

A Surface Enhanced Raman Scattering Nanotag Library based on Cucurbit[7]uril Controlled Nanoparticle Aggregates for Fast Cell Imaging

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Abstract: Surface Enhanced Raman Spectroscopy (SERS) nanotags offer ultrahigh sensitivity and multiplexing capability over and above traditional fluorescence dyes for bioimaging but suffer from more drawbacks of more complex synthesis and measurement. Here we report a facile method to create libraries of nanotags with diverse Raman reporters for sensitive SERS labelling, whilst addressing complexity and reproducibility of synthesis that many nanotags suffer from. We create and demonstrate a library of SERS nanotags synthesized via incorporation of different thiolated and non-thiolated Raman reporters (including barcoded combinations) into silver nanoparticle (Ag NP) aggregates, mediated by water soluble cucurbit[7]uril (CB7) as a molecular glue. These controlled aggregates achieve significant SERS enhancement regardless of whether the Raman reporter possesses functional groups for metal adsorption, greatly increasing the choice of potential Raman reporters for creating libraries of SERS nanotags with massive barcoding and multiplexing depth. Further coating of the SERS nanotag aggregates with functional polyethylene glycols results in long-term stability, excellent biocompatibility, and versatile functionality to attach biomolecules. We demonstrate the utility of our SERS nanotags in fast imaging of cell-particle interactions and multiplexed (‘multicolor’) surface biomarker mapping at 10 ms per point using a 1.5 mW laser or less. Our method is not only simple to implement but offers flexible and reproducible libraries of SERS nanotags with great potential for a broad range of biological labelling challenges requiring sensitivity, speed and multiplexing.

Keywords: Surface enhanced Raman scattering, Single-cell imaging, Cucurbit[7]uril , Plasmonic nanoparticles, Barcoding, Multiplexing.

1.Introduction

Raman spectroscopy is widely used for analyzing materials and biological samples but suffers from inherently weak signals. Surface Enhanced Raman Scattering (SERS) techniques can enhance Raman signals by up to 14 orders of magnitude, making them a highly sensitive tool for biosensing and bioimaging.¹ SERS nanotags, where plasmonic nanoparticles are functionalized with Raman active molecules and biorecognition moieties, have been used as labels to study intracellular pH,^{2, 3} biomarker profiling for diagnostics,⁴⁻⁶ and cancer therapies *etc.*^{7, 8} Such nanotags demonstrate excellent photostability, suitable for repeated imaging,⁹ and the narrow bandwidth of Raman peaks leads to a huge multiplexing potential,⁶ compared to conventional fluorescence-based labeling.¹⁰ Whilst advances in Resonance Raman probes have added new tools to the nanotag toolbox, SERS remains the current simplest and most flexible approach.¹¹

To achieve ideal attributes of SERS nanotags, such as high signal-to-noise, reproducibility, and long-term stability, substantial efforts have been focused on creating desirable “hotspots”, which are vital for SERS enhancement.¹² One common approach is to create anisotropic shapes to increase localized field strength to enhance signals, such as nanorods,¹³ nanocubes,¹⁴ nanostars,^{15, 16} nanocavities,¹⁷ nanoflowers, *etc.*¹⁸ Another promising approach is to use nanogaps within a core-shell particle structure to create interior hotspots.^{19, 20} Although these nanostructures can yield highly intense SERS signals, they require complex, challenging, and often multistep, nanoparticle synthesis.²¹

In contrast, spherical or pseudospherical Au or Ag nanoparticles (NPs) are widely available, but individual spherical nanoparticles have limited SERS enhancement. However, assembling Au or Ag nanoparticles into aggregates can create interparticle nanogaps for signal enhancement.²²⁻²⁴

Due to the strong affinity between thiol molecules and Au or Ag surface, thiolate molecules (such as double DNA strands²⁵ and 1,4-benzenedithiol²⁶) have been widely used to form aggregates and control interparticle distances. However, to create multiplexed, multi-reporter SERS nanoprobe requires multiple different thiolated Raman reporters leading to the challenge of finding enough suitably different molecules and still controlling nanoparticle aggregation reproducibly.²⁷ Thus, multiplexed SERS nanoprobe has been prepared by embedding Raman molecules (often a mixture of Raman dyes) either onto the surface of individual nanoparticles, with lower signal enhancement, or within the gap of a multilayer core-shell structures,^{6, 16, 28-32} with challenging and inefficient synthesis, leaving significant room for improvement.

Here, we developed a robust and simple method to create libraries of ultrasensitive SERS nanotags with combinatorial barcoding ability for multiplex sensing. Our method utilizes a so-called “molecular glue” - cucurbit[7]uril (CB7) to form a rigid, interparticle gap between Ag NPs, and a self-assembled monolayer (SAM) to protect and functionalize the aggregates (Figure 1). Cucurbit[n]urils (CBns) are barrel-shaped cavitands are well known to cross-link metal and semiconductor nanoparticles via their two carbonyl lined portals,³³⁻³⁵ and can function as “molecular spacers” to generate well-controlled and intense hotspots (the strong plasmonic field that arises when two metal nanoparticles are brought close together) from the separation of the two carbonyl-lined portals which gives a near-ideal interparticle gap of 0.9 nm.³⁶⁻³⁸ The cavitand can under certain conditions, encapsulate small analytes directly into the “hot spots” within the cavity of the CBn, or locate or trap molecules very close to the hotspots, and these particle assemblies have great potential for highly sensitive detection of soluble molecules.^{36, 39} CB7, comprising of 7 glycoluril units in the cyclic oligomer, is especially highly prized in SERS applications for its balance of good water solubility and large hydrophobic cavity. Whilst controllable aggregation of

nanoparticles with CB7 and other CBn has been demonstrated,³⁹⁻⁴¹ to the best of our knowledge, CBn mediated nanoparticle aggregates have not been reported as functional SERS nanotags.

Compared with the existing methods to create SERS nanotags, our approach offers distinct advantages: the ability to use the majority of Raman active reporters both with and without addition of functional links (e.g., thiols), offering a simple and robust route to create diverse libraries of SERS nanotags for barcoding and multiplexing detection; strong and reproducible SERS enhancement due to the rigid, fixed interparticle gaps from CB7; and SERS nanotags with different sizes and surface functionalities engineered via controlling Ag NPs aggregation and changing surface functionalization. We have synthesized and characterized a variety of nanotags from our library to demonstrate the potential for labelling and multiplexing and applied these in cell imaging validation studies. We demonstrate that the SERS nanotags reported here provide a versatile tool for cell sensing, analyzing, and sorting.

