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Supporting Information

Complex cellular environments imaged by SERS nanoprobes using sugars as all-in-one vector

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I. Instrumentation

Transmission Electron Microscopy images were collected using a Hitachi H-7000 TEM at 75 kV with 50 000X magnification. Inductively coupled plasma mass spectroscopy (ICP-MS) was performed using a PerkinElmer's NexION 350. Darkfield images were collected using a Nikon TE2000 inverted microscope containing a CCD camera (DS- Fi1, Nikon) using an objective iris and a darkfield stop inside the condenser. SERS spectra were obtained using a motorized Raman spectrometer (Renishaw) coupled to a Leica DMI6000 inverted microscope containing a deep-depletion silicon CCD array with 600/1200/1800 l/mm grating and a solid-state excitation source (638 nm, diode laser, 15 mW).

II. Preparation of coated gold nanoparticles

Synthesis of thiol-derivatized monosaccharides. Chemical functionalization of acetobromo-α-D-glucose (GlcAc) and acetobromo-α-D-galactose (GalAc) was performed to insert thiol groups to the molecules, allowing it coordination to the surface of the AuNPs.[55-57] For that, both bromo-carbohydrates were treated with potassium thioacetate, giving the corresponding thioacetate derivatives AcSGlcAc and AcSGalAc, in quantitative yields. Briefly, a mixture of each carbohydrate (acetobromo-α-D-glucose and acetobromo-α-D-galactose, Sigma-Aldrich) and potassium thioacetate (Sigma-Aldrich) in acetone was kept stirring during 2h at RT. Thereafter, the reaction solvent was evaporated, diluted with ethyl acetate and washed with water. Purification by silica column chromatography using toluene/ethyl acetate (4:1) gave the compounds AcSGlcAc and AcSGalAc in quantitative yields. Secondly, these intermediates were dissolved in a mixture of tetrahydrofuran/methanol (1:1) allowed to react overnight at 40 ºC with sodium metoxide (excess, 5 eq.) and neutralized with Amberlyst® 15 ion-exchange resin, giving rise to the final thiol-carbohydrates SGal and SGlc in quantitative yields.

AcSGlcAc: ^{1}H NMR (300.13 MHz, Chloroform-d_6): δ 2.00 (s, 3H), 2.01 (s, 3H), 2.02 (s, 3H), 2.01 (s, 3H) 2.38 (s, 3H, -SAc), 3.83 (ddd, J = 10.1, 4.4, 2.2 Hz, 1H), 4.09 (dd, J = 12.5, 2.2 Hz, 1H), 4.26 (dd, J = 12.5, 4.4 Hz, 2H), 5.05-5.17 (m, 2H), 5.21-5.33 (m, 2H); ^{13}C NMR (75.47 MHz, Chloroform-d_6): δ 20.59, 20.61, 20.74, 31.86, 62.66, 68.84, 69.94, 74.94, 81.18, 170.36, 170.41, 171.06, 171.67, 193.06.

AcSGalAc: ^{1}H NMR (300.13 MHz, Chloroform-d_6): δ 1.97 (s, 3H), 2.01 (s, 3H), 2.02 (s, 3H), 2.14 (s, 3H), 2.33 (s, 3H, -SAc), 4.01-4.16 (m, 1H), 5.09 (dd, J = 9.3, 3.4 Hz 1H), 5.20-5.34 (m, 1H), 5.44 (dd, J = 3.4, 0.9 Hz, 1H); ^{13}C NMR (75.47 MHz, Chloroform-d_6): δ 20.66 (d, J = 1.5), 30.85, 61.23, 66.33, 67.19, 71.90, 74.99, 80.55, 169.53, 169.89, 170.16, 170.36, 192.11.

HSGlc: ^{1}H NMR (300.13 MHz, D_2O): δ 3.59-3.78 (m, 16H), 3.96-3.96 (m, 4H), 4.48 (d, J=9.8, 1H). ^{13}C NMR (75.47 MHz, D_2O): δ 60.49, 60.75, 69.11, 69.44, 73.27, 73.47, 76.93, 77.15, 89.32, 89.48, 171.11.

HSGal: ^{1}H NMR (300.13 MHz, D_2O): δ 3.39-3.51 (m, 10H), 3.67-3.93 (m, 9H), 4.55 (d, J=9.4, 1H). ^{13}C NMR (75.47 MHz, D_2O): δ 60.35, 60.57, 69.01, 69.25, 73.19, 73.38, 76.74, 77.10, 89.29, 89.39, 171.10.

