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Supplementary information

Raman Reporters Derived From Aryl Diazonium Salts for SERS Encoded-Nanoparticles

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I. Experimental section

I.1. Synthesis of aryl diazonium salts (D-COOH, D-CN and D-NO_2)

The aryl diazonium salts were synthesized as described previously.[1] For the synthesis of D-COOH, 2 g of 4-aminobenzoic acid (≥99%, Sigma-Aldrich) was added into 12mL of tetrafluoroboric acid solution (48% in H_2O, Sigma-Aldrich) in a flask, followed by adding 10mL of acetonitrile (≥99.9%, Sigma-Aldrich). The transparent solution was cooled down to *ca.* 2°C in ice/water bath under stirring. Then 2mL of *tert*-butyl nitrite (90%, Sigma-Aldrich) was added dropwise into the flask. The solution was kept cooled under stirring for 30 min. The obtained diazonium salt was washed by diethyl ether (≥99.7%, Sigma-Aldrich) for 3 times, then dissolved in acetone (≥99.9%, Sigma-Aldrich) and dried at room temperature. The synthesis of D-CN and D-NO_2 followed the same protocol, except using 4-aminobenzonitrile (98%, Sigma-Aldrich) and 4-nitroaniline (≥99%, Sigma-Aldrich) as precursors, respectively.

I.2. Synthesis of citrate-capped AuNPs

50mL of gold(III) chloride trihydrate (≥99%, Sigma-Aldrich) at a concentration of 10^{-3} mol L^{-1} was heated to boiling. Then 5mL of aqueous sodium citrate (anhydrous, ⩾99.5%, Sigma-Aldrich) solution was injected into the boiling solution under vigorous stirring for 15 min. Soon, the solution turned to red. It was then cooled down to room temperature and stored at 4°C.

I.3. Spontaneous functionalization of AuNPs by aryl diazonium salts

The functionalization reaction was performed in 1mL of citrate-capped AuNPs by mixing the colloidal solution obtained above, with 5µL of aqueous solutions of D-NO_2 or D-CN (10^{-2} mol.L^{-1}) for 1h at room temperature. The solution was then centrifuged at 5000 rpm for 30 min. The supernatant was discarded and replaced by deionized water. The reaction was stopped by repeating 3 times of centrifugation/redispersion process, and the resulting samples were noted Au@CN and Au@NO_2. For Au@CN@COOH and Au@NO_2@COOH, the second layer was grafted by adding 5 µL of D-COOH (10^{-2} mol L^{-1}) into Au@CN and Au@NO_2 solutions and let the reaction proceed for 1h, followed by 3 times of centrifugation/redispersion process in water.

I.4. Post-functionalization by AMP

The double-layer grafted AuNPs were stabilized by adding 0.0001-1 wt.% of polyvinylpyrrolidone (PVP, average mol. Wt. 10,000, Sigma-Aldrich). After 1h at room temperature, 10µL of an aqueous solution of adenosine 5'-monophosphate disodium salt

(AMP, 10^{-2} mol.L^{-1}, from Sigma-Aldrich) was added in the stabilized AuNPs suspension, followed by the addition of 1 µL of N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC, 50 g.L^{-1}, ≥99%, Sigma-Aldrich) and 2 µL of N-hydroxysuccinimide (NHS, 50 g.L^{-1}, 98%, Sigma-Aldrich). The reaction was carried out at room temperature during 2h and then at 4°C overnight. After three cycles of centrifugation/redispersion in water, Au@CN@COOH@AMP and Au@NO_2@COOH@AMP were obtained.

I.5. Cellular uptake studies

The cellular uptake study was performed by exposing Au@CN@COOH@AMP with cholangiocarcinoma EGI-1 cells and Au@NO_2@COOH@AMP with CT-26 cells. EGI-1 and CT-26 cells were seeded in 6-well plates with coverslips at a density of 1.2×10^6 cells/well by using RPMI1640 (Gibco, Life Technologies Corporation, U.S.A) with 15% Fetal Bovine Serum (Gibco, Life Technologies Corporation, U.S.A) as the culture medium. The cells were cultured in an atmosphere of 5% CO_2 at 37℃ for 24 h. The culture medium was then replaced by 2 mL of medium containing 1 nM NPs and incubated with cells for 24 h. After incubation, cells were washed with ice cold DPBS (Dulbecco's Phosphate Buffered Saline [-] $CaCl_2$, [-] $MgCl_2$, Gibco, Life Technologies Corporation, U.S.A) three times and then fixed with 1% PFA.

