Supporting Information

Discrimination of Antibiotic-Resistant Gram-Negative Bacteria with a Novel 3D Nano Sensing Array

Jian-Yu Yang†, Xin-Di Jia†, Xiao-Yan Wang, Meng-Xian Liu, Ming-Li Chen*, Ting Yang*, Jian-Hua Wang

Research Center for Analytical Sciences, Department of Chemistry, College of Sciences, Box 332, Northeastern University, Shenyang 110819, China

Corresponding Author

*E-mail: yangting@mail.neu.edu.cn (T. Yang); chenml@mail.neu.edu.cn (M.-L. Chen).

Tel: +86 24 83688944; Fax: +86 24 83687659

Table of contents

Experimental details

Materials and Chemicals. Cetyltrimethylammonium bromide (CTAB) and vancomycin (Van) were purchased from Aladdin Reagent Co., Ltd. (Shanghai, China). Chloroauric acid hydrate ($HAuCl_4 \cdot 4H_2O$), anionic and metal ion salts were obtained from Sinopharm Chemical Reagent Co. (Beijing, China). Artificial urine was received from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). All the reagents were used as received without further purification. Ultrapure water of 18.2 MΩ·cm was used to prepare all the solutions. *Escherichia coli* (*E. coli O157:H7*), *Bacillus subtilis* (*B. subtilis*, *CICC10071*) and *Pseudomonas aeruginosa* (*P. aeruginosa CICC10204*, *P. aeruginosa CICC21954*) were provided by China Center of Industrial Culture Collection (Beijing, China). *Escherichia coli* (*E. coli DH5α*) was the product of Beijing Zoman Biotechnology Co., Ltd. (Beijing, China). The tetracycline-resistant *Escherichia coli* (*E. coli ER2738*) was obtained from NEB (New England Bio-Labs, US). The kanamycin-resistant *Escherichia coli* (*E. coli pET-30a(+)*) and the ampicillin-resistant *Escherichia coli* (*E. coli pHSP70-EGFP*) were obtained from Fenghui Biotechnology Co., Ltd. (Hunan, China). *Yersinia mollaretii* (*Y. mollaretii*, *CGMCC 1.6197*), *Pseudomonas putida* (*P. putida*, *CGMCC 1.2309*) and *Proteus vulgaris* (*P. vulgaris CGMCC 1.1651*) were obtained from China General Microbiological Culture Collection Center (Beijing, China). *Staphylococcus aureus* (*S. aureus*, *CMCC(B) 26003*) was provided by National Center for Medical Culture Collections (Beijing, China).

Apparatus. Fluorescence and light-scattering measurements were carried out on an F-7000 fluorescence spectrophotometer (Hitachi High-Technologies Corporation, Japan) equipped with a quartz cell (optical path: 4 mm × 4 mm). UV−vis absorption spectra were recorded on a U-3900 UV−vis spectrophotometer (Hitachi High-Technologies Corporation, Japan). High resolution transmission electron microscope (HRTEM) images of Van-AuNCs and the (+)AuNPs/AuNCs composite were collected on a JEM-ARM 200F transmission electron microscope (JEOL Ltd., Japan) operated at 200 kV. TEM image of (+)AuNPs was acquired on a G20 transmission electron microscope (FEI Ltd., USA) at an accelerate voltage of 200 kV. The cell images were acquired on an FV-1200 confocal fluorescence microscope (Olympus, Japan). Scanning electron microscopy (SEM) images were obtained from a field emission scanning electron microscope at an accelerating voltage of 5 kV (SU8010, Hitachi High-Technologies Corporation, Japan). The hydrodynamic diameters of (+)AuNPs, Van-AuNCs and their electrostatic composite were acquired from Malvern Nano ZS90 nanosizer in phosphate buffer (pH 7.0, 10 mM) at 25 °C (Malvern Instruments Ltd., England). The zeta potential of (+)AuNPs, Van-AuNCs and the Gram-negative bacteria were also measured by the Malvern Nano ZS90 nanosizer.

Bacteria Cultivation The containers and glassware were sterilized at 121 °C for 30 min. 0.5 g sodium chloride, 1.0 g peptone and 0.5 g yeast extract were dissolved in 100 mL ultra-pure water to prepare LB medium (pH 7.0). 0.5 g sodium chloride, 1.0 g peptone and 0.3 g beef extract were dissolved in 100 mL ultra-pure water to prepare nutrient broth (pH 7.0). In order to obtain the LB agar and broth agar, 1.5 g granulated