Sugar conjugation with Gold (Au) NP. To AuNP with 60 nm in diameter (1 mL, Citrate caped, Sigma-Aldrich), a solution of thiol-derivatized monosaccharides (galactose – Gal and glucose – Glc,**)** was added dropwise, at a molar ratio of 4.3×10^{17} monosaccharide molecules per nanoparticle (7 mM in 100 μL of ultra-pure water). Self-assembly was facilitated by allowing the solution to stir for 72h at room temperature giving rise to NP @SGal (AuNP functionalized with galactose) and @SGlc (AuNP functionalized with glucose).[58] The unreacted carbohydrate was then removed via centrifugation (15 minutes at 16 000 rpm) washed 3 times with water and stored at 4 ºC in PBS in a final concentration of 1mg mL^{-1} NP.

Characterization of functionalized AuNP. Plasmon resonance profiles of functionalized AuNP were assessed by ultraviolet−visible spectroscopy (UV-1650 PC; Shimadzu

Corporation, Kyoto, Japan) and compared to the non-functionalized ones. In turn, the ability to produce SERS were evaluated using a motorized Raman spectrometer (Renishaw).

III. **Stability of functionalized AuNP in physiologic media.**

The stability of the Raman signal produced by the SERS-active AuNP were assessed in several concentrations of fetal bovine serum (FBS, 10, 25 and 50%) in phosphate buffer solution (PBS) and in cell culture media (DMEM) with or without 10% FBS. Both samples (@SGlc and @SGlc) were diluted in each set of conditions to a final concentration of 10 μg mL^{-1} and incubated during 5 and 24h at 37 ºC. Control experiments were performed in parallel for each condition. The Raman signal from a single drop (10 μL) on top of a glass coverslip was collected from each condition, using a 20x lens with an integration time of 10 s and 10 accumulations.

IV. **Biocompatibility.**

9L^{luc} rat glioma cell line was maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 350 μg mL^{-1} G418 (antibody). The cell culture medium was renewed every two days and cells passaged at 80% confluence. To assess cellular viability, cells were seeded in clear bottom 96-well plates, at a density of 15,000 cells per well, and allowed to attach overnight. SERS-active AuNP (@SGal and @SGlc) were added to the culture medium at the appropriate concentration (10 μg mL^{-1}) and incubated for 24 h at 37 ºC. Viability was then measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Invitrogen) assay according to the manufacturer’s instructions. Three independent experiments were conducted, and each condition was represented in quadruplets. Cells unexposed to AuNP were used as control. Results are presented as the mean relative percentage of cell viability compared to non-treated cells ± standard error of the mean.

V. **Expression levels of GLUT1 and galectin-1.**

GULT1 and galectin-1 gene expression was evaluated by Western blotting. For this, others cancer cells (KB, U87 and PC3) known to express good levels of GLUT1 and galectin-1 were used as comparison. After growth, cells were washed twice with PBS and harvested in RIPA buffer. After centrifugation at 13,000 rpm for 20 min at 4 ºC, supernatants were used for protein quantification using the BCA Protein Assay. For the Western blotting analysis, 20 μg proteins were loaded per well on sodium dodecyl sulphate-polyacrilamide gels (SDS-PAGE). Following electrophoresis and transfer to PVDF membranes (Bio-Rad, Hercules, CA, USA), the blots were incubated in blocking buffer for 1h and then probed with the first rat anti-galectin-1 1:2,000 (Abcam, Cambridge, UK) or rat anti-GLUT1 1:500 (Chemicon, Boston, MA, USA) antibodies overnight at 4 ºC. After washing, the membranes were probed with the secondary anti-rabbit (1:2,000) or HRP-conjugated anti-biotin (1:30,000, Bio-Rad) antibodies. Immunoreactive bands were detected by enhanced chemiluminiscence (ECL) substrate using an imaging system (VersaDoc 4000 MP, Bio-Rad) followed by densitometric analysis.

VI. **Cellular internalization**

Visualization of internalized SERS nanoprobes**.** Dark-field (DF) scattering using a microscope equipped with an oil-immersion condenser and an objective (100× lens) were used to visualize the internalization effectiveness of the SERS-active AuNP. Microscope and camera light intensities, as well as integration settings, were kept constant among all images used for Rayleigh intensity measurements. Briefly, $9L^{luc}$ rat cells were seeded in round coverslips (13 mm, previously coated with collagen I, 1:30 for 2h at room temperature) in 24-well plates, at a density of 30,000 cells per well and allowed to spread at 37 ºC overnight. Cells were then washed twice with PBS and allowed to incubate with each SERS-active AuNP (10 μg mL^{-1}) for another 12 h at 37 ºC. After that, cells were washed with PBS and coverslips mounted in slides using Vectashield™ as mounting media.

