# Electronic Supporting Information

## Bimetallic Nanoparticles against Multi-Drug Resistant Bacteria

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## Experimental Section

### 1. Materials

$HAuCl_4 \cdot 4H_2O$, $Na_3RhCl_6 \cdot 12H_2O$, $H_2IrCl_6 \cdot 6H_2O$, $RuCl_3$, and $OsCl_3 \cdot 3H_2O$ are from Beijing Innochem technology co. LTD, China. $CuCl_2$, $NiCl_2$, $CoCl_2$, $FeCl_3$, and $CuCl_3$ are from Beijing Chemical Works. We purchase the standard strains, *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. a*), and *Klebsiella pneumoniae* (*K. p*), from Beijing Runzekang Biotechnology co. LTD China. MDR strains we used including multidrug-resistant *Escherichia coli* (MDR *E. coli*), multidrug-resistant *Klebsiella pneumoniae* (MDR *K. p*), polymyxin-resistant *Klebsiella pneumoniae* (PR *K. p*), polymyxin-resistant *Pseudomonas aeruginosa* (PR *P. a*) and polymyxin-resistant *Escherichia coli* (PR *E. coli*). We purchase ATP assay kit and total ROS assay kit from Beyotime Institute of Biotechnology, China. We obtain the cell counting kits (CCK-8) from Beijing Solarbio Life Sciences Technology Co. LTD.

### 2. Synthesis of Bimetallic NPs

We take 250 μL of $HAuCl_4 \cdot 4H_2O$ (0.1 mM) and 250 μL of the second metal salt solutions (0.1 mM) including $Na_3RhCl_6 \cdot 12H_2O$, $H_2IrCl_6 \cdot 6H_2O$, $RuCl_3$, $OsCl_3 \cdot 3H_2O$ $CuCl_2$, $NiCl_2$, $CoCl_2$, $FeCl_3$ or $CuCl_3$, and add them into a round-bottomed flask with 10 mL $H_2O$ in an ice-water bath. We add 50 mg Tween 80 as stabilizer into the mixture and stir it at 800 rpm for 20 min. We adjust the stirring speed to 1500 rpm and dropwise add 2.5 mL $NaBH_4$ (2.4 mg/mL) as a reductant. After 1 h, we put the liquid in the dialysis bag (14 kDa MW cut off, Millipore) and dialyze it with deionized water for 24 h. We use a 0.22 μm filter (Millipore) to sterilize the liquid and store it at 4 $^oC$. We synthesize bimetallic NPs with different proportions (50:1, 20:1, 10:1, 5:1, 4:1, 2:1, 1:1, 1:2, 1:4, 1:5, 1:10) using the same method by keeping the amount of $HAuCl_4 \cdot 4H_2O$ (0.1 mM) of 250 μL.

### 3. Antibacterial and Bactericidal Properties of Bimetallic NPs

We estimate the antibacterial activities of bimetallic NPs by measuring the minimum inhibitory concentration (MIC) using the microtiter broth dilution method. We dilute the bimetallic NPs for 2-128 times in 96-well plates and add 10 μL bacterial suspensions ($10^4$~$10^5$ CFU/mL) in each well. We record the MICs after incubating the plate at 37 °C for 24 h. The concentration of bimetallic NPs is the total concentration of two metals (Au and Rh/Ru). In this work, we use the concentration of bimetallic NPs to calculate the MICs.

To further evaluate the antibacterial activity of bimetallic NPs, we conduct the turbidity assay in Luria-Bertani (LB) liquid medium. We culture bacteria (*E. coli*, *K. p*, MDR *E. coli*, MDR *K. p*, PR *E. coli* and PR *K. p*) treated with AuRh NPs (28 μg/mL), monometallic NPs (40 μg/mL) and three antibiotics (polymyxin B, imipenem and penicillin, 16 μg/mL) at 37 °C. After 12 h, we observe the turbidity and measure optical density at 600 nm ($OD_{600}$) of the bacterial suspensions by a microplate reader. The group without LB liquid medium is the control group.