I.6. Instrumentation

The colloidal solutions were diluted 4 times for UV-vis spectroscopy. The extinction spectra were recorded on a Shimadzu 2700 UV-vis spectrometer, using 1x1cm quartz cuvettes. The SERS were recorded on a Horiba XploRA PLUS Raman microscope. All the measurements were performed on dried drops of the samples deposited on glass microscope slides, using a laser at 638 nm, an exposition time of 10s and an accumulation of 10 spectra. The SERS mappings (NPs and cells) were recorded on a Horiba LabRAM HR Evolution Raman microscope with an excitation wavelength of 633 nm. The exposition time for mappings was 0.8s per spectrum for the reference map and 2s per spectrum on the cell map. The XPS spectra were recorded using a Thermo Scientific K-Alpha spectrometer equipped with a monochromatic Al-Kα X-ray source (hv = 1486 eV). The spot size was of 400 µm, while the pass energy of surveys and high-energy resolution were fixed at 200 and 50 eV, respectively. The charge compensation was performed by a combination of an electron flood gun with an argon ion gun. The TEM images were recorded with a Tecnai microscope (120 kV) and a 4kx4k Eagle camera (Thermofisher, USA), after depositing 4 µL of each sample on carbon coated grids (primarily glow discharged).

II. Complementary results

II.1. TEM images

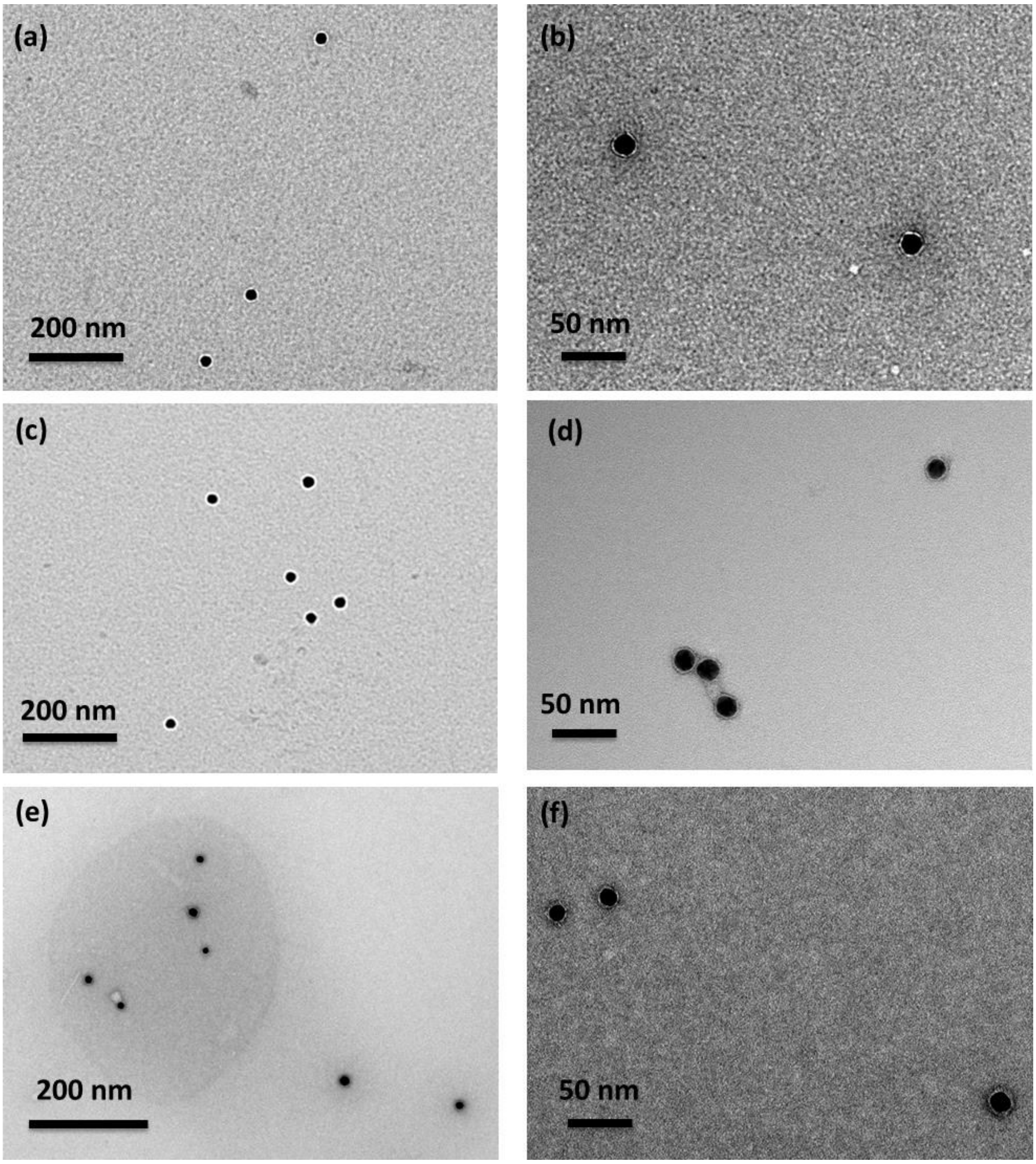

Figure S1. TEM images of (a) and (b) AuNPs; (c) and (d) Au@NO_2@COOH@AMP; (e) and (f) Au@CN@COOH@AMP.

