Electronic Supporting Information

Levonorgestrel protected Au_8 and Au_{10} clusters with different Antimicrobial Ability

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Experimental section

Materials

Levonorgestrel was purchased from Hubei Goto Biopharm Co., Ltd. Me_2SAuCl was prepared according to a previously reported literature.[1] Dichloromethane (DCM), acetonitrile (CH_3CN), triethylamine (Et_3N), ethanol (C_2H_5OH) and dimethyl sulfoxide (DMSO) were obtained from Tianjin Fengchuan Chemical Reagent Co., Ltd., and used without any additional purification. All solvents were analytical grade reagents. Melatonin was purchased from Shanghai Macklin Biochemical Co., Ltd. Sodium chloride (NaCl) was purchased from China National Medicines Co., Ltd. Phosphate-buffered saline (PBS), Trypsin-EDTA solution were purchased from Beijing Solarbio Science & Technology Co., Ltd. HeLa and NG108-15 cells were obtained from the China Center for Type Culture Collection (Wuhan, P.R. China). RPMI-1640 and DPBS (no calcium, no magnesium) were purchased from Mediatech, Inc., a Corning subsidiary. Trypsin-EDTA solution, agar and reduced GSH assay kit were purchased from Beijing Solarbio Science & Technology Co., Ltd. Fetal bovine serum (FBS) was purchased from Zhejiang Tianhang Biotechnology Co., Ltd. Cell culture plates were purchased from Nest Biotechnology Co., Ltd. Cell Counting Kit-8 (CCK-8) and GR assay kit were purchased from Beyotime Biotechnology Corporation. Tryptone, yeast extract, LIVE/DEAD™

BacLight™ Bacterial Viability Kit and CellROX® Deep Red Reagent were purchased from Thermo Fisher Scientific. Milli-Q grade water (18.2 MΩ) was used to prepare solutions throughout the experiments.

Instruments

UV–vis spectroscopy was conducted using a Hitachi UH4150 UV-Vis-NIR spectrophotometer. Steady-state excitation and emission spectra of the compounds were analyzed using a Horiba FluoroLog-3 spectrofluorometer. ESI-TOF-MS spectra of the Au_8NCs and Au_{10}NCs were acquired using an AB Sciex X500R Q-TOF spectrometer. Fourier transform infrared (FT-IR) measurements were conducted on a Bruker ALPHA II spectrometer in the range of 4000-400 cm^{-1}. TEM images were acquired with a FEI Talos F200 transmission electron microscope. Confocal imaging was performed with a Leica TCS SP8 confocal fluorescence microscope. ICP-MS analysis was performed with inductively coupled plasma mass spectrometer (PerkinElmer NexION 300X). Optical density at 600 nm (OD_{600}) of bacterial cells and absorbance of dyes were measured on multifunctional microplate reader PerkinElmer EnSpire™ HH3400.

Synthesis of Au_8NCs and Au_{10}NCs

The synthesis of Au_8NCs and Au_{10}NCs was according to a reported

method.[2, 3] Levonorgestrel (78 mg, 250 μmol) was dissolved in a mixed system of 20 mL of dichloride and acetonitrile (DCM/CH_3CN = 1:1) and then 80 μL neat NEt_3 and Me_2SAuCl (74 mg, 250 μmol) were added under stirring to yield a yellow-green transparent solution. The resultant solution was stirred for 5 min and allowed to evaporate slowly in the dark at room temperature for 1 day to yield yellow-greenish block crystals (Au_8NC). Au_8NC (10 mg) crystals was dissolved in DMSO (3 mL) in an oven at 45 ℃ for six days to get yellow flake crystal $Au_{10}NC$.

Bacterial Cell Culture

Escherichia coli (ATCC 8739) and *Staphylococcus aureus* (ATCC 6538) were cultured in an orbital shaker at 37 °C and 220 rpm. For each experiment, some colonies from the LB agar plate were transferred to 5mL liquid LB medium and cultured at 37 °C and 220 rpm for 12 h. When the logarithmic growth phase was reached, the bacterial solution was diluted to the required concentration with normal saline.

