# Supporting Information

## SERS Surface Selection Rules for the Proteomic Liquid Biopsy in Real Samples: Efficient Detection of the Oncoprotein c-MYC

*Elena Pazos,[1] Manuel Garcia-Algar,[1,2] Cristina Penas,[3] Moritz Nazarenus,[2] Arnau Torruella, [1,2] Nicolas Pazos-Perez,[2] Luca Guerrini,[2] M. Eugenio Vázquez, [3] Eduardo Garcia-Rico,[4]* José L. Mascareñas[3]* and Ramon A. Alvarez-Puebla[1,5]**

[1] Universitat Rovira i Virgili and Centro de Tecnología Química de Catalunya, Carrer de Marcellí Domingo s/n, 43007 Tarragona, Spain.

[2] Medcom Advance S.A., Av. Roma, 08840 Barcelona, Spain.

[3] Centro Singular de Investigación en Química Biolóxica e Materiais Moleculares (CIQUS) and Departamento de Química Orgánica, Universidade de Santiago de Compostela, 15782 Santiago de Compostela, Spain.

[4] Department of Clinical Oncology, Hospital Universitario Hm Madrid-Torrelodones, 28250 Madrid, Spain

[5] ICREA, Passeig Lluís Companys 23, 08010 Barcelona, Spain.

## Experimental Section

**Materials.** All peptide synthesis reagents and amino acid derivatives were purchased from *GL Biochem* (Shanghai), *Novabiochem* or *Iris Biotech Gmbh*; amino acids were purchased as protected Fmoc amino acids with the standard side chain protecting scheme: Fmoc-Lys(Boc)-OH, Fmoc-Ile-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Glu(*t*Bu)-OH, Fmoc-Leu-OH, Fmoc-Ala-OH, Fmoc-Phe-OH, Fmoc-Asp(*t*Bu)-OH, Fmoc-Gln(Trt)-OH, and Fmoc-Lys(Alloc)-OH. DMF and TFA were purchased from *Scharlau,* $CH_2Cl_2$ from *Panreac,* and $CH_3CN$ from *Merck.* Dihydrated trisodium citrate (≥99.5%), L-ascorbic acid (≥99.0%), silver nitrate (≥99.9999%), magnesium sulfate (99.5%), polyethylenimine branched (PEI, 25000), and the rest of the reagents were acquired from *Sigma-Aldrich*. Silica beads of 7.75 μm in diameter were acquired from *Microparticles GmbH*. Recombinant Human c-MYC was purchased from *Creative BioMart*. All solvents were dry and synthesis grade, unless specifically noted, and all reactants were used without further purification. Milli-Q water (18 MΩ $cm^{-1}$) was used in all aqueous solutions, and all glassware was cleaned with aqua regia before the experiments.

**Instrumentation.** Reactions were monitored by analytical RP-HPLC with an *Agilent 1100* series LC/MS using an *Eclipse* XDB-$C_{18}$ (4.6 × 150 mm, 5 μm) analytical column. Compounds were detected by UV absorption (220 and 270 nm); the standard conditions for analytical RP-HPLC consisted on an isocratic regime during the first 5 min, followed by a linear gradient from 5 to 95% of solvent B for 30 min at a flow rate of 1 mL/min (A: water with 0.1% TFA, B: acetonitrile with 0.1% TFA).

Reversed phase HPLC purification was performed on an *Agilent 1100* series equipped with a binary pump system and a UV-vis detector using a *Phenomenex Luna* C18 100A (250 × 21.2 mm, 10 μm) semi-preparative column. Purification was carried out using an isocratic regime during the first 5 min at 5% of solvent B and then linear gradients from 5% to 75% of solvent B for 30 min at a flow rate of 3 mL/min (A: water with 0.1% TFA, B: acetonitrile with 0.1% TFA). The fractions containing the products were freeze-dried, and their identity confirmed by ESI-MS.

UV–vis spectra were recorded using a *Thermo Scientific Evolution 201* UV–vis spectrophotometer. Peptide concentration was calculated by the Lambert−Beer law using an extinction coefficient of 11862 $M^{-1}$ $cm^{-1}$ at 310 nm. Silver concentration for AgNPs was calculated using extinction coefficient of $6.61\times10^{10}$ $M^{-1}$ $cm^{-1}$.[1-3]

Electron micrographs were recorded with a transmission electron microscopy (*JEOL JEM-1011* operating at 80 kV) and an environmental scanning electron microscopy (*JEOL 6400*) for the structural characterization of the samples.