agar was separately added into the above LB medium (100 mL) and (nutrient) broth 100 mL. The bacteria models were collected with inoculating loops from the agar slant culture medium. Then the bacteria model *S. aureus*, *E. coli DH5α, E.coli O157:H7, E.coli ER2738, E.coli pET-30a(+), E.coli pHSP70-EGFP, P. aeruginosa CICC 10204, P. aeruginosa CICC 21954*, *B. subtilis*, and *Y. mollaretii* were inoculated in the LB medium. For the culture of antibiotic-resistant strains, corresponding antibiotic were added into the LB medium (For *E. coli ER2738*, 40 μL tetracycline (20 μg mL^{-1}) was added, for *E. coli pET-30a(+)*, 20 μL kanamycin (100 μg mL^{-1}) was added, for *E. coli pHSP70-EGFP*, 10 μL ampicillin (50 μg mL^{-1}) was added). Meanwhile, bacteria model *P. vulgaris* and *P. putida* were inoculated in the nutrient broth. The above bacteria were cultured at 37 °C for 12 h (1×108 cfu mL-1), and the solutions were stored at 4 °C. Meanwhile, bacteria model *P. vulgaris* and *P. putida* were inoculated in the nutrient broth. The above bacteria were cultured at 37 °C for 12 h (1×10^8 cfu mL^{-1}), and the solutions were stored at 4 °C.

The bacterial cells were collected by centrifugation (6000 rpm for 10 min) and re-suspended in PBS buffer solution (pH 7.4, 10 mM). This washing process is repeated for three times to ensure that metabolism products on the surface are washed away.

Preparation of Van-AuNCs. All glassware was thoroughly washed with aqua regia (caution: aqua regia is a strong oxidizing reagent, and it should be handled with extreme care) and rinsed extensively with ultrapure water before use. Van-AuNCs were prepared according to the literature with minor modifications. Firstly, 1 mL of 1%

(w/v) $HAuCl_4$ solution was added into 99 mL ultrapure water. The mixture was boiled at 100 °C for 2 min with vigorous stirring. Then, 0.5 mL vancomycin (w/v) solution was quickly added into the solution and allowed to react at 100 °C for 50 min. Finally, the product was lyophilized after dialysis against ultrapure water (MWCO: 1000 Da) for 24 h to remove the unreacted species.

Preparation of CTAB-AuNPs. CTAB-AuNPs were prepared based on a previously reported procedure with minor revisions. In a typical experiment, 2 mL of aqueous CTAB solution (10 mM) was added into 15 mL of $HAuCl_4$ solution (1.0 mM) with vigorous stirring for 15 min, followed by addition of 2 mL freshly prepared $NaBH_4$ solution (0.1 M) drop-wisely. The reaction mixture was stirred at room temperature for 1 h until the color of the solution turned from pale yellow to wine red. The solution was filtered through a 0.22 μm filter membrane for the removal of large particulate matter. Ultimately, the solution was stored in brown glass reagent bottle at 4°C for future use. Due to the positively charged feature, the CTAB-AuNPs were labeled as (+)AuNPs.

Bacteria identification. Firstly, 150 μL Van-AuNCs (0.01 mg mL^{-1}, pH 7.0, 30 mM phosphate buffer solution) was added into 150 μL of the positively charged (+)AuNPs solution and incubated for 10 min at room temperature, resulting in the (+)AuNPs/AuNCs composite. Then, 150 μL Gram-negative bacteria at various concentration levels were added into the mixture separately. After incubation at room temperature for 10 min, the mixture was centrifuged at 6000 rpm for 5 min. The fluorescence intensity ($\lambda_{ex/em}$ 290 nm/400 nm), UV−vis absorption (λ_{max}=525 nm),

and light-scattering signal (λ=390 nm) of the supernatant were measured. All measurements were repeated to generate four replicates for each analyte, so that for a given concentration, a 3 channels ×10 analytes × 4 replicates data matrix could be generated. Finally, the obtained data were processed using linear discriminant analysis (LDA) and hierarchical cluster analysis (HCA) with SPSS V 17.0 software.