Minimum Inhibitory Concentration (MIC) Determination

MIC of Au_8NCs and $Au_{10}NCs$ were determined using a broth microdilution method. Briefly, bacterial cells were cultured overnight at 37 °C to logarithmic growth phase and then were collected by centrifugation at 5000 rpm for 5 min, and resuspended with sterile saline. The bacterial suspension obtained was diluted to 10^6 CFU mL^{-1} in liquid LB medium for later use.

Add 50 μL of preconfigured LB medium with different concentrations of Au_8NCs or Au_{10}NCs, and add 50 μL of prepared bacterial suspension to the 96-well plate. The mixed components of blank LB medium and bacterial suspension were used as control group. The absorbance of bacterial suspension at 600 nm at 0 h was measured with a multifunctional microplate reader. After that, the plate was placed in an orbital shaker at 37 ℃ and 220 rpm for 18 h. Then the absorbance of bacterial suspension at 600 nm (OD_{600}) was measured by multifunctional microplate reader for 18 h culture, and the inhibition rate of Au_8NCs and Au_{10}NCs at different concentrations was calculated according to the following formula, and the minimum inhibitory concentration (MIC) and half inhibitory concentration (IC_{50}) were obtained by regression analysis with SPSS software.

$$\text{Inhibition rate} = 1 - \frac{S_t - S_0}{C_t - C_0} \times 100\%$$

where S_0 and S_t represent the OD_{600} values before and after AuNCs treatment of bacteria, respectively; C_0 and C_t represent the OD_{600} values before and after bacteria in the blank control group, respectively.

Growth Curve of Bacteria Treated with Au_8NCs and Au_{10}NCs

LB medium and bacterial solution (50 μL, 10^6 CFU mL^{-1}) containing Au_8NCs, Au_{10}NCs (50 μL, 240 μg mL^{-1}) and Ligands (50 μL, 147 μg mL^{-1}) were successively added to the 96-well plate. The mixed components of

blank medium and bacterial solution without any materials were used as the control group, and the mixtures were incubated in an orbital shaker at 37 ℃ and 220 rpm. The OD_{600} value of the mixed bacterial solution was recorded by multifunctional microplate reader with a time interval of 2 h, which was used to analyze the inhibition effect of the nanoclusters and ligands on bacterial growth. All experiments were repeated three times.

Colony-Forming Analysis

The experiment was divided into four groups: bacteria, bacteria + Au_8NCs, bacteria + Au_{10}NCs, bacteria + Ligands. The final concentrations of Au_8NCs, Au_{10}NCs were 120 μg mL^{-1} and the final concentration of bacteria was 10^6 CFU mL^{-1}. The above treatments were incubated at 37 °C under 220 rpm for 8 h. The mixed components of blank medium and bacterial solution without any materials were used as the control group. Then, the bacterial suspension was diluted 10^6 times, and the 100 μL diluted bacterial suspension was spread on solid medium and cultured at 37 °C for 24 h. All the above experiments were repeated three times.

Cell Uptake of Au_8NCs and Au_{10}NCs

The bacteria (10^8 CFU mL^{-1}) were treated with Au_8NCs and Au_{10}NCs (120 μg mL^{-1}) for 12 h and then washed with PBS three times to remove any residual NCs species which had not been internalized by the bacteria. After that, the bacteria were collected and freeze-dried. The freeze-dried cells

were weighted, and then acid digested with aqua regia and analyzed for the metal content by ICP-MS. The measured Au content was converted to cluster content and then normalized back to the dry weight of the cell pellet.

SEM Observation

0.5 mL bacterial suspensions (10^6 CFU mL^{-1}) were treated with 0.5 mL of AuNCs medium suspension (240 μg mL^{-1}) and ligands medium suspension (147 μg mL^{-1}) for 2 h at 37 °C. The mixed components of blank medium and bacterial solution without any materials were used as the control group. Then the bacteria were collected and washed with PBS buffer, and fixed with 2.5% glutaraldehyde for 4 h at 4 °C. After being washed with PBS, bacterial cells were dehydrated by sequential treatment with 50%, 70%, 80%, 90%, 95% and 100% ethanol for 15 min each. Then, a drop of 5 μL dehydrated bacterial cell suspension was added on a silica wafer and airdried. Finally, the morphology of the samples was observed using SEM.

