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Electronic Supplementary Information

Aminophenol-modified gold nanoparticles kill bacteria with minimal ototoxicity

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Experimental

Materials

We purchased different aminophenol isomers (A, *ortho*-position (2A), *meta*-position (3A), *para*-position (4A), Mw=109.13), tetrachloroauric acid ($HAuCl_4$), tween 80, and gentamycin (Gen, Mw=1390.71) from Sigma-Aldrich (USA).

Preparation and Characterization of AGNPs

We prepared aqueous solutions of AP (0.05 mmol), triethylamine (50 μL) and tween 80 (30 mg) in 10 mL water under ice-water bath. We added $HAuCl_4$ (0.05 mmol, 400 μL) into the mixture with stirring (1000 rpm) for 2 h to synthesize AGNPs by the one-pot method. We removed the unreacted chemicals by dialyzing AGNPs in double-distilled water for 24 h and used 0.22 μm filter (Millipore) to sterilize the solution. We characterized the size and the Zeta potential of the AGNPs using a transmission electron microscopy (TEM, TECNAI G2F30, FEI company, USA) and a Zetasizer Nano ZS (Malvern Company, Malvern Company, England) respectively. We employed energy dispersive spectrometry (EDS) to characterize the elements of AGNPs. We tested the concentrations of the AGNPs by inductively coupled plasma (ICP, iCAP 6300, Thermo Scientific, USA) and calculated the weight of gold (Au) in the AGNPs. The yield of the AGNPs was calculated according to the following formula: YR (%)=(W_0-W_t)/W_0×100%, where W_0 represents the original weight of Au in the $HAuCl_4$ and W_t represents the weight of Au in the AGNPs. The purity of the AGNPs was calculated according to the following formula: PR (%)=[W/(W+W_1+W_2+W_3+W_4)]×100%, where W, W_1, W_2, W_3, and W_4 respectively represents the weight of AGNPs, $HAuCl_4$, aminophenol, triethylamine, and tween 80 in the dialysate solution.

Antibacterial Activity of AGNPs

We cultured bacteria in the Luria-Bertani (LB) medium at 37 ^oC and tested the optical density at 600 nm (OD_{600nm}) of the bacterial suspension to measure the concentration of the bacteria. We diluted the AGNPs 2-128 times and incubated with different tested bacteria (1×10^5 CFU/mL) for 24 h in 96-well plates (Corning, Costar 3599). We determined the minimal inhibitory concentration (MIC) of AGNPs by tested OD_{600nm} and set Gen (gentamycin, one of the clinical aminoglycosides) as a positive control.

AGNPs-Induced Permeability Change

We explored the antibacterial mechanism of the AGNPs by testing the permeability of bacterial cell wall and morphological change. We used 4AGNPs (16 μg/mL) to treat bacteria at 37 °C for 4 h, stained the bacteria with propidium iodide (PI, excitation wavelength: 568 nm, excitation wavelength: 633 nm) and SYTO 9 (L-13152, Invitrogen, excitation wavelength: 488 nm, excitation wavelength: 530 nm), and observed the samples by a laser scanning confocal microscopy (Zeiss LSM 710).

Biosafety and Toxicity Evaluation

For potential clinical applications, we tested the blood compatibility of the 4AGNPs. We centrifugated fresh blood (Sprague Dawley rat, Beijing HFK Bioscience Co., Ltd) at 1500 rpm for 15 min to collect erythrocytes. We diluted 4AGNPs in saline to different concentrations and added 4% erythrocyte suspension into Au NPs dispersions (v/v 1/1). After incubation at 37 °C for 4 h, we centrifugated the samples at 1500 rpm for 15 min and tested the optical density at 540 nm (OD_{540nm}) by a microplate reader (Tecan infinite M200). Saline was set as a negative control and deionized water (18 MΩ) was set as a positive control.

