Supporting Information

Gold Nanostars for the Detection of Foodborne Pathogens via Surface-Enhanced Raman Scattering Combined with Microfluidics

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1. **Gold nanostars for in-flow average SERS measurements.**

The use of GNSs in suspension offers the attractive simplicity of the experimental design (i.e. cost reduction, less purification steps, etc) and average SERS enhancement under fairly reproducible experimental conditions. As the direction contact of nanoparticles with the environment can produce important changes in terms of colloidal stability and electrokinetic properties and as consequence to affect the enhancement in SERS experiments.\(^1\) For this, we selected encoded-GNSs to protect with a silica shell the plasmonic surface. This avoids the increase of the variability in the SERS signal due to the formation of “new” hot-spots (e.g. aggregation) during in-flow measurements, which can increase the variability of the SERS signal. The silica shell acts as a dielectric spacer and thus avoids any additional plasmonic coupling between closely packed GNSs.\(^2\)

2. **The optofluidics approach**

A detection strategy based on microfluidics and hydrodynamic flow focusing was selected to facilitate an accumulation of the SERS signal during in-flow detection. In this way, an average SERS signal was acquired at a specific volume given by the objective, the flow and the microfluidic geometry. Therefore, while encoded GNSs were “homogeneously” distributed in the whole sensing volume, the encoded GNSs that interact with the bacterium were specifically accumulated on the area of a single bacterium, increasing the average SERS signal. This increase was proportional to the number of GNSs per unit area of the bacterium. Thus, the lowest average SERS signal was acquired with encoded GNSs alone and the highest average SERS signal was observed when the sensing volume was saturated by bacteria (in this case *L. monocytogenes*) conjugated with specific antibody-functionalized encoded GNSs. As the antibody specific for *L. monocytogenes* was selected (mAb C11E9) at the same cfu mL\(^{-1}\) concentration, the average SERS
signals of *L. innocua* and *S. Typhimurium* were lower and statistically different than the average signal of *L. monocytogenes* as shown in Figure 4D for two 1NAT peaks (1371 and 1551 cm⁻¹). The variability of the SERS signal in Figure 4 is the cause of a the contribution of the in-flow detection rather than of the gold nanostars. For the sample having a concentration of 1×10⁴ cfu mL⁻¹ the statistical analysis performed in Figure 4D shows that the area under the curve of the 1NAT peaks, reporting for the presence/absence of the bacteria, was not significantly different than that of the signal corresponding to either the negative control for the bacteria *S. Typhimurium* or of just the GNSs alone in the channel, used as threshold reference of the NPs signal. The sensitivity of this method may be further increased by either increasing the acquisition time during the measurements accumulating more data, and also by increasing the concentration of the sensing NPs used in future studies. It is worth noting that by using 20× instead of 10×, the spatial resolution could be reduced from ca. 1.10 µm to 0.69 µm, which may allow to reach the saturation of the sensing volume at lower bacteria concentration. Therefore, the detection limit of this strategy may be further improved. The authors present this work herein to make a prompt release of these results to the nanomaterials, lab-on-a-chip and optofluidics community, in order to expand the applications of these type of automated and miniaturized platforms.

3. Future work

Using this in-flow strategy, it is possible as well to avoid washing steps after incubation, as the average SERS signal for the single nanoparticles is much lower than the accumulated SERS signal on the bacteria as shown in Figure 4, reducing the sample preparation process. The further use of this method in food samples though, will have to include, as usual for foodborne pathogen detection, a pre-sample processing using either centrifugation,³ filtering⁴ or degradation⁵ of the food matrix prior to analysis. Also, clog-free microchannels will have to be included in the design
for a proof-of-concept of this methodology.\textsuperscript{6} For the validation with real samples, there will be a comparison with respect to the classical method (ISO 11290).

Finally, despite the lowest concentration discriminated was still high for foodborne pathogen applications, more than demonstrating that high sensitivity could be reached, this work was aimed towards the demonstration of the combination of SERS and microfluidics and its potential to be used in automated analysis of foodborne pathogens.

**EXPERIMENTAL SECTION**

**Materials.** All chemicals were purchased from Sigma-Aldrich unless otherwise stated. All glass labware was washed with *aqua regia* and Milli-Q® ultrapure (18.2 MΩ·cm) for the nanoparticle synthesis.

