

Silica encapsulated SERS nanoprobe conjugated to the bacteriophage tailspike protein for targeted detection of *Salmonella*

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Experimental

Materials

Ultrapure water (18.2 MΩ cm⁻¹) was used to prepare all aqueous solutions. Hydrogen tetrachloroaurate (III) trihydrate (99.9 %, Sigma-Aldrich) and sodium citrate tribasic dihydrate (99.0 %, Sigma-Aldrich) were used in the preparation of the gold nanoparticles. Tetraethyl orthosilicate (TEOS) (99.0 %, Aldrich), sodium hydroxide (97.0 %, Merck), and (3-mercaptopropyl)-trimethoxysilane (MPTMS) (95.0 %, Aldrich) were used for silica coating. Ammonia hydroxide (28-30 wt-%, EMD), ethanol anhydrous, and N, N-dimethylformamide (DMF) (99.8 %, EMD) were used as received. N-[3-(trimethoxysilyl)-propyl]diethylenetriamine (Aldrich) and succinic anhydride (99.0 %, Aldrich) were used for functionalization of NAEBs. 3'-Diethylthiadicarbocyanine iodine (DTDC) (98.0%, Aldrich) was used as Raman reporter. Sodium dihydrogen orthophosphate (98.0 %, BDH), disodium hydrogen phosphate (99.0 %, BDH), and sodium chloride (99.0 %, BDH) were used to prepare phosphate-buffered saline (PBS) buffer. N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) (Sigma) and N-hydroxysuccinimide (NHS) (98.0 %, Aldrich) were used for antibody conjugation. Bovine serum albumin (BSA) was purchased from Sigma-Aldrich. HisProbe-HRP was purchased from Thermo Scientific (Thermo Scientific Cat. Number 15165). 6-Maleimidoheptanoic acid N-hydroxysuccinimide ester (NHS-maleimide) was purchased from Sigma Aldrich (> 98%, Aldrich M9794).

Synthesis of NAEB

Gold nanoparticles with a mean diameter of 12 nm were synthesized as specified in our previous publications.^{1, 2} The pH of 4.0 mL of Au sol was adjusted to 10 by adding 320 μL of 100 mM NaOH_(aq). A solution of 100 μL of 10⁻⁴ M DTDC_(aq) was then added to the colloidal sol under vigorous stirring. This solution was allowed to equilibrate for 15 minutes. To the equilibrated solution, 20 μL of 5.0 x 10⁻⁵ M of MPTMS in ethanol was then added and allowed to equilibrate for another 15 minutes. The as-prepared solution of aggregated gold nanoparticles with an adsorbed Raman reporter was mixed with 16 mL ethanol in a 50 mL falcon tube. Under vigorous shaking, 0.5 mL of 33 wt.-% ammonium

hydroxide_(aq) was added to the falcon tube, and followed by the addition with 0.6 mL of 47.5 mM TEOS in ethanol eight times within 4 h (at a time interval of 0.5 h). After injection of TEOS ethanol solution, the reaction mixture was allowed to react for 12 h. The reacted mixture was then centrifuged at 12000 rpm for 5 min and the Au/DTDC@SiO₂ core-shell particles precipitate was redispersed into ethanol for further washing. The as synthesized NAEB surface was terminated with a –OH functional group.

NAEB Surface modification and protein cross-linking

I. TSP conjugation onto NH₂-terminated NAEB (Method I)

Before immobilizing TSPs onto the NAEBs, the surfaces of –OH terminated NAEB were chemically modified to –NH₂ termination. To form the amine-functionalized group on the NAEBs surface, 12.0 mL of 8.0 x 10¹²/mL NAEB was diluted to 24 mL by anhydrous ethanol, and then reacted with 53.8 µL of diethylenetriamine (DETA) under continuous stirring for overnight at room temperature. Then the solution was transferred into two necks round-bottom flask. Under refluxing, the solution was heated to 70°C for 1 h. These amine-functionalized NAEB were thoroughly washed four times with ethanol. 4 mL of 2.4 x 10¹³/mL –NH₂ terminated NAEB was diluted to 6.0 mL by DMF, and then reacted with 644 µL of 100 mg/mL NHS-maleimide under N₂ gas overnight at room temperature with stirring. Then the maleimide-functionalized NAEB was thoroughly washed with DMF, ethanol, and PBS buffer. NHS-Maleimide activated NAEB (640 µL of 2.4 x 10¹³ /mL) was then reacted with 48 µL of 0.8 mg/ml of TSP and stored overnight at 4 °C. The resulting nanoparticles were centrifuged twice at 12,000 rpm for 6 min and redispersed in 300 µL of PBS buffer.

