Supporting information

Surface charge modulates the internalization vs penetration of gold nanoparticles: A comprehensive scrutiny on monolayer cancer cells, multicellular spheroids and solid tumor by SERS modality

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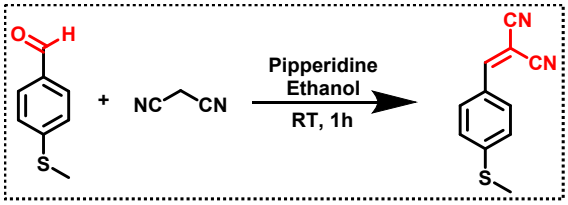
Materials and methods:

All chemicals and solvents were brought from Sigma Aldrich, Alfa Aesar, TCI and Merck and were used without further purification. ^{1}H-NMR spectra were recorded on Bruker Advance 500 NMR spectrometer, and chemical shifts are expressed in parts per million (ppm). Mass spectra were recorded under ESI/HRMS at 61800 resolution using Thermo scientific exactive, mass spectrometer. Absorption spectra were measured on a shimadzu (UV-2450) UV-Vis spectrophotometer, and the data analysis was performed using Microsoft excel and Origin 7. SERS measurements were carried out in a WITec Raman microscope (WITec Inc. Germany, alpha 300R) with a laser beam directed to the sample through 20x objective and a Peltier cooled CCD detector. Samples were excited with 633 nm excitation wavelength laser and Stokes shifted Raman spectra were collected in the range of 400 to 4000 cm^{-1} with 1 cm^{-1} resolution. Prior to every measurement, a calibration with a silicon standard (Raman peak cantered at 520 cm^{-1}) was performed. WITec Project plus (v 2.1) software package was used for data evaluation. TEM measurements were performed (JEOL 2010) with an accelerating voltage of 200 KV. The sample was prepared by pipetting a drop of the aqueous solution of nanoparticles onto a 230 mesh copper grid coated with carbon and the sample was allowed to dry in air before the measurement.

Synthesis of CNBN



500 mg (3.28 mM) thiomethyl benzaldehyde and 217 mg (3.28 mM) malanonitrile were allowed to react in presence of piperidine at room temperature for 1 hour in ethanol.(1)The reaction was instantaneous and the yellow precipitate formed was washed repeatedly using ethanol (yield 77%). ^{1}H NMR (500 MHz; $CDCl_3$): δ 7.75(d, 2H, J=10Hz), 7.59(s, 1H),7.24 (d, 2H, J=10Hz), 2.48 (s, 3H). ^{13}C NMR (100 Hz; $CDCl_3$): δ158.8, 149.4, 131.0, 127.1, 125.5, 114.2, 113.1, 14.6. ESI-MS (+ve mode) m/z Calcd for $C_{11}H_8N_2S$ 200.25; Found:200.04

Synthesis of gold nanoparticles

Gold nanoparticles were synthesised by the well-established citrate- reduction method. (2) In short, 300 mL of deionised water was heated to 100^{o}C with continuous stirring to which 300 µL of gold chloride solution (250 mM) in deionised water was added and allowed to stir for 5 min after that 750 µL tri sodium citrate solutions (100 mM) in water was added and waited until the colour changes to purple. After this heating was stopped and allowed to cool to room temperature with continuous stirring. After 3 hours the solution was kept in refrigerator for further use.

Surface modification of gold nanoparticles for three different surface charges

Negative: 10 mL as synthesised gold nanoparticles taken in 15 mL centrifuging tubes and 100 μL CNBN solution (1 mM in acetonitrile) was added followed by continuous stirring at 60 rpm for 1 hour using a rotator. Solution was centrifuged for 10 min at 8000 rpm, the pellet collected was dispersed in to 2 mL using deionised water. 50 μL SH-PEG (100 mM) solutions was added and allowed to stir for 3 hours. The nanoparticles so obtained were centrifuged again 8000 rpm for 10 min and the unattached PEG was removed. The pellet was re-dispersed in to 2 mL using deionised water and the solution was kept in refrigerator for further use.

