A Bone-Targeting Enoxacin Delivery System to Eradicate Staphylococcus Aureus-Related Implantation Infections and Bone Loss

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Abstract

Post-operative infections in orthopaedic implants are severe complications that require urgent solutions. Although conventional antibiotics limit bacterial biofilms formation, they ignore the bone loss caused by osteoclast formation during post-operative orthopaedic implant-related infections. Fortunately, enoxacin exerts dual antibacterial and osteoclast inhibitory effects, playing a pivot in limiting infection and preventing bone loss. However, enoxacin lacks specificity in bone tissue and low bioavailability-relate side effects, which hinders translational practice. Herein, we developed a nanosystem (Eno@MSN-D) based on enoxacin (Eno)-loaded mesoporous silica nanoparticles (MSN), decorated with the eight repeating sequences of aspartate (D-Asp8), and coated with polyethylene glycol (PEG). This Eno@MSN-D delivery nanosystem exhibited both antibacterial and anti-osteoclast properties in vitro. More importantly, Eno@MSN-D allowed the targeted release of enoxacin in infected bone tissues and prevented implant-related infection and bone loss in vivo. Therefore, our work highlights the significance of novel biomaterials that offer new alternatives to treat and prevent orthopaedic staphylococcus aureus-related implantation infections and bone loss.

1. Introduction

Owing to the beneficial application of orthopaedic implants and improvement in treatments, patients affected by several orthopaedic diseases, including fractures, congenital malformations, acquired deformities, and osteoarthritis, can achieve satisfactory therapeutic effects. However, like a “double-edged sword,” the widespread use of implants implied new medical complications, and post-operative implant-related infections are one issue that needs to be addressed. The incidence of infection in orthopaedic surgery is reportedly about 5%, of which post-operative infection rates of closed fractures make up 3.6–8.1%. By contrast, that of open fracture could be as high as 21.2% [1]. Antibiotics are still the primary treatment method for bone and joint infections; however, because of the limited penetration of antibiotics in bone tissues, sufficient blood concentrations cannot be obtained [2]. Additionally, the lack of blood supply around the implant, and the formation of bacterial biofilm [3, 4], usually results in ineffective anti-infection treatments. This not only increases the length of hospital stays and the total cost but also reduces the effectiveness of rehabilitation.

The pathogenesis of post-operative infections due to implant presence differs from that of general post-operative infections. The gap between the body tissue and the implant is a fibroinflammatory area where host immunity is suppressed, resistance is low, and bacteria can quickly colonise to form infections [5]. Besides, after bacteria invade the bone muscle system, due to intra-operative soft tissue injury and destruction of the blood supply, bacteria can colonise the proximities of the implants and form biofilms [6]. Bacterial biofilms limit antibiotic diffusion into the infected area, further reducing the concentration of antibiotics that can enter the infected area [7, 8]. Therefore, the efficient use of antibiotics is the key to limiting infections and inhibiting biofilm formation. Improving antibiotic treatment efficiency and increasing the concentration of drugs in bony tissues is particularly crucial for treating bone and joint infections. On the other hand, abnormal bone metabolism caused by bacterial erosion and bone
destruction can activate large numbers of osteoclasts, resulting in bone degradation and absorption [9, 10]. Simple anti-infection treatments of post-operative implant infections cannot eliminate the excessive osteoclast activation due to the inflammatory environment, which results in bone loss and bone destruction. The inhibition of osteoclast formation and bone resorption is an effective strategy to reduce bone loss and maintain long-term implant stability.

Enoxacin, a third-generation fluoroquinolone antibiotic, has a broad-spectrum and robust bactericidal effect. Additionally, enoxacin has an inhibitory effect on osteoclasts [11, 12], and our previous research reported that its mechanism is to occupy the ATP binding domain of the JNK protein, inhibit the phosphorylation of JNK, and activate the JNK/MAPKs signalling pathway [13]. In terms of bacterial erosion of post-operative implant infections and the pathological state of a large number of osteoclasts activated in inflammatory environments, enoxacin exerts both antibacterial and anti-osteoclastic activities, making it an ideal drug candidate for preventing and treating post-operative implant-related infections. However, poor bioavailability due to its poor bone-targeting specificity and systemic toxicity [14] limits enoxacin clinical applicability in treating orthopaedic implant-related post-operative infections.

In recent years, due to their high specific surface area and large pore volume, mesoporous silica nanoparticles (MSN) offered advantages that include excellent loading capability and biocompatibility, making them ideal candidates for drug delivery systems [15–17]. Nevertheless, despite the promising applicability of MSN for patients with post-operative infection related to orthopaedic endophytes, the lack of specific targeting to the infected bone tissue reduced the therapeutic effect of encapsulated antibiotics, simultaneously promoting drug resistance. Reportedly, eight repeating sequences of aspartate (D-Asp8) preferably bound to highly crystalline hydroxyapatite, and D-Asp8 could successfully bind to bone resorption surfaces to target osteoclasts [18]. Based on these findings, to improve target specificity, we deployed MSN as a carrier for the transport of enoxacin equipped with the bone-targeting D-Asp8 and coated with polyethylene glycol (PEG) to prevent premature release of enoxacin before reaching the target tissue. A bone-targeted delivery system containing enoxacin (Eno@MSN-D) was prepared for the targeted release of enoxacin in infected bone tissue. We hypothesised that Eno@MSN-D has antibacterial properties and can inhibit osteoclast activation, thereby preventing staphylococcus aureus-related implantation infections and consequent bone loss.