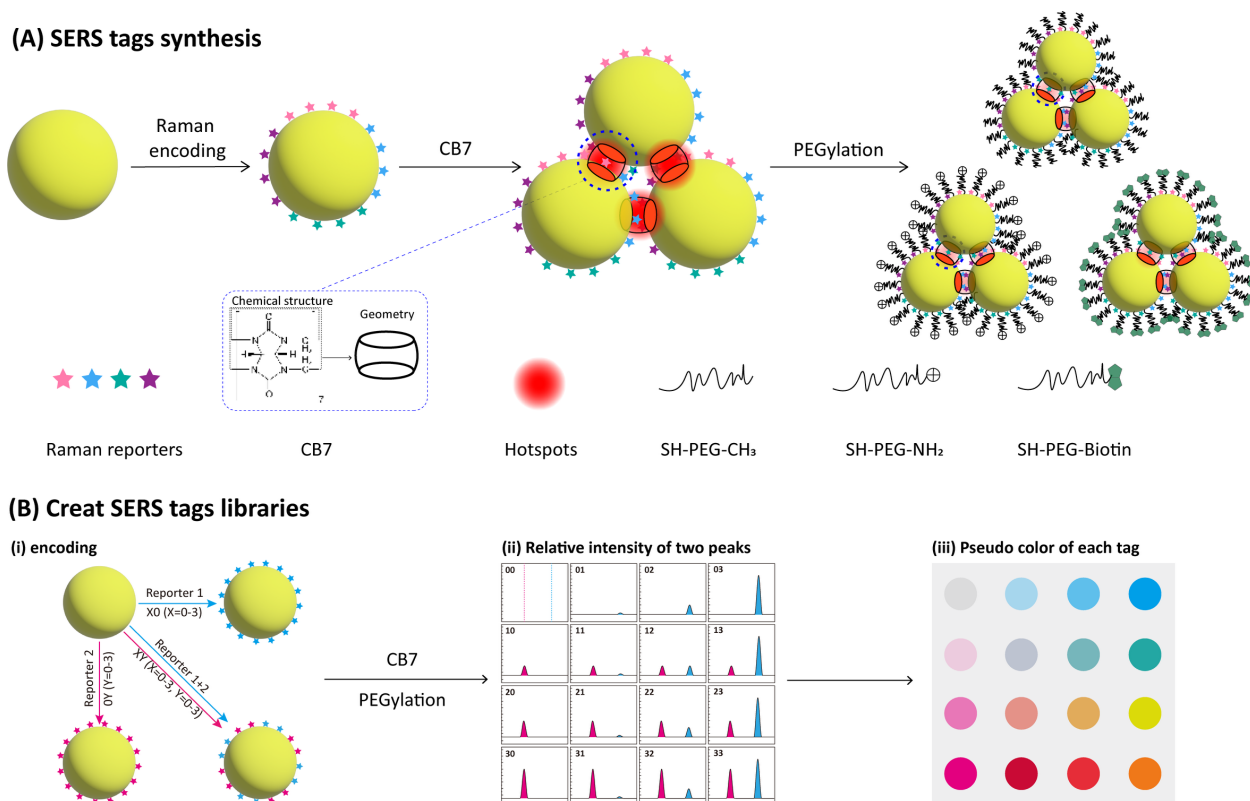


Figure 1. Schematic illustrations. **(A).** SERS nanotag synthesis, including Raman reporter chemi/physisorption during CB7 mediated aggregation, and finally PEGylation. Raman reporters trialed here include 4-mercaptobenzonitrile (MBN), 4-ethynylbiphenyl (EB), 2-aminothiophenol (ATP), and 4-nitrothiophenol (NTP), and their molecular structures can be found in Figure S1. **(B).** Creating a SERS nanotag library using a combinatorial barcoding method.

2. Experimental section

2.1 Synthesis of Ag NPs

Ag NPs were synthesized according to a previous method with slight modifications.⁴² Typically, 10 mM AgNO_3 was prepared by dissolving 170 mg AgNO_3 (1 mmol) into 100 mL H_2O . A 1.7 mM solution of $\text{NH}_2\text{OH} \cdot \text{HCl}$ was prepared by adding 11.6 mg $\text{NH}_2\text{OH} \cdot \text{HCl}$ (0.17 mmol) into a 100 mL flask, followed by the addition of 3.3 mL NaOH (0.1 M) and water to a final volume of

100 mL. To produce nanoparticles, 9 mL of the $\text{NH}_2\text{OH}\cdot\text{HCl}$ solution was added to a polypropylene centrifuge tube, quickly followed by 1 mL of AgNO_3 solution. The reaction mixture was gently agitated for a few seconds, and the produced yellow/green colloids were stored in the dark at 4 °C.

2.2 Synthesis of SERS nanotags

The overall synthesis protocol is illustrated in Figure 1. A certain amount (0-15 μL) of 1.0 mM 4-mercaptobenzonitrile (MBN) solution was added into 1 mL Ag NPs colloids in a 1.5 mL Eppendorf tube and agitated overnight to graft MBN on the Ag NPs surface via Ag-S bonding (Ag-MBN). Then, a given amount (0-22 μL) of CB7 solution (43 μM) was pipetted into the Ag-MBN colloids to induce aggregation for 0-300 min under agitation (Ag-MBN-CB7). Afterwards, 25 μL of 100 μM SH-PEG-R (R: CH_3 , NH_2 , Biotin) solution was added without agitation, to stabilize the nanoaggregates. The obtained PEG-modified colloids (Ag-MBN-CB7@PEG-R) were washed twice and dispersed into DI water before being stored in a fridge. For other SERS nanotags of Ag-R-CB7@PEG, where R is 4-nitrothiophenol (NTP), 4-ethynylbiphenyl (EB) or 2-aminothiophenol (ATP), the protocol is similar. Based on trials, NTP at 5 μM , EB at 1 μM , and ATP at 0.5 μM were used, respectively. Although EB has no thiol group, it was still robustly trapped in the aggregates (see below).

2.3 Cell preparation for SERS nanotags uptakes

Cell lines (MCF-7, MDA-MB-231 cells) were cultured in a humidified incubator (37 °C, 5% CO_2) and then harvested with trypsin/EDTA after growing to confluence. The cell suspension (200 μL) was seeded into each well (about 6×10^4 cells/well) of an 8-well ibidi chamber for 24 – 48 h and cultured (37 °C, 5% CO_2) to confluence. SERS nanotags (Ag-MBN-CB7@PEG- CH_3 or - NH_2)

suspended in DMEM (200 μ L) were added into each well, and the cells were returned to the 37 °C incubator for 5 hours. After incubation, cells were washed thoroughly using cell culture medium and PBS buffer. The obtained cell samples were fixed with 4% formaldehyde at room temperature for 25 min, followed by further thorough washing with PBS and DI water.

2.4 Cell preparation for multiplexed SERS immunostaining

Cell lines (MCF-7, MDA-MB-231 cells) were cultured in a humidified incubator (37 °C, 5% CO₂) and then harvested with trypsin/EDTA after growing to confluence. The cell suspension (200 μ L) was seeded into each well (about 6×10^4 cells/well) of an 8-well plate ibidi chamber for 24–48 h culture (37 °C, 5% CO₂) to grow to confluence. The obtained cell samples were fixed with 4% formaldehyde at room temperature for 25 min, followed by extensive washing with PBS and DI water, before blocking with 1.5% BSA for 15 min at room temperature. Biotinylated anti-human CD44 antibody (5 μ L, 0.5 mg/mL) was added to each well, and the plate incubated for 3 hours at room temperature, followed by thorough washing using PBS. Streptavidin (5 μ L, 5 mg/mL) was added into each well followed by 30 min incubation and further washing with PBS at room temperature. SERS nanotags (200 μ L) in DMEM (Ag-MBN-CB7@PEG-Biotin) were added into each well and incubated for 45 min at room temperature, followed by extensive washing with PBS and DMEM. Biotinylated anti-human CD24 Antibody/Ag-EB-CB7@PEG-Biotin, and biotinylated anti-human EpCAM Antibody/Ag-NTP-CB7@PEG-Biotin were labeled onto the cells in sequence, using the same protocol.

During each labeling run, after the cells were labeled with biotinylated antibodies and washed, excess streptavidin was used to label all biotinylated antibodies on cell membranes. Similarly, excess SERS nanotags were added to label all streptavidin that was anchored on the antibody

during each labeling run. Extensive washing after each reagent addition was used to ensure complete removal of unbound antibodies, streptavidin, and SERS nanotags from each labeling run and avoid non-specific labeling.

