Electronic supporting information

Hypoxia Targeting Lutetium-177 Labeled Nitroimidazole Decorated Gold Particles as Cancer Theranostic Nanoplatforms

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Materials and methods

Gold (III) chloride hydrate ($AuHCl_4.3H_2O$) was purchased from Sigma Aldrich, USA. Chemicals of analytical grade such as 2-nitroimidazole, N-boc-bromopropyl amine, trifluoroacetic acid (TFA), lipoic acid, fluorescein-5-isothiocyanate (FITC) and sodium borohydride were procured from Sigma chemical Inc., USA. Anhydrous potassium carbonate was purchased from Fluka, Germany. Lipoic acid modified DOTAGA (TA-DOTAGA) was purchased from Chematech, France. Silica gel plates (silica gel 60 F_{254}) used for analytical TLC as well as silica gel (60–120 mesh) used for column chromatography were obtained from Merck, India. The PD-10 (Sephadex G-25) columns and Amicon Ultra centrifugal filter devices (MWCO 3KDa) used for purification purposes were obtained from GE Healthcare Life Sciences, USA and Millipore, India, respectively. Lutetium-177 as $[^{177}Lu]LuCl_3$ solution used for radiolabeling was produced in-house following a procedure reported elsewhere. Specific activity of $[^{177}Lu]LuCl_3$ used was ~20 mCi/µg (~740 MBq/µg). Well-type NaI(Tl) detector (ECIL, India) was used for radioactivity measurements in vitro. Integral line flat-bed type NaI(Tl) detector (ECIL, India) was used for determining radioactivity associated with various organs and tissues after biodistribution studies. HPLC analysis were performed on a JASCO HPLC system (JASCO, Japan) coupled to a NaI(Tl) radioactivity detector (Raytest, Germany) and a PU 1575 UV/Vis detector (JASCO, Japan) using a TSK gel column (G3000 SWXL; 30 cm × 7.8 mm; 5 µm) from TOSOH Bioscience, USA. Whatman No.1 chromatography paper was used for the paper chromatography (PC) and analyzed by MiniGITA TLC scanner from Elysia Raytest with BGO detector. All the solvents used for HPLC were filtered and degassed prior to use and were of HPLC grade. UV/Vis-spectra were recorded using Bruker UV/Vis spectrophotometer, Japan. Infrared spectra of synthesized compounds were recorded on a JASCO FT/IR-420 spectrophotometer, Japan. ^1H NMR spectra were recorded on a 300 MHz Varian VXR 500 S spectrophotometer, USA. Low

resolution mass spectra were recorded on Advion Mass Spectrometer, USA, in electron spray ionization (ESI) mode. Dynamic light scattering (DLS) measurements were carried out using Malvern 4800 Autosizer (Malvern Instruments, UK), equipped with 15 mW He/Ne laser (632.8) nm, APD detector and 7132 digital correlator. The zeta potential measurements were carried out using Zetasizer nanoseries (Malvern instruments, UK) by phase analysis light scattering. Images of the surface modified nanoparticles were obtained on a Zeiss-Carl-Libra-120 Transmission electron microscope (TEM) using a LaB6 filament and an acceleration potential of 200 kV. The sample for TEM analysis were prepared by depositing a drop of nanoparticles suspension on a carbon coated copper grid. TEM images were processed using ImageJ software. Cyclic voltammograms were recorded on a CH instrument (CHI760D), USA. Guava easyCyte^{TM} Flow Cytometer System (Merck Millipore, Germany) was used for carrying out cytotoxicity studies. Guava Flow cytometer kits were purchased from Merck KGaA (Darmstadt, Germany). Dissolved oxygen (in ppm) in CHO cell suspension during in vitro cell uptake studies was measured using portable waterproof dissolved oxygen meter (Model HI9143M) procured from Hanna Instruments, Portugal.

Synthesis of 5-(1,2-dithiolan-3-yl)-N-(3-(2-nitro-1H-imidazol-1-yl)-propyl) pentanamide (2-NIM-TA, 3)

*(i) Synthesis of tert-butyl-3-(2-nitro-1H-imidazol-1-yl)propyl carbamate (**1**)*

To a solution of 2-nitroimidazole (0.22 g, 2.0 mmol) in acetonitrile (15 mL), crushed anhydrous K_2CO_3 (1.20 gm, 8.6 mmol) was added followed by the drop-wise addition of *tert*-butyl *N*-(3-bromopropyl)carbamate (0.44 g, 1.8 mmol). The reaction mixture was subsequently refluxed for 12h with continuous stirring. The residue obtained on removal of the solvent was dissolved in water (30 mL) and the aqueous layer was extracted with chloroform (15 mL x 3). Combined organic extracts were dried over anhydrous Na_2SO_4.

After drying, the organic extract was filtered and concentrated under vacuum. Crude product thus obtained was purified by silica gel column chromatography using diethyl ether to obtain compound **1** (0.39 g, 80%). R_f= 0.7 (diethyl ether), IR (neat, ν_{max}/cm^{-1}) 3336 (m), 3111 (s), 2920 (m), 1684 (s), 1536 (s), 1486 (s), 1451 (m), 1360 (s), 1273 (m), 1250 (m), 1164 (s). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.46 (s, 9H, -C(C**H**$_3$)$_3$), 2.04 (quin, *J* = 6.9 Hz,, 2H, -CH_2C**H**$_2$$CH_2$NHCO-), 3.18 (t, *J* = 6.9 Hz, 2H, -CH_2CH_2C**H**$_2$NHCO-), 4.44 (t, *J* = 6.9 Hz, 2H, -C**H**$_2$$CH_2CH_2$NHCO-), 7.16 (s, 1H, Ar**H**), 7.54 (s, 1H, Ar**H**); ESI-MS *m/z*: 271.0 $(M+H)^+$, (*m/z* calcd for $C_{11}H_{18}N_4O_4$: 270.13).

*(ii) Synthesis of 3-(2-nitro-1H-imidazol-1-yl)propan-1-amine hydrochloride (**2**)*

Compound **1** (0.32 g, 1.2 mmol) was added to the mixture of methanol (2 mL) and 6N HCl (5 mL). The mixture was then heated at 50°C for 12h with continuous stirring. Subsequently, the solvent was removed under vacuum to obtain compound **2** in quantitative yield as hydrochloride salt. IR (neat, ν_{max}/cm^{-1}): 3338 (br, m), 2971 (s), 2928 (m), 1536 (s), 1507 (s), 1487 (s), 1365 (s), 1280 (m), 1159 (m), 1106 (s). ^{1}H NMR (300 MHz, D_2O): δ = 2.20 (quin, *J* = 6.8 Hz, 2H, -CH_2C**H**$_2$$CH_2NH_2$), 3.04 (t, *J* = 6.8 Hz, 2H, -CH_2CH_2C**H**$_2NH_2$), 4.50 (t, *J* = 6.8 Hz, 2H, -C**H**$_2$$CH_2CH_2NH_2$), 7.14 (s, 1H, Ar**H**), 7.42 (s, 1H, Ar**H**)); ESI-MS *m/z*: 171.1 $(M+H)^+$, (*m/z* calcd for $C_6H_{10}N_4O_2$: 170.08).