2. Materials And Methods

2.1. Materials

Hexadecyl trimethyl ammonium bromide (CTAB), tetraethyl orthosilicate (TEOS), silane-polyethylene glycol-carboxyl (Silane-PEG-COOH), MES buffer, aqueous ammonia, hydrochloric acid, ethanol, ethyl acetate, crystal violet solution, phosphate-buffered saline (PBS), 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (EDC) and N-hydroxy succinimide (NHS) were obtained from Sinopharm Chemical Reagent Co., China. Enoxacin was obtained from Sigma Aldrich, St Louis, USA. Dialysis bags were obtained from UC Union Carbide, Danbury, USA. S. aureus (ATCC25923), S. epidermidis
(ATCC12228), and methicillin-resistant *S. aureus* (ATCC43300) were obtained from American Type Culture Collection, Manassas, VA, USA. Trypsin soy broth (TSB) and trypsin soybean agar plate was obtained from Shanghai Chengsheng Biotechnology Co., Ltd.

### 2.2. MSN synthesis

MSN were synthesised as previously reported, with minor modification [19]. NaOH aqueous solution (2 M, 0.35 ml) was mixed with water (50 ml) containing CTAB (100 mg), and the solution was heated to 70°C while stirring. Afterward, TEOS (0.5 ml) was introduced dropwise to the reaction mixture. After 3 min, ethyl acetate (0.5 ml) was added, and the mixture was stirred for 30 s, followed by an ageing procedure at 70°C for 2 h. The precipitate was collected through centrifugation and washed with ethanol. The collected products were extracted for 6 h using a solution of hydrochloric acid (HCl) in ethanol (10% v/v) at 78°C by refluxing to remove CTAB and obtain the MSNs.

For enoxacin loading, 50 mg of MSN were dispersed in 10 mL of a methanol solution mixed with enoxacin (25 mg), and the mixture was shaken at 25°C for 4 h. Then, the dispersion solution was centrifuged at 10,000 rpm to collect the enoxacin-loaded MSN (Eno@MSN). Next, the Eno@MSN were washed with distilled water to remove enoxacin from the exterior surface.

### 2.3. Synthesis of Eno@MSN-D

Briefly, Eno@MSN was dispersed in 10 ml of water mixed with Silane-PEG-COOH (28 mg). Afterward, 0.2 ml of aqueous ammonia was added to the mixture and stirred for the next 4 h. The dispersion solution was centrifuged at 10,000 rpm, and the nanoparticles were washed with water three times to obtain Eno@MSN-PEG. Next, 30 mg of Eno@MSN-PEG was dispersed in 5 ml of MES buffer, then 5.0 mg EDC and 3.8 mg of NHS were added to the solution. After the excitation reaction occurred for 30 min, 5 mg of D-Asp8 moiety was added to the mixture for a 2 h reaction. Subsequently, the solution was then centrifuged at 10,000 rpm and washed with water three times to obtain Eno@MSN-D. Finally, to monitor the cellular uptake of Eno@MSN-D by confocal microscopy, the red fluorescent dye Cy7 was loaded into the Eno@MSN-D for tracking and labelling. Figure 1 shows a scheme of Eno@MSN-D production and its mechanism.

### 2.4. Characterisation of nanoparticles

Transmission electron microscope (TEM) images were obtained using a JEM2010 instrument (JEOL, Tokyo, Japan) with 200 kV acceleration voltage to investigate the ultrastructure of drug-free MSN, Eno@MSN, and Eno@MSN-D. The nitrogen adsorption-desorption was measured using a Micromeritics porosometry analyser (Micromeritics, Norcross, GA). For the surface area, pore volume was calculated from the Brunauer–Emmett–Teller (BET) and Barrett–Joyner–Halenda (BJH) analyses, respectively. Additionally, the hydrodynamic size of the samples was measured by dynamic light scattering (DLS) using a Zetasizer Nano instrument (Malvern Instruments, Malvern, UK) at 298 K. Finally, the thermal stability and composition of the solid samples were determined by TGA. TGA was performed on a TG
209F1 thermal analyser (NETZSCH, Germany) at a heating rate of 10°C/min in a continuously moving N₂ atmosphere.

### 2.5. Drug release determination

The *in vitro* pH-sensitive enoxacin release pattern of Eno@MSN-D was evaluated in PBS with pH = 7.4 and pH = 6.0. The Eno@MSN-D dispersion solution was loaded in a dialysis bag with a molecular weight cut-off of 8 kDa. Follow the dialysis bag was placed in 8 ml of PBS and gently shaken at 37°C. One milliliter of supernatant was collected at predetermined time points, and the amount of drug released was determined by an ultraviolet spectrophotometer at \( \lambda = 232 \) nm. Then, 1 ml of fresh PBS was added to the dialysate to maintain a constant volume.

### 2.6. Bacterial strains and cultures

*S. aureus* (ATCC25923), *S. epidermidis* (ATCC12228), and methicillin-resistant *S. aureus* (ATCC43300) were stored on a TSA plate at -4°C. Place the three bacterial colonies in a centrifuge tube containing TSB and cultured overnight at 37°C in an oscillator incubator at 150 rpm, respectively. After an overnight culture of TSB, which was placed in an EP tube and centrifuged at 5000 rpm for 5 min. After centrifugation, the supernatant was discarded. Then, TSB was used to adjust the bacterial precipitation concentration to 1×10⁶ CFU/mL for storage.

### 2.7. Minimum inhibitory concentration (MIC)

Each well of the 96-well plates was added 100 µL of ATCC25923, ATCC12228, ATCC4330 bacteria cells (1×10⁶ CFU/mL). Different concentration gradients of TSB (as the blank control), Eno, and Eno@MSN-D were separately added into 96-well plates. After incubating at 37°C for 24 h, the bottom of the plate was closely inspected for biofilm formation. The lowest concentration without biofilm formation was determined as MIC, and it was determined using a microtiter plate dilution assay.

### 2.8. Determination of bacterial biofilm formation

A crystal violet assay was performed using 96-well microtiter plates to observe the effects of Eno@MSN-D on bacterial biofilm formation. Briefly, ATCC25923 with a concentration of 1×10⁶ CFU/ml were added to 96-well plates at 100 µl/well in triplicate and cultured for 24 h in 37 °C. Then, each well of plates was added 100 µL of TSB (10 µg/ml), MSN (10µg/ml), Eno(10µg/ml), L-Eno@MSN (5µg/ml), and H-Eno@MSN-D (10µg/ml). Next, each well was washed with PBS three times and stained with 0.1% crystal violet solution. The plates were then incubated at room temperature for 15 min and then gently washed with PBS three times to remove excess crystal violet. Afterward, 33% of acetic acid was added to each well and placed in the incubator at 37°C for 30 min. The absorbance at 570 nm was determined using a microplate reader.

### 2.9. Morphological characterisation of bacteria and bacterial biofilm
The morphology of bacteria and the formation of biofilm were observed by SEM. ATCC25923 with a concentration of \(1 \times 10^6\) CFU/ml were added to 12-well plates at 1 mL/well in triplicate and cultured for 24 h in 37 °C. TSB (10 µg/ml), MSN (10 µg/ml), and Eno@MSN-D (5, 10 µg/ml) were added to 12-well plates at the same time, and cover slides were placed in the wells. Next, the cover slides were gently washed with PBS and fixed in 2.5% glutaraldehyde for 12 h. After washing with PBS, the sample was dehydrated in an ethanol series (50, 60, 70, 80, 90, and 100%). After the samples were freeze-dried and gold-sputtered, they were observed by SEM (SU8010, Hitachi, Japan).

### 2.10. Determination of antibacterial properties of Eno@MSN-D in vitro

The effects of Eno@MSN-D on bacterial biofilm were observed using a fluorescence inversion microscope system. ATCC25923 was diluted to \(1 \times 10^6\) CFU/ml by TSB, and 1 ml was placed into a confocal petri dish with a glass bottom and incubated at 37°C for 24 h. After incubation, the supernatant in the dish was removed, and the biofilm was treated with 5 or 10 µg/ml Eno@MSN-D. The control group included MSN (10 µg/ml) and TSB (10 µg/ml). Petri dishes were then washed with aseptic PBS to remove loosely bound bacteria. Bacteria in the biofilm were stained with a LIVE/DEAD® BacLight bacterial viability kit (L7007) at room temperature for 15 min in the dark. The dye was cleaned with aseptic PBS, and the biofilm was observed using a fluorescence inversion microscope. Bacteria with intact and damaged cell membranes were obtained by scanning under excitation using the green (488 nm) and red (543 nm) channels, respectively.

### 2.11. Cell viability and osteoclast differentiation assay in vitro

To evaluate the cytotoxic effect of Eno@MSN-D, we used the Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer’s instructions. Briefly, bone marrow macrophages (BMMs) were added to 96-well plates at \(8 \times 10^3\) cells/well in triplicate and cultured for 24 h in alpha modification of Eagle’s medium (α-MEM, Gaithersburg, MD, USA) containing 30 ng/mL macrophage colony-stimulating factor (M-CSF; PeproTech, Rocky Hill, NJ, USA), 10% foetal bovine serum (FBS; Gibco-BRL, Sydney, Australia), and 1% penicillin/streptomycin. BMMs were then separately treated with different concentrations of Eno@MSN-D (0, 0.625, 1.25, 2.5, 5, 10, 20, 40, 80 and 160µg/ml) for 48 or 96h. Next, 10 µL of CCK-8 substrate was added to each well, and the plate was incubated at 37 °C under 5% CO₂ for 2 h. The absorbance of each well was measured at 450 nm with an ELX800 microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA). On the other hand, BMMs were seeded in a 96-well plate at a density of \(8 \times 10^3\) cells/well in α-MEM with 30 ng/mL M-CSF, 50 ng/mL RANKL (PeproTech, Rocky Hill, NJ, USA), and different concentrations of Eno@MSN-D (0, 2.5, 5, 10µg/ml). BMMs were supplemented with fresh medium every 2 days until mature osteoclasts were observed. Next, the cells were fixed with 4% paraformaldehyde and stained for tartrate-resistant acid phosphatase (TRAP;
Sigma Aldrich, St. Louis, MO, USA) activity. The number of mature osteoclasts (TRAP-positive cells with ≥ 3 nuclei) was counted, and their spread area was measured.

2.12. Bone-targeting properties of Eno@MSN-D in vivo

All animal experiments were performed in the Department of Animal Experimental Sciences of Nanchang University, under the approval and guidance of the Animal Experimental Ethics Committee of the First Affiliated Hospital of Nanchang University. Targeting of common fluorescent MSN and fluorescent Eno@MSN-D was evaluated in Sprague Dawley (SD) rats (Shanghai SLAC Laboratory Animal Co., Ltd. (China). Twelve 3-month-old female SD rats were divided into two groups: MSN control groups and Eno@MSN-D experimental groups. Six animals from each group were injected in the tail vein with identical doses of nanoparticles. After injection, all animals had free access to food and water. The animals were euthanised after 4 and 72 h, and the main organs (heart, liver, spleen, lung, kidney, femur) were removed. A fluorescence imaging system was used to detect the fluorescence of each organ in each group.

2.13. Antibacterial properties of Eno@MSN-D in vivo

Fifty specific pathogen-free-grade 12-week-old female SD rats were used and randomly assigned to five independent groups. All animals were housed in clean plastic cages with a 12h light dark cycle and free access to fresh food and water. Five groups of rats were anaesthetised by intraperitoneal injection with 10% chloral hydrate (4 ml/kg). After complete anaesthesia, the supine position was used to remove hair from the left knee joint, subsequently sterilised with 75% ethanol. A 15 mm incision was made along the lateral end of the femur. Subcutaneous tissues and muscles of the lateral femoral condyle were incised, the joint capsule and lateral collateral ligaments were retained, and the femoral condyle was fully exposed. The bone marrow cavity of the femur was opened and expanded to a depth of 10 mm using an electric drill with a diameter of 1 mm. Subsequently, a 1-mm diameter titanium rod with a length of 10 mm was implanted. ATCC25923 concentration was set to $1 \times 10^6$ CFU/ml, and 100 µl was injected into the bone marrow cavity. The hole in the femoral condyle was blocked with bone wax. A saline solution was used for flushing the wound, and a medical suture was used to close the wound. Berberine was then applied to the wound. In the Sham group, only the condyle of the femur was exposed before closing the incision. After the operation, rats were resuscitated under a fan heater and put back in their cages. The rats were kept in separate cages and could eat and drink at will.

In the first week post-operation, body temperature and weight were examined every day. After one week of observation, each experimental group was injected with different drugs. Group A: Sham group (4 mg/kg of normal saline was injected); group B: NS group (no treatment); group C: MSN group (4 mg/kg of MSN was injected); group D: Eno group (4 mg/kg of enoxacin was injected); group E: Eno@MSN-D group (4 mg/kg of Eno@MSN-D was injected). Then, body weight and temperature were recorded every three days. The drugs were then intraperitoneally injected every day for a total of four weeks, and the animals were
euthanised four weeks later. The femurs were separated from the skin and subcutaneous tissue under aseptic conditions. Soft tissues were removed, and the femurs were prepared for further experiments. All titanium rods were collected and processed for analysis.

Bacteria attached to titanium rods were detected by SEM, fluorescence staining, plate colony counting method, and plasma coagulase test. After the titanium rods were removed from the distal femur and washed with PBS, five bars of each group were randomly placed in 2.5% glutaraldehyde for 12h. Subsequently, they were dehydrated with an ethanol series (50, 60, 70, 80, 90, 100%). The samples were freeze-dried and gold-sputtered. The surfaces of the titanium rods were then observed by SEM. Five titanium rods were randomly selected and fixed for 12h according to the steps above. After washing with PBS, they were stained with a LIVE/DEAD ®BacLight bacterial viability kit (L7007) at room temperature for 15 min in the dark. They were then observed using a fluorescence inversion microscope. Meanwhile, titanium rods were washed, put into 1 ml of TSB, and ultrasonicated for 15 min. After a 10-fold dilution, 100 µl of suspension was evenly applied to a TSA plate and incubated at 37°C for 24 h. Then, the CFU was calculated according to the colony count on the plates. Additionally, a plasma coagulase test was performed to determine whether the bacteria attached to the titanium rods were S. aureus. A single colony was picked from the TSA plate and suspended in 50 µl PBS before adding 50 µl rabbit plasma. The occurrence of agglutination indicated that the bacteria were S. aureus.

2.14. Micro-computed tomography

After euthanasia, the femur of each group was removed entirely under sterile conditions. After the titanium rod was removed, the femur was fixed with 4% paraformaldehyde for two days and washed with tap water for 24 h. The peripheral bone structure of the distal femoral implant was then evaluated 28 days after the injection of different drugs. The femurs were examined using a desktop micro-X-ray computed tomography (micro-CT Skyscan1076, Aartselaar, Belgium) machine equipped with a 40 kV X-ray source with a camera pixel size of 12.60µm. A reconstructed data set with an image pixel size of 18.26 µm was generated via scanning. To determine the axial trabecular volume of interest, we selected a region of interest (ROI) with a length of 5.578 mm closest to the growth plate edge. Micro-CT images of the transverse, sagittal, and coronal sections of the area around the implants were obtained. Object volume, total VOI volume, bone evolution fraction, bone mineral density, trabecular thickness, trabecular spacing, and trabecular number were used as indices to measure trabecular bone mass and its distribution.

2.15. Histology and histomorphometry

Bone histology was used to assess infection and bone structure changes around the distal femoral implants. The femurs of the rats were fixed with 4% paraformaldehyde at room temperature for 24 h. Subsequently, 10% of ethylenediamine tetra-acetic acid was fully decalcified and dehydrated with an ethanol series (50, 75, 80, 85, 90, 95, 100%). The bone tissue, after transparent treatment in xylene, was
embedded in paraffin. The sample was longitudinally cut into 5-µm-thick slices. After slicing, histological sections were prepared for TRAP and H&E staining. Slices were observed with an optical microscope. Representative images were randomly obtained from the distal femur implanted with titanium rods. We used Image-Pro Plus 6.0 software (Media Cybernetics, MD, USA) to process TRAP-stained pictures and counted the number and area of osteoclasts per field of view.

2.16. Statistical analysis

IBM SPSS statistics 22 (SPSS Inc., USA) software was used for statistical analysis. The results are presented as means ± SD. Experiments were conducted at least three times. One-way analysis of variance (ANOVA) with Bonferroni post hoc test was performed to determine group differences. p-values < 0.05 were considered statistically significant.

3. Results

3.1 Synthesis and characterisation of the Eno@MSN-D

The protocol for the preparation of Eno@MSN-D is shown in Fig. 1. Nanoparticle mesoporous carriers were prepared using TEOS as a hydrolytic inorganic precursor and the surfactant CTAB as the poreogenic substance. Bare MSN was obtained by solvent extraction of surfactant removal and was then loaded with Eno by free diffusion. Subsequently, PEG was further grafted onto silica surfaces and channels to act as a gatekeepers for drug delivery. Finally, Eno@MSN-PEG was constructed by fusing D-Asp8 to Eno@MSN-D.

From the TEM images (Fig. 2A), we observed the size and morphological characteristics of the three nanoparticle types. MSN maintained a highly ordered mesoporous structure, which disappeared after enoxacin loading and PEG/D-Asp8 immobilisation, and the average particle size of Eno@MSN-D was also larger than that of MSN and Eno@MSN, in consistent with the results obtained from DLS analysis (Fig. 2D). The average particle diameters of the MSN, Eno@MSN, and Eno@MSN-D were 113.9, 133.8, and 179.7 nm, respectively. The particle dispersion index (PDI) and the zeta potential of the nanoparticles were obtained through DLS analysis. The MSN, Eno@MSN, and Eno@MSN-D had a PDI of 0.213, 0.272, and 0.202nm and zeta potentials of -24.3, -22.4, and −19.3mV, respectively.

The Nitrogen adsorption-desorption isotherm curves further exhibited the classical Langmuir type IV isotherm with H2 type hysteresis loops, indicating the presence of a mesoporous structure in the MSN (Fig. 2B). Furthermore, the Relative Pressure (P/P0) has been shown to increase rapidly in the range 0.8 ~ 1.0, reflecting the magnitude of the mesoporous pore size, and its variation width can be used as a basis for measuring mesoporous pore uniformity. Pore size distribution was studied using the Barrett–Joyner–Halenda method and the isotherm desorption branch (Fig. 2C). The specific surface area and pore size of MSN were 875.21 m$^2$/g and 3.57 nm, respectively. In addition, Fig. 2E shows the TGA curves of all
nanoparticles, indicating that during analysis, the weight loss of MSN, Gen@MSN, and Eno@MSN-D was close to 7.71%, 15.62%, and 25.65%, respectively.

3.2 Drug loading and release in vitro

It is well known that controlled release performance is an indispensable key step for the expected nanoparticles. Under simulated physiology conditions (pH = 7.4) and acid microenvironment caused by implant infection (pH = 6.0); two different pH buffer solutions were simulated for pH response release pattern. In Eno@MSN-D, burst release was not found after stirring for 150 min at pH 7.4, and approximately 40% of enoxacin was released (Fig. 2F). Interestingly, when the pH was decreased from 7.4 to 6.0, the cumulative release of enoxacin increased to approximately 60% (Fig. 2F).

3.3 Antibacterial properties of Eno@MSN-D in vitro

As previously reported, MIC values of enoxacin against *S. aureus* (ATCC25923), *S. epidermidis* (ATCC12228), and methicillin-resistant *S. aureus* (ATCC43300) were 2, 2, and 4 µg/ml, respectively [20]. Our results show that the MIC values of Eno@MSN-D against ATCC25923, ATCC12228, and ATCC43300 were 4, 4, and 8µg/ml, respectively.

ATCC25923 was co-cultured with different concentrations of Eno@MSN-D for 24 h, and the absorbance values were measured by crystal violet staining to determine the amount of biofilm formation (Fig. 3A). We established a TSB blank control group, TSB and bacteria (TSB + B) co-culture-positive control group, 10 µg/ml MSN and bacteria (MSN + B) co-culture-positive control group, 10 µg/ml enoxacin and bacteria (Eno + B) co-culture-positive group, low concentration of 5 µg/ml Eno@MSN-D and bacteria (L-Eno@MSN-D + B) co-culture-positive group, and high concentration of 10 µg/ml Eno@MSN-D and bacteria (H-Eno@MSN-D + B) co-culture-positive group. Our results showed that biofilm formation was significantly lower in the TSB groups compared to the TSB + B groups. However, both Eno@MSN-D + B and Eno + B groups decreased biofilm formation after treatment. Compared to the group treated with L-Eno@MSN-D + B, superior antibacterial effects were observed in the H-Eno@MSN-D + B group. Additionally, H-Eno@MSN-D + B groups showed superior compared to the groups with Eno + B, and there was a significant difference between them (Fig. 3C). This result indicates that the bacterial biofilm formation decreased with Eno@MSN-D, which further proved that synthesised Eno@MSN-D has an antibacterial effect.

The antibacterial ability of Eno@MSN-D to each strain was verified by the bacterial colony counting plate method. ATCC25923, ATCC12228, and ATCC43300 were co-cultured with TSB, MSN, enoxacin, L-Eno@MSN-D, and H-Eno@MSN-D. Figure 3B, D, E, and F show no colony formation at an H-Eno@MSN-D concentration of 10 µg/ml. At an L-Eno@MSN-D concentration of 5 µg/ml, only a few colonies were formed in ATCC25923 and ATCC12228, while there were relatively more colonies in ATCC43300. However, many colonies were formed in the MSN and TSB groups. Interestingly, compared with the Eno group, the L-Eno@MSN-D group had a greater CFU number, while the H-Eno@MSN-D group showed fewer CFUs. In summary, Eno@MSN-D has visible antibacterial effects.
We observed the surface of treated glass slides by SEM. Figure 4A shows that the integrity of some bacterial morphology was destroyed at L-Eno@MSN-D and H-Eno@MSN-D groups. In the MSN and TSB groups, significant adhesions formed between the bacteria, indicating the formation of biofilm. This observation also shows that Eno@MSN-D could destroy bacterial integrity, playing an antibacterial role, and inhibiting biofilm formation.

Live and dead bacteria were marked by green and red fluorescence, respectively. From the fluorescence inverted microscope pictures (Fig. 4B), it can be seen that with increasing drug concentrations of Eno@MSN-D, the red fluorescence of dead bacteria was gradually increased. In contrast, the corresponding green fluorescence gradually weakened.

3.4. Eno@MSN-D suppressed RANKL-induced OC differentiation without any cytotoxicity \textit{in vitro}

Our previous studies have reported that enoxacin could inhibit osteoclast differentiation and function [13]. However, the effect of Eno@MSN-D on the formation of osteoclasts needs to be explored. BMMs were stimulated with M-CSF and RANKL in the presence of different concentrations of Eno@MSN-D (0, 2.5, 5,10 µg/ml). Interestingly, the BMMs treated with Eno@MSN-D showed a significant concentration-dependent decrease in mature osteoclast formation (Fig. 5C–E). Additionally, to determine if the inhibitory effects of Eno@MSN-D were due to cytotoxicity, we used the CCK-8 assay to measure the effect of Eno@MSN-D on BMM proliferation and survival. Our data shows that cell viability will not be significantly affected when the concentration is lower than 20 µg/ml Eno@MSN-D (Fig. 5A and B).

3.5. Bone-targeting properties of Eno@MSN-D \textit{in vivo}

As shown in Fig. 6, after 4 h, the fluorescence signals of the different organs in the MSN group and Eno@MSN-D group were almost the same, while the fluorescence signal of the femur in the Eno@MSN-D group was stronger. After 72 h, almost no fluorescence remained in the organs in the MSN group, whereas the fluorescence in the Eno@MSN-D group was mainly concentrated in the liver, kidney, and femur. The MSN group was not loaded with enoxacin in the bone-targeting group with D-Asp8, and the drug was metabolised by the liver and kidney. Thus, the Eno@MSN-D group had a strong liver and kidney fluorescence signal. Most importantly, the femoral fluorescence was also strong, indicating that Eno@MSN-D also accumulated in the femur and exhibited bone-targeting abilities \textit{in vivo}.

3.6. Antibacterial properties of the Eno@MSN-D \textit{in vivo}

We determined the animal model infection by measuring the rats' body temperature and weight over five weeks post-operation. From Fig. 7A and B, the bodyweight in each group of the rats gradually decreased within one week after the operation, while the body temperature gradually increased. One week later, rat bodyweight began to increase in each group, whereas the Sham group body temperature began to decrease. However, the body temperature of each experimental group did not change. There was no significant difference in body weight between the experimental and Sham groups (p > 0.05), but the body temperature differed between the experimental and Sham groups (p < 0.0001).
For the plate colony count of titanium rods after the ultrasound treatment, although colony number in the Eno@MSN-D group significantly differed from that in the Eno group, colony numbers of the MSN between NS groups was not statistically significant (Fig. 7C, and D). The titanium rods were fixed with glutaraldehyde, dehydrated with ethanol, gold plated, and observed by SEM. As shown in Fig. 8A, compared with other groups, including NS, MSN, and Eno groups, the bacterial biofilm on the surface of the Eno@MSN-D injection titanium rod was looser, with larger gaps in the biofilm. Furthermore, in other groups, the titanium rod surface was not significantly different, and the biofilms on the surface of the other groups were more closely connected than that in the Eno@MSN-D group. We used the living/dead bacterial staining method to observe the bacterial biofilm on the titanium rod surface under an inverted fluorescence microscope. Figure 8B shows that the red fluorescence signal in the Eno@MSN-D group was more intense than that in the MSN, NS, and Eno groups. There was no significant difference in red fluorescence among the Eno, MSN, and NS groups, which shows that the Eno@MSN-D group was more substantial than other groups in terms of antibacterial performance in vivo.

3.7. Bone morphometry assay

We used micro-CT to obtain two- and three-dimensional images from the femur group. We also acquired coronal, sagittal, and transverse images of the two-dimensional images and the overall, longitudinal, and transverse three-dimensional images (Fig. 9A). Compared with those in the Sham control group, the femur morphologies in the four experimental groups changed significantly, and osteolysis of different degrees was present in all four groups. However, compared with the NS and MSN groups, there was evident bone formation around the titanium rod pores in the Eno@MSN-D and Eno groups. Besides, no significant difference between the NS and MSN groups was observed. By performing parameter analysis on the bone structure of the ROI and using one-way variance analysis, we obtained some quantitative statistical graphs of the bone parameters. The bone volume fraction (BV/TV), trabecular number (Tb.N), and trabecular thickness (Tb.Th) were higher in the Eno@MSN-D and Eno groups than in the NS group, and the Eno@MSN-D group is superior to the Eno group. However, the trabecular bone separation (Tb.Sp) was remarkably lower in both the Eno@MSN-D and Eno groups than in the NS group. Otherwise, referring to BV/TV, Tb.N, Tb.Th, and Tb.Sp, no noticeable difference was present between NS and MSN groups (P > 0.05). Altogether, these results indicate that both Eno-@MSN-D and Eno groups effectively prevented osteolysis in vivo. Of note, the Eno-@MSN-D group was more effective than the Eno group.

3.8. Histopathology

Figure 10A and B show the longitudinal decalcified sections of different groups. The morphological changes in the left femur were detected by HE staining (Fig. 10A), and the osteoclasts around the pores of the titanium rod were detected by TRAP staining (Fig. 10B). Figure 10A shows more extensive bone cortex destruction and more abundant bone tissue death in the NS and MSN groups. Although there were signs of intramedullary inflammation in all four experimental groups, these were more severe in the MSN and NS groups. As shown in Fig. 10B, the number of osteoclasts around the titanium rod pores was less in the Eno@MSN-D and Eno groups compared to the NS group. Moreover, the number and area of osteoclasts in each group were measured, and quantitative analysis was performed (Fig. 10C and D).
Noteworthy, compared with the NS group, the number and area of osteoclasts in the Eno@MSN-D group and the ENO group were relatively fewer. Furthermore, the number and area of osteoclasts in the Eno@MSN-D group were lower than those in the ENO group.

4. Discussion

MSNs have been widely used in nanomedicine because they can be functionalised with chemical groups delivered to specific target sites to alter external organ organisation to accommodate specific target tissues [21]. However, despite these advances, challenges remain in effectively and sustainably controlling local bone drug release, calibrating the long-term stability and activity of drugs, while minimising cytotoxicity [22, 23]. Regarding orthopaedic implant-related infections, nanoparticles are a promising strategy for overcoming biofilm formation and drug resistance [24].

In our study, we loaded enoxacin into MSN and coated the nanoparticles with PEG, which is phagocytised by macrophages. Simultaneously, bone-targeting D-Asp8 was connected to the nanoparticles to achieve targeted drug release in the bone tissue [25, 26]. Usually, bone adsorption molecules, such as bone-targeting ligands, are coupled to nano-carrier surfaces. Those nano-carriers can chelate HA to achieve local drug deposition in bone tissue. After administration, a strong interaction between the bone-targeted ligands and HA leads to rapid retention and accumulation of the nano-carriers in the bone tissue. Therefore, this approach reduces drug leakage in the circulation and ensures highly localised antibiotic concentration [27]. From the fluorescence signals in rats (Fig. 6), it is evident that we have synthesised Eno@MSN-D nanoparticles that have excellent bone-targeting properties. Our data showed that the present MSN had ultra-high specific surface areas and suitable pore sizes for enoxacin loading. During synthesis, the average particle size increased from MSN to Eno@MSN-D. The increase in the size can be attributed to integrating the drugs and polymers into the mesoporous silica matrix. The results are in accordance with those in the literature [28], which show that the synthesised carrier size must be larger than that of the pure carrier material when the drugs and polymers are mixed with mesoporous silica nanoparticles.

The PDI reflects particle size uniformity, which is an important index for particle size characterisation. Within a specific distribution range, the smaller the distribution coefficient, the more uniform the particle size [29]. Our data shows that the PDI of the synthesised Eno@MSN-D is the smallest compared to MSN and Eno@MSN, so it has a uniform particle size. The zeta potential of the carrier material can reflect the system’s stability, and it has an important influence on the release and circulation of body fluids. The carrier material stability in the surrounding fluid is an important index to overcome aggregate formation in the nano-drug delivery system [28]. The higher the zeta potential (positive or negative) of the particles, the more stable the system is against aggregation [30]. Our results show that the synthesised Eno@MSN-D is -19.3mV, which the negative zeta potential is the smallest, so it has good dispersion properties. Besides, the TGA curves (Fig. 2E) showed that the weight loss of Eno@MSN was higher than that of MSN, which indicated that the enoxacin was loaded on MSN. In addition, the weight loss of Eno@MSN-D was higher than that of MSN and Eno@MSN; this was due to the loading of the enoxacin and the
immobilisation of PEG and D-Asp8 on the surface of the nanoparticles. From the \textit{in vitro} drug release curves of Eno@MSN-D in different pH response release patterns (Fig. 2F), we analyse the causes of this phenomenon in two main reasons: firstly, bacterial infections generally feature very low pH values due to their hypoxic nature \cite{31}. The acidic environment of bacterial infectious sites can be harnessed in the design of pH sensitive drug delivery systems \cite{32}. Moreover, via protonation at acidic pH, these chemistries result in greater drug release due to electrostatic repulsion within the nanocarriers \cite{33, 34}. These nanoparticles prevent non-specific interaction at physiological pH 7.4, thereby increasing the therapeutic activity with declining pH \cite{32}. Secondly, the reason is due to enoxacin molecular structure, as studies have reported that enoxacin is least water soluble at neutral pH and greater at acidic pH \cite{35}.

From the crystal violet staining, bacterial colony counting plate method unveiled the integrity of some bacterial morphology by SEM and live/dead bacterial staining, a series of \textit{in vitro} experiments, showed that Eno@MSN-D had good antibacterial properties. From the application of Eno@MSN-D \textit{in vivo}, we know that the monodisperse silica microspheres were biocompatible with low toxicity, lacked immunogenicity, and degraded into non-toxic compounds (mainly silicic acid) in a short amount of time \cite{36}. Additionally, our data show that Eno@MSN-D can inhibit osteoclast formation and function when the concentration is lower than the safe concentration of 20 µg/ml Eno@MSN-D.

The successful establishment of a rat infection model is the key to our confirmation that Eno@MSN-D has antibacterial properties \textit{in vivo}. From SEM (Fig. 8A), live/dead bacterial staining (Fig. 8B), and bacterial colony plate count on the surface of femoral titanium rods (Fig. 7C), we observed that Eno@MSN-D had a certain antibacterial effect. In the quantitative bacterial colony plate count graphs (Fig. 7D), compared the Eno@MSN-D and Eno groups, the difference between the two groups was statistically significant, which indirectly indicates that Eno@MSN-D had a better therapeutic effect. Similarly, we observed that, although there was no significant difference between the MSN and NS groups, the colony number in the MSN group was slightly lower; however, this was related to the antibacterial properties of the MSN. The inherent antibacterial activity of the nanoparticles was due to one of the three mechanisms: induction of oxidative stress, the release of metal ions, or non-oxidative stress mechanism \cite{37}.

In the two- and three-dimensional rat femur images (Fig. 9A), we observed the osteolysis phenomenon in the experimental group, which also confirmed the success of the rat bone infection model. Based on our data (Fig. 9B-D), we found that the BV/TV, Tb.N, Tb.Th, and Tb.Sp were significantly different between the Eno@MSN-D and Eno groups, which indicated that Eno@MSN-D does play a role in preventing osteolysis \textit{in vivo}. Of note, the Eno-@MSN-D group was more effective than the Eno group.

Bioactive silicon-based nanoparticles reportedly promote differentiation and mineralisation of osteoblasts \cite{38}. Additionally, silicon ions released from MSNs have been shown to promote the formation of mineralised nodules, the synthesis of COL1, and the expression of osteogenic genes in osteoblasts \cite{39}. These observations suggest that MSN plays a role in osteogenesis, and thus may prevent osteolysis. Nevertheless, there was no significant difference in bone parameters between the
MSN and NS groups, which indicates that the osteogenic capacity of MSN is still limited, and thus enoxacin still plays a significant role. Besides, the Eno@MSN-D had better osteogenesis abilities to prevent osteolysis, which is consistent with the histomorphological femoral HE (Fig. 10A). The number and area of osteoclasts (Fig. 10B-D) in the Eno@MSN-D and Eno groups was smaller than that of the NS groups. Furthermore, the Eno@MSN-D and EN groups significantly differed in terms of osteoclast number and area. Therefore, the Eno@MSN-D group could more effectively than the Eno group to prevent osteolysis.

The in vitro and in vivo studies of most nanoparticles-based sustained-release systems show that these materials show better antibacterial and antibiofilm activity, which is mainly related to reducing bacterial adhesion to grafts, higher effectiveness in drug-resistant strains, and/or controlling the antibiotic release. By obtaining sustained-release performance [40–42], we can achieve better local antibiotic concentrations and prolong the contact time between antibiotics and bacteria, which is essential for removing biofilms. Additionally, carriers offer advantages in terms of therapeutic drug release kinetics, ease of manufacture, biodegradability, chemical binding relative to physical binding, and excellent transport characteristics. Thus, these biomaterials will play a more critical role in medical diagnosis and treatment processes.

5. Conclusions

In this study, we successfully prepared and characterised Eno@MSN-D nanoparticles-based on MSN. Our data show that the synthesised Eno@MSN-D is a safe drug delivery system that enables sustained-release of specific antibiotics to target staphylococcus aureus-related post-operative implantation infections. Eno@MSN-D not only have excellent antibacterial properties and osteoclast inhibitory properties in vitro but also allows the specific targeting of bone tissues to prevent bone loss in vivo. Therefore, this method provides a new way to treat and prevent post-operative, orthopaedic staphylococcus aureus-related implantation infections and bone loss.

Declarations

Availability of data and materials

All experimental data to support the findings from this study will be made available to interested investigators. All data generated or analysed during this study are included in this manuscript and its Additional file.

Ethics approval and consent to participate

All animal experiments were performed in the Department of Animal Experimental Sciences of Nanchang University, under the approval and guidance of the Animal Experimental Ethics Committee of the First Affiliated Hospital of Nanchang University.
Consent for publication

Not applicable

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Not applicable

Authors' contributions

Cong Yao: Methodology, Investigation, Writing-original draft. Qiang Xu: Investigation, Writing - original draft. Xiuguo Han: Methodology, Validation. Qianyuan Tao: Formal analysis. Xuwen Luo: Formal analysis. Feilong Li: Data curation.. Fuqiang Wang: Data curation. Zhiping Gu: Writing - review & editing. Huaen Xu: Writing - review & editing. Bin Zhang: Writing - review & editing. Min Dai: Writing - review & editing, Conceptualization, Funding acquisition. Tao Nie: Writing - review & editing, Conceptualization, Funding acquisition. Xuqiang Liu: Supervision, Writing - review & editing, Funding acquisition.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figures
Figure 1

Schematic diagram of the process flow for synthesising Eno@MSN-D nano slow-release particles based on mesoporous silica nanomaterial (MSN) and schematic diagram of the action mechanism of Eno@MSN-D nanoparticles.
Figure 2

(A) Typical TEM images of MSN, Eno@MSN, and Eno@MSN-D. (B) N2 adsorption–desorption isotherms curves of the MSN. (C) Pore size distribution curves of the MSN. (D) The dynamic light scattering (DLS) curves of MSN, Eno@MSN, and Eno@MSN-D. (E) TGA curves recorded for MSN, Eno@MSN, and Eno@MSN-D. (F) Accumulative release curve of Eno@MSN-D in different pH PBS (pH = 7.4, 6.0).
Figure 3

(A, C) TSB (10 µg/ml), MSN (10 µg/ml), Eno (10 µg/ml), L-Eno@MSN (5 µg/ml), H-Eno@MSN-D (10 µg/ml) were co-cultured with bacteria to form a biofilm, respectively and then the absorbance was measured by crystal violet staining. (B) Determination of antimicrobial properties bacterial by colony counting plate method. (D) The number of CFU after co-culture of ATCC25923 with Eno@MSN-D. (E) The number of CFU after co-culture of ATCC12228 with Eno@MSN-D. (F) The number of CFU after co-culture of ATCC43300 with Eno@MSN-D.
Figure 4

(A) SEM images of TSB (10 µg/ml), MSN (10 µg/ml), Eno (10 µg/ml), L-Eno@MSN (5 µg/ml), H-Eno@MSN-D (10 µg/ml) and bacteria co-cultured on cover slides, respectively. (B) The fluorescence inverted microscope images of TSB (10 µg/ml), MSN (10 µg/ml), Eno (10 µg/ml), L-Eno@MSN (5 µg/ml), H-Eno@MSN-D (10 µg/ml), and bacteria co-cultured on confocal petri dishes, respectively.
Figure 5

(A, B) BMMs were plated in 96-well plates and stimulated with M-CSF (30 ng/mL) and different concentrations of Eno@MSN-D (0-160 µg/ml) for 48 h or 96 h. Cell viability was measured using the CCK-8 assay. (C) Bone marrow macrophages (BMMs) were treated with various concentrations of Eno@MSN-D (0, 2.5, 5, 10 µg/ml) followed by M-CSF and RANKL stimulation for 5 days. Cells were then fixed with 4% paraformaldehyde and subjected to tartrate-resistant acid phosphatase (TRAP) staining. (D, E) The number and spread area of TRAP-positive multinuclear cells was measured.
Figure 6

In vivo targeted fluorescence experiment: fluorescence diagrams of various organs 4 and 72 h after injection of MSN and Eno@MSN-D nanoparticles.
Figure 7

(A) The change curve of body temperature in rats. (B) The curve of body weight change in rats. (C) Plate colony count after ultrasonic concussion of bacteria on the surface of a titanium rod. (D) Statistical chart of plate colony count after the ultrasound treatment.
Figure 8

(A) SEM images of biofilm on the surface of the titanium rod. (B) The Live dead bacteria staining of the biofilm on the surface of the titanium rod was observed under an inverted microscope.

Figure 9
(A) Coronal, sagittal, and transverse two-dimensional micro-CT and integral, longitudinal, transverse three-dimensional micro-CT images of the femur. (B-E) Statistic diagram of bone volume fraction, number of bone trabeculae, trabecular bone thickness, and trabecular bone separation.

Figure 10

(A) The representative images of HE staining of the middle part of the femur. Yellow arrows indicate dimensional intracortical abscesses or inflammatory cells. (B) The representative images of TRAP
staining of the middle part of the femur. Black arrows represent osteoclasts. (C) Statistical graph of the number of osteoclasts in each field of view. (D) Statistical graph of the area of osteoclasts in each field of view.

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