Live/Dead Imaging

The bacteria cells (10^6 CFU mL^{-1}) were treated with Au_8NCs, Au_{10}NCs (120 μg mL^{-1}) and ligands (74 μg mL^{-1}) for 2 h at 37 °C. The mixed components of blank medium and bacterial solution without any materials were used as the control group. And then bacteria were collected. The viability of bacterial cells was determined using LIVE/DEAD BacLight Bacterial Viability Kit which contained PI and SYTO9 dye. Briefly, cells

were incubated with LIVE/DEAD reagent for 30 min at 37 °C, then were imaged using confocal laser scanning microscopy.

In Situ Detection of ROS Production

ROS production in bacterial cells treated with Au NCs was detected using the ROS indicator CellROX® Deep Red Reagent. Briefly, bacteria were treated with Au_8NCs, Au_{10}NCs (120 μg mL^{-1}) and ligands (74 μg mL^{-1}) for 2 h at 37 °C. The mixed components of blank medium and bacterial solution without any materials were used as the control group. Then, bacterial cells were washed with PBS, followed by the addition of CellROX® Deep Red Reagent at a final concentration of 5 μM and incubated at 37 °C for 1 h. Next, cells were centrifuged at 10000 rpm for 2 min and washed with PBS for three times. Then, intracellular ROS levels in cells were imaged using confocal laser scanning microscopy with excitation wavelength at 647 nm.

Melatonin Rescue Experiment

Different concentration of AuNCs and melatonin were added to a 96-well plate respectively. Then, Bacteria (10^6 CFU mL^{-1}) were introduced into the mixtures. Next, the plate was placed in the orbital shaker (220 rpm) at 37 °C. Among them, *E. coli* and *S. aureus* were incubated for 8 h and 24 h, respectively. After incubation, the OD_{600} value of each component was recorded with a microplate reader, and the survival rate of bacteria was

calculated according to the following formula：

$$\text{Bacterial survival (\%)} = \frac{S_t - S_0}{C_t - C_0} \times 100\%$$

where S_0 and S_t represent the OD_{600} values before and after AuNCs treatment of bacteria, respectively; C_0 and C_t represent the OD_{600} values before and after bacteria in the blank control group, respectively.

Detection of Enzymatic Activity of GR and GSH Levels in Bacteria

Bacteria (10^9 CFU mL^{-1}) were treated with different concentration of Au_8NCs and Au_{10}NCs (0, 150, 300, 450, 600 μg mL^{-1}) for 2 h at 37 °C and 220 rpm. Following treatment, bacteria were centrifuged at 12000 rpm for 5 min, washed with PBS twice, the collected bacteria were resuspended in 50 mM Tris-HCl buffer (pH = 8), and lysozyme was added to make the final concentration reach 1mg mL^{-1}. After an ice bath for 1h, bacteria solution was lysed by ultrasonic crushing for 5 min at 300 W (10 s pulse and 10 s rest) under an ice bath. The obtained lysed cells were centrifuged again and the clear supernatant was collected for the following analysis. The enzymatic activity of GR was identified using a GR assay kit. For GSH detection, bacteria solution was lysed by ultrasonic crushing for 10 min at 400 W (3 s pulse and 7 s rest) under an ice bath. The centrifuged supernatant was used for GSH detection. The GSH levels were quantified using a reduced GSH assay kit.

Cytotoxicity Assay of Au_8NCs and Au_{10}NCs

Cell toxicity of Au_8NCs and Au_{10}NCs was evaluated by CCK-8 assay. Briefly, Hela cells were seeded into 96-well plate at a density of 2×10^4 cells per well. Then, the 96-well plates were placed in the incubator for 12 h at 37 °C and 5% CO_2. Next, supernatant was removed, and different doses of Au_8NCs and Au_{10}NCs dissolved in medium (0, 5, 10, 25, 50 and 100 μg mL^{-1}) were added to the cells and incubated for 24 h. After 24 h, a 10% volume of CCK-8 solution in medium was introduced into the cells. After incubation for 20 min at 37 °C, the absorption value at 450 nm of each well was measured using a multifunctional microplate reader. The cytotoxicity of different treatment groups was calculated as $(A_{test}/A_{control}) \times 100$.