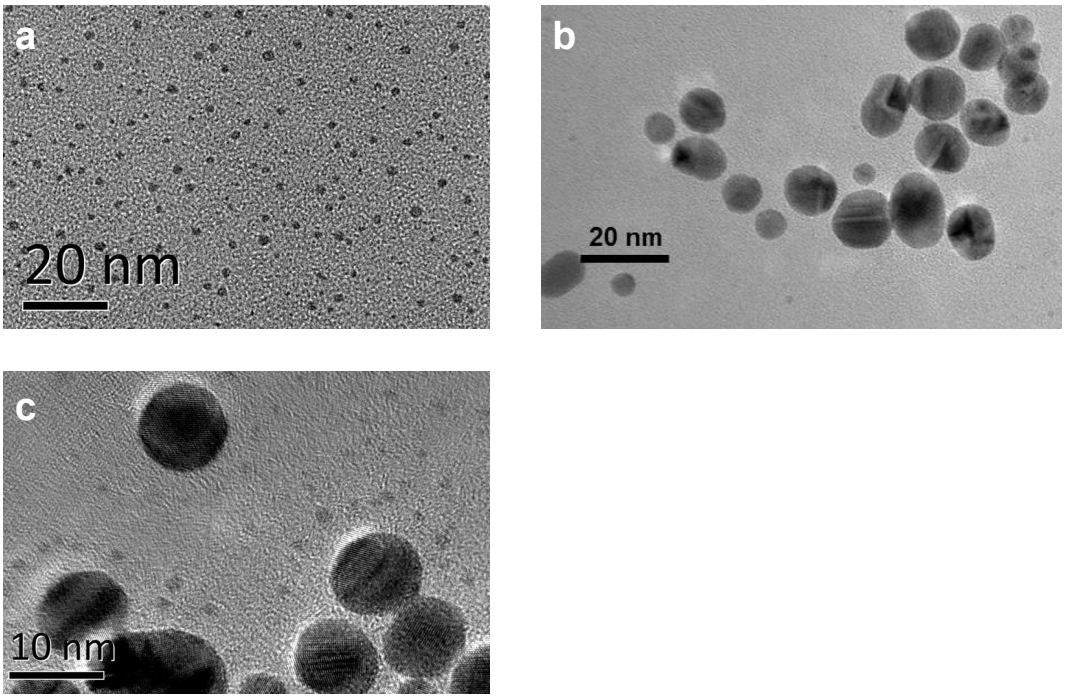

Fig. S1. (a) HRTEM image of Van-AuNCs and (b) TEM image of (+)AuNPs and (c) HRTEM image of (+)AuNPs/AuNCs composite.

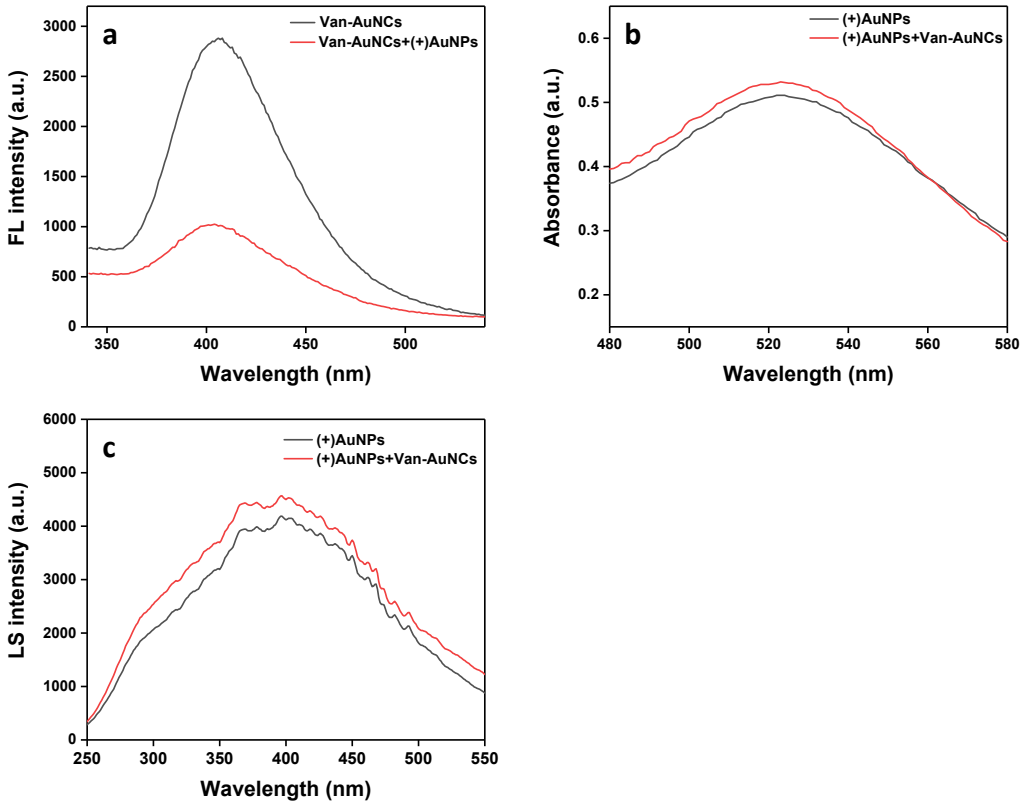

Fig. S2. (a) Fluorescence spectra of Van-AuNCs after addition of (+)AuNPs in phosphate buffer solution (pH 7.0, 10 mM). (b) UV-vis absorption spectra of (+)AuNPs after addition of Van-AuNCs in phosphate buffer solution (pH 7.0, 10 mM). (c) Light-scattering spectra of (+)AuNPs after addition of Van-AuNCs in phosphate buffer solution (pH 7.0, 10 mM).

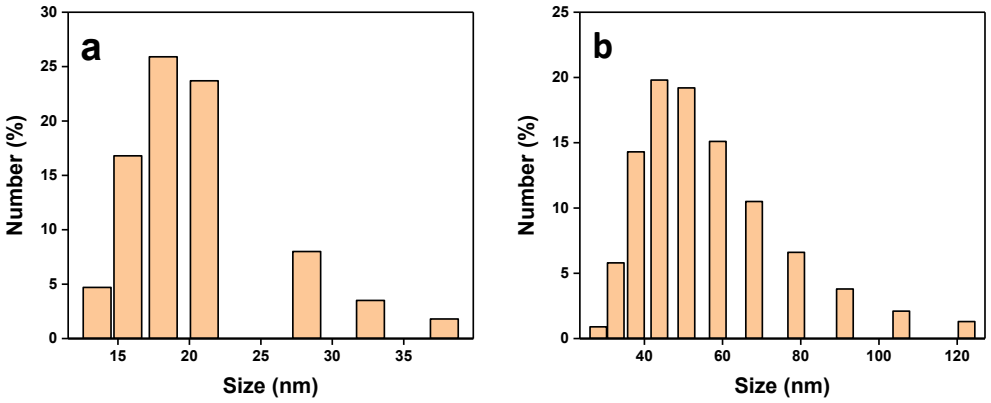

Fig. S3. Hydrodynamic diameters of (a) (+)AuNPs and (b) (+)AuNPs/AuNCs composite in phosphate buffer solution (pH 7.0,10 mM).

