**Supporting Information**

**Investigation of Apoptotic Events at Molecular Level Induced by SERS Guided Targeted Theranostic Nanoprobe**

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**List of contents:**

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Contents</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Materials and Methods</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>Synthesis of squaraine dye (SQ)- MMP peptide substrate (PLGLAGS) conjugated gold nanorod , MMP-SQ@GNR</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>Synthesis of doxorubicin tethered gold nanorod, LAH-DOX @GNR</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>Cell viability assay</td>
<td>14</td>
</tr>
<tr>
<td>5</td>
<td>SERS spectra of MMP-SQ@GNR</td>
<td>15</td>
</tr>
<tr>
<td>6</td>
<td>TEM image of MMP-SQ@GNR</td>
<td>16</td>
</tr>
<tr>
<td>7</td>
<td>Zeta potential</td>
<td>17</td>
</tr>
<tr>
<td>8</td>
<td>DOX release from MMP-SQ@GNR@LAH-DOX</td>
<td>18</td>
</tr>
<tr>
<td>9</td>
<td>SERS spectra of MMP-SQ@GNR in presence of MMP-9</td>
<td>19</td>
</tr>
<tr>
<td>10</td>
<td>SERS spectra of MMP-SQ@GNR in presence of MMP-7</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>------------------------------------------------------------------------------------</td>
<td>---</td>
</tr>
<tr>
<td>11</td>
<td>Cell viability assay in 3T3-L1 cells</td>
<td>21</td>
</tr>
<tr>
<td>12</td>
<td>Colocalization study of MMP-SQ@GNR@LAH-DOX in HT1080 cells</td>
<td>21</td>
</tr>
<tr>
<td>13</td>
<td>Apoptotic DNA fragmentation study</td>
<td>22</td>
</tr>
<tr>
<td>14</td>
<td>Time dependent and dose dependent SERS profile evaluation</td>
<td>23</td>
</tr>
</tbody>
</table>
1. Materials and Methods

All the chemicals (synthetic building blocks) and solvents were purchased from Sigma Aldrich, Alfa Aesar, Fluka, Merck, and 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. Surface plasmon absorption spectra were measured on a SpectraMax M2 spectrophotometer (Molecular Devices), and the data analysis was performed using GraphPad Prism 5.0/ 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 centered at 520 cm$^{-1}$) was performed. WITec Project plus (v 2.1) software package was used for data evaluation. TEM measurements were performed on a JEOL 2010 high-resolution transmission electron microscope 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. The fluorescence emission spectra were recorded using a Shimadzu RF-5301PC spectrofluorophotometer with an excitation wavelength at 480 nm for DOX release.
Preparation of GNRs. GNRs was prepared by adding around 5 mL of cetyltrimethylammonium bromide (CTAB) solution (200 mM CTAB in deionized water) to 5 mL deionised water. 40 μL of 250 mM HAuCl₄ aqueous solution was added to above solution then its colour changes to light yellow. Then 500 μL of 4mM silver nitrate solution was added followed by the addition of 250 μL 80 mM ascorbic acid solution. The solution immediately changes colorless followed by the addition of freshly prepared 5 μL 10 mM sodium borohydride solution and kept undisturbed for 30 mins, centrifuged to remove excess CTAB.

Cell Culture. The human cancer cell lines HTI080 (fibrosacroma), was obtained from American Type Culture Collection (ATCC, USA). The cells were maintained in DMEM media supplemented with 10% fetal bovine serum and antibiotics (100U mL⁻¹ penicillin/100 μg mL⁻¹ streptomycin mixture) in a 5 % CO₂ incubator at 37°C.

Cell viability study. HT1080 cells were seeded at a density of 10⁴ cells/well in two 96 well plates and incubated at 37°C for sufficient growth. Control, GNR, MMP-SQ@GNR@LAH-DOX were added to the wells separately in each plates. The treated cells were the incubated for 4 hours. Then one of the plates were irradiated with 808 nm laser for 1 minutes.100 μl MTT (0.5mg/ml) was added to each well and the purple color formation was measured calorimetrically at 570 nm.

