

Au Nanoclusters Ameliorate *Shigella* Infectious Colitis by Inducing Oxidative Stress

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Background: *Shigella* infection has always been a global burden, and it particularly threatens children between the ages of 1 and 5 years. Economically underdeveloped countries are dominated by *Shigella flexneri* infection. The most effective method to treat *Shigella* is antibiotics, but with the abuse of antibiotics and the prevalence of multidrug resistance, we urgently need a relatively safe non-antibiotic treatment to replace it. Ultrasmall Au nanoclusters (NCs) have special physical and chemical properties and can better interact with and be internalized by bacteria to disrupt the metabolic balance. The purpose of this study was to explore whether Au NCs may be a substitute for antibiotics to treat *Shigella* infections.

Methods: Au NCs and *Shigella* Sf301, R2448, and RII-1 were cocultured in vitro to evaluate the bactericidal ability of Au NCs. The degree of damage and mode of action of Au NCs in *Shigella* were clearly observed in images of scanning electron microscopy (SEM). In vivo experiments were conducted to observe the changes in body weight, clinical disease activity index (DAI) and colon (including length and histopathological sections) of mice treated with Au NCs. The effect of Au NCs was analysed by measuring the content of lipocalin-2 (LCN2) and *Shigella* in faeces. Next, the changes in *Shigella* biofilm activity, the release of reactive oxygen species (ROS), the changes in metabolism-related and membrane-related genes, and the effect of Au NCs on the body weight of mice were determined to further analyse the mechanism of action and effect.

Results: Au NCs (100 μ M) interfered with oxidative metabolism genes, induced a substantial increase in ROS levels, interacted with the cell membrane to destroy it, significantly killed *Shigella*, and effectively alleviated the intestinal damage caused by *Shigella* in mice. The activity of the biofilm formed by *Shigella* was reduced.

Conclusion: The effective antibacterial effect and good safety suggest that Au NCs represent a good potential alternative to antibiotics to treat *Shigella* infections.

Keywords: *Shigella*, Au nanoclusters, antibacterial effect, reactive oxygen species, oxidative stress

Introduction

Shigella is a facultative anaerobic gram-negative bacilli without flagella and with fimbriae. Kiyoshi Shiga confirmed that it was the culprit causing bacillary dysentery in 1897.¹ Since then, *Shigella* has become the main pathogen causing bacillary dysentery worldwide. A retrospective investigation of cases from 1966 to 1997 by Kotloff et al showed that among 164.7 million cases, more than 99% occurred in developing countries, and 80% of them occurred in Asia. The review by Bardhan et al concluded that the number of cases in Asia was still high, with approximately 125 million cases per year from 1990 to 2009.^{2,3} Most importantly, bacillary dysentery is more harmful to children and is one of the main causes of death in

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children.^{4,5} It is also the most widely distributed intestinal infectious disease with the highest incidence in China.

Shigella is divided into four serotypes, namely, *Shigella dysenteriae*, *Shigella flexneri* (*S. flexneri*), *Shigella boydii* and *Shigella sonnei*. Bacterial dysentery is mainly caused by *S. flexneri*, especially in developing countries and countries with low economic levels. This disease is related to health, the environment, resources and other factors.^{6,7} Faecal-oral transmission is the most important transmission mode of *Shigella*,⁸ while invasiveness and endotoxin are the pathogenic factors of *Shigella*. *Shigella* adapts to gastric acid,⁹ the bacteria pass through the stomach and small intestine and finally reach the large intestine to promote infection. Endotoxin increases the permeability of the intestinal mucosa and destroys it.¹⁰ In addition, *Shigella* enters the intestinal epithelium through microfolded cells, rapidly induces the death of macrophages, and spreads to neighbouring cells under the cover of actin.^{11,12} The NF- κ B pathway activated by the chlamydial type III secretion apparatus upregulates inflammatory factors,¹³ initiates neutrophil infiltration and destroys the integrity of intestinal epithelial cells, allowing more *Shigella* to easily enter the submucosa and produce an infection.^{11,14} In addition, *Shigella* changes the composition of connexins, weakening the sealing of endothelial cells, which aggravates the inflammatory response¹⁵ and ultimately manifests as watery diarrhoea, mucus pus and blood in the stool and other inflammatory reactions. Moreover, *Shigella* can cause invasive infections such as meningitis and osteomyelitis.^{16,17} In young children, intussusception or necrotizing enterocolitis is a common infection.¹⁸ Currently, the most reliable treatment for these bacteria is antibiotics. In 2017, the World Health Organization selected ciprofloxacin as the first choice for treatment. However, with the evolution and mutation of the pathogen, multidrug resistance has become a stumbling block to *Shigella* treatment. Multidrug resistance poses a great threat to most special populations with low immunity and even contributes to *Shigella* infection.^{19–21} Scientists have gradually shifted the treatment of *Shigella* infections to other areas. Probiotics are also the focus of research.²² Short-chain fatty acids have been shown to assist therapy,²³ and bacteriophages have also suggested as a treatment,²⁴ but longer follow-up of the treatment is required. Many ethical guidelines restrict vaccine development, and no licenced vaccines have been developed.²⁵ Therefore, a new treatment for *Shigella* with more promise is needed.

