Electronic Supplementary Information (ESI)

Supporting Information File

Chemicals and Instruments

The chemicals and reagents used in this study including diethylenetriamine pentaacetic acid dianhydride (cDTPA), *N, N*-Disiopropyl ethylamine (DIPEA; Hunig's base), triethylamine (TEA), trifluoroacetic acid (TFA), O-(7-azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluron hexafluorophosphate (HATU; coupling agent), dimethyl sulfoxide (DMSO), dimethylformamide (DMF), and sodium borate were purchased from Sigma-Aldrich (Germany). Cyanine 5.5 *N*-Hydroxysuccinimide ester (Cy5.5 NHS: ≥ 95% purity; ex/em: 650 nm/ 700 nm) was purchased from Biocone Biotechnology Co. Ltd. Chengdu, China. Head-to-tail cyclic heptapeptide having Arg-Gly-Asp-Lys(Boc)-Leu-Ala-Lys [RGDK(Boc)LAK; Mr = 869.41 g/ mole] sequence was obtained from KareBay Biochem, Inc. New Jersey, USA. Other chemicals including acetic acid (CH_3COOH), sodium hydroxide (NaOH), hydrochloric acid (HCl), deionized water, deuterated dimethyl sulfoxide (d^6-DMSO), stannous chloride dihydrate ($SnCl_2.2H_2O$), sodium borohydride ($NaBH_4$), acetonitrile (HPLC Grade), Milli-Q ultrapure water (Millipore), and Phosphate buffer saline (PBS; pH 7.4) were purchased from Sigma-Aldrich, Germany. All the reagents used in the experiments were of analytical grade and used without further purification. Thin-layer chromatography coated with silica-gel (TLC-SG; 60 F_{254} 20 × 20 cm), Whatman paper no. 3 (3MM), and pH paper were bought from Merck, Germany. The locally produced $^{99}Mo/^{99m}Tc$ generator (PAKGEN) was purchased from PINSTECH, Nilore, Islamabad-Pakistan, and radioactivity (^{99m}Tc; Half-life 6.0 h, energy 140 keV, ≥ 98% purity) was eluted from a generator using elution solvent (0.9% saline; sterilized vials) in the form of sodium pertechnetate ($Na^{99m}TcO_4$).

The qualitative measurements of Cy5.5 NHS, cDTPA, and cyclic peptide were carried out before and after modifications using High-Performance Liquid Chromatography (HPLC) Primaide ORG, Hitachi interconnected with 1410 UV detector and NaI(Tl) detector (for radioactive measurements) along with C-18 column (4.6 × 250 mm ID; 5 µm particle size) as stationary phase as well as isocratic mobile phase (acetonitrile: water with 0.1% TFA) system was used for all analysis. High-resolution mass spectrometer (HRMS; Thermo Scientific, Orbitrap ELITE) and Fourier-transform infrared spectrometer-attenuated total reflectance (FTIR-

ATR; Thermo Scientific) was used for quantitative measurements after modification of peptide analogs. Fluorescence intensity measurements were performed on Fluorescence Spectrophotometer (RF-5301PC; SHIMADZU, USA). Particle size, shape, and morphology of nanoparticles were determined using Dynamic laser light scattering (DLS, BI-200SM, USA), and Transmission electron microscope (TEM, Tecnai G2 TF30, USA). A cell viability study was carried out using Microplate reader SPARK 10M (TECAN, Switzerland). Cell imaging studies were performed using a confocal fluorescence microscope connected with Imager.Z2 Axiocam 506 color/mono ZEISS camera and functionalized with ZEN V2.3 pro software. Apoptosis assay was performed using the BD FACSCanto™ flow cytometer instrument (BD Biosciences, USA). For the animal imaging study, a Dual-headed SPECT/CT camera (Discovery NM/CT 670 Pro, GE Healthcare USA) interconnected with DICOM V3.0 software was used for acquiring static images.

Characterizations

Fluorescence

The final product Cy5.5@SAPD nanoparticles were diluted with sodium phosphate buffer (Na-PB; pH 7.4) with a concentration of 0.5 μM/ mL, filled in a quartz cuvette (1 cm path length), and subjected to measure the emission spectra. The excitation wavelength was set at 650 nm and emission wavelength set from 675-750 nm with slit width 5/ 5 nm and intensity 500 mV. The same protocol was used to measure the spectra after dilution with deionized water (DW).

Dynamic light scattering (Particle size)

The diluted solution of Cy5.5@SAPD nanoparticles (0.5 mg/ mL DW) was analyzed using a dynamic laser light scattering device. The measurements were carried out at 25°C with a detection angle of 90° on Delsa Nano C Particle Analyzer (Beckman coulter) interconnected with 25 mW He-Ne solid-state laser (660 nm). The size of hydrophilic samples was obtained by calculating mean values from three to five measurements.

Ultra-centrifugation

The increasing concentrations of the radiotracer Cy5.5@SAPDN-$^{99m}Tc(CO)_3$ (0 – 100 μL) were added in saline up to the total volume of 1 mL and vortex for 30 sec followed by sonication for 30 min at room temperature. After that, the suspension was centrifuged at 13000 rpm for 5 min and the supernatant was separated from the pellet. The radioactivity of pellet and supernatant

was measured using NaI(Tl) well-type gamma-counter to observe the unbound activity present in the supernatant.

Transmission electron microscopy (TEM)

The sample solutions of Cy5.5@SAPD nanoparticles and Cy5.5@SAPDN-$^{99m}Tc(CO)_3$ were prepared in deionized water with a concentration of 0.5 mg/ mL. About