Three-Dimensional Plasmonic Trap Array for Ultrasensitive Surface-Enhanced Raman Scattering Analysis of Single Cells

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Supporting Information

ABSTRACT: Single-cell analysis provides an important strategy to evaluate cellular heterogeneity. Although surface-enhanced Raman scattering (SERS) has been considered as a promising label-free technique for single-cell analysis, it remains at the early stage for characterizing the extracellular metabolites of single cells. Herein, we developed a convenient, flexible, and straightforward three-dimensional (3D) plasmonic trap array for simultaneously compartmentalizing and sensitively detecting single-cell metabolites. The 3D trap was spontaneously self-formed by an interfacial-energy-driven process when a liquid droplet was covered with an immiscible oil liquid (polydimethylsiloxane, PDMS). When a droplet of pure AgNO₃ solution was immersed into PDMS, Ag⁺ ions were automatically reduced by the residual Si−H groups in PDMS. Snowflake-like nanoparticles of Ag could be formed on the inner surface of the 3D traps by tuning the concentration of Ag salt precursors and then assembled to flower-like microstructures, endowing the traps with remarkable plasmon enhancement. The established 3D traps exhibited considerably enhanced surface plasmon resonance signals for Raman reporting, and a low detection limit at the aM level was achieved for p-aminophenol. Moreover, these 3D traps can serve as an efficient tool for single-cell SERS measurement. As a proof-of-concept, dipicolinic acid, a common biomarker of bacterial spores, was successfully detected from a single cell. The presented approach provides a versatile tool for label-free and sensitive detection of single-cell environments.

Intracellular or extracellular single-cell analysis plays an important role in elucidating cellular diversity and heterogeneity, and can help to unravel the pathways associated with cellular heterogeneity and disease states, which are masked by bulk measurements. Various single-cell analysis techniques have been developed, including capillary electrophoresis, mass spectrometry, spectroscopy, DNA sequencing, protein arrays, and electrochemical detection. Surface-enhanced Raman scattering (SERS) has been considered as a promising and powerful label-free technique for real-time in situ monitoring of single cells because of its high sensitivity, flexible excitation wavelengths, high spectral resolution, noninvasiveness to biological samples, and resistance to autofluorescence and photobleaching. For biotechnological phenotyping of single cells, one of the key bottlenecks is real-time and label-free determination of the products of cellular secretion or consumption. Although several SERS sensors have been developed to locally probe the intracellular contents of cells, the technique remains at the early stage for characterizing the extracellular metabolites of single cells. To address this limitation, many efforts have been made to exploit the metabolic behavior of single cells by SERS sensing. Recently, a plasmonic nanosensor decorated with metallic nanostructures has been developed for probing metabolite secretion in the extracellular environment using a SERS optophysiology technique analogous to the patch clamp. Developing a convenient and facile approach, with high-throughput potential, for sensitive SERS-based single-cell metabolite probing is still an area of active innovation.

SERS-based single-cell extracellular analysis protocols require a highly sensitive SERS substrate but offer the benefit of effectively encapsulating individual cells. Microwells, micropatterns, microfluidic devices, and traps have been employed with varying degrees of success for cell confinement and manipulation. Endowing the confined single cells with an endogenous hot spot factor for signal enhancement is an effective approach to improve SERS-based sensors. Recently, a novel shell-based spectroscopic platform, known as mechanical trap SERS, was constructed by coating gold nanostars on the inner surfaces of patterned bilayer mechanical traps, making it feasible for simultaneous capture, profiling, and three-dimensional (3D) microscopic mapping of the surface molecular expressions of single living cells. The plasmonic traps exhibited high SERS signal enhancement because of the existence of multiple hot spots with a large combined volume for analyte molecules providing a platform for integrating cell capture and measurement. Most commonly plasmonic
traps have been fabricated on silicon substrates by techniques such as reactive ion etching,\textsuperscript{17} direct laser writing lithography,\textsuperscript{20} photolithography,\textsuperscript{21} plasma-enhanced chemical vapor deposition,\textsuperscript{22} thermal evaporation,\textsuperscript{23} and electron beam lithography.\textsuperscript{24} However, these techniques are usually expensive and require elaborate preparation methods. Thus, an urgent need remains to establish a simple and convenient method for construction of a plasmonic trap array for single-cell analysis.