Nanoparticles have become a hot topic in medicine and biology. Using various technologies, such as electrostatic bonding and physical adsorption, nanoparticles are coupled with different ligands and biomolecules to allow them to exert different effects.²⁶ The detection of Au nanoclusters (Au NCs) relies on surface plasmon resonance (SPR), which specifically marks and quantifies macromolecules under the microscope, and Au NCs have an important auxiliary role in the treatment of diseases.²⁷ In addition, lateral flow assays using labelled Au NCs and biosensors for their applications can also be applied to the detection of *Shigella*.^{28,29} In the treatment of diseases, Au NCs can be used as drug carriers to specifically target and attack target cells. Au NCs have proven to be promising delivery vectors in the treatment of bacterial infections and tumours,^{30,31} such as *Staphylococcus aureus* infections.³² More importantly, Au NCs are a “drug” with great potential. After being modified by small molecules, they exhibit a high specific surface area and high surface chemical activity. Au NCs attack bacterial membranes and interfere with cellular metabolism, effectively killing many gram-negative bacteria and gram-positive bacteria, such as *Staphylococcus epidermidis*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*.^{33,34} Au NCs also show significant antifungal activity toward some fungi, such as *Aspergillus* and *Penicillium*. Due to the membrane structure, Au NCs will be more effective against gram-negative bacteria.³⁷ In addition, gold is a stable inert metal, and Au NCs have been proven to display good biocompatibility, low cytotoxicity, and considerable safety.^{34–36} We have previously proven that Au NCs effectively kill *Clostridium difficile*,³⁶ but their function in killing *Shigella* remains to be studied.

Shigella infectious dysentery has become a global burden that cannot be ignored. With the prevalence of antibiotic abuse, *Shigella* resistance has become increasingly intense. This study explored the bactericidal effect of Au NCs on *Shigella* for the first time. At the same time, experiments in mice were carried out to further prove and explain the antibacterial effect of Au NCs and explore the possible mechanism underlying the antibacterial effect of Au NCs. In summary, Au NCs are very likely to become the focus as the next treatment for *Shigella* infection.

Materials and Methods

Synthesis and Modification of Au NCs

First, 0.25 mL of hydrogen tetrachloroaurate (III) hydrate (HAuCl₄) (20 mM), 1 mL of 10 mM 6-mercaptohexanoic

acid (MHA) and 3.35 mL water were mixed and stirred to obtain an Au-MHA complex. Then, 0.3 mL of NaOH (1 M) was added to dissolve it, and 0.1 mL of a newly prepared 112 mM NaBH₄ solution was added. After 3 h, the antigen was collected and treated with stirred cells (model 8010, Millipore Corporation, USA) by ultrafiltration. The size and quality of Au NCs were characterized using transmission electron microscopy (TEM) (JEM-2100, Japan), dynamic light scattering (DLS) (Malvern Zetasizer Nano ZS90, UK) and ultraviolet-visible (UV-Vis) spectrometry (Shimadzu, UV-3600, Japan). Au NCs modified with MHA ligands were finally obtained.³⁶

Growth and Culture of Strains

The *Shigella* strain used in this study was donated by Xu³⁸ as *Shigella* Sf301-gfp (ampicillin screening marker). In this study, *S. flexneri* was stored at -80°C and resuscitated before use. The standard strain Sf301 and clinically resistant strains R2448 and RII-1 (the results of the susceptibility analysis of strains R2448 and RII-1 are shown in [Tables S1](#) and [S2](#)) were grown in trypticase (tryptic) soy broth (TSB) (Aoboxing, Beijing, China) and cultured at 37°C for 24 h. Sf301-GFP was grown in TSB medium containing 100 µg/mL ampicillin and cultured at 37°C for 24 h.

Detection of the Antibacterial Activity of Au NCs Toward Strains Sf301, R2448, and RII-1

We conducted antibacterial tests on the three strains (Sf301, R2448 and RII-1) to determine whether Au NCs had antibacterial activity against *Shigella*. The bacteria were cultured in TSB liquid medium at 37°C for up to 24 h and then added to bacterial culture tubes with Au NC concentrations of 0, 50 and 100 µM. Sf301 was initially incubated with AuNCs for 1 h and 2 h, and R2448 and RII-1 were incubated for 2 h. Then, cultures were incubated for 48 h at 37°C in an anaerobic environment. Finally, the cfu of *Shigella* was counted, and the bacterial growth curve was plotted.

Scanning Electron Microscopy (SEM) Characterization of Sf301

Sf301 was cocultured with 0 µM and 100 µM Au NCs in the dark for 2 h, and then the supernatant was collected by centrifugation and the pellet was washed twice with normal saline. After adding 100 µL of fixative solution, it was placed at 4°C for 12 h, washed with 0.1% phosphate-

buffered saline (PBS) (3 times, 10 min each), and then dehydrated with 25% ethanol/50% ethanol/70% ethanol/80% ethanol/90% ethanol/95% ethanol (2 times, 15 min each). The film was then dried, coated, observed and photographed under an SEM (Hitachi-S4800, Japan).

Induction of *S. flexneri* Enteritis and Administration of Au NCs

Male C57BL/6J mice (16–18 g) were purchased from Zhejiang Vital River Company. All animal experiments were performed according to the standards in the Guide for the Care and Use of Laboratory Animals published in 2011 (Institute of Laboratory Animal Resources of National Research Council, United States).³⁹ All mouse studies were evaluated by the Laboratory Animal Ethics Committee of Xuzhou Medical University (IACUC number: 202012A200), Xuzhou, China. The room temperature was 25°C, and the humidity was 40–70%. The mice were housed on a 12 h alternating day and night cycle, and standard laboratory sterilized feed and water were freely available.

The mice were divided into the normal control group (NC), *S. flexneri* infection group (SF) and Au NC treatment group (SF-Au NCs), with 10 mice in each group. First, the SF group and the SF-Au NC group were pretreated for 7 days. Mice in the SF group were gavaged with 200 µL of sterile water for 7 days, and mice in the SF-Au NC group were gavaged with 200 µL of Au NCs (200 µM) for 7 days. After pretreatment, mice were provided antibiotic-containing drinking water for 3 days. The antibiotic concentrations in drinking water were as follows: metronidazole 215 mg/L, colistin 850 µg/mL, gentamicin 35 mg/L, and vancomycin 45 mg/L. During the 4 days, mice in the SF group and SF-Au NC group were treated with the same treatment as the pretreatment. Then, mice in the SF and SF-Au NC groups were gavaged with 5×10⁸ CFU *S. flexneri* on day 0, followed by continuous gavage with 200 µL of sterile water or 200 µM Au NCs for 3 days, respectively. During the entire post infection period, clinical scores were recorded for each mouse. The mice were euthanized on day 3, and the colon length of the mice was observed and measured. The faeces were collected and frozen at -80°C.