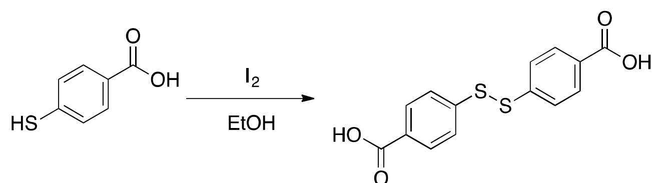
SERS experiments were conducted using a *Renishaw InVia Reflex* confocal microscope equipped with a 1200 grooves $mm^{-1}$ grating for the NIR wavelengths, additional band-pass filter optics, and a 2D-CCD camera. All spectra were acquired using 785 nm laser excitation by focusing the laser onto the sample with ×5 or ×50 objectives.

## General peptide synthesis procedures

**H1** was synthesized as C-terminal amide (0.05 mmol scale using the resin Rink Amide-ChemMatrix from *Biotage*: 0.46 mmol/g) on a *PS3 Peptide Synthesizer* (*Protein Technologies*) following standard Fmoc solid phase synthesis protocols. Peptide bond-forming couplings were conducted for 50 min by using *N,N,N′,N′*-Tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU, 8 equiv), *N,N*-Diisopropylethyl amine (DIEA, 8.8 equiv) in DMF (2.5 mL) and 8 equivalents of the amino acids. Each amino acid was incubated for 2 min in the coupling mixture before being added onto the resin. After washing with DMF, the deprotection of the temporal Fmoc protecting group was performed by treating the resin with 20% piperidine in DMF solution for 15 min.

**Cleavage and deprotection of the resin bound peptide.** The resin-bound peptide dried under argon (ca. 0.025 mmol) was placed in a 50 mL falcon tube and suspended in 2 mL of the cleavage cocktail (100 µL of $CH_2Cl_2$, 50 µL of water, 50 µL of triisopropylsilane (TIS) and TFA to 2 mL), and the resulting mixture was shaken for 3 h. The resin was filtered, and the TFA filtrate was concentrated under an argon current to a volume of approximately 1 mL, which was then added to ice-cold diethyl ether (20 mL). After 10 min, the precipitate was centrifuged and washed again with 10 mL of ice-cold ether. The solid residue was dried under argon, redissolved in $CH_3CN/H_2O$ 1:1 (1 mL) and purified by semi-preparative reversed-phase HPLC. The collected fractions were lyophilized and stored at −20 °C.

## Synthesis of 4,4'-disulfanediyldibenzoic acid




**Scheme S1.** Synthesis of 4,4'-disulfanediyldibenzoic acid.

4-mercaptobenzoic acid (20 mg, 0.13 mmol) was dissolved in EtOH (1.3 mL), a saturated solution of $I_2$ in EtOH was added dropwise until a precipitate appeared. The solution was centrifuged and the precipitate was dissolved in DMSO. The product was precipitated again in EtOH, centrifuged and dried under high vacuum. 4,4'-disulfanediyldibenzoic acid was identified as the desired product by ESI-MS (15 mg, 75% yield). ESI-MS [$MNa^+$] calcd for $C_{14}H_{10}O_4S_2$ = 329.36, found = 329.0.

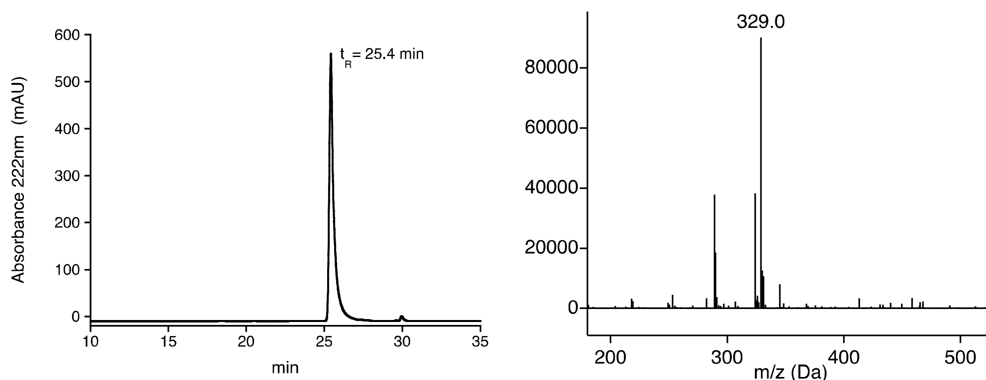
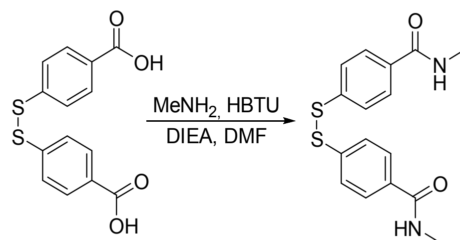



**Figure S1**. *Left*: HPLC trace of the pure product. *Right*: ESI-MS spectra corresponding to the peak at c.a. 25.4 min.

