Electronic Supporting Information

Re-engineered Theranostic Gold Nanoparticles for Targeting Tumor Hypoxia

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1. Experimental

1.1. Materials and method

Hydrogen tetrachloroaurate(III) trihydrate ($HAuCl_4.3H_2O$) was purchased from Sigma Aldrich, USA. Chemicals of analytical grade such as 2-nitroimidazole (2-NIM), tert-butyl bromoacetate, dimethyl aminopyridine (DMAP), Fluorescein Isothiocyanate (FITC), 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), sodium borohydride ($NaBH_4$) and SH-PEG-2K-NH_2 were procured from Sigma Chemical Inc., USA. Hydroxybenzotriazole (HOBt) was purchased from Fluka, India. Anhydrous potassium carbonate (K_2CO_3) was purchased from Fluka, Germany. Bz-DOTA was purchased from Macrocyclics, USA. 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 Amicon Ultra centrifugal filter devices (MWCO 3KDa) used for purification purposes were obtained from Millipore, India. 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 during in-vitro cell studies. Integral line flat-bed type NaI(Tl) detector (ECIL, India) was used for determining radioactivity associated with various organs and tissues after biodistribution studies. ITLC-SG was used for chromatography and the developed strips were analyzed on MiniGITA TLC scanner with BGO detector (Elysia Raytest, Germany). 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. 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 nano series (Malvern instruments, UK) by phase analysis light scattering. Images of the surface modified nanoparticles were obtained on a EOL JEM 1400Plus TEM Transmission electron microscope (TEM) at an acceleration potential of 120 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 iTEM software. 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. Guava® easyCyte Flow Cytometer System (Merck Millipore, Germany) was used for carrying out cytotoxicity studies. Guava Flow cytometer kits were purchased from Merck KGaA (Darmstadt, Germany). Cell imaging studies were carried out in a confocal microscope (Leica Microsystems CMS GmbH, Mannheim, Germany) equipped with LEICA-DFC-7000T camera, and LAS (Leica Application Suit) software (Leica Microsystems CMS GmbH, Friedensplatz, Mannheim, Germany) at 10X magnification.

1.2. Synthesis

1.2.1. *Synthesis of 2-(2-nitro-1H-imidazol-1-yl)-tert-butyl acetate (**1**)*

To a solution of 2-nitroimidazole (0.34 g, 3 mmol) in acetonitrile (5 mL) crushed anhydrous K_2CO_3 (1.6 gm, 12 mmol) was added. To the above mixture, a solution of tert-butyl bromoacetate (0.58 g, 3 mmol) in acetonitrile was added dropwise followed by refluxing of the reaction mixture for 6 h. The progress of reaction was monitored by TLC. After completion of the reaction, solvent was removed under vacuum and the residue thus obtained was dissolved in chloroform (15 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 thus obtained was passed through a small silica column using diethyl ether to obtain compound **1** (0.57 g, 85 %). R_f = 0.8 (diethyl

ether), IR (neat, ν_{max}/cm^{-1}): 3121 (w), 2980 (w), 2933 (w), 1741 (s), 1540 (m), 1489 (s), 1362 (s), 1292 (m), 1238 (s), 1153 (s), 1138 (s), 945 (m), 855 (s). ^{1}H NMR (800 MHz, $CDCl_3$): δ =1.47 (s, 9H, -C**H**$_3$), 5.01 (s, 2H, NIM-C**H**$_2$CO), 7.09 (s, 1H, Ar**H**), 7.18 (s, 1H, Ar**H**);^{13}C-NMR: 27.9, 51.6, 84.19, 126.5, 128.3, 144.9, 164.9; ESI-MS *m/z*: 228.09 $(M+H)^+$, (*m/z* calcd for $C_9H_{13}N_3O_4$: 227.09).

1.2.2. *Synthesis of 2-(2-nitro-1H-imidazol-1-yl)acetic acid (**2**)*

The compound **1** (0.30 g, 1.3 mmol) was dissolved in dry chloroform (1 mL) and TFA (1 mL) was added followed by room temperature stirring for 3 h. Subsequently, the solvent was removed under vacuum and the crude obtained was recrystallized using diethyl ether and ethyl acetate solvent (1:1 v/v). The product could be obtained in near quantitative yield. IR (neat, ν_{max}/cm^{-1}): 3160 (w), 3146 (w), 2971 (w), 1729 (s), 1548 (m), 1495 (s), 1417 (s), 1369 (s), 1292 (m), 1222 (m), 1213 (m), 1160 (m), 1138 (m). ^{1}H NMR (800 MHz, DMSO-d6): δ = 5.32 (s, 2H, NIM-C**H**$_2$-), 7.31 (s, 1H, Ar**H**), 7.74 (s, 1H, Ar**H**); ^{13}C NMR: 51.13, 128.18, 128.93, 145.14, 169.05; ESI-MS *m/z*: 172.03 $(M+H)^+$, (*m/z* calcd for $C_5H_5N_3O_4$: 171.03).

1.2.3. *Preparation of PEGylated gold nanoparticles (AuNP-PEG-2K-NH_2, **3**)*

The PEGylated gold nanoparticles (AuNP-PEG-2K-NH_2) were prepared by the in situ reduction of hydrogen tetrachloroaurate(III) trihydrate ($HAuCl_4.3H_2O$) in presence of SH-PEG-2K-NH_2. To a 5 mL aqueous solution of $HAuCl_4.3H_2O$ (6.00 mg, 16.0 μmol), an aqueous solution (5 mL) of SH-PEG-2K-NH_2 (100 mg, 0.05 mmol) was added slowly 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 room temperature, ice-cold aqueous solution (5 mL) of $NaBH_4$ (6.00 mg, 0.16 mmol,) was added drop-wise. A further change of color from orange to brownish red was observed. The reaction was continued at room temperature for 3 h followed by centrifugal

filtration using Amicon Ultra centrifugal filter (MWCO 3 KDa). The nanoparticles (Au-PEG-2K-NH_2) thus obtained were lyophilized and stored at 4°C.