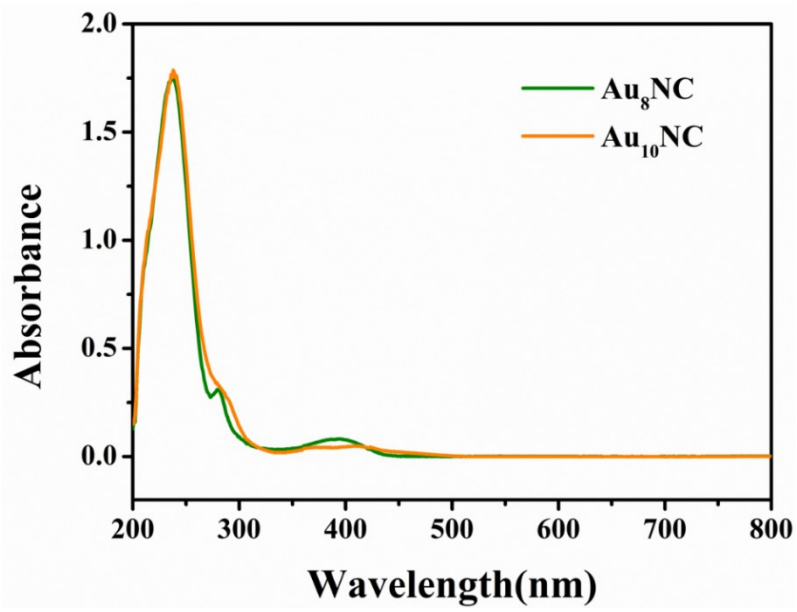

Fig. S1 UV-vis spectra of Au_8NCs and Au_{10}NCs.

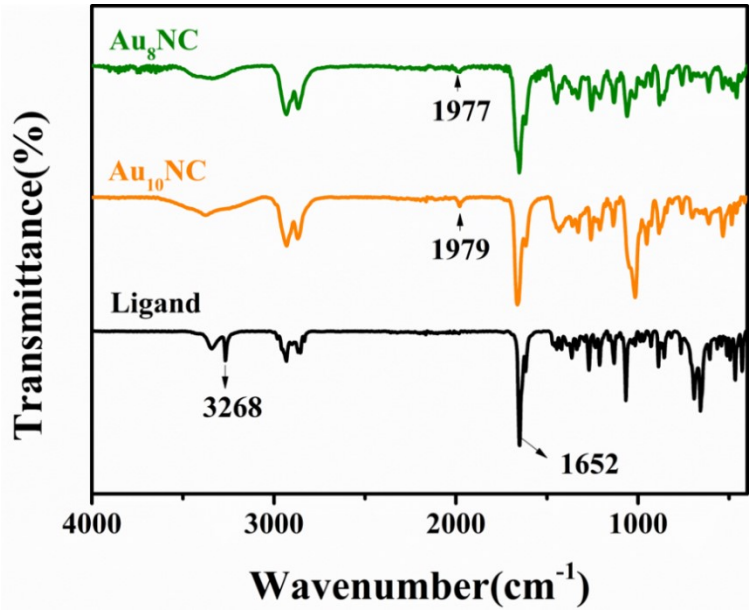

Fig. S2 FT-IR analysis of Au_8NCs and Au_{10}NCs.

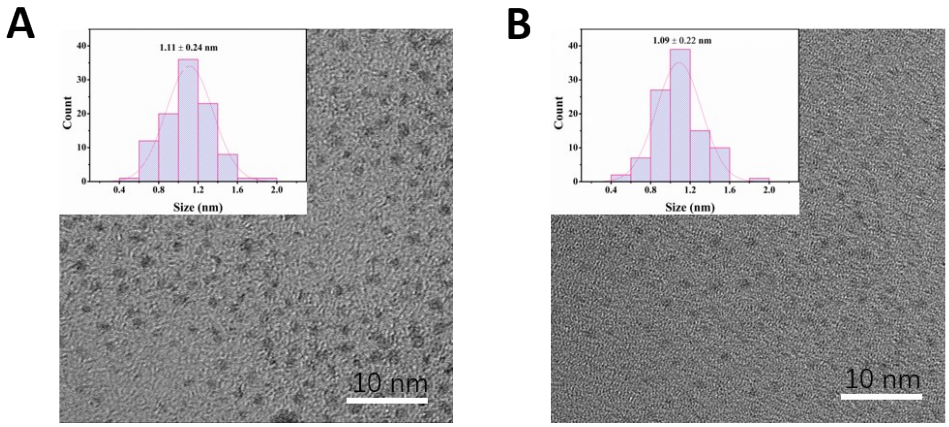

Fig. S3 Typical TEM images and corresponding size distribution histograms of (**A**) Au_8NCs and (**B**) Au_{10}NCs.