We evaluated the viability of HUVECs (10^5 cells/mL), HAFs (10^5 cells/mL), NIH-3T3 (10^5cells/mL), house ear institute-organ of corti 1 cells (HEI-OC1, 2×10^5 cells/mL) and Madin-Darby canine kidney cells (MDCK, 10^5 cells/mL) to test the *in vitro* cytotoxicity of the 4AGNPs. We incubated the cells with 4AGNPs for different periods in Dulbecco's Modified Eagle Media (DMEM) containing 10% fetal bovine serum (FBS, Invitrogen, USA) and 1% penicillin-streptomycin (PS, MP Biomedicals, USA) at 37 °C with 5% CO_2, we measured the absorbance of the samples at 450 nm by a cell counting kit (CCK, Dojindo, Japan). HEI-OC1 cells were cultured under permissive conditions (33 °C, 10 % CO_2). We extracted cochleae from C57BL/6 wild-type mice at postnatal day 4-5 and incubated the explant in DMEM/F12 culture medium. We observed the morphologies of the HUVECs (10^5 cells/mL) under the treatment of 4AGNPs for 3 d. We stained the cells with phalloidin-FITC/hoechst (Dojindo, Japan) to observe the morphologies of the cells and the cell density by LSCM. We treated HAFs cell suspension with 4AGNPs for 3 d and stained the cells with Calcein AM/PI (Dojindo, Japan) to evaluate the cytotoxicity by LSCM. We fixed the explants treated by 4AGNPs or Gen (10 mM) for 24 h and incubated with a mouse monoclonal anti-myosin7A antibody overnight at 4 °C. We stained the explants with 4,6-diamidino-2-phenylindole (DAPI 1 mM) and phalloidin (33 nM) at room temperature. We characterized the

pyknotic and surviving hair cells to evaluate the ototoxicity by LSCM. Hair cells with normal nuclei were viable surviving hair cells. After cochlea hair cell incubated with 4AGNPs or Gen (10 mM) for 24h, we stained them with FM1-43 (6 mM) for 20 min for uptake observation. We delivered the 4AGNPs (56 mg/mL) on mice cochlea locally for 7 d. To evaluate the function of hair cells, we changed the hearing threshold gap at 8, 16 and 32 kHz to test the auditory brainstem response (ABR) audiology. We measured the vestibulo-ocular reflex (VOR) of the 4AGNPs-treated mice to verify the function of the semicircular canal. We used the Caspase-Glo® 3/7 Assay (Promega, Madison, WI) according to the manufacturer's protocol to test the caspase level.

At 6 dpf, we divided the zebrafish larvae into three groups (n=20): control (0 μg/mL), 4AGNPs (56 mg/mL or 5.6 mg/mL), and Gen (2.8 mg/mL or 0.56 mg/mL) treatment. We cocultured the zebrafish larvae with different materials for 1 h and then rinsed with clean culture medium for three times for startle response test. In parallel with the startle response test, the body balance damage was confirmed by observing the degree of torso bending at rest. We set the red line between the head and tail of the normal zebrafish larvae as the central position to visualize the bending position. We detected the serum biochemical biomarkers for the liver metabolism (alanine aminotransferase, ALT; aspartate aminotransferase, AST; alkaline phosphatase, ALP), kidney metabolism biomarkers (urine acid, UA; creatinine, CR; Urea) and protein metabolism biomarkers (albumin, Alb; total protein TP) to evaluate the biosafety of the 4AGNPs by using the ELISA kit (NEOBIOSCIENCE) according to the manufacturer’s instructions. We collected blood samples of the mice via retro-orbital to determine the routine blood evaluation of mice at different time points treated by 4AGNPs.

Table S1. DLS analysis of the bare GNPs.

	Diameter (nm)	Zeta potential (mV)
GNPs	5.26 ± 0.31	-13.82

Table S2. Antibacterial activities of AGNPs indicated by MIC (μg/mL).

		2AGNPs	3AGNPs	4AGNPs	Gen
Gram-negative bacteria	*E. coli*	8	8	8	1
	MDR *E. coli*	8	8	8	>64
	A. b	2	8	4	2
	MDR *A. b*	2	2	4	>64
	P. a	6	8	4	4
	MDR *P. a*	6	6	4	>64
Gram-positive bacteria	*S. a*	4	8	2	2
	MRSA	8	8	8	16
	S. e	8	6	8	8
	MDR *S. e*	16	16	12	32
	E. f	12	12	8	4
	MDR *E. f*	8	4	4	>64

Table S3. Antibacterial activity of aminophenol and their different isomers and bare GNPs indicated by MIC (μg/mL).