## Synthesis of 4,4'-disulfanediylbis(*N*-methylbenzamide)




**Scheme S2.** Synthesis of 4,4'-disulfanediylbis(*N*-methylbenzamide).

250 mg of 4,4'-disulfanediyldibenzoic acid (0.82 mmol) were dissolved in DMF. Then, HBTU (744 mg, 1.96 mmol), DIEA (838 μL, 4.9 mmol) and methylamine were added over the previous solution and the mixture was shaken at room temperature for 2 hours. After confirming the obtaining of the desired product by HPLC-MS analysis, DMF was evaporated under vacuum. Then, the reaction crude was directly purified by preparative reversed-phase chromatography (15→75% MeOH, 0.1% TFA / $H_2O$, 0.1% TFA, 30 min) and the appropriate fractions were collected and freeze-dried to provide the desired product as a white solid (52 mg, 0.16 mmol, 19% yield). ESI-MS [$MH^+$] calcd for $C_{16}H_{16}N_2O_2S_2$ = 333.07, found 333.1 $[MH]^+$, 355.0 $[MNa]^+$, 665.1 $[M_2H]^+$.

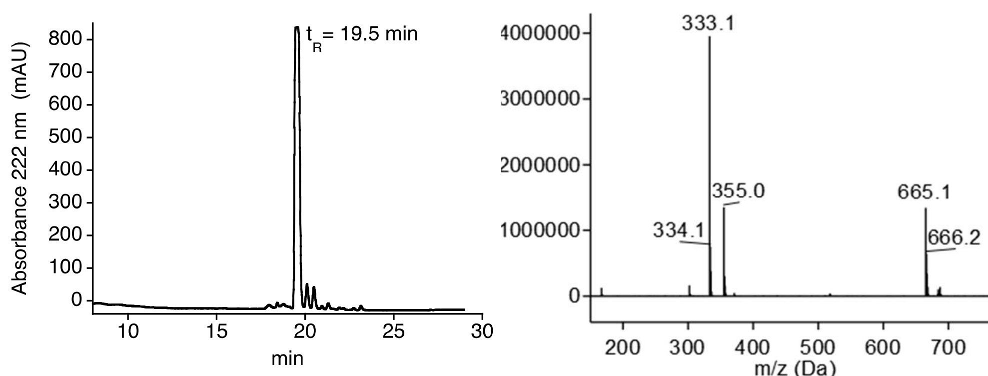
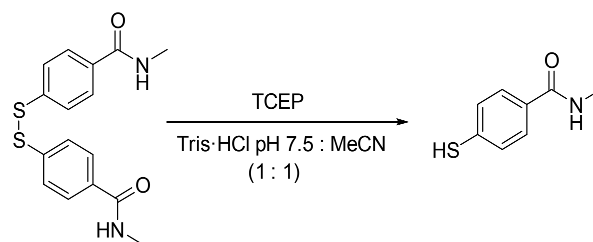



**Figure S2**. *Left*: HPLC trace of the pure product. *Right*: ESI-MS spectra corresponding to the peak at c.a. 19.5 min.

## Synthesis of 4-mercapto-*N*-methylbenzamide




**Scheme S3**. Synthesis of 4-mercapto-*N*-methylbenzamide.

For the synthesis of 4-mercapto-*N*-methylbenzamide, 8 mg of 4,4'-disulfanediylbis(*N*-methylbenzamide) (0.024 mmol) were dissolved in 1 mL of a 0.2 M solution of tris(2-carboxyethyl)phosphine hydrochloride (TCEP) in 20 mM Tris-HCl pH 7.5 : $CH_3CN$ (1:1). The mixture was shaken at rt for 1 h. The crude was then purified by semi-preparative reverse phase HPLC and the appropriate fractions were collected and freeze-dried to provide the desired product as a white solid (3 mg, 0.018 mmol, 75% yield). ESI-MS $[MH^+]$ calcd for $C_8H_9NOS$ = 168.04, found 168.1 $[MH]^+$, 190.1 $[MNa]^+$.