*(iii) Synthesis of 5-(1,2-dithiolan-3-yl)-N-(3-(2-nitro-1H-imidazol-1-yl)-propyl)pentanamide (**3**)*

To the solution of TA (0.05 g, 0.3 mmol) in DMF (5 mL), HATU (0.1 g, 0.3 mmol) and TEA (0.06 g, 0.6 mmol) was added. The reaction mixture was stirred for 10 min and then compound **2** (0.05 g, 0.3mmol) was added. The reaction was continued at room temperature for 16h. Subsequently, the solvent was removed under vacuum and the residue was dissolved in ethyl acetate (20 mL). The organic layer was washed with water (30 mL x 3) and dried

over anhydrous Na_2SO_4. Subsequently, the organic layer was filtered and concentrated under vacuum. The crude product was purified by silica gel column chromatography using 5% methanol in chloroform (v/v) to obtain pure compound (**3**) (0.07 g, 70%). R_f = 0.7 ($CHCl_3$/MeOH, 9:1 v/v), IR (neat, ν_{max}/cm^{-1}): 3435 (br, s), 2971 (s), 2928 (m), 1634 (s), 1525 (w), 1485 (w), 1360 (m), 1280 (m), 1150 (m), 1106 (s). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.55-1.57 (m, 2H, -CO-$(CH_2)_2$-C**H**$_2$-CH_2-), 1.74-1.81 (m, 4H, -CO-CH_2-C**H**$_2$-CH_2-C**H**$_2$-), 1.96-2.05 (m, 1H, -CH-S-S-CH_2-C**H**H-), 2.16 (m, 2H, 2-NIM-CH_2-C**H**$_2$-CH_2), 2.31 (t, 2H, -CO-C**H**$_2$-$(CH_2)_3$-), 2.54 (m, 1H, -CH-S-S-CH_2-C**H**H-), 3.25 (m, 2H, S-S-C**H**$_2$-CH_2), 3.45 (m, 2H, 2-NIM-CH_2-CH_2-C**H**$_2$), 3.66 (m, 1H, S-S-C**H**-CH2), 4.55 (t, 2H, 2-NIM-C**H**$_2$-CH_2-CH_2), 7.23 (s, 1H, Ar**H**), 7.42 (s, 1H, Ar**H**); ^{13}C NMR (300 MHz, $CDCl_3$): δ = 173.68, 128.66, 126.83, 56.60, 47.97, 40.42, 38.64, 36.50, 36.35, 34.72, 31.29, 29.02, 25.47; ESI-MS *m/z*: 397.1 $(M+K)^+$, (*m/z* calcd for $C_{14}H_{22}N_4O_3S_2K$: 397.21).

*Preparation of DOTAGA-AuNP-2-NIM (**4**)*

To prepare 2-NIM-decorated, DOTAGA-conjugated gold nanoparticles, hydrogen tetrachloroaurate(III) trihydrate ($HAuCl_4.3H_2O$, 6.00 mg, 16.0 µmol) was dissolved in methanol (5 mL) and the solution was stirred at room temperature. To this, aqueous solution of DOTAGA-TA (16.00 mg, 23.0 µmol, 2.5 mL) and methanolic solution of 2-NIM-TA (**3**) (9.00 mg, 23.0 µmol, 2.5 mL) were added together, drop-wise, with continuous stirring. Immediate change in color of the reaction mixture from light yellow to deep orange indicated the formation of thiol–Au complex. After stirring the reaction mixture for 10 min at 25°C, ice-cold aqueous solution of $NaBH_4$ (6.00 mg, 0.16 mmol, 5 mL) was added drop-wise. A further change of color of the reaction mixture from orange to deep purple was observed. The reaction was continued for 2h. Subsequently, nanoparticles were separated by centrifugal filtration using Amicon Ultra centrifugal filter (MWCO 3 KDa). The DOTAGA-AuNP-2-NIM obtained was lyophilized and stored at 4°C.

*Preparation of DOTAGA-AuNP (**5**)*

DOTAGA-AuNP used as control for in vitro experiments was prepared following the same procedure mentioned earliesr using an aqueous solution of DOTAGA-TA.

*2.2.4. Preparation of FITC-tagged AuNPs[DOTAGA-AuNP(FITC)-2-NIM](**6**)*

The DOTAGA-AuNP-2-NIM prepared earlier was further functionalized with FITC to impart florescence. Briefly, DOTAGA-AuNP-2-NIM nanoparticles (1.00 mg) were dispersed in water (5 mL). To this solution, about 100 µL of FITC in water (500 µg/mL) was added and the solution stirred overnight. Subsequently, the nanoparticles were purified using Amicon Ultra centrifugal filter (MWCO 3 KDa), lyophilized and stored at 4°C.

Electrochemical studies

Cyclic voltammogram of nitroimidazole decorated gold nanoparticles was carried out using a glassy carbon working electrode, platinum counter electrode and an Ag/Ag^{+} as reference electrode, following a procedure previously reported by Lei Mei et al. Solutions for analysis were freshly prepared in anhydrous DMF and contained ~5 mM analyte and tetrabutylammonium perchlorate (TBAP) (0.1 mol/mL). Ferrocene was used as a reference standard for which the one electron reduction process occurs at $E_{1/2}$ = 0.47 V in DMF against standard calomel electrode. Before recording the voltammograms, the test solution was thoroughly purged with high purity argon gas to remove any tracers of dissolved oxygen. Scan rate was maintained at 50 mV/s.

Radiolabeling of DOTAGA-AuNP-2-NIM with ^{177}Lu

To a solution of DOTAGA-AuNP-2-NIM (0.20 mg) in water (100 µL), 1.5 M NaOAc buffer (200 µL, pH 5.6) was added followed by [^{177}Lu]$LuCl_3$ (20 µL, 148 MBq). The mixture was incubated in a water bath at 100°C for 30 min. After cooling the reaction mixture to ambient temperature, the radiolabeled preparation was purified by passing through PD-10 column pre-conditioned with phosphate buffered saline (PBS). The column was eluted with PBS and

different fractions (1 mL) were collected. [^{177}Lu]Lu-DOTAGA-AuNP-2-NIM nanoparticles were obtained in 3rd and 4th fractions which were pooled together. Free [^{177}Lu]$LuCl_3$ remained trapped in the column.

Quality control

The radiochemical purity (RCP) of [^{177}Lu]Lu-DOTAGA-AuNP-2-NIM was determined both by PC and HPLC. For this purpose, 5 µL of the preparation was spotted on a PC strip 1.5 cm from the bottom. The PC strip was developed in 10 mM DTPA solution. Subsequently, the PC strip was dried and radioactivity distribution was analyzed on a TLC Scanner. As a control, PC of [^{177}Lu]$LuCl_3$ solution was also performed in 10 mM DTPA. In PC, [^{177}Lu]Lu-DOTAGA-AuNP-2-NIM remained at the point of spotting while [^{177}Lu]$LuCl_3$ moved with the solvent front. The radiochemical purity of [^{177}Lu]Lu-DOTAGA-AuNP-2-NIM was determined from peak area measurement using GINA star TLC scanner software and expressed as the percentage of the total activity associated with gold nanoparticles.

The HPLC analysis of [^{177}Lu]Lu-DOTAGA-AuNP-2-NIM was carried out using 0.05 M phosphate buffer (pH = 7.4) with 0.05% NaN_3 as the mobile phase in isocratic mode. Flow rate of the solvent was maintained at 0.5 mL/min. About 10 µL of the radioactive preparation was injected in the column and elution was monitored by observing the radioactivity profile. The RCP of the [^{177}Lu]Lu-DOTAGA-AuNP-2-NIM was determined by peak area integration from the chromatogram.

In vitro stability of radiolabeled nanoparticles

In vitro stability of [^{177}Lu]Lu-DOTAGA-AuNP-2-NIM nanoparticles was determined in PBS and human serum over a period of one week using PC at different time points. For this purpose, the radiolabeled preparation (200 µL) was incubated with PBS/human serum at room temperature for a period of 1 week. The solution was analyzed periodically by PC using 10 mM DTPA solution as described in earlier Section.