Neutral: 1 mL of negative nanoparticles as synthesised was taken in 2 mL centrifuging tubes and mixed with 5 μL poly allyl amine hydrochloride solutions (3 mg/mL) in deionised water. The solution was stirred at 60 rpm for 1 hour. After this the solution was centrifuged at 8000 rpm for 10 min and collected the pellet which was further dispersed to 1 mL and the solution was kept in refrigerator for further use.

Positive: 1 mL of negative nanoparticles as synthesised was taken in 2 mL centrifuging tubes and mixed with 50 μL poly allyl amine hydrochloride solution (3 mg/mL) in deionised water. The solution was stirred at 60 rpm for 1 hour. After this the solution was centrifuged at 8000 rpm for 10 min and collected the pellet which was further dispersed to 1 mL and the solution was kept in refrigerator for further use.

Cell culture

Human cervical cancer cells HeLa was cultured in DMEM Medium supplemented with 10% fetal bovine serum (FBS, Invitrogen), and 1% antibiotics (containing penicillin and streptomycin, Invitrogen). The medium was changed every other day, and the cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

SERS mapping on 2D cell culture model

Cells were cultured in 4 well chamber slide made of glass at a seeding density of 10^4 cells /mL. Cells were incubated with same concentration of all the three nanoparticles in presence and absence of serum proteins and incubated for 4 hours. Further the cells were washed in PBS three times and the cells were subjected to SERS spectral mapping mode of the confocal Raman microscope (alpha300R, WITec Inc. Germany). Spectrum at every pixel was taken (Scan range 60 µm X 60µm, 150 X 150 pixel) was taken by 20X objective. Sample was excited with a power of 20 mW from 633 nm laser and mapping was done using 600 g/mm grating is an integration time of 0.022 s using 1580 cm^{-1} peak and the intensity modulated images were prepared by image processing option in control four software.

SERS mapping on 2D cell for inhibitor based studies

The cells grown in chamber slides were pre incubated with different inhibitors chlorpromazine (20 mg/mL), genistien (300 mg/mL), sodium azide (150 mg/mL), amiloride (30 mg/mL) for 1 hour and washed with PBS for three times and then incubated with gold nanoparticle solution of same concentration. After 4-hour incubation the mapping was done and the images were processed by control 4 software and image J for SERS quantification.

ICP-MS measurements for nanoparticle internalization in cells.

For ICP-MS analysis, HeLa cells were first seeded in 6 well plates (1×10^5 cells/well), which were subjected to different treatments as described above. Prior to analysis, the cells were washed with PBS thrice to wash off the unbound nanoparticles. The cells were then trypsinized and lysed with PBS. The cell lysate was then subjected to digestion in a microwave reactor (Anton Paar, Multiwave 3000) by mixing the sample with aquaregia in 0.5:4.5 v/v ratio. The samples were held at zero ramp time at a power of 400 W for 25 min, which was then retained at 5 ramp and 500 W power for 30 min. After cooling, the sample vials were opened and the samples were diluted to 25 mL with type 1 water (Thermo Scientific, Barnstead, Smart2Pure). The vials were closed and shaken vigorously to complete the dissolution, which were finally subjected to ICP-MS analysis using Thermo Scientific iCAP RQ spectrometer.

SERS evaluation on tumor spheroids

Tumor spheroids were subjected to different treatments with three nanoparticles and carefully transferred in to a glass slide surface. SERS mapping was conducted using 633 nm laser with power of 10 mW. Details of different scanning modes opted are given as follows:

Line scanning

Line scan was done from the top of the spheroid to the core through the Z axis of the microscope. Since the average size range of tumour spheroids was observed to be 200 μm, line scan was opted for a depth of 100 μm in which number of points on the line was opted to be 50 with an integration time of 2 s and accumulation of 5. The varying distribution of peak intensity was assessed using control 4 software.