We choose MDR *E. coli* to test the bactericidal abilities of bimetallic NPs. We use PBS to wash the overnight culture bacteria and re-suspend the bacteria to an $OD_{600}$ is 0.5. We dilute the bacteria 1000 times with PBS, and add different concentrations of AuRh (0, 1, 2, 4, 8, and 16 x MIC) into bacterial suspensions and incubate them at 37 °C. At 12 h and 24 h, we take bacterial suspensions on the agar plates and count the number of bacteria.

## 4. Metal Release

We incubate 100 μg/mL bimetallic NPs (AuRh and AuRu NPs) in $H_2O$ and PBS at 37 °C. After 96 h, we use ultrafilters (MWCO 10000 Da and 3000 Da) to centrifuge the bimetallic NPs solution in sequence. We determine the metal content of the liquid in the ultrafiltration tubes by ICP-MS (Agilent 7700x).

## 5. Characterization of Bimetallic NPs

We observe the bimetallic NPs by transmission electron microscopy (TEM, Tecnai G2 F20, FEI). The elemental concentration is tested by inductively coupled plasma optical emission spectrometry (ICP, iCAP 6300, Thermo Scientific). The surface charges of bimetallic NPs are measured by dynamic light scattering (DLS, Zetasizer Nano ZS, Malvern). We measure the UV absorbance of bimetallic NPs and monometallic NPs by UV spectrophotometer (UV-2600).

## 6. Effects of Nanoparticles on the Morphologies of Bacteria

We choose *E. coli* as an example to observe the morphologies of bacteria treated with bimetallic NPs. We incubate bacteria onto sterile coverslip at 37 $^{o}$C. After 12 h incubation, we add AuRh NPs (20 μg/mL), and AuRu NPs (40 μg/mL) at 37 $^{o}$C for 12 h. We use 2.5 % glutaraldehyde to fix the bacteria for 12 h and use graded ethanol (30, 50, 70, 80, 90 and 100 % v/v, in water) to dehydrate the bacteria. We observe the morphologies of bacteria with scanning electron microscopy (SEM, Hitachi-SU8220, Hitachi). The group without NPs is the control group.

We incubate *E. coli* treated with, AuRh NPs (20 μg/mL), and AuRu NPs (40 μg/mL) respectively. After culturing the bacterial suspensions at 37 $^{o}$C for 4 h, we centrifuge them (8000 rpm, 3 min) and remove the liquid supernatant. We fix the samples with 2.5 % glutaraldehyde for 12 h, and 0.1% osmic acid for 30 min. We wash the samples with PBS for three times and dehydrate them with graded ethanol. After the samples were polymerized, we cut the samples into superthin slice and stain them with 0.2% lead citrate and 2% uranyl acetate. The group without NPs is the control group.

## 7. Membrane Potential Test

We evaluate the membrane potential of bacteria using a membrane potential dye, $DiSC_3(5)$. We collect the bacteria and dilute bacteria with HEPES/glucose buffer (5 mM HEPES, 5 mM glucose, pH 7.0). We incubate the bacteria treated with AuRh and AuRu NPs at 37 $^{o}$C for 2 h. We stain them with $DiSC_3(5)$ for 1 h and add 0.1 M KCl into each sample. After incubating them for 1h, we detect the fluorescence intensity (Excitation: 622 nm, Emission: 670 nm) by a microplate reader. The group without NPs is the control group.

## 8. ATP Level Assay

We determine the bacterial ATP level using ATP assay kit. We collect the *E. coli* and dilute bacteria with PBS. We incubate bacteria treated with PBS (control), AuRh (1x MIC), and AuRu NPs (1x MIC) at 37 $^{o}$C. After 4 h, we collect bacteria and follow the steps of the ATP assay kit to determine the ATP level.

## 9. Total ROS Test

We determine the total ROS in bacteria treated with bimetallic NPs using the commercial ROS assay kit. We collect *E. coli* and wash them with PBS. We stain the bacteria with DCFH-DA for 30

min and wash them with PBS once. We stimulate the bacteria with PBS (control), AuRh and AuRu NPs. After 3 h, we measure the fluorescence intensity (Excitation:488 nm, Emission: 525 nm).

