Supplementary Information

Development of Nanoparticle Probes for Multiplex SERS Imaging of Cell Surface Proteins

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Materials:

d_7-benzyl chloride, d_2-glycine and d_5-phenylalanine (Cambridge Isotopes); silver nitrate, dithiobis(succinimidyl propionate) (DTSP), 4-(chloromethyl)benzonitrile), propargyl amine, 4-(chloromethyl)nitrobenzene) and 4-ethynyl benzyl alcohol (Sigma); and 4-cyano-phenylalanine (Chem Impex) were used as received. 2-(2-(2-methoxyethoxy)ethoxy)ethanethiol (EG_3SH) was synthesized as previously described.[15] Proton and carbon nuclear magnetic resonance (^1H and ^{13}C NMR) spectra were recorded using a Bruker AMX 400 spectrometer. Electrospray Ionization mass spectrometry was performed on a Waters system consisting of a Waters 996 Photodiode Array Detector, an Alliance HT – Waters 2795 Separations Module, and a Waters Micromass Z_Q 2000 unit equipped with a pneumatically-assisted electrospray ionization source.

Nanoparticle synthesis:

Silver NPs were synthesized with the citrate reduction protocol. A solution of $AgNO_3$ (50.0 ml, 1.0 mM) in 18.2 MΩ deionized water was heated until it begin to boil. A 1.0 ml of 51.0 mM sodium citrate solution was added to the boiling $AgNO_3$ solution. The colour of the solution slowly turned into grayish-yellow. The solution was maintained at boiling condition and under reflux for another 60 minutes. The Ag sol was cooled to room temperature before storage at 4°C.

Ligand Synthesis:

General procedure for mercaptomethylbenzene thiol based ligand synthesis.[1] In a small round bottom flask the benzyl chloride (1 mol eq) was dissolved in ethanol and heated to 50°C. Thiourea (1 mol eq) was added and the reaction as stirred vigorously overnight and left to cool to room temperature. The solid was filtered off and dissolved in water, if no precipitate was formed, the solvent was evaporated by rotary evaporation, and the resulting solid was dissolved in water. Na_2CO_3 was added until the reaction was basic (pH ~ 8). The resulting precipitate was then filtered and dissolved in 0.2M NaOH. The insoluble sulfide was filtered, and the filtrate was acidified with 1M HCl. The resulting precipitate was collected.

General procedure for DTSP-based ligand synthesis.[2] In a 4 ml vial, DTSP (1 mol eq) was added to a mixture of the amine (2 mol eq) and N_2CO_3 (2 mol eq) in acetonitrile/H_2O (1:1). The reaction was stirred at 50°C and monitored by LC-MS. Once complete (1 - 12 hours) the reaction was cooled to 0°C and acidified with HCl (1M aq). The resulting suspension was stirred at 0°C for 30 min and the precipitate collected and washed with water. If no precipitate was formed, the reaction was extracted twice with ethyl acetate and once with brine solution. The organic layers were combined, dried over magnesium sulfate and the solvent was removed by rotary evaporation to afford the product.

Nanoparticle Functionalization for Spectroscopy:

Synthesis of Ag NPs is described above. To 1 mL of Ag NP solution in a 4 mL glass vial, was added 100 μL of 50 mM sodium borate buffer solution (pH 9). After mixing, 40 μL of the Raman reporter ligand in the case of the DTSP-derived ligands (2mM stock solution) otherwise, 2 μL DTSP, 18 μL 2-(2-(2-methoxyethoxy)ethoxy)ethanethiol (EG_3SH) and 20 μL of reporter (benzylthiol derived ligands) were added. The reaction was stirred vigorously at room temperature. After 4 hours, the solutions were transferred to 1.5 mL plastic eppendorf tubes and spun at 8000 rpm for 10 min to form a pellet. The supernatant was removed and the pellet was resuspended in 1 mL MeOH. To obtain the thiol-coordinated MMByne nanoparticles, 100 uL of a $NaBH_4$ stock solution (10 mg/mL) was added to the NP solution prior to adding MMByne. The resulting thiol-bound NP was not used in the multiplexed experiment as it was not as stable as the C-bound NP in solution following resuspension in MeOH.