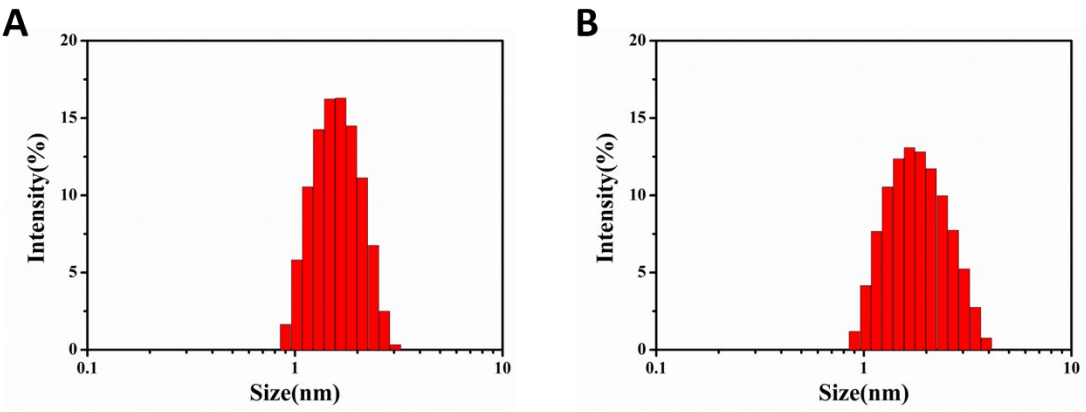

Fig. S4 Hydrodynamic size distribution of (**A**) Au_8NCs and (**B**) Au_{10}NCs in PBS buffer.

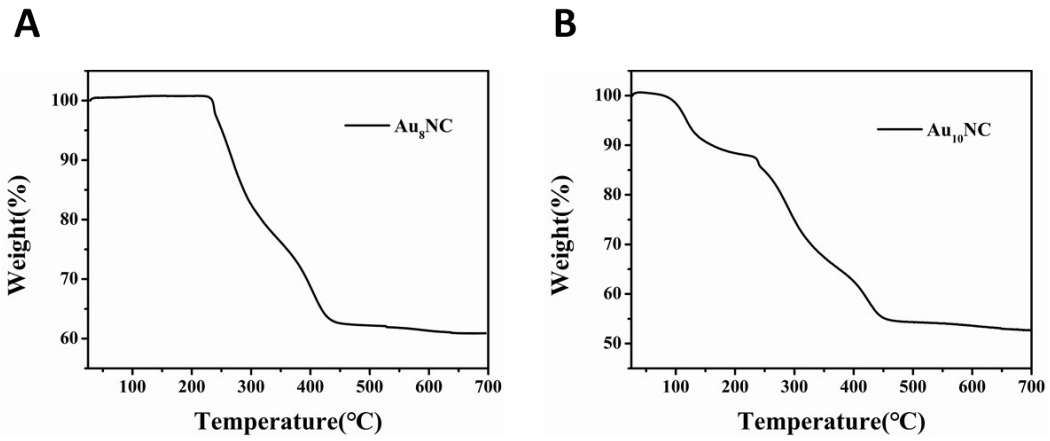

Fig. S5 The TG curve of the as-synthesized (**A**) Au_8NCs and (**B**) Au_{10}NCs.