Cytotoxicity studies of DOTAGA-AuNP-2-NIM by flow cytometry

Cytotoxicity of DOTAGA-AuNP-2-NIM in CHO cells was evaluated by flow cytometry. Approximately, 1×10^6 pre-seeded CHO cells were incubated with different concentrations of DOTAGA-AuNP-2-NIM (0.004-1600 ng/ml) along with vehicle control (PBS) for 24h at 37°C. Post incubation, cells were harvested by trypsinization and the cell number was maintained at 1×10^5 per tube (50 µL). Guava® ViaCount™ Reagent (350 µL) was added into each tube and incubated in dark for 25 min at room temperature. Samples were acquired on a flow cytometer and analyzed using Guava Soft 3.1.1. The Via Count assay distinguishes viable and non-viable cells based on differential permeability of two DNA-binding dyes in the flow cytometer. The nuclear dye stains only nucleated cells, while the viability dye brightly stains dying cells. Debris is excluded from results based on negative staining with the nuclear dye**.** During analysis, apoptotic marker was set up according to analysis protocol for distinguishing apoptotic and necrotic populations.

In vitro cell uptake studies under hypoxic and normoxic conditions

Cellular uptake of [^{177}Lu]Lu-DOTAGA-AuNP-2-NIM in CHO cells under normoxic and hypoxic conditions was studied following a protocol reported earlier. About 15 mL aliquot of CHO cells (1 x 10^6 cells/mL) in suspension culture at 37°C in RPMI medium, supplemented with 2 mM L-glutamine and 10% heat-inactivated fetal bovine serum, was kept stirring in a glass vial. The glass vial was flushed with a gentle, continuous flow of warm, humidified gas mixture of 95% air/5% carbon dioxide (aerobic exposure) or 95% nitrogen/5% carbon dioxide (hypoxic exposure). After equilibration for 45 min, the dissolved oxygen level reduced to ~800 ppm in medium exposed to 95% nitrogen/5% carbon dioxide (hypoxic exposure). Dissolved oxygen level in medium exposed to 95% air/5% carbon dioxide (aerobic exposure) was ~88440 ppm. Subsequently about 0.1 mL (5 MBq, ~40 ng) of radioactive preparation was added to the vial such that final activity of ~0.3 MBq/mL was

obtained. About 1 mL aliquots in triplicate were withdrawn from the vial at 2h, 3h and 4h post incubation. Cell aliquots withdrawn from vial under hypoxic exposure were immediately covered with a layer of mineral oil (100 μL) to prevent direct contact of cells with ambient atmosphere. The cells in suspension were spun down (at 250 g) into a pellet and supernatant was separated. Subsequently, the activity associated with the cell pellet and in supernatant was determined in a well-type gamma counter. The % cellular uptake was calculated using the following equation.

Percentage cellular uptake = [activity associated with cell pellet/(Sum of activity in supernatant and cell pellet)]*100

Similar procedure was followed to determine cellular uptake of [^{177}Lu]Lu-DOTAGA-AuNP-2-NIM under normoxic conditions. In this case, covering the cell aliquot with mineral oil is not necessary. Hypoxia selectivity of the radiotracer is determined by calculating the hypoxic/normoxic ratio, which is the ratio of % cellular uptake under hypoxic condition to that under normoxic condition.

Cell internalization study by Flow cytometry

Cellular accumulation of nitroimidazole tagged gold nanoparticles was studied using fluorescent analogue DOTAGA-AuNP(FITC)-2-NIM in CHO cell under hypoxic and normoxic conditions following similar procedure described in earlier Section. Cells were treated with DOTAGA-AuNP(FITC)-2-NIM (~40 ng) and incubated under normoxic and hypoxic conditions. At 3h post incubation, 1 mL aliquot (~1×10^6 CHO cells) was withdrawn in triplicate from the 15 mL bulk solution under hypoxic/normoxic condition. To estimate the cell uptake of DOTAGA-AuNP(FITC)-2-NIM, the cells were analyzed using Guava easyCyte^{TM} Flow Cytometer System and the mean fluorescence intensities were recorded.

In vivo studies

The distribution of [^{177}Lu]Lu-DOTAGA-AuNP-2-NIM in vivo was studied in Swiss mice bearing fibrosarcoma tumors. All animal studies were conducted following the protocols approved by the Institutional Animal Ethics Committee of Bhabha Atomic Research Centre, Trombay, Mumbai. Solid tumor models were developed in Swiss mice by implantation of HSDM1C1 murine fibrosarcoma cells obtained from National Centre for Cell Science (NCCS), Pune, India. About 1×10^6 cells were injected subcutaneously into the dorsum of each Swiss mouse. The tumors were allowed to grow till they were approximately 10 mm in diameter. Subsequently, the animals were used for experiment. Each animal was administered with the purified radioactive preparation (~3.7 MBq per animal in 100 μL volume) intravenously via lateral tail vein. After the injections, animals (n = 3) were incubated for different time intervals (3 h, 1 d, 2 d and 3 d). At the end of respective time intervals, the animals were sacrificed and relevant organs/tissue excised for the measurement of associated radioactivity. The organs were weighed and activity associated with them were measured in a flat-bed type NaI(Tl) counter with energy window adjusted for ^{177}Lu. Results were expressed as percentage injected activity per gram of organ (%ID/g ± s.d.).

Statistical analysis

Statistical analysis of relevant data was performed by one-way analysis of variance (ANOVA). Confidence level of 95% (p<0.05) was taken for statistical significance.

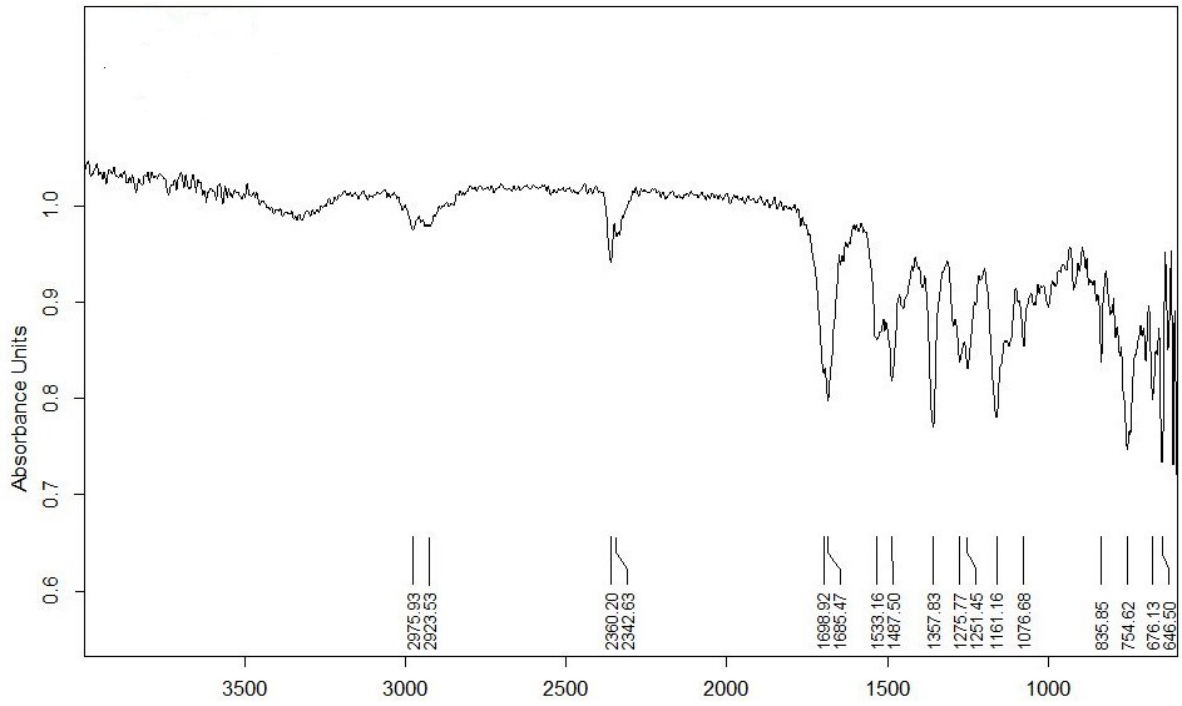

Fig. S1. FT-IR spectrum of tert-Butyl-3-(2-nitro-1H-imidazol-1-yl)propylcarbamate (**1**)