Raman and Surface Enhanced Raman Scattering Spectroscopy:

Raman spectra were acquired with a commercial microRaman system (LabRAM HR, Horiba Jobin Yvon) equipped with a software controlled XYZ stage and a thermal-electric cooled CCD detector. All SERS samples were excited with 632.8 nm radiation at a power density of ~ 10^3 W/cm^2 while the Raman scattering of all ligands (non-SERS measurements), were acquired with a power density of 10^5 W/cm^2. Incident radiation was coupled into an Olympus BX51 optical microscope and focused to ~ 1 μm diameter spot through a 100X objective. The same objective also collects the retro-reflected radiation and guides it to a notch filter which removes the Rayleigh radiation.

Nanoparticle Functionalization for Imaging:

To 1 mL of Ag particles (10^{13}/mL) was added 100 μL of 50 mM Na borate buffer (pH=9) in a glass vial (plastic tubes resulted in particle precipitation during the ligand exchange process). To this was added 4 μL of 2mM DTSP. After 5 minutes, 18 μL of 2mM EG_3SH and 20 μL of 2mM MMBN or DMMB were added. The resulting solution was left stirring for 4 h. The pH was then raised by adding 400 μL of 50 mM Na borate buffer (pH=9). To this solution, 80 μL of the secondary antibody solution (Affinipure goat anti-rabbit or donkey anti-goat IgG (H+L) 2.4 mg/mL (Jackson Immunoresearch)) was added and the resulting solution mixed by pipetting it slowly several times. This solution was left in the fridge at 4^{o}C overnight (16 hrs). After 16 h, the solution was warmed to rt and 30 μL of 30% bovine serum albumin (BSA) (Sigma) was added. This solution was mixed with a pipette and left to stand for 30 min at rt. After 30 min, the solution was transferred to a 1.5 mL Eppendorf micro-centrifuge tube and spun at 13.4 k rpm for 20 min to pellet the particles. The supernatant was then removed and the particles resuspended in 500 μL of PBS. Attempts to pellet and resuspend particles either without BSA blocking or prior to antibody treatment were not successful. The resulting solutions were determined to be approximately 20 nM by UV-Vis and were stored at 4^{o}C. Samples were stable for over one month under these conditions. NP solutions could be collected after use and used at least 5 times before a decrease in effectiveness of labelling was observed.

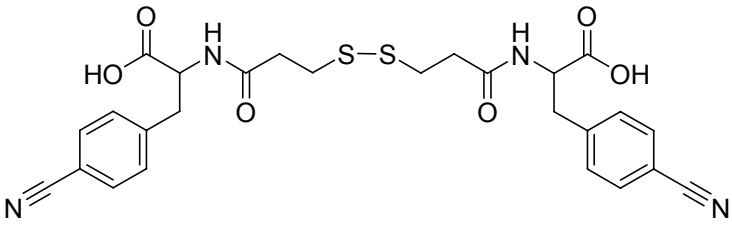
Cell culture:

H9c2 cells (ATCC, Manassas, VA) were grown in Dulbecco's modified Eagle's medium (Invitrogen, Burlington, ON) supplemented with 10% fetal bovine serum (FBS) (NorthBio, Toronto, ON) under standard culture conditions (37 °C, 5% CO_2).

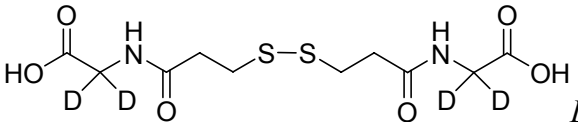
Sample preparations for Imaging:

H9c2 cells were plated onto photo-etched cover slips (Electron Microscopy Sciences, Hatfield, PA). After 24 h, the cells were fixed and then rinsed and stored in PBS at 4°C. The cells were then treated with a primary antibodies against the β_2-adrenergic and caveolin-3 receptors (β_2-AR (H-73) rabbit polyclonal IgG and cav3 (E-12) goat polyclonal IgG (Santa Cruz Biotechnology) 0.2 mg/mL, 4 µL used per mL PBS) for 16 hrs at 4°C, then rinsed twice with PBS and 1 mL of Ag NP solution was added. The cells were incubated with the NP solution for 24 h and then rinsed twice with PBS to remove unbound particles before imaging. The NP solution was recovered after the 24 h treatment and could be re-used for subsequent labelling studies (at least 5 times).