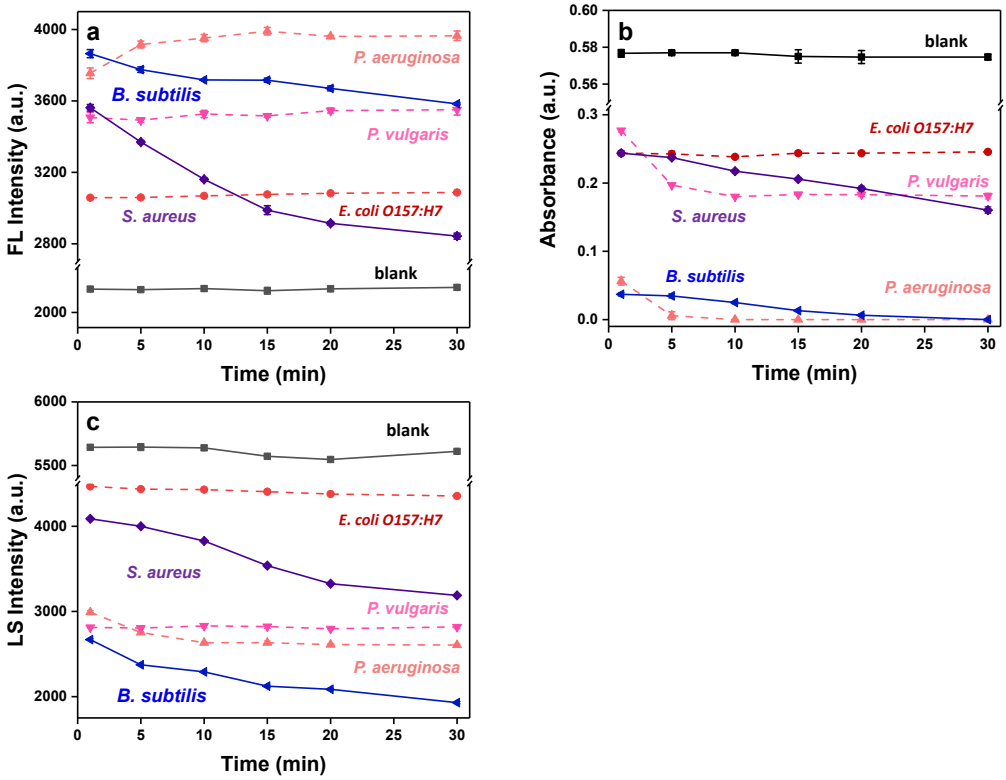

Fig. S4. Fluorescence (a), UV-vis absorption (b) and light-scattering (c) signals of the (+)AuNPs/AuNCs composite after addition of *E. coli O157:H7*, *P. aeruginosa*, *P. vulgaris*, *S. aureus* and *B. subtilis* (OD_{600}=0.015) in phosphate buffer solution (pH 7.0, 10 mM) with different incubation time. The results for Gram-positive bacteria are labeled in solid line, whereas those for Gram-negative ones are labeled in dash line.