Herein, we developed a convenient, flexible and label-free method for preparation of a 3D plasmonic trap array for SERS sensing of single-cell secretion. The 3D trap was spontaneously self-formed when a liquid droplet was covered with an immiscible oil liquid (polydimethylsiloxane, PDMS). PDMS has attracted much attention as a support matrix for SERS substrates because of its biocompatibility, chemical inertness, flexibility, and optical transparency.\textsuperscript{25,26} Moreover, PDMS can reduce metal ions to nanoparticles in one step without the requirement of additional reducing/stabilizing agents.\textsuperscript{26} When an AgNO\textsubscript{3} droplet was dripped onto PDMS, Ag\textsuperscript{+} ions were reduced to form an interfacial plasmonic layer, which was coated on the inner surface of the trap. The formed 3D traps served not only to encapsulate the cells but also offered a label-free and sensitive SERS sensing platform for directly probing single-cell extracellular secretion.

\section*{Experimental Section}

Reagents and Materials. \textit{p}-Aminothiophenol (\textit{p}-ATP, 98%), 2,6-pyridinedicarboxylic acid (DPA, 98%), and Luria broth (LB) medium were obtained from Sigma-Aldrich (U.S.A.). All other reagents were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) with analytical grade and used as received without any further pretreatment. \textit{p}-ATP solution was prepared with methanol and further diluted with water. All other solutions were prepared using Milli-Q grade water obtained from a Milli-Q Gradient water purification system (Millipore, Bedford, MA).

\textbf{Instruments.} Scanning electron microscopy (SEM) images were acquired using an Ultra SS field emission scanning electron microscope (Zeiss, Germany) with an accelerating voltage of 5 kV. Transmission electron microscopy (TEM) images and energy-dispersive spectra (EDS) were taken with a Tecnai G2 Spirit TWIN transmission electron microscope (FEI, U.S.A.) at an accelerating voltage of 200 kV. The solution at the water–PDMS interface of the 3D trap was collected for TEM analysis. Atomic force microscope (AFM) images were recorded with an XploRA laser Raman spectrometer (HORIBA Jobin Yvon, France) with a laser power of 2 mW and excitation wavelength of 532 nm.

Fabrication of 3D Plasmonic Trap Array. First, the prepared AgNO\textsubscript{3} solutions with varying concentrations (from 1 to 5 mM) were dripped onto the surface of a cell culture dish (35 mm × 10 mm, Corning) with an average contact angle of 102.8 ± 1.6°. In the second step, a commercially available RTV615 PDMS kit (Momentive) containing the liquid PDMS base and curing agent at their recommended ratio of 10:1 (w/w) was prepared. Then, the PDMS mixture was degassed for 1 h to remove bubbles and poured into the cell culture dish. The culture dishes were slowly cured at room temperature for 12 h to allow the formation of microstructural 3D plasmonic trap arrays. Once solidified, the arrays were cut with a sterile surgical blade and stored for use in detection experiments. The fabrication process of 3D plasmonic trap array was shown in Scheme 1.

\textit{Bacillus subtilis} Cell Culture and Preparation. \textit{Bacillus subtilis} cells (CICC 10732) were purchased from the China Center of Industrial Culture Collection (CICC). Cell cultures were performed according to the standard protocol.\textsuperscript{27} Bacterial cells cultured in 25 mL of LB medium overnight grew to an optical density at 600 nm (OD\textsubscript{600}) of 1.0. The number of B. subtilis cells was determined by optical density measurements.
*B. subtilis* cells was measured by the plate-counter method, by which it was found that a 1 mL sample contained approximately $3 \times 10^8$ cells. Then, the bacterial cells were washed three times by centrifugation (12000 g for 3 min) with 1 mL water. Different concentrations of DPA extractions were prepared through adding different volumes of 0.02 M HNO$_3$ into the cell suspension followed by sonication for 10 min. Green fluorescent protein (GFP)-tagged *B. subtilis* cells (BS168 strain, ATCC23857) were purchased from Biovector Science Lab, Inc. (Beijing, China). The transformation of the GFP-tagged *B. subtilis* cells was carried out using standard laboratory techniques. The tagged cells were cultured according to standard methods. Next, 100 μL transfer cell suspension was inoculated into an Erlenmeyer flask containing 10 mL LB medium with a concentration of 50 μg mL$^{-1}$ kanamycin. Then, the cell cultures were incubated under vigorous shaking at 37 °C for 3 h and grew to an OD 600 of 1.0. Finally, 10 μL of isopropyl-β-D-thiogalactopyranoside (IPTG, 1 M) was added and incubated for 3 h. Then, the cell suspension was washed three times with 1 mL of water by centrifugation (12000 g for 3 min) and resuspended in water.

