An Expanded Surface-Enhanced Raman Scattering Tags Library by Combinatorial Encapsulation of Reporter Molecules in Metal Nanoshells

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Section 1. Plasmonic nanocapsules characterization

1.1. Plasmonic nanocapsules synthesized from 40 nm Ag seed nanoparticles (UV-Vis and TEM characterization)

Figure S1. (A) UV-Vis-NIR spectra of ca. 40 nm silver spherical nanoparticles before (black) and after Galvanic replacement reaction with decreasing Ag:Au molar ratios; 0.15 (red spectrum), 0.09 (blue) and 0.06 (green). Representative TEM images of silver nanoparticles (B) and nanoparticles obtained at different Ag:Au molar ratios; 0.15 (C), 0.09 (D) and 0.06 (E). The numbers indicate the average size together with the standard deviation.
1.2. Plasmonic nanocapsules synthesized from 80 nm Ag seed nanoparticles (UV-Vis-NIR and TEM characterization)

![Graph](image)

**Figure S2.** (A) UV-Vis-NIR spectra of ca. 80 nm silver spherical nanoparticles before (black) and after Galvanic replacement reaction with decreasing Ag:Au molar ratios; 2.36 (red spectrum), 1.37 (blue) and 0.88 (green). Representative TEM images of silver nanoparticles (B) and nanoparticles obtained at different Ag:Au molar ratios; 2.36 (C), 1.37 (D) and 0.88 (E). The numbers indicate the average size together with the standard deviation.
1.3. Plasmonic nanocapsules synthesized from 40 nm and 80 nm Ag seed nanoparticles (additional HAADF-STEM, EDXS and electron tomography characterization).

**Figure S3.** Representative HAADF-STEM images, EDXS maps and visualizations of 3D electron tomography reconstructions of nanocapsules obtained from 40 nm (A) and 80 nm (B) Ag seeds using Ag:Au molar ratios of 0.15 and 0.88, respectively. For the electron tomography results, three orthogonal views of an isosurface and one slice through the reconstruction are depicted for each particle.
1.4. SEM characterization of the plasmonic nanocapsules.

**Figure S4.** Representative SEM micrographs of nanocapsules obtained from 60 nm Ag seeds.
Section 2. Simulations of extinction spectra and electric field distribution for hollow nanocapsules

2.1- Extinction spectra

Extinction cross sections were estimated assuming a spherical shape for the nanocapsules and using morphology parameters obtained from previously described TEM and STEM studies. Experimental nanocapsule external diameter is maintained constant while the hole diameter and Ag shell layer thickness are varied until the simulated extinction peak position matches the experimental result. Table S1 shows experimental diameters of seeds and nanocapsules and the parameters obtained from the simulations which match best the experimental results. Figure S5 shows the experimental extinction (panel A) and simulated extinction cross sections (panel B). The simulated extinction cross sections are normalized to experimental values at 400 nm for comparison purposes.

Table S1. Experimental morphology measurements of nanocapsules and morphology parameters used in FDTD simulations with extinction peak at the same wavelength.

| Sample | Seed diameter (nm) (exp) | Nanocapsule diameter (nm) (exp) | Ag shell (nm) (simulations) | Au shell (nm) (simulations) | Hole diameter (nm) (simulation) |
|--------|--------------------------|---------------------------------|-----------------------------|-----------------------------|--------------------------------|
| 40 nm  | 37.8±3.8                 | 63.2±6.3                        | 2.4                         | 12.70                       | 33.0                           |
| 60 nm  | 63.2±6.3                 | 89.4±9.1                        | 2.0                         | 15.75                       | 53.9                           |
| 80 nm  | 81.0±12.7                | 118.9±14.9                      | 2.7                         | 18.95                       | 75.6                           |
Figure S5. A) Experimental extinction spectra for plasmonic nanocapsules obtained from seeds of 40, 60 and 80 nm. B) Simulated extinction spectra obtained by FDTD modeling using the morphological parameters obtained from TEM and STEM (Table S1) analysis and considering a spherical shape for the nanocapsules.

The experimental and simulated extinction spectra follow the same trend, where the extinction peak shifts to longer wavelengths as the size of the hole and the nanocapsule are increased. Simulations show a narrower optical response than experimental spectra. This difference could be associated to the non-perfect spherical shape of the nanocapsules and the polidispersity of the samples.