Stack scanning

Selected spheroids were focused on the centre and 4 layers of mapping was done using 1580 cm^{-1} peak. Points per line and lines per image was opted to be 50 X 50 with 633 nm laser with power 20 mW with a grating of 600 g/mm and 1580 cm^{-1} mapping was done using integration time of 0.1s

***In vivo* analysis**

Animal experiments were performed according to the CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) guidelines and were approved by the Institutional Animal Ethics Committee (IAEC) of the Regional Cancer Centre (Accreditation number: 657/Go/Re/02/CPCSEA), Trivandrum, India.The murine transplantable lymphoma cell lines, EAC and DLA, were maintained in the peritoneal cavity of mice by ip transplantation of 1x106 cells per mouse. Balb/c mice were injected (sc) with EAC cells to develop solid tumors. Immuno-deficient 8-12 weeks old NCr Nude female mice (CrTac: NCr-Foxn1nu) weighing 18 to 22 g were maintained on a 16:8 h light-dark cycle in microinsulator cages in a pathogen-free insulation facility. Nude mice were injected with HeLa cells to develop xenograft of human solid tumors.

Hydrodynamic size of three nanoparticles in different conditions

Medium	Negative (nm)	Positive (nm)	Neutral (nm)
MilliQ	82±1.5	75±2.5	80±1.8
PBS (pH 7.5)	78±1.3	89±.9	89±1.25
PBS (pH 5.5)	79±2.5	80±2.6	76±.8
PBS (pH 4.5)	78±1.9	78±1.9	86±1.2
Plain Medium	93±2.4	100±1.7	108±2.3
Full medium	100±2.6	104±2.6	105±2

Table S1: DLS analysis of three different nanoparticles in different conditions.