1.2.4. *Preparation of AuNP-PEG-2K-(2-NIM)(**4**)*

To a dispersion of AuNP-PEG-2K-NH_2 (**3**) in Milli Q water (300 µL), 100 µL solution of 2-(2-nitro-1H-imidazol-1-yl)acetic acid (5.4 mg, 0.03 mmol), DMAP (3.9 mg,0.03 mmol), HOBt (5.4 mg, 0.04 mmol) and EDC (6.2 mg, 0.04 mmol) in DMF was added. The solution was stirred at room temperature for 16 h. Subsequently, nanoparticles were separated by centrifugal filtration using Amicon Ultra centrifugal filter (MWCO 3 KDa).

1.2.5. *Preparation of (DOTA)AuNP-PEG-2K-(2-NIM)(**5**)*

To a dispersion of nanoparticles AuNP-PEG-2K-(2-NIM) (**4**) in carbonate buffer (0.01 M, pH 9.5) a solution of Bz-DOTA (13 mg, 0.02 mmol) in same buffer was added and the solution was stirred overnight at room temperature. Subsequently, nanoparticles were separated by centrifugal filtration using Amicon Ultra centrifugal filter (MWCO 3 KDa) and stored at 4°C temperature.

1.2.6. *Preparation of (DOTA)AuNP-PEG-2K-(2-NIM)(FITC)(**6**)*

To prepare fluorescent nanoparticles, the amino group of free PEG units of (DOTA)AuNP-PEG-2K-(2-NIM) (**5**) nanoparticles were further conjugated with fluorescein isothiocyanate (FITC). In brief, to the water dispersed solution of (DOTA)AuNP-PEG-2K-(2-NIM), a solution of FITC in water (10 µL, 50 µg) was added and the resulting mixture was stirred at room temperature for 5 h. Subsequently, any unreacted FITC was removed by Amicon Ultra centrifugal filter (MWCO 3 KDa). The purified fluorescent nanoparticles were stored at 4°C in dark.

1.2.7. *Preparation of (DOTA)AuNP-PEG-2K (**7**)*

(DOTA)AuNP-PEG-2K without the 2-nitroimidazole moiety was used as control for the in vitro cell internalization studies. The preparation was carried out following the same procedure mentioned in Section 1.2.3 and 1.2.5 using AuNP-PEG-2K and Bz-DOTA.

1.2.8. *Radiolabeling of (DOTA)AuNP-PEG-2K-(2-NIM) (**5**) with [^{177}Lu]LuCl$_3$*

To a dispersion of (DOTA)AuNP-PEG-2K-(2-NIM) (**5**) (0.3mg) in water (100 µL), 0.05 M NaOAc buffer (200 µL, pH 5.6) was added followed by [^{177}Lu]LuCl$_3$ (20 µL, 185 MBq). The mixture was incubated in a water bath at 37°C for 40 min. Subsequently, the radiolabeled preparation was purified by passing through a PD-10 column, pre-conditioned with 25 mL of phosphate buffered saline (PBS). The radiolabeled preparation (200 µL) was loaded in the column followed by elution with PBS. The fractions (1 mL) were collected separately. [^{177}Lu]Lu-(DOTA)AuNP-PEG-2K-(2-NIM) nanoparticles obtained in 3rd and 4th fractions were pooled together. Free [^{177}Lu]LuCl$_3$ remained trapped in the column.

1.3. Quality control

The radiochemical purity (RCP) of the radiotracer [^{177}Lu]Lu-(DOTA)AuNP-PEG-2K-(2-NIM) was determined by radio-TLC. About 5 µL of the preparation was spotted on a TLC strip 1.5 cm from the bottom. The radio-TLC was developed using 10 mM DTPA solution as mobile phase. The radioactivity distribution on the developed TLC strip was recorded on a TLC Scanner. As a control, the TLC of [^{177}Lu]LuCl$_3$ solution was also performed in 10 mM DTPA. In TLC, [^{177}Lu]Lu-(DOTA)AuNP-PEG-2K-(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-(DOTA)AuNP-PEG-2K-(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.

1.4. In vitro studies

1.4.1. *In vitro stability studies*

The stability of [^{177}Lu]Lu-(DOTA)AuNP-PEG-2K-(2-NIM) nanoparticles was ascertained in PBS and human serum by radio-TLC assay over a period of one week. Briefly, the radiolabeled nanoparticles (50 μL, ~100 μCi) were incubated with excess of PBS/human serum (1:20, v/v) and incubated at 37°C for a period of 1 week. The radioactive solution was analyzed periodically by radio-TLC using 10 mM DTPA solution as described in the Section 1.3.

1.4.2. *In vitro cell uptake studies under normoxic and hypoxic conditions*

The in vitro cell uptake studies of [^{177}Lu]Lu-(DOTA)AuNP-PEG-2K-(2-NIM) in CHO cells under normoxic and hypoxic conditions was carried out using 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 kept in a water jacket at 37°C. 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 (6 MBq, ~60 ng) of radioactive preparation was added to the vial such that final activity of ~0.4 MBq/mL was obtained. About 1 mL aliquots in triplicate were withdrawn from the vial at 2 h, 3 h and 4 h 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-(DOTA)AuNP-PEG-2K-(2-NIM) under normoxic and hypoxic conditions. Hypoxia selectivity of the radiotracer was determined by calculating the hypoxic/normoxic ratio, which is the ratio of % cellular uptake under hypoxic condition to that under normoxic condition.