		2A	3A	4A	GNPs
G- bacteria	*E. coli*	>64	>64	>64	>64
	MDR *E. coli*	>64	>64	>64	>64
G+ bacteria	*S. a*	>64	>64	>64	>64
	MRSA	>64	>64	>64	>64

Table S4. Liver and kidney function, immunological and inflammatory evaluation of mice at different time points treated by 4AGNPs.

	Ref	Normal	4AGNPs		
			24 h	48 h	72 h
ALB	24-45 (U/L)	28.13	30.46	27.23	26.63
ALT	8-90 (U/L)	48.29	61.08	53.73	49.26
ALP	70-110 (U/L)	84.10	86.49	85.44	83.52
AST	75-145 (U/L)	102.65	108.39	105.92	103.48
UA	70-410 (μmol/L)	308.21	119.28	270.51	293.64
Urea	2.5-7.1 (mmol/L)	5.52	5.21	4.60	5.46
CR	44-110 (μmol/L)	79.46	55.27	61.9	73.23
TP	45-70 (g/L)	57.14	48.23	54.44	59.36

Table S5. Routine blood evaluation of mice at different time points treated by 4AGNPs.

	Ref	Normal	4AGNPs		
			24 h	48 h	72 h
Neu	0.1-2 (10^9/L)	1.47	1.32	1.42	1.54
WBC	2-13 (10^9/L)	8.43	4.74	6.23	8.47
Lym	1-8 (10^9/L)	5.71	4.11	5.04	5.62
RBC	6-12 (10^{12}/L)	8.05	8.73	7.99	8.07
PLT	540-1540 (10^9/L)	1068.21	1112.37	1159.49	1025.30
HGB	100-190 (g/L)	148.22	140.45	145.92	143.12

Table S6. Biocompatibility of 4AGNPs and different aminoglycosides antibiotics.

Materials	LD_{50}	Route	Ref.
Paromomycin	90 mg/kg	Mouse intravenous	[1]
Tobramycin	72.5 mg/kg	Mouse intravenous	[2]
Ribostamycin	300 mg/kg	Mouse intravenous	[3]
Kanamycin A	115 mg/kg	Mouse intravenous	[4]
Gentamycin	43.5 mg/kg	Mouse intravenous	[5]
4AGNPs	600mg/kg (No mice died)	Mouse intravenous	In this paper

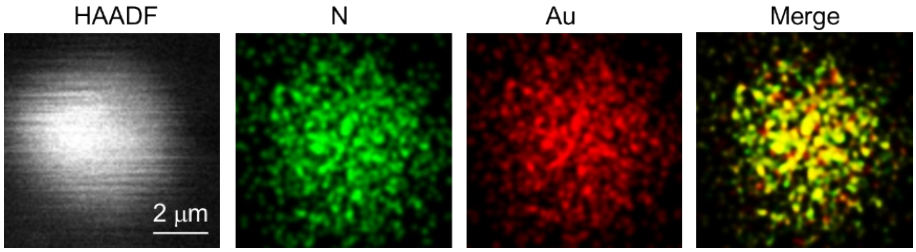

Figure S1. Energy-dispersive spectroscopy (EDS) mapping of 4AGNPs by TEM.

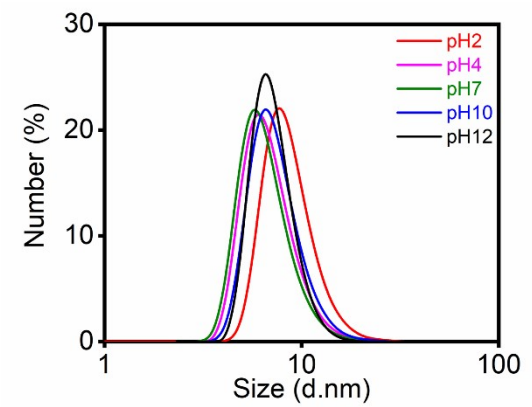

Figure S2. The stability of 4AGNPs in PBS solution with different pH values.

