SUPPORTING INFORMATION

Fluorescent Glyco-Gold Nanocluster Induced EGFR Mediated Targeting of Cancer Cells

Ankita Chandra, Preeti Ravindra Bhoge, Remya K. R. Chethan D. Shanthamurthy, Raghavendra Kikkeri*

Indian Institute of Science Education and Research, Dr. Homi Bhabha Road, Pashan, Pune-411008, Maharashtra, India

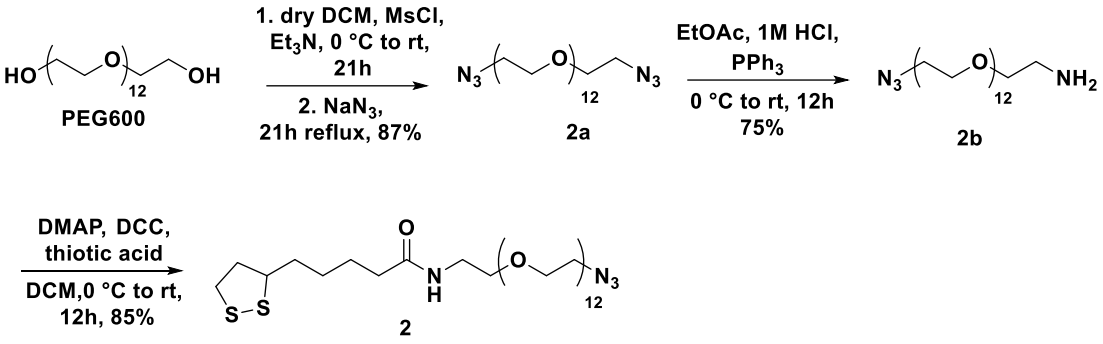
Email. rkikkeri@iiserpune.ac.in

Table of contents

1. General Information

All chemicals were reagent grade and used without further purification unless otherwise noted. Reactions were carried out in anhydrous solvents under a nitrogen atmosphere. Reaction progress was monitored by analytical thin-layer chromatography (TLC) on Merck silica gel 60 F_{254}. Spots on TLC plate were visualized under UV light or dipping the TLC plate in CAM/ninhydrin/PMA solution followed by heating. Column chromatography was carried out using Fluka kieselgel 60 (230-400 mesh). ^{1}H and ^{13}C NMR spectra of compounds were measured with Bruker 400 MHz, Bruker 600 MHz and Jeol 400 MHz using residual solvents as an internal reference ($CDCl_3$ δH 7.26 ppm, δC 77.3 ppm, CD_3OD δH 3.31 ppm, δC 49.0 ppm, and D_2O δH 4.79 ppm). The chemical shifts (δ) are reported in ppm and coupling constants (J) in Hz. UV-visible measurements were performed with Evolution 300 UV-visible spectrophotometer (Thermo Fisher Scientific, USA). Fluorescence spectra were measured with FluoroMax-4 spectrofluorometer (Horiba Scientific, U.S.A.). The time resolved (TR) fluorescence decay data were collected and analysed using a time correlation single photon counting (TCSPC) system integrated in FLS 980 system. Zeta Potential measurement was performed using Nano ZS-90 setup (Malvern instrument). For colocalization studies mitotracker green (M7514), ER tracker blue (E12353), EEA1 antibody (MA5-31575, early endosomal marker), LAMP2 antibody (MA1-165, late endosomal marker), Donkey anti Rat IgG (H+L)highly cross absorbed secondary antibody, Alexa fluor 488 (A-21208) purchased from eBioscience™. All microscopy images were captured using Leica SP8 confocal microscope and processed using Image J software. All cancer cell lines were obtained from NCCS, Pune cell bank.

2. Synthesis of Linkers and DBCO-conjugated sugars:

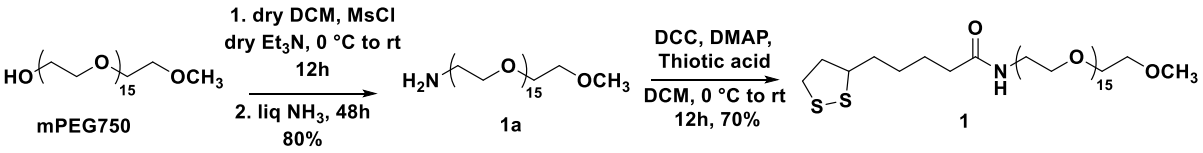


Synthesis of compound 2a. PEG (average MW 600) (4 g, 6.6 mmol) dissolved in dry DCM, followed by addition of methanesulfonyl chloride (3 g, 26.09 mmol) and allowed to cool to 0 °C. Then triethylamine (6 ml, 40 mmol) was added dropwise and kept stirring for 21 h at room temperature. After completion of the reaction, the reaction mixture was transferred to a separating funnel. DCM layer was initially washed with water (3 × 50 ml), then saturated brine solution (50 mL). Finally the collective organic layer was dried over Na_2SO_4 and concentrented with rota evaporator. The crude product MsO-PEG600-OMs was used for next step without purification (Yeild – 4.8g , 87%). MsO-PEG600-OMs (4.8 g, 6.3 mmole) was dissolved in ethanol (50 mLl) and sodium azide (1.6 g, 2.4 mmole) was added. The reaction mixture was kept for reflux for 21 h. After completion of the reaction, ethanol was evaporated with rota evaporator. Then the reaction mixture was transferred to a separating funnel, DCM was added and DCM layer was initially washed with water (3 × 60 ml), then saturated brine solution (60 mL). Finally the collective organic layer was dried over Na_2SO_4 and concentrated with rota evaporator to get compound **2a** which was used further without purification (Yield- 80%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.68 – 3.60 (m), 3.37 (t, J = 5.1 Hz, 4H). IR (CH_2Cl_2 solvent): 2,869; 2,098; 2025 1,453; 1,095 cm^{-1}.

Synthesis of compound 2b. Compound **2a** (2 g, 3.07mmol) was taken in a 100 ml two-necked round-bottomed flask, EtOAc (24 mL) and 1 M HCl (10 mL, 0.06 mol) was added to it. Then triphenylphosphine (824 mg, 3.1 mmol) dissolved in 16 mL of EtOAc and was added dropwise at 0°C to above solution. Once the addition was complete the reaction mixture gradually warmed up to rt and stirred for 12 h. After completion of the reaction the reaction mixture was transferred to a separatory funnel and collected the aqueous layer. Aqueous layer was washed with EtOAc (30 mL ×2). To the aqueous layer KOH (5 g, 8.9 mmol) was added slowly and stirred the mixture until KOH dissolved followed by extraction with EtOAc. The organic layers were dried over Na_2SO_4 and evaporated the solvent using rotary evaporator and dried the product **2b** under vaccum (Yield – 1.44g, 75%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.68 – 3.62 (m), 3.50 (t, J = 5.2 Hz, 2H), 3.38 (t, J = 5.1 Hz, 2H), 2.85 (t, J = 5.2 Hz, 2H). IR (CH_2Cl_2 solvent): 3,418; 2,871; 2,106; 1,637; 1,456; 1,095 cm^{-1}.

Synthesis of compound 2. Compound **2b** (1.4 g, 2.2 mmol) taken in a 100 ml round-bottomed flask, 4-(*N*,*N*-dimethylamino)pyridine (55 mg, 0.4 mmol), N,N-dicyclohexylcarbodiimide (475 mg, 2.3 mmol) and DCM (10 mL) was added to it followed by addition of thioctic acid (2.2 mmol) dissolved in (5 mL) DCM dropwise at 0 °C. The reaction mixture gradually warmed to rt and was stirred for 12 h. After completion of the reaction the mixture was filtered through celite bed and evaporated the solvent using a rotary evaporator. To the residue, water was added and the aqueous mixture was washed with ether (2 × 30 mL). The aqueous layer was saturated with $NaHCO_3$ and the product was extracted with DCM (3 × 30 mL). The organic layer was dried over Na_2SO_4 and solvent was evaporated under reduced pressure. Crude product was purified by chromatography on silica gel (eluent 20:1 DCM:MeOH) to get pure compound **2** with 85% of yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.33 (br s, 1H), 3.67 – 3.60 (m), 3.54 (t, J = 5.6 Hz, 2H), 3.44 (t, J=5.0 Hz,

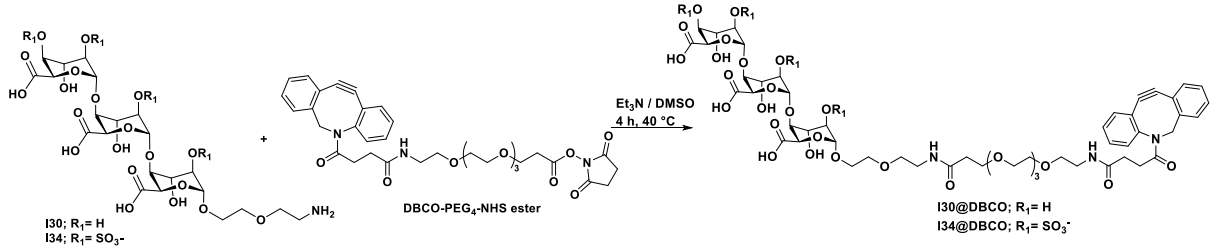
2H), 3.38 (t, J = 5.1 Hz, 2H), 3.20 – 3.06 (m, 2H), 2.50 – 2.40 (m, 1H), 2.18 (t, J = 7.2 Hz, 2H), 1.94 – 1.85 (m, 1H), 1.73 – 1.61 (m, 4H), 1.51 – 1.39 (m, 2H). IR(CH_2Cl_2 solvent): 3,328; 3,3075; 2,918; 2,867; 2,105; 1,653; 1,543; 1,456; 1,092 cm^{-1}.