2.2- Electric field distribution

Figure S6 shows the electric field distributions on nanocapsules at all the Raman excitation wavelengths studied in this work, in a plane perpendicular to the propagation direction of a linear polarized beam. A dipolar field with local maxima in the vertical direction of the polarization is observed in the external water-gold interface of the nanocapsule. However, a nearly homogeneous electric field distribution is obtained inside the nanocapsule interior.
Figure S6. Electric field map distributions of a cross section plane at the center of the nanocapsules and perpendicular to the propagation direction of an incident beam linearly polarized in the vertical direction. A1, A2 and A3 correspond to a 40 nm nanocapsule at 532 nm, 633 nm and 785 nm respectively. Panels B and C are the results obtained for 60 nm and 80 nm nanocapsules. All electric field maps were normalized to the same electric field scale from 0 to 6.

2.3.- Raman enhancement factors

Raman efficiency of SERS-encoded nanoparticles are usually associated to the maximum Raman enhancement achieved at the metal-dielectric (water) interface. Figure S8.A shows the maximum value of the Raman enhancement obtained at the center of the hole in the nanocapsules for all geometries and wavelengths studied in this work. Taking into account the maximum values of Raman enhancement, the most efficient are the 40 nm nanocapsules at 532 nm. However, these capsules have the smallest hole volume. Therefore, an accurate estimate of the most efficient geometry using the same concentration of Raman reporters during the synthesis, requires estimating the total
Raman enhancement (enhancement*volume) experienced taken into account the volume associated to each geometry (see Figure S8.B). When we take into account the volume of the hole, the total Raman enhancement in the larger nanocapsules increases, thus being the nanocapsules of 60 nm at 633 nm the optimum geometry for SERS-encoded nanoparticles.

**Figure S7.** (A) Maximum value of Raman enhancement factor inside the hole of the nanocapsules for different seed sizes. (B) Total Raman enhancement estimated taking into account the volume of the hole (average Raman enhancement value multiplied by the hole volume).
Section 3.- SERS characterization with different laser lines and stability analysis of nanocapsule

Figure S8. SERS characterization with 532, 633 and 785 nm laser lines. (A-C) SERS (with 633 nm excitation) and SERS (with 532 and 785 nm excitations) spectra obtained for plasmonic nanocapsules encoded with Malachite Green (A), Astra Blue (B) and Nile Blue (C). All spectra were normalized by intensity power and acquisition time. The relative intensities of several peaks varied depending on the excitation wavelengths due to surface selection rules. The enhancement of different peaks varied with respect to excitation wavelength, due to variation in the coupling with extinction spectra of hybrid particles, although the relative intensities varied depending on the excitation wavelength (note the different scales for each spectrum) being the 633 nm laser line the most efficient one, since we are under resonant Raman conditions.
Figure S9. SERS tags sensitivity. (A) Representative SERRS spectra of RaR-encoded nanocapsules, as indicated. The shadowed regions highlight the Raman peak selected to estimate the limit of detection and its time-dependent variation. (B) SERRS intensity representation of the most intense band for each dye, as a function of nanocapsule concentration. The excitation laser line was 633 nm.
**Figure S10. Analysis of the SERS tags stability.** UV-Vis-NIR spectra of plasmonic nanocapsules doped with AB (A) and MG (B) recorded after preparation and 180 days later. (C) Representation of the SERS intensity of the most intense signal for AB (open squares) and MG (open circles), as a function of time.
Figure S11. Dye encapsulation. In order to indirectly demonstrate successful dye encapsulation, we performed a control experiment where initially the plasmonic nanocapsules were synthesized in the presence of Astra blue (AB). After washing the excess of AB, the SERS spectrum was measured, still indicating the presence of AB (black spectrum). To this colloidal dispersion of nanocapsules, 20 µL of $10^{-2}$M Malachite Green (final concentration 0.2mM) was added. SERS characterization (red spectrum) reveals the presence of both dyes (AB and MG with its main characteristic SERS peaks indicated by shadowed areas), AB being encapsulated within the capsules and MG adsorbed onto its outer surface (see schematic representation on the right). Subsequently, the colloidal dispersion was subjected again to washing and redispersion, leading to complete removal of adsorbed MG molecules, as demonstrated by SERS characterization, whereas the characteristic peaks of AB are retained (blue spectrum).
Figure S12. Influence of dye concentration. The encapsulation efficiency could be improved by increasing the amount of dye present in the reaction medium, without affecting the final size and morphology of the particles. (A) NIR-Vis-NIR spectra of plasmonic nanocapsules synthesized in the presence of 4.1 (black), 8.2 (red), 16.2 (blue) and 24.1 (green) µM Astra Blue. (B) SERS characterization of the colloidal dispersions shown in A. (C) Variation of the main Raman peaks at 745, 1340 and 1541 cm\(^{-1}\) with Astra Blue concentration. In all cases, prior to the SERS characterization the excess of AB was removed by several centrifugation and redispersion cycles.
Section 4. SERS encoding possibilities

Encoding of SERS tags relies on the characteristic spectra and narrow bandwidth of the Raman peaks of RaRs. Thus, two main approaches were proposed for SERS spectral encoding; wavenumber-based encoding and wavenumber coupled to signal intensity encoding.