3. Results and Discussion

3.1 Modulating CB7 attachment on nanoparticles enables SERS nanotag formation

Ag nanoparticles (Ag NPs) were synthesized via well-established hydroxylamine hydrochloride mediated reduction of silver salts. The concentration of the Ag NPs was estimated to be 1.35×10^{-9} M using the molar extinction coefficient of $0.8 \times 10^{10} \text{ M}^{-1}\text{cm}^{-1}$.⁴³ CB7 serves as a well known molecular “glue” to induce the aggregation of metal (Au, Ag) NPs via interactions between the two symmetrical carbonyl-lined portals and the metal NP surface, and the degree of aggregation can be tuned by the amount of CB molecules added per unit surface area.^{36, 44} Such NP aggregates have been shown to be highly versatile in SERS, where the target or reporter can form an inclusion complex in the CB7 at or near the junction of two particles, or just become kinetically trapped in the aggregate. Two approaches for incorporating Raman reporters and CB7 on Ag NPs were evaluated, using MBN as an example: 1) MBN was allowed to adsorb to Ag NPs first, followed by CB7 addition & attachment, denoted as Ag-MBN-CB7 and 2) a mixture of MBN and CB7 was added to Ag NPs solution together, denoted as Ag-(MBN+CB7).

For the Ag-MBN-CB7 approach, an MBN monolayer was formed first on Ag NPs (denoted as Ag-MBN). For all the tested MBN concentrations (i.e., 0-13 μM), the Ag-MBNs obtained have a similar hydrodynamic particle size to the uncoated ones (Ag NPs) (Figure 2 A(i) and Supplementary Figure S3), suggesting that, as expected, MBN does not induce aggregation by itself. This was further confirmed by the similar absorbance spectra between all Ag-MBN and

uncoated Ag NPs (Figure 2 A(ii) & Supplementary Figure S4). Upon the addition of 0.72 μ M CB7, a broad absorbance peak between 500 nm and 700 nm appeared, indicating the formation of Ag-MBN-CB7 aggregates in the solution (Figure 2 A(ii)), which was also verified by a measured increase in particle hydrodynamic size beyond that measured for Ag-MBN (Figure 2 A(i)). The Raman intensity of Ag-MBN-CB7 aggregates prepared at all MBN concentrations was higher than that of the Ag-MBN samples (Figure 2 A(iii)), demonstrating significant enhancement due to CB7 mediated assembly. In addition, Raman intensity remains stable over a prolonged incubation time of 5 hours (Supplementary Figure S5). Ag-MBN-CB7 obtained at 9 μ M MBN and 0.72 μ M CB7 showed the highest SERS intensity (Supplementary Figure S5) and the strongest aggregate absorbance peak (Supplementary Figure S6 & S7).

Raman intensity was compared between independent batches of particles and aggregated SERS nanotags (Supplementary Figure S9), where 15 independent batches of the same aggregated reporters were created and showed an overall Relative Standard Deviation (RSD) of <9% across all batches. When five independent batches of Ag NPs were pooled and used to prepare a further 20 batches of SERS nanotags an RSD of less than 7% was observed demonstrating highly reproducible aggregation for future applications.^{45, 46}

TEM was employed to further evaluate aggregate formation. Before CB addition, Ag NPs predominantly existed as individual particles with an average size of 22.6 ± 4.6 nm (N = 76) (Figure 2 A(iv) & Supplementary Figure S2), which is comparable to the hydrodynamic size of ~ 24 nm (Figure 2 A(i)). The addition of MBN did not induce any aggregation (Figure 2 A(iv) and created better spaced particles on the TEM grid, thanks to the stable SAM, resisting drying artefacts. However, upon addition of CB7, substantial aggregates were observed, consistent with the measurements of hydrodynamic size.

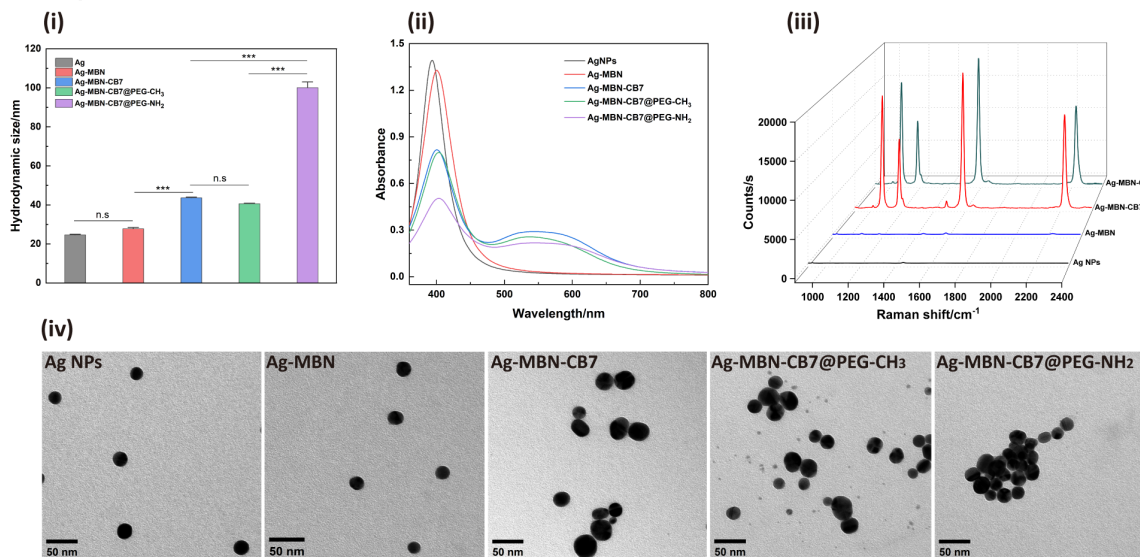
For the Ag-(MBN+CB7) approach, it was expected that controlling the ratio of MBN to CB7 in the solution could modulate CB7's coverage on Ag NPs, and consequently, the assembly of Ag NPs. Upon adding the mixture of MBN and CB7, the 2nd broad absorbance peak between 500 nm and 700 nm also appeared, indicating the formation of Ag-(MBN+CB7) aggregates (Figure 2 B(i), Supplementary Figure S8). The SERS intensity of Ag-(MBN+CB7) also increased substantially compared to Ag-MBN (Figure 2 B(ii)).

To evaluate how the two approaches modulate CB7 attachment and consequently the SERS enhancement, a series of conditions with a fixed 9 μ M MBN but various CB7 concentrations (between 0.5 μ M and 1.0 μ M) were evaluated. For the Ag-MBN-CB7 approach, strong and stable SERS enhancement was achieved with CB7 concentrations of up to 0.94 μ M (Figure 2 C(i)) and SERS signals remained stable over a long period of time (red line of Figure 2 C(ii), and Supplementary Figure S5). However, for the Ag-(MBN+CB7) approach, high SERS enhancement was observed at CB7 concentrations <0.77 μ M and above that, the SERS intensity rapidly decreased (Figure 2C(i), black line). The characteristic UV absorbance rapidly weakens due to significant flocculation/precipitation (Supplementary Figure S8). Concurrently, Ag-(MBN+CB7) also showed a rapid loss of SERS intensity over a prolonged time of incubation (Figure 2 C(ii), black line), suggesting the formation of excessively large aggregates, due to reduced competition with the surface adsorbed species (where there is no pre-adsorbed Raman reporter) and decreased stability.

