Supporting Information

Label-Free in Situ Discrimination of Live and Dead Bacteria by Surface-Enhanced Raman Scattering

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EXPERIMENTAL SECTION

Chemicals, Biochemcials and Materials. Methanol, ethanol, 25% ammonia (NH_4OH), 98% sulfuric acid (H_2SO_4), 37% hydrochloric acid (HCl), sodium hydroxide (NaOH), sodium choride (NaCl), hydroxlamine hydrochroide ($NH_2OH{\cdot}HCl$), sliver nitrate ($AgNO_3$), trimethoxy(propyl)silane ($CH_3CH_2CH_2Si(OCH_3)_3$), chloramphenicol, polymyxin B, ampicillin, formalin, and trimethoprim were purchased from Sigma Aldrich (Taufkirchen, Germany) and used without further purification. *E. coli* K-12 DSM 1116, *E. coli* TOP-10 K-12 and *S. epidermidis* shock frozen strains were purchased from DSM nutritional products (GmbH Grenzach, Germany). Glass slides (26 mm × 76 mm × 1 mm) were purchased from Carl Roth (Karlsruhe, Germany). Activation of glass slides, cleaning steps and the hydrophobic procedure were performed in plastic containers from Carl Roth. Hellmanex solution was purchased from Hellma (Muellheim, Germany). Milli-Q water (18.2 MΩ cm) was produced using a Millipore water purification system.

Phosphate buffered saline (PBS, pH=7.6) contained 1.36 g (10 mmol) KH_2PO_4, 12.20 g (70 mmol) K_2HPO_4 and 8.50 g NaCl (145 mmol) in 1 L Milli-Q water and was filtered before use.

Glass Slides Hydrophobic Treatments. *Cleaning*. The normal glass slides were put in a chip reservoir containing 2% Hellmanex solution and left for 1 h in ultrasonic bath to rinse. Subsequently, the chips were put on a shaker over night (15 h) at room temperature followed by 1 h in an ultrasonic bath. The treated slides were thoroughly washed five times in 200 mL Milli-Q water and dried under nitrogen flow. The prepared glass slides were dipped in 200 mL of methanol/hydrochloric acid solution (1:1) and left shaking for 1 h at room temperature. A washing step in water followed, where the chips were rinsed five times in 200 mL Milli-Q water. Subsequently, they were dipped in concentrated sulfuric acid and left shaking for 1 h at room temperature. An additional washing step in water followed and the slides were again dried under a nitrogen flow.[1]

Hydrophobic Treatment. The cleaning slides were put in a chip reservoir containing a 150 mL methanol and 150 mL water mixed solution under magnetic stirring, then 2 mL trimethoxy(propyl)silane was added dropwise followed by addition of 3 mL 25% ammonia. The solution was stirred overnight. Finally, the chips were thoroughly washed five times in 300 mL absolute ethanol and the slides were again dried under a nitrogen flow.[2]

Microorganism Preparation. *Wild Type Bacteria Preparation.* Shock-frozen *E. coli* DSM 1116, *E. coli* TOP-10 K-12 and *S. epidermidis* cells were cultivated in LB Media (Lennox) (100 mL in 250 mL flasks) in gyratory shaker at 100 rpm and 37 °C for 16 h. 10 mL of bacteria were harvested and washed twice in H_2O ("Bacteria(H_2O)") or PBS ("Bacteria(PBS)") by centrifugation at 4500 rpm and 4 °C. Then the bacteria were immediately used in the following experiments. Prior to analysis, all cell suspensions were diluted in PBS to reach the desired concentrations. The stock cell concentration was determined by flow-cytometry using SYTO9.