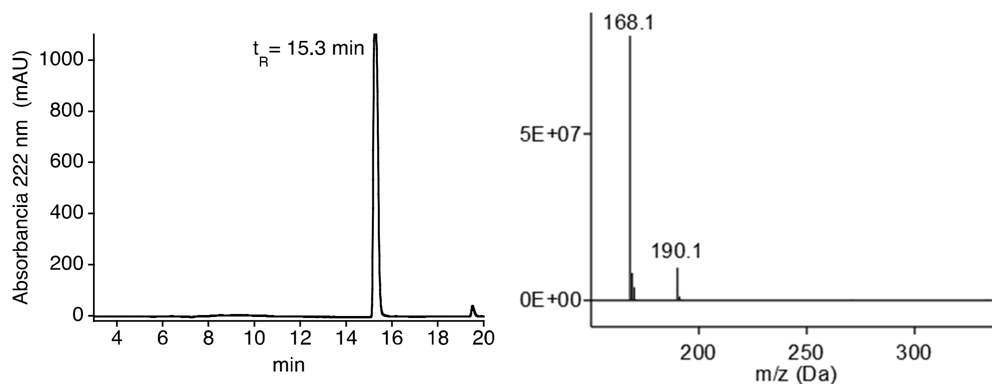
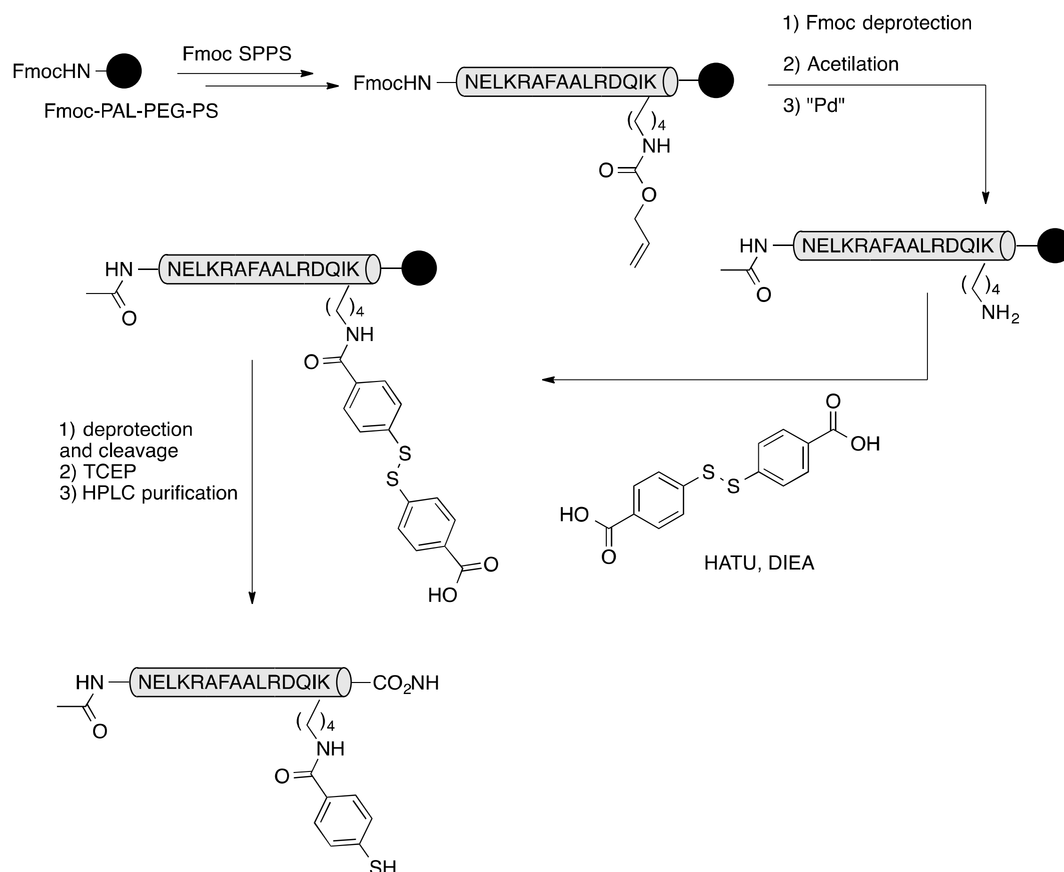



**Figure S3.** *Left:* HPLC trace of the pure product. *Right:* ESI-MS spectra corresponding to the peak at c.a. 15.3 min.