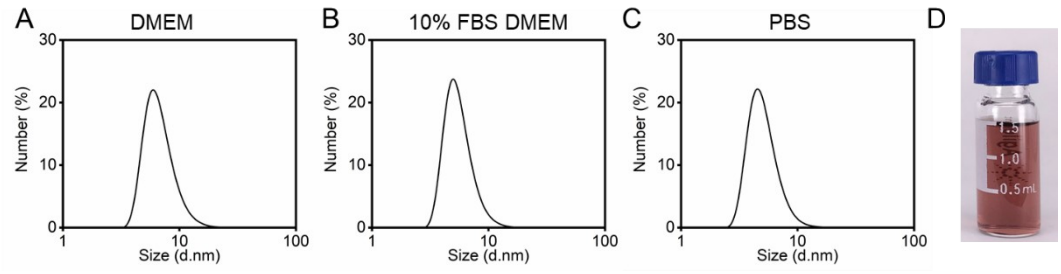

Figure S3. The long-term stability of 4AGNPs. Variation of 4AGNPs size in (A) serum-free DMEM, (B) 10% FBS-containing DMEM, and (C) PBS is shown in the graphs. (D) 4AGNPs solution shows clear dark-brown appearance under normal light conditions after the storage at 4°C for 6 months.

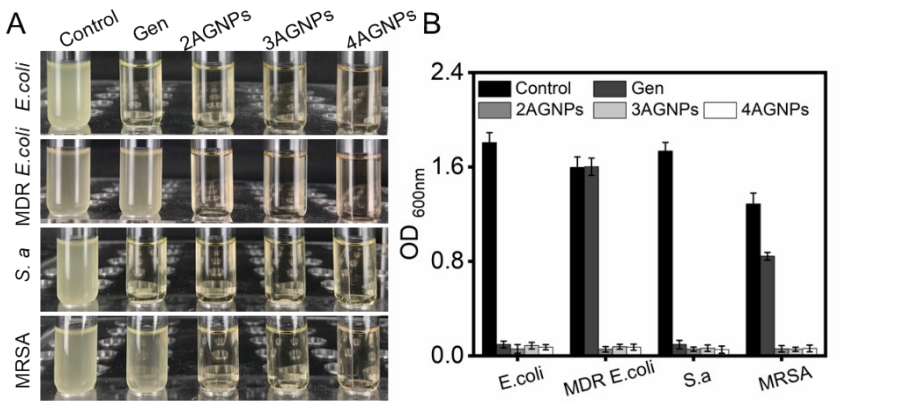

Figure S4. Antibacterial activity of the AGNPs. (A) The macrophotographs of the bacterial suspensions treated by the AGNPs or Gen. (B) Optical density at 600 nm (OD_{600nm}) of bacterial suspensions treated with AGNPs or Gen.