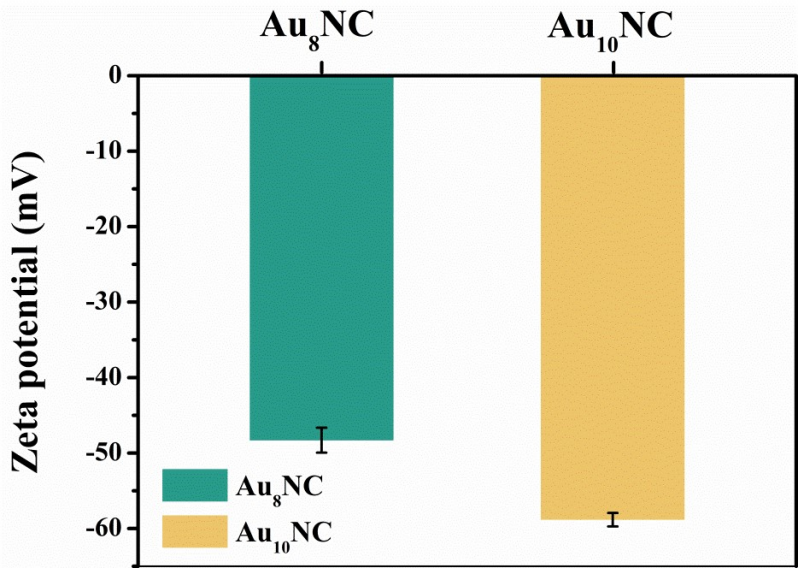

Fig. S6 Zeta potential of Au_8NC and $Au_{10}NC$ in aqueous solution.

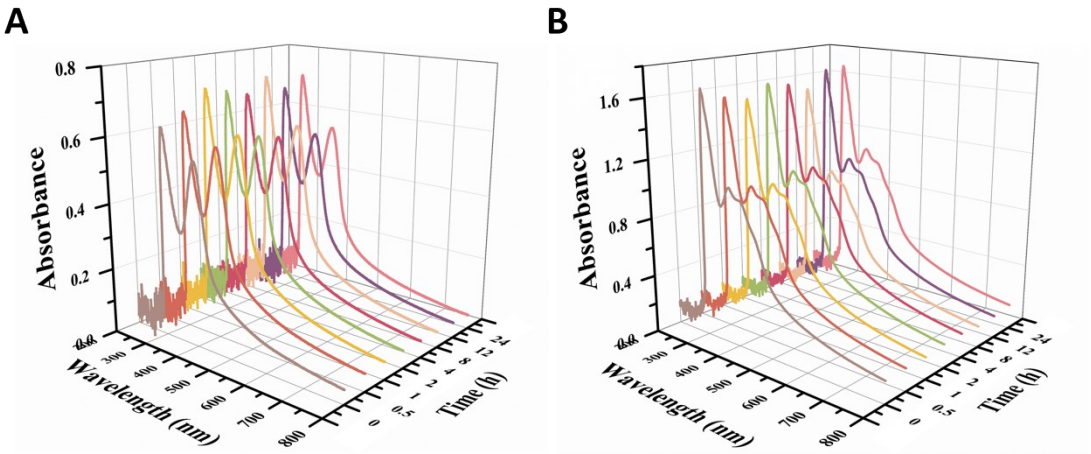

Fig. S7 Time-course UV−vis absorption of (**A**) Au_8NCs and (**B**) $Au_{10}NCs$ in medium.

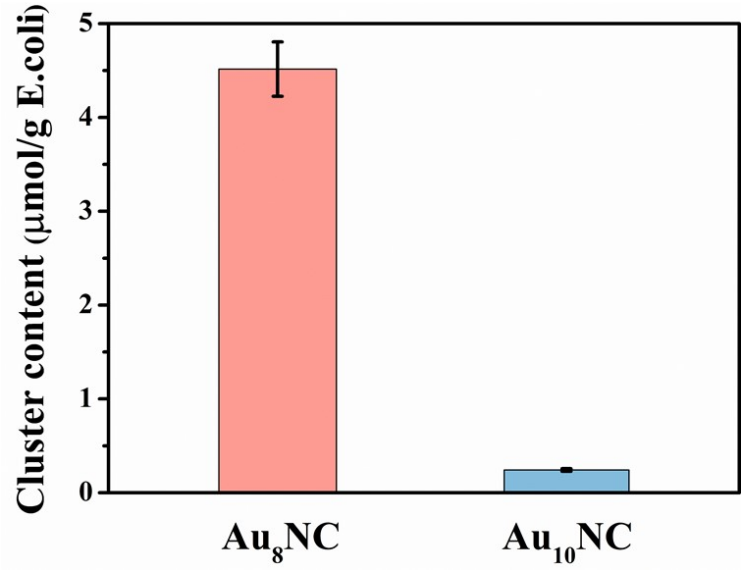

Fig. S8 Bacterial uptake results of *E. coli* for Au_8NCs (red column) and Au_{10}NCs (blue column) after incubation of 12 h based on ICP-MS analysis.