II. TSP conjugation onto Zn²⁺ implanted NAEB (Method II)

Zn²⁺ implanted NAEBs were synthesized by a modified protocol from reference ^{3,4}. 80 mg of zinc acetate was added into 8 mL of 8.0 x 10¹²/mL of (as synthesized) NAEB. This solution was sonicated for 3 hours. The resultant nanoparticles were centrifuged 4 times at 12000 rpm for 6 minutes, washed and re-dispersed in PBS buffer. The concentration of the Zn²⁺-NAEB was adjusted to 2.4 x 10¹³ /mL. To every 100 µL of 2.4 x 10¹³ /mL Zn²⁺-NAEB, 46.1 µL of 0.52 mg/ml of TSP was added and incubated overnight at 4 °C. The resulting TSP-conjugated NAEBs were centrifuged twice at 12000 rpm for 6 minutes and redispersed in PBS buffer.

III. TSP conjugation onto Ni²⁺-HRP activated NAEB (Method III)

4 mL of 2.4 x 10¹³/mL –NH₂ terminated NAEB (as prepared in I) was diluted to 6.0 mL by DMF and reacted with 0.6 g of succinic anhydride in N₂ atmosphere overnight at room temperature. The carboxylate-terminated NAEBs were thoroughly washed 4 times by DMF, ethanol, PBS buffer and re-dispersed in PBS buffer. 200 µL of 2.4 x 10¹³/mL of carboxylate-terminated NAEB was activated with 170 µL of 100 mg/ml EDC and NHS for an hour at room temperature under stirring. The resulting NAEBs was centrifuged at 12000 rpm for 6 min twice (in PBS buffer) and re-dispersed in 200 µL of PBS buffer. The NHS-activated NAEB was reacted with 50 µL of 6.25 mg/mL of Ni²⁺-HRP (HisProbe-HRP, Thermo Scientific) and stored overnight at 4 °C. The

Ni²⁺-HRP-activated NAEBs were again centrifuged twice at 12000 rpm for 6 minutes and re-dispersed in 200 μ L of PBS buffer. 200 μ L of 2.4×10^{13} /mL of Ni²⁺-HRP-NAEB was incubated with 80 μ L of 0.3 mg/ml of TSP and stored at 4 °C for overnight. TSP conjugated NAEBs were centrifuged twice at 12000 rpm for 6 minutes and re-dispersed in 100 μ L of PBS buffer.

Transmission electron microscopy (TEM) and energy dispersive X-ray spectroscopy (EDX)

Samples were prepared by drop casting a 1 μ L of 2.4×10^{13} /mL Zn²⁺-NAEB on a formvar coated Cu grid and allowed to dry in the N₂ atmosphere for 24 hours before imaging. The transmission electron microscopy (TEM) was performed on a JEOL JEM-2100F field emission source transmission electron microscope equipped with a scanning unit (STEM) operating at 200 kV. For the present study, bright field TEM (BF-TEM) images (e.g. Fig. 1a) were obtained using a Gatan CCD camera, and bright field scanning TEM (BF-STEM) images (e.g. Fig. 2a) were obtained using a Gatan bright field STEM detector attached to the JEM-2100F. The chemical composition of NAEB was further studied by the energy dispersive X-ray spectroscopy (EDX) (e.g. Fig. 2b) in the STEM mode (STE-EDX) using an Oxford INCA Energy TEM 200 attached to the JEM-2100F.