## MB-H1 peptide synthesis

For the synthesis of the 4-mercaptobenzamide-conjugated peptide we used a modified Fmoc SPPS strategy with an extended protecting group scheme. The Lys residue is introduced as an Alloc-protected derivative, which allows its selective side chain deprotection (Pd catalysis), and derivatization with 4,4'-disulfanediyldibenzoic acid in the solid phase as shown below:




**Scheme S4.** Synthetic route for the obtaining of the Raman-labeled c-MYC receptor.

**Acetylation of the N-terminal end of the peptide and deprotection of the orthogonally protected Lys(Alloc) side chain.** Once the peptide was fully assembled in the solid phase, the Fmoc group was deprotected following the conditions described in the general methods, and then the N-terminal amine was acetylated. For this purpose 0.05 mmol of the resin were treated with 3 mL of a solution of 20% $Ac_2O$ in DMF/0.195M DIEA in DMF (2:1) for 20 min. After N-terminal acetylation, the side chain of the Lys(Alloc) residue was selectively deprotected for specific attachment by

treating 0.05 mmol of peptide attached to the solid support with a mixture of $Pd(OAc)_2$ (0.3 eq), $PPh_3$ (1.5 eq), *N*-Methylmorpholine (NMM, 10 eq), and $PhSiH_3$ (10 eq) in $CH_2Cl_2$ (2.5 mL) at rt for 12 h. The resin was then filtered and washed with DMF (1 × 5 mL × 2 min), diethyldithiocarbamate (DEDTC) (25mg in 5 mL of DMF, 2 × 5 min), DMF (2 × 5 mL × 2 min) and $CH_2Cl_2$ (2 × 5 mL × 2 min).

**4,4'-disulfanediyldibenzoic acid coupling.** 4,4'-disulfanediyldibenzoic acid (92 mg, 0.3 mmol), HATU (114 mg, 0.3 mmol) and DIEA (51.3 µL, 0.3 mmol) were dissolved in 3 mL of DMF. After 2 min, the mixture was added over the alloc-deprotected peptide (0.05 mmol), attached to the resin, and the suspension was shaken for 2 h. After filtration, the resin was washed with DMF (3 × 1 mL × 3 min) and $CH_2Cl_2$ (3 × 1 mL × 3 min), and dried under argon.

**MB-H1:** (4.6 mg, 10% based on the original resin loading). ESI-MS (m/z): $[MH]^+$ calcd for $C_{87}H_{140}N_{26}O_{23}S$ = 1950.03, found 975.7 $[MH_2]^{2+}$, 650.7 $[MH_3]^{3+}$.

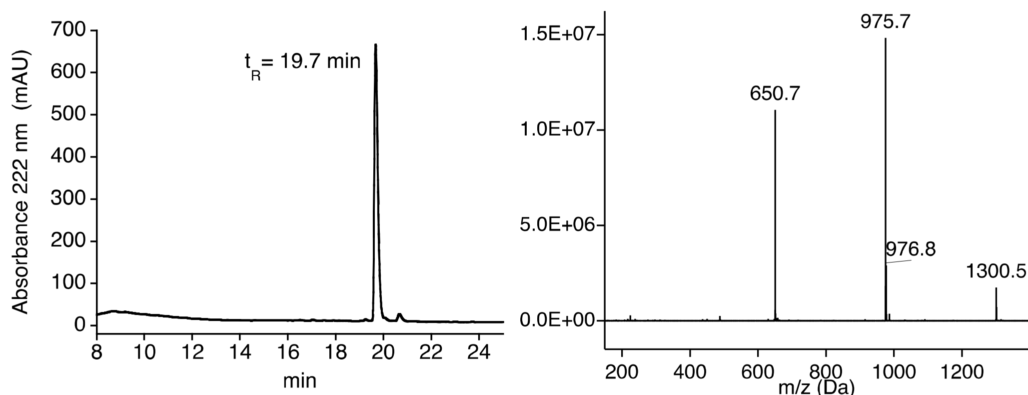



**Figure S4.** *Left*: HPLC trace of the pure product **MB-H1**. *Right*: ESI-MS spectra corresponding to the peak at c.a. 19.7 min.

## Synthesis and functionalization of $SiO_2$@Ag beads

**Synthesis of citrate-stabilized spherical silver nanoparticles** Spherical silver nanoparticles of approximately 65 nm in diameter were produced by a modification of the previously reported protocol.[1,2] Briefly, 250 mL of Milli-Q water were heated to reflux under strong magnetic stirring. Once it boils energetically, a mixture containing ascorbic acid (250 µL, 0.1 M) and trisodium citrate (3.41 mL, 0.1 M) was added. After 1 minute, a solution containing $AgNO_3$ (744 µL, 0.1 M) and $MgSO_4$ (559 µL, 0.1 M), previously incubated for 5 min at room temperature, was injected into the reaction vessel under vigorous stirring. Boiling and stirring were continued during 30 min to ensure the precursors reduction. During this time, the color of the solution quickly changed from colorless to yellow, and gradually into dark orange.