1.4.3. *Cell internalization study by Flow cytometry*

Cellular accumulation of fluorescent gold nanoparticles, (DOTA)AuNP-PEG-2K-(2-NIM) (FITC), was studied in CHO cells under hypoxic and normoxic conditions following the same procedure described in Section 1.4.2. Cells were treated with (DOTA)AuNP-PEG-2K-(2-NIM) (FITC) (~40 ng) and incubated under normoxic and hypoxic conditions. At 4 h 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 (DOTA)AuNP-PEG-2K-(2-NIM) (FITC), the cells were analyzed using Guava® easyCyte Flow Cytometer System and the mean fluorescence intensities (MFI) were recorded.

1.4.4. *Slide preparation and confocal microscopic examination/imaging*

Slides were prepared by smearing 20 µl aliquot of the CHO cells treated with (DOTA)AuNP-PEG-2K-(2-NIM)(FITC) under hypoxic/normoxic condition on the top of the glass slide. The sample was overlaid with a cover-slip and sealed with rubber cement. Slides were scanned with Leica SP8, laser scanning super resolution, confocal microscope. Images were acquired at 10X magnification under bright field and fluorescence (green filter, Alexa flor 488) mode, using fluorescence-DIC. Subsequently the images were superimposed using LAS-X software inbuilt in the microscope.

1.5. In vivo studies

Biodistribution study of [^{177}Lu]Lu-(DOTA)AuNP-PEG-2K-(2-NIM) was carried out 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 (~5.5MBq per animal in 100 µL volume) intravenously via lateral tail vein. After the injections, animals (n = 3) were incubated for different time intervals (2 h, 4 h, 24 h). At the end of respective time intervals, the animals were sacrificed and relevant organs/tissues 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]Lu. Results were expressed as percentage injected activity per gram of organ (%ID/g ±s.d.).

1.6. Statistics

Statistical analysis of relevant data was performed by *t*-test. Confidence level of 95 % ($p<0.05$) was taken for statistical significance.

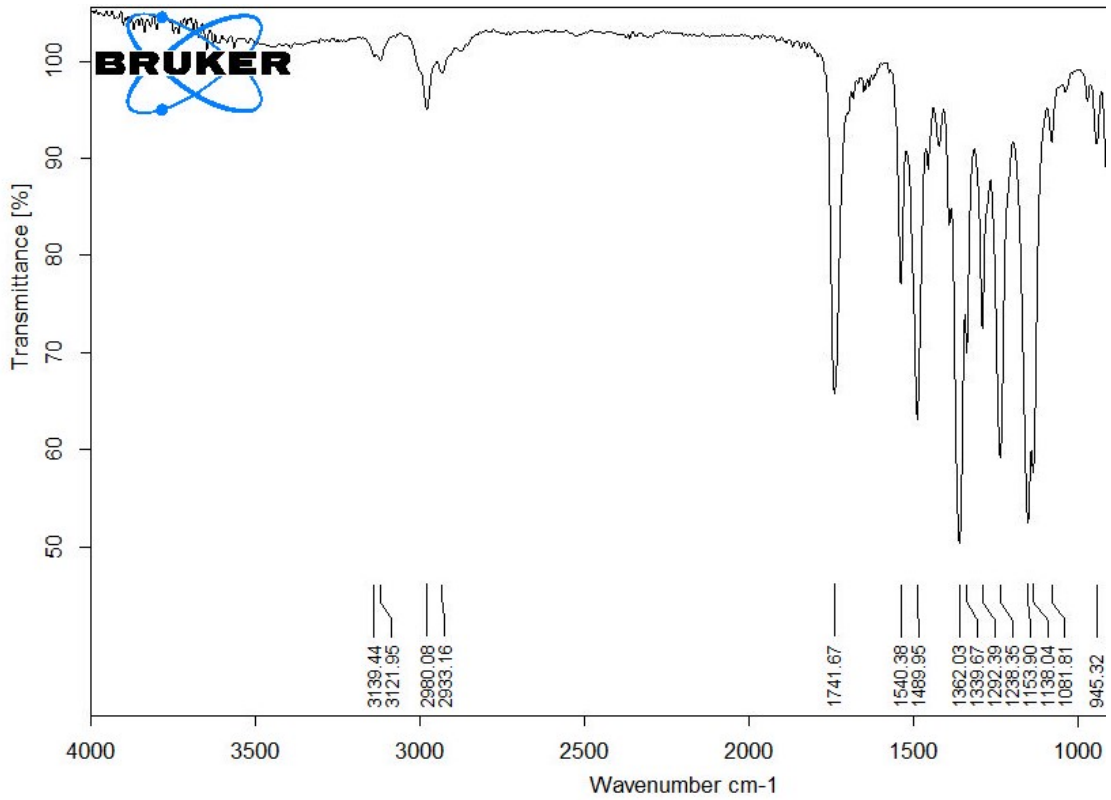


Fig.S1. FT-IR spectrum of 2-(2-nitro-1H-imidazol-1-yl)tert-butyl acetate (**1**)