## 10. Cytotoxicity of Bimetallic NPs

We evaluate the cytotoxicity of bimetallic NPs using human umbilical vein endothelial cells (HUVECs). We culture HUVECs at 96-well plate (100 μL, $1\times10^5$ cells per milliliter). After the cells completely attach on the plate, we add the different concentrations (10, 20, 40 and 80 μg/mL) of AuRh NPs into the plate and incubate the plate at 37 $^{o}$C. After 24 h, we wash the cells by sterile phosphate-buffered saline (PBS) and stain them with CCK-8 (10%, v/v). We incubate the plate at 37 $^{o}$C for 2 h and measure the values of $OD_{450}$ by a microplate reader (Tecan infinite M200).

## 11. Hemolysis Test

We test the hemolysis of bimetallic NPs using the blood of rats. We dilute the blood by 10 times with normal saline, collect the red blood cells (RBCs) by centrifugation 12000 rpm, 3min), and wash them with normal saline three times. We dilute the AuRh NPs with saline to different concentrations and add 120 μL the diluted bimetallic NPs into each well of a 96-well plate. The water and normal saline with equal volume are used as positive control and negative control. We added 30 μL of the diluent RBCs to each well with a final RBCs concentration of 4 %. After incubating the samples at 37 $^{o}$C for 2 h, we take the supernatant by centrifuging and measure the $OD_{567}$ of the supernatant.

## 12. Rat Wound Infection Model

We evaluate the antibacterial activities of bimetallic NPs *in vivo* via a rat wound infection model. We choose Sprague-Dawley female rats (200 g) for animal experiments, and animal experiments were approved by the Institutional Animal Care and Use Committee, Institute of Process Engineering, Chinese Academy of Sciences. We cut two round wounds with the same size (d = 2 cm) after shaking the hair on the back of the rat. We use MDR *E. coli* (~$10^8$ CFU/mL, 200 μL) to infect the wounds for 30 min. We put gauze to cover all wounds, and the left wound was added 100 μL saline as control and the right wound was added 100 μL AuRh NPs as the experimental group. After 7 and 14 days, we cut off the wound tissues and dip them in 4% paraformaldehyde. We obtain slices of the wound tissues stained by HE and Masson from Beijing Bioss Biotechnology Co. LTD., China, and photograph and analyze them.

**Table S1.** Antibacterial activities of monometallic NPs against *E. coli* and *S. aureus* (minimal inhibitory concentration, MIC, μg/mL).

| NPs | MIC [μg/mL] *E.coli* | NPs | MIC [μg/mL] *S. aureus* |
|---|---|---|---|
| Fe | >128 | Fe | >128 |
| Co | >128 | Co | >128 |
| Ni | >128 | Ni | >128 |
| Cu | >128 | Cu | >128 |
| Ru | >128 | Ru | >128 |
| Rh | >128 | Rh | >128 |
| Pd | >128 | Pd | >128 |
| Os | >128 | Os | >128 |
| Ir | >128 | Ir | >128 |
| Au | >128 | Au | >128 |

**Table S2**. The release amount and percentage of metal ions from 100 μg/mL of AuRh and AuRu NPs in $H_2O$ and PBS at 37 °C for 96 h.

| **Medium** | **NPs** | **Au release, (ng/mL)** | **Rh/Ru release, (ng/mL）** | **Au, %** | **Rh/Ru, %** |
|---|---|---|---|---|---|
| In $H_2O$ | AuRh | 0.23 | 31.33 | 0.00023 | 0.03133 |
| | AuRu | 0.31 | 5.16 | 0.00031 | 0.00516 |
| In PBS | AuRh | 0.22 | 32.82 | 0.00022 | 0.03282 |
| | AuRu | 0.32 | 4.98 | 0.00032 | 0.00498 |