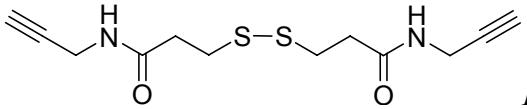
Experimental data for DTSP-derived compounds:



DTSP-PheCN. 32 mg of white powder (26% yeild). ^{1}H NMR (400 MHz, *CDCl$_3$)* δ ppm 12.81 (s, 2 H), 8.34 (d, *J*=8.20 Hz, 2 H), 7.73 (d, *J*=8.17 Hz, 4 H), 7.42 (d, *J*=8.18 Hz, 4 H), 4.48 (dt, *J*=4.96, 9.09, 8.95 Hz, 2 H), 3.15 (dd, *J*=4.91, 13.71 Hz, 2 H), 2.93 (dd, *J*=9.62, 13.72 Hz, 2 H), 2.76 (t, *J*=7.01, 7.01 Hz, 4 H), 2.43 (dt, *J*=3.64, 7.18, 7.45 Hz, 4 H). ^{13}C NMR (101 MHz, *d$_6$DMSO*) δ ppm 172.46 (2 C), 169.95 (2 C) 143.59 (2 C), 131.92 (4 CH), 130.17 (4 CH), 118.82 (2 CN), 109.21 (2 C), 52.75 (2 C), 36.69 (2 CH_2), 34.58 (2 CH_2), 33.45 (2 CH_2). MS (pos. ESI, M+1) *m/z* 555, (neg ESI, M-1) *m/z* 553.

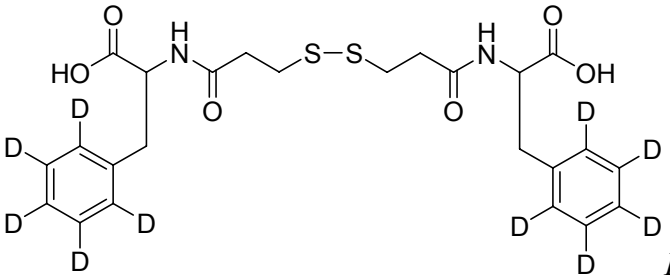


DTSP-d$_2$Gly. 28 mg of white powder (34% yield). ^{1}H NMR (400 MHz, *d$_6$DMSO*) δ ppm 12.53 (s, 2 H), 8.27 (s, 2 H), 2.90 (t, *J*=7.23, 7.23 Hz, 4 H), 2.55 (d, *J*=7.23 Hz, 4 H). ^{13}C NMR (101 MHz, *d$_6$DMSO*) δ ppm 171.17 (2 C), 170.36 (2 C), 39.64 (m, 2 CD_2), 34.62 (2 CH_2), 33.57 (2 CH_2). MS (pos. ESI, M=1) *m/z* 329, (neg. ESI, M-1) *m/z* 327.



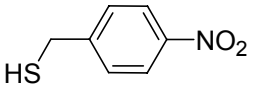
DTSP-alkyne. 3 mg white powder (8% yield). ^{1}H NMR (400MHz, *d$_6$DMSO*) δ ppm 8.39 (t, J=5.12, 5.12 Hz, 2 H), 3.86 (dd, J=2.51, 5.44 Hz, 4

H), 3.11 (t, J=2.50, 2.50 Hz, 2 H), 2.88 (t, J=7.15, 7.15 Hz, 4 H), 2.48 (m, 4 H). ^{13}C NMR (100 MHz, *d_6DMSO*) δ ppm 172.56 (2 C), 80.97 (2 C), 72.93 (2 CH), 34.57 (2 CH_2), 33.50 (2 CH_2), 32.89 (2 CH_2). MS (neg. ESI, M-1) *m/z* 283.