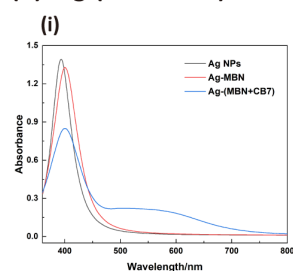
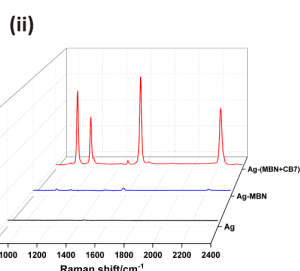
Both approaches show that CB7 is vital for ideal hotspot formation, to generate substantial signal enhancement, but that the pre-formation of a Raman reporter layer may enable more control in subsequent attachment of CB7 on the surface and reduces the formation of excessively large

aggregates. Thus, the Ag-MBN-CB approach was selected for the subsequent SERS tags formation.

(A). Ag-MBN-CB7



(B). Ag-(MBN+CB7)



(C). Effect of adding order of CB7 on SERS

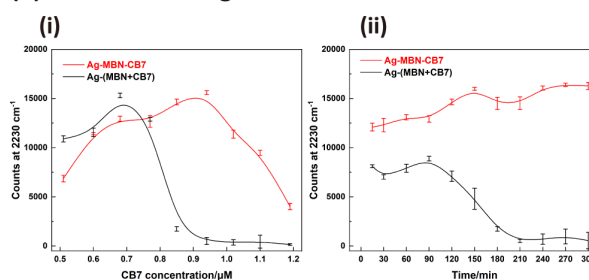


Figure 2. Morphology, size and Raman characterization of SERS nanotag formation. **(A).** Nanotag formed via Ag-MBN-CB7. **(i)** hydrodynamic size, one batch of Ag NPs was prepared, followed by addition of MBN, CB7, and SH-PEG-CH₃ or SH-PEG- NH₂ in sequence described in the Materials and Methods. At each step of reagent addition, the sample was measured at least 4 times for statistical analysis. Focusing on temporal sampling of one batch provided more directly comparable impacts for each reagent on aggregation. **(ii)** Representative UV-Vis absorbance. **(iii)** SERS spectra at different stages. **(iv)** TEM images of SERS Nanotag formed at different stages. Wide field images are provided in the SI. **(B).** Nanotag formed via Ag-(MBN+CB7) approach. **(i)**

Size and **(ii)** Raman characterization. **(C)**. The effect of MBN and CB7 addition order on SERS signal stability. **(i)** At different CB7 concentrations and a fixed condition of 9 μM MBN, 150 min vortex at 2000 RPM. At each concentration, one batch of Ag-MBN-CB7 and Ag-(MBN+CB7) was prepared and at least 20 SERS spectra were acquired, correspondingly to give statistics. **(ii)** With different incubation times (0–300 min vortex at 2000 RPM) and a fixed condition of 9 μM MBN and 0.85 μM CB7. One batch of Ag-MBN-CB7 and Ag-(MBN+CB7) was prepared, and at least 20 SERS spectra were acquired consecutively at each agitation time. Lines plotted are B-splines to show the trend. For all cases, Ag-MBN-CB7: Ag NPs were incubated with MBN first, followed by CB7 addition. Ag-(MBN+CB7): a mixture of MBN and CB7 was added to an Ag NP solution. One-way ANOVA and Tukey tests were used for statistical analysis. n.s: not significant, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

3.2 Surface functionalization of the SERS nanotags provides stability and utility

Surface functionalization of the SERS nanotags is indispensable for bioimaging and sensing. PEGylation has been a well-established method for coating NPs with a wide range of PEGylated functional molecules.^{4, 47, 48} Thiolated PEG with a distal end group of either $-\text{CH}_3$ or $-\text{NH}_2$ was used to functionalize Ag-MBN-CB7 nanotags, allowing modulation of the interaction between cells and SERS nanotags. The $-\text{NH}_2$ groups also facilitates further conjugation with biomolecules.⁴⁷

Successful grafting of thiolate PEG molecules in the Ag-MBN-CB7@PEG- CH_3 or Ag-MBN-CB7@PEG- NH_2 with minimal disruption of the aggregates was illustrated by the slight change of the 2nd broad peak around 600 nm in the UV-vis absorbance spectra (Figure 2 A(ii)) and a non-significant decrease in hydrodynamic radius for Ag-MBN-CB7@PEG- CH_3 (Figure 2 A(i)), which

was also demonstrated in TEM images (Figure 2 A(iv)). However, the size of Ag-MBN-CB7@PEG-NH₂ was markedly larger compared to Ag-MBN-CB7 (Figure 2 A(i)), with increased aggregation visible in the UV-vis (Figure 2 A(ii)). These data suggested amine-induced aggregation linking multiple nanoaggregates into larger clusters, given amines are well known to interact with Au and Ag metal surfaces, as well as potential reduction in small aggregate stability due to partial cancellation of negative surface charge by the positive ammonium groups, leading to further clustering.⁴⁹⁻⁵¹ TEM images of Ag-MBN-CB7@PEG-NH₂ also revealed larger aggregate formation in comparison to the other conditions (Figure 2 A(iv)). To further probe the role of the amine group we prepared SERS nanotags of Ag-MBN-CB7@PEG-CH₃/NH₂ coated with a mixture of SH-PEG-CH₃/NH₂ and measured their hydrodynamic size and zeta potential (Figure S10). With a decreasing ratio of -CH₃ to -NH₂, the hydrodynamic size of Ag-MBN-CB7@PEG-CH₃/NH₂ increased gradually, along with a gradual decrease of zeta potential to less negative values under the same conditions, although the aggregates remained stable in solution.

Incorporating the PEG layer was vital to improve the stability of the aggregates as SERS nanotags.^{39, 52} Before PEGylation, the SERS intensity of Ag-MBN-CB7 nanotags was stable in H₂O for several days, but it rapidly decreased in cell culture medium (DMEM) and PBS within a day (Figure 3 A and Supplementary Figure S11 A). In contrast, PEGylated SERS nanotags maintained the peak SERS intensity after 9 days of storage in H₂O, DMEM, or PBS (Figure 3 B&C, Supplementary Figure S11 B&C). They also remained stable after centrifugal washing (Figure 3 E&F, Supplementary Figure S12 B&C) compared with uncoated Ag-MBN-CB7 (Figure 3 D, Supplementary Figure S12 A).

Although PEGylation could replace some pre-adsorbed MBN (and CB7) on the surface of Ag-MBN-CB7, this only resulted in a slight decrease in SERS intensity (Figure 3 A-C). This suggested

that the Raman reporters within the internal hotspots contributed most to the observed signal and were preserved during PEG coating, long-term storage, and washing steps.