**Table S3.** Antibacterial properties of different proportions of AuRu NPs against Gram-negative bacteria (minimal inhibitory concentration, MIC, μg/mL)

| NPs | MIC [μg/mL] antibiotic-sensitive strains | | | clinical MDR isolates | | | | |
|---|---|---|---|---|---|---|---|---|
| | *E. coli* | *K. p* | *P. a* | MDR *E. coli* | PR *E. coli* | MDR *K. p* | PR *K. p* | PR *P. a* |
| $Au_{50}Ru_1$ | >128 | >128 | >128 | >128 | >128 | >128 | >128 | >128 |
| $Au_{20}Ru_1$ | >128 | >128 | >128 | >128 | >128 | >128 | >128 | >128 |
| $Au_{10}Ru_1$ | >128 | >128 | >128 | >128 | >128 | >128 | >128 | >128 |
| $Au_5Ru_1$ | >128 | >128 | >128 | 68 | >128 | >128 | >128 | >128 |
| $Au_4Ru_1$ | >128 | >128 | >128 | 30 | >128 | >128 | >128 | 60 |
| $Au_2Ru_1$ | >128 | 69 | 34 | 34 | >128 | 69 | 34 | 34 |
| $Au_1Ru_1$ | 20 | 40 | 20 | 20 | 80 | 80 | 20 | 20 |
| $Au_1Ru_2$ | 53 | 106 | 53 | 53 | 106 | 106 | 106 | 53 |
| $Au_1Ru_4$ | 77 | >128 | >128 | 77 | >128 | >128 | >128 | >128 |
| $Au_1Ru_5$ | >128 | >128 | >128 | >128 | >128 | >128 | >128 | >128 |
| $Au_1Ru_{10}$ | >128 | >128 | >128 | >128 | >128 | >128 | >128 | >128 |

**Table S4.** Zeta potentials of monometallic NPs and Bimetallic NPs in water at 25 °C.

| Feeding ratio | Zeta potential (mV) | |
|---|---|---|
| Au: X | AuRh NPs | AuRu NPs |
| Au | -9.8±0.2 | -9.8±0.2 |
| 10 : 1 | -8.7±1.1 | -5.1±1.1 |
| 5 : 1 | -9.8±1.4 | -5.0±0.3 |
| 4 : 1 | -11.7±2.6 | -5.1±0.1 |
| 2 : 1 | -11.0±1.2 | -8.7±0.6 |
| 1 : 1 | -12.0±0.9 | -15.7±1.8 |
| 1 : 2 | -12.9±1.3 | -16.1±0.8 |
| 1 : 4 | -17.7±0.9 | -15.7±1.0 |
| 1 : 5 | -17.7±1.8 | -16.5±0.9 |
| 1 : 10 | -27.6±1.0 | -15.0±1.6 |
| X | -19.6±1.3 | -19.3±0.4 |

**Table S5** Elemental molar ratio of bimetallic NPs synthesized with different feeding ratio (AuRh and AuRu NPs).

| Feeding ratio | Elements ratio | |
|---|---|---|
| Au: X | AuRh NPs | AuRu NPs |
| 10 : 1 | 10:1 | 42:1 |
| 5 : 1 | 6:1 | 14:1 |
| 4 : 1 | 5:1 | 11:1 |
| 2 : 1 | 2:1 | 4:1 |
| 1 : 1 | 1:1 | 1:1 |
| 1 : 2 | 1:2 | 1:2 |
| 1 : 4 | 1:3 | 1:4 |
| 1 : 5 | 1:5 | 1:5 |
| 1 : 10 | 1:8 | 1:8 |