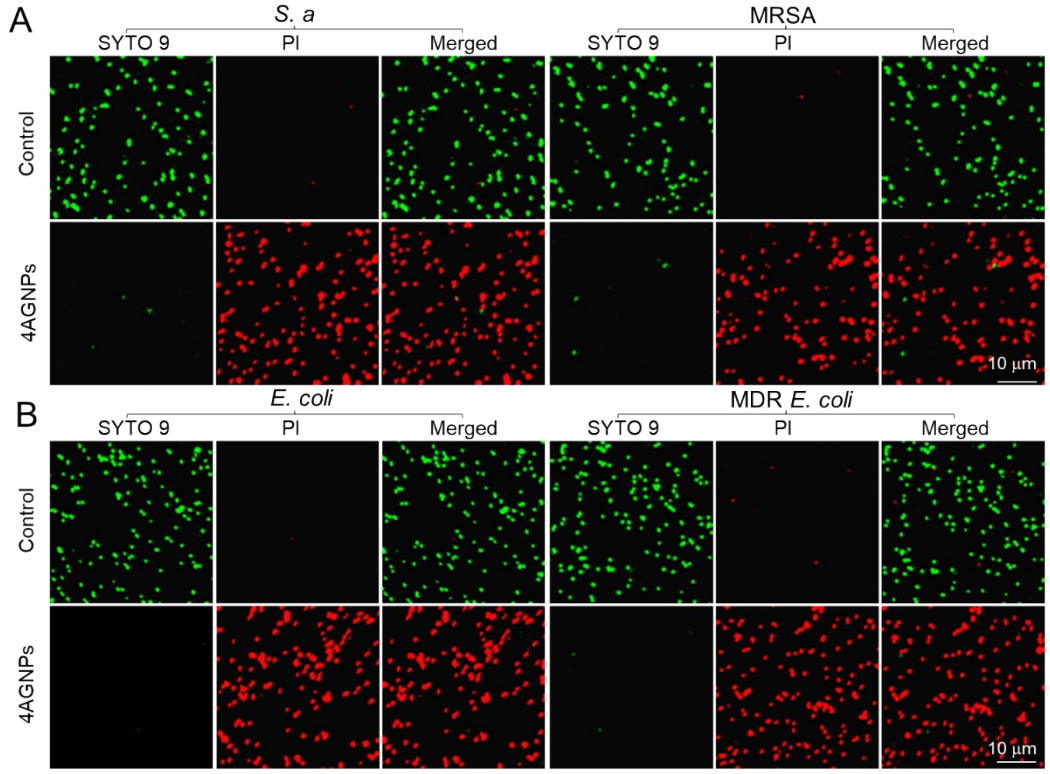

Figure S5. Antibacterial activity of the 4AGNPs. Monitoring 4AGNPs-induced cell wall permeability change of different bacteria by SYTO9/PI staining. Scale bar: 10 μm.

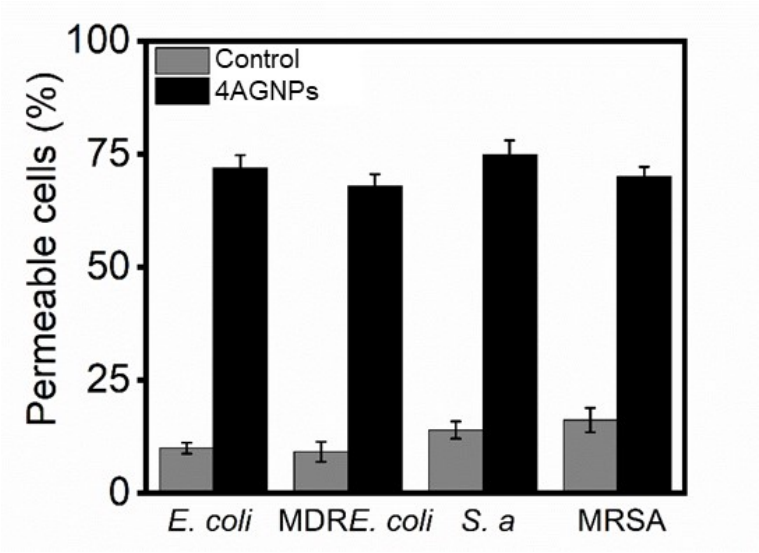

Figure S6. Observation of 4AGNPs-induced permeability of different bacterial cell membranes by PI staining.