TEM images (Fig. S1) show individually dispersed particles indicating that the reaction of the AuNPs with the diazonium salts does not disturb their colloidal stability. The AuNPs average diameter was estimated using 100 NPs from different TEM images. Interestingly, the overall NPs size appears to increase after surface functionalization (see Table S1) and the functionalization process leads to the appearance of a thin layer covering the NPs, likely due to the presence of the polyaryl layers and AMP.

Table S1. Evolution of the average NPs diameter estimated by TEM for Au, Au@CN@COOH@AMP and Au@NO_2@COOH@AMP.

Sample	NPs diameter (TEM) /nm
Au	15 ± 2
Au@CN@COOH@AMP	18 ± 2
Au@NO_2@COOH@AMP	19 ± 2

II.2. Extinction spectra

Table S2. Wavelength of the plasmon band (λ_{max}) observed in the extinction spectra of the different AuNP samples.

Sample	***Plasmon band λ_{max} (nm)***
Au	520
Au@CN	522
Au@CN@COOH	524
Au@CN@COOH@AMP	531
Au@NO_2	523
Au@NO_2@COOH	525
Au@NO_2@COOH@AMP	530

II.3. Effect of the PVP concentration on the post-functionalization reaction by AMP

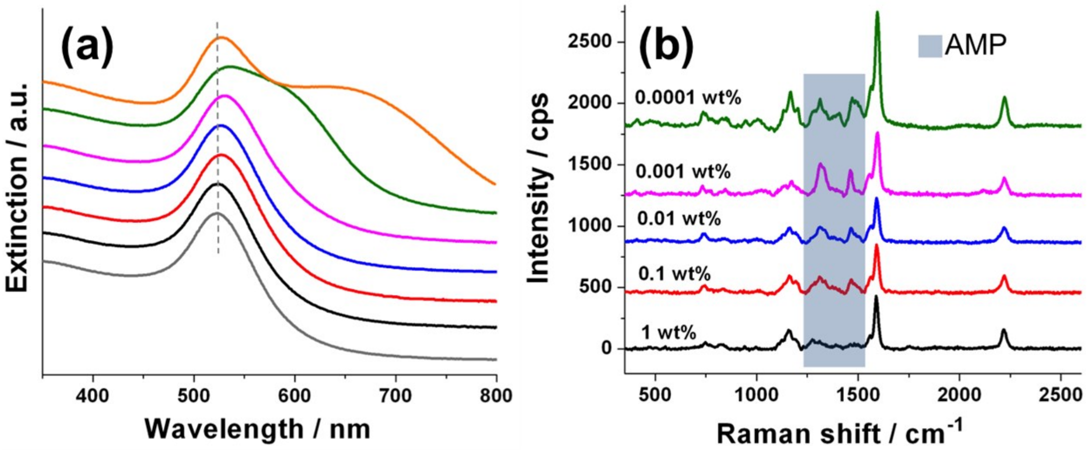

Figure S2. (a) Normalized extinction spectra and (b) SERS spectra for AuNPs (grey), Au@CN@COOH@AMP prepared in 1 wt.% PVP (black), 0.1 wt.% PVP (red), 0.01 wt.% PVP (blue), 0.001 wt.% PVP (pink), 0.0001 wt.% PVP (green), and without (orange) PVP solution. The blue shadow highlights the SERS bands belonging to AMP.