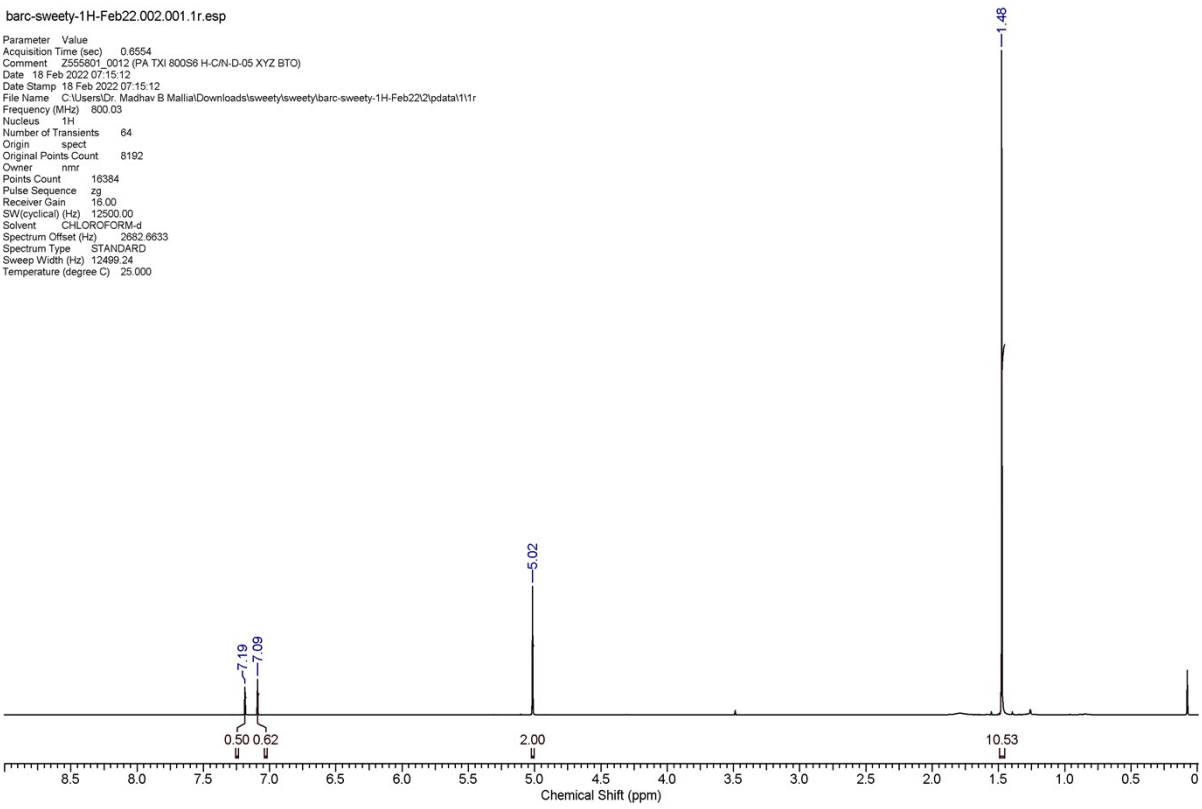


Fig.S2. ^{1}H-NMR spectrum of 2-(2-nitro-1H-imidazol-1-yl)tert-butyl acetate (**1**)

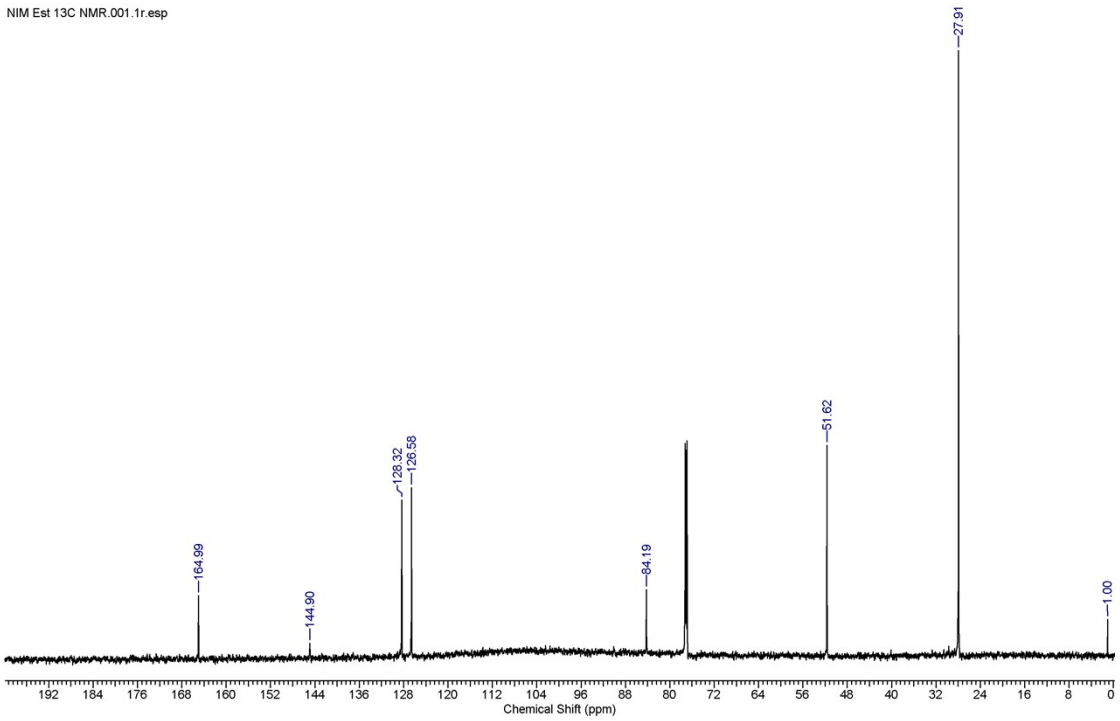


Fig.S3. ^{13}C NMR spectrum of 2-(2-nitro-1H-imidazol-1-yl)tert-butyl acetate (**1**)