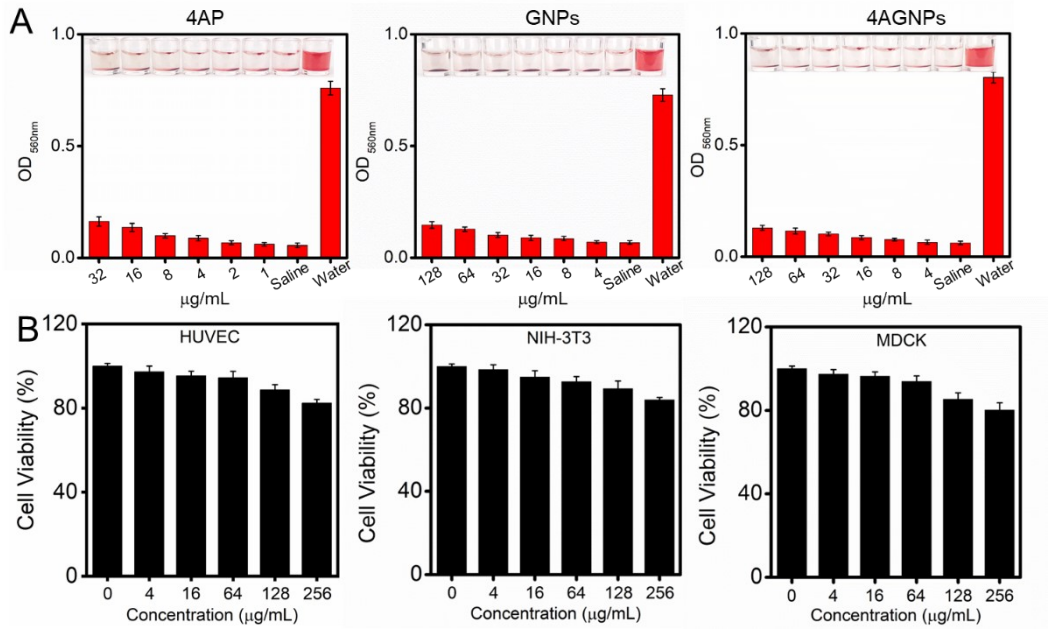

Figure S7. Biocompatibility of 4AP, GNPs, and 4AGNPs. (A) Fresh rat erythrocytes are incubated with different concentrations of 4AP, GNPs, and 4AGNPs. Saline is used as a negative control and water as a positive control. (B) Concentration-dependent cytotoxicity of 4AGNPs is characterized by a CCK-8 kit, 4AGNPs are incubated with HUVEC, NIH-3T3 and MDCK cells for 24 h before the test. The bars represent the average results of three parallel samples, and the error bars indicate one standard deviation of the means (Mean ± SD).