In order to stabilize the AuNP samples during the post-functionalization reaction, different concentrations of PVP were mixed with the Au@CN@COOH and Au@NO_2@COOH samples before activation by EDC/NHS. Figure S2(a) displays the extinction spectra recorded when using different concentrations of PVP, ranging from 0.0001 to 1 wt%. Below 0.001% PVP, the NPs aggregate during post-functionalization as confirmed by the appearance of a shoulder in the extinction spectra, above 600 nm. In contrast, above this concentration value, the shape of the plasmon band remains unchanged after post-functionalization, emphasizing the stability of the colloidal dispersion. The SERS spectra, displayed in Fig. S2(b) reveal that when the concentration of PVP is too high, the post-functionalization reaction cannot proceed, probably due to a poor access of the reactants to the surface. Indeed, the signal of AMP could not be detected on the SERS spectra when the PVP concentration reaches 1%. Finally, a good compromise between colloidal stability and post-functionalization capacity was found using a concentration of PVP of 0.001 wt%.

II.4. Evaluation of the nanoprobe colloidal stability in various media

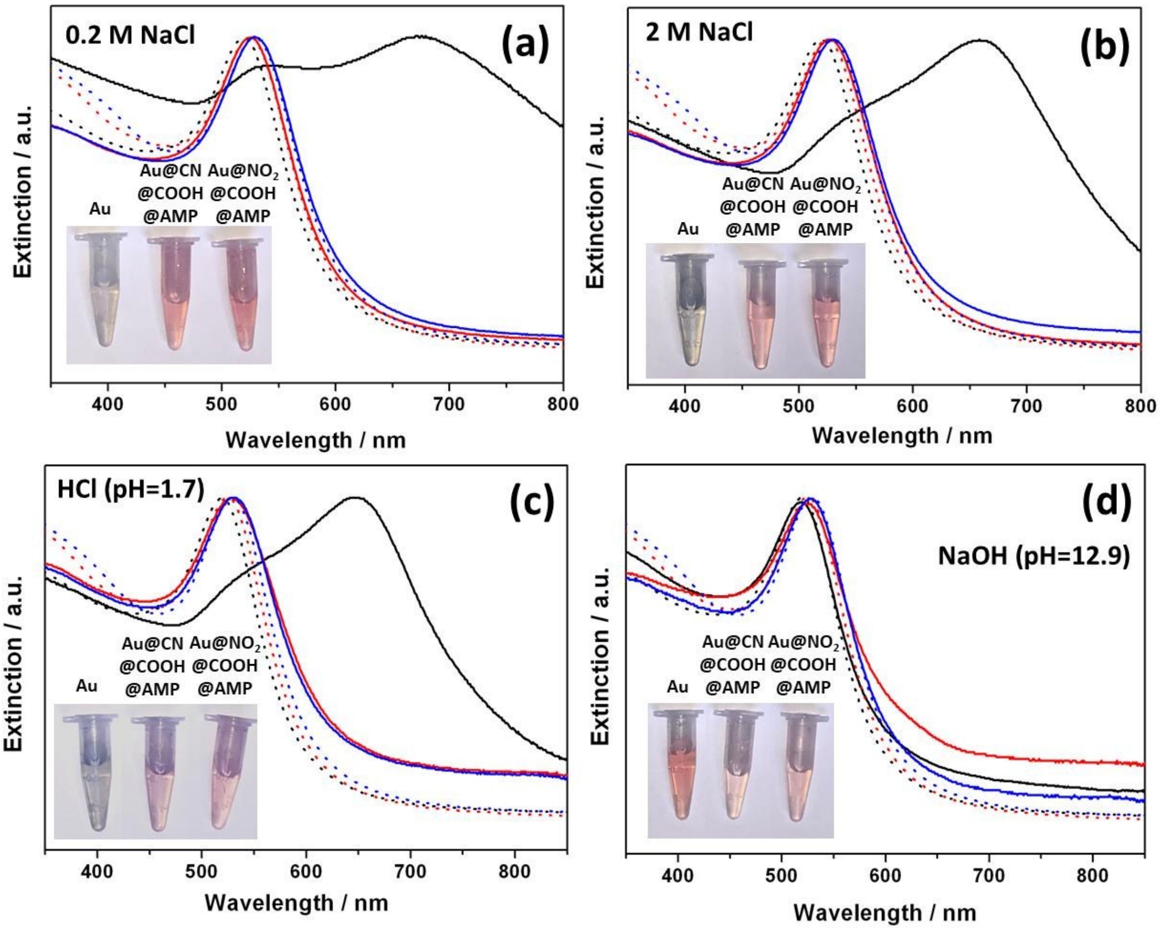

Figure S3. Extinction spectra of AuNP samples dispersed in various aqueous media: (a) NaCl 0.2 M; (b) NaCl 2 M; (c) HCl at pH = 1.7 and (d) NaOH at pH = 12.9. The black solid line corresponds to the spectra of bare AuNPs, the red line to Au@CN@COOH@AMP and the blue spectra to Au@NO_2@COOH@AMP. The spectra of the as-prepared AuNPs, Au@CN@COOH@AMP and Au@NO_2@COOH@AMP were used as references (dotted lines).