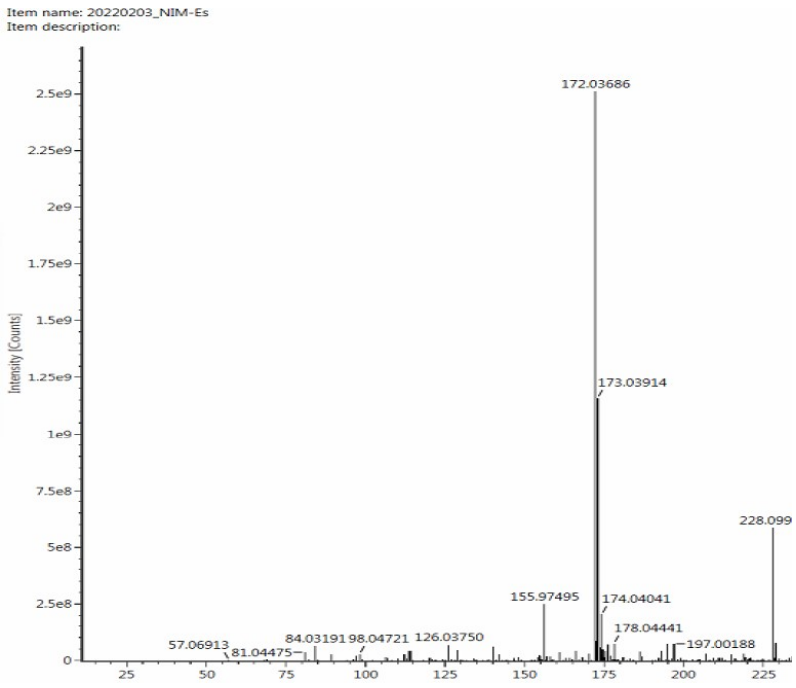


Fig.S4.ESI-MS of 2-(2-nitro-1H-imidazol-1-yl)tert-butyl acetate (**1**)

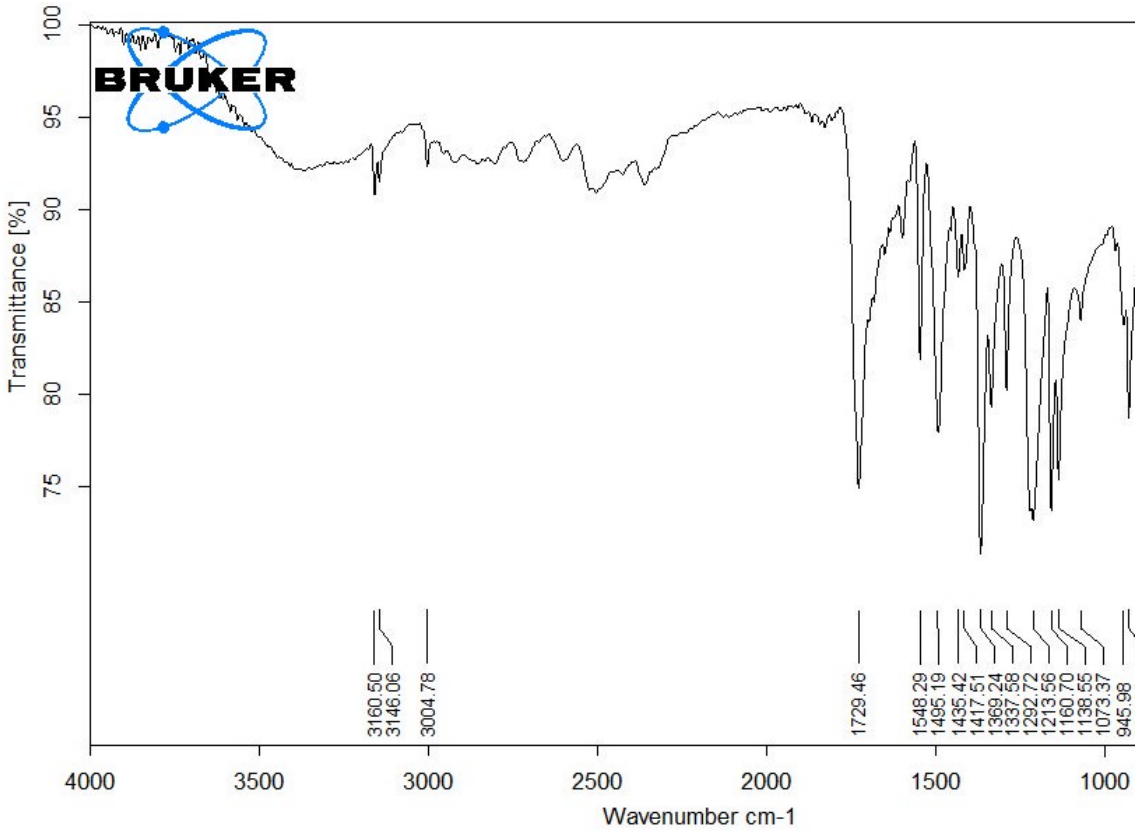


Fig.S5.FT-IR spectrum of 2-(2-nitro-1H-imidazol-1-yl)acetic acid (**2**)