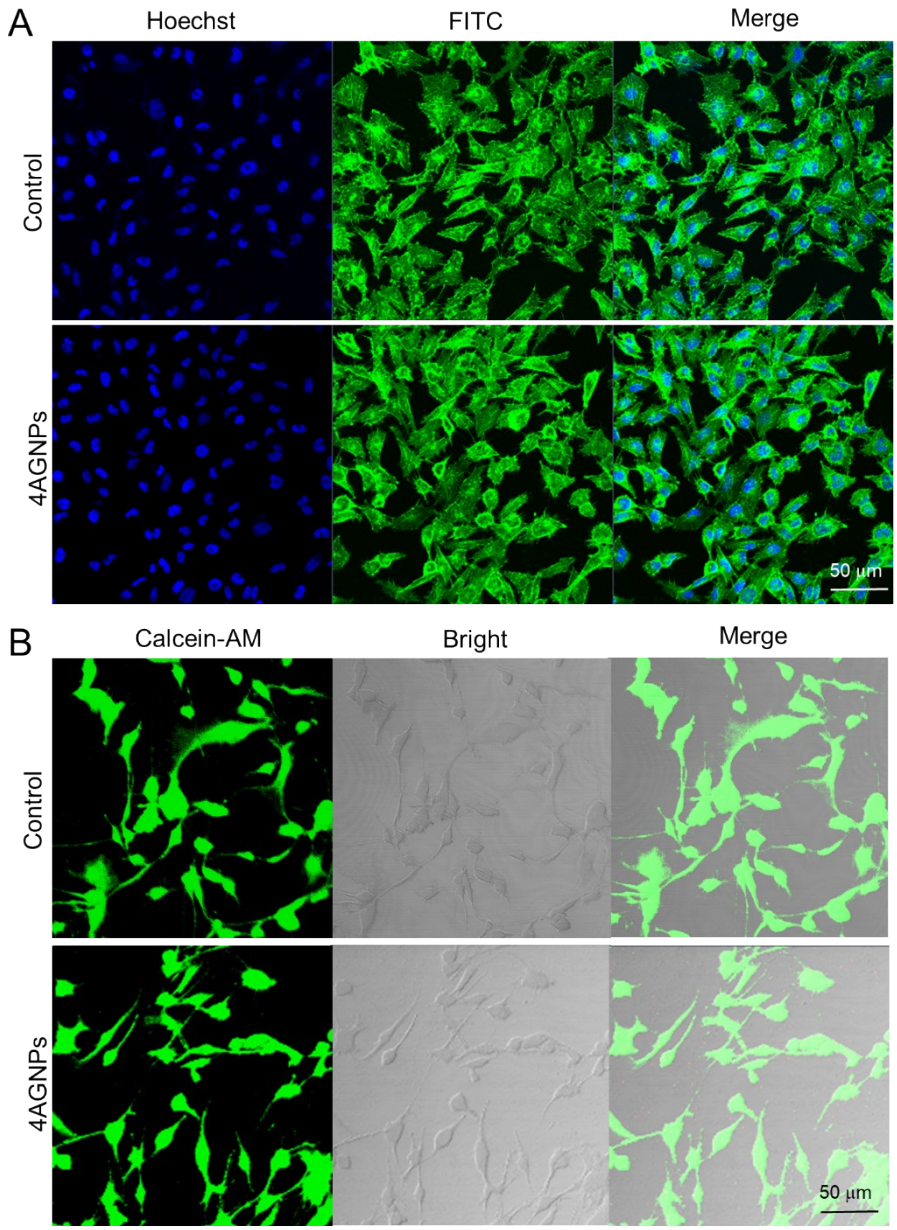

Figure S8. Biocompatibility of 4AGNPs. (A) Confocal images of HUVECs treated with 4AGNPs. The cell nuclei are stained by hoechst 33342 and the cytoskeleton are stained by phalloidin-FITC. (B) Confocal images of HAF cells treated with 4AGNPs. The cells are stained by Calcein-AM.

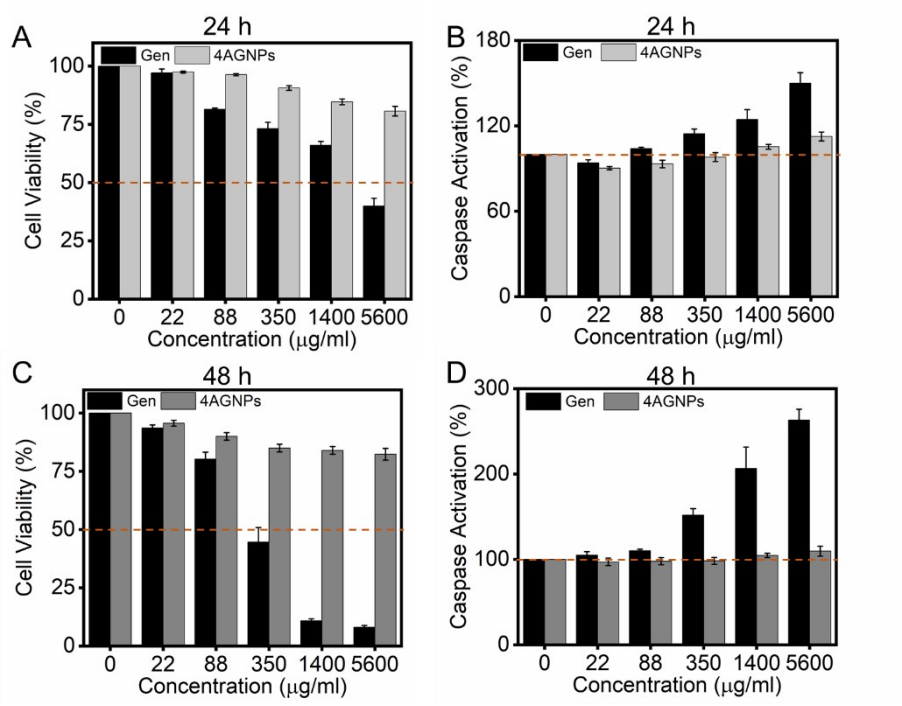

Figure S9. Responses of HEI-OC1 cells exposed to different antibacterial agents. (A) Cell viability evaluated with the CCK-8 assay at 24 h. (B) Antibacterial agents-induced cell death evaluated with the activation of executioner caspases 3/7 at 48 h. (C) Cell viability evaluated with the CCK-8 assay at 24 h. (D) Antibacterial agents-induced cell death evaluated with the activation of executioner caspases 3/7 at 48 h.

