL^{-1}, and then the wound surface was coated with preparation materials. We used an 808 nm laser irradiated the wound site of mice with power of 2.5 W cm^{-1} for 5 min. Meanwhile, the temperature change of the wound site was recorded with the FORTRIC225 IR thermographic camera (Shanghai Thermal Image Electromechanical Technology Co. Ltd., China).

Antimicrobial Activity and Wound Healing Evaluation *in Vivo*

The laboratory preserved bacteria were inoculated into the sterilized LB liquid culture medium at a ratio of 1:1000, and cultured overnight at the shaking rate of 37 ℃ and 250 rpm. The bacteria cultured overnight were inoculated again in the LB liquid culture medium with the ratio of 1:100, and cultured at 37 ℃ at the shaking rate of 250 rpm. The bacteria were washed and suspended with sterile 0.9% normal saline. BALB/c mice at 6 weeks of age were anesthetized by intraperitoneal injection of uratan solution with a concentration of 20% (w/v) of 150 μL. Hair was removed from the back of the anesthetized mice with a hair shaver and disinfected with iodine volts and 70% alcohol, respectively. Use a scissors to make a full-thickness skin cut on each mouse. Each wound was first added with the above treated bacterial suspension of 20 μL, the concentration of bacteria was 5×10^8 CFU mL^{-1}, and then the wound surface was coated with preparation materials. All mice were divided into six groups according to different treatments (i.e. bacteria, Fe_3O_4 + bacteria, RBC+bacteria, RBC@Fe_3O_4+bacteria, Fe_3O_4+bacteria+NIR, and RBC@Fe_3O_4+bacteria+NIR), respectively. All mice wore sterile medical tape and were kept alone in cages. After 1, 3, 5, 7, 11, and 14 days of treatment, take photos of the wound, and skin tissues were stained with hematoxylin and eosin (H&E). In addition, we also recorded changes in the mice's weight over 14 days.

Recycling of nanoparticles

The composite materials were added into the MARS bacteria suspension (10^7-10^8 CFU

mL^{-1}), After mixing, they were incubated at low speed in a shaking bed at 37 ℃ for 3 h, and then we used an 808 nm laser to irradiated the bacteria suspension at 2.5 W cm^{-2} for 5 min, meanwhile, using an infrared camera to record the temperature change. Next, put the bacterial suspension on the magnetic force to isolate the magnetic materials, collect and centrifugal supernatant to centrifuge for collected the bacteria at the bottom. And then culture the collected bacteria in a shaking bed at 37℃, 2500 rpm for 72 h, and the bacterial activity was calculated. The separation of nanometer material washing three times to remove the surface adsorption of proteins, suspended in PBS again for next time use. The photothermal sterilization experiment was repeated for 3 times.

Toxicity Evaluation *in Vivo*

To assess the toxicity of the nanoparticles *in vivo*, we injected 200 μL 1xPBS, PBS containing RBC, RBC@Fe_3O_4, and Fe_3O_4 (Fe, 5 mg mL^{-1}). And then the mice were euthanized and their major organs (i.e., heart, liver, spleen, lung, and kidney) and blood were taken for following analysis to detect major liver and kidney indicators (i.e., alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (CRE), and blood urea nitrogen (BUN)). Meanwhile, we used hematoxylin and eosin (H&E) to stain the major organs.

Animal Procedures

All the animal procedures were performed according to the guidelines of the Institutional Animal Care and Use Committee at Wuhan University.