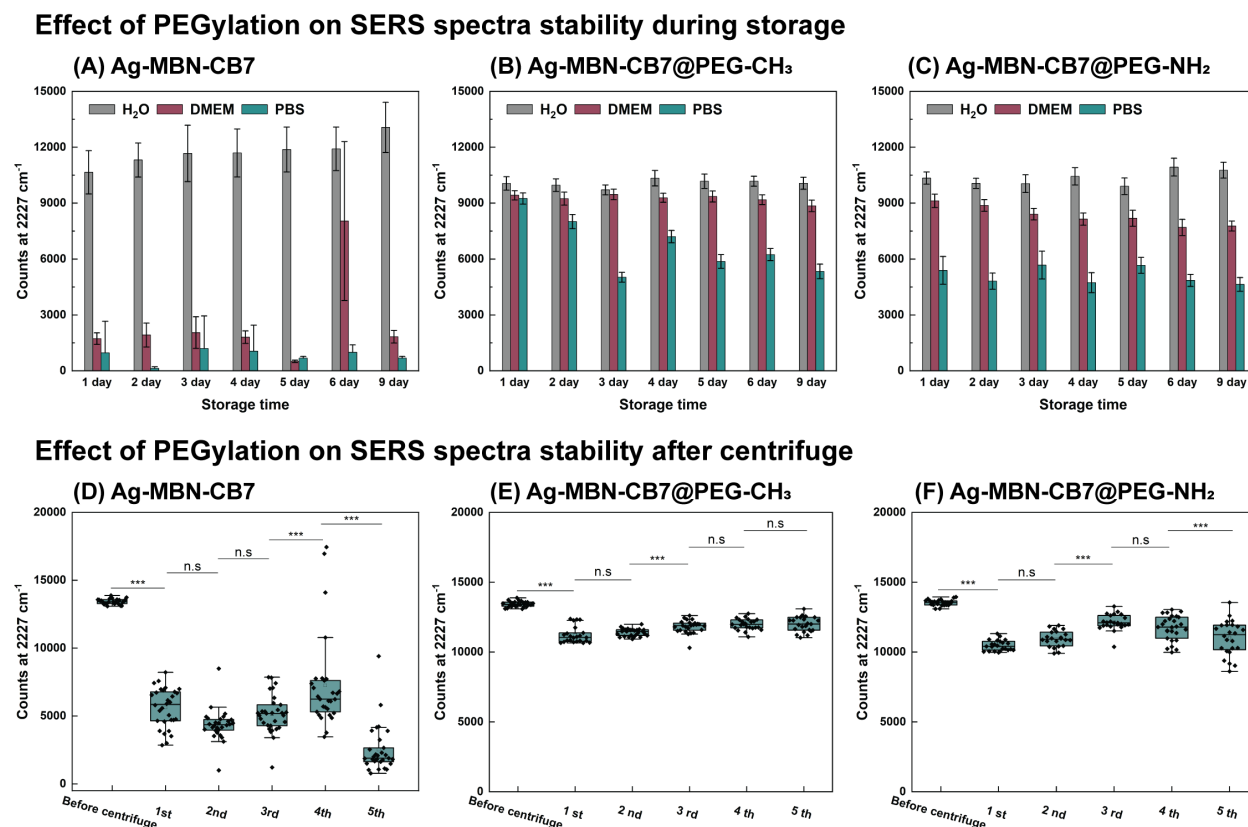


Figure 3. Stability test of the SERS intensity in various media (A-C); after the centrifugal washing in H₂O (D-F). SERS nanotags were prepared in the conditions of 1 mL Ag NPs, 9 μ M MBN, 0.72 μ M CB7, 2000 RPM 120 min, 2.5 μ M HS-PEG-CH₃ or -NH₂. One batch of sample was prepared per condition and 20-25 SERS spectra were acquired for each sample whilst the aggregates freely diffused across focal volume in a well, to obtain aggregate statistics. One-way ANOVA and Tukey tests were used. n.s: not significant, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

3.3 Creation of SERS nanotag libraries via barcoding with reporter combinations

Currently, the commercially available Raman reporters incorporated in SERS nanotags are mainly limited to a few Raman-active compounds with thiol functional groups. However, being able to use non-functionalized Raman reporters offers great flexibility in creating libraries of SERS nanotags with near-unlimited barcoding capability. Our approach offers such a capability by incorporating a mixture of Raman reporters on SERS nanotags without needing a specific functionality. To demonstrate this capability, three thiol-containing small molecules (MBN, ATP, NTP, chemical structures in Supplementary Figure S1) and a non-thiolate small molecule (EB, chemical structure in Supplementary Figure S1) were used to create SERS barcodes with CB7 mediated Ag NPs aggregation. The SERS nanotags made from each molecule demonstrate its characteristic and distinguishable Raman shift at 2227 cm^{-1} , 1961 cm^{-1} , 1435 cm^{-1} , and 1340 cm^{-1} , respectively, for Ag-MBN-CB7@PEG-CH₃, Ag-EB-CB7@PEG-CH₃, Ag-ATP-CB7@PEG-CH₃, and Ag-NTP-CB7@PEG-CH₃, respectively (Figure 4 A).

More thiolated and non-thiolated Raman reporter molecules could be incorporated into nanotags with this method without overlapping diagnostic Raman bands, and previously published work suggests signals from up to 26 reporters might be combined and successfully unmixed.^{28, 29, 53} Thus, to further extend the library, it is possible to combine two or more of the reporters onto the same SERS nanotag for ‘barcoded’ detection based on the relative intensity of the reporter’s characteristic peaks.⁵⁴ To demonstrate this capability, MBN and EB were selected (because their peaks fell in the Raman silent region of cells to avoid interference with cellular components) and combined at different relative concentrations into a range of nine nanotags. Relative concentrations of MBN:EB (9 μM : 0.5 μM) to MBN:EB (1 μM : 2.5 μM) were trialed, and at least 6 easily discernible pairings (by eye) from the 9 were found (Figure 4 B), which was verified by the

distinguishable peak ratios at $I_{1961\text{ cm}^{-1}}/I_{2227\text{ cm}^{-1}}$ for all Tags 1-9 (Figure 4 C). SERS tags at all ratios demonstrated a good reproducibility, implied by the RSD of less than 5% (Figure 4 D & Supplementary Figure S13). The nano-aggregates diffusing freely in and out of the focal region show excellent intra-batch homogeneity of signal (Figure 3 and Figure 4). This is crucial as one set of barcoded aggregates would likely be prepared and applied within a barcoding experiment so the crucial metric is whether the aggregates from that batch maintain a statistically different barcode from another, which we show to be the case here (Figure 4 C, Figure S13, RSD within a given batch is $< 4\%$, and all of the 9 separate barcodes are significantly different from each other). Our data suggests that, conservatively, hundreds of distinct multiplexed, barcoded SERS nanotags could be created when up to three molecules from our four tested are combined at different ratios (i.e., 4 native tags, 6 pairs of double reporters assuming at 6 levels = 216, 4 sets of three reporters assuming 6 possible levels = 864 tags) and this would scale rapidly as more, different reporters are integrated, with the potential for even thousands of tags with the right reporters and good ratiometric measurement. Consequently, the method developed in this paper is simple and robust to create an abundant Raman encoding library via selecting different Raman reporters and modulating their ratios.