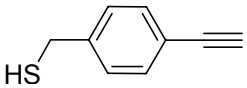


DTSP-d_5Phe. 100 mg of yellow powder (77% yield). ^{1}H NMR (400 MHz, *d_6DMSO*) δ ppm 12.70 (s, 2 H), 8.29 (d, *J*=8.06 Hz, 2 H), 4.42 (dt, *J*=5.15, 8.72, 8.60 Hz, 2 H), 3.04 (dd, *J*=4.98, 13.78 Hz, 2 H), 2.85 (dd, *J*=9.48, 13.71 Hz, 2 H), 2.76 (t, *J*=7.06, 7.06 Hz, 4 H). ^{13}C NMR (101 MHz, *d_6DMSO*) δ ppm 172.86 (2 C), 169.94 (2 C), 137.27 (2 C), 128.57 (t, J_{CD}=24.45, 22.68 Hz,4 CD), 127.56 (t, J_{CD}=27.41, 21.28 Hz,4 CD), 125.83 (m, 2 CD), 53.36 (2 C), 36.62 (2 CH_2), 34.66 (2 CH_2), 33.55 (2 CH_2). MS (pos. ESI, M+1) *m/z* 515, (neg ESI, M-1) *m/z* 513.

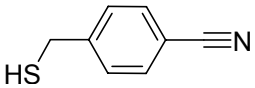
Experimental data for mercaptomethylbenzenethiol-derived compounds:



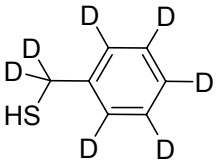
MMBNO.[3] 95mg of white powder (19% yield). ^{1}H NMR (400 MHz, *$CDCl_3$*) δ ppm 8.19 (d, J=8.72 Hz, 2 H), 7.50 (d, J=8.70 Hz, 2 H), 3.82 (d, J=6.62 Hz, 2 H), 1.83 (t, J=7.66, 7.66 Hz, 1 H). MS (neg ESI, M-1) *m/z* 168.



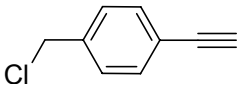
MMByne. 65mg of yellow oil (33% yield). ^{1}H NMR (400 MHz, *$CDCl_3$*) δ ppm 7.47 (d, *J*=8.11 Hz, 2 H), 7.20 (d, *J*=8.13 Hz, 2 H), 3.61 (s, 2 H), 3.11 (s, 1 H). MS (EI, M) *m/z* 148.



MMBN.[4] 44mg of white powder (90% yield). ^{1}H *NMR (400 MHz, $CDCl_3$)* δ ppm 7.61 (d, J=8.20 Hz, 2 H), 7.44 (d, J=8.17 Hz, 2 H), 3.77 (d, J=7.87 Hz, 2 H), 1.80 (t, J=7.87, 7.87 Hz, 1 H). MS (neg ESI, M-1) *m/z* 148.



DMMB.[5] 13mg of yellow oil (32% yield). ^{2}H NMR (400 MHz, CH_2Cl_2) δ ppm 7.35 (m, 5H), 3.71 (s, 2H). MS (EI, M) *m/z* 131.



Ethynyl benzyl chloride. Ethynylbenzyl alcohol (198 mg, 1.5 mmol) and pyridine (324 μL, 4 mmol) were combined in dichloromethane and slowly added to $SOCl_2$ (182 μL, 2.5 mmol) at 0°C. The reaction mixture was brought to room temperature and left to stir overnight. Once the reaction was complete, the solution was diluted with dichloromethane and washed twice with HCl (0.2M aq), twice with $NaHCO_3$ (saturated solution aq) and once with brine solution. The organic phase was dried over magnesium sulfate and concentrated by rotary evaporation. The resulting oild was purifed by flash chromatography (9:1 hexanes : ethyl acetate) to afford the 35 mg (16% yeild) of yellow oil. ^{1}H NMR (400 MHz, *CDCl*$_3$) δ ppm 7.49 (d, *J*=8.27 Hz, 2 H), 7.35 (d, *J*=8.33 Hz, 2 H), 4.57 (s, 2 H), 3.10 (s, 1 H). MS (EI, M) *m/z* 150.