Live dead assay. 100 μg/ mL Acridine orange (AO) and 100 μg/mL ethidium bromide (EB) were mixed in 1:1 ratio. 200 μL of the final mixture was added to 24 well plate. Washed twice with PBS after 2 mins. Fluorescence images were taken. Live cells were stained green and dead cells stained orange/red.

Annexin V assay. Additionally, evaluation of apoptosis by FITC Annexin V staining (FITC Annexin V apoptosis detection kit from BD Phamingen #556547, BD Biosciences,
San Jose, CA) was also confirmed by flow cytometry, using kit specified instructions. Signals were then detected using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA) and the data was analyzed with the CellQuest Pro software.

**DNA isolation before and after Photothermal chemotherapy.** The DNA isolation kit for the study was bought from Geneaid. HT 1080 cells were grown in 6 well plates. Control, GNR, GNR DOX and MMP-SQ@GNR@LAH-DOX were treated in triplicate wells. HT1080 cells with and without laser irradiation in 10% DMEM medium was scraped using cell scraper followed by centrifugation and resuspension of the cells in Lysis Buffer. It was then incubated at 60°C for 10 minutes 5 µl of RNase A (10 mg/ml) was added. After several steps of centrifugation by addition of provided reagents, purified DNA was eluted out and quantified for further experiments. Agarose gel electrophoresis for apoptotic DNA fragmentation study. For confirming apoptotic cell death due to photothermal-chemotherapy by the nanoconstruct, agarose gel electrophoresis was carried out. Genomic DNA isolated from with and without laser irradiated HT1080 cells were run on 0.8% agarose gel at 80V power for about 45 min. The gel was then examined in a gel documentation system for the visualization of bands.

**SERS spectral analysis.** Cells were cultured in 4 well chamber slide made of glass at a seeding density of $10^4$cells/mL. MMP-SQ@GNR@LAH-DOX was added to the wells separately including control (media alone) and was incubated at 37 °C for 4 h. Further the cells were irradiated with 808nm laser (0.1 W/cm², 1 min). These cells were investigated with the spectral Imaging Mode of the confocal Raman Microscope (alpha300R, WITec Inc. Germany). A spectrum at every pixel was taken (Scan Range: 40 x 40 µM², 100 x 100 pixel, 10,000 spectra) using a 20 x Nikon objective. The sample was excited with a10 mW power 633 nm frequency doubled Nd:YAG laser. Using the integrated video camera, a suitable cell was focused and scanned with an integration time of 0.5s.
2. Synthesis of squaraine dye (SQ), peptide substrate (PLGLAGS) conjugated gold nanorod, MMP-SQ@GNR

2.1. Synthesis of squaraine dye (SQ)

**Synthesis of 1-(3-aminopropyl) – 2, 3, 3- trimethyl – 3H - indolium:**

\[
\text{Br-NH}_2\text{HBr} \quad \text{Toluene} \quad \text{N}^+\text{Br}_2\text{N}_2\text{H}_2\text{Br}
\]

3-bromopropylamine hydrobromide (2.71 g, 12.5 mmol) was added in a seal tube containing 2, 3, 3-trimethyl-3H-indolium (2.2 ml; 12.5 mmol; 1 eqv.) under N\textsubscript{2} atmosphere, and was gently heated up to 110 °C in an oil bath. The mixture was kept at 120 °C for 10 h with stirring. After the reaction was completed, the mixture was cooled down to room temperature to form a solid cake that was washed with Et\textsubscript{2}O and a chloroform-Et\textsubscript{2}O solution. The resulting solid was then dried under high vacuum to obtain it as a solid (yield 84%). \textsuperscript{1}H NMR (500 MHz; CDCl\textsubscript{3}) \(\delta\)7.99–7.66 (m, 1H), 7.26–7.24 (m, 1H), 7.18–7.14 (m, 2H), 3.71 (s, 1H), 3.53–3.47 (m, 2H), 3.23–3.20 (m, 2H), 2.51–2.43 (m, 2H), 1.54 (s, 6H), 1.25 (s, 3H); HRMS (FAB): m/z (C\textsubscript{14}H\textsubscript{21}N\textsubscript{2}+) calculated: 217.32, found: 217.3022.