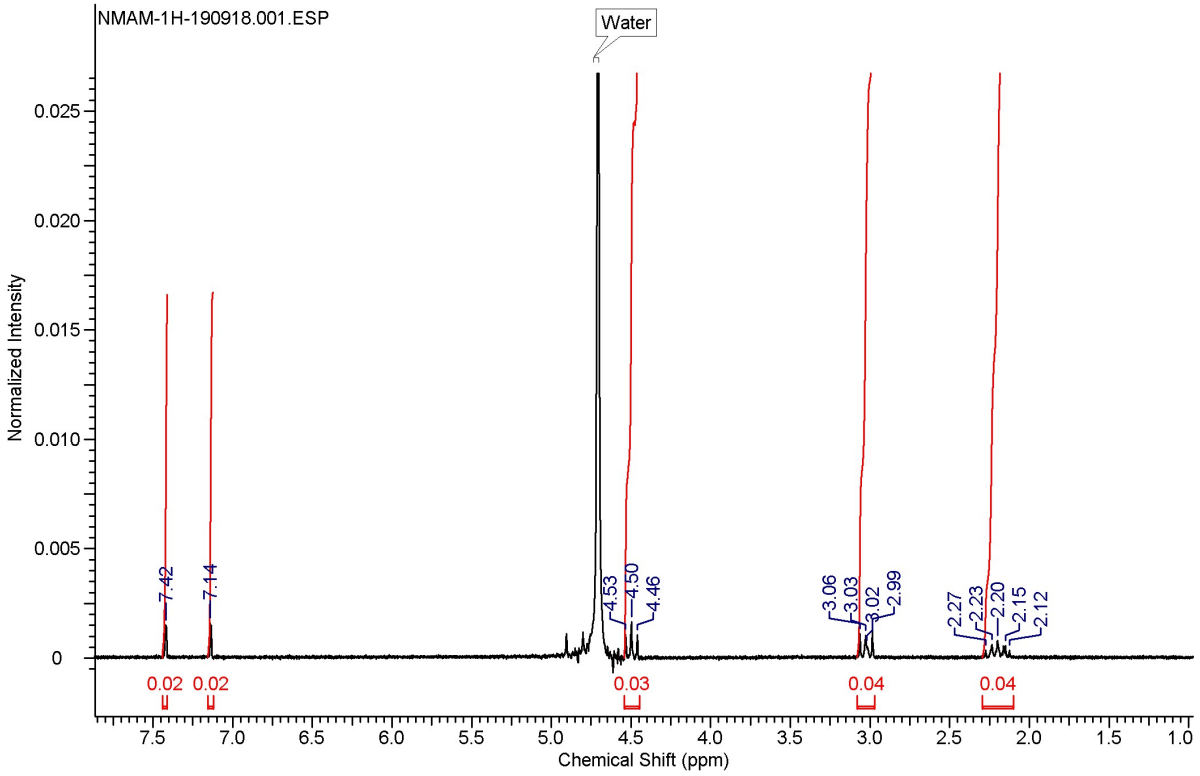

Fig. S2. ^{1}H-NMR spectrum of tert-Butyl-3-(2-nitro-1H-imidazol-1-yl)propylcarbamate (**1**)

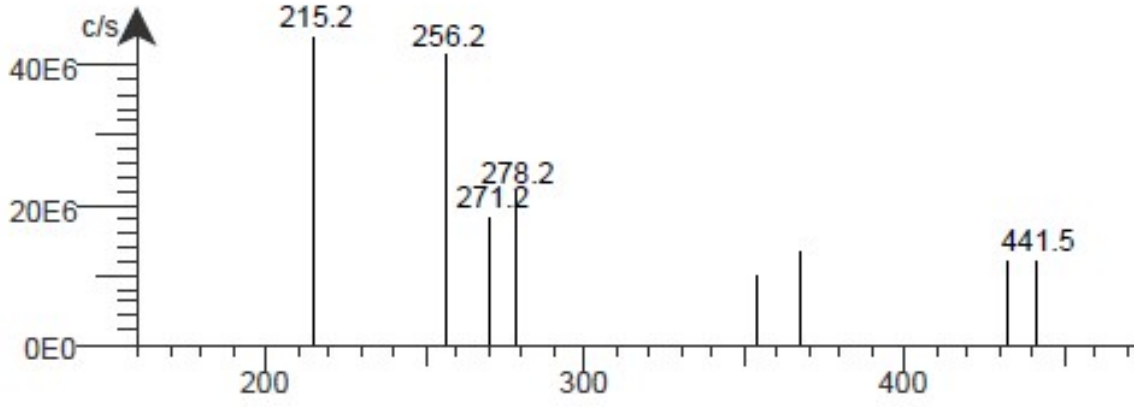

Fig. S3. Low resolution ESI-MS of tert-Butyl-3-(2-nitro-1H-imidazol-1-yl) propylcarbamate (**1**)

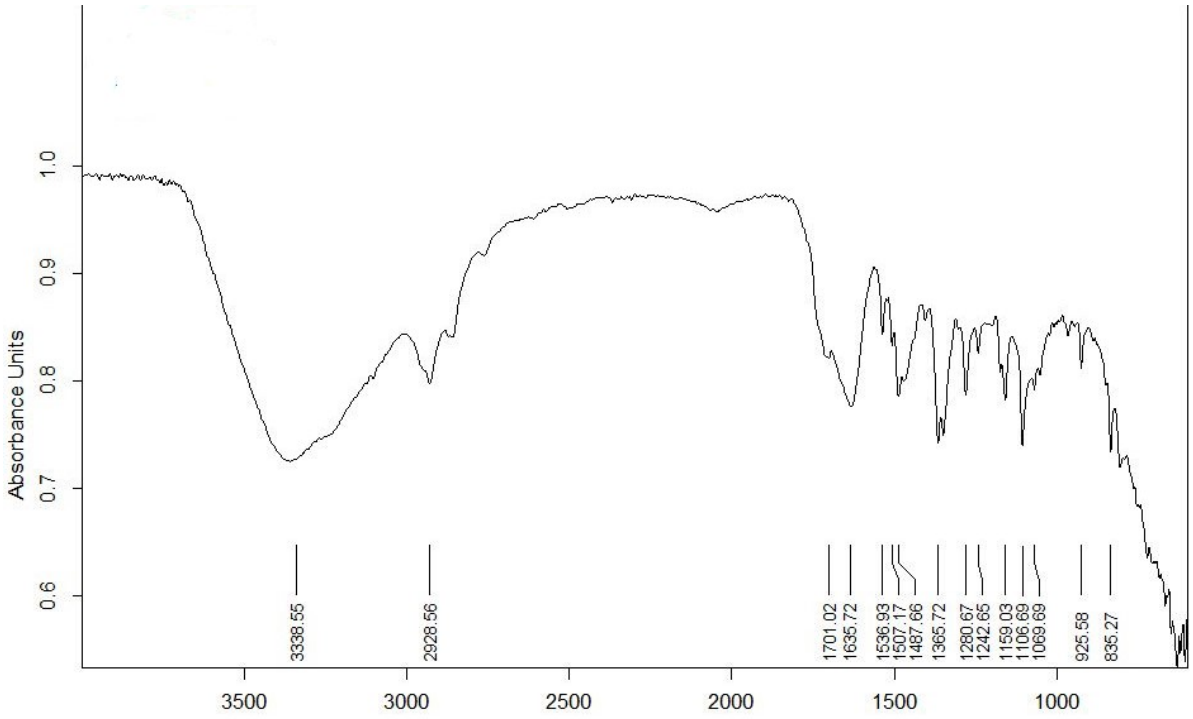

Fig. S4. FT-IR of 3-(2-nitro-1H-imidazol-1-yl) propan-1-amine hydrochloride (**2**)