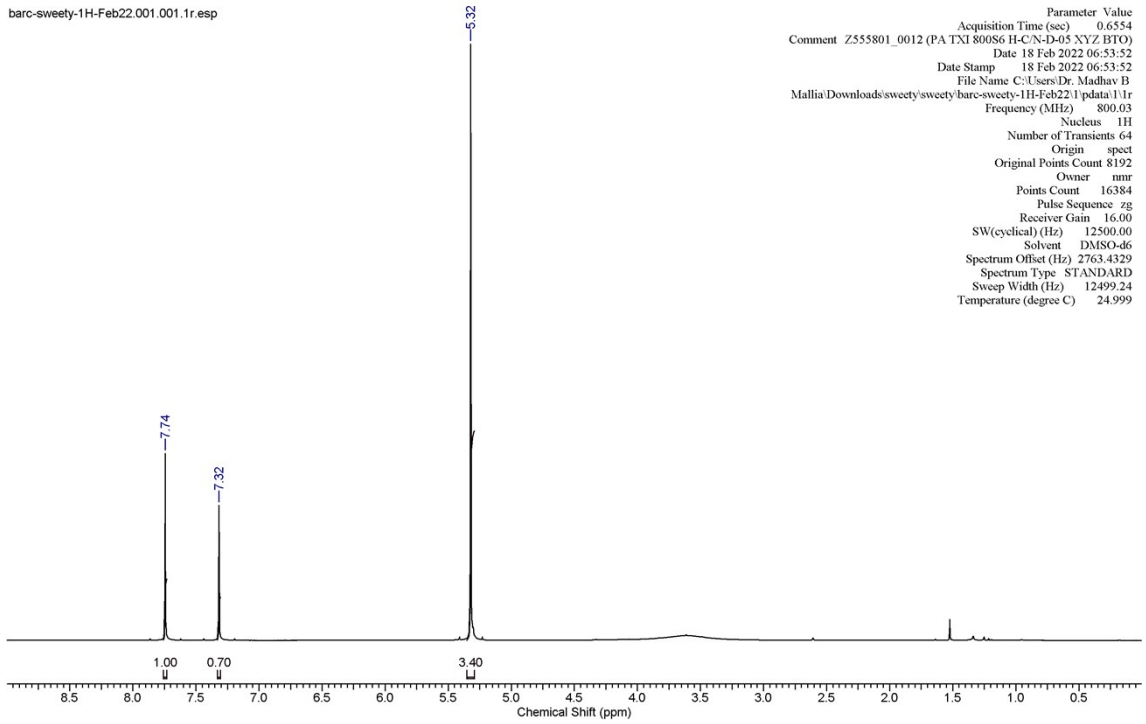


Fig.S6.[1]H-NMR spectrum of 2-(2-nitro-1H-imidazol-1-yl)acetic acid (**2**)

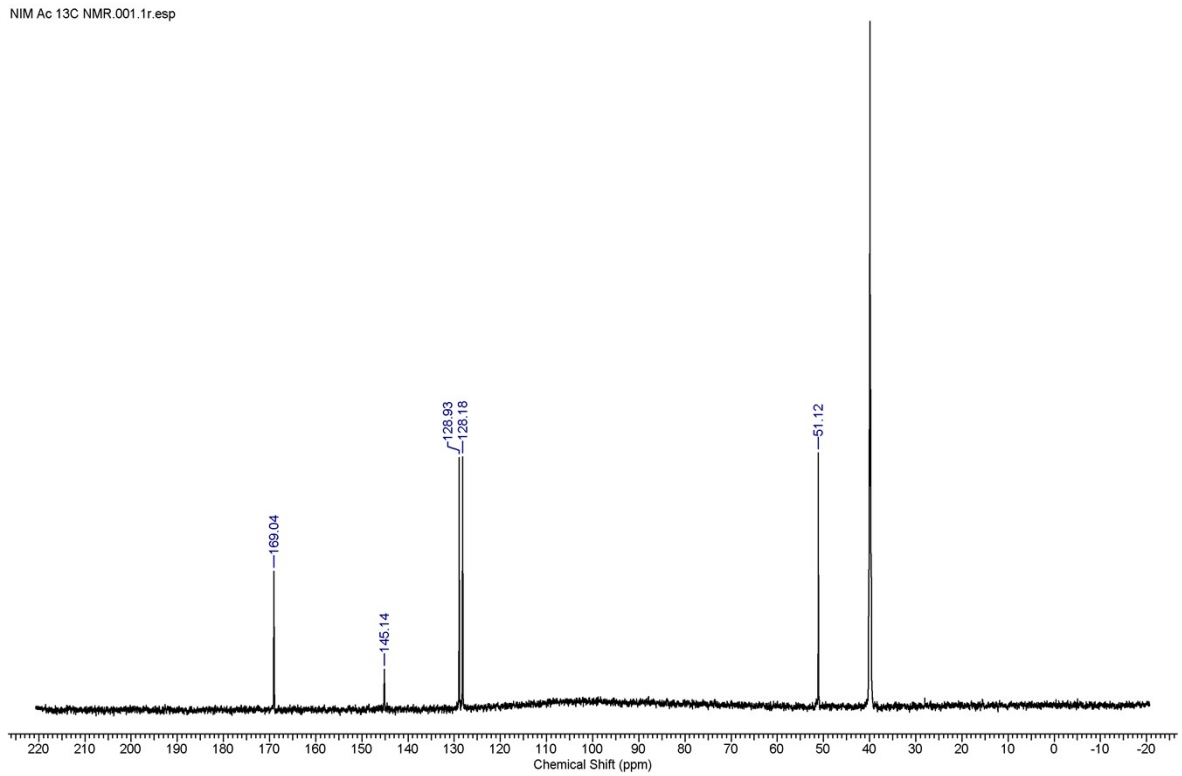


Fig.S7.^{13}C- NMR spectrum of 2-(2-nitro-1H-imidazol-1-yl)acetic acid (**2**)