Concentration dependent SERS study using CNBN reporter

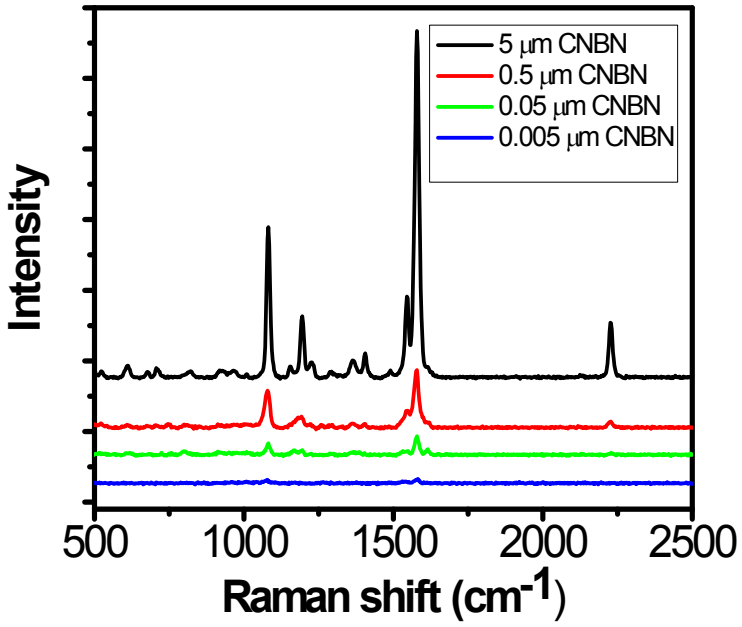


Fig S1. Concentration-dependent SERS spectrum using CNBN Raman reporter

Representative TEM images of the three nanoparticles

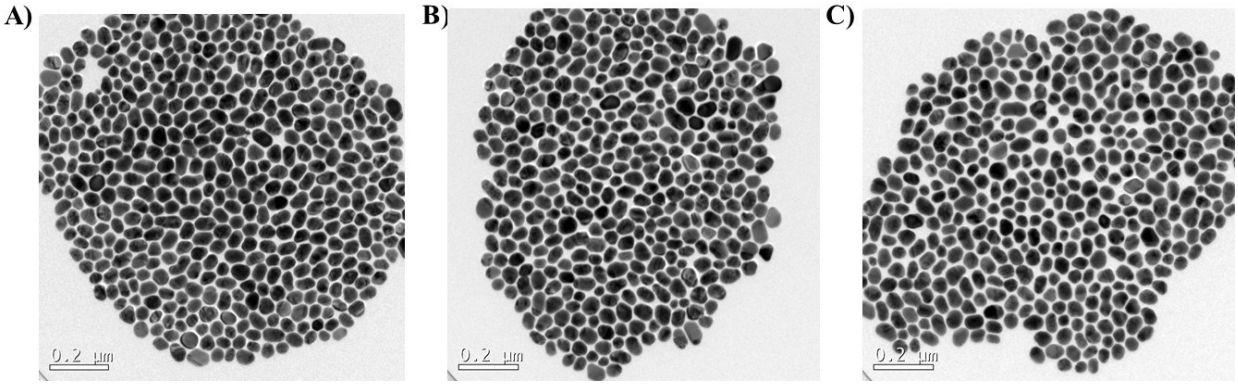


Fig S2: TEM images of A) Positive B) Negative and C) Neutral gold nanoparticles synthesized.

Absorption spectrum nanoparticles in different conditions.

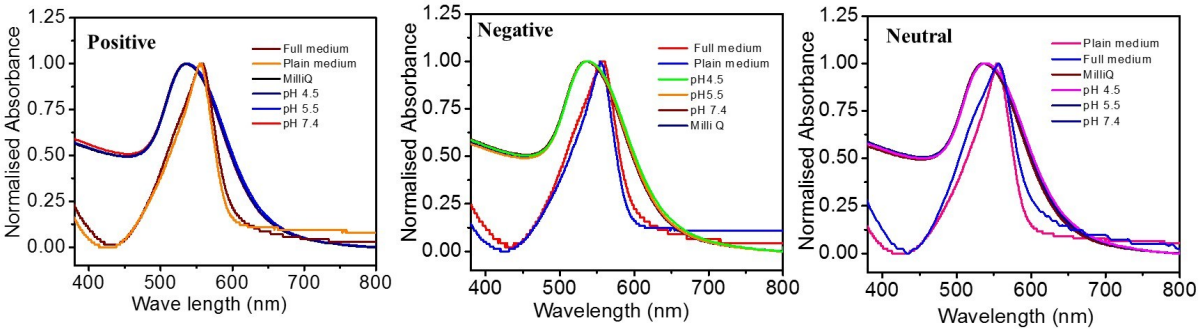


Fig S3: Absorption spectra of gold nanoparticles in different conditions

SERS intensity from three different nanoparticles in different medium.

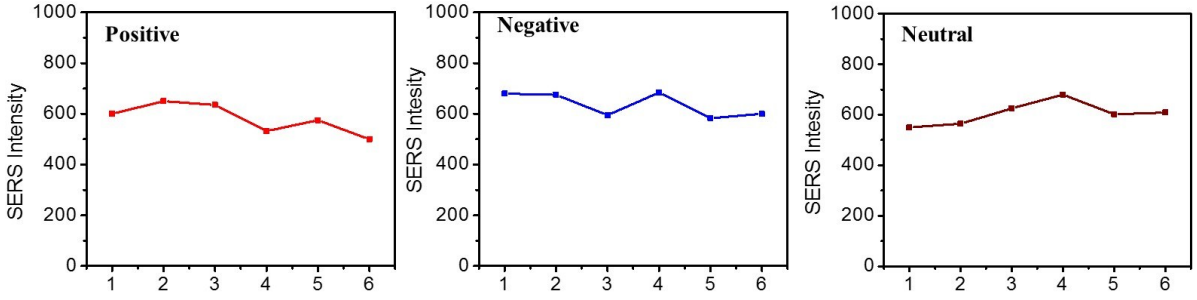


Fig S4: SERS intensity value of 1580 cm^{-1} peak from three different nanoparticles at different conditions. 1) MilliQ, 2) PBS (pH 7.4), 3) PBS (pH 5.5), 4) PBS (pH 4.5), 5) Plain medium, 6) Full medium.