Optical spectroscopy and microscopy

UV-vis absorption and fluorescence spectra were recorded using a Varian Cary 100 Scan Spectrophotometer and a PTI Luminescence Spectrophotometer, respectively. Raman spectroscopy and microscopy was acquired with a commercial microRaman system (LabRAM HR, Horiba Jobin Yvon) equipped with a software controlled XY stage and a thermoelectrically cooled CCD detector. SERS detection is carried out by spectroscopic imaging of a 1 μ L drop of solution on a clean Si wafer. Raman excitation was carried out with 632.8 nm laser at a power of 17 μ W focused down to $\sim 1 \mu\text{m}^2$ spot. The focused beam is raster scanned over the area of interest while collecting a complete SERS spectrum from each pixel. Acquisition time for each pixel is 0.5 second. A Raman intensity map was regenerated by fitting each the Raman spectra to remove the associated background or fluorescence components. This was achieved by the Labspec 5.17 software (Horiba Jobin Yvon).

Cell Growth and Microagglutination Assay

Growth of cells: *Staphylococcus aureus* (ATCC 12598) and *Salmonella* (ATCC 19585) were ordered from American Type Culture Collection (Manassas, VA). *Salmonella* cells were inoculated in a 10 mL nutrient broth (Becton, Dickinson and Company, Sparks, MD) and grown overnight at 37 °C, 150 rpm. The next day, the culture was spun down in a fixed rotor, Sorval RT6000B refrigerated centrifuge at 6000 rpm for 10 minutes. The cell pellet was resuspended in PBS buffer and adjusted to OD₆₀₀ of 1 which is equivalent to 3×10^8 cells/mL.

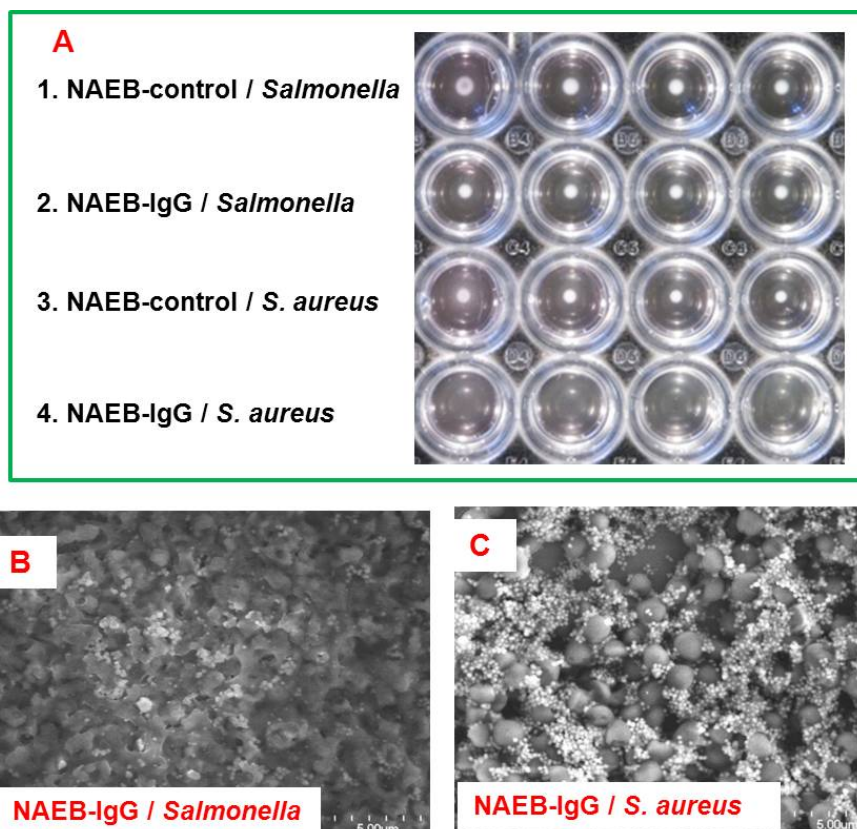
A single colony of *S. aureus* from a Brain Heart Infusion (BHI) plate (EMD Chemicals Inc., Darmstadt, Germany) was inoculated into 10 mL of BHI broth and grown overnight

at 37 °C, 200 rpm. The next day, the culture was spun down in a fixed rotor, Sorval RT6000B refrigerated centrifuge at 5,000 rpm for 10 min. The cell pellet was resuspended in PBS, pH 7.0, and the cell density was measured at OD₆₀₀. The titer was determined by spreading serial dilutions of the cultures on BHI plates and incubating the plates overnight at 37 °C. An OD₆₀₀ of 1.0 is equivalent to 1x10⁸ cells/mL.