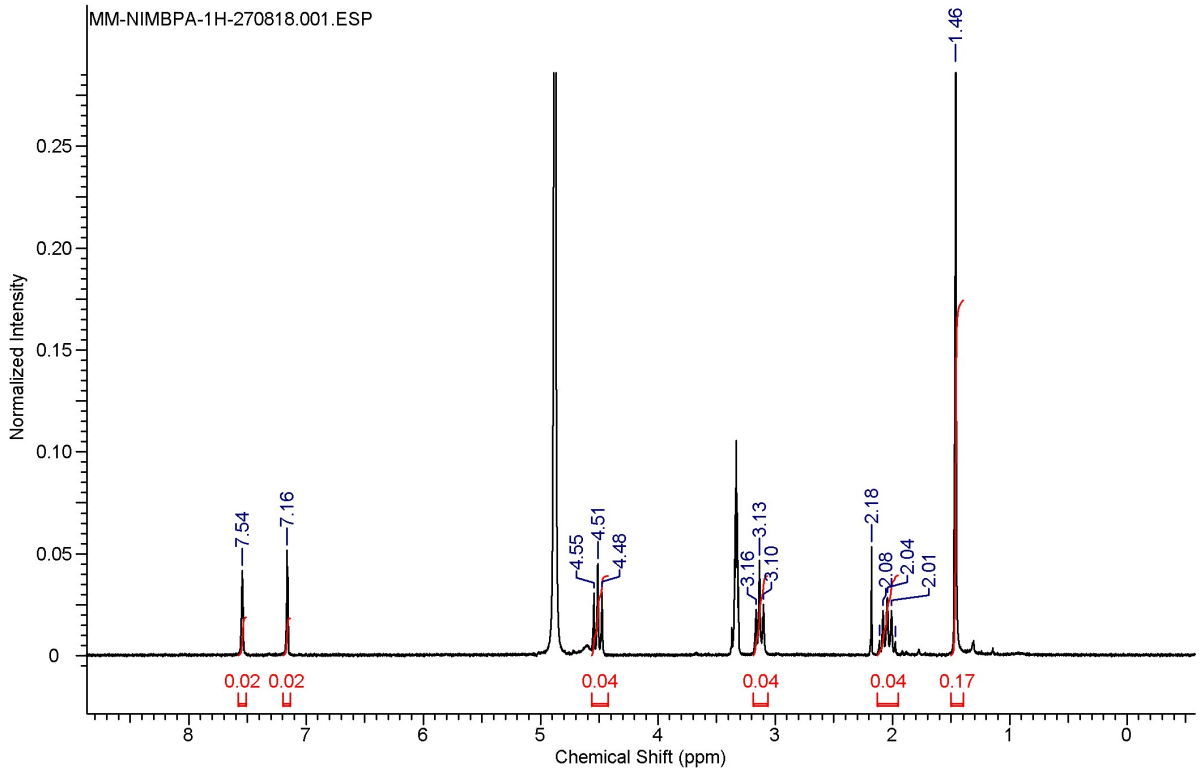

Fig. S5. [1]H-NMR of 3-(2-nitro-1H-imidazol-1-yl) propan-1-amine hydrochloride (**2**)

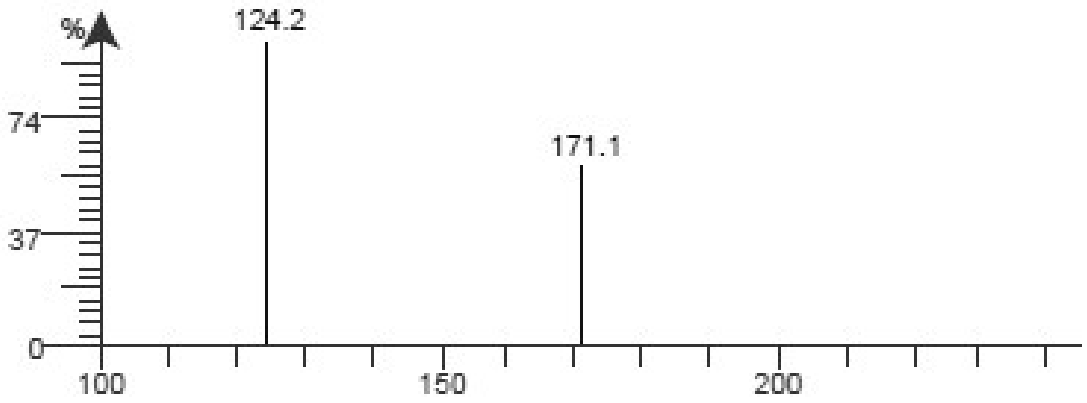

Fig. S6. Low resolution ESI-MS of 3-(2-nitro-1H-imidazol-1-yl) propan-1-amine hydrochloride (**2**)