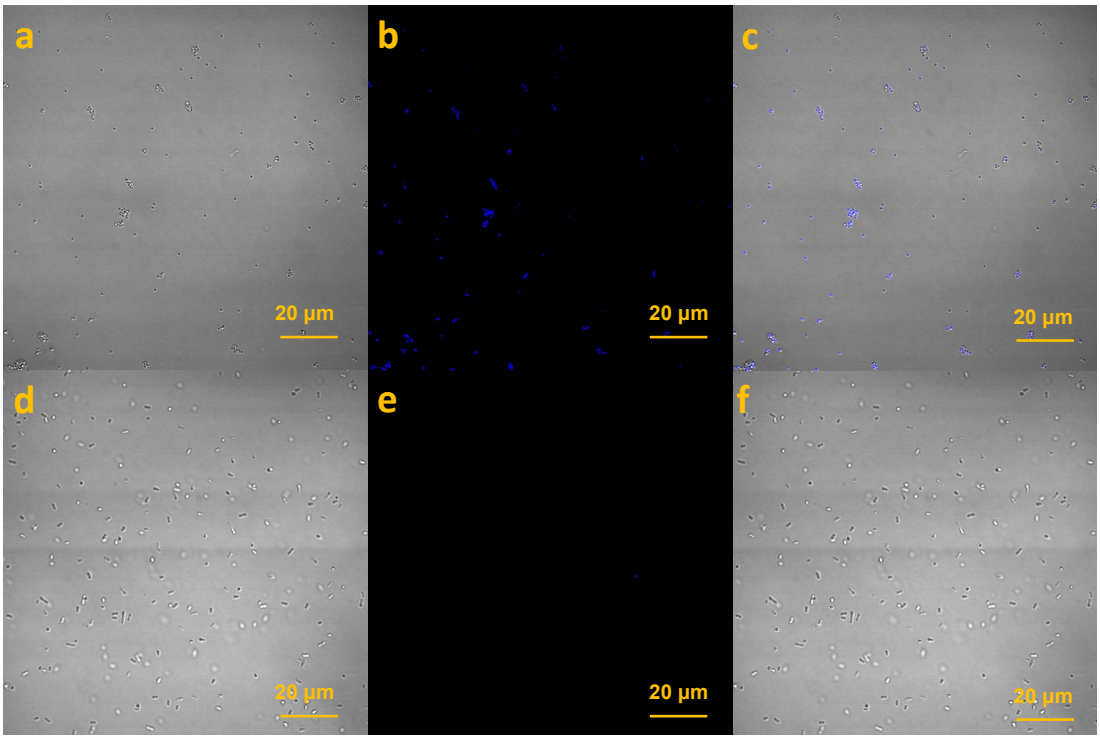

Fig. S5. Confocal laser scan microscopy images of the (+)AuNPs/AuNCs composite with *S. aureus* in phosphate buffer solution (pH 7.0, 10 mM). (a) bright-field image, (b) fluorescence image, (c) merged image. Confocal laser scan microscopy images of the (+)AuNPs/AuNCs composite with *E. coli O157:H7* in phosphate buffer solution (pH 7.0, 10 mM). (d) bright-field image, (e) fluorescence image, (f) merged image (Scale bars: 20 μm).

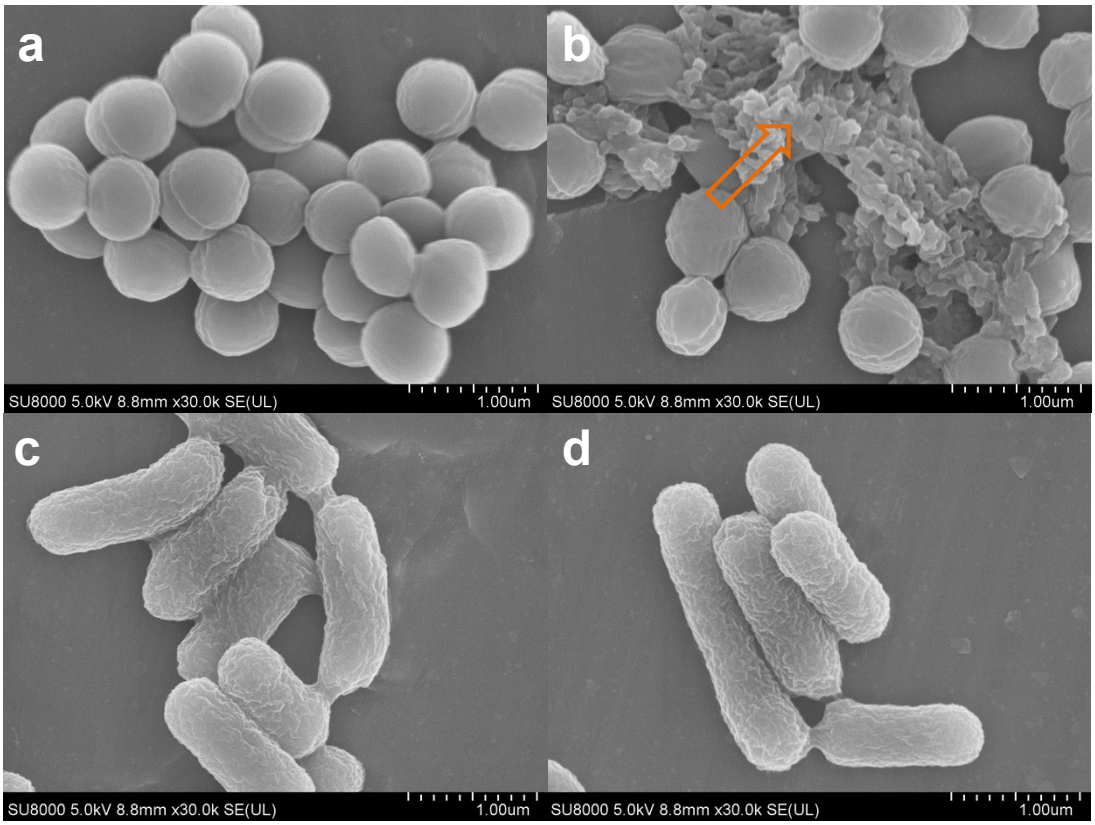

Fig. S6. (a) SEM image of the *S. aureus* in phosphate buffer solution (pH 7.0,10 mM). (b) SEM image of the *S. aureus* in the presence of Van-AuNCs in phosphate buffer solution (pH 7.0,10 mM). (c) SEM image of the *E. coli O157:H7* in phosphate buffer solution (pH 7.0,10 mM). (d) SEM image of *E. coli O157:H7* in the presence of Van-AuNCs in phosphate buffer solution (pH 7.0, 10 mM). Orange arrow indicates intracellular substances come out from *S. aureus* cells.