**Bacteria culture.** *L. monocytogenes* WDCM00021, *L. innocua* WDCM00017 and *Salmonella enterica* serovar Typhimurium WDCM00031 (*S. Typhimurium*) were selected as reference strains. Reference values of viable microorganisms were obtained by culturing the bacteria in Nutrient Broth (NB, Biokar Diagnostics S.A., France) at 37 °C overnight, then each bacterium were ten-fold serially diluted in NB, the *Listeria* spp. were plated on Tryptic Soy Yeast Extract Agar (TSYEA, Biokar Diagnostics S.A., France) while the *Salmonella* on Tryptic Soy Agar (TSA, Biokar Diagnostics S.A., France). The plates were incubated at 37 °C overnight.

**SERS tags synthesis and biofunctionalization.** GNSs were prepared by using a modification of the seed-mediated growth method previously reported by Kumar et al.\textsuperscript{7} Gold spheres of 15 nm were synthesized by the Turkevich method, and further used as seeds.\textsuperscript{8} Briefly, 5 mL of a 1% solution of sodium citrate dihydrate was added to 100 mL of boiling chloroauric acid (HAuCl$_4$),
at 0.5 mM, under vigorous magnetic stirring. Upon the addition of the sodium citrate, the solution was kept under magnetic stirring and boiling until the colour changed from yellow to dark red. Following, citrate was replaced by polyvinylpyrrolidone 10K (PVP MW = 10,000 g mol⁻¹), using 60 molecules of PVP per nm². One mL of PVP (5.6 mM) was added to 25 mL of gold nanospheres (0.557 mM) under vigorous stirring and left overnight. The resulting dispersion was centrifuged for 90 minutes at 7000 rpm, re-dispersed in 25 mL of isopropanol and stored in the fridge until further use. For the seed-mediated growth of the GNSs, 25 g of PVP-10K were dissolved in 250 mL of DMF for 15 minutes by using an ultrasound sonicator. Following, 1 mL of an aqueous solution of 0.126 M HAuCl₄ was added and the mixture was vigorously magnetically stirred for two minutes in order to allow the Au³⁺ to be reduced to Au⁺¹. After, 1.3 mL of 1.8 mM Au@PVP seeds were added to the reaction flask and the reaction was left 1 h under magnetic stirring. The resulting GNSs were washed six times (4000 rpm, 30 min) with isopropanol to remove excess PVP and DMF and stored in isopropanol protected from the light until further use. The methodology for the calculation of this size distribution of the GNSs (Figure S1) was adopted from Vo-Dihn and co-workers. This specific synthetic approach was selected as it provides very stable GNSs with enhanced stability regarding reshaping and aggregation. Further, this synthetic methodology does not involve the use of CTAB (cetyltrimethylammonium bromide), which is not only toxic, but also increases the risk of reshaping.

For the Raman labeling of GNSs, 1-napthalenethiol (1NAT) was used as Raman reporter and 11-mercaptoundecanoic acid (MUA) was used for stabilisation and silica growth promotion. 1NAT (10⁻⁴ M) and MUA (1 µM) were added to the GNSs under magnetic stirring for 2 hours, centrifuged (2500 rpm, 30 min) and re-dispersed in isopropanol. Following, the resulting GNSTs@1NAT were coated with a mesoporous silica shell by adapting the Stöber controlled silica
growth method.\textsuperscript{12} For this, 14 µL of NH\textsubscript{4}OH and 0.167 mL of H\textsubscript{2}O were added to 2.5 mL of the GNSs@1NAT under magnetic stirring. After 2 minutes to allow homogenisation, 16.7 µL of tetraethyl orthosilicate (TEOS) was added to the mixture and left under magnetic stirring for 8 hours. The silica-coated GNSs were centrifuged (2500 rpm, 15 min) and re-dispersed in ethanol.