Colon Histological Score

The distal colon tissue of the mouse was fixed with a 4% paraformaldehyde solution and sent to Wuhan Servicebio Technology Co., Ltd. (Wuhan, China) to prepare

haematoxylin-eosin (H&E)-stained sections. The lesions of the colon tissue were observed under a microscope and scored according to the scoring criteria listed in [Table S3](#). Histological injury score = inflammatory infiltration + crypt damage + crypt damage degree + the absence of oedema.

Detection of the Faecal Lipocalin-2 (LCN2) Content

When the mice showed symptoms of infection, such as weight loss and loose stools, the mouse faeces were collected and stored at -80°C . Before testing, the supernatant was collected after homogenization, and the LCN2 content in faeces was detected using the protocol of the LCN2 ELISA Kit (Biotyscience) to assess the level of colitis in mice.

Measurement of the Tissue Bacterial Load

The collected faeces were weighed, sterile PBS was added to prepare a homogenate, and the bacteria were counted using the plate counting method.

Detection of Sf301 Biofilm Activity

A turbidimetric metre (DensiCHEKTM Plus, USA) was used to configure a 0.5 McKenzie concentration bacterial solution that was co-cultured with 0 or 100 μM Au NCs in a 96-well plate at 37°C for 24 h. The samples were centrifuged, the supernatant was discarded, and the pellet was washed 3 times with PBS. One hundred microliters of MTT were added, incubated for 30 min before DMSO (100 μL /well) was added, and then the absorbance was measured at 570 nm using the iMarkTM microplate reader (Bio-Rad, USA) to determine the OD value.

Detection of ROS Release from Sf301

One millilitre of 5×10^8 CFU/mL bacterial solution was added to two Eppendorf (EP) tubes, centrifuged, and the supernatant was discarded. One millilitre of normal saline was added to each tube, along with Au NCs or the same amount of normal saline. The samples were mixed, and 400 μL of each mixture were incubated in the dark at 37°C for 2 h. Then, 20 μL of DCFH-DA (8 mg/mL) were added in the dark, and incubated for 30 min in the dark at room temperature. Then, the cells were centrifuged, the supernatant was discarded, and the cells were washed twice with PBS (350 μL). The samples were mixed and placed in a 96-well plate (100 μL /well) with three

replicate wells per concentration, and then the OD600 value was measured with an iMark microplate reader.

Shigella Transcriptome Sequencing Analysis

Total RNA was extracted from the tissue using TRIzol[®] reagent according to the manufacturer's instructions (Invitrogen), and genomic DNA was removed using DNase I (TaKara). The RNA-seq transcriptome library was prepared from 2 μg of total RNA using the TruSeqTM RNA sample preparation kit from Illumina (San Diego, CA). The processing of original images to sequences, base calling, and quality value calculations were performed using the Illumina GA Pipeline (version 1.6), in which 150 bp paired-end reads were obtained. A Perl program was written to select clean reads by removing low-quality sequences, reads with more than 5% N bases (unknown bases) and reads containing adaptor sequences.

The data generated from the Illumina platform were used for the bioinformatics analysis. All analyses were performed using the I-Sanger Cloud Platform (www.i-sanger.com) from Shanghai Majorbio. After correction for multiple testing, we chose pathways with a P-value ≤ 0.05 that were significantly enriched in DEGs.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software). Data are reported as the means \pm SD. Comparisons between two groups were assessed using Student's *t*-test or the Mann–Whitney test, depending on whether the data were normally distributed. Statistical significance between multiple groups was tested using one-way multiple analysis of variance (ANOVA) or the Kruskal–Wallis test followed by Tukey's test. The level of statistical significance was set to $p < 0.05$.

Results

The Antibacterial Activity of Au NCs Toward Strains Sf301, R2448, and RII-1

We synthesized Au NCs with MHA ligands with a diameter of 9 nm using the NaBH_4 reduction method^{34,36} (Au NC particle size of 2 nm, with good dispersibility) to evaluate the antibacterial activity of Au NCs. Different absorption peaks were observed at 670 and 760 nm.^{36,40} We incubated the standard strain Sf301 and the clinically resistant bacterial strains R2448 and RII-1 with 0, 50, or 100 μM Au NCs and used the colony-forming unit (CFU) method to

quantitatively analyse the viability of *Shigella*. The growth of *Shigella* in the presence of different concentrations of Au NCs is shown in Figure 1 (the standard strain Sf301 was analysed after 1 h and 2 h, clinically resistant bacteria R2448 and RII-1 were analysed after 2 h). The plate counts of the three strains all showed that Au NCs almost completely inhibited the growth of *Shigella* (Figure 1), especially the clinically resistant bacteria R2448 (Figure 1B).

The Antibacterial Activity of Au NCs

The antibacterial effect of Au NCs on *Shigella* is reflected in the morphology of bacteria, as shown in Figure 2. Compared with the control group, the *Shigella* membrane was dissolved and the bacterial structure was disintegrated in the Au NC group. The internalization of Au NCs is the basis for killing *Shigella*, and the SEM image also

indicates the inevitable effect of Au NCs on the cell membrane.