Synthesis of compound 1a. Poly(ethylene glycol) methyl ether (average MW 750 Da) (2 g, 2.6 mmol) dissolved in anhydrous DCM to which methanesulfonyl chloride (412.8 µl, 5.3mmoles) was added at 0°C in dropwise manner. The mixture was stirred at 0°C for 10mins followed by slow addition of dry triethylamine. The reaction mixture was stirred for 2 hr at 0°C and then kept at room temperature for 12 hours. On completion of the reaction the organic layer was washed with 2N HCL (50ml ×2) followed by brine (50ml) and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the residue was dissolved in 100ml ammonia. The mixture was stirred for 48 hours at rt and then extracted with DCM (3 ×50ml). The organic layer was dried over Na_2SO_4 and concentrated on rotatory evaporator. The crude product was purified by column chromatography (eluent 1:10 MeOH:DCM) to get compound **1a** in 80% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.63 – 3.59 (m), 3.53 – 3.49 (m, 4H), 3.34 (s, 3H), 2.86 (t, J = 5.1 Hz, 2H).

Synthesis of compound 1. To a solution of compound **1a** (2.5 g, 3.3 mmol), 4-(*N*,*N*-dimethylamino)pyridine (82.6 mg, 0.6 mmol) and N,N-dicyclohexylcarbodiimide (703 mg, 3.4 mmol) in DCM (20 mL), thioctic acid (0.698g, 3.3mmol) was added dropwise at 0°C. The reaction mixture was gradually warmed to rt and was stirred for 12 h. With the completion of the reaction by monitoring TLC, the mixture was filtered through celite bed and evaporated the solvent using a rotary evaporator. To the residue water was added and the

aqueous mixture was washed with ether (2 × 50mL). The aqueous layer was saturated with $NaHCO_3$andextracted the product with DCM (3 × 50mL). The organic layer was dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. The crude product was purified by column chromatography on silica gel with 25:1 DCM:MeOH as the eluent to get final compound **1** with 70% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.34 (s, 1H), 3.65 – 3.59 (m), 3.54 – 3.51 (m, 4H), 3.43 (t, J = 5.2 Hz, 2H), 3.36 (br s, 3H), 3.19 – 3.06 (m, 2H), 2.48 – 2.39 (m, 1H), 2.17 (t, J = 7.5 Hz, 2H), 1.93 – 1.84 (m, 1H), 1.72 – 1.61 (m, 4H), 1.49 – 1.39 (m, 2H). IR (CH_2Cl_2 solvent): 3,3548; 2,866; 1,652; 1,542; 1,455; 1,348; 1,296; 1,248; 1,091; 947; 848 cm^{-1}.



Synthesis of compound I30@DBCO (3) and I34@DBCO (4). I30 or **I34** (3.16 mmol) was first dissolved in dry DMSO solvent (1ml) and the reaction mixture was allowed to warm at 40°C. To this solution DBCO-PEG4-NHS ester (3.7 mmol) was added and allowed to stir at 40°C for 4hrs. After completion of reaction, the reaction mixture was extracted out with $CHCl_3/H_2O$ (1ml × 4) to remove unreacted DBCO-PEG4-NHS ester and DMSO partially. Water layer was lyophilized and the residue was passed through Bond Elute C-18 column to get pure desired product. ^{1}H NMR of **I34@DBCO (3)** (400 MHz, D_2O): δ 7.70 – 7.65 (m, 1H), 7.63 – 7.36 (m, 7H), 5.25 (d, J = 11.7 Hz, 3H), 5.17 – 5.08 (m, 5H), 4.67 (s, 1H), 4.47 (s, 1H), 4.24 – 4.20 (m, 4H), 4.15 (s, 2H), 4.10 (s, 1H), 3.87 – 3.83 (m, 2H), 3.80 – 3.74 (m, 4H), 3.71 – 3.70 (m, 2H), 3.69 – 3.68 (m, 2H), 3.66 (s, 3H), 3.65 (s, 3H), 3.64 – 3.63 (m, 1H), 3.62 – 3.59 (m, 4H), 3.42 – 3.35 (m, 4H), 3.24 – 3.19 (m, 2H), 2.56 – 2.50 (m, 3H), 2.28 – 2.22 (m, 2H). ^{1}H NMR of **I30@DBCO (4)** (400 MHz, D2O) δ 7.86 – 7.01 (m, 8H), 4.99 –

4.92 (m, 5H), 4.74 (t, J = 2.7 Hz, 1H), 4.71 – 4.68 (m, 1H), 4.66 (d, J = 2.7 Hz, 1H), 4.16 – 4.11 (m, 3H), 3.99 (t, J = 3.8 Hz, 2H), 3.94 (t, J = 4.6 Hz, 2H), 3.91 (t, J = 4.8 Hz, 2H), 3.84 (t, J = 5.1 Hz, 2H), 3.79 – 3.74 (m, 4H), 3.72 (br s, 1H), 3.70 – 3.66 (m, 6H), 3.65 – 3.58 (m, 11H), 3.43 – 3.33 (m, 4H), 3.23 – 3.18 (m, 2H), 2.67 – 2.64 (m, 1H), 2.58 – 2.50 (m, 2H).

Synthesis of AuNC@OMe. 30 µmol of compound **5** was dissolved in 20ml of deionized water containing 50µL of 2M NaOH, followed by addition of 200 µL of 50 mM gold (III) chloride hydrate ($HAuCl_4$. xH_2O). The mixture was stirred for 5 mins and then 400 µL of 50mM $NaBH_4$ was added slowly. The reaction mixture was allowed to stir for 15 h at rt, followed by purification using membrane filtration device with a molecular weight cutoff of 10 kDa.

Synthesis of AuNC@N_3. Purified AuNC@OMe dispersed in 5ml of water, followed by addition of 2.4 ml of 12.3 mM stock solution of compound **3**. The mixture was stirred for 3 h at rt and then purified by applying three cycles of centrifugation using 10 kDa cutoff filter. The purified sample was then lyophilized and characterized by FT-IR to verify introduction of azide groups on cluster surface.

Synthesis of AuNC@I34 and AuNC@I30. Synthesized **AuNC@N_3** and **I30@DBCO or I34@DBCO** where mixed together in 1 mL MilliQ water and kept stirring for 12 hrs at rt to promote strain promoted azide alkyne cycloaddition reaction. The reaction mixture was purified and washed with water by three cycles of centrifugation using 10 kDa cutoff centrifugal filters (Millipore) to remove unreacted sugar moieties.

3. Surface Plasmon Resonance binding kinetics.

HS biomimetics (**I34**) was covalently immobilized on CM5 sensor surface using active ester and amine coupling reaction. For binding profile, a known concentration of growth factors were injected in HBS-EP buffer at a flow rate of 50 µl/min at 25 ºC for 150 sec followed by running buffer (HBS-EP without growth factors) which then flowed for another 100 sec to enable dissociation. The sensor surface then regenerated by washing with 0.1% SDS and 0.085% H_3PO_4 injected for 3 min at a flow rate of 100 µL/min. For K_D experiments, immobilized HS biomimetics (**I34**) were treated with different growth factors at a flow rate of 50 µL/min and 25 ºC. HBS-EP buffer without growth factor was then flowed over the sensor surface for 3 min to enable dissociation. Kinetic analysis was performed using the BIAevaluation software for T100. Association and dissociation phase data were globally fitted to a simple 1:1 interaction model.

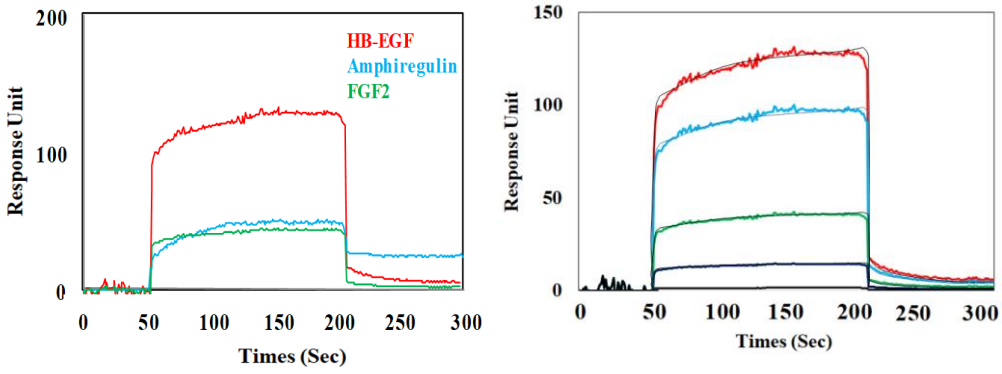


Figure S1: SPR analysis of growth factors binding profile on sensor chip having **I34** ligand.