Binding analysis by microagglutination assays: The microagglutination assay was performed similarly to our earlier established protocols.¹ NAEB in PBS solution was serially diluted down the row of the titer plate. To each well, one OD₆₀₀ unit of the appropriate cell sample in 50 µL buffer was added. The plate was incubated overnight at 4 °C. In the morning, pictures of the plates were taken for further analysis.

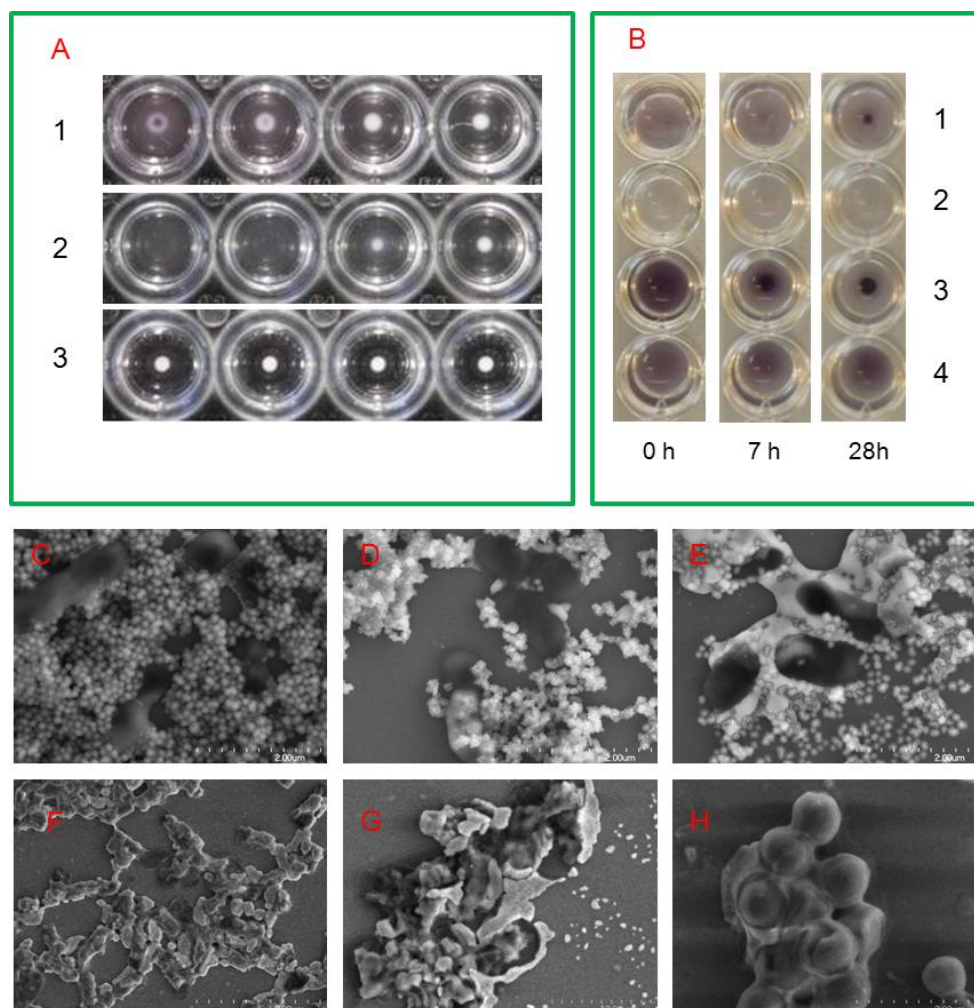
Figure S1. Agglutination Assay of polyclonal IgG-NAEB

NAEB conjugated to a commercial Rabbit polyclonal IgG (Abcam, ab35156), a *Salmonella* primary antibody, showing significant cross-reactivity with *S. aureus*.