The insets show photos of the different samples in NaCl, HCl and NaOH containing aqueous media.

II.5. XPS analysis

The surface chemical composition of the modified AuNPs was investigated by XPS, revealing large modifications after functionalization, as summarized in Table S3. Particularly, the oxygen signal, very intense in the spectrum of the initial AuNPs due to the citrate capping layer, was strongly attenuated after reaction with the different diazonium salts due to the ligand exchange at the surface of the NPs. In the meanwhile, the intensity of the nitrogen signal increased markedly, in agreement with the presence of CN or NO_2 groups. After immobilization of AMP, the carbon content increased while a new peak assigned to phosphorus appeared. Peak fitting of the C1s signal, displayed in Fig. S4, further reflects the surface modifications introduced by the grafting of AMP, confirming the successful covering of the gold cores by the functional polyaryl layers and AMP.

Table S3. Surface chemical composition (At %) of initial AuNPs, Au@CN, Au@CN@COOH@AMP, Au@NO_2, Au@NO_2@COOH@AMP.

Material[a]	Au	C	O	N	P
Citrate-capped AuNPs	4	59	36	1	-
Au@CN	27	50	19	4	-
Au@CN@COOH@AMP[a]	11	64	18	5	2
Au@NO_2	21	54	21	4	-
Au@NO_2@COOH@AMP	12	61	19	6	2

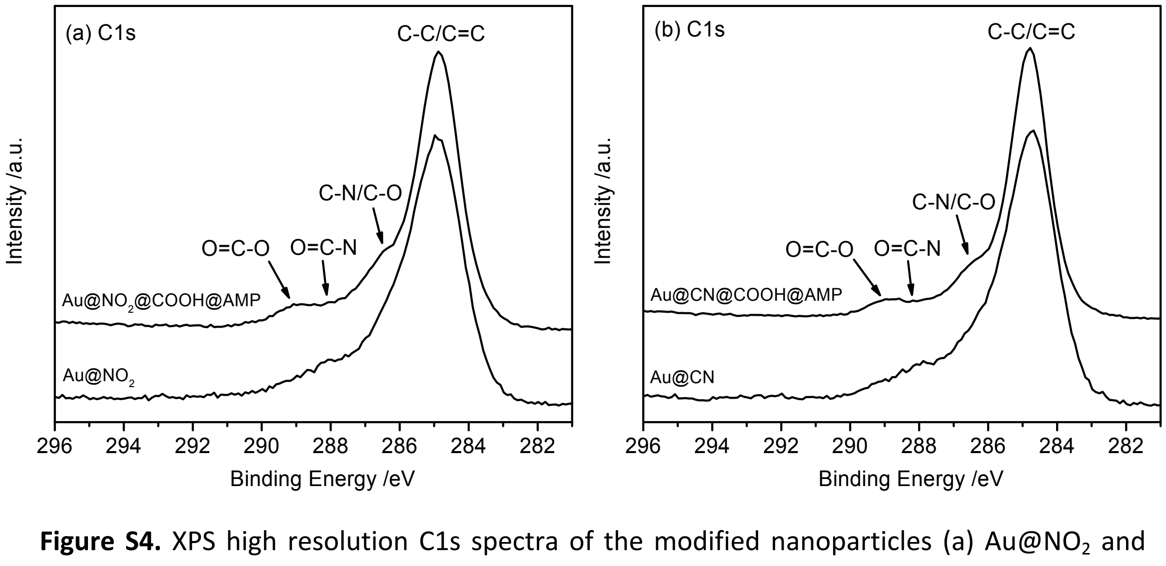

Figure S4. XPS high resolution C1s spectra of the modified nanoparticles (a) Au@NO_2 and Au@NO_2@COOH@AMP; (b) Au@CN and Au@CN@COOH@AMP.