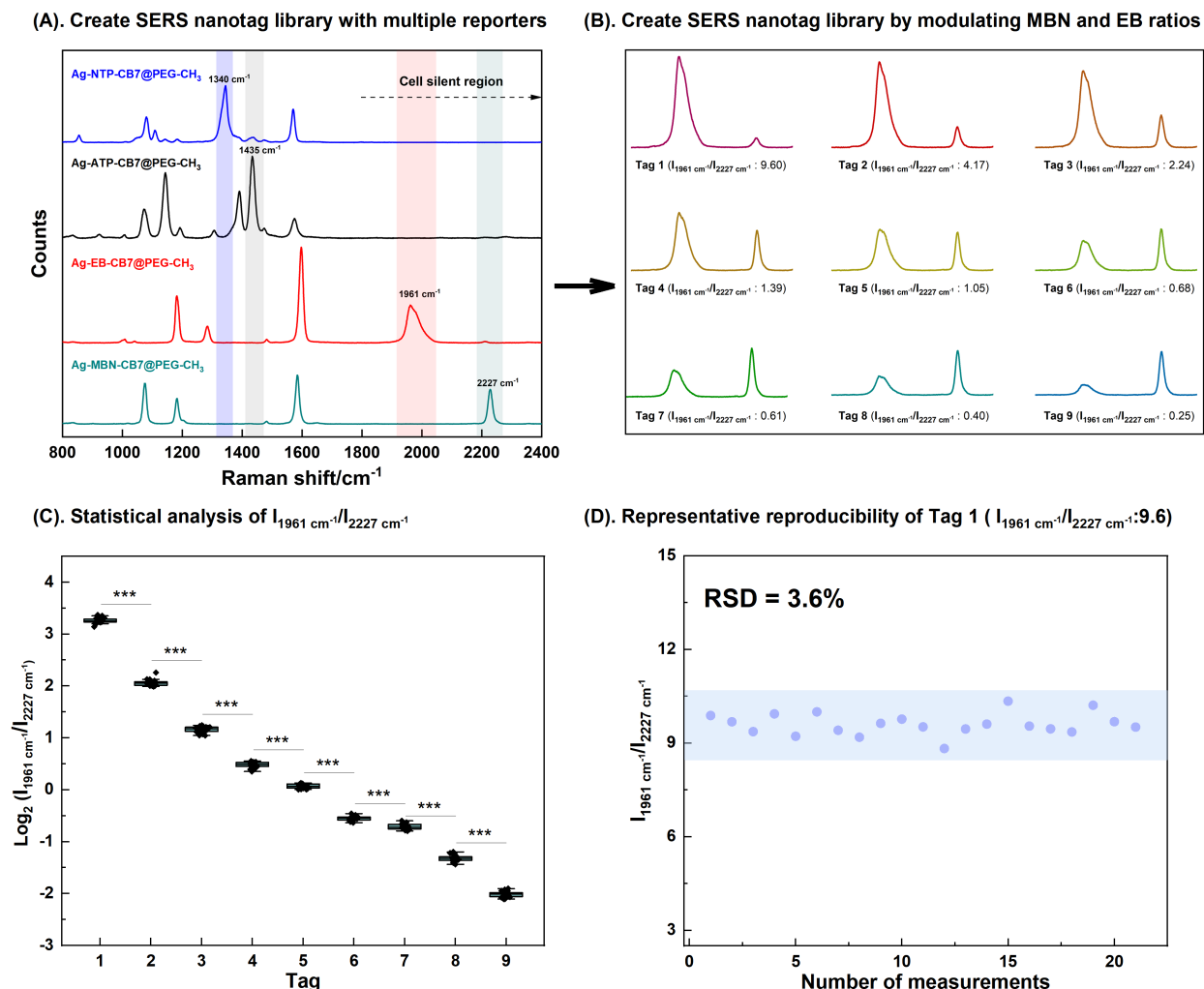


Figure 4. Methods for creating SERS nanotag libraries. **(A).** SERS nanotags incorporating different Raman reporters: 4-mercaptobenzonitrile (MBN), 4-ethynylbiphenyl (EB), 2-aminothiophenol (ATP), and 4-nitrothiophenol (NTP). **(B).** Creating multiplex SERS nanotags using a combination of different ratios of two Raman reporters (i.e., MBN and EB). **(C).** Statistical analysis of the ratios of Tag 1-9, represented as $\log_2$ of the relative intensity of the diagnostic bands to better display the ratiometric trend. **(D).** Representative reproducibility analysis of Tag 1 by RSD. For **(C)** and **(D)**, one sample of each Tag was prepared, and 20-25 SERS spectra were acquired consecutively at fixed point whilst the aggregates freely diffused across focal volume in a well, to obtain aggregate statistics. One-way ANOVA and Tukey tests were used. n.s: not

significant, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. Samples: 1.0 mL Ag NPs, 0.5 μM -9 μM Raman reporters (For tags 1-9, the concentrations of MBN and EB are: 1 μM -2.5 μM , 3.0 μM -2.5 μM , 5.0 μM -2.5 μM , 7 μM -2.5 μM , 9 μM -2.5 μM , 9 μM -2.0 μM , 9 μM -1.5 μM , 9 μM -1.0 μM , 9 μM -0.5 μM), 0.72 μM CB7, 2000 RPM 0-120 min, 2.5 μM CH₃-PEG-SH.

3.4 SERS nanotags enable fast mapping of cell-particle interactions

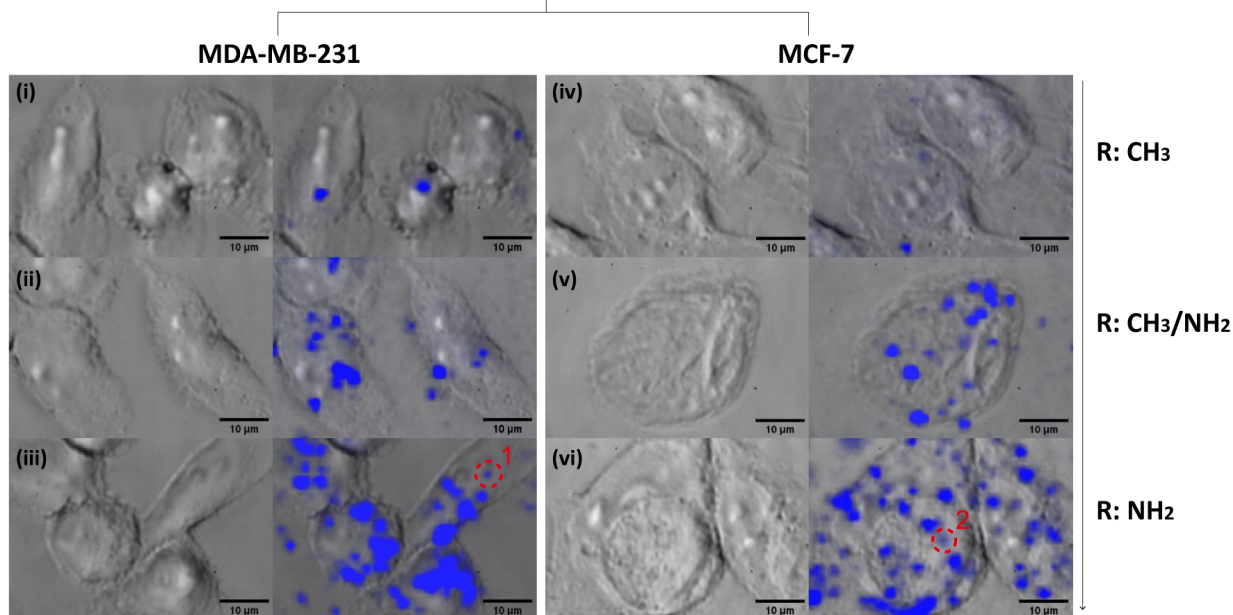
SERS nanoparticles can be taken up by living cells and are often used for tracking biological processes (e.g., proliferation⁵⁵) and effects of therapies.⁴⁷⁻⁵⁰ To evaluate the biocompatibility of our SERS nanotags, cell viability was tested using MDA-MB-231 cell for SERS nanotags modified with functional PEG layers (i.e., Ag-MBN-CB7@PEG-CH₃, Ag-MBN-CB7@PEG-NH₂). There was no significant difference in cell viability after cells were incubated with the SERS nanotags for 12 h, 24 h, and 40 h, in comparison with the control without SERS nanotags (Supplementary Figure S14), indicating the excellent biocompatibility of the developed SERS nanotags.