A monoclonal antibody (mAb) C11E9, originally produced by Bhunia el at.,\textsuperscript{13} was selected for the functionalization of the GNSs. The fixed-orientation conjugation of mAb C11E9 to the resulting GNSs@1NAT@SiO\textsubscript{2} was carried out via Protein G (PG). First, 1 mM of GNSs@1NAT@SiO\textsubscript{2} was transferred to 10 mM filtered phosphate buffer (PB) pH 7.4 by centrifuging at 4600 rpm for 10 min and functionalized with 0.5 mM carboxyethyl-silanethiol (CST). After 10 h incubation at room temperature (RT), the functionalized particles were centrifuged at 4000 rpm for 10 min and redispersed in 10 mM PB to reach a gold concentration of 4 mM. Then, a solution of 20 µM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and 50 µM N-Hydroxysuccinimide sodium salt (NHS) was mixed with prepared GNSs@1NAT@SiO\textsubscript{2}-CST suspension and the total volume was brought to 0.5 mL with PB. The suspension was incubated at RT under orbital shaking for 15 min to activate the carboxylic groups. Then, the particles were incubated with 1.5 µg of PG (280 PG/GNS) at RT under orbital shaking for 2 h. The remaining activated carboxyl group was blocked with 0.1 mg mL\textsuperscript{-1} NH\textsubscript{2}-PEG 5 kDa. The suspension was then incubated at RT under shaking for 60 min. The resulting GNSs@1NAT@SiO\textsubscript{2}-PG were centrifuged at 4000 rpm for 5 min and redispersed in PB. Finally, the PG-functionalized GNSs were incubated with 15 µg of mAb C11E9 for 1 h under orbital shaking. The oriented-C11E9-conjugated GNSs were centrifuged (1000 rpm for 2 min) and redispersed in 250 µL PB.
**Bacteria incubation with SERS tags.** Bacterial concentration was adjusted to $10^6$ cfu mL$^{-1}$ by diluting in PB (10 mM, pH 7.4) the fresh cultures prepared as described above in section “Bacteria culture”. For the labeling of the bacteria with the NPs, 400 µL of bacteria at specific densities and 40 µL of the mAb C11E9 functionalised GNSs were mixed and incubated for 30 min. After incubation, the resulting mixture was used directly in SERS experiments on-chip. The matrix of experiments is shown in Table 1.

**Microfluidics device fabrication.** For the fabrication of the polydimethylsiloxane (PDMS) microfluidic devices, a soft-lithography method previously described was adapted. Briefly, the geometry of the microfluidic channels was designed with Autocad 2013 (Autodesk), and a dark-field mask was printed (JD-Photo Data, UK). SU-8 2025 negative photoresist (MicroChem) was spin-coated onto a silicon wafer (diameter: 76.2 mm) at 500 rpm for 5 s and then ramped to 1000 rpm at an acceleration of 300 rpm s$^{-1}$ for 33 s. A final thickness of 80 µm, as measured by profilometry on the finished master (KLA-Tencor). After spinning, the wafer was prebaked (3 min at 65 °C, then 9 min at 95 °C) and exposed to UV light through the acetate mask on a mask aligner for 10 s (lamp intensity 50 mW cm$^{-2}$; MA6BA6, Suss Microtech). After post-baking (1 min at 65 °C and 4 min at 95 °C) and development in SU-8 developer (PGMEA, Sigma Aldrich), the master was hard-baked for 2 min at 170 °C. For the fabrication of the PDMS (Sylgard 184) replicas, a mixture of PDMS and cross-linker (ratio 10:1, w/w) was poured on top of the master and on top of an empty petri-dish, degassed and then cured for 1 h at 65 °C. The cured device was cut and peeled off from the master, and holes for tubing were made with a biopsy punch (diameter = 1 mm; Kai Medical). The cured PDMS blank part on the empty petri-dish was peeled-off and used as a base for bonding for obtaining the final PDMS/PDMS devices.
Optical, morphological and SERS characterization. For the characterization of the resulting GNSs and GNSs@1NAT@SiO$_2$ both UV-visible spectrophotometry (SHIMADZU UV-2550) and transmission electron microscopy (JEOL 2100 200kV) were used. TEM samples were prepared by drop casting 10 µL of the suspensions of the NPs on 400 mesh copper grids (Ted Pella, INC). For the SERS characterization of the GNSs@1NAT@SiO$_2$ a confocal Raman (Alpha 300R Witec) was used. For this, 200 µL of the material were measured in average Raman mode for 10s and 1 scan, using 785 nm excitation laser line and the 10× objective. A 600 lines mm$^{-1}$ grating and a CCD camera were used for all spectra acquisition detailed in the manuscript.