II.6. SERS analysis

SERS spectra of Au@CN and Au@NO$_2$ and Raman spectra of D-NO$_2$ and D-COOH

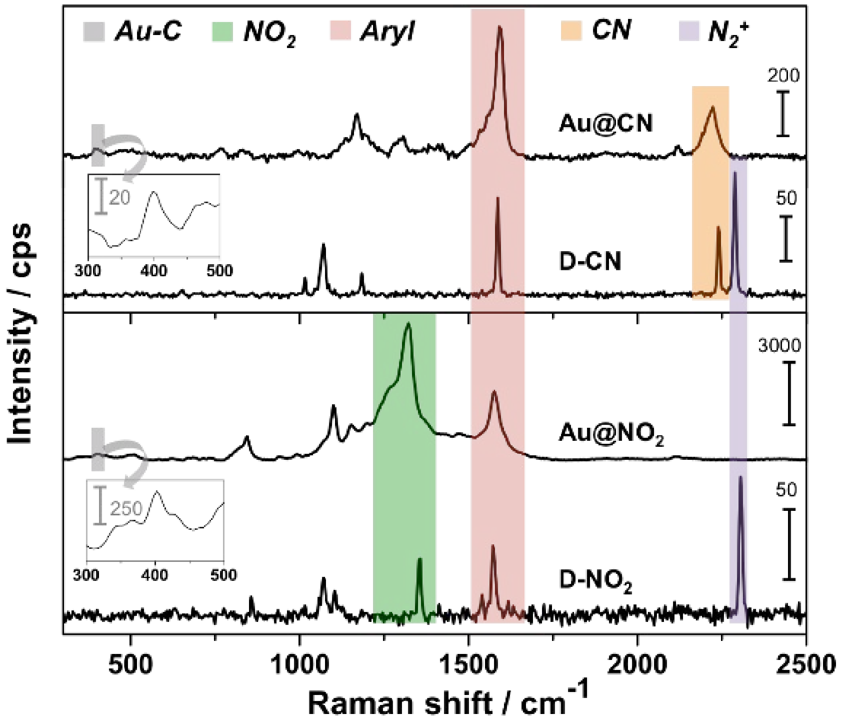

Figure S5. Comparison between the SERS spectra of nanoprobes Au@CN and Au@NO$_2$ and the Raman spectra of the corresponding diazonium salt powders, D-NO$_2$ and D-COOH. Inset: zoom in the spectral range 300-500 cm^{-1}, revealing the presence of $\nu_{Au\text{-}C}$ peak.

Evolution of the SERS spectra versus time of reaction

D-NO$_2$ or D-CN (final concentration 5x10^{-5} mol.L^{-1}) were added in 1mL of AuNPs and an aliquot of the solution was taken out at various times after addition of the diazonium salts (5, 10, 15, 20 and 40 min). Each aliquot was washed by repeating 2 cycles of centrifugation/redispersion in water. After 60 min, D-COOH (final concentration 5x10^{-5} mol.L^{-1}) was added into the initial solution. New aliquots were then taken out at 60, 80, 100 and 120 min. The SERS spectra of each aliquot were recorded by depositing a drop of the washed solutions on glass slides and let them dry at air (see Fig. S6 and S7). 5 spectra of each sample were recorded to obtain average band intensities.