Considering the ease of incorporation of different functional groups on our SERS nanotags and their ultrahigh intensity for fast imaging, we next evaluated the potential of mapping the nanotags within cells as a measure to investigate different cell responses. It is known that cell internalization of nanoparticles can be affected by many factors,^{56, 57} including cell-particle interactions (e.g., cell types and the surface coating of nanoparticles). Two human breast cancer cell lines, namely a highly tumorigenic and invasive MDA-MB-231 and non-invasive MCF-7, were selected as model systems. They were incubated with SERS nanotags coated with different functional PEG (i.e., SH-PEG-CH₃, SH-PEG-CH₃/NH₂ (1:3), and SH-PEG-NH₂) for 5 hours, followed by thorough washing and fixing prior for Raman imaging. Before SERS measurement, the slides with cells

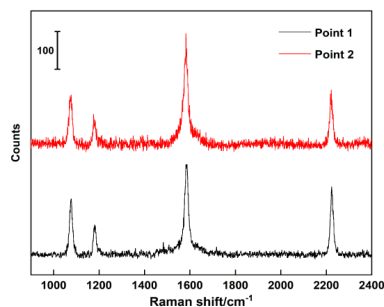
were flushed with water/PBS extensively to remove any physically absorbed NPs on the surface, indicated by the absence of SERS signals in the non-cell area of the substrate (Supplementary Figure S14). It was found the majority MCF-7 cells contained SERS tags after 5 hours of incubation as indicated by high SERS intensities (Supplementary Figure S14).

(A) Cell imagings

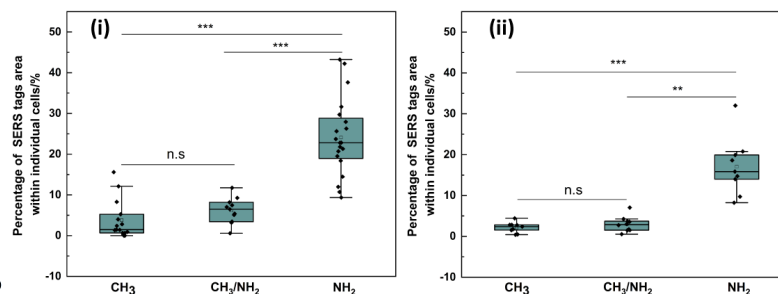
Ag-MBN-CB7@PEG-R



(B) SERS spectra at weak points



(C) Percentage of SERS tags area within individual cells



(D) Effect of surface charge on cell-nanoparticle interaction

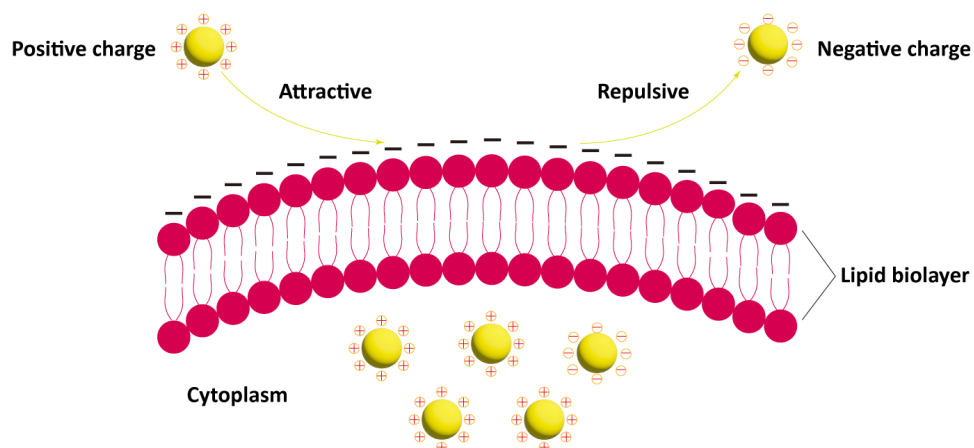


Figure 5. Rapid cell imaging for studying the effect of surface coating of SERS nanotags on cell uptake. **(A).** Bright field and SERS images of cells containing different SERS nanotags. Cell lines: MDA-MB-231 and MCF-7. **(B).** SERS spectra of two weak points in **(A)**. **(C).** Percentages of SERS nanotag area within individual cells for (i) MDA-MB-231 and (ii) MCF-7 after incubating with SERS nanotags. All cells were incubated with Ag-MBN-CB7@PEG-R for 5 hours before washing, fixing, and Raman measurements, R=CH₃, CH₃/NH₂ (1:3), NH₂. For each case of cells incubated with different SERS tags, three wells of cells were cultured and incubated, and at least 9 cells were mapped randomly from these wells. T-tests were used. n.s: not significant, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. **(D).** Illustration of cell's uptake tendency for nanoparticles with positive or negative surface charge. SERS mapping conditions: 63X 0.75 NA objective lens, 532 nm laser of 2.0 mW, 0.01 s accumulation time, step size of $1\ \mu\text{m} \times 1\ \mu\text{m}$ in XY plane.

Raman cell images were obtained at 10 ms per pixel (Figure 5 A), and even the weakest points (points 1 and 2 in Figure 5 A(iii) & A(vi)) show SERS spectra of high signal-to-noise ratio at 2227 cm^{-1} ($s/n > 12$) (Figure 5 B). It should be noted that 10 ms is the fastest response speed of the camera used (here imaging $35\ \mu\text{m} \times 50\ \mu\text{m}$ in 17.5 s, containing approximately 1-5 cells), and the acquisition time per pixel can be shortened further with an appropriate camera. Given that Raman intensity depends on acquisition time and laser power, fast Raman imaging is often accompanied by high laser power, which can compromise cell viability and physiological function. Thus, the potential of these bright SERS nanotags for fast cell imaging at low laser power was evaluated with Ag-MBN-CB7@PEG-NH₂ in MDA-MB-231 and MCF-7 cells. At a low laser power of ~0.25 mW, SERS imaging of cells can be obtained at 0.1 s per pixel (Supplementary Figure S16). These results demonstrate the promising potential of our SERS nanotags in applications requiring high-speed and live cell imaging.

The peak of 2227 cm^{-1} in the Raman silent region was selected to identify the distribution of SERS nanotags in the cell (Figure 5 A). For cells incubated with Ag-MBN-CB7@PEG-CH₃, very few SERS signal or only individual points were found within cells (Figure 5 A(i) & 5 A(iv)). In contrast, many SERS signal spots were found when cells were incubated with Ag-MBN-CB7@PEG-NH₂ (Figure 5 A(iii) & 5 A(vi)), whereas the number of SERS signal points for cells incubated with Ag-MBN-CB7@PEG-CH₃/NH₂ was in between those for Ag-MBN-CB7@PEG-CH₃ and Ag-MBN-CB7@PEG-NH₂ (Figure 5 A(ii) & 5 A(v)). This trend was further verified by the percentage of the SERS tags area within individual cells (Figure 5 C) when Ag-MBN-CB7@PEG-NH₂ was used for MDA-MB-231 (Figure 5 C(i)) and MCF-7 (Figure 5 C(ii)). These results indicate that a strongly negative surface charge would inhibit nanoparticles from entering cells and reducing the surface charge from negative to neutral can promote the cell to uptake nanoparticles (Figure 5D), which is consistent with previous publications where amines promote cellular adhesion and particle uptake.⁵⁸⁻⁶⁰ It appeared that MDA-MB-231 cells took up more SERS nanotags than MCF-7 cells under the same conditions, indicated by the higher percentage of SERS nanotags area within MDA-MB-231 cells. The SERS nanotags developed in this work can therefore be used to image and characterize cell uptake differences between cell types. Compared with previously reported SERS tags that require delicate synthesis to achieve strong SERS signals with low laser power and fast imaging speed (Supplementary Table S1); the Ag-MBN-CB7@PEG-CH₃/NH₂ nanotags developed in this work demonstrate comparable or higher sensitivity and barcoding capacity for cell imaging at low laser power, from a simplified, robust preparation procedure.