Preparation of resistant E. coli *strains.* Two resistant *E. coli* K-12 strains were prepared. First, ampicillin resistant *E. coli K-12* DSM 1116 (Amp-R K-12 *E. coli*) was generated by electroporating a beta-lactamase enzyme coding plasmid into the mother strain. Transformants were selected by plating on Ampicillin 100µg/ml containing LB media petri dishes. Second, polymyxin B resistant *E. coli* K-12 Top-10 (PolyB-R K-12 *E. coli*) was generated by confronting the mother strain with increasing amounts of polymyxin B in liquid media and subsequent cultivation on non-selective LB media dishes. The procedure was stopped when a strain with a minimal inhibitory concentration (MIC) against polymyxin B of above 32µg/ml was reached.
For use in the experiments, the strains were reactivated from frozen stocks and cultured on LB-media dishes containing the appropriate antibiotic. For further use, a small amount was scratched off the fresh grown plate and inoculated into 50 mL LB medium with 10 µg/mL polymyxin B or 100 µg/mL ampicillin, respectively. Finally, the cells were cultivated in a in a gyratory shaker at 100 rpm and 37 ºC for 16h. 10 mL of bacterial culture was harvested and washed twice in H_2O by centrifugation at 4500 rpm and 4 °C. Then the bacteria were immediately used for the experiments involving antibiotic resistance.

Bacteria@AgNPs Suspensions Preparation. "Bacteria@AgNPs" suspensions were prepared by our previously reported method.[3] In brief, a 1 mL sample liquid was centrifuged at 4500 rpm, and the supernatant was discarded. Then 100 µL of 10 mM $AgNO_3$ solution was added, and the mixture was vortexed and then an interaction time of 5 min was allowed. Subsequently, 900 µL of $NH_2OH{\cdot}HCl$ solution was pipetted into the prepared mixture and the mixture was again vigorously vortexed. Finally, we stored the suspension in the dark at 4°C until it was analyzed. The resulting hybrid bacterial suspension is designated as Bacteria@AgNPs.

Preparation of Suspension with Different Percentage of Dead Bacteria and Bacteria@AgNPs. Typically, two 10 mL batches of bacteria were washed twice with Milli-Q

water at 4500 rpm for 10 min at 4 °C. One batch of bacteria was autoclaved to achieve complete killing. Solutions with varying percentages of dead bacteria were generated by mixing different volumes of live and dead bacterial suspensions. The total volume of bacterial suspensions used was always 1 mL. Different percentages of dead bacteria of Bacteria@AgNPs suspensions were prepared according to the above mentioned Bacteria@AgNPs suspension preparation method.

Test Chemicals. Six different chemicals were used to confront bacteria (*E. coli* DSM 1116 and *S. epidermidis*) to elucidate further the mechanism of vanishing signals after AgNP addition. Chemicals used were in detail: NaCl (0.9%), chloramphenicol (50 µg/mL), trimethoprim (75 µg/mL), polymyxin B (10 µg/mL), ampicillin (100 µg/mL), and formalin (3.7%). All of these solutions were prepared in physiological NaCl solution. After confronting the bacteria with the respective chemical, samples were analysed at different time points (5 min, 20 min, 60 min, and 240 min). Finally, we detected the corresponding SERS signals of these killed bacteria by our Bacteria@AgNPs method. For example, a 1 mL 1×10^7 cell/mL of the "Bacteria(H_2O)" were centrifuged at 4500 rpm and 4 °C for 10 min, and the supernatant was discarded, then 1 mL of the 50 µg/mL chloramphenicol was added, and the mixture was vigorously vortexed. Subsequently, 200 µL of above mixture was pipetted into another tube at different time points. The solution was washed twice with water to remove traces of the used chemical and obtain pure bacteria. Then 20 µL of 10 mM $AgNO_3$ solution was added, the mixture was vortexed and an interaction time of 5 min was allowed. Subsequently, 180 µL of $NH_2OH{\cdot}HCl$ solution was pipetted into the prepared mixture and the mixture was again vigorously vortexed. Finally, we measured corresponding Raman spectra. The same procedure was used for all chemicals and antibiotic resistant bacteria (PolyB-R K-12 *E. coli* & Amp-R K-12 *E. coli*).

SERS Measurements. *SERS Detection of Bacteria in Suspension.* 3 µL of the sample suspension, already treated with different antibiotics, was pipetted onto the normal glass slide. The recording of the Raman spectra was started right after this sample preparation, using the 633 nm line of a He-Ne with a maximum power of 14 mW at the sample and a 10× objective. The Raman spectra were continuously (one spectrum every 40 s) collected with the auto repeat function until the droplet had dried. If not explicitly stated, the exposure time was 1 s and the number of accumulations was 10, and the confocal slit width was 100 µm, detecting a spectral region of 50 to 3000 cm^{-1}.