Substrate	**Growth factor**	**K_D (µM)**	**K_{on} ($M^{-1}S^{-1}$)**	**K_{off} (S^{-1})**
I34	HB-EGF	4.98 ± 0.6	$3.4 \pm 0.3 \times 10^4$	$1.7 \pm 0.38 \times 10^{-1}$
	Amphiregulin	16.06 ± 0.21	$1.21 \pm 0.12 \times 10^4$	$1.94 \pm 0.25 \times 10^{-1}$
	FGF2	14.7 ± 0.37	$1.28 \pm 0.26 \times 10^4$	$1.89 \pm 0.25 \times 10^{-1}$

Table S1: SPR analysis of kinetic rate constants and equilibrium affinities for HS ligands binding to chemokines

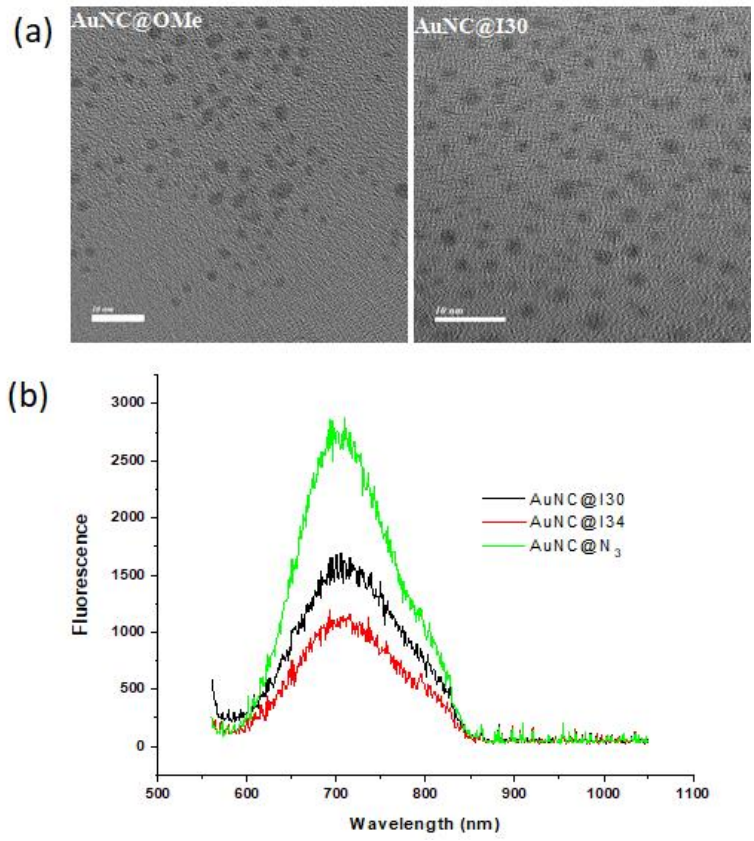


Figure S2. (a) TEM images; (b) Fluorescence spectra of AuNCs at 1mg/mL concentration.

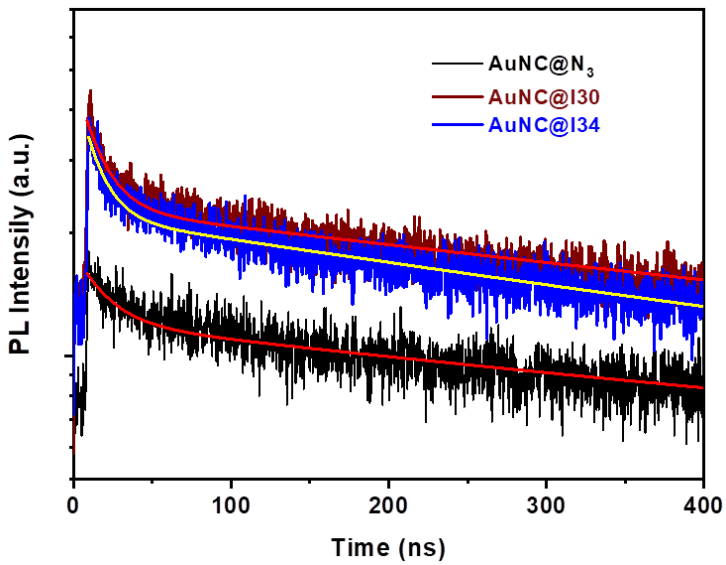


Figure S3. Time-resolved fluorescence decay spectra. Excitation laser source used at 405 nm. The fluorescence deacy of the AuNCs (emission at 710nm) were analysed using TCSPC system fitted to a three-exponentia l function: $\tau_{avg} = \frac{\sum A_i \tau_i^2}{\sum A_i \tau_i}$

Where A_i is a weighting parameter corresponding to each decay time and τ_{avg} is average amplitude weighted lifetime.

4. Cell lines: MDA-MB 231, MDA-MB-468, T-47D, MCF-7, SK-BR-3 cell lines were grown at 37 ºC in 5% CO_2 in DMEM medium containing 10 % fetal bovine serum and 0.1 % streptomycin.

5. Confocal Imaging. 2×10^4 cells were seeded 8 well confocal imaging plates and incubated at 37 ºC overnight. Then cells were incubated with AuNC@I34 or AuNC@I30 (5 µg/ml) for different time intervals of 4 h and 24 h. Then the cells were washed with the PBS and nuclei were stained with Hoechst 33342. The fluorescent images were taken using Leica sp8 microscope.

6. Cellular uptake mechanism studies. To study cellular internalization mechanism MDA-MB 468 cells were grown in an 8-well chamber plate (2×10^4 cells per well) overnight and treated with the specific inhibitor for 30 min followed by treatment with AuNC@I34/I30 (5 µg/ml) for 4 h at 37 °C. For the energy dependent, dynamin-mediated, clathrin mediated, caveolae mediated and EGFR mediated study cells were incubated for 30 min with NaN_3 (50 mM), dynasore hydrate (50 µM), chlorpromazine (25 µM) and methylated-β-cyclodextrin (10 mM), gefitinib (30 µM) respectively. After 4 h of treatment of AuNC@I34/I30, the cells were washed to remove unbound materials and stained with Hoechst 33342 before procceding for imaging.

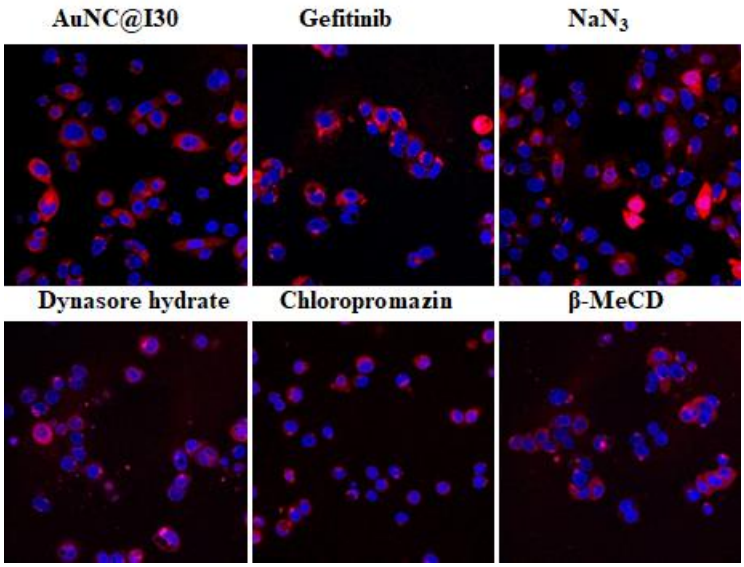


Figure S4. Confocal images of uptake of AuNC@I30 by MDA-MB468 cells in presence of different endocytotic pathway inhibitors (scale bar: 20 mm)

7. Cellular trafficking studies. To study cellular co-localization of AuNC@I34 MDA-MB 468 cells were grown in an 8-well chamber plate (1×10^4 cells per well) overnight. For early endosome co-localization cells treated with EEA1 antibody for 45 min then AuNC@I34 (5µg/mL) were added and incubated further for 4h. For other organelle co-localization studies first cells treated with AuNC@I34 (5 µg/mL) for 4h, then LAMP2 antibody for late endosome, mitotracker green and ER tracker blue was added and incubated further for 30 min. For early and late endosome secondary Goat anti rat Alexa 488 antibody was added and incubated further for 30 min. Finally all cells stained with Hoechst, washed and taken for imaging.