Quantification of internalized SERS nanoprobes. Inductively coupled plasma mass spectrometry (ICP-MS) was used to assess the amount of SERS-active AuNP (@SGal and @SGlc) internalized by the cells. $9L^{luc}$ cells were seeded in 12-well plates at a density of 50,000 cells per well and allowed to spread at 37 ºC overnight. Incubation with both AuNPs was carried out as described above. Cells were twice washed with PBS, detached with Trypsin (200 μL) and centrifuged (5 min, 1,000 rpm). The supernatant was discarded, an acidic solution of HNO_3/HCl (4:1, 200 μL) was added, and AuNPs were allowed to digest overnight at room temperature. Before each analysis, samples were diluted in ultra-pure water to a final volume of 5 mL. The bare 60 nm AuNP were incubated in parallel to compare the effect of surface functionalization in the internalization efficiency by the cells. Three independent experiments were conducted, and each condition was represented in triplicates. Standard samples were prepared before each analysis in order to assess the amount of Au in lysates.[59] The number of AuNP contained in each cell was calculated as a total number of AuNP/total number of cells.

Localization of internalized SERS nanoprobes. Intracellular localization was assessed by transmission electron microscopy (TEM). $9L^{luc}$ cells were seeded in 6-well plates at a density of 1 000,000 cells per well and allowed to spread at 37 ºC overnight. Incubation with both AuNP samples was carried out as described above. After incubation, cells were twice washed with PBS, detached with Trypsin (200 μL) and centrifuged (5 min at 1,000 rpm). The supernatant was discarded, and 1 mL of 2% glutaraldehyde was added as a fixative. Pellets were carefully rinsed with PBS and post-fixed using 1% aqueous solution of osmium tetroxide (OsO_4, 0.5 mL) for 1 h. Subsequently, the cells were washed with DI water, 30% ethanol solution and stained with 0.5% uranyl acetate (0.5 mL, in 30% ethanol) for 1 h. Cells were then gradually dehydrated using a series of ethanol solutions (30, 60, 70, 80, and 100%) and embedded in epoxy resin. After resin polymerization at 60 °C for 48 h, ultra-thin sections (70–100 nm) were cut using a diamond knife on a Leica Ultramicrotome and mounted on Formvar coated copper grids. Sections were then post-stained with 5% uranyl acetate in 50% ethanol and 2% aqueous citrate solution and imaged with TEM at 200 kV.

VII. In vivo experiments

Subcutaneous tumor growth. All animal procedures were carried out with institutional approval (University Health Network, Toronto, Canada) on 8-week-old female nude mice. $9L^{luc}$ cells (5,000 000 cells per tumor) were injected subcutaneously in both mice legs and allowed to grow (~3 weeks). General anesthesia with ketamine was used for the injection sessions.

Intratumoral injections. After grown, xerograft tumors were injected with the SERS nanoprobes (@Gal or @Glc). For that 75 pM of each nanoprobe was incubated (with the mice alive) and allowed to distribute for 4 h, after whish mice were scarified and the tumors explanted. General anesthesia with ketamine was used for the injection sessions.

VIII. **Confocal Raman imaging.**

Cells labelling. $9L^{luc}$ cells were seeded onto 8-chambered coverslips at a density of 20,000 cells per well and allowed to spread overnight at 37 ºC. After incubation with either of nanoprobes (@SGal and @SGlc), cells are washed twice with PBS, fixed with 5% formalin during 20 min (150 μL per well), and VectaShield™ used as mounting media. Cellular mapping was achieved with a laser set to 25% of its maximum intensity (3.8 mW) and 0.1 s of integration time. Reconstructed Raman mapping were originated from direct classical least squares (DCLS) analysis. DCLS finds the linear combination of spectra from the pure components within the sample that most closely matches the mixed spectrum acquired from each pixel of the sample. Control analysis (just cells) ensured that the cell autofluorescence did not contribute to the signal obtained from the nanoprobes. A 63x magnification lens was used in all cases.

Tumoral tissue analysis: Explanted tumors were sliced in half and embedded in a plastic mould with optimal cutting temperature (OCT) medium and frozen at -80 ºC for slides preparation. The frozen blocks were cut in 20 μm slices with 5 μm slices intercalation. The thicker slices (20 μm) were mounted in quartz slides without Hematoxylin/Eosin (H&E) staining. The SERS signal was assessed without any mounting medium or coverslip to improve the received signal. All mapping images were acquired with 50% of the laser power (7.5 mW), and each spectrum was collected with 1 s integration time. Tumour slides without Au NPs injection were used as background. A 19x magnification lens was used in all cases.

***Solid tumour analysis*:** The *ex-vivo* mapping required the tumours to be sliced in half after the 4 h incubation period with each nanoprobe and fixed in a formalin solution. Prior analysis, the smoothest part of the tumour slice was placed onto a quartz slide, and an area was mapped. Images were acquired with 25% of the laser power (3.8 mW), and each spectrum was collected with 3 s integration time. A 19x magnification lens was used in all cases.

Depth profiling: Tumours were placed onto a quartz slide, and SERS spectra were collected using Renishaw's depth mapping [between two XY coordinates (i.e. in a line)] along 7 mm depth. The analysis was performed with 25% the intensity of the laser power (3.8 mW) and each spectrum collected with 1 s integration time. The reconstruction of the depth map was done from the intensity to baseline between 1500 and 1700 cm^{-1}.