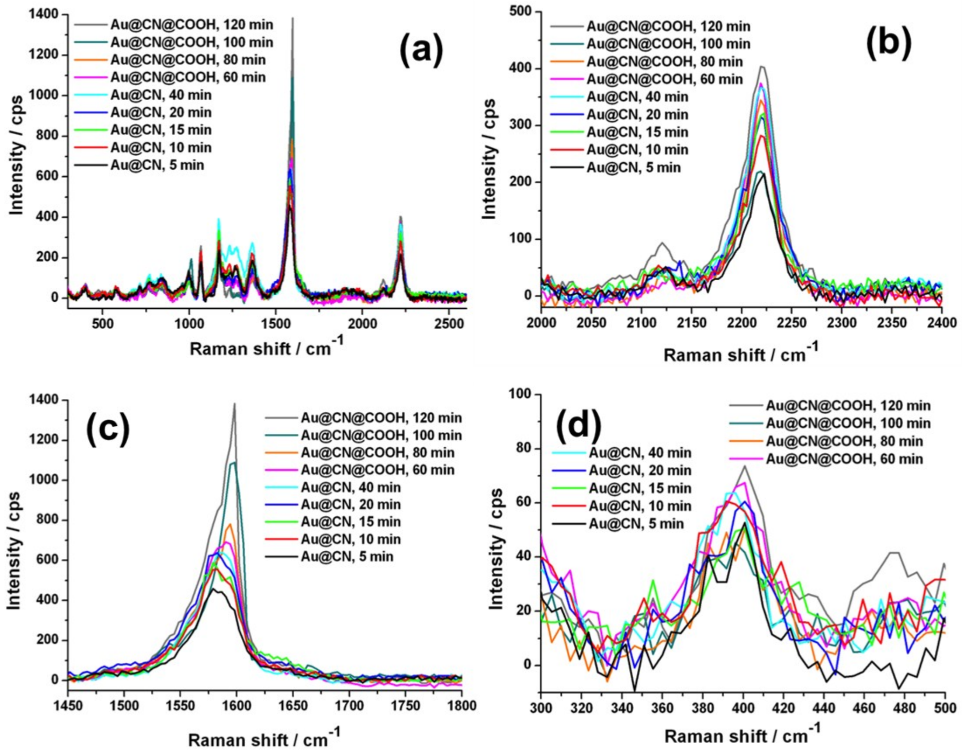

Figure S6. SERS spectra of Au@CN and Au@CN@COOH recorded after different times of the functionalization reaction. (a) in the spectral range 300-2600 cm^{-1}, (b) 2000-2400 cm^{-1} (CN stretch), (c) 1450-1800 cm^{-1} (aryl peak) and (d) 300-500 cm^{-1} (Au-C stretch).

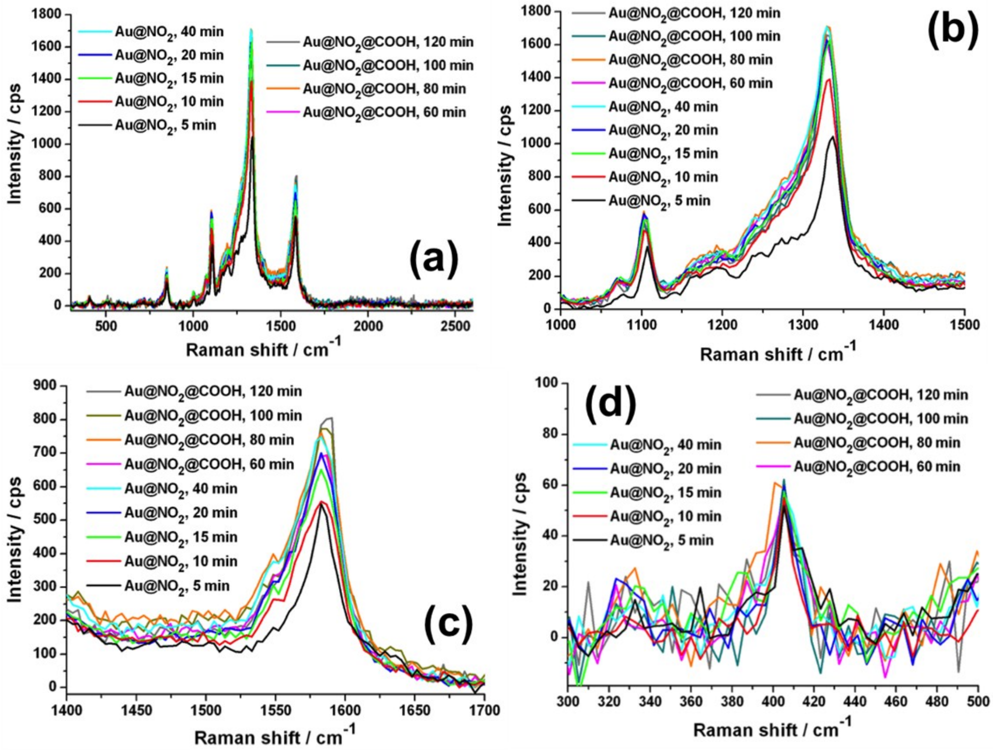

Figure S7. SERS spectra of Au@NO$_2$ and Au@NO$_2$@COOH recorded after different times of the functionalization reaction. (a) in the spectral range 300-2600 cm^{-1}, (b) 1000-1500 cm^{-1} (NO$_2$ peak), (c) 1400-1700 cm^{-1} (aryl peak) and (d) 300-500 cm^{-1} (Au-C stretch).