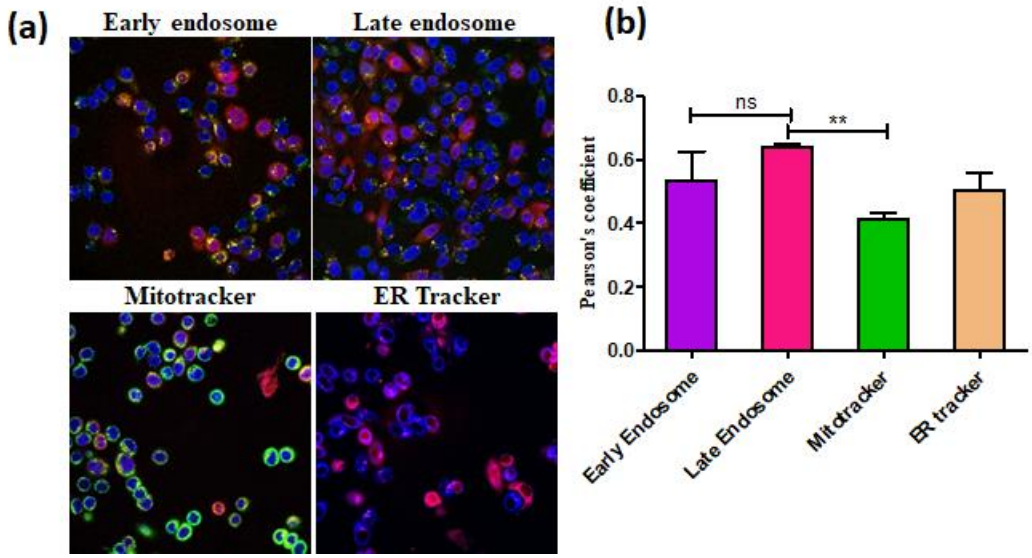


Figure S5. (a) Confocal microscopic images for trafficking of AuNC@I34 (Red) in mitochondria (Mitotracker green), early (EEA1 antibody), late (LAMP2 antibody) endosomal compartments and Endoplasmic reticulum (ER tracker blue) of MDA-MB648 after 4 h of treatement (scale bar: 20 μm); (b) Time point was assessed for >20 cells per treatment using Pearson's coefficient as determined by the just another colocalization plugin in ImageJ. Asterisks indicate statistically significant differences (**p < 0.01; ns, not significant).

8. 3D spheroid formation. 3D spheroids of MDA-MB-468 cells were formed using Matrigel (ECM Gel from EngelbrethHolm-Swarm murine sarcoma) according to manufacturer's protocol. First a monolayer of 100 % matrigel (~55 μL) was coated on 8-well chamber imaging plate and kept at 37 °C for 15 min for polymerization. Then fresh MDA-MB-468 (1 X 10^4 cells/well) in growth medium containing 2% matrigel was plated on top of the polymerized matrigel layer and incubated at 37 °C for 7-8 days. Growth Medium was replaced every 3nd day. Once sufficient nuber of spheroid formation was seen AuNC@34/I30 (20 μg/mL) were added and incubated for 4 h. Later, the cells were washed with PBS buffer, nuclei were stained with Hoechst 33342 and taken for imaging.

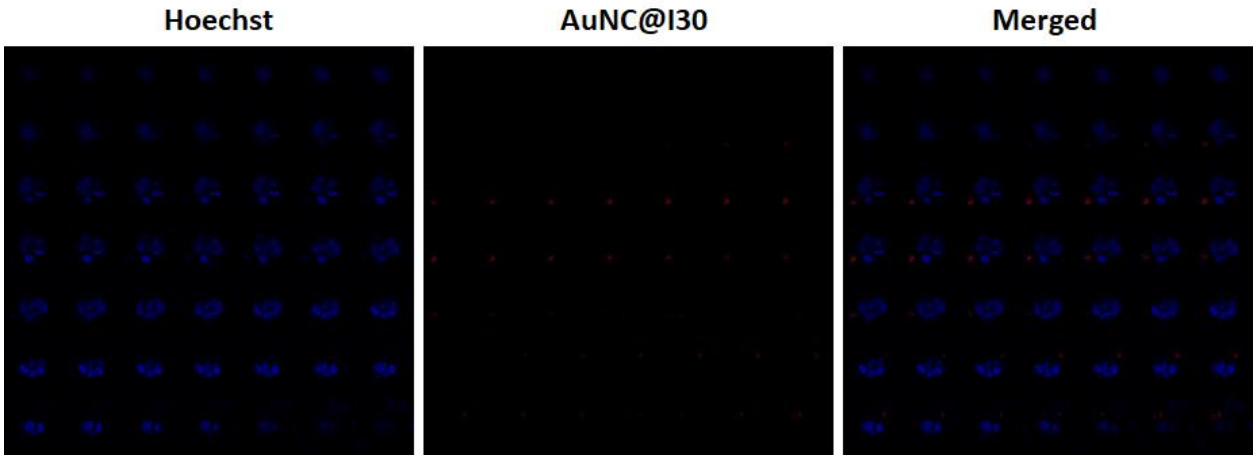
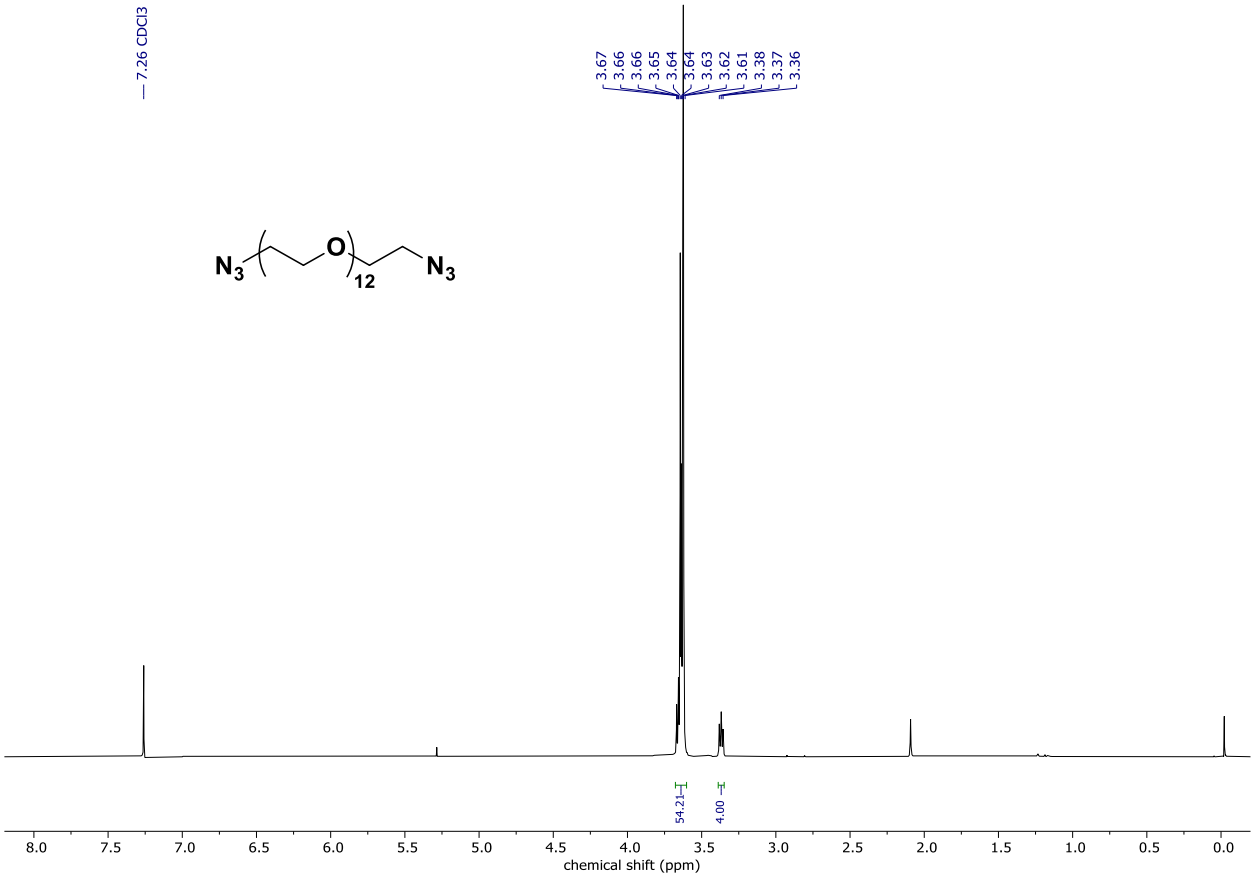


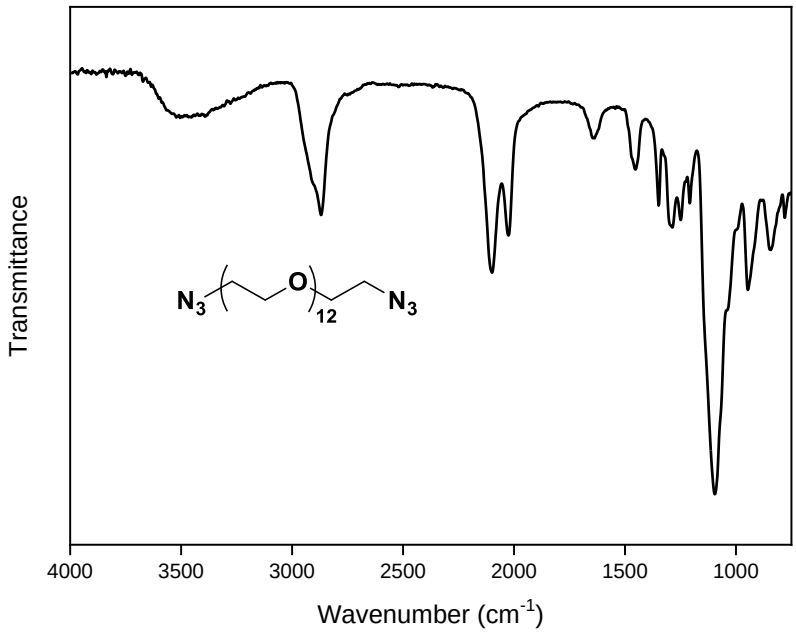
Figure S6. Z-stack montage for MDA-MB-468 spheroids (total slices: 49); (a) nuclei staining (blue channel); (b) AuNC@I30 (red channel); (c) merged (blue and red (scale bar: 20 mm).