Au NCs Alleviate the Clinical Symptoms of *S. flexneri* Enteritis

We treated mice with Au NCs for 14 days and induced murine *Shigella* infectious colitis by administrating *Shigella* to clarify the role of Au NCs in *Shigella* infectious colitis (Figure 3A). The most important consideration for the development of new drugs is that their benefits to the human body outweigh the harms. We have concluded that Au NCs do not exert toxic side effects on human cells.³⁶ In this experiment, we administered 100 μ M Au NCs to healthy mice by gavage, observed the weight change in the mice, and obtained a 14-day mouse body weight curve (Figure 3B). No

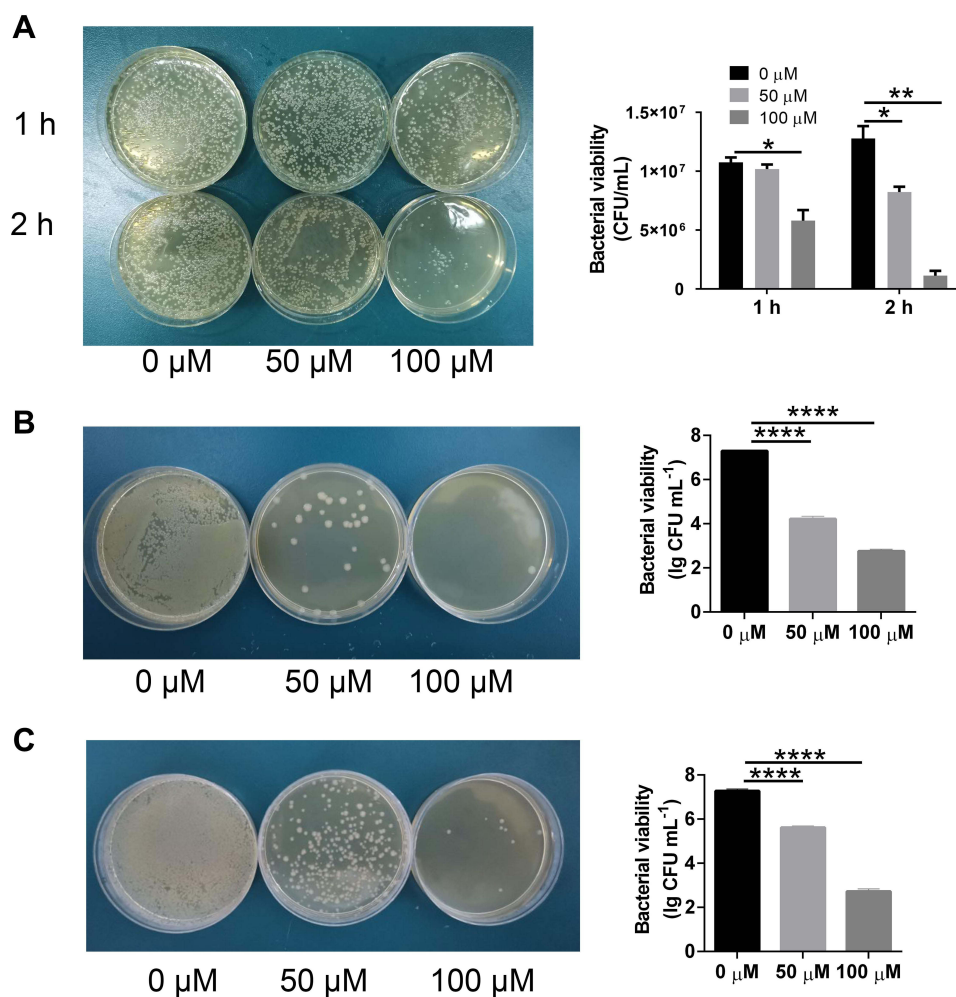


Figure 1 Antibacterial effect of Au NCs on the standard strain Sf301 (A), clinically resistant *Shigella* R2448 (B), and clinically resistant *Shigella* RII-1 (C). Data are presented as the means \pm SEM (n=6). Statistical significance was determined with one-way ANOVA followed by Tukey's test, and *p*-values were calculated as follows: **p*<0.05, ***p*<0.01, ****p*<0.0001.

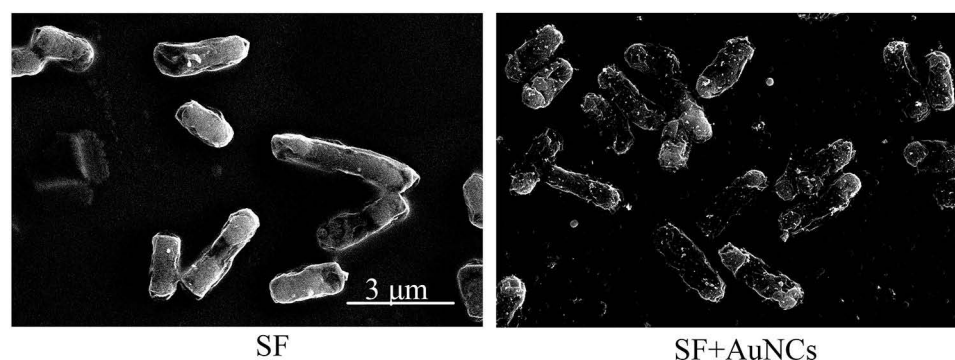


Figure 2 Au NCs exert a strong killing effect on *Shigella*. *Shigella* were incubated with 100 μ M Au NCs for 2 h. SEM images of *Shigella* before and after treatment with Au NCs and changes in the cell structure of *Shigella*. The scale bar represents 3 μ m.