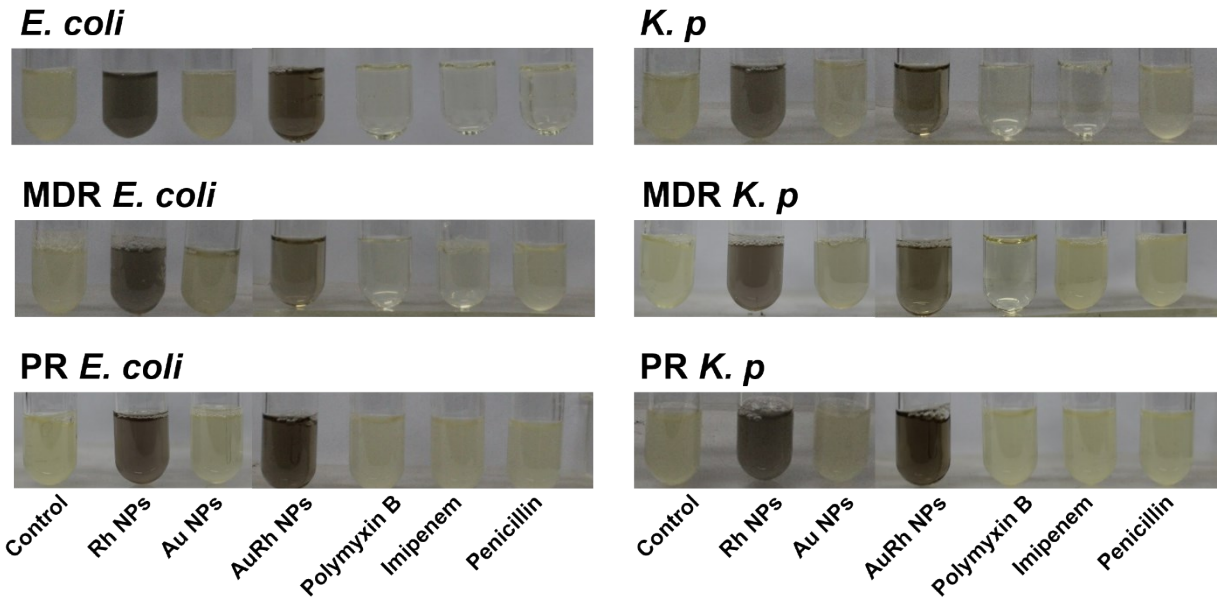


**Fig. S1** Photographs of bacterial suspensions treated with LB liquid medium, Rh NPs, Au NPs, AuRh NPs, Polymyxin B, Imipenem, Penicillin.

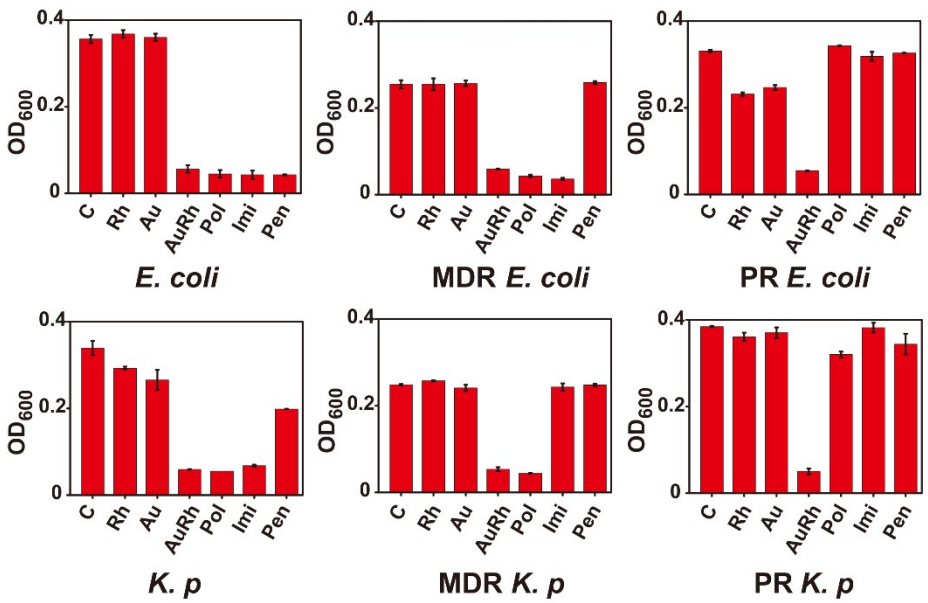


**Fig. S2** Antibacterial activities of six bacteria treated with AuRh NPs and three antibiotics. The $OD_{600}$ values of bacterial suspensions treated with, Au NPs, Rh NPs, AuRh NPs, and three antibiotics (C: control, Rh: Rh NPs, Au: Au NPs, AuRh: AuRh NPs, Pol: polymymxin B, Imi: imipenem, Pen: penicillin).