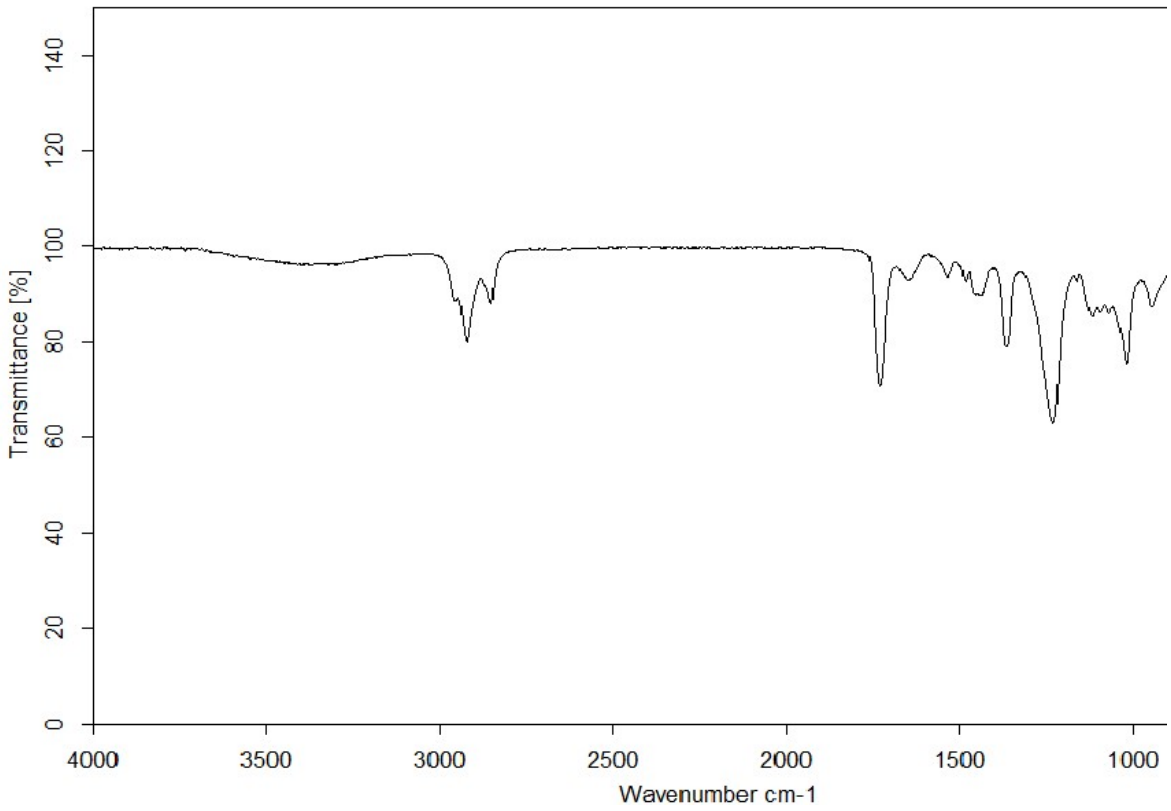

Fig. S7. FT-IR of 3-(2-nitro-1H-imidazol-1-yl) propan-1-amine Lipoic acid (**3**)

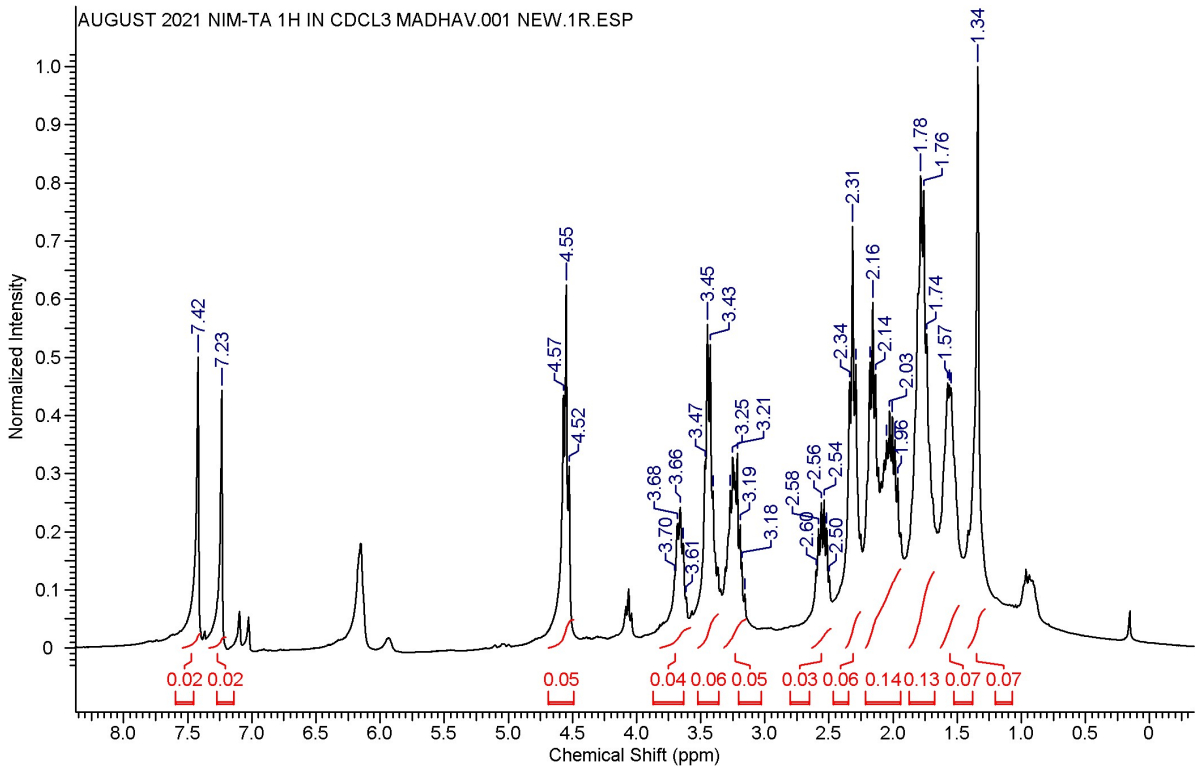

Fig. S8. ^{1}H-NMR of 3-(2-nitro-1H-imidazol-1-yl)propan-1-amine Lipoic acid (**3**)

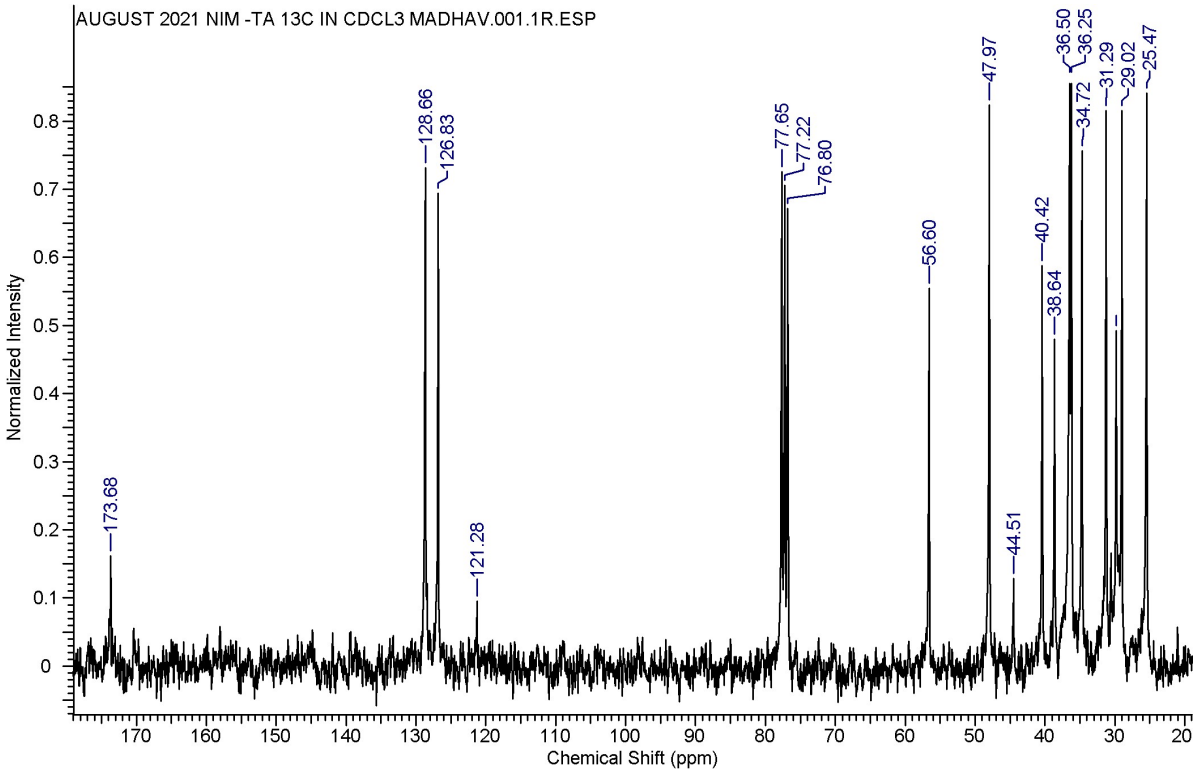

Fig. S9. ^{13}C NMR of of 3-(2-nitro-1H-imidazol-1-yl)propan-1-amine Lipoic acid (**3**)