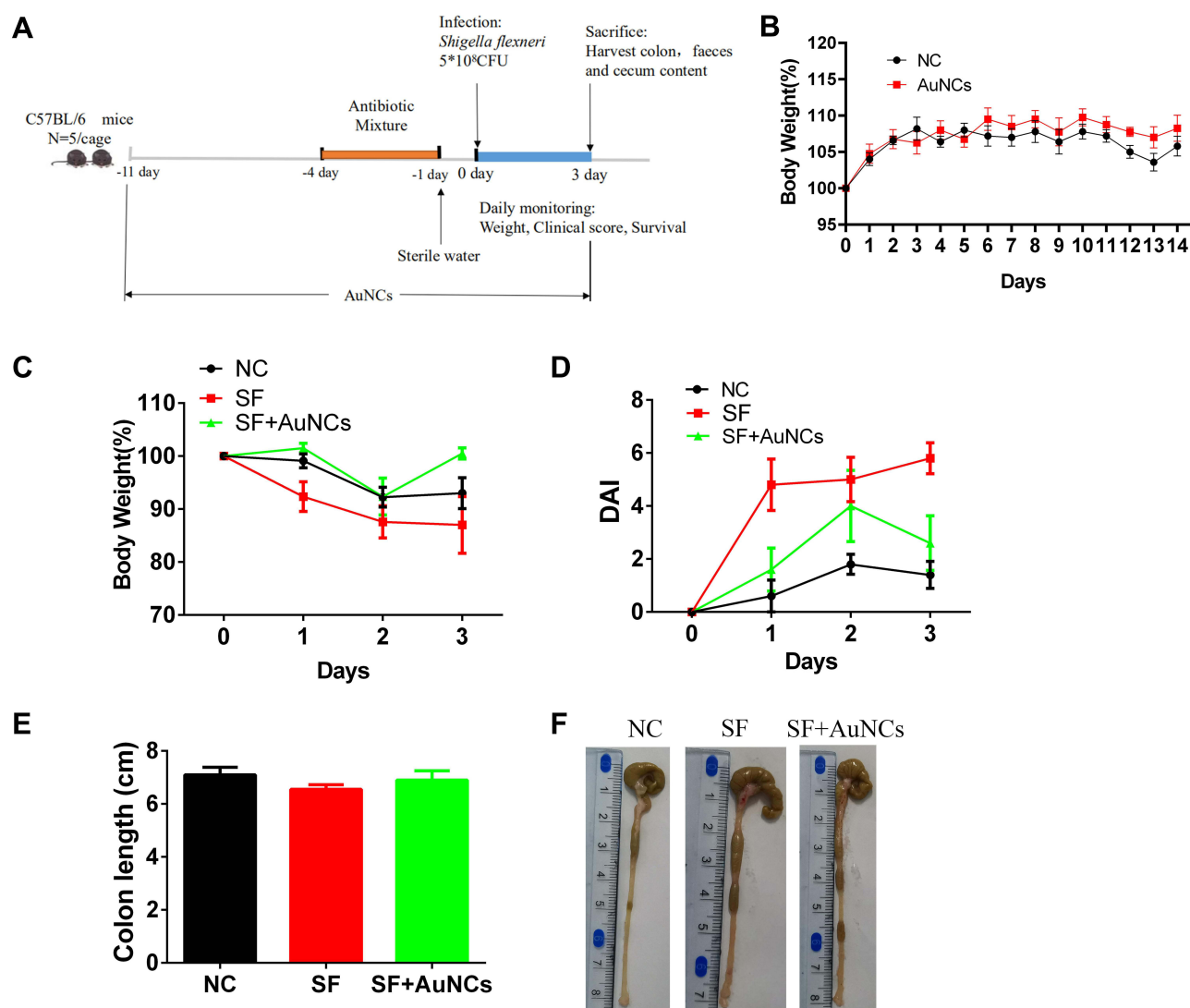


Figure 3 Au NCs alleviate the clinical symptoms of *S. flexneri* enteritis. (A) Mouse model of *Shigella* infection, (B) body weight changes of the NC group and the SF-Au NCs, (C) changes in body weight of mice from day 0 (the day of infection), (D) DAI scores of mice from day 0 (the day of infection), (E) statistical chart of mouse colon length, and (F) colon appearance of the NC group, the SF group and the SF-Au NCs group. Data are presented as the means \pm SEM (n=10). Statistical significance was determined with one-way ANOVA followed by Tukey's test.

significant difference in body weight was observed between Au NC-treated mice and control mice, which further proved the biocompatibility of Au NCs.

The body weight of *S. flexneri*-infected mice decreased substantially after *Shigella* infection. In the SF+Au NC group, weight loss did not occur immediately after *Shigella* infection, and weight recovered significantly on day 2. During the entire post infection period, the body weight of Au NC-treated mice was always greater than that of untreated mice, indicating a beneficial effect of Au NCs on *Shigella* enteritis (Figure 3C).

Due to *Shigella* infection, *S. flexneri*-infected mice presented clinical symptoms such as weight loss, diarrhoea and bloody stools, and the clinical disease activity index (DAI) increased. Au NC-treated mice showed a relatively smaller decrease in body weight and lower clinical scores than the SF group (Figure 3D). In conclusion, Au NCs improved the clinical symptoms of *Shigella* enteritis in mice.

Gross observation of the colon segment showed that Au NCs relieved *Shigella* enteritis, and the length of the colon in the SF+Au NC group was slightly increased compared with that in the SF group, which was not significantly different from that of the NC group (Figures 3E and F).

Au NCs Relieve Intestinal Damage Caused by *Shigella* Infection

Shigella-infected mice showed signs of colitis, such as weight loss, loose stools, colonic mucosa erosion, and crypt structure destruction. The colon glands of the mice treated with Au NCs were arranged neatly, the crypt structure was slightly damaged, and inflammatory cells were locally infiltrated, similar to the colon of the control group (Figure 4A). The histological damage score of the colon also showed that Au NCs significantly reduced colitis caused by *Shigella* infection (Figure 4B).

When the mice showed symptoms of infection, the faeces were collected to detect the LCN2 content. The results are shown in Figure 4C. Compared with mice in the SF group, the faecal LCN2 content of mice in the SF+Au NC group was significantly decreased, indicating that colitis was effectively relieved.