Spectroscopic Data

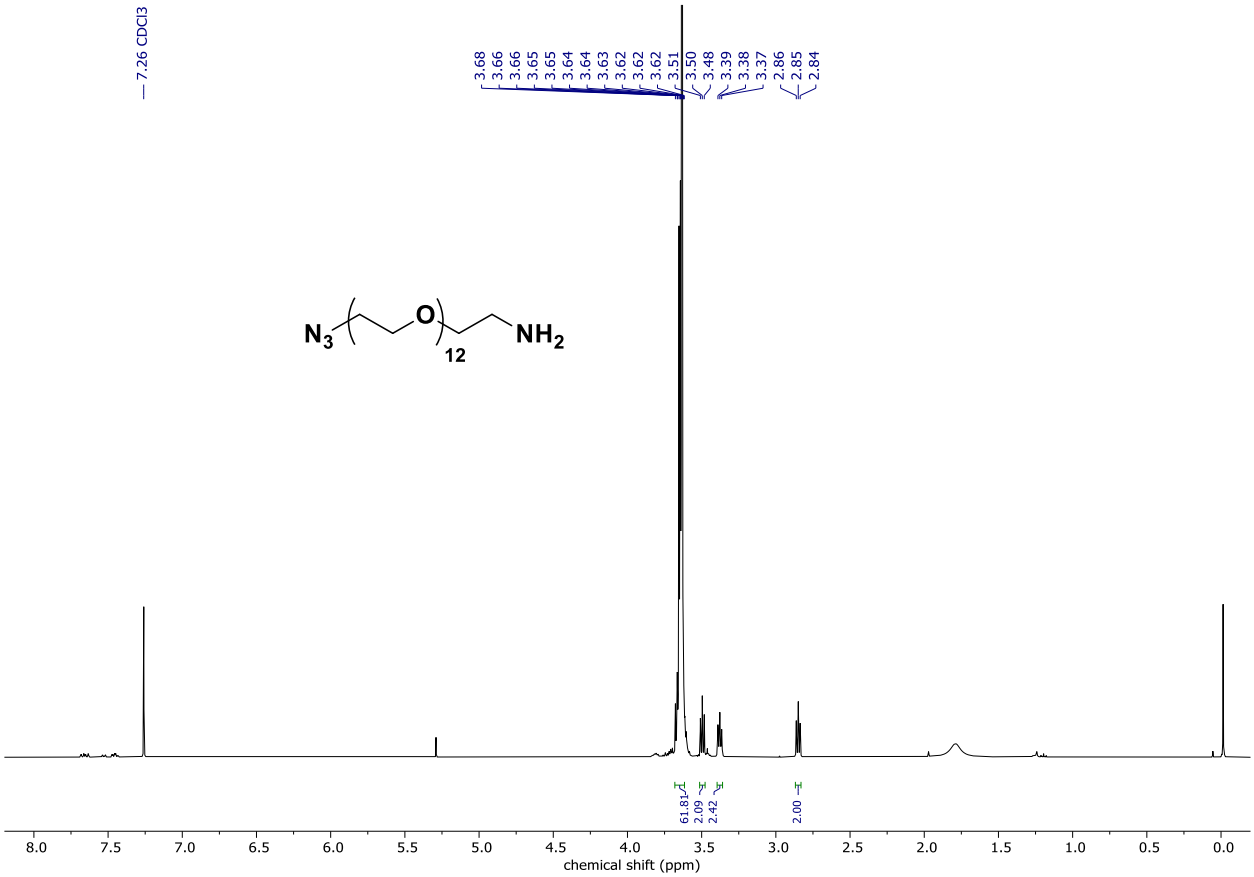
^{1}H NMR of compound 2a



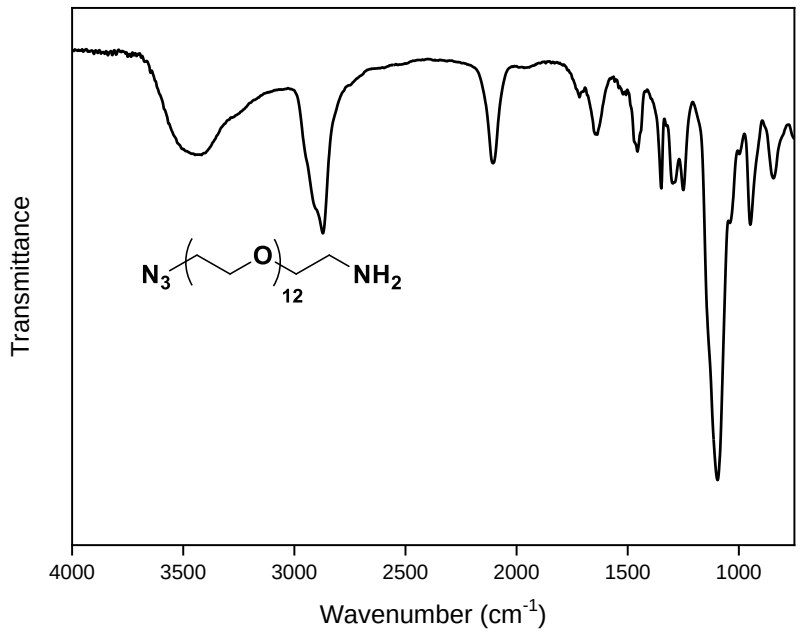
AT-IR spectra of compound 2a



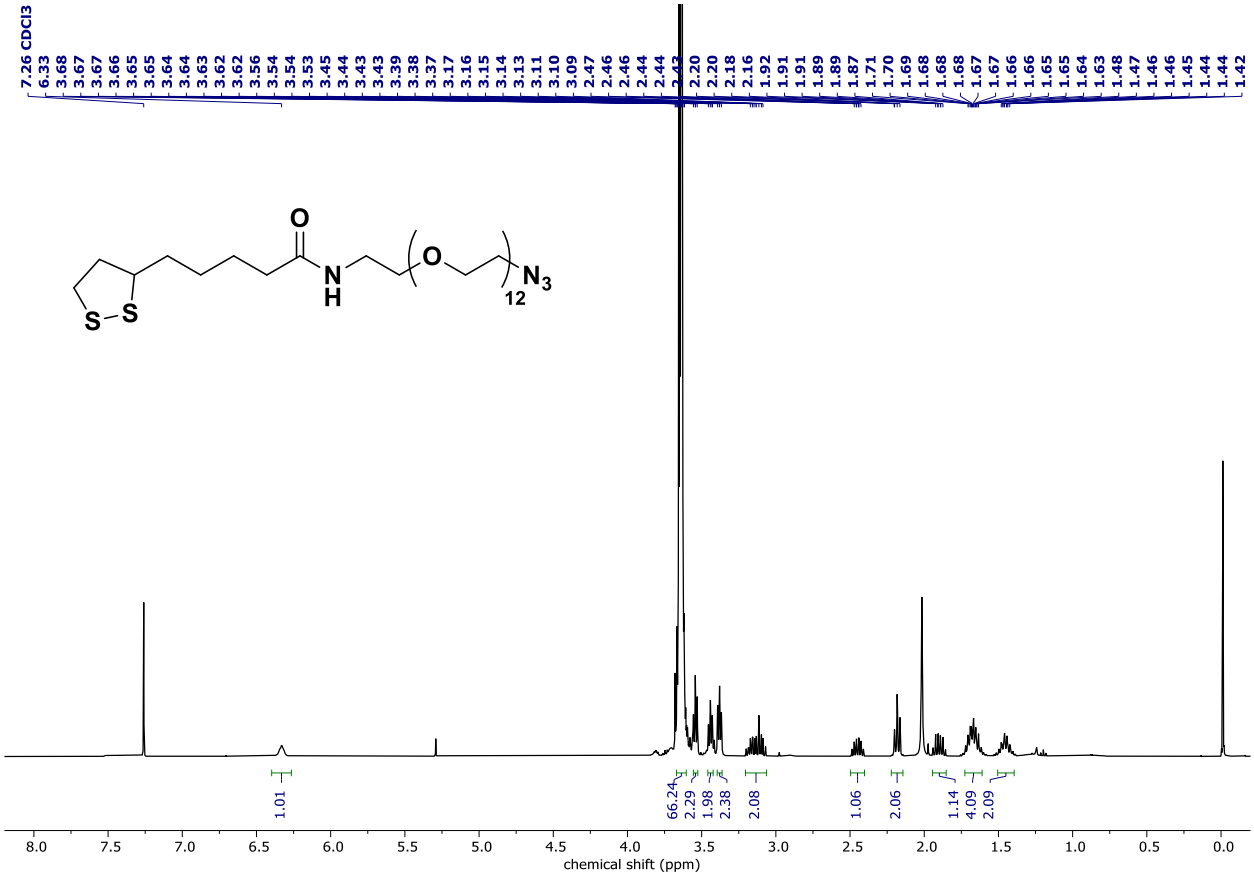
^{1}H NMR of compound 2b



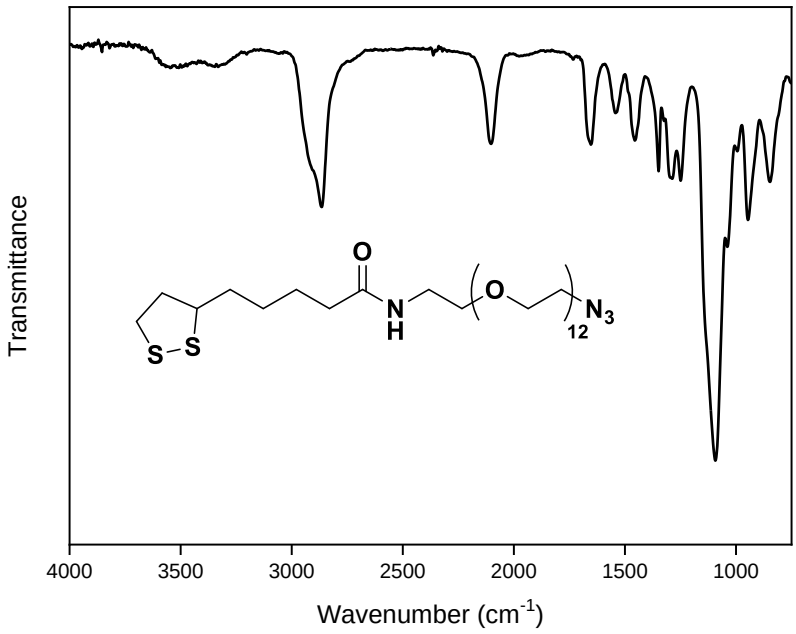