**Silica beads coating with polyelectrolytes.** The well know layer-by-layer (LbL)[4] technique was used to coat the $SiO_2$ beads with 3 alternating layers of PEI, PAA and PEI providing a positive net charge on the particle surface. To achieve this, 0.5 mL of a $SiO_2$ beads solution (50 mg/mL) were added drop-wise, meanwhile stirring, to 25 mL of a PEI aqueous solution (2 mg/mL) previously sonicated during 30 min. The mixture was vigorously stirred for 2 h and then centrifuged twice (3190 rcf, 20 min) and redispersed with Milli-Q water to remove the polyelectrolyte excess. The same procedure was applied for the other 2 layers of poly(acrylic acid) (PAA) and PEI, using 2 mg/mL PAA, 6 mM NaCl, and 2 mg/mL PEI solutions. Cyclic repetition of both adsorption steps (+ and - polymers) leads to the formation of multilayer structures with higher charge density, while a single deposition cycle only generates a submonolayer coverage.[4] Finally, the $SiO_2$ beads were redispersed in 10 mL of Milli-Q water to achieve a final concentration of 2.5 mg/mL.

**Silver nanoparticles assembly onto silica beads.** AgNPs were cleaned by centrifugation (2770 rcf, 20 min) and redispersed in Milli-Q water, to a final $[Ag^0]$ = $2.3\times10^{-4}$ M. This washing cycle allows to remove the excess of citrate that partially hinders the adhesion of AgNPs on silica beads, while preserving the overall colloidal stability. The polyelectrolyte wrapped silica beads were coated with AgNPs by adding drop by drop, under sonication, 800 µL of the silica beads solution into a flask containing a AgNPs solution (133 mL, $[Ag^0]$ = 0.23 mM). Then, the mixture was vortexed for 30 s and left sedimenting overnight. After that, the sample was centrifuged

3 times (95 rcf, 5 min) and redispersed in 2 mL of Milli-Q water to achieve a final $SiO_2$ beads concentration of 1 mg/mL.

**MB-H1 conjugation to $SiO_2$@Ag beads.** 5 mL of **MB-H1** solutions (200, 20, 2 nM, 200, 20, and 2 pM, respectively) in 10 mM Tris·HCl pH 7.5, 100 mM NaCl, were added over 5 mL aliquots of a 20 µg/mL $SiO_2$@Ag beads solution in 10 mM Tris·HCl, pH 7.5, 100 mM NaCl, and the resulting mixtures were incubated overnight at room temperature. The following morning, the final $SiO_2$@Ag bead concentration of the solutions (ca. $2.4\times10^4$ beads/mL) was calculated with a hemocytometer.

**Samples for SERS characterization**. Silver nanoparticles (20 µL $10^{-6}$ M in silver) were spin coated on glass slide at low particle concentration. Also, they were aggregated into a film by casting a drop of a concentrated solution (10 µL $10^{-3}$ M in silver) and air-dried.

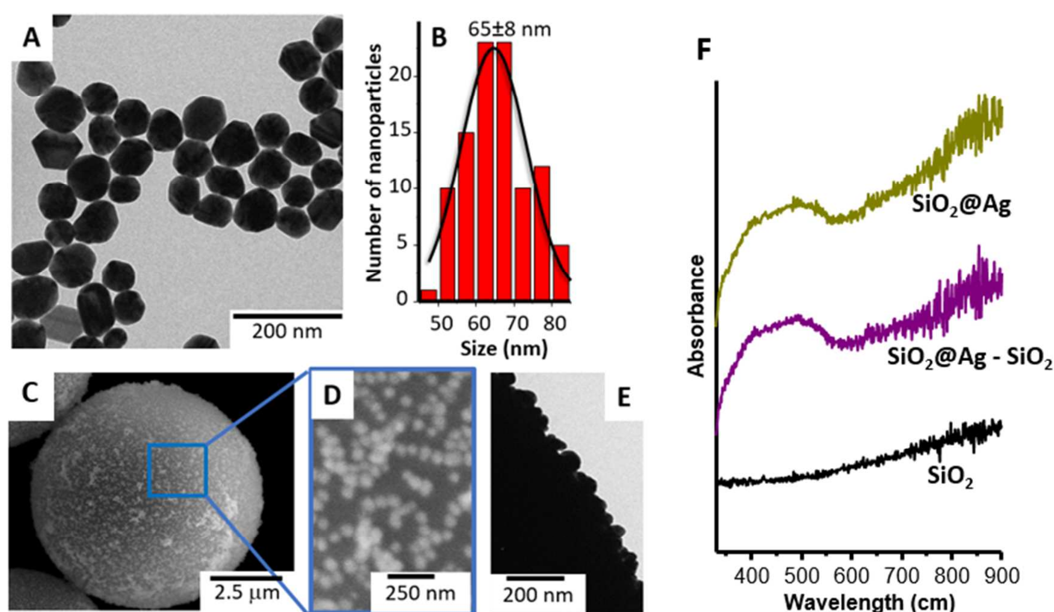



**Figure S5.** (A) TEM image and (B) size distribution of the AgNPs as prepared. (C and D) SEM and (E) TEM images of AgNPs coating silica beads of 8 µm. (F) UV-Vis spectra of $SiO_2$ and $SiO_2$@Ag (0.2 mg/mL) and the corresponding difference spectrum.