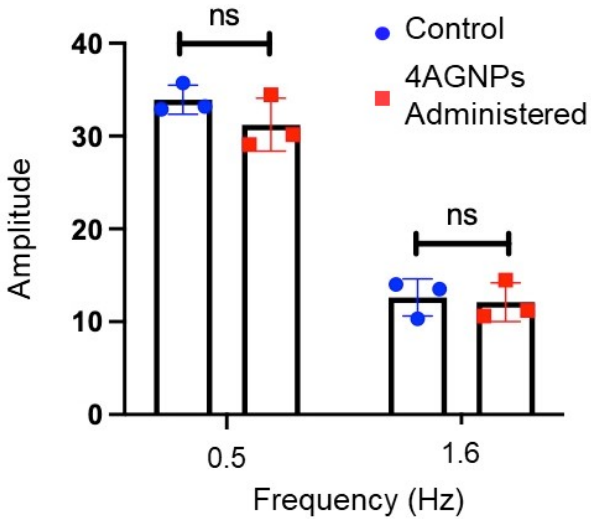

Figure S10. Vestibulo-ocular reflex (VOR) testing of mice treated by 4AGNPs.

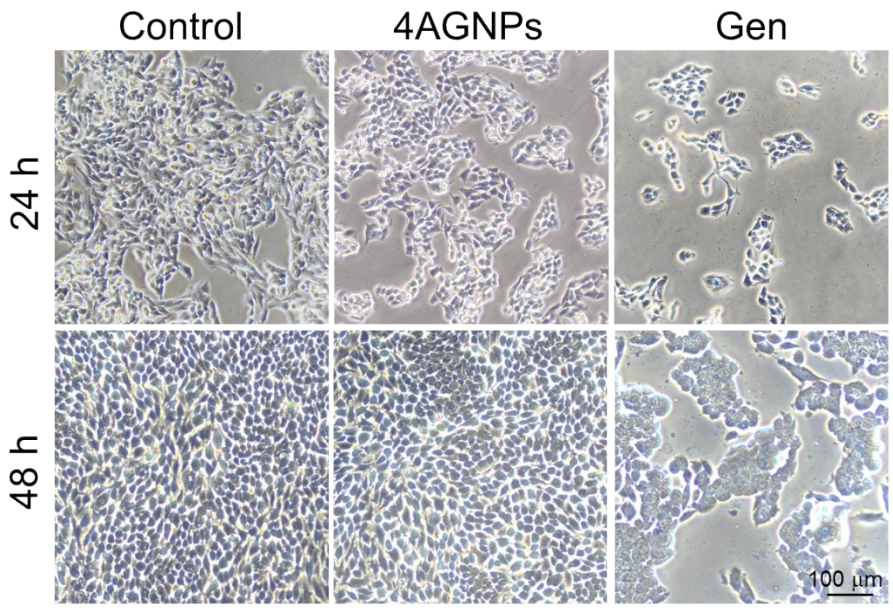

Figure S11. Cytotoxicity assay on HEI-OC1 cells. The cells were treated by 5600 µg/mL of 4AGNPs or Gen for different incubation times.

References

[1] A. J. Bret, S. Simon, *La Presse medicale* **1962**, *70*, 127.

[2] I. V. Iakushkina, S. T. Filippos'iants, L. A. Shevniuk, *Antibiotiki* **1984**, *29*, 361.

[3] K. Rinsho, *Clinical Report.* **1970**, *4*, 2464.

[4] C. Caruso, *Minerva Farm*. **1962**, *11*, 108.

[5] M. A. Bykova, V. N. Solv'ev, V. V. Berezhinskaia, G. G. Egorenko, GV. K. Dolgova, *Antibiotiki* **1976**, *21*, 532.