Au NC Treatment Reduces the *Shigella* Load in Faeces

Au NCs showed strong antibacterial effects in vitro and relieved *Shigella* infection. Therefore, we further investigated whether they exerted an antibacterial effect on

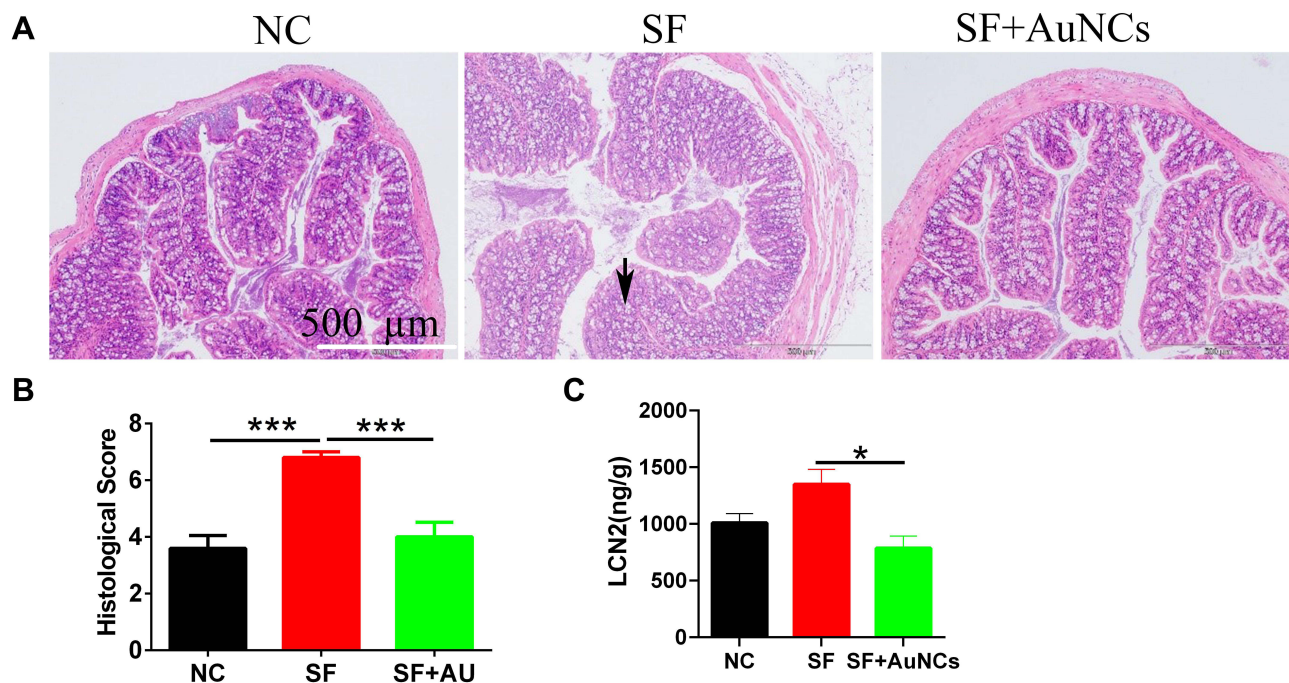


Figure 4 The effect of Au NCs on colon tissue damage and the faecal LCN2 content in mice infected with *Shigella*. (A) H&E-stained pathological sections of the colon tissue (100×), (B) histological score, and (C) faecal LCN2 content. Data are presented as the means±SEM (n=10). Statistical significance was determined with one-way ANOVA followed by Tukey's test, and *p*-values are as follows: **p*<0.05 and ****p*<0.001.

Shigella in vivo. The results of the bacterial load analysis in mice showed that Au NCs significantly reduced the CFU of *Shigella* in faeces compared with the SF group (Figure 5), indicating that Au NCs inhibited *Shigella* and alleviated *Shigella* enteritis.

Au NCs Reduce *Shigella* Biofilm Activity

A biofilm is a common infection mode of *Shigella*, a food-borne bacterium, and it is also a protective umbrella for *Shigella*. Biofilms have natural resistance to antibiotics, which substantially reduces their sensitivity to antibiotics.⁴¹

We tried to study whether the biofilm formed by *Shigella* was also an impenetrable wall for Au NCs. As shown in Figure 6, 100 μ M Au NCs reduced the biofilm activity of *Shigella* to approximately 60%, suggesting that the application of Au NCs will not encounter resistance due to biofilm formation.

Au NCs Affect Metabolism-Related Genes and Induce ROS Release in *Shigella*

After being internalized by bacteria, Au NCs affect the metabolism of bacteria and induce excess ROS release. As shown in Figure 7A, Au NCs increased the level of ROS metabolized by *Shigella* by approximately 5 times. This effect is the main antibacterial pathway of Au NCs, as excessive ROS directly kill bacteria.³⁴ After testing the expression of metabolism-related genes, as shown in Figure 7B, we found that Au NCs downregulated antioxidant genes such as *ompA*, *gshA*, *bssR*, *elaB*, *alkB*, *grxA*, *nth*, *relB*, and *nuoF* and upregulated oxidative stress genes such as *copA*, *htrA*, *soxS*, and *hha* (Table S4), which may have led to a substantial increase in ROS production. We decomposed the predicted metagenomic information into KEGG (Kyoto Encyclopedia of Genes and Genomes) and GO (Gene Ontology) pathways. Treatment with Au NCs mainly caused changes in the tricarboxylic acid cycle and cell metabolism (including pyrimidine metabolism, purine

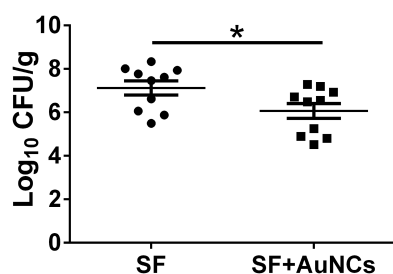


Figure 5 The effect of Au NCs on the *Shigella* load (faeces). Data are presented as the means \pm SEM (n=10). Statistical significance was determined with one-way ANOVA followed by Tukey's test, and *p*-values are as follows: **p*<0.05.

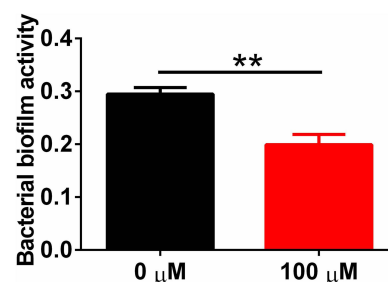


Figure 6 The effect of Au NCs on *Shigella* biofilm activity. Data are presented as the means \pm SEM (n=6). Statistical significance was determined with one-way ANOVA followed by Tukey's test, and *p*-values are as follows: ***p*<0.01.

metabolism, and nucleotide metabolism), cell membrane transport, signal transduction and other pathways and changes in gene abundance (Figures 7C and D, Supplementary Figure 1), which may be involved in the release of ROS and cell membrane integrity.