SERS Mapping On Glass Slides. A droplet of 3 μL of the sample suspension was added on the hydrophobic glass slides and then dried at room temperature. If not explicitly stated, all SERS mapping data were obtained by using 633 nm He-Ne laser line with 0.14 mW power and 50× objective. The exposure time was chosen to be 1 s and the accumulation was 5. The slit width was 100 μm, detecting the spectral region of 50 to 2000 cm^{-1}. The mapping area was 40 × 40 μm^2 with a step size of 2 μm. 441 Raman spectra were collected for each map. To distinguish single live & dead bacteria, normal hydrophilic glass slides were used for the sake of well dispersing bacteria, making sure that one mapping spot (4 μm^2) would only cover one bacterium (for example, *E. coli* DSM 1116, length: 2 μm, diameter: 1.2 μm), so that double counting of a single bacterium can be ruled out. The other experimental parameters were kept constant except for using the 100× objective.

Characterization and Instruments. TEM images were taken with JEM 2010 (JEOL, Munich, Germany) with an accelerating voltage of 200 kV. SERS measurements were conducted with a Raman microscopy spectrometer (LabRAM HR, HORIBA Scientific, Japan).

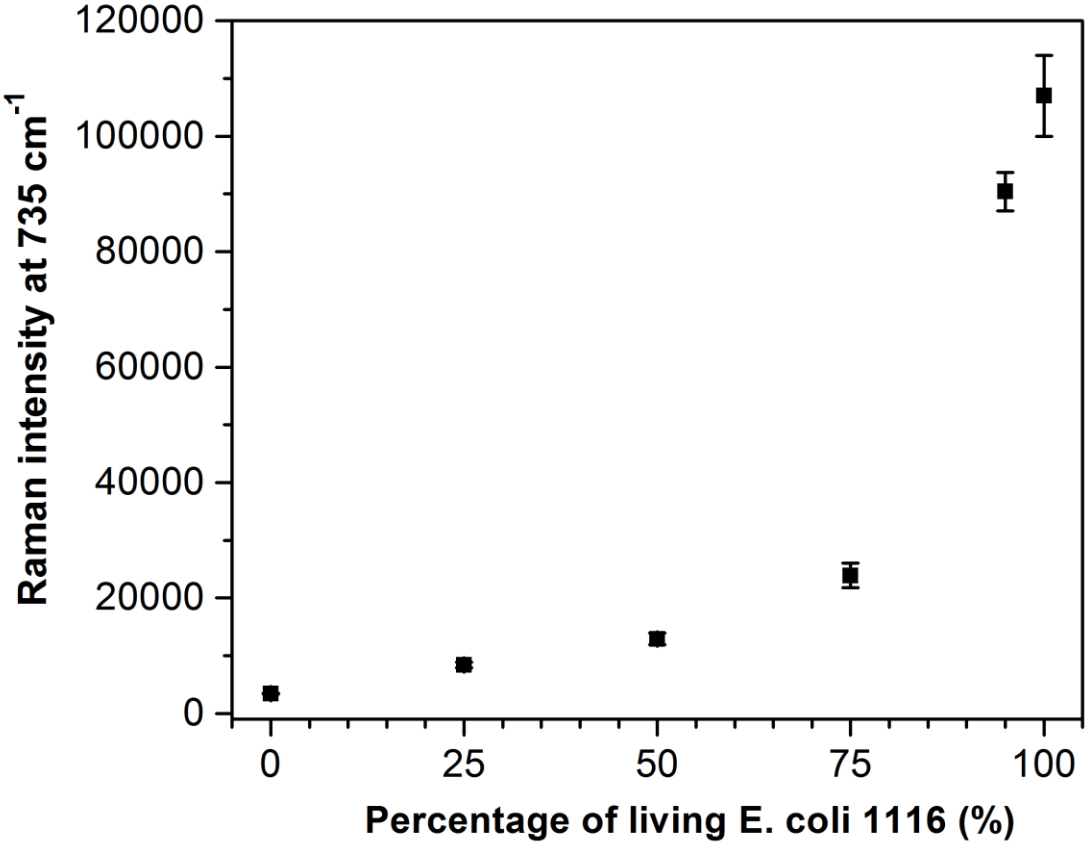

Figure S1. SERS intensity of *E. coli* DSM 1116 at 735 cm^{-1} vs. the corresponding percentage of live *E. coli*.