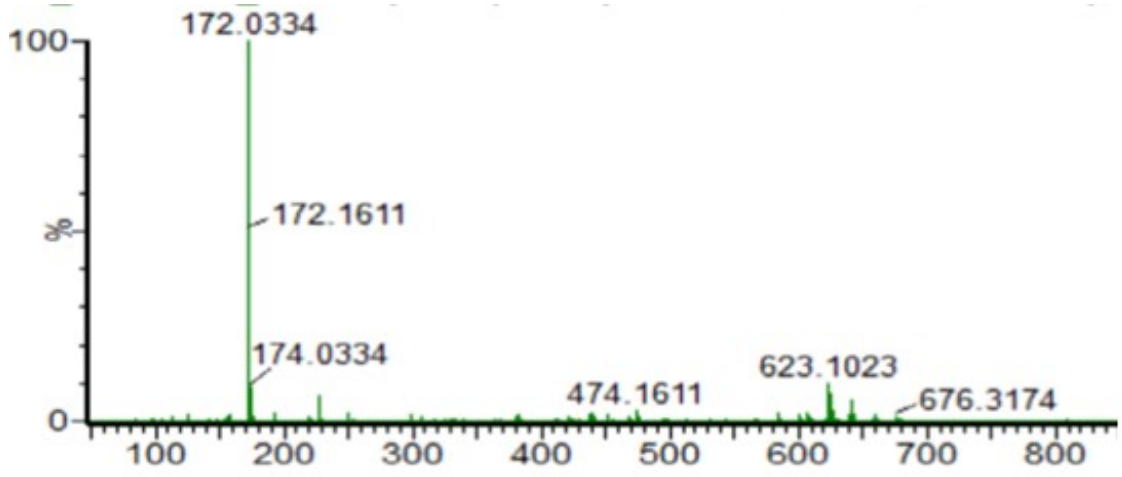


Fig.S8.ESI-MS of 2-(2-nitro-1H-imidazol-1-yl)acetic acid (**2**)

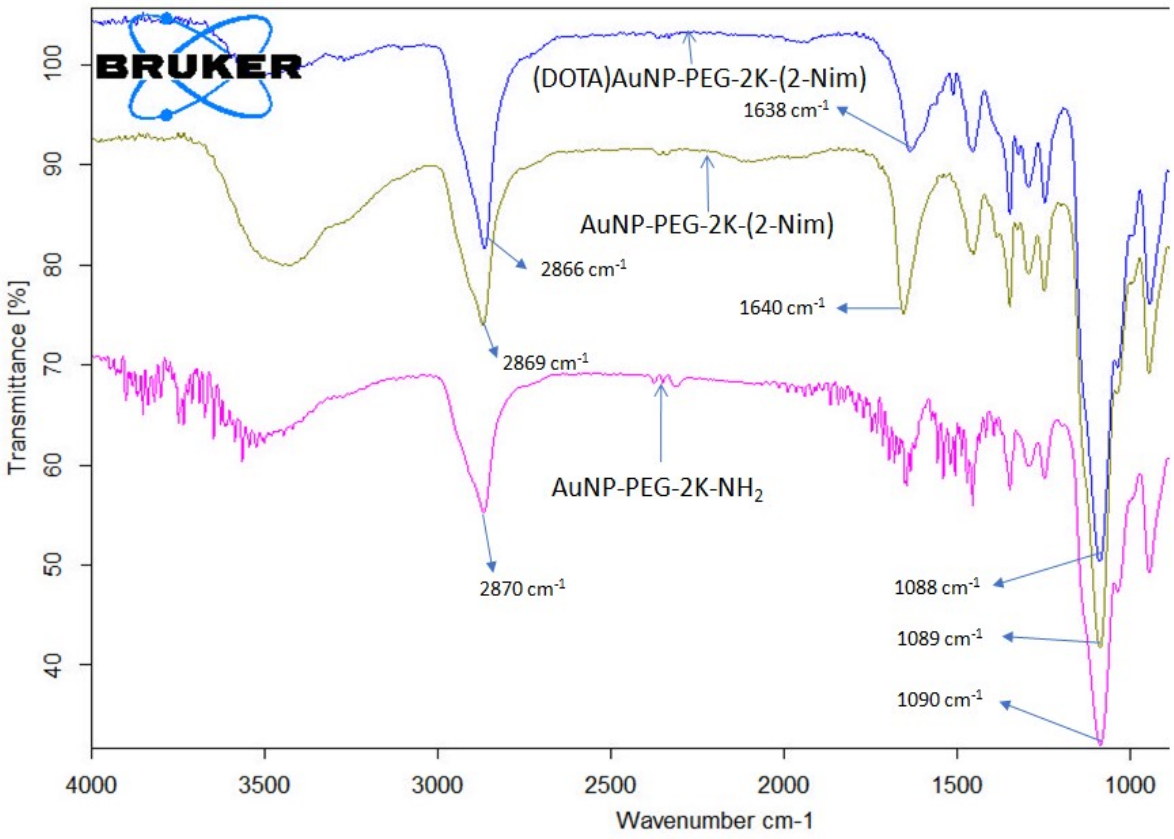


Fig. S9 Overlaid FT-IR spectra of AuNP-PEG-2K-NH_2, AuNP-PEG-2K-(2-Nim) and (DOTA)AuNP-PEG-2K-(2-Nim)