AT-IR spectra of Compound 2b



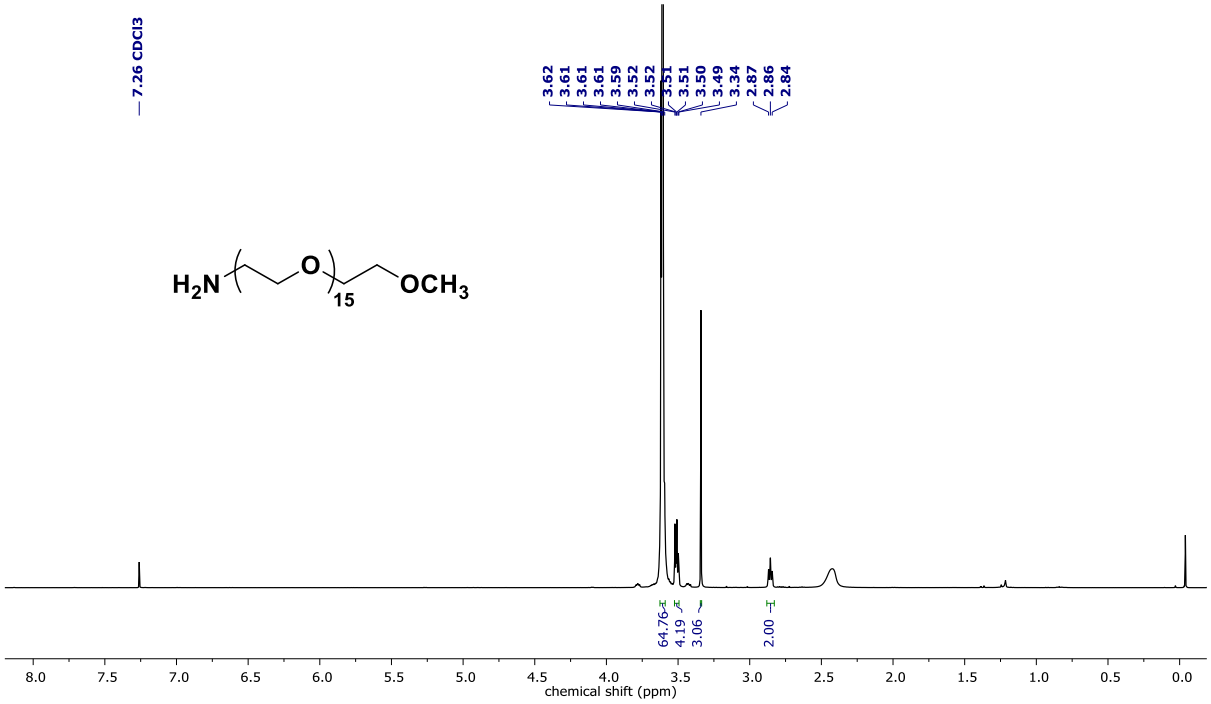
^{1}H NMR of Compound 2



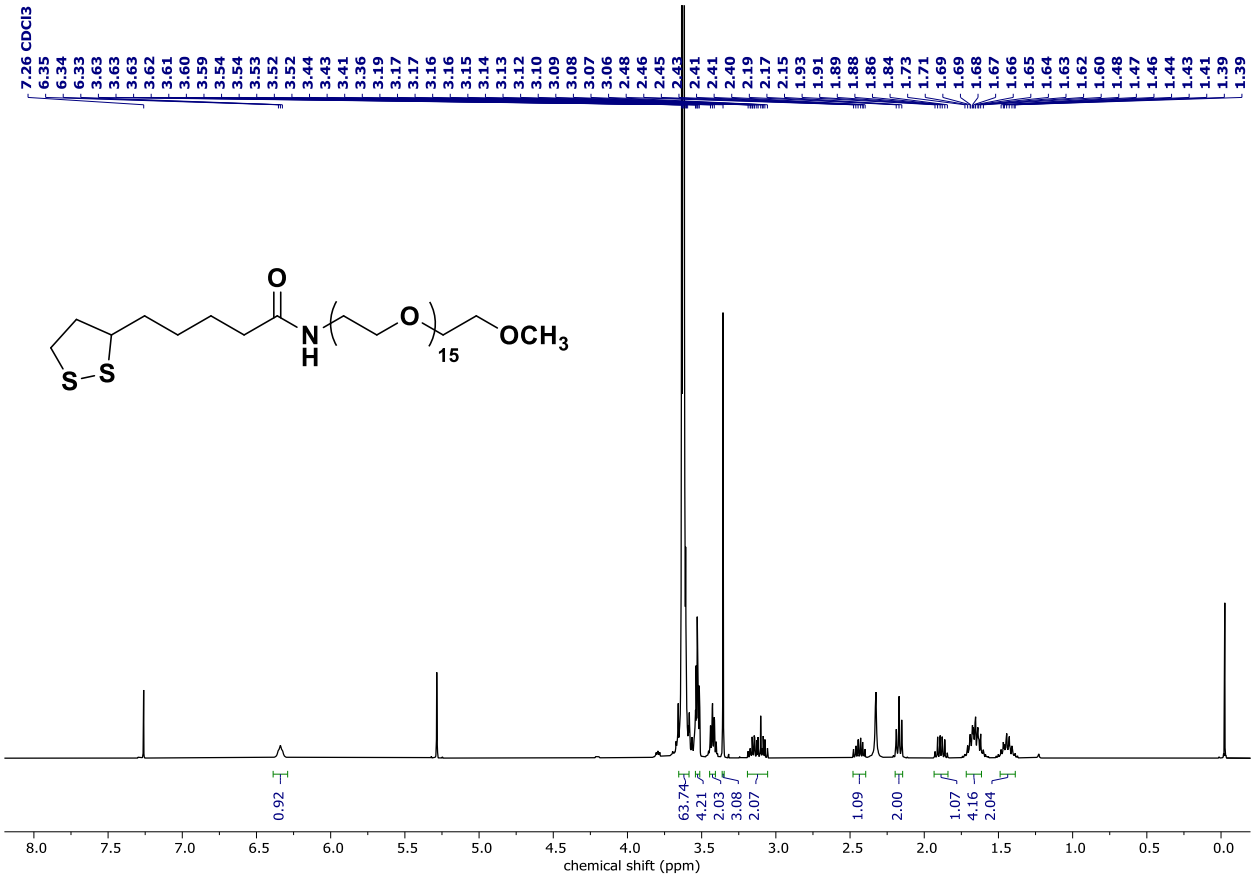
AT-IR spectra of Compound 2



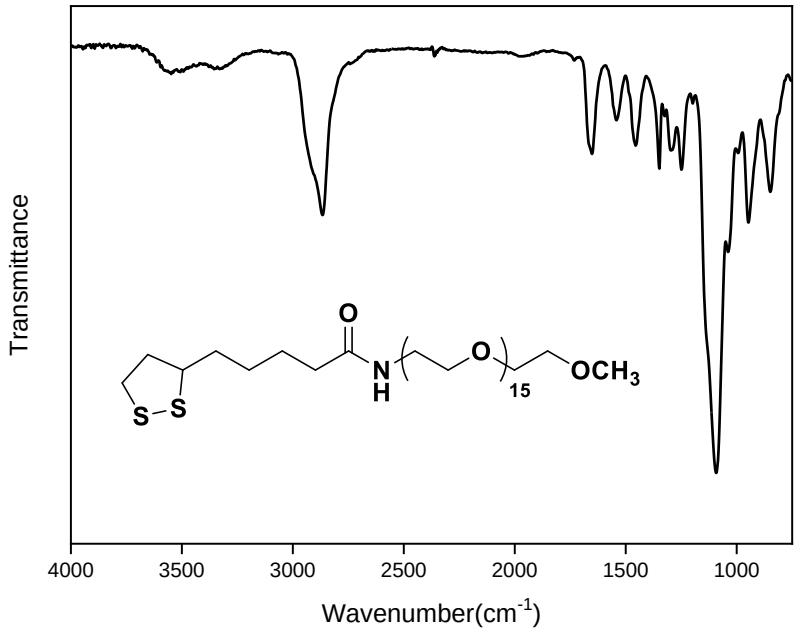
^{1}H NMR of Compound 1a



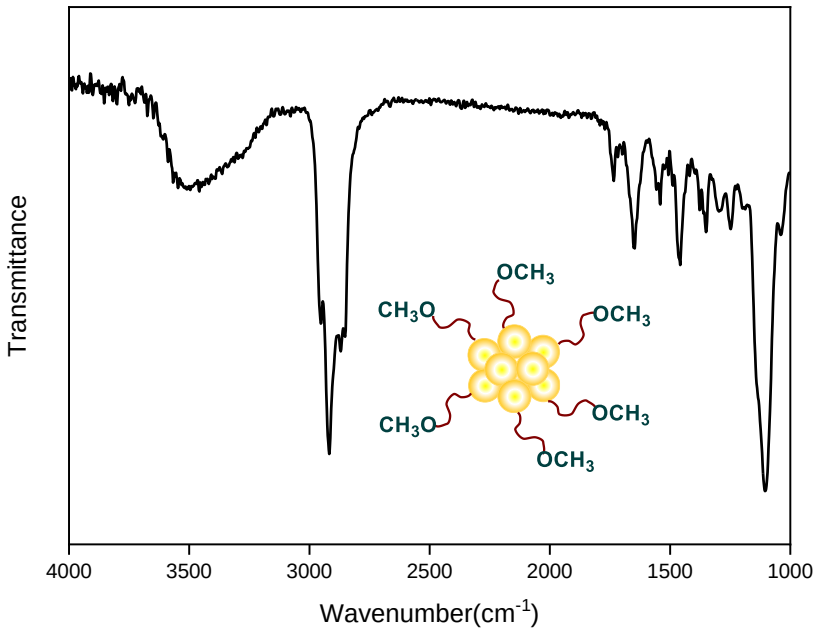