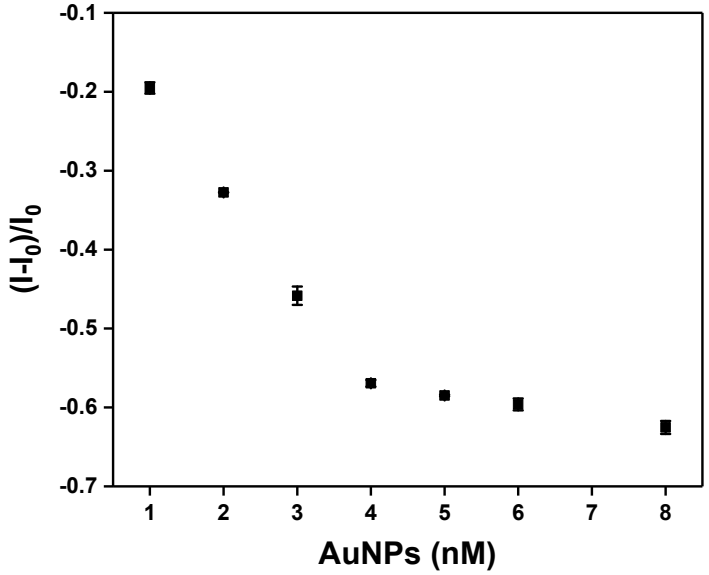

Fig. S7. The fluorescence response $(I-I_0)/I_0$ of Van-AuNCs (0.01 mg mL^{-1}) with addition of (+)AuNPs at various concentrations within 0−8 nM in phosphate buffer solution (pH 7.0, 10 mM).

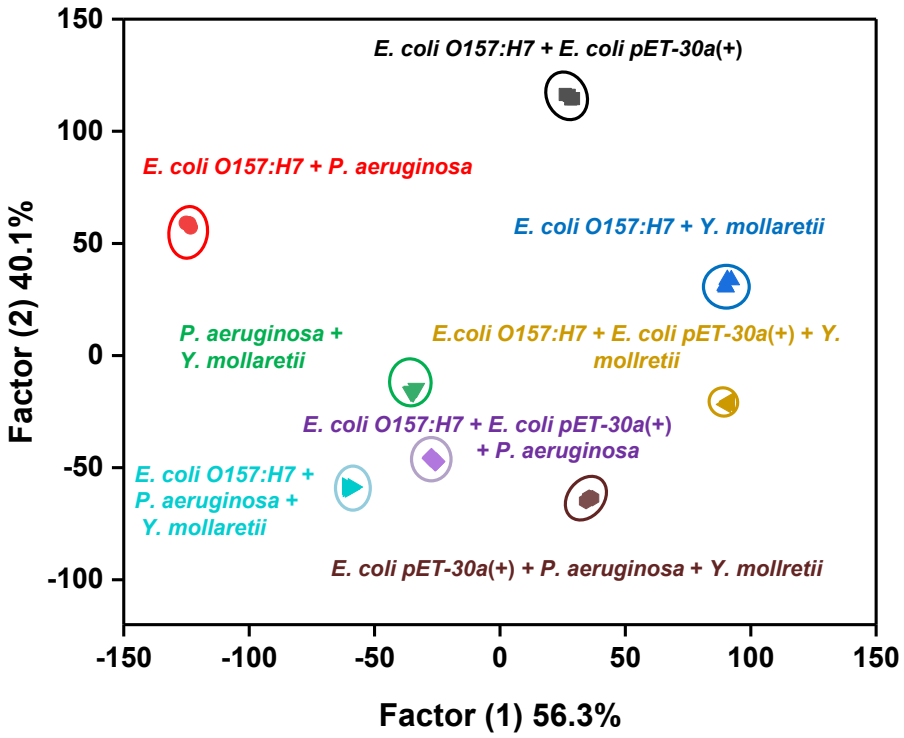

Fig. S8. Canonical score plot against eight sets of bacteria mixtures by LDA. The concentration ratio of bacteria was set at 1:1 or 1:1:1. The total concentration of the mixture was set at OD_{600}=0.015.