4.1. Wavenumber-based encoding

The SERS spectra of the various RaRs should be easily distinguished from each other to facilitate the decoding process. Consequently, a binary code can be applied for each Raman reporter reflecting either its presence or its absence. Alternatively, the SERS spectrum of each possible combination could be differentiated through a multivariate analysis based on principal component analysis (PCA). Thus, the potential number of codes (N) can be easily estimated, as a function of the number of RaRs (n), through the following combination

\[ N = \sum_{i=1}^{n} C^n_i = 2^n - 1 \]

In the present case, since we are using five different RaRs, we can synthesize up to 31 different codes.

4.2. Raman frequency combined with signal intensity

The number of potential codes can be increased by combining Raman frequency with signal intensity. In the present case, as a proof of concept, we combined Astra Blue, Malachite Green and Nile Blue in different ratios. Considering the characteristic peaks at 1539 cm\(^{-1}\) for Astra Blue, 1617 cm\(^{-1}\) for Malachite Green and 591 cm\(^{-1}\) for Nile Blue normalizing the SERS spectra to the highest peak and recognizing up to ten levels, it is possible to generate up to 300 different codes. Figure S12 shows the characteristic SERS spectra, as well as the barcode, for 37 (out of 300) different SERS tags.
Figure S13. Representative SERS spectra of 26 (out of 31) different combinations of five Raman reporters (MB, CV, NB, AB and MG).
Figure S14. PCA loadings plot from PC1, PC2 and PC3 illustrating which spectral features are important for separation in each case.
Figure S15. Representative SERS spectra of 37 (out of 300) codes made by combination of three dyes (AB, MG and NB), normalizing to the highest peak and recognizing up to ten levels for each reporter. Top, medium and low rows show SERS spectra and codes normalizing by MG, AB and NB, respectively. Note that there is a break in the X-axis to better display the three representative bands for each dye.
**Section 5. Cell imaging**

5.1. Performance of SERS tags in biological media

![Figure S16](image)

**Figure S16. Study of the SERS tags performance in biological media.** *Green spectra:* Representative SERS spectra of three different SERS tags encoded with MG (A), NB (B) or AB (C), bioconjugated to antibodies against EGFR, EpCAM and CD44, respectively. *Red spectra:* Representative SERS spectra of three different SERS tags measured on A431 cells after incubation and washing. *Black spectra:* Representative SERS spectra measured outside the cells in each case. The fact that no additional SERS signals are observed in the spectra is an indication of interference-free detection in complex biological samples.
5.2. - Cell imaging of A431 or 3T3 cells, separately, with SERS tags

We first assessed the specificity of the antibody-conjugated SERS tags towards the corresponding target antigens, by incubating each SERS tag (NB-encoded anti-EpCAM SERS tags, MG encoded anti-EGFR SERS tags and AB encoded anti-CD44 SERS tags) with both cell lines separately (Figure S16-18). A statistical analysis comprising three SERS spectra per cell in at least 100 individual cells, showed that anti-EGFR (Figure S16), anti-EpCAM (Figure S17) and anti-CD44 (Figure S18) SERS tags effectively bound to their target cells with high percentages ranging from 80.1 % to 90 %. This result demonstrated the outstanding selectivity and specificity of the SERS encoded nanoparticles.

5.2.1.- Cell imaging of A431 or 3T3 cells with NB-encoded anti-EpCAM SERS tags
**Figure S17.** Cell SERS mapping (A1 and B1) and large area single-point SERS analysis (A2 and B2) for statistics of selectivity and specificity of NB-encoded anti-EpCAM SERS tags against A431 (A) and 3T3 (B) cell lines, separately. A detection probability of 85% for A431 and 7% for 3T3 were estimated measuring 281 A431 cells and 307 3T3 cells from different areas. The graphs on the right show representative SERS spectra measured at the points labeled with numbers in the confocal images.
5.2.2.- Cell imaging of A431 or 3T3 cells with MG encoded anti-EGFR SERS tags