**Synthesis of 1-(3-(tert-butoxycarbonylamino) propyl)-2, 3, 3 -trimethyl-3H-indolium:**

\[
\text{1DiBoc} \quad \text{2 CHCl}_3 \quad \text{3 DIPEA}
\]

1- (3-aminopropyl) – 2, 3, 3- trimethylindolium (4 g; 18.43 mmol) and di-tert-butyl dicarbonate (10.1 g; 46.1 mmol) were added to a mixture of dry chloroform and diisopropyl ethylamine. The reaction mixture was gently heated to reflux temperature and stirred for 4h. Afterwards, the organic layer was extracted with Et\textsubscript{2}O, dried over anhydrous Na\textsubscript{2}SO\textsubscript{4}, and
concentrated under reduced pressure. Purified the crude residue on a silica gel column using dichloromethane and methanol. Then the product is obtained as a brown liquid (yield: 72%).

\[ \text{H NMR (500 MHz; CDCl}_3\text{): } \delta 7.12 - 7.07 \text{ (m, 2H), 6.76 (t, 1H, } J = 7.5 \text{ Hz), 6.52 (d, 1H), 4.62 (s, 1H), 3.85 (t, 2H, } J = 10 \text{ Hz), 3.27 (d, 2H, } J = 5 \text{ Hz), 1.57 (s, 9H), 1.42 (s, 6H), 1.32 (s, 3H); HRMS (FAB): m/z (C}_{19}H_{29}N_{2}O_{2}^{+} \text{ calculated: 317.4458, found: 317.4410.} \]

**Synthesis symmetrical squaraine dye SQ**

1-(3-(tert-butoxycarbonylamino)propyl)-2,3,3-trimethyl-3H-indolium (250 mg, 0.99 mmol) was added to a solution of squaric acid (56 mg, 0.49 mmol) in a mixture of dry n-butanol (6 ml) and dry toluene (4 ml) in an round bottom flask equipped with a Dean Stark apparatus.\(^1\) Reaction mixture was refluxed for 12 h under N\(_2\) atmosphere. A deep green coloured reaction mixture was concentrated and the crude product was purified by column chromatography using silica gel. Elution of the column with a mixture of DCM/ methanol (1:9) afforded the desired squaraine dyes as green solid (yield = 49 %). \(^1\)H NMR (500 MHz; CDCl\(_3\)): \( \delta 7.35, 7.26 \text{ (m; 2H), 7.15 (t, 1H, } J = 7.25 \text{ Hz), 7.01 (d, 1H, } J = 8 \text{ Hz), 5.94 (s, 1H), 3.27 (d, 2H, } J = 5.5 \text{ Hz), 2.03 (m, 2H), 1.76 (s, 6H), 1.44 (s, 9H), } \)

\[ \text{^13C NMR (125 MHz, CDCl}_3\text{): } \delta 206.97, 156.13, 142.36, 127.89, 123.86, 122.27, 109.41, 79.26, 50.34, 49.30, 38.18, 30.86, 28.33, 27.00, 24.56; HRMS (FAB): m/z (C}_{42}H_{54}N_{4}O_{6} \text{ calculated: 710.9014 found: 711.9031 [M + 1]. Deprotection of Boc was carried out by treating the squaraine dye intermediate with 10% TFA in DCM (2 mL) for 3h.} \]

7
2.2. Synthesis of Matrix metalloproteinases (MMP 2) peptide sequence (PLGLAGS)