Table T1. The tentative band assignment of the SERS spectra of the *E. coli* DSM 1116.[4]

Raman Shift (cm^{-1})	**Assignment***
563	carbonhydrates
624	aromatic ring skeletal
652	δ(COO-)
735	adenine, glycosidic ring mode
808	υ(CN) tyrosin, porine, valin
955	υ(CN)
1128	amide III, adenine, polyadenine and DNA
1250-1310	amide III
1268	δ(CH_2) amide III
1330	υ(NH_2) adenine, polyadenine, DNA
1360-1440	υ(COO-) symmetric
1368	υ(COO-) and δ(C-H) proteins
1440-1460	δ(CH_2) saturated lipids
1540-1645	amide II, υ(CN), γ(NH)
1640-1680	amide I

* Approximate description of the modes (υ, stretch; δ and γ, bend).

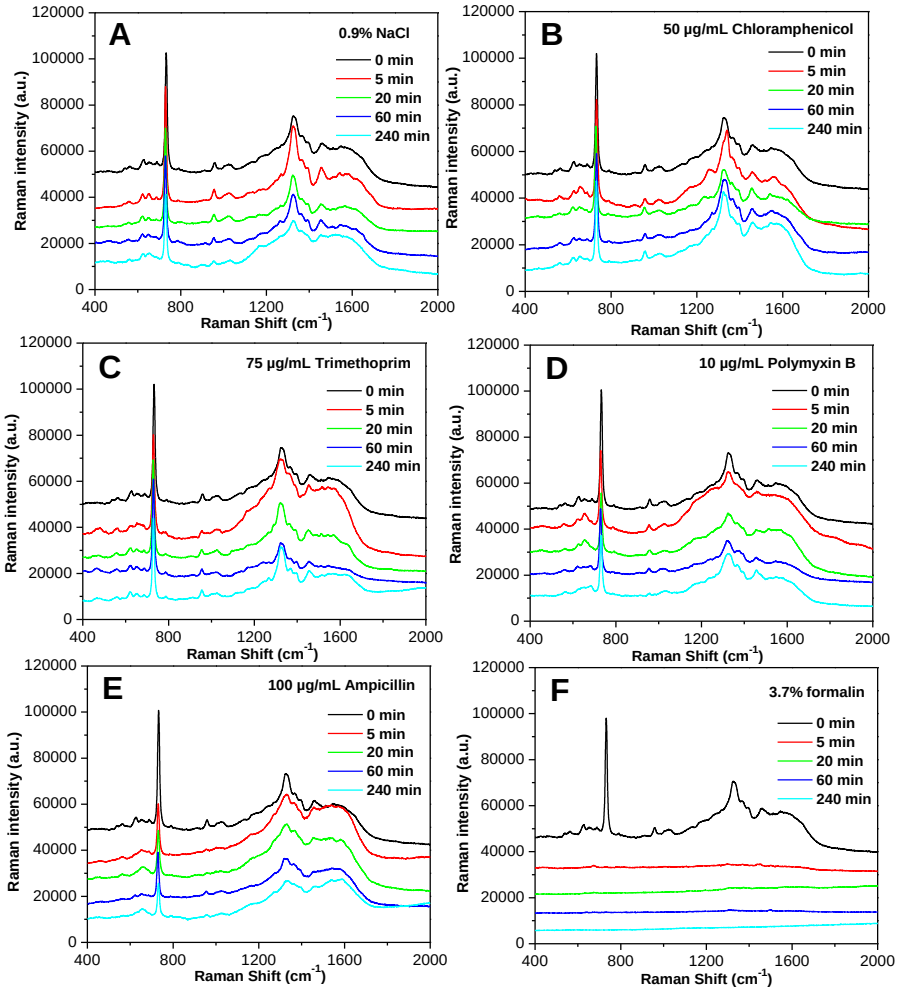

Figure S2. The SERS spectra of bacteria (1×10^7 cell/mL *S. epidermidis*) confronted with different chemicals. Each measured at 0, 5, 20, 60, and 240 min, respectively. The substances used were 0.9% NaCl (A), 50 μg/mL chloramphenicol (B), 75 μg/mL trimethoprim (C), 10 μg/mL polymyxin B (D), 100 μg/mL ampicillin (E), and 3.7% formalin (F).

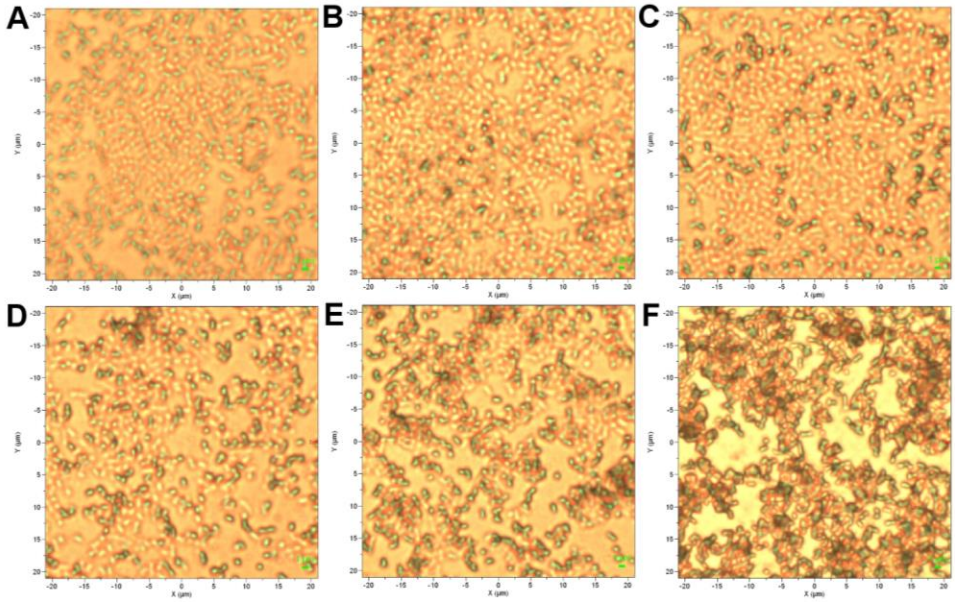