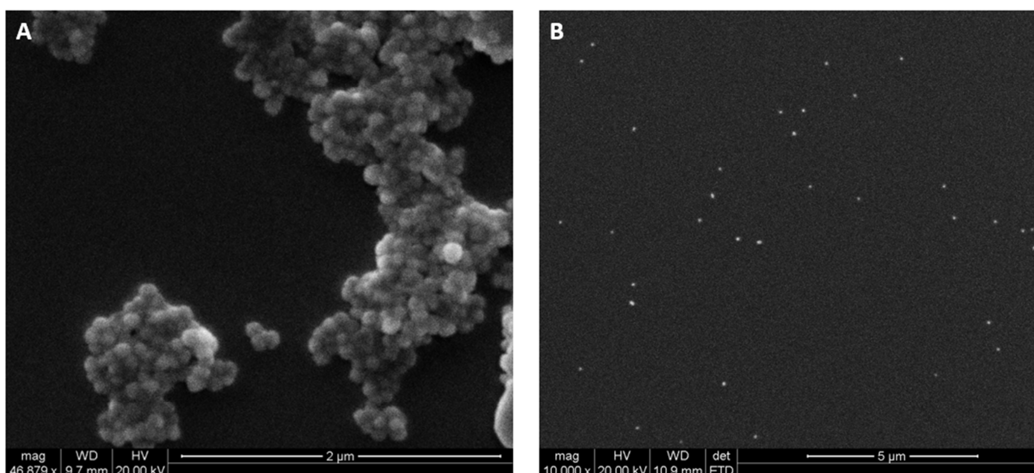



**Figure S6.** SEM images of (A) aggregated (cast and air-dried) and (B) non-aggregated (spin coated) films of AgNPs.

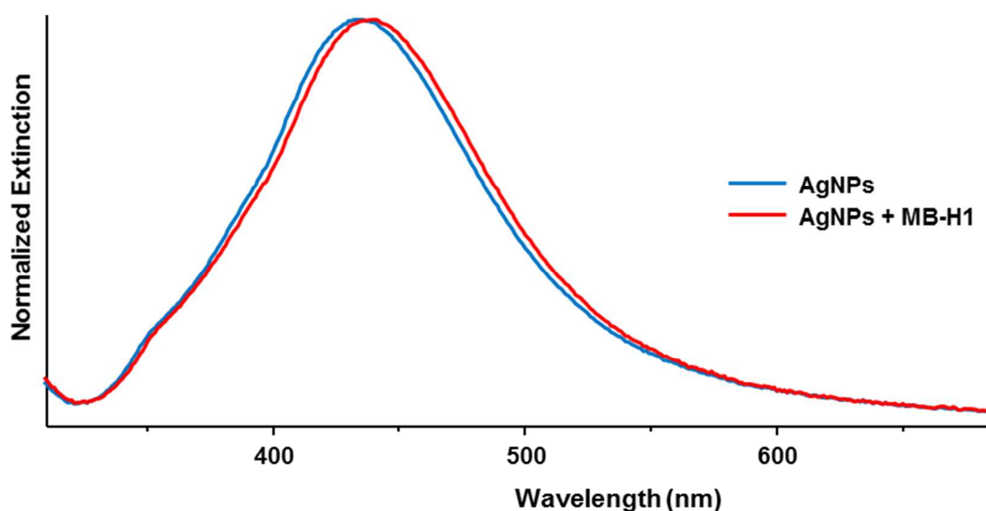



**Figure S7.** UV-Vis spectra of AgNPs before and after functionalization with MB-H1. AgNPs were centrifuged (2770 rcf, 20 min) and redispersed in Milli-Q water to a final concentration of [NP] ca. 85 pM. Then, two aliquots of 100 µL of colloids were placed in different Eppendorfs and mixed with: (a) 3 µL of Tris-buffer (10 mM Tris·HCl pH 7.5, 100 mM NaCl); (b) 3 µL of MB-H1 solution 1.57 mM in Tris-buffer. We refer to the first sample as "AgNPs" and to the second one as "AgNPs + MB-H1". The volume of buffered solutions added to the colloids was kept low enough to avoid salt-induced nanoparticle aggregation. A ca. 6 nm redshift of the LSPR is observed upon functionalization of the AgNPs with the MB-H1 chemoreceptor.