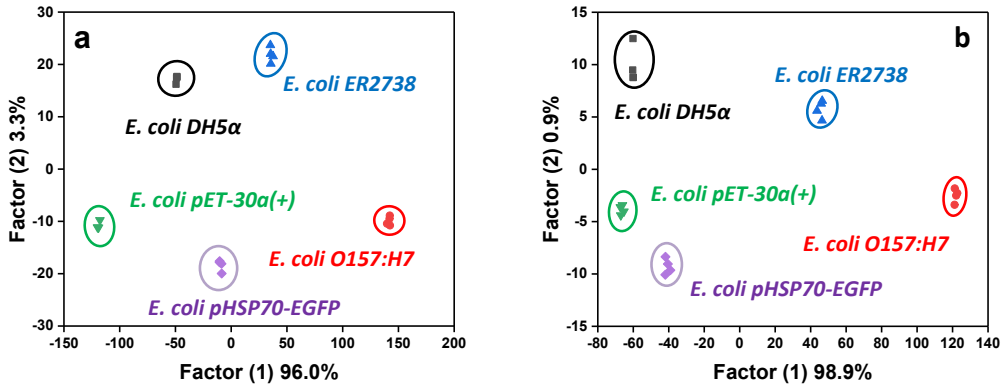

Fig. S9. (a) Canonical score plots for the three optical response patterns obtained from the (+)AuNPs/AuNCs composite-based sensor array toward five different strains of *E. coli* by LDA in phosphate buffer solution (pH 7.0, 10 mM). (b) Canonical score plots against five different strains of *E. coli* by LDA after three days.

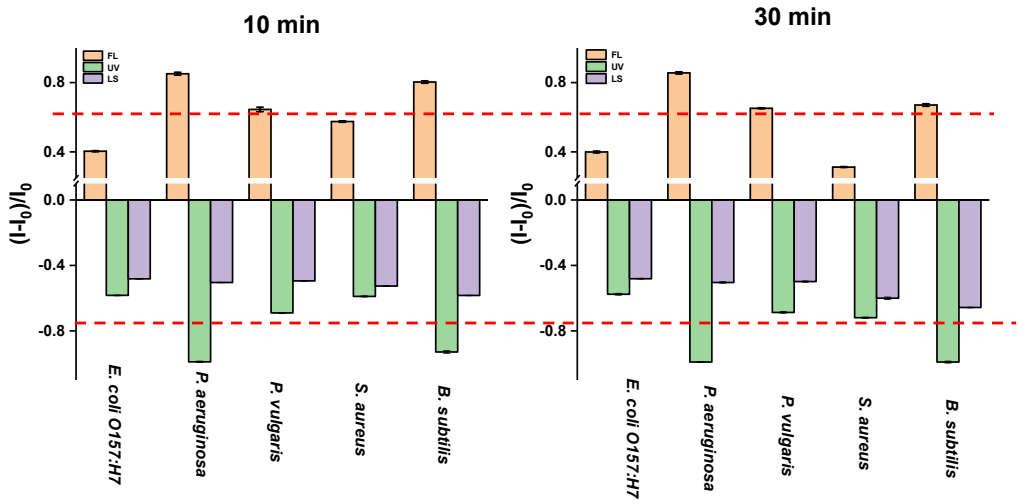

Fig. S10. The triple optical response pattern (fluorescence, UV-vis absorption and light-scattering) $(I-I_0)/I_0$ obtained by the (+)AuNPs/AuNCs composite treated with five different kinds of bacteria (OD_{600}=0.015) in phosphate buffer (pH 7.0, 10 mM) at different incubation time (10 min and 30 min).

Table S1. Table R1 Details of bacteria used in this article.

Microorganism	Zeta potential (mV)	Gram property	Antibiotic resistance	No. of bacteria at OD_{600} = 0.1 (per mL)
E. coli O157:H7	-13.4	negative	non-resistant	8.1×10^7
E. coli DH5α	-10.6	negative	non-resistant	6.6×10^7
E. coli pET-30a(+)	-9.8	negative	kanamycin	9.1×10^7
E. coli pHSP70-EGFP	-10.1	negative	ampicillin	7.2×10^7
E. coli ER2738	-33.0	negative	tetracycline	7.4×10^7
P. aeruginosa 10204	-20.9	negative	non-resistant	6.2×10^7
P. aeruginosa 21954	-21.1	negative	non-resistant	6.5×10^7
Y. mollaretii	-2.1	negative	non-resistant	9.2×10^7
P. putida	-11.1	negative	non-resistant	8.1×10^7
P. vulgaris	-15.9	negative	non-resistant	5.9×10^7
S. aureus	-39.9	positive	non-resistant	1.2×10^7
B. subtilis	-49.6	positive	non-resistant	7.7×10^7

Table S2. Detection and identification of Gram-negative bacteria (OD_{600}=0.015) in phosphate buffer (pH 7.0, 10 mM) using linear discriminant analysis (LDA). According to the verification, only 1 out of 40 unknown samples was misclassified, representing an accuracy of 97.5 %.