Figure S3. Optical images of different percentage of dead *E. coli* DSM 1116 on hydrophobic glass slides. The percentages of dead bacteria are 0% (A), 5% (B), 25% (C), 50% (D), 75% (E) and 100% (F), respectively. The concentration of *E. coli* used was 1×10^8 cell/mL; 50× objective.

References

[1] A. Wolter, R. Niessner, M. Seidel, *Anal. Chem.* **2008**, *80*, 5854-5863.

[2] D. M. Gao, Z. Y. Wang, B. H. Liu, L. Ni, M. H. Wu, Z. P. Zhang, *Anal. Chem.* **2008**, *80*, 8545-8553.

[3] H. Zhou, D. Yang, N. P. Ivleva, N. E. Mircescu, R. Niessner, C. Haisch, *Anal. Chem.* **2014**, *86*, 1525-1533.

[4] a) M. Knauer, N. P. Ivleva, X. J. Liu, R. Niessner, C. Haisch, *Anal. Chem.* **2010**, *82*, 2766-2772; b) A. Walter, A. Marz, W. Schumacher, P. Rosch, J. Popp, *Lab Chip* **2011**, *11*, 1013-1021; c) A. Sengupta, N. Brar, E. J. Davis, *J. Colloid Interf. Sci* **2007**, *309*, 36-43; d) N. P. Ivleva, M. Wagner, A. Szkola, H. Horn, R. Niessner, C. Haisch, *J. Phys. Chem. B* **2010**, *114*, 10184-10194.