SERS measurements on-chip. The PDMS/PDMS device was placed into the Raman stage (Alpha 300R Confocal Raman, Witec) and the different fluids were connected via polyethylene tubing (Smiths Medical, 0.38 mm inner diameter) and pumped using syringe pumps (New Era NE-4000). Milli-Q water was used in the outer inlet to narrow the central stream containing the GNSs@1NAT@SiO$_2$@Ab labeled bacteria for detection in a flow-focusing geometry device. The flow rates were 100 and 50 µL h$^{-1}$ for the Milli-Q water and targeted bacteria, respectively. Using this in-flow strategy, we are able to avoid washing steps after incubation, as the average SERS signal for the single nanoparticles is much lower than the accumulated SERS signal on the bacteria as shown in Figure 4, reducing the sample preparation time and steps.

Using this flow rates above specified, the width of the central stream $w_s$ could be controlled by the relative flow rates for both the sample (central) and focusing (lateral) inlets. The equation governing this effect is,$^{15,16}$

$$\frac{w_s}{w} = \frac{1}{g(\lambda)} \cdot \frac{Q_s}{Q_s + Q_f}$$  \hspace{1cm} (1)
where \( w \) is the total physical channel width. \( Q_s \) is the sample flow rate and \( Q_f = Q_{f1} + Q_{f2} \) is the total focusing flow rate. Finally, \( g(\lambda) \) is a factor depending on the aspect ratio of the channel. \( \lambda = h/w \) with \( h \) being the channel height. In the limit case when \( \lambda \to 0 \) (\( h << w \)), \( g(\lambda) = 1 \). For the rest of the cases:

\[
g(\lambda) = (1 + \lambda) \left( 1 - 1.3553\lambda + 1.9467\lambda^2 - 1.7012\lambda^3 + 0.9564\lambda^4 - 0.2537\lambda^5 \right), \quad \lambda < 1
\]

For our specific flow-focusing device, \( \lambda = 0.8 \) \( \mu \text{m} \) and applying equation 2, \( g(\lambda) = 1.94 \). According to equation 1 the calculated \( w_s = 17.2 \) \( \mu \text{m} \), whereas the experimental \( w_s \), measured at Figure 3D is 21.8 \( \mu \text{m} \).

The laser spot (785 nm laser line) was focused using a 10× objective at a distance of 100 \( \mu \text{m} \) from the T-junction. The SERS signals were recorded using 1 s integration times and 10 scans for each of the samples. Time series were recorded for all the different solutions (target bacteria solution and controls) for 100 s at a frequency of 1 Hz. The resulting data were treated with the Witec software Project 4 (cosmic ray removal and baseline correction) and Spectragryph (area integration under the peak 1371 and 1551 cm\(^{-1}\) with baseline for the 1NAT).

**PDMS peak assignments:** Si-O-Si symmetric stretching (488 cm\(^{-1}\)), Si-CH\(_3\) symmetric rocking (687 cm\(^{-1}\)), Si-C symmetric stretching (708 cm\(^{-1}\)), CH\(_3\) asymmetric rocking + Si-C asymmetric stretching (787 cm\(^{-1}\)), CH\(_3\) symmetric rocking (862 cm\(^{-1}\)), CH\(_3\) symmetric bending (1262 cm\(^{-1}\)), CH\(_3\) asymmetric bending (1412 cm\(^{-1}\)).\(^{17}\)

**1NAT peak assignments:** The vibrational fingerprint of 1NAT is dominated by the ring stretching (1553, 1503, and 1368 cm\(^{-1}\)), CH bending (1197 cm\(^{-1}\)), ring breathing (968 and 822 cm\(^{-1}\)), ring deformation (792, 664, 539, and 517 cm\(^{-1}\)), and CS stretching (389 cm\(^{-1}\)).\(^{18}\)
Figure S1. (A) Representative TEM micrographs of GNSs. Histograms showing the particle diameter (B) and tip-to-tip (C) distribution of GNSs.
Bibliography


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