**SERS Measurements.** SERS measurements were carried out by dripping 2 μL of test solution onto the 3D trap array and putting the array on a glass slide. After static sorption for 10 min, the Raman spectra were collected for five exposure times in the range of 200−2000 cm$^{-1}$. Taking p-ATP as the test molecule, the enhancement factor (EF) was estimated according to eq 1. $^{19−31}$

$$EF = \frac{I_{\text{SERS}}/N_{\text{bulk}}}{I_{\text{Raman}}/N_{\text{bulk}}} \tag{1}$$

where $I_{\text{SERS}}$ is the SERS intensity (at ~1445 cm$^{-1}$) of p-ATP molecules adsorbed on the substrate (the 3D trap), $I_{\text{Raman}}$ is the intensity (at ~1138 cm$^{-1}$) of p-ATP solution with the same concentration as that used to measure the normal Raman spectrum. $N_{\text{bulk}}$ is the number of p-ATP molecules excited under normal Raman conditions and $N_{\text{SERS}}$ is the number of surface-adsorbed p-ATP molecules excited by the laser beam under SERS conditions. Using eq 1, the EF value was estimated from the average of three measurements.

## RESULTS AND DISCUSSION

**Characterization of 3D Plasmonic Trap.** The 3D trap was spontaneously self-formed by an interfacial-energy-driven process when a liquid droplet was covered with an immiscible oil phase, PDMS. When PDMS was added into a dish containing a water droplet adhered on the dish surface, the water did not float on top of the PDMS because of the adhesion between the dish surface and water. Owing to the surface tension of the water droplet (72.8 mN/m) was much higher than that of the liquid PDMS (22−25 mN/m),$^{32}$ after curing at room temperature, but not at higher temperature, high-quality traps were formed, which avoided the fast streaming of water, while allowing the tested samples to be easily added through the relatively large open mouth of the trap cavity. If the water droplets were dripped onto PDMS, rather than vice versa, the droplets tended to sink completely into the liquid PDMS, and the open pore (entrance) of the formed cavity was too small to allow samples through. The diameters of the cavities ranged from dozens of microns to a few centimeters, depending on the volume of the droplets. When AgNO$_3$ droplets came into contact with PDMS, the transparent 3D traps gradually became yellow in color after 10 min, indicating the formation of plasmonic Ag nanoparticles (AgNPs). On the basis of previous reports, the mechanism of self-assembly of the 3D traps can be described as follows: first, Ag$^+$ ions slowly diffused into the bulk of the PDMS liquid; then, without any additional reducing/stabilizing agents, the residual Si−H groups in the PDMS acted as a direct reduction reagent, and reduced the Ag$^+$ ions to AgNPs. $^{18,19,26,32}$ Subsequently, the AgNPs self-assembled at the water−oil interface and self-integrated into a plasmonic thin film layer on the inner surface of the traps, which took advantage of the inherent self-assembly of AgNPs at liquid−liquid interfaces and aggregated at the interface during emulsification. $^{32−35}$ This layer can be expected to improve the signal sensitivity of plasmon-based detection. Normally, the size and density of metal NPs largely affect the total number of “hot” spots. Therefore, controllable growth of AgNPs is the key to maximizing the SERS sensitivity. $^{36}$ Various concentrations of AgNO$_3$ solution ranged from 1 to 5 mM were investigated in order to control the growth of AgNPs for the preparation of 3D plasmonic trap array.

The colors of the 3D traps visibly varied depending on the concentration of AgNO$_3$ solution in contact with PDMS, indicating the synthesis of nanoparticles with different size or morphology. The surface morphologies of the 3D traps with varying concentrations of AgNO$_3$ solution (from 1 to 5 mM) were investigated by SEM. With the increase of AgNO$_3$ concentration, the surface gradually became rougher and the density of AgNPs was increased (Figure S1). Interestingly, as shown in Figure 1A, the reduced AgNPs self-assembled into a flower-like microstructure when the concentration of AgNO$_3$ was 2 mM. To further estimate the size and morphology of the AgNPs formed in the plasmonic layer, nanoparticles formed at the water−PDMS interface were placed onto TEM grids. With the increase of AgNO$_3$ concentration, the average diameters of the AgNPs increased (A, 1 mM: 36.7 ± 3.2 nm; B, 3 mM: 50.5 ± 13.5 nm; C, 4 mM: 64.2 ± 16.9 nm; D, 5 mM: 89.3 ± 10.5 nm; Figure S2). In particular, Ag snowflakes were generated, for which the average diameter of a typical individual branch was 820 nm when using a concentration of 2 mM AgNO$_3$ (Figure 1B,C). Additionally, the flower-like microstructure were characterized by AFM, which found that the average diameter of each branch was approximately 800 nm (Figure 1D).
microstructure with large area-to-volume ratio when the concentration of AgNO₃ was 2 mM, thereby generating more hot spots for SERS analysis of p-ATP. Thus, 2 mM AgNO₃ solution was identified as optimal for the construction of the 3D plasmonic trap array.