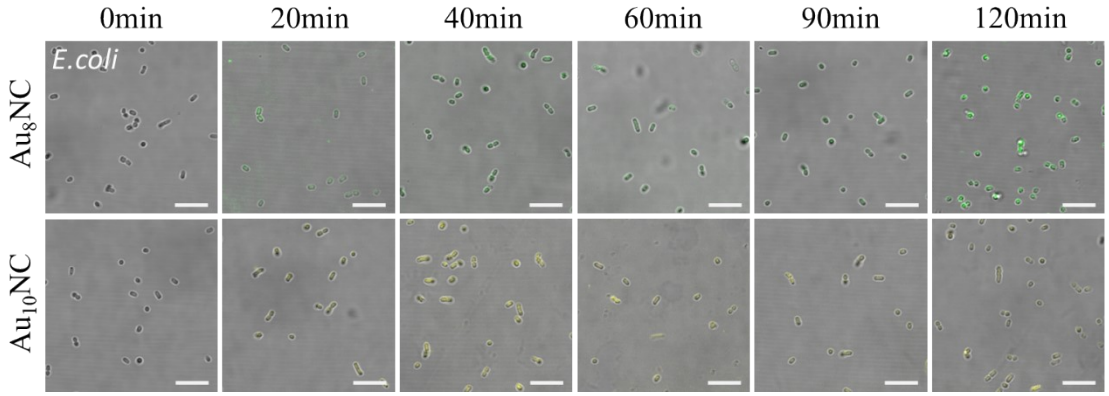

Fig. S9 Confocal images of *E. coli* incubated for 0, 20, 40, 60, 90 and 120 min after treated with Au_8NCs and Au_{10}NCs. Scale bar: 7 μm.