**Figure S18.** Cell SERS mappings (A1 and B1) and large area single-point SERS analysis (A2 and B2) for statistics of selectivity and specificity of MG encoded anti-EGFR SERS tags against A431 (A) and 3T3 (B) cell lines, separately. A detection probability of 90% for A431 and 80% for 3T3 cell was estimated measuring 321 A431 cells and 114 3T3 cells from different areas. The graphs on the right show representative SERS spectra measured at the points labeled with numbers in the confocal images.
5.2.3.- Cell imaging of A431 or 3T3 cells with AB encoded anti-CD44 SERS tags

**Figure S19.** Cell SERS mapping (A1 and B1) and large area single-point SERS analysis (A2 and B2) for statistics of selectivity and specificity of AB encoded anti-CD44 SERS tags against A431 (A) and 3T3 (B) cell lines, separately. A detection probability of 80.1% for A431 and 5.3% for 3T3 were estimated measuring 101 A431 cells and 239 3T3 cell from different areas. The graphs on the right show representative SERS spectra measured at the points labeled with numbers in the confocal images.
5.3.- Cell imaging of A431 and 3T3 2.2 cells in co-culture with MG encoded anti-EGFR SERS tags and AB encoded anti-CD44 SERS tags

The multiplexing capabilities of the SERS tags were evaluated towards the detection of cellular EGFR, and CD44 in a co-culture of A431 and 3T3 2.2 cells. The cell co-culture was incubated with anti-EGFR and anti-CD44 conjugated SERS tags, encoded with MG and AB, respectively. Figure S19C and D shows the SERS analysis of four cells. SERS mappings were recorded at 1537 cm\(^{-1}\) and 1617 cm\(^{-1}\) to check the spatial distribution of AB (that is, CD44) and MG (that is, EGFR), respectively. The results revealed that three of the cells were A431 cells since they expressed both EGFR and CD44, while the fourth one was an EGFR-negative 3T3 2.2 cell since it expressed CD44 only. Figure 19F-G shows representative SERS spectra recorded in a A431 cell (labeled with a dot in A) expressing CD44 and EGFR (and in a 3T3 2.2 cell (labeled with an asterisk in A) expressing EGFR. Figure 19 B shows fluorescence images of the same group of cells after their immunostaining with mouse anti-EGFR and a fluorescently labeled anti-mouse secondary antibody to reveal the presence of EGFR-expressing A431 cells. The results are in good agreement with the SERS analysis (Figure 19D)

Figure S20. (A) Bright field microscope image of co-cultured A431 and 3T3 2.2 cells (B) Fluorescence microscopy image showing EGFR positive A431 and EGFR negative 3T3 2.2 cells. (C-D) SERS mappings recorded in the same area using the 1537 cm\(^{-1}\) peak of AB (C), 1617 cm\(^{-1}\) peak of MG (D). (F,G) SERS spectra obtained at the spots labeled with a dot and an asterisk in panel (A). The shadowed regions denote the peaks used for mapping. (F) The presence of MG and AB characteristic peaks indicates A431 cells. (G) The absence of MG characteristic peak indicates 3T3 cells.
5.4.- Cell imaging of A431 and 3T3 2.2 cells in co-culture with MG encoded anti-EGFR SERS tags, NB encoded anti-EDCAM SERS tags and AB encoded anti-CD44 SERS tags.

Figure S21. (Top) Schematic representation of the SERS immunophenotype detection of A431 and 3T3 cells. (A) Bright field confocal image of a mixture of A431 and 3T3 cells. (B-D) SERRS mappings recorded in the same area using the 1617 cm\(^{-1}\) peak of MG (B), 1537 cm\(^{-1}\) peak of AB (C), 1640 cm\(^{-1}\) of NB (D), and (E) merged image of the three mappings. (F, G) SERRS spectra obtained at the areas labeled with numbers in panel (A). The shadowed regions denote the peaks used for mapping. (F) The presence of MG, AB and NB characteristic peaks indicates A431 cells. (G) The absence of MG and AB characteristic peaks indicates 3T3 cells.
Figure S22. (A) Bright field confocal image of a mixture of A431 and 3T3 cells. (B-D) SERRS mappings recorded in the same area using the (B) 1617 cm\(^{-1}\) peak of MG, (C) 1537 cm\(^{-1}\) peak of AB, (D) 1640 cm\(^{-1}\) of NB, and (E) merged image of the three mappings. (F) SERRS spectra obtained at the areas labeled with numbers in panel (A). Shadowed regions denote the peaks used for mapping. The absence of MG and AB characteristic peaks in cell labeled with 1 indicates 3T3 cells.

References

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