Preparation of PLGLAGS was initiated with Fmoc-Pro-OH (383mg, 1.13 mmol) which was taken in dry dichloromethane (10mL) and DIC (0.218mL, 0.071mmol) was added. This was stirred at 0-5 °C for 1h under N₂ atmosphere. The Fmoc-Pro-OH activated DIC complex was concentrated and then dissolved in DMF along with 2-3 drops of DIPEA and charged into the resin bed of HMPB-MBHA resin (200mg, 0.0142mmol) which was swelled up in dry dichloromethane (6mL) for 30 min. The reaction was continued for 8 h with shaking. The progress of the reaction was monitored by the Kaiser test. After completion of the coupling, the resin was washed with DMF (3 x 3 mL), and the Fmoc protection group was removed by treatment with piperidine in DMF (20%, 3 x 2 ml, 3 x 15 min). The reaction cycle was continued in a similar manner with Fmoc-Leu-OH (150mg, 0.426mmol), Fmoc-Gly-OH (0.16mg, 0.426 mmol), Fmoc-Leu-OH (150mg, 0.426mmol), Fmoc-Ala-OH(132mg, 0.426 ), Fmoc-Gly-OH(0.16mg, 0.426 mmol) and Fmoc-Cys-OH(585mg, 0.426mmol) amino acids charged to the resin. The resulting resin-bound peptide was washed with DMF (3 x 5 ml), dichloromethane (7 x 3 mL) and methanol (3 x 3 mL), dried in vacuum (10 h), re-swelled in dichloromethane (5ml), and filtered. Finally desired peptide sequence was released from the resin by treatment with 2% trifluoroacetic acid in dichloromethane (10 x 2 mL). The resin washing was combined and concentrated under reduced pressure, and the residue co-evaporated with toluene. The residue was
precipitated with cold ether (3 mL) and filtered the residue peptide that afforded of white solid (yield: 89%). $^1$H-NMR (500MHz, CDCl$_3$) : $\delta$ 8.90 (d, 1H), 7.99 (s, 5H), 7.43-7.40 (m, 6H), 7.38-7.23(m, 9H), 4.49-4.50( m, 1H), 4.29-4.27(m, 2H), 4.12- 4.11(m,1H), 3.91(s, 2H), 3.74-3.71(m, 1H), 2.66-2.65(m,1H), 2.60-2.57(m,1H), 1.97(s,3H), 1.70-1.67(m, 2H), 1.39-1.28(m, 2H), 0.94-0.90 (m,7H).

2.3. Synthesis of squaraine dye (SQ) conjugated MMP substrate (MMP-SQ)

![Diagram of synthesis process]

Squaraine dye (15mg, 0.029 mmol) MMP substrate (54 mg, 0.058 mmol) were added to dry dichloromethane. Solution was stirred over night after adding HATU (22mg, .058 mmol) DIPEA (4.5mg, .035mmol) at room temperature to yield SQ-MMP as sticky blue solid.(Yield=63%). $^1$H-NMR (500MHz, CDCl$_3$) : $\delta$ 8.86 (s, 1H), 8.01-7.90 (m, 10 H), 7.42-7.36(m, 18H), 7.30-7.27(m, 1H), 7.22-7.20 (m, 1H), 7.14-7.11(m, 2H), 5.84(s, 1H), 4.20 (s, 2H), 4.16 (s, 2H), 3.65-3.59(m, 2H), 3.01(s, 3H), 2.10-2.07(m, 2H), 1.67(s, 6H).

2.4. Synthesis of MMP-SQ substrate conjugated gold nanorod, MMP-SQ@GNR

Squaraine dye, SQ was conjugated to MMP substrate by amide coupling. GNR was functionalized with MMP-SQ by simple mixing method. First, CTAB stabilized GNR was centrifuged (9000 rpm, 12 min) and washed twice with double distilled water. MMP-SQ (30 μM) solution was added in GNR colloid and it was kept in the absence of light at 25°C under
stirring for 24 h. The solution was purified by repeated centrifugation (9000 rpm, 30 min). To calculate the amounts of MMP-SQ attached per GNR, the absorption spectrum of the supernatant was measured using a UV-vis spectrophotometer. The CTAB-coated GNR has molar absorption coefficients of $1.3 \times 10^9$ and $4.6 \times 10^9 \text{ M}^{-1} \text{ cm}^{-1}$ at 510 and 785 nm, respectively. Molar absorption coefficient of MMP-SQ is calculated to be $1.5 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ at 620 nm. These values were used to calculate the average number of MMP-SQ bound per GNR in MMP-SQ@GNR.