(A). Agglutination assay of control NAEB (without IgG) and IgG-NAEB against *Salmonella* (rows 1 & 2) and *S. aureus* (rows 3 & 4). IgG-NAEB agglutinates *Salmonella* mildly as shown in row 2, but agglutinates *S. aureus* strongly (row 4) likely due to the strong interaction between the F_c binding domain of IgG and protein A of *S. aureus*. SEM images confirm the cross-reactivity of IgG-NAEB binding to both *Salmonella* (B) and *S. aureus* (C). In line with the observation of the agglutination assay, Fig. S1 C shows noticeably more NAEBs on the *S. aureus* cells while Fig. S1 B has fewer NAEB present on the *Salmonella* surface.

Figure S2. Agglutination Assay of TSP-NAEB Agglutination assays and SEM images of control-NAEB and TSP-NAEB (prepared by methods I, II and III) against *Salmonella* (target organism) and *S. aureus* (control organism).



(A). Row 1, Control NAEB showed negative reactivity against *Salmonella* cells. Row 2, TSP-NAEB prepared by maleimide conjugation (method I) successfully agglutinates *Salmonella*. Row 3, TSP-NAEB (Maleimide, method I) shows no reactivity against the control organism, *S. aureus*.

(B). Time lapsed agglutination of TSP-NAEB and control-NAEB against *Salmonella*. Note that this agglutination does not show the serial dilution but rather shows the response of a single well over 28 hours of reaction time. Rows 1 and 2 shows the control-NAEB (no TSP) and TSP-NAEB (as prepared by method III) treated against *Salmonella*. In row 1, the control-NAEB shows no agglutination response towards *Salmonella*. While the concentration of row 2 is too low to be visually inspected, SEM images E confirms positive binding between TSP-NAEB and *Salmonella*. Row 3 and 4 shows the time lapsed agglutination response of control-NAEB (no TSP) and TSP-NAEB (as prepared

by method II). Again, control NAEB (row 3) shows a negative agglutination response while TSP-NAEB (row 4) agglutinates *Salmonella* effectively which is also confirmed by SEM image D.

SEM images C, D and E show the positive binding of TSP-NAEB (prepared by method I, II and III, respectively) and *Salmonella* cells. Images F, G correspond to the negative binding response of control NAEB (no TSP) against *Salmonella* while image H shows the negative binding response of TSP-NAEB against control organism *S. aureus*.

Figure S3. SERS detection of NAEB-labeled *Salmonella*

Images A, B and C show the corresponding SEM, optical and SERS intensity map of a group of TSP-NAEB (prepared by method I) labeled *Salmonella* cells. Images D, E and F shows the corresponding SEM, optical and SERS intensity map of two *Salmonella* cells labeled with TSP-NAEB (prepared by method II). Scale bars are 1 μm .