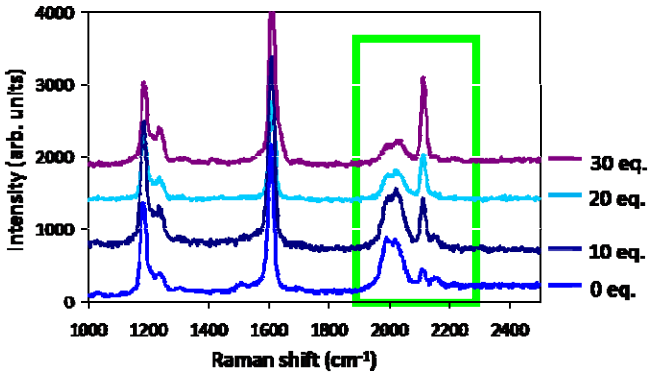


Figure S1. Addition of $NaBH_4$ to the reaction mixture changes the ratio of the two alkyne peaks, favouring the free alkyne to the bound alkyne at higher borohydride concentrations. At thirty equivalents of borohydride with respect to the ligand, the MMByne molecule is almost exclusively thiol-bound as opposed to alkyne bound in the absence of sodium borohydride.

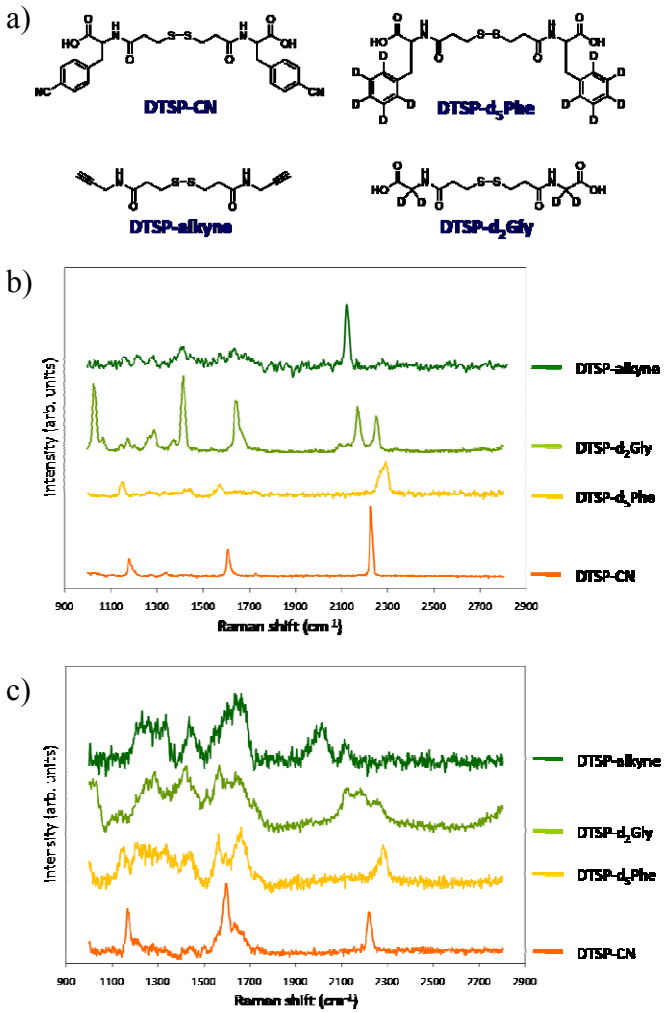


Figure S2. a) Structures of 4 DTSP-derived reporter molecules. b) Raman spectra of the 4 DTSP-derived reporter molecules. c) SERS spectra of functionalized Ag NPs derived using the 4 DTSP-reporter molecules.

References:

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