3.5 Rapid multiplex imaging of cell surface markers

The expression of biomarkers in tumors and other complex tissue samples is heterogeneous.⁶¹ Simultaneous imaging the distribution of multiple cancer-related biomarkers on cell surface can facilitate accurate identification of cell phenotypes and be used in label-assisted cell sorting. Using MDA-MB-231 and MCF-7 breast cancer cell lines as a model system, the potential of simultaneously imaging multiple markers for phenotype identification was evaluated using different SERS nanotags (Ag-NTP-CB7@PEG-biotin, Ag-EB-CB7@PEG-biotin, and Ag-MBN-CB7@PEG-biotin). Three consecutive runs of labelling were used to transform the SERS nanotags onto corresponding biomarkers on cell membranes. During each run of SERS cell labelling, antibodies, streptavidin, and SERS nanotags were added in sequence with extensive washing after each step (details in Methods). The SERS intensities at 1340 cm^{-1} of NTP, 1961 cm^{-1} of EB, and 2227 cm^{-1} of MBN were represented by the red, green, and blue channel for Raman mapping, corresponding to the expression of EpCAM, CD24, and CD44 respectively (Figure 6). Detailed distributions of the SERS nanotags across the cells were obtained at high resolution imaging at the shortest acquisition time (i.e. 10 ms/point). An extensive spread of MBN-nanotags, but limited numbers of EB-nanotags, was observed across MDA-MB-231 cells, indicating the overexpression of CD44 biomarker. In contrast, more NTP-nanotags and EB-nanotags but fewer MBN-nanotags were detected for MCF-7 cells (Figure 6), indicating high expression of CD24 marker but low expression of CD44 biomarkers. Both observations agree well with previous studies,^{4, 62} and simultaneous comparison of different CD markers can offer useful information in pathology. It is worth noting that, while the sequential labeling procedure limits the multiplexing ability and specificity of different SERS nanotags, optimization of the conjugation strategy to link antibodies directly to nanotags' surface, via bioconjugation routes, will improve the specificity and versatility of the SERS nanotag library for multiplexing and barcoding.

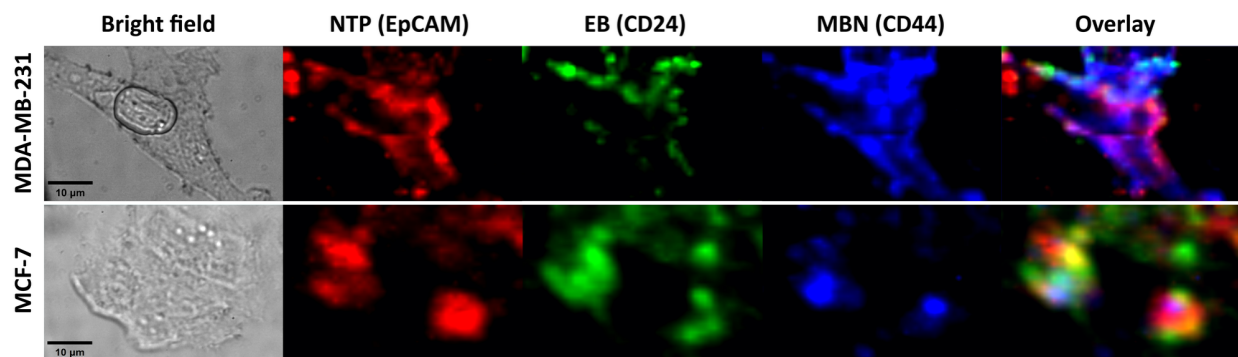


Figure 6. Multiplexed SERS cell imaging using MDA-MB-231 and MCF-7 breast cancer cells. EpCAM, CD24, and CD44 were labelled with Ag-NTP-CB7@PEG-biotin, Ag-EB-CB7@PEG-biotin, and Ag-MBN-CB7@PEG-biotin, respectively, corresponding to red, green, and blue, respectively. SERS mapping conditions: 63X 0.75 NA objective lens, 532 nm laser of 1.5 mW, 0.01 s accumulation time.

4. Conclusions

We have developed a simple, robust, and effective method for synthesizing a library of SERS nanotags for rapid cell imaging, offering strong, reproducible, and stable SERS intensity under physiological conditions. We employ the well-known CB7-mediated metal nanoparticle aggregation to create reproducible, and tuneable plasmonic Ag NP aggregates with strong SERS enhancement. This method enables easy creation of a SERS nanotag library by barcoding a variety of Raman molecules and their combinations into an aggregate. The produced SERS nanotags are modified with functionalized polyethylene glycol to enhance stability, biocompatibility and add specific biorecognition capabilities. Importantly, our method is broadly applicable to incorporating both thiolated and non-thiolated Raman active molecules, providing flexibility to generate extensive SERS nanotag libraries with huge barcoding potential (thousands of different tags), when coupled with the latest de-mixing techniques. Whilst deeply encoded SERS nanotags have

been previously reported (with up to 26 dyes per nanotag) these systems to date have typically relied on single coated particles, giving lower signal to noise, or challenging core-shell or galvanic replacement strategies to generate hotspots for high enhancement, which can lead to batch-to-batch variation and limited encoding depth.

The excellent Raman intensities generated by our, comparably simple, aggregated nanotags allow for rapid, low power imaging, revealing detailed distribution of multiplex SERS nanotags within or on cells. This will lead to more detailed studies, employing our nanotags, to measure cell phenotype and responses and further efforts will be devoted to improving cell labelling strategies with bio(orthogonal) conjugation chemistry and exploring in-situ living cell imaging. We will also seek to employ the nanotags in applications requiring high sensitivity, multiplexing and speed,⁶³ such as Raman flow cytometry for high throughput cell screening and sorting.

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Author contributions

Conceptualization: Y.Z., H.Y. Methodology: all authors. Investigation: Y.Z., X.L., S.M., N.B., Q.P., O.D, S.L, J.G., X.Y., Data Curation and formal analysis: Y.Z., Writing – Original Draft

Preparation: Y.Z, W.J.P., H.Y. Writing – Review & Editing: all authors. Supervision – W.J.P., H.Y.

Declaration of Competing Interest

The authors declare no competing financial interest.

Supporting Information

Further information can be found in supporting information. Molecular structures of the used Raman reporters (Figure S1). TEM images of Ag-MBN-CB7@PEG during preparation (Figure S2). Hydrodynamic size of Ag-MBN under different MBN concentrations (Figure S3). UV-vis spectra of Ag-MBN under different MBN concentrations (Figure S4). Effect of the MBN concentration and CB7 concentration on SERS enhancement and aggregation (Figure S5-S7). Effect of adding order of MBN and CB7 on aggregation (Figure S8). Batch-to-batch variation of the CB7 induced aggregation and SERS intensity (Figure S9). Effect of PEG on aggregate sizes (Figure S10). Effect of PEG coating on SERS spectra stability during storage (Figure S11). Effect of PEG coating on SERS spectra stability during centrifugal washing (Figure S12). Reproducibility analysis of Tag 2-9 based on ratio at $I_{1961\text{cm}^{-1}}/I_{2227\text{cm}^{-1}}$ (Figure S13). Cell viability test (Figure S14). SERS cell imaging using a 20X 0.45NA objective lens (Figure S15). SERS cell imaging using a low-power laser (Figure S16). Performance comparison of single cell imaging between other SERS tags and this work (Table S1).

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