SERS mapping using 1580 cm^{-1} peak using bare gold nanopartilces

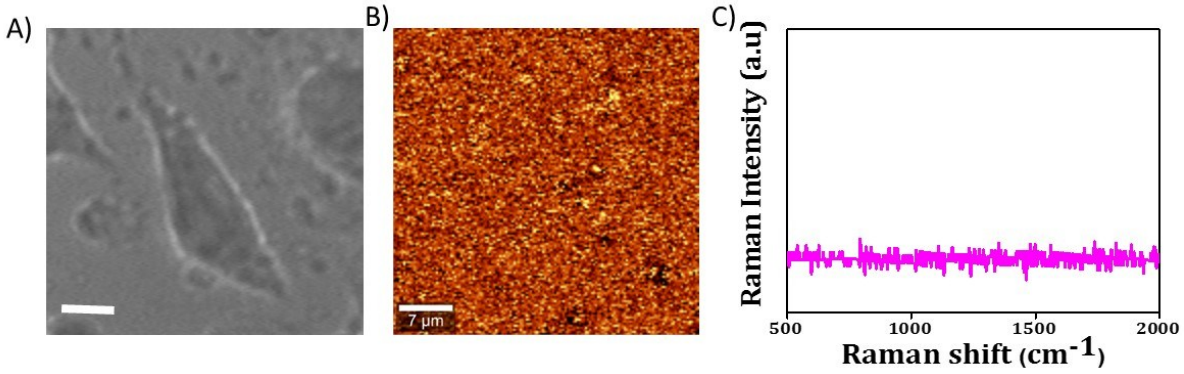


Fig S5: SERS mapping of HeLa cells using bare nanoparticles through 1580 cm^{-1}.

SERS mapping on 2D cell culture model in presence of serum proteins

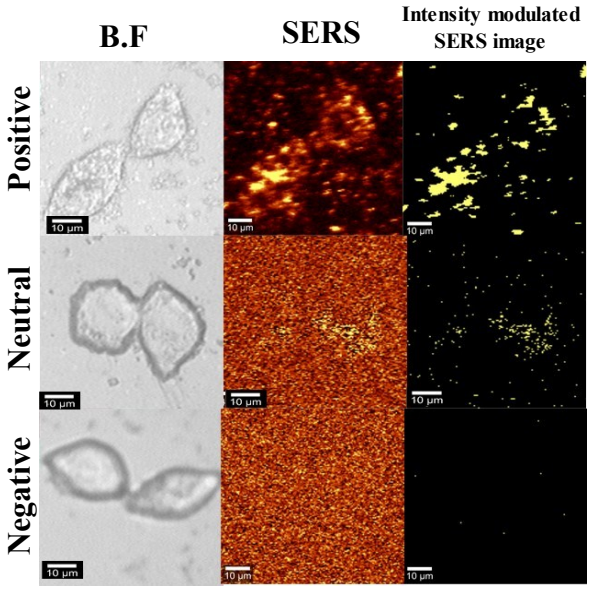


Fig S6: SERS mapping of HeLa cells in presence of serum proteins.

SERS stack scanning images of tumour spheroids treated with neutral gold nanoparticles