## Cell and human samples preparation

**MCF-7 cell line culture.** MCF-7 cancer cell line was used as a positive c-MYC expression control. MCF-7 cells were maintained in EMEM media supplemented with 10% fetal bovine serum and 0.1% human insulin and incubated at 37 °C, 5% $CO_2$ in a humidified atmosphere.

**Human samples.** Samples were provided by a patient with stage IV ovarian adenocarcinoma, in progression at the time of the sample collection. The presence of a tumor was confirmed histologically in compliance with common standards.

**Peripheral blood mononuclear cells extraction.** Blood samples were obtained from and healthy donors and processed within the following 24h. 8 mL of blood from each donor were diluted 1:2 with Hank's balanced salt solution (HBSS) and disposed carefully onto a 15 mL layer of Ficoll-Paque Plus (from Ge Healthcare LifeSciences). Samples were centrifuged for 40 min at 400 g to separate blood content. Peripheral blood mononuclear cells (PBMCs) were collected and washed with 1x PBS and finally, cultured in RPMI media supplemented with 10% fetal bovine serum at 37 °C, 5% $CO_2$ in a humidified atmosphere.

**Protein extraction.** PBMCs and MCF-7 cells were similarly counted and harvested from flask using trypsin 0.25% EDTA, and centrifuged at 170 g for 5 min. Cell pellets were suspended in 0.2 mL of lysis buffer, containing 50 mM HEPES, 0.5 M NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 10% v/v glycerol, 1% Triton X-100, and 0.01 mL of protease inhibitor cocktail (from Sigma-Aldrich). Lysis step was carried out for 1h at 4 °C, spinning tubes every 15 min to disrupt cell membranes. Lysates were centrifuged at 13,000 g for 10 min at 4 °C and protein containing supernatants were kept at −80 °C until use.

**Total protein and c-MYC levels quantification.** Protein concentration of lysates were quantified with Bradford protein assay; human c-MYC oncoprotein levels were determined with a total-human c-MYC enzyme-linked immunoabsorbed assay (ELISA) kit (from LifeSciences). Samples were diluted 1/10 – 1/100 and incubated for 3h with a primary anti-c-MYC antibody, followed by 30 min incubation with a secondary HRP labeled antibody. HRP substrate was added for 30 min, and absorbance was measured with a Multimode Plate Reader Tristar 2S. All standards and lysates were assayed in triplicate and protein concentrations were extrapolated from calibration curves.

**SERS characterization of the MB-H1 functionalized beads and quantification of c-MYC.** An aliquot of each suspension of $SiO_2$@Ag@MB-H1 was cast on a glass slide. Before drying, beads were first localized with a ×5 objective and, later, investigated by SERS using a ×50 objective to focus 2.5 mW of 785 nm laser line for 1s. For each bead, 10 measurements were collected. For each sample, 10 beads were measured. Notably, time was incremented to 10 s in the samples with 10 and 1 pM MB-H1 concentration. The same protocol was repeated also for a bead concentration of ca. 240 beads/mL. We also attempted to perform the identical study for 24 beads/mL but due to the difficulty of locating the beads (and their low amount for statistical purpose) we could not use these diluted samples for detection.

SERS quantification of c-MYC was performed as follows. First, a calibration curve was generated using the same standards than for ELISA. Briefly, 100 µL of a 2400 beads/mL suspension of $SiO_2$@Ag@MB-H1, diluted 1/10 in 10 mM Tris·HCl pH 7.5, 100 mM NaCl from a 24000 beads/mL-100 pM MB-H1 solution (corresponding to ca. $2.5\times10^6$ molecules of MB-H1 per bead), was added per mL of sample, reaching a final bead concentration of ~218 beads/mL. After waiting for 1h to ensure the interaction between H1 and c-MYC, aliquots of 100 µL of sample ere cast onto a glass slide. Then, before drying, beads were first localized with a ×5 objective and then mapped with the Raman microscope using a ×50 objective and 785 nm laser line. For statistical purpose, 10 spectra of 10 seconds were recorded for each bead (with a power laser at the sample of 2.5 mW). For each sample, between 10 to 15 beads were measured. Each experiment was replicated three times. The same protocol was followed for human samples.

For all calculations, bands were fitted by applying a Voigt algorithm with a linear baseline with Grams 9.

**Theoretical calculations.** B3LYP-functional density functional theory (DFT) calculations were performed using a Gaussian 09. B01 suite of programs with the 6–311G(p,d) basis set.[5]

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