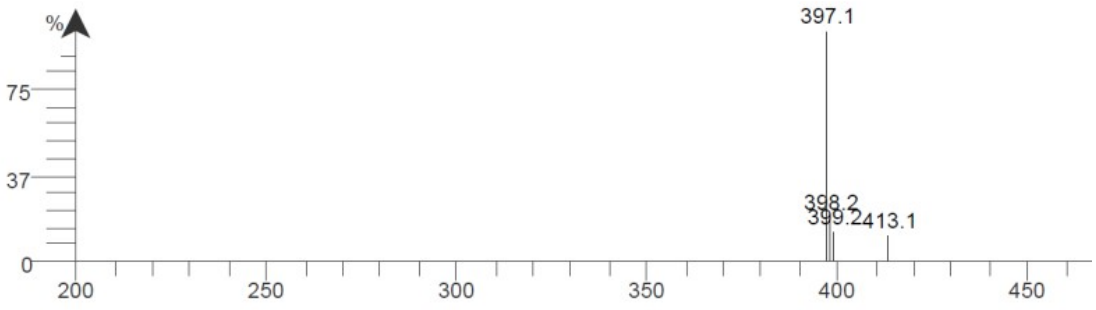

Fig. S10. Low resolution ESI-MS of 3-(2-nitro-1H-imidazol-1-yl)propan-1-amine Lipoic acid (**3**)

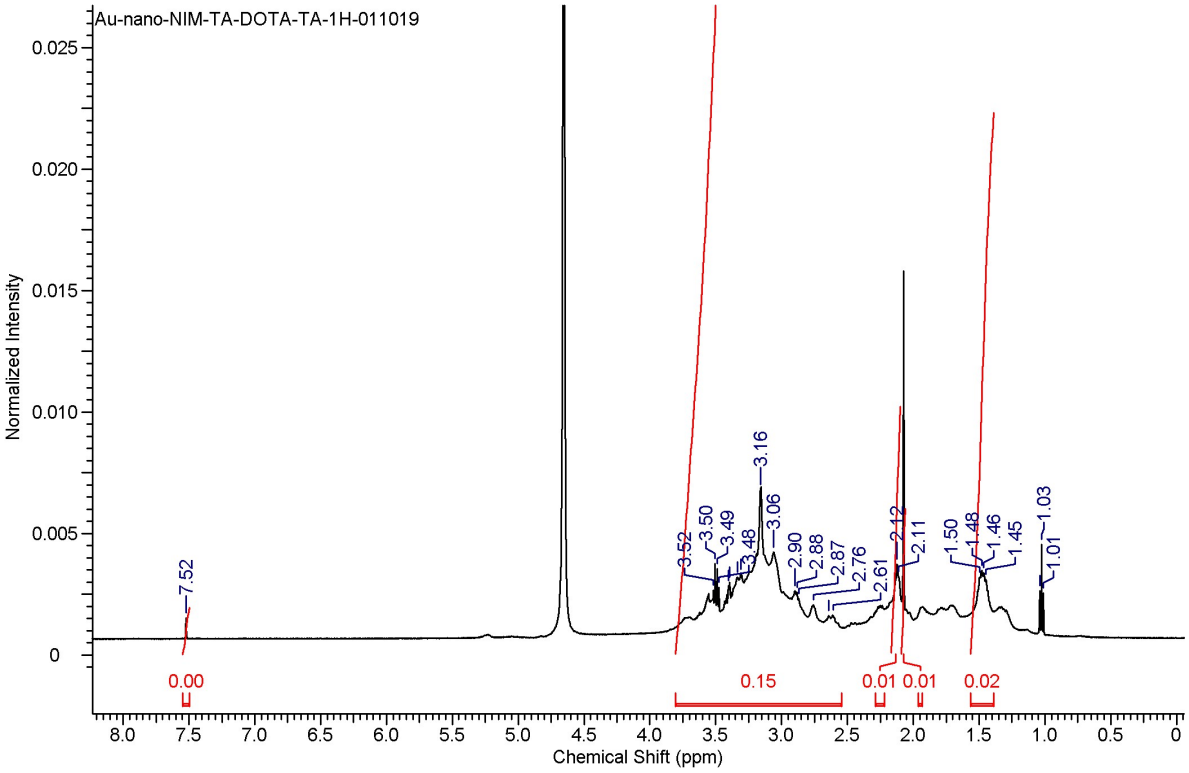

Fig. S11. ^{1}H-NMR spectra of Au-NIM-DOTAGA

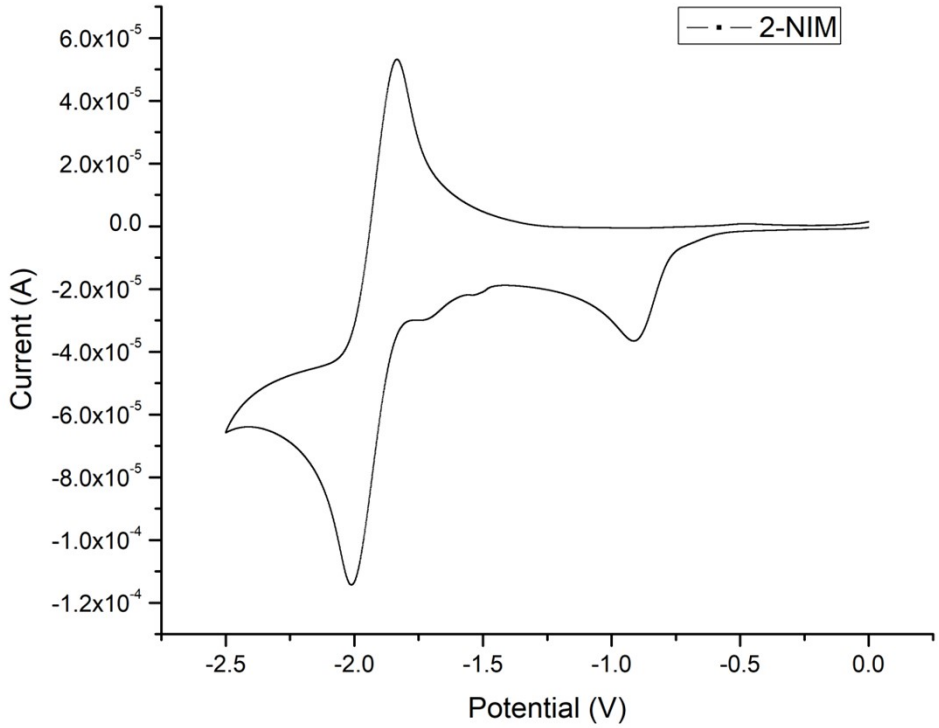

Fig. S12. Cyclic voltammogram of 2-nitroimidazole

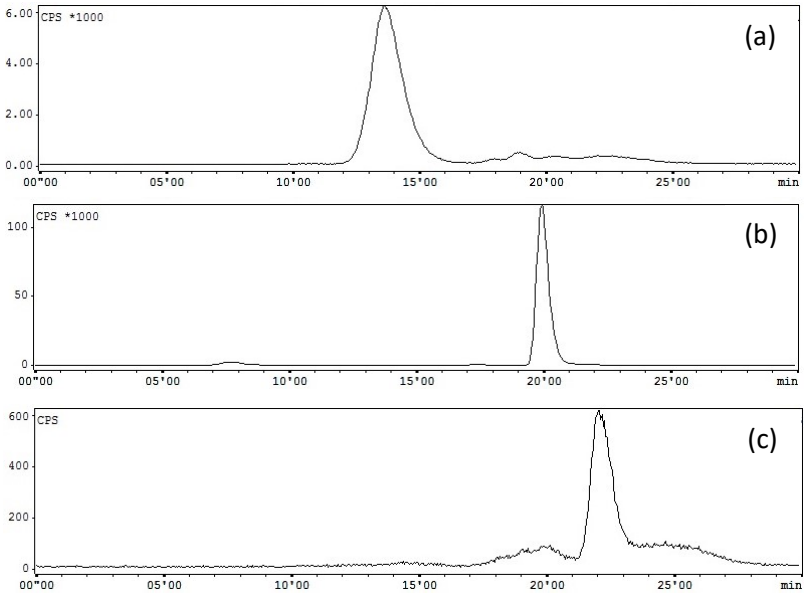

Fig. S13. Typical Size exclusion HPLC profiles (chromatogram) of (a) [^{177}Lu]Lu-DOTAGA-AuNP-2-NIM (b) [^{177}Lu]Lu-DOTAGA and (c) $^{177}LuCl_3$