^{1}H NMR of Compound 1



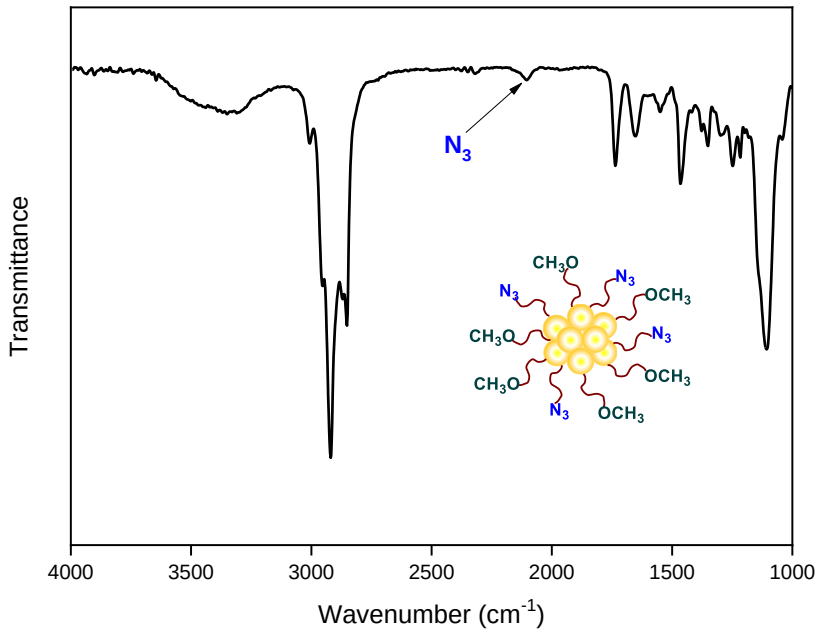
AT-IR spectra of Compound 1



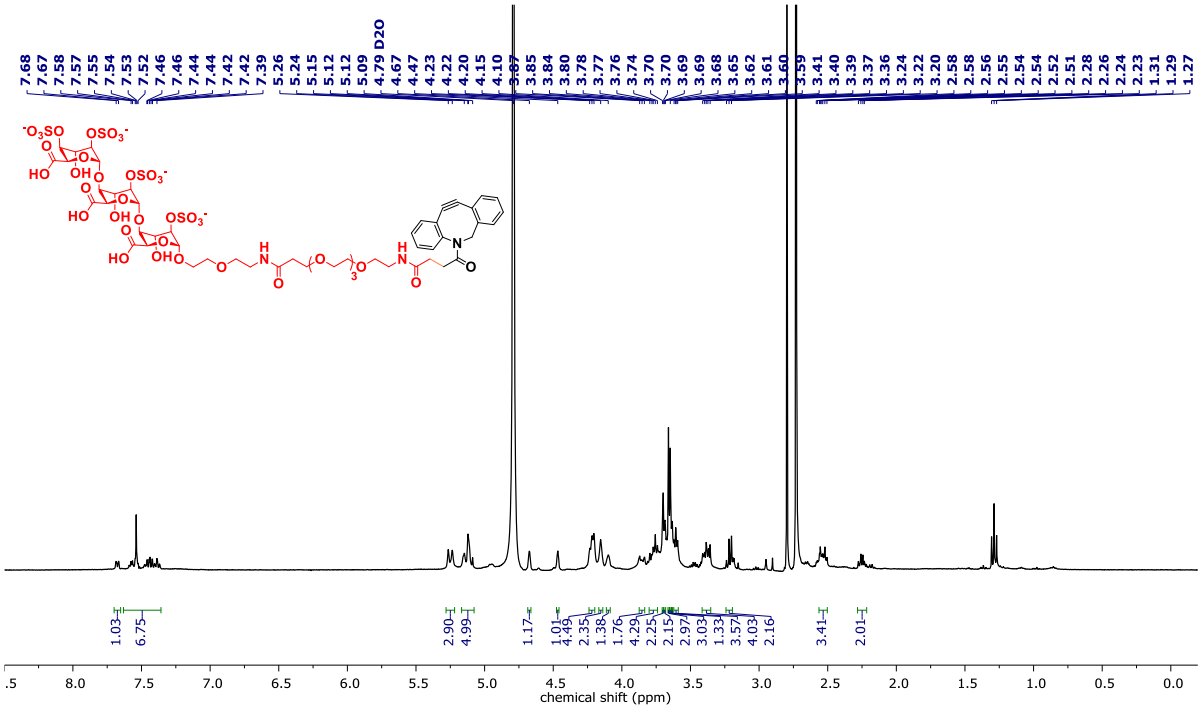
AT-IR spectra of AuNC@OMe



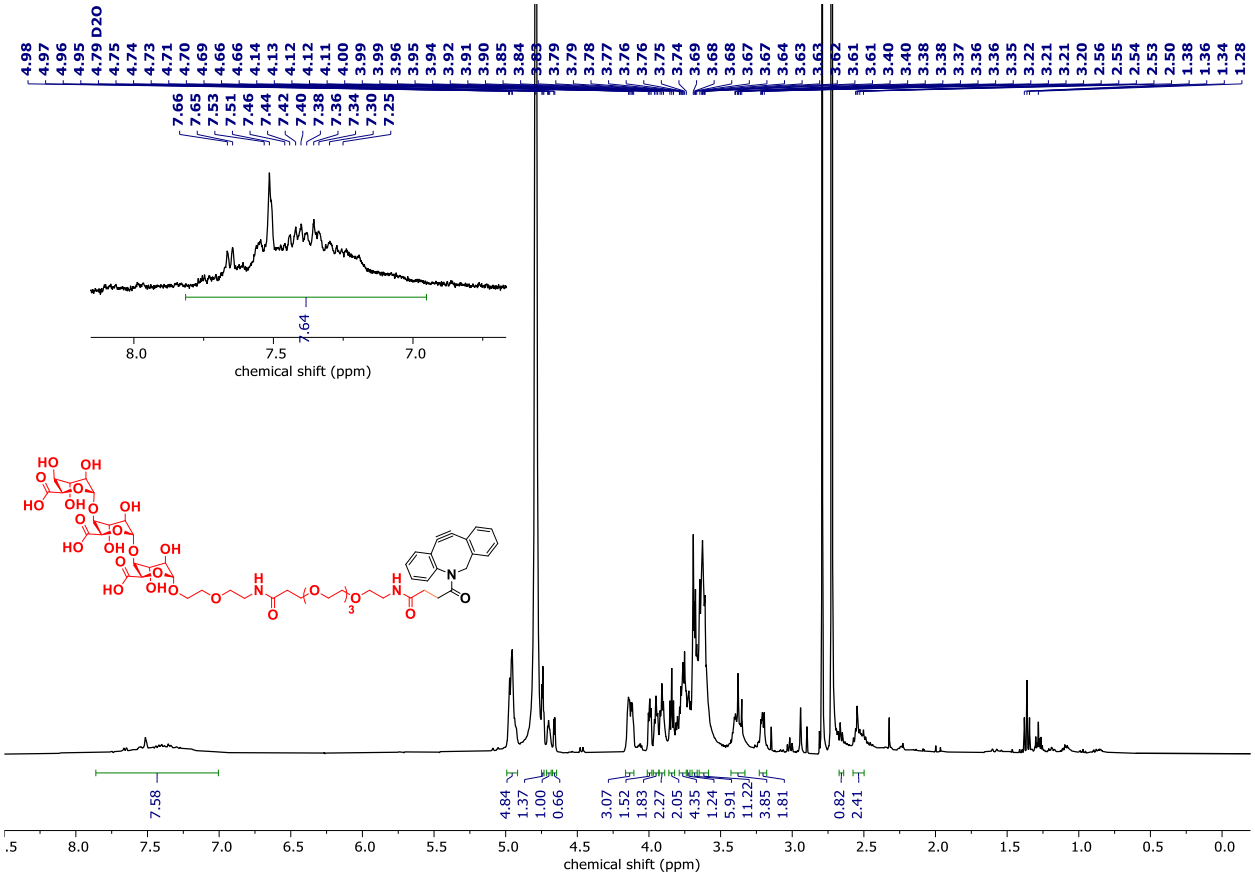
AT-IR spectra of AuNC@N_3