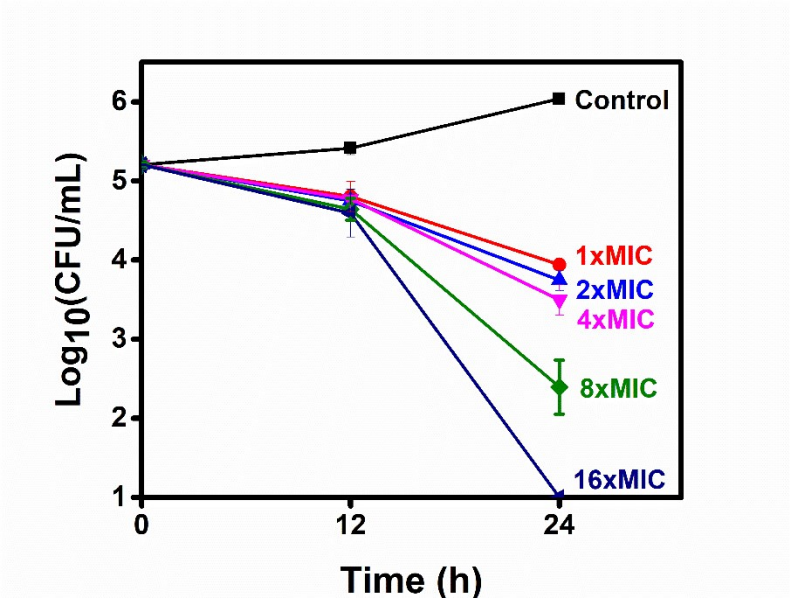


**Fig. S3** The concentration-dependent killing of MDR *E. coli* by AuRh NPs at 0, 1, 2, 4, 8, and 16 x MIC.

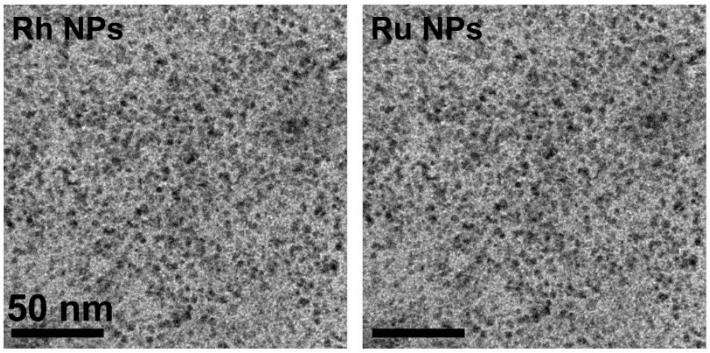


**Fig. S4** The TEM images of Rh and Ru NPs.

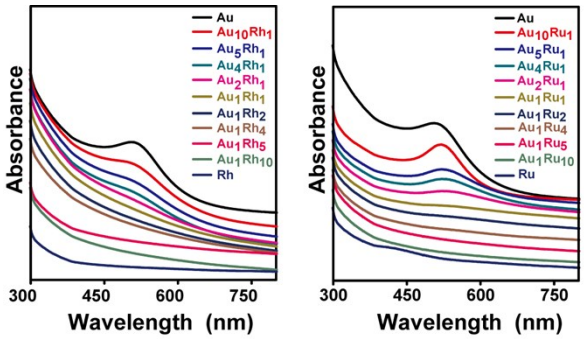


**Fig. S5** UV-Vis spectra of bimetallic NPs and monometallic NPs (AuRh, AuRu, Au, Rh, and Ru NPs).

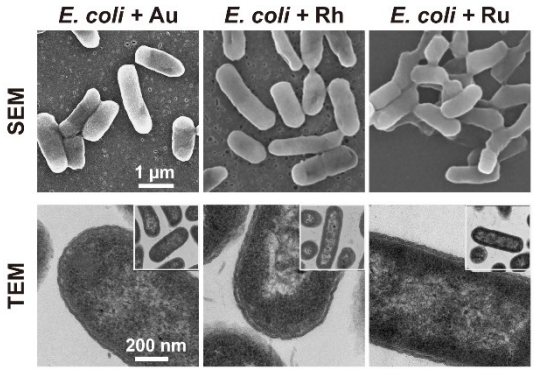