To evaluate the SERS activity of the 3D plasmonic trap array, the EF was estimated for the case of p-ATP as the Raman reporter. Without the 3D trap, the Raman scattering signal of 10 μM p-ATP solution was nearly undetectable (Figure 2C (curve a) and Figure S3). When using the 3D trap, the vCS vibration shifted from 1086 cm⁻¹ (Figure S3) to 1080 cm⁻¹ (Figure 2C (curve b)), indicating that the thiol group in p-ATP was directly bonded to the internal surface of 3D trap. Remarkably, the SERS intensity of p-ATP in the 3D trap increased by about 145 times relative to the bulk solution without the trap, implying a large SERS enhancement by the 3D trap, with its high hot spot density. In this system, the laser spot radius was ~2.6 μm and the penetration depth was ~2 μm, while the density of p-ATP is 1.18 g cm⁻³ and its molar mass is 125.19 g mol⁻¹. Nbulk was estimated to be 6.0 × 10¹⁰, and Nads was calculated to be 8.2 × 10⁹ by dropping 2 μL of p-ATP solution (10 μM) on the 3D trap. To simplify the calculation, p-ATP molecules were assumed to be uniformly adsorbed on the surface of the 3D trap and the effect of the surface roughness was ignored. Thus, EF was calculated as 1.1 × 10⁷ using eq 1. The huge enhancement was attributed to the abundance of SERS hot spots in the closely packed flower-like microstructures and the large 3D area imparted by the plasmonic traps, which provided a large-area, ultrasensitive SERS platform for single-cell analysis.

Sensitivity, reproducibility, and stability are crucial to any SERS platform. To estimate the sensitivity of the 3D trap, p-ATP was selected as a probe molecule. As shown in Figure 2D, the SERS intensities gradually increased with the increase of p-ATP concentration, and the main Raman peaks could be clearly seen even when the concentration of p-ATP was as low as 10 aM, presenting an excellent sensitivity for SERS measurement. In contrast, no signal of p-ATP was obtained from flat PDMS or a PDMS trap without nanoparticle modification, revealing that PDMS contributed only a weak background interference in SERS detection.

To access the reproducibility of the 3D trap, six random spots on the surface of six different trap platforms were selected and the SERS spectra at those spots were investigated (Figure 3A). The peak intensities of p-ATP at 1146 cm⁻¹ were measured and the relative standard deviation (RSD) was 5.6% (Figure 3B), suggesting the good uniformity and reproducibility of the fabricated 3D trap. Compared with other SERS substrates, such as quasi-periodic SERS substrate, core–shell SERS substrate, and hybrid nanostructure SERS substrate, the signal reproducibility of the prepared substrate was similar to those values reported for SERS substrates. To evaluate the stability, the SERS spectra were measured after different storage times at room temperature (Figure 3C). After 7 weeks, the SERS signal still retained 83.2% of its initial intensity (Figure 3D), demonstrating the long-term stability of the 3D trap. These good performances endorse the use of the 3D plasmonic trap array as a powerful tool for rapid and label-free SERS detection.

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3D trap, the SERS signals from 10μM 3′,5′-ATP (10 μM) were measured by Raman spectroscopy under 532 nm laser excitation. An aliquot of 2 μL of p-ATP solution (10 μM) was dropped onto the 3D trap array for SERS measurement. Figure 2A presents the SERS spectra of 10 μM p-ATP solution in 3D plasmonic trap array with varying concentrations of AgNO₃ solution (from 1 to 5 mM). Two characteristic fingerprint peaks of 3′,5′-ATP were assigned to the νCS vibration shifted from 1086 cm⁻¹ (Figure S3) to 1080 cm⁻¹ (Figure 2C (curve a) and Figure S3). When using the 3D trap, the νCS vibration shifted from 1086 cm⁻¹ (Figure S3) to 1080 cm⁻¹ (Figure 2C (curve b)), indicating that the thiol group in p-ATP was directly bonded to the internal surface of 3D trap. Remarkably, the SERS intensity of p-ATP in the 3D trap increased by about 145 times relative to the bulk solution without the trap, implying a large SERS enhancement by the 3D trap, with its high hot spot density. In this system, the laser spot radius was ~2.6 μm and the penetration depth was ~2 μm, while the density of p-ATP is 1.18 g cm⁻³ and its molar mass is 125.19 g mol⁻¹. Nbulk was estimated to be 6.0 × 10¹⁰, and Nads was calculated to be 8.2 × 10⁹ by dropping 2 μL of p-ATP solution (10 μM) on the 3D trap. To simplify the calculation, p-ATP molecules were assumed to be uniformly adsorbed on the surface of the 3D trap and the effect of the surface roughness was ignored. Thus, EF was calculated as 1.1 × 10⁷ using eq 1. The huge enhancement was attributed to the abundance of SERS hot spots in the closely packed flower-like microstructures and the large 3D area imparted by the plasmonic traps, which provided a large-area, ultrasensitive SERS platform for single-cell analysis.