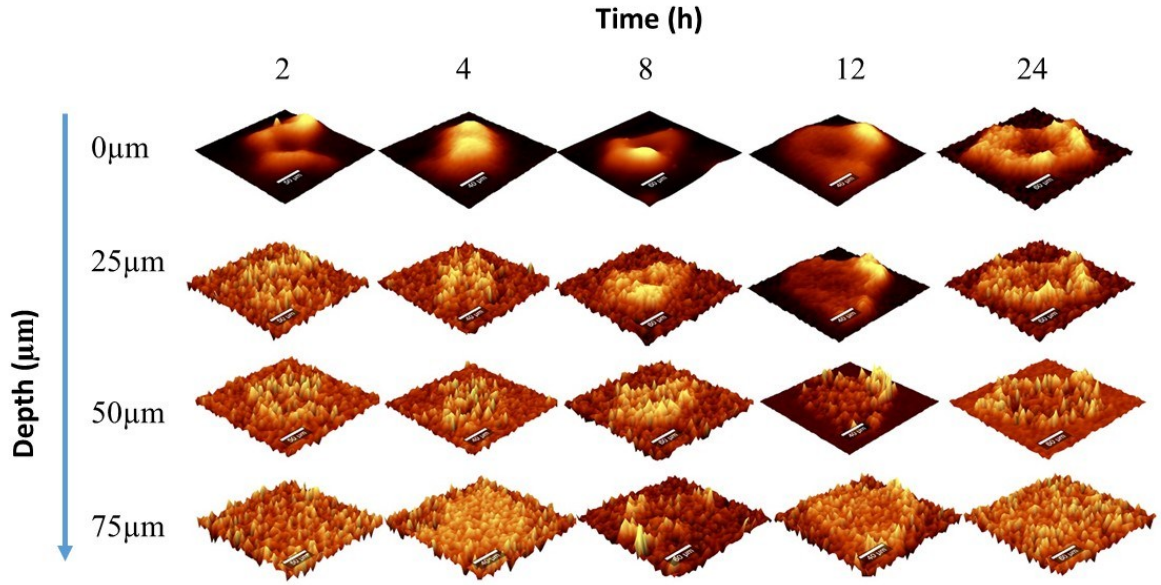


Fig S7: SERS stack scanning images of tumour spheroids treated with neutral gold nanoparticles.

SERS stack scanning images of tumour spheroids treated with positive gold nanoparticles