**Fig. S6** SEM and TEM images of *E. coli* treated with Au, Rh, and Ru NPs.

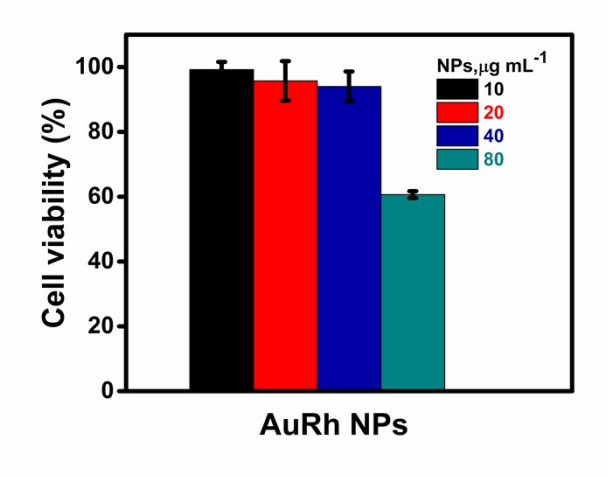


**Fig. S7** Concentration-dependent cytotoxicity of AuRh NPs cultured with HUVEC cells for 24 h and their viability were tested with a CCK-8 kit.

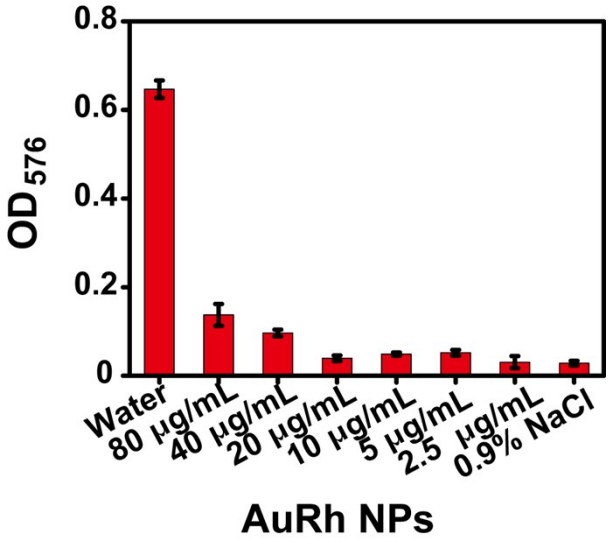


**Fig. S8** Hemolysis of different concentrations of AuRh NPs.

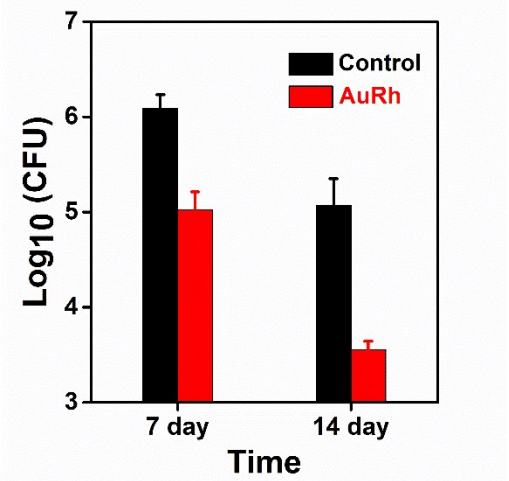


**Fig. S9** The bacteria counts at the infected wound at different time points.