FL	UV-vis	LS	Identification	Verification
0.327	-0.274	-0.418	*E. coli DH5α*	*E. coli DH5α*
0.325	-0.272	-0.417	*E. coli DH5α*	*E. coli DH5α*
0.327	-0.271	-0.420	*E. coli DH5α*	*E. coli DH5α*
0.329	-0.269	-0.420	*E. coli DH5α*	*E. coli DH5α*
0.585	-0.575	-0.471	*E. coli O157:H7*	*E. coli O157:H7*
0.584	-0.574	-0.470	*E. coli O157:H7*	*E. coli O157:H7*
0.588	-0.574	-0.470	*E. coli O157:H7*	*E. coli O157:H7*
0.588	-0.577	-0.469	*E. coli O157:H7*	*E. coli O157:H7*
0.362	-0.321	-0.428	*E. coli ER2738*	*E. coli ER2738*
0.363	-0.323	-0.428	*E. coli ER2738*	*E. coli ER2738*
0.364	-0.320	-0.428	*E. coli ER2738*	*E. coli ER2738*
0.362	-0.322	-0.428	*E. coli ER2738*	*E. coli ER2738*
0.285	-0.198	-0.463	*E. coli pET-30a(+)*	*E. coli pET-30a(+)*
0.286	-0.201	-0.462	*E. coli pET-30a(+)*	*E. coli pET-30a(+)*
0.286	-0.200	-0.461	*E. coli pET-30a(+)*	*E. coli pET-30a(+)*
0.287	-0.200	-0.462	*E. coli pET-30a(+)*	*E. coli pET-30a(+)*
0.366	-0.319	-0.419	*E. coli pHSP70-EGFP*	*E. coli ER2738*
0.377	-0.307	-0.414	*E. coli pHSP70-EGFP*	*E. coli pHSP70-EGFP*
0.378	-0.308	-0.415	*E. coli pHSP70-EGFP*	*E. coli pHSP70-EGFP*
0.378	-0.305	-0.416	*E. coli pHSP70-EGFP*	*E. coli pHSP70-EGFP*
0.855	-0.911	-0.536	*P. aeruginosa 10204*	*P. aeruginosa 10204*
0.855	-0.910	-0.535	*P. aeruginosa 10204*	*P. aeruginosa 10204*
0.849	-0.911	-0.537	*P. aeruginosa 10204*	*P. aeruginosa 10204*

0.851	-0.911	-0.537	*P. aeruginosa 10204*	*P. aeruginosa 10204*
0.577	-0.779	-0.556	*P. aeruginosa 21954*	*P. aeruginosa 21954*
0.579	-0.781	-0.556	*P. aeruginosa 21954*	*P. aeruginosa 21954*
0.582	-0.779	-0.557	*P. aeruginosa 21954*	*P. aeruginosa 21954*
0.582	-0.782	-0.557	*P. aeruginosa 21954*	*P. aeruginosa 21954*
0.351	-0.497	-0.548	*P. vulgaris*	*P. vulgaris*
0.351	-0.499	-0.549	*P. vulgaris*	*P. vulgaris*
0.350	-0.496	-0.550	*P. vulgaris*	*P. vulgaris*
0.350	-0.498	-0.549	*P. vulgaris*	*P. vulgaris*
0.510	-0.612	-0.417	*P. putide*	*P. putide*
0.508	-0.615	-0.417	*P. putide*	*P. putide*
0.510	-0.612	-0.417	*P. putide*	*P. putide*
0.509	-0.612	-0.417	*P. putide*	*P. putide*
0.233	-0.238	-0.504	*Y. mollaretii*	*Y. mollaretii*
0.234	-0.238	-0.504	*Y. mollaretii*	*Y. mollaretii*
0.236	-0.238	-0.503	*Y. mollaretii*	*Y. mollaretii*
0.235	-0.234	-0.503	*Y. mollaretii*	*Y. mollaretii*

Table S3 Detection and identification of Gram-negative bacteria or Gram-positive bacteria (OD_{600}=0.015) in phosphate buffer (pH 7.0, 10 mM). According to the verification, all the unknown samples were classified, representing an accuracy of 100 %.

FL (%)	UV (%)	LS (%)	Identification	Verification
-2.619	2.469	-3.142	*E. coli O157:H7*	Gram-negative
-2.99	1.646	-2.732	*E. coli O157:H7*	Gram-negative
-2.49	1.235	-1.229	*E. coli O157:H7*	Gram-negative
-2.74	2.058	-2.766	*E. coli O157:H7*	Gram-negative
-0.375	0	-3.167	*P. aeruginosa*	Gram-negative
0.482	0	-1.078	*P. aeruginosa*	Gram-negative
0.054	0	-1.281	*P. aeruginosa*	Gram-negative
0.616	0	-0.404	*P. aeruginosa*	Gram-negative
-0.168	-1.786	-0.550	*P. vulgaris*	Gram-negative
1.425	0.714	0.206	*P. vulgaris*	Gram-negative
0.224	-1.071	-0.619	*P. vulgaris*	Gram-negative
-0.168	1.429	-0.172	*P. vulgaris*	Gram-negative
-10.971	-25.619	-12.112	*S. aureus*	Gram-positive
-11.735	-23.141	-13.415	*S. aureus*	Gram-positive
-11.529	-21.901	-16.634	*S. aureus*	Gram-positive
-12.618	-21.488	-15.561	*S. aureus*	Gram-positive
-7.322	-41.176	-13.702	*B. subtilis*	Gram-positive
-7.717	-45.098	-13.010	*B. subtilis*	Gram-positive
-8.164	-52.941	-13.703	*B. subtilis*	Gram-positive
-8.454	-47.059	-13.433	*B. subtilis*	Gram-positive