3. Synthesis of doxorubicin tethered gold nanorod, LAH-DOX @GNR

3.1. Synthesis of Lipoic acid attached hydrazone linked doxorubicin

Scheme 1: Synthetic route towards LAH-DOX
Synthesis of lipoic acid succinimidyl ester

To a solution of EDC.HCl (1.1g, 0.005 mmol) in dichloromethane, Diisopropyl ethylamine (0.750g, 0.005 mmol) was added and stirred for 10 minutes. N- Hydroxysuccinimide (0.78g, 0.006mmol) was added followed by DL-Lipoic acid(1g, 0.004mmol). The reaction mixture was stirred in an ice bath for half an hour and then slowly continued in room temperature for overnight. The reaction mixture was washed with dilute HCl(5% 50 ml x 2) and water. The organic layer was dried over Na$_2$SO$_4$, filtered and concentrated. The crude residue was purified by silica gel column using ethyl acetate and hexane(yield 63%). $^1$HNMR (500 MHz, CDCl$_3$) δ ppm: 3.557-3.612 (m, 2H), 3.178-3.212 (m, 1H), 3.097-3.133 (m, 2H), 2.617-2.647 (m, 2H), 2.442-2.5.0 (m, 2H), 1.897-1.964 (m, 2H), 1.750-1.824 (m, 2H), 1.665-1.720 (m, 2H), 1.571-1.624 (m, 2H).

Synthesis of LA-Pr-NPC

1- amino3-propanol (10 µl, 0.141 mmol) was dissolved in dry DCM 0.2 ml of TEA was added to it. Lipoic acid succinimidyl ester (150 mg, 0.05 mmol) was added after 10 minutes of stirring. Product obtained as white solid after overnight stirring. It was concentrated and precipitated in diethyl ether twice to produce LA-Pr-OH with a yield of 61%. $^1$H-NMR (500MHz, CDCl$_3$): δ 5.91(s, 1H), 3.63(t,
2H, J=5 Hz), 3.44-3.40(m, 2H), 2.49-2.43(m, 3H), 1.93-1.89(m, 2H), 1.69-1.67(m, 8H), 1.43-1.44(m, 2H). The hydroxyl group of LA-Pr-OH was activated by p-nitrophenyl chloroformate. LA-Pr-OH (0.4 g, 0.115 mmol) and triethylamine (27 mg, 0.267 mmol) were dissolved in 20 mL anhydrous dichloromethane and stirred at 0 °C. To this solution was added drop wise p-nitrophenyl chloroformate (71 mg, 0.352 mmol) in 10 mL dichloromethane. The reaction was further performed at 0 °C for 1 h and at room temperature for 24 h under N₂ atmosphere. The resultant solution was diluted by dichloromethane and washed with brine solution for three times. The organic phase was collected, dried with anhydrous sodium sulphate, concentrated and precipitated into diethyl ether to give LA-Pr-NPC with a yield of 74%. ¹H-NMR (500MHz, CDCl₃) : δ 8.15 (d, 2H, J=5Hz), 6.92(d, 2H, J= 5Hz), 3.59-3.54(m,2H), 3.45-3.41(m, 2H), 2.48-2.43(m, 2H), 2.24-2.20(m, 2H), 1.93-1.88(m, 2H), 1.70-1.67(m, 8H), 1.49-1.44(m, 2H).

**Synthesis of LA-Pr-Hyd**

LA-Pr-NPC (30mg, 0.07mmol) was dissolved in 20 mL dichloromethane and reacted with hydrazine monohydrate (0.93 mmol). The solution was reacted for 24h at room temperature. The resultant solution was washed with brine solution. The organic phase was collected and dried under vacuum after removing the solvent with a yield of 68%. ¹H-NMR (500MHz, CDCl₃): δ6.20(s,1H),3.72-3.70(m, 2H),3.18-3.08(m,2H), 2.48-2.42(m, 3H),2.28-2.24(m, 2H), 1.92-1.86(m, 2H),1.72-1.66 (m, 8H),1.49-1.44(m, 2H). ESIMS: m/Z(C₁₂H₂₃N₅O₃S₂) calcd: 321.118, found: 344.1085 (M+Na).
Synthesis of LAH-DOX

LA-Pr-Hyd (100 mg, 0.027 mmol) and doxorubicin (15 mg, 0.027 mmol) were dissolved in 25 mL anhydrous methanol. The mixture was reacted in the presence of a drop of trifluoro acetic acid at 60°C overnight. After removal of methanol under vacuum purified through zeolite column. ESI-MS: m/Z (C_{39}H_{50}N_{4}O_{13}S_{2}) calcd: 846.2816, found: 845.6616(M-1).