Figure S8 displays the evolution of band intensity ratios selected to monitor the successive grafting of the different layers for Au@CN@COOH and Au@NO_2@COOH, as a function of reaction time with diazonium salts. The variations in relative intensity of the characteristic CN (I_{CN} at 2220 cm^{-1}) and NO_2 peaks (I_{NO2} at 1330 cm^{-1}) were selected to measure the first layer growth, taking the Au-C peak intensity (I_{Au-C}) as the reference. The intensity ratios I_{CN}/I_{Au-C} and I_{NO2}/I_{Au-C} were shown to increase during the first 20 minutes of reaction, revealing the grafting and polymerization of the first CN- or NO_2-terminated polyaryl layer. After 20 min, the reaction seems to be complete as no more evolution of the SERS spectra is detectable. It is noteworthy that the introduction of D-COOH after 60 min does not bring any additional changes to I_{CN}/I_{Au-C} and I_{NO2}/I_{Au-C} intensity ratios. To assess the growth of the second poly(aryl) layer derived from D-COOH, the intensity ratio I_{aryl}/I_{Au-C} was selected as the appropriate indicator. It was shown to increase at the beginning of the reaction, after addition of the Raman reporters, and then to remain constant after 20 min, thus following a similar trend than the intensity ratios I_{NO2}/I_{Au-C} and I_{CN}/I_{Au-C}. However, after addition of D-COOH, the intensity ratio I_{aryl}/I_{Au-C} increased again, confirming the grafting of the second carboxyl-terminated polyaryl layer.

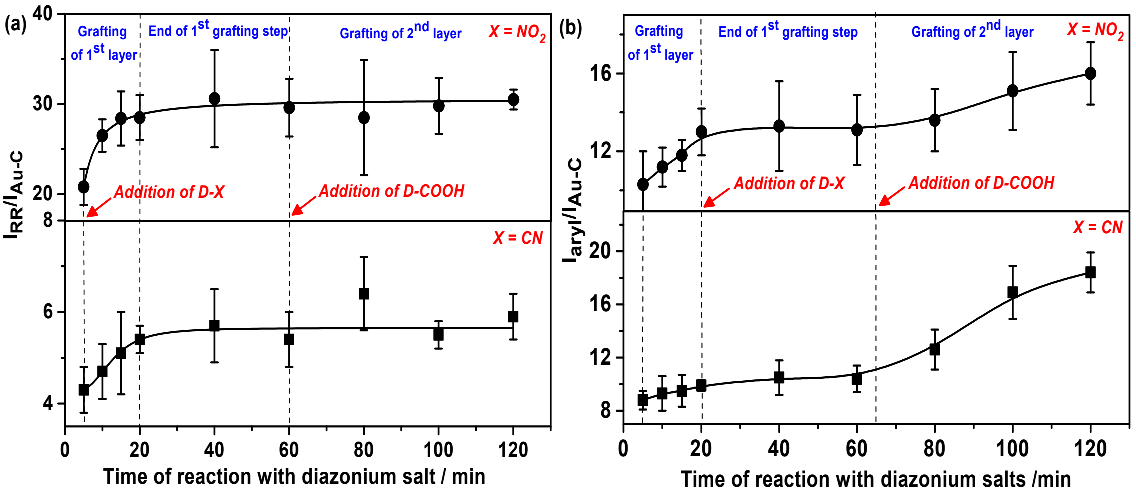

Figure S8. SERS band intensity ratios *vs* time of reaction after the two-step addition of first, the Raman reporter diazonium salts (D-CN and D-NO_2) and second, D-COOH: (a) I_{NO2}/I_{Au-C} (circles) and I_{CN}/I_{Au-C} (squares); (b) I_{aryl}/I_{Au-C} for Au@NO_2@COOH (circles) and Au@CN@COOH (squares).

References

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