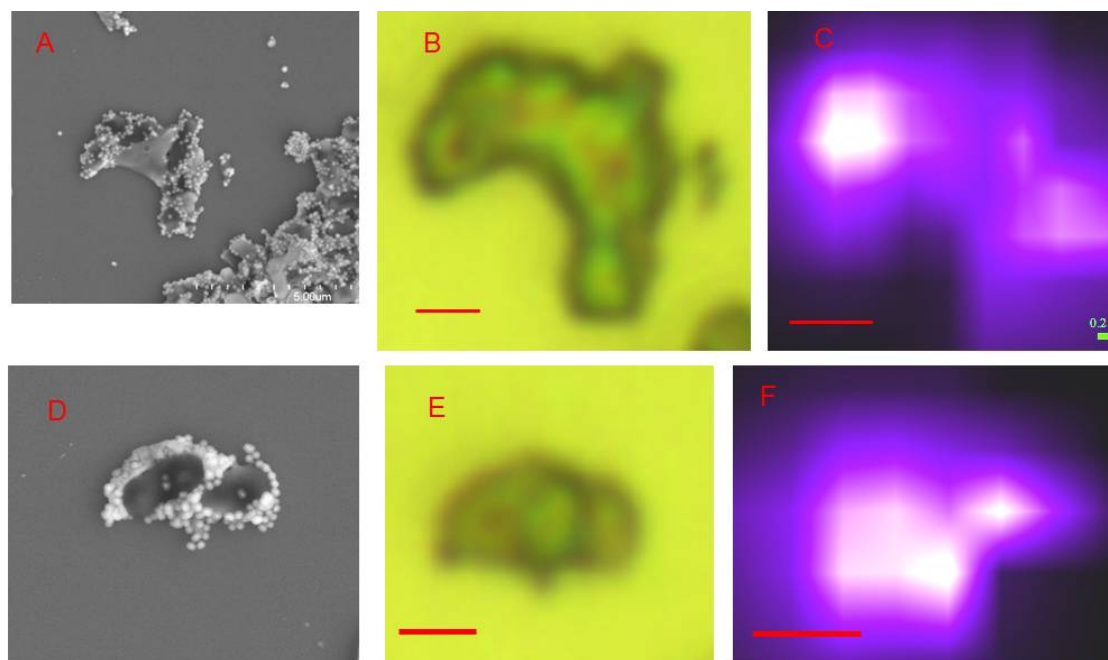
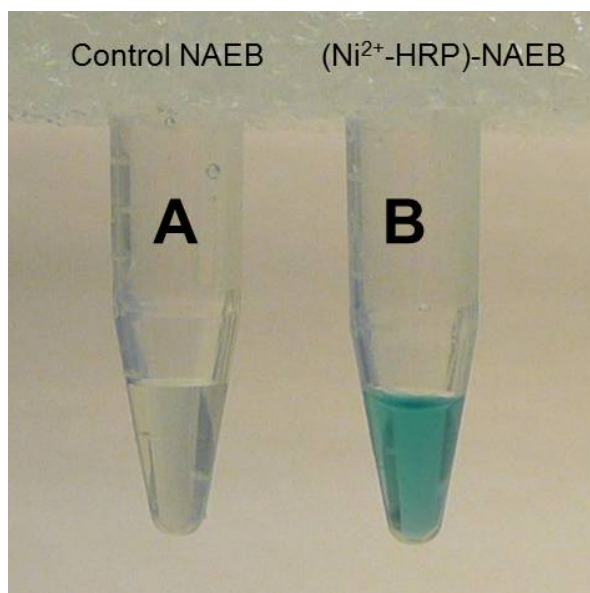


Figure S4. Verification of Ni²⁺-HRP conjugated to NAEB (Method III, Scheme I)

Reactions of the chromogenic reagent TMB in H₂O₂ of (A) control NAEB with COOH surface termination and (B) Ni²⁺-HRP-NAEB. Tube B shows the distinct colour change indicating the presence of Ni²⁺-HRP on NAEB.



A commercially available His-probe (ESI) which incorporates a Ni²⁺ activated derivative of horseradish-peroxidase (Ni²⁺-HRP) is immobilized on the COOH-terminated NAEB as outlined in Method III of Scheme 1. This requires the conversion of NH₂-NAEB into –COOH termination which is detailed in page 2 of ESI. The COOH-NAEBs were activated with EDC/NHS before conjugating to the Ni²⁺-HRP through the exposed Lys residue. One advantage in the Ni²⁺-HRP conjugated NAEB is that the conjugation can be verified easily by a chromogenic reagent for HRP such as 3,3',5,5'-tetramethylbenzidine (TMB ELISA, Pierce: 34022). Figure S4 (ESI) shows both control NAEB and Ni²⁺-HRP conjugated NAEB that were verified with TMB substrate in the presence of H₂O₂. A positive conjugation was confirmed by a distinct colour change (Fig. S4, ESI) in the Ni²⁺-HRP-NAEB. The Ni²⁺-HRP-NAEB is then incubated with TSP to produce TSP-NAEB conjugate as shown in Method III of Scheme 1.

References:

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