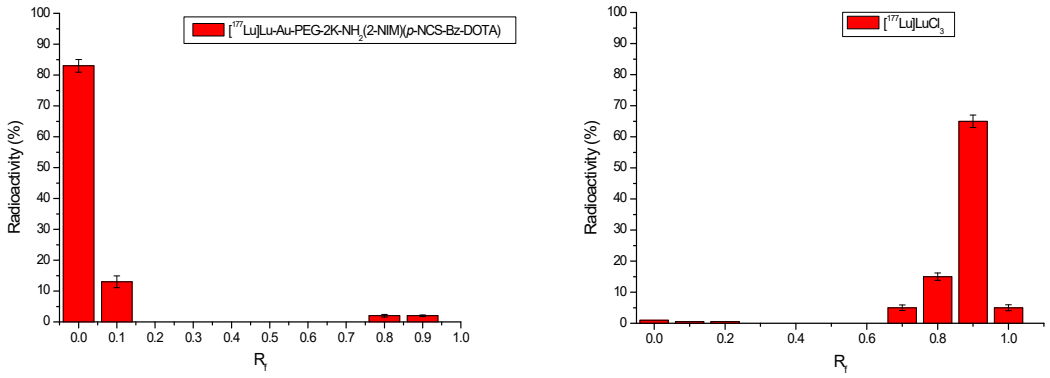


Fig. S10. (a) Radio-TLC pattern of [^{177}Lu]Lu-Au-PEG-2K-NH_2(2-NIM)(*p*-NCS-Bz-DOTA) developed with 10 mM DTPA (b) Radio-TLC pattern of free [^{177}Lu]$LuCl_3$under same condition

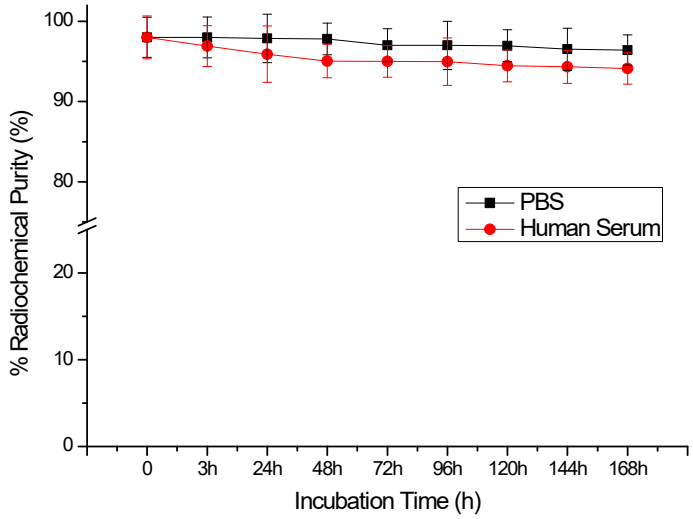
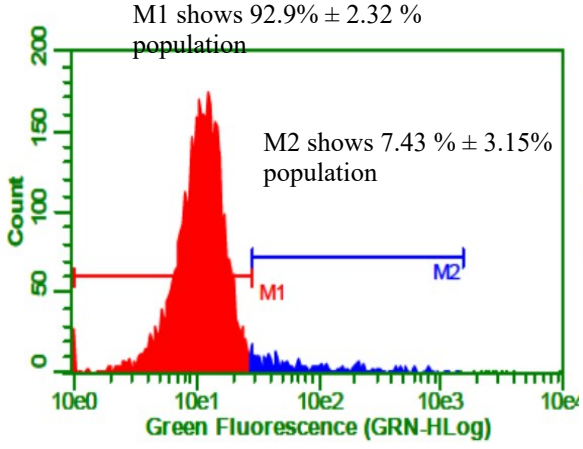


Fig. S11. In vitro radiochemical stability of [^{177}Lu]Lu-(DOTA)Au-PEG-2K-(2-NIM) in PBS and Human Serum over the period of 7 days

	Count	%Total
M1	4609	92.18%
M2	380	7.60%
All Events	5000	100.00%

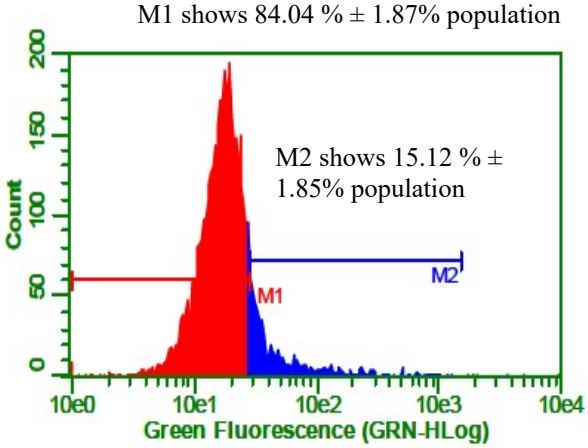
	Count	%Total
M1	4255	85.10%
M2	736	14.72%
All Events	5000	100.00%

M1 shows 92.9% ± 2.32 % population



Flow histogram under normoxic conditions

M1 shows 84.04 % ± 1.87% population



Flow histogram under hypoxic conditions

Fig. S12. Quantified flow histogram profile of CHO cells incubated with FITC tagged gold nanoparticles [(DOTA)AuNP-PEG-2K-(2-NIM)(FITC)] under normoxic and hypoxic conditions for 4 h