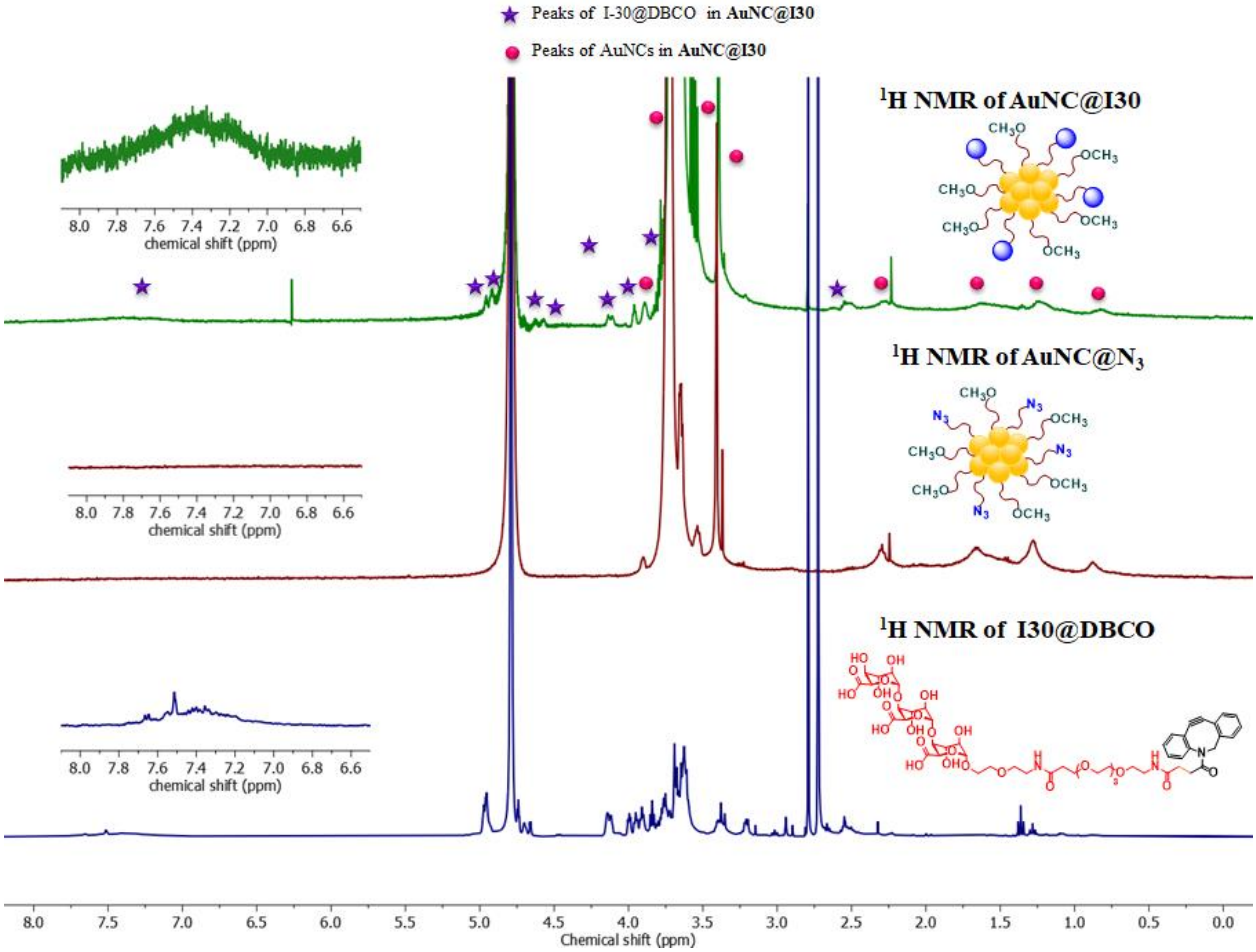
^{1}H NMR of I34@DBCO (4)



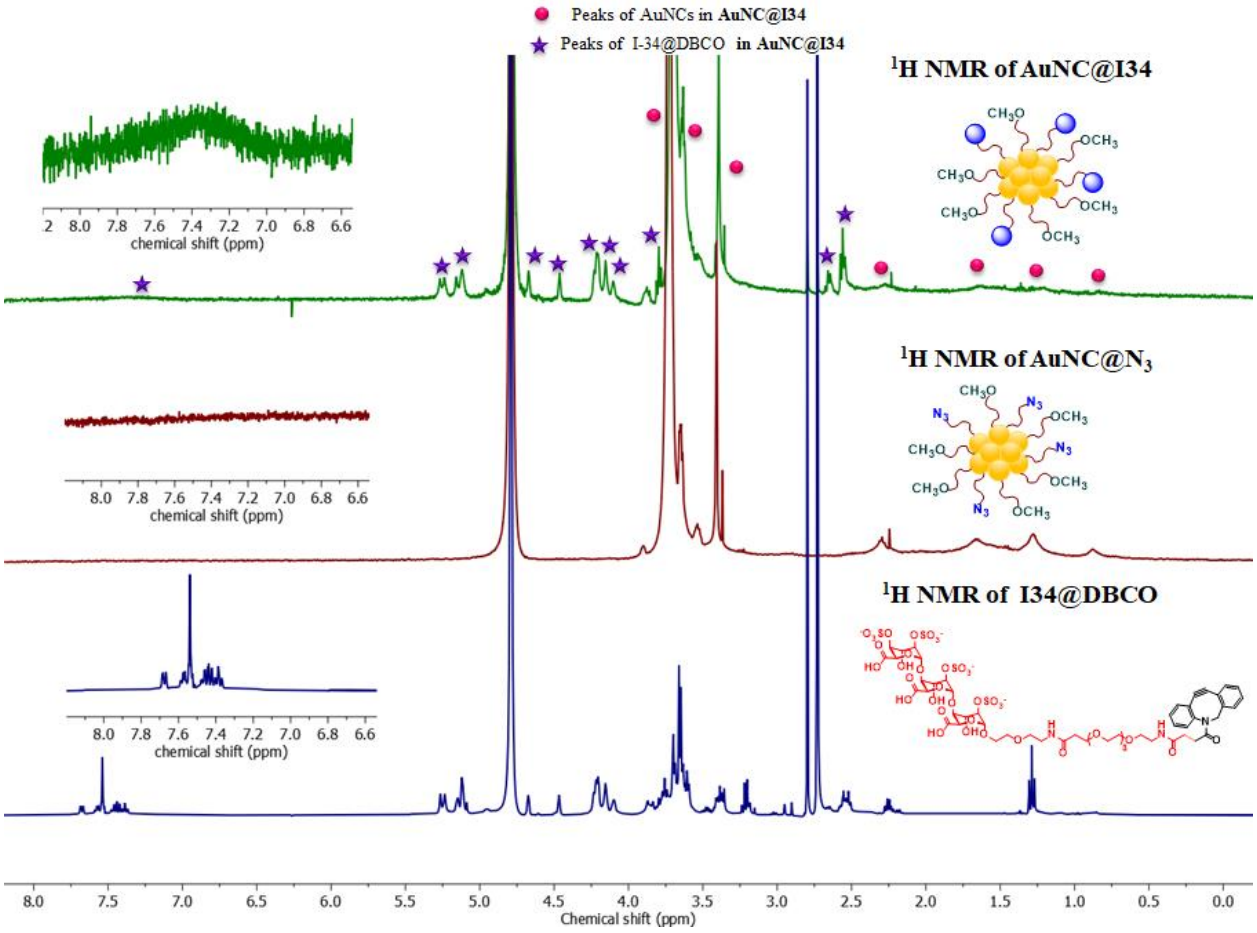
^{1}H NMR of I30@DBCO (3)



^{1}H NMR comparision for AuNC@I30



^{1}H NMR comparision for AuNC@I34



Comparision of AT-IR spectra of AuNC@N_3 and subsequent click reactions