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array was employed to perform quantitative detection of DPA. The carboxylate groups of DPA display strong binding affinity to Ag nanomaterials, making the Ag SERS substrate an ideal candidate for bacillus spore detection. As shown in Figure 4A, the SERS intensities increased with the increase of DPA concentration from $10^{-6}$ to 100 ppm. The peak at 1008 cm$^{-1}$ originated from pyridine, while the peak at 1050 cm$^{-1}$ was the symmetrical stretching vibration of NO$_3$.$^{44,45}$ Figure 4B shows the calibration curve between the logarithm of the SERS intensity of the 1008 cm$^{-1}$ peak and the logarithm of DPA concentration. A wide linear relationship was observed with a correlation coefficient of 0.989. The characteristic peak at 1008 cm$^{-1}$ could still be clearly identified at a concentration as low as 5.9 pM (Figure 4A (inset)), and the limit of detection (LOD) of 1.2 pM was correspondingly reduced to 0.001 ppb. The proposed 3D trap exhibited a wider response range and lower LOD for the determination of DPA compared with previously reported SERS substrates (Table S1), providing a high density of hot spots, achieving considerable signal enhancement of surface plasmon resonance, leading to high SERS activity. An EF of 1.1 $\times$ 10$^7$ and a low detection limit at the aM level were obtained for the Raman reporter p-ATP. Moreover, these 3D traps can serve as a convenient and efficient tool for direct SERS measurement of DPA from single cells, with a minimum LOD of 1.2 pM. With more precise control over the water drop distribution, such as inkjet printers, it may be possible to create microcavities with volumes in the pL–nL range and scale-up the deposition of the aqueous droplets. The resulting high sensitivity would allow detection of chemical secretions from single cells in the zepto- to attomole range. We envision that our findings, with further refinement, will lead to solutions of the current bottleneck for detecting single-cell secretions at low expression levels, and open many possibilities for application in clinical diagnosis, environmental monitoring, and food safety.

■ CONCLUSIONS

In summary, we developed a flexible, label-free, and straightforward method for preparation of a 3D plasmonic trap array for simultaneous compartmentalization and measurement of single-cell secretion. The trap was prepared by interfacial-energy-driven spontaneous self-formation of a film of plasmonic Ag snowflakes from a small water droplet containing metal ions placed on an immiscible liquid, PDMS. The flower-like Ag microstructures on the trap surface provided a high density of hot spots, achieving considerable signal enhancement of surface plasmon resonance, leading to high SERS activity. An EF of 1.1 $\times$ 10$^7$ and a low detection limit at the aM level were obtained for the Raman reporter p-ATP. Moreover, these 3D traps can serve as a convenient and efficient tool for direct SERS measurement of DPA from single cells, with a minimum LOD of 1.2 pM. With more precise control over the water drop distribution, such as inkjet printers, it may be possible to create microcavities with volumes in the pL–nL range and scale-up the deposition of the aqueous droplets. The resulting high sensitivity would allow detection of chemical secretions from single cells in the zepto- to attomole range. We envision that our findings, with further refinement, will lead to solutions of the current bottleneck for detecting single-cell secretions at low expression levels, and open many possibilities for application in clinical diagnosis, environmental monitoring, and food safety.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.8b02252.

SEM images of 3D plasmonic trap array surface with varying concentrations of AgNO$_3$ solution, TEM images of AgNPs solution at the water–PDMS interface of 3D
plasmonic trap array with varying concentrations of AgNO₃ solution, normal Raman spectrum of 10 μM p-ATP solution, fluorescent images of GFP-tagged B. subtilis with different numbers of cells, SERS spectrum of B. subtilis adsorbed on the 3D plasmonic array with single cell of B. subtilis, and table of our proposed SERS substrate that comparison with other reported substrates for the determination of DPA (PDF).

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