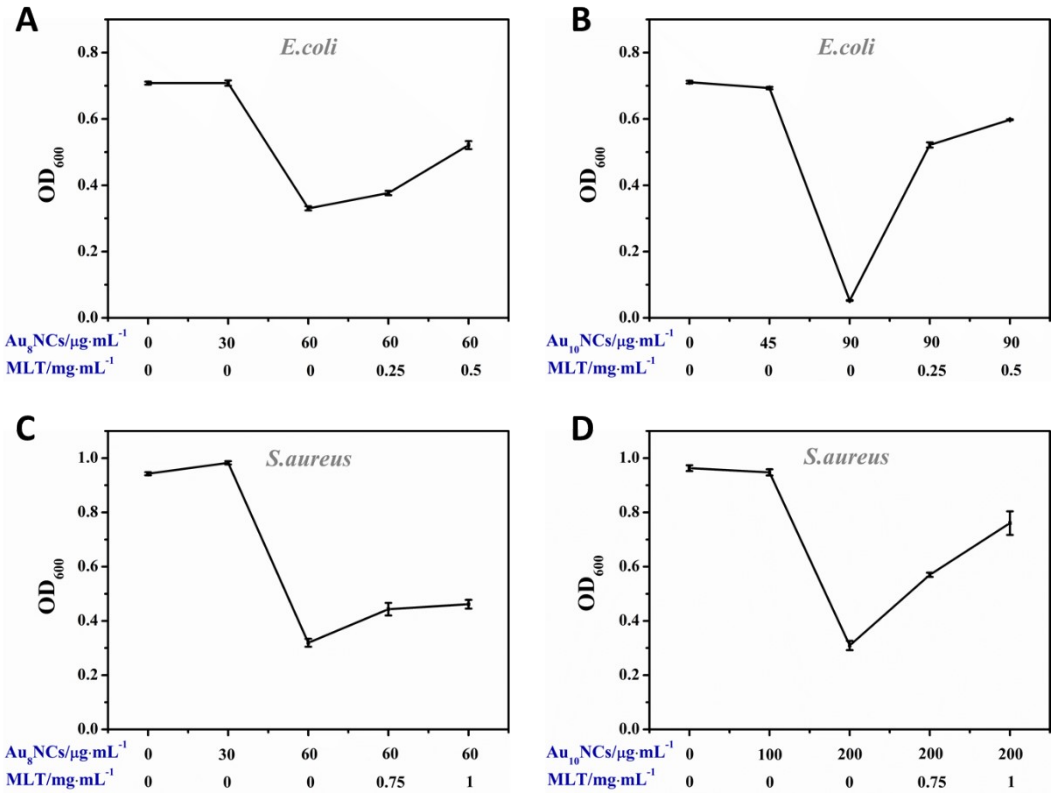

Fig. S10 Inhibition of different concentrations of melatonin on the growth of *E. coli* with (**A**) Au_8NCs and (**B**) Au_{10}NCs; Inhibition of different concentrations of melatonin on the growth of *S. aureus* with (**C**) Au_8NCs and (**D**) Au_{10}NCs.

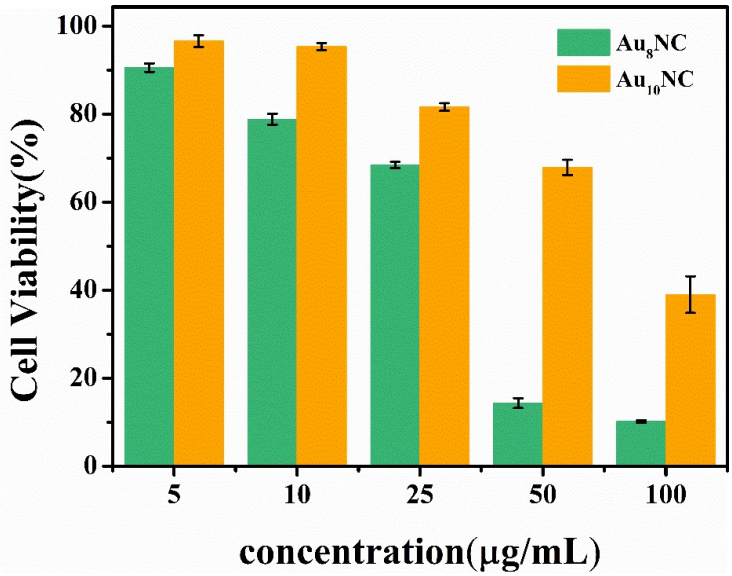

Fig. S11 Viability of Hela cells incubated with different concentrations of Au_8NCs and Au_{10}NCs.

Table S1 EA (%) data of Au_8NC and Au_{10}NC.

Sample	Element	Calculated (%)	Experimental (%)
Au_8(Levonorgestrel)$_8$	Carbon	49.61	49.88
	Hydrogen	5.35	5.30
Au_{10}(Levonorgestrel)$_{10}$	Carbon	49.61	49.19
	Hydrogen	5.35	5.32

Table S2 Minimum inhibitory concentrations (MICs) of Au_8NCs and Au_{10}NCs.

Bacterial	Au_8NC	Au_{10}NC
E. coli	16.077μg/mL	125.687μg/mL
S. aureus	460.654μg/mL	978.068μg/mL

Table S3 Half inhibitory concentration (IC_{50}) of Au_8NCs and Au_{10}NCs.

Bacterial	Au_8NC	Au_{10}NC
E. coli	10.042 μg/mL	111.026 μg/mL
S. aureus	75.655 μg/mL	382.901 μg/mL

1. T. N. Hooper, C. P. Butts, M. Green, M. F. Haddow, J. E. McGrady and C. A. Russell, *Chem. Eur. J.*, 2009, **15**, 12196-12200.
2. T. T. Jia, G. Yang, S. J. Mo, Z. Y. Wang, B. J. Li, W. Ma, Y. X. Guo, X. Chen, X. Zhao, J. Q. Liu and S. Q. Zang, *ACS Nano*, 2019, **13**, 8320-8328.
3. M. M. Xu, T. T. Jia, B. Li, W. Ma, X. Chen, X. Zhao and S. Q. Zang, *Chem. Commun.*, 2020, **56**, 8766-8769.