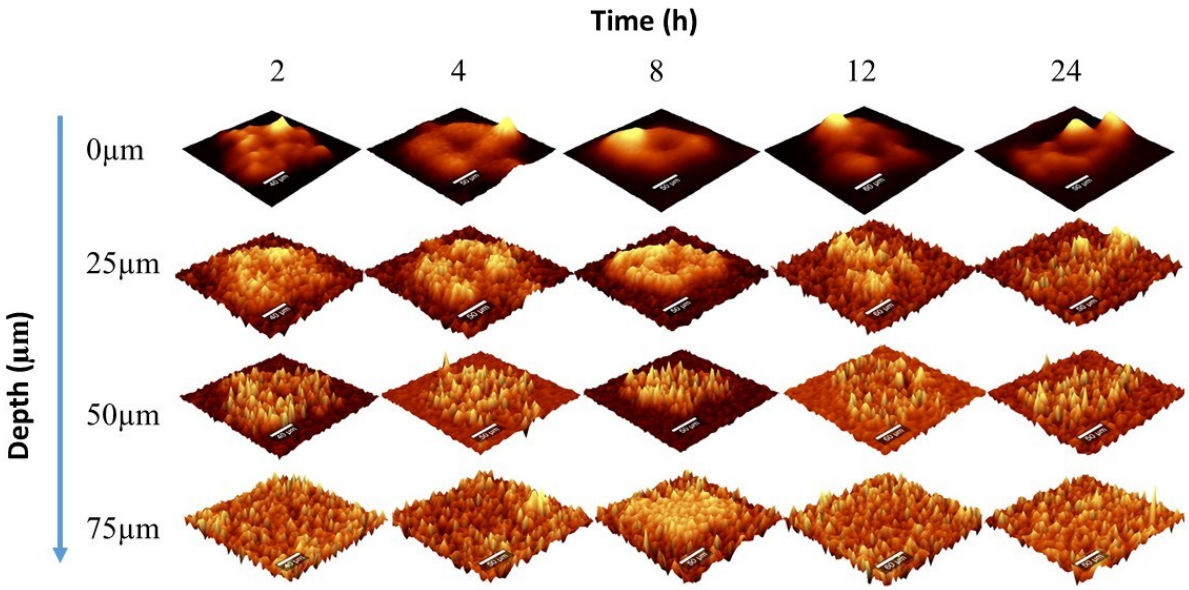
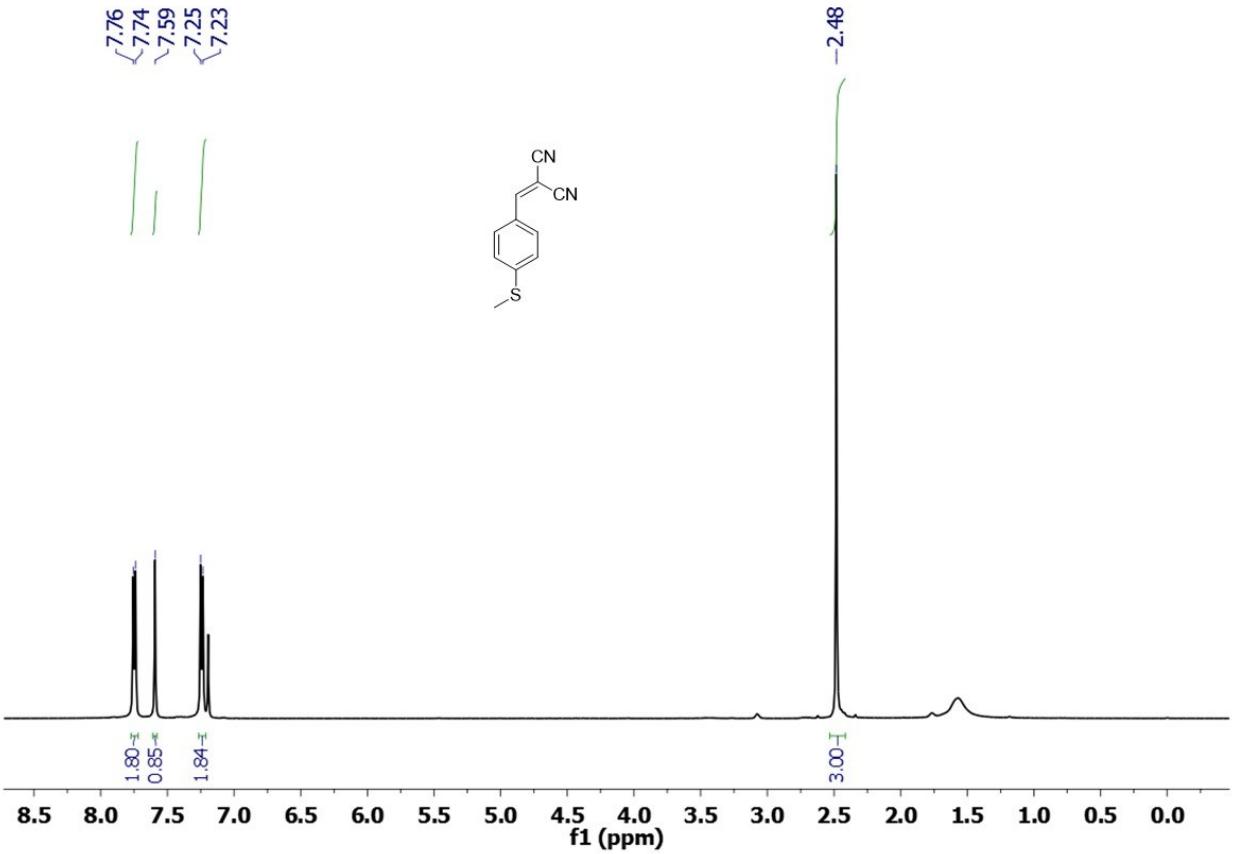
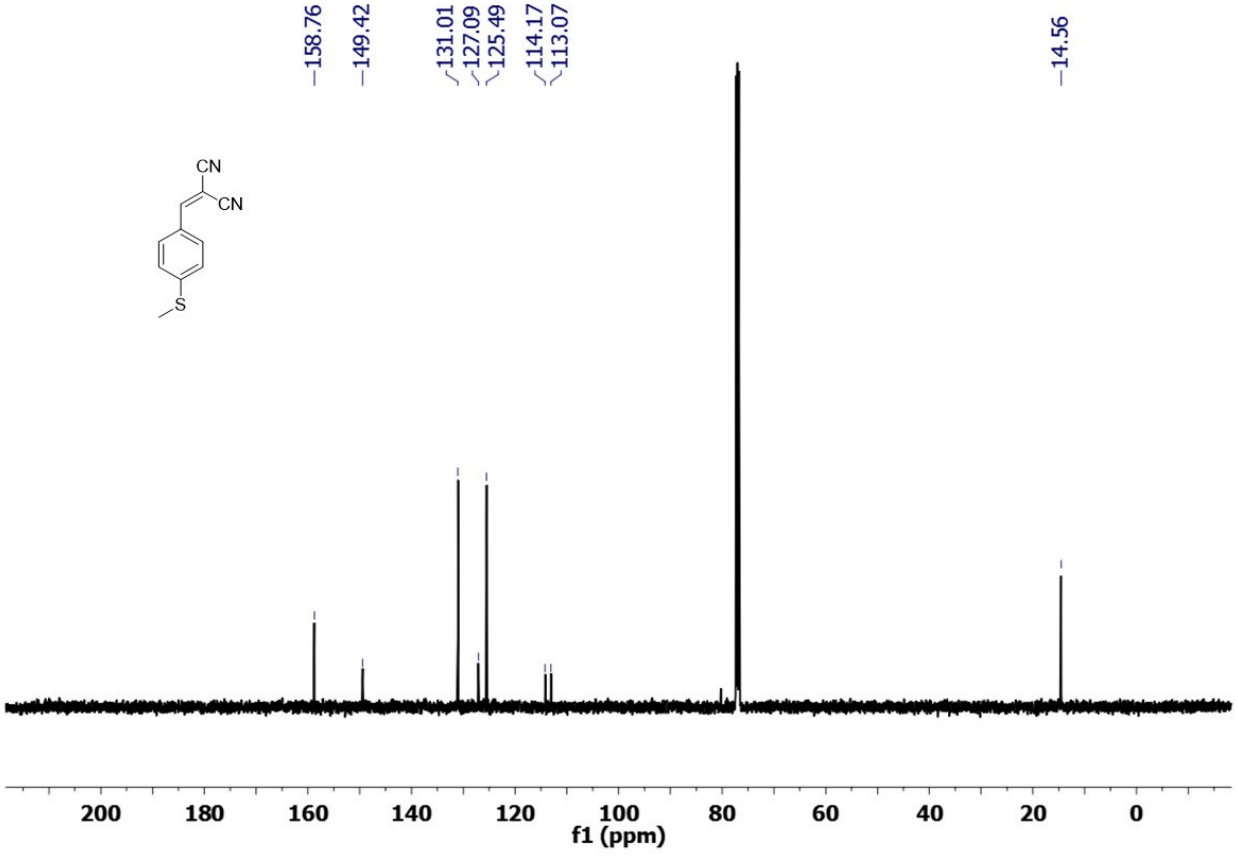


Fig S8: SERS stack scanning images of tumour spheroids treated with positive gold nanoparticles.

[1]H NMR spectrum of CNBN



^{13}C NMR of CNBN



Reference

[1] J. D. Bauer, M. S. Foster, J. D. Hugdahl, K. L. Burns, S. W. May, S. H. Pollock, H. G. Cutler, S. J. Cutler, *Med Chem Res*. 2007, **16**, 119–129

[2] T. T. Ahner, F. Delissen, S. Sokolov, *J. Am. Chem. Soc.* 2010, **132**, 1296.