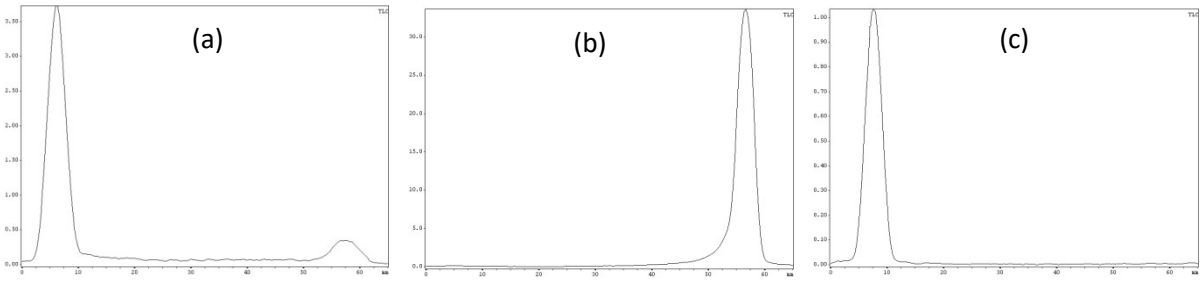

Fig. S14. (a) PC of [^{177}Lu]Lu-DOTAGA-AuNP-2-NIM before purification (b) PC of $^{177}LuCl_3$ and (c) PC of [^{177}Lu]Lu-DOTAGA-AuNP-2-NIM after purification

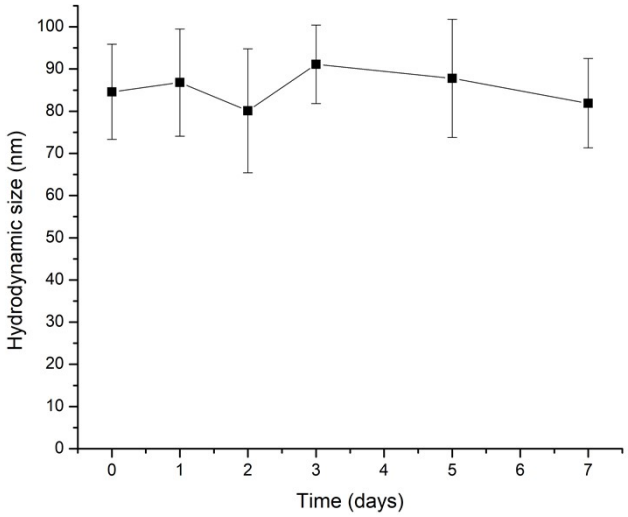

Fig. S15. Variation in hydrodynamic size of the radiolabeled nanoparticles with time

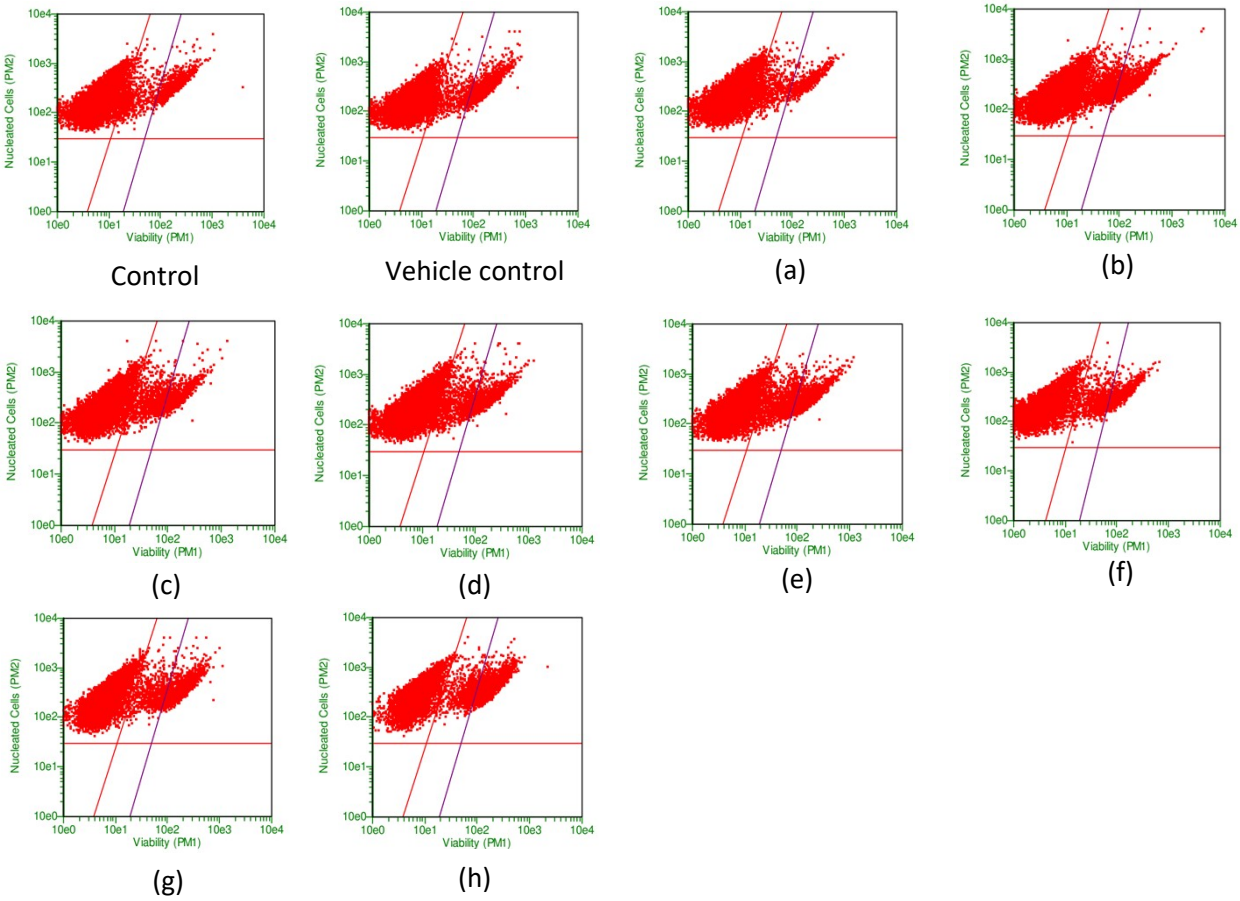

Fig. S16. Dot-plots showing viable, apoptotic and dead population of CHO cells treated with different concentrations (0.004-1600 ng/ml) of DOTAGA-AuNP-2-NIM obtained by flow cytometry