3.2. Preparation of DOX-Tethered GNRs.

LAH-DOX (100μM) was mixed with CTAB stabilized GNRs in 3 mL of ultra-purified water at pH 8.0. The mixture was stirred in the dark for 12 h at room temperature. Thereafter, the nanoparticles were centrifuged at a speed of 10000 rpm for 30 min to remove unconjugated LAH-DOX and washed once with Milli-Q water. The concentration of unconjugated LAH-DOX was determined by UV analyses as described above, and the amount of conjugation at the surface of GNRs was thus calculated.
4. Cell viability assay

**Figure S2:** HT1080 cell viability after 4 h of incubation with increasing concentration of GNR and MMP-SQ@GNR
5. SERS spectra of MMP-SQ@GNR

**Figure S3**: SERS spectra of MMP

**Figure S4**: SERS spectrum of SQ
Figure S5: SERS spectrum of MMP-SQ@GNR.

6. TEM image of MMP-SQ@GNR

Figure S6: TEM image of MMP-SQ@GNR at different MMP-SQ: GNR molar ratio A) 1:9, B)3:7, C)1:1, D) 9:1.
7. Zeta potential

![Zeta potential graph](image)

**Figure S7:** Zeta potential of GNR, MMP-SQ@GNR and MMP-SQ@GNR@LAH-DOX.

![Hydrodynamic Radius graph](image)

**Figure S8:** Hydrodynamic Radius of GNR, MMP-SQ@GNR and MMP-SQ@GNR@LAH-DOX
8. DOX release from MMP-SQ@GNR@LAH-DOX

Figure S9: DOX release from MMP-SQ@GNR@LAH-DOX at different pH.
9. SERS spectra of SQ-MMP@GNR in presence of MMP-9

Figure S10. SERS spectra of SQ-MMP@GNR in presence of MMP-9 enzyme measured under confocal Raman microscope.
10. SERS spectra of SQ-MMP@GNR in presence of MMP-7

![SERS spectra graph](image-url)

**Figure S11.** SERS spectra of SQ-MMP@GNR in presence of MMP-7 enzyme measured under confocal Raman microscope.
11. Cell viability assay in 3T3-L1 cells

![Graph showing cell viability assay results](image)

Figure S12. Cell viability assay in 3T3-L1 cells treated with GNR, MMP-SQ@GNR and MMP-SQ-DOX@GNR under NIR laser irradiation (0.1 W/cm², 2 min)

12. Colocalization study in HT1080 cells

![Images of HT1080 cells](image)

Figure S13. Colocalization study in HT1080 cells. A) Bright field image B) Nuclei stained with hoechst C) Fluorescence image of HT1080 cells incubated with MMP-SQ@GNR@LAH-DOX after 3 hours. D) Merged image of A &C.
13. Apoptotic DNA fragmentation study

Figure S14. DNA fragmentation study without laser irradiation 1) ladder 2) DNA isolated from HT1080 cells 3) from cells incubated with GNR 4) DNA isolated from HT1080 cells incubated with MMP-SQ@GNR@LAH-DOX.
14. Time dependent and dose dependent SERS profile evaluation

**Figure S15.** Time dependent SERS spectral evaluation of HT1080 cells incubated with MMP-SQ@GNR@LAH-DOX for 4 hour.

**Figure S16.** Time dependent SERS spectral evaluation of HT1080 cells incubated with MMP-SQ@GNR@LAH-DOX for 4 hour after laser irradiation.
Figure S17. Dose dependent SERS spectral evaluation of HT1080 cells incubated with MMP-SQ@GNR@LAH-DOX for 4 hour after laser irradiation

Figure S18. SERS spectra of MMP-SQ@GNR@LAH-DOX incubated with MMP 2 enzyme for 2 days.
Reference