Discussion

The global financial burden caused by *Shigella* infection cannot be ignored, and its multidrug resistance has hindered effective antibiotic treatment. The remarkable antibacterial properties and excellent therapeutic effects of Au NCs reported in this study have once again ushered in the dawn of the treatment of *Shigella* infectious diseases. We used the NaBH₄ reduction method to synthesize Au NCs. The process is energy saving, and the synthesis is fast, making it highly stable. The synthesized Au NCs have an ultrasmall size and large specific surface area, rendering the surface highly active.^{34,37}

We incubated Au NCs with Sf301 and the clinically resistant strains R2448 and RII-1 to develop a drug with antibacterial activity that does not produce drug resistance. These two strains of resistant bacteria are resistant to almost all antibiotics and are representative strains. Remarkably, 100 μ M Au NCs exerted obvious antibacterial effects on these three strains and basically inhibited the growth of 100% of the colonies, indicating that Au NCs may not be affected by the resistance mechanism of *Shigella*. SEM was used to determine the structure of *Shigella* in order to prove the effectiveness of Au NCs, and studies have shown that after nanoparticles are in contact with microorganisms, nanoparticles first attack the outermost structure of the microbe cells.⁴² Similar to the role of Au NCs in *Clostridium difficile*, the SEM images we obtained and the detection of the changes in the abundance of membrane-related genes proved that Au NCs dissolved the *Shigella* membrane. This effect may be

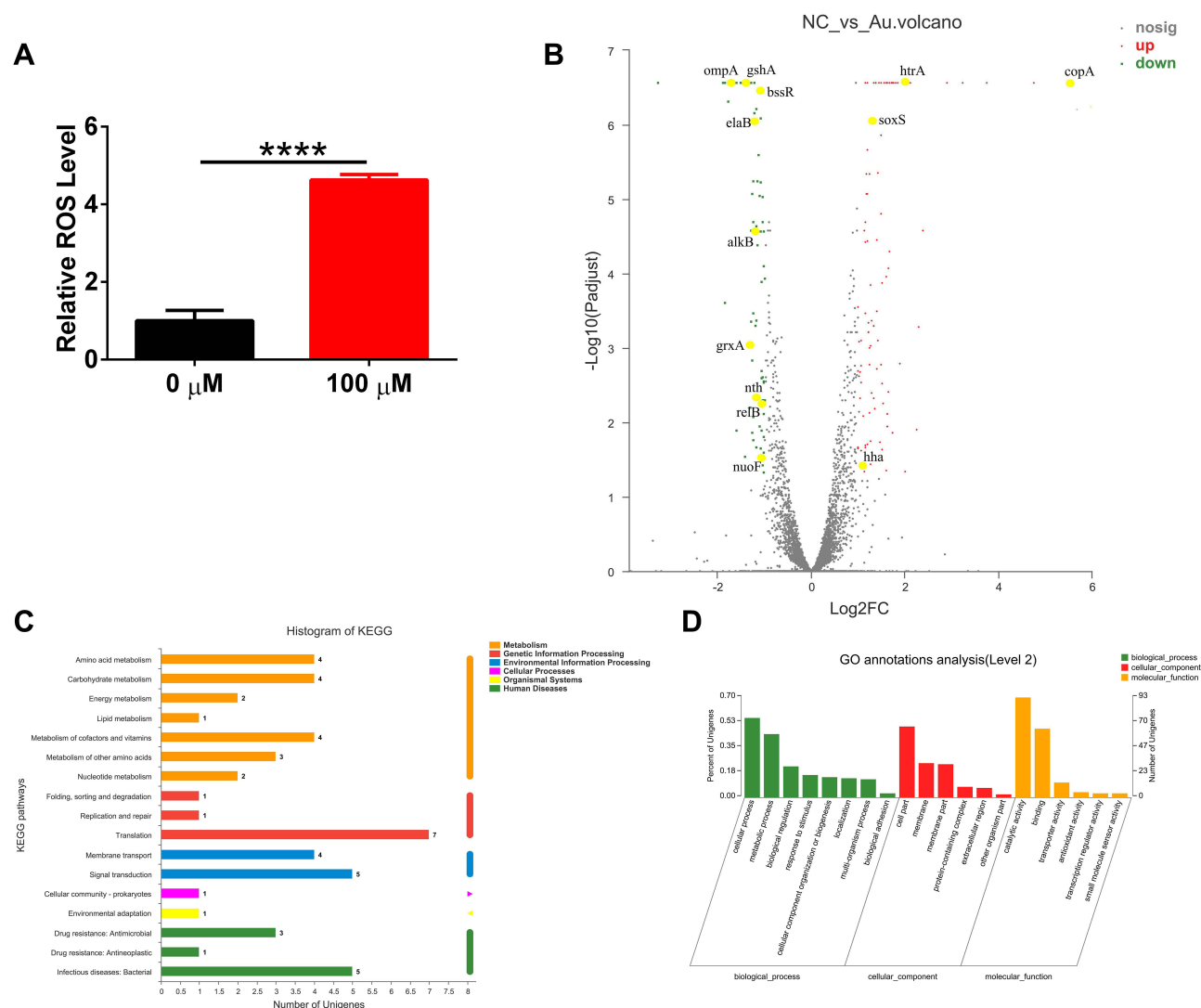


Figure 7 Au NCs affect the cellular functions of *Shigella* and kill the cells. Au NCs induced an increase in intracellular ROS levels (A). Volcano map of changes in ROS metabolism-related gene expression after Au NC treatment. Red indicates that the gene is upregulated, and green indicates that the gene is downregulated (B). Diagram of the KEGG analysis of genes related to cell metabolism, substrate transport, membrane integrity and cell transcriptome processes after Au NC treatment (C). After the Au NC treatment, a GO analysis of genes related to cell metabolism, substrate transport, membrane integrity and cell transcriptome processes was performed (D). Data are presented as the means \pm SEM (n=6). Student's *t*-test was used for statistical analyses, and *p*-values are as follows: *****p*<0.0001.

mediated by the free radicals generated in response to Au NCs, which is a characteristic of metals.^{36,43} Most interestingly, scientists have shown that the stronger effect of Au NCs on gram-negative bacteria is because gram-negative bacteria lack the hard cell wall network structure of the peptidoglycan layer of gram-positive bacteria,⁴⁴ making them more susceptible to mechanical damage by Au NCs that further interact with the membrane. *Shigella* is exactly this type of bacteria.

We established *Shigella*-induced colitis mouse models and compared the therapeutic effects of Au NCs between the SF group and SF-Au NC group to prove the feasibility of Au NCs. Judging from the clinical signs of the mice,

including the mouse body weight, DAI, and colon length, the mice almost recovered their health after treatment with Au NCs. We investigated the curative effect more deeply. The tissue damage score basically returned to normal. The intestinal mucosa and epithelial cells damaged by *Shigella* gradually recovered in mice treated with Au NCs. The increase in goblet cells was evidence of recovery (the mucus secreted by goblet cells provides the protective layer of the intestinal barrier).⁴⁵ The bacterial load in mouse faeces also decreased significantly after treatment with Au NCs. In individuals with colitis, the content of LCN2 is usually increased, and LCN2 exerts an anti-inflammatory effect.⁴⁶ Au NCs reduced the faecal LCN2

content, which further proves that Au NCs represent a treatment for *Shigella*-induced colitis in mice.

We then conducted an in-depth study of the antibacterial mechanism of Au NCs. Surprisingly, Au NCs affected *Shigella* biofilms and reduced their activity. Biofilm refers to a special bacterial structure attached to the surface of an object and wrapped by bacterial extracellular macromolecules. Biofilms protect invading bacteria from being discovered by the immune system. In addition, biofilms cause chronic infections. Moreover, ordinary antibiotics do not penetrate the biofilm to attack *Shigella*. The existence of biofilms makes bacteria 1000 times more resistant.^{47–49} Biofilms are an obstacle that must be overcome in the treatment of *Shigella* infection. Studies have shown that nanoparticles prevent the production of biofilms,⁵⁰ and thus we explored whether Au NCs affected *Shigella* biofilms. The results proved the unexpected antibacterial power of Au NCs against *Shigella*. Au NCs are also expected to pass through the biofilms of *Pseudomonas aeruginosa* and *Escherichia coli* to kill pathogens,^{51,52} potentially because tiny particles destroy the stability of the biofilm through some mechanisms.⁵³ We further explored the mechanism by focusing on ROS, which are essential for antibacterial activity. Many effective antibiotics achieve antibacterial effects by increasing the ROS level in bacteria.⁵⁴ ROS include superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^{\cdot}), singlet oxygen, and lipid hydroperoxides. Among them, H_2O_2 plays an important antibacterial role;⁵⁵ therefore, we used DCFH reagent to detect the ROS H_2O_2 . The mechanism of antibiotics is to interfere with the tricarboxylic acid cycle through the electron transport chain, thereby affecting cellular metabolism and inducing ROS generation.⁵⁶ Our detection of the metabolic gene abundance also proves this pathway. ROS are a natural by-product of energy produced by mitochondria that play an important role in cellular signal transduction and homeostasis. However, during periods of environmental stress, ROS levels increase substantially,⁵⁷ especially H_2O_2 , and bacteria must use H_2O_2 to balance stimulation.⁵⁸ However, once ROS are released in large quantities and exceed a certain limit, they produce oxidative stress, killing bacteria by interfering with proteins, DNA, lipids and other macromolecular substances.^{59,60} In addition, ROS can control the bacterial load and destroy biofilms, and studies have proven that nanoparticles have the characteristics of antimicrobial drug resistance, which mainly depends on ROS.⁶¹ The antimicrobial drug resistance of Au NCs also mainly depends on

ROS, and studies have shown that ROS even become an antibacterial substance alone.⁶² Moreover, the potential healing effect of Au NCs on a variety of cancer cells, such as prostate cancer,^{63,64} is mostly based on ROS. We quantitatively detected ROS-related genes and observed decreased expression of *ompA*, *gshA*, *bssR*, *elaB*, *alkB*, *grxA*, *nth*, *relB*, *nuoF* and other antioxidant genes,^{65–72} whereas the expression of *copA*, *htrA*, *soxS*, *hha* and other oxidative stress genes^{71,73–77} was upregulated. These changes also contribute to the mechanism by which Au NCs induce ROS production.

Overall, in the era of antibiotic abuse and universal drug resistance of pathogenic microorganisms, Au NCs will have a place in the treatment of microbial infections in the near future due to their antibacterial activity and antidrug resistance, as well as their biological safety. Au NC treatment of *Shigella* infectious diseases is expected in the near future. However, further experimental studies are needed to determine whether Au NCs are resistant to other probiotics in vivo and the dosage of Au NCs.

Abbreviations

NCs, nanoclusters; SEM, scanning electron microscope; DAI, disease activity index; LCN2, lipocalin-2; ROS, reactive oxygen species; *S. flexneri*, *Shigella flexneri*; SPR, surface plasmon resonance; MHA, mercaptohexanoic acid; TEM, transmission electron microscopy; DLS, dynamic light scattering; UV-Vis, ultraviolet-visible; TSB, Trypticase (Tryptic) Soy Broth; PBS, phosphate buffered solution; NC, normal control group; SF, *S. flexneri* infection group; SF-Au NCs, Au NCs treatment group; H&E, hematoxylin-eosin; EP, eppendorf; CFU, colony-forming unit; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology.

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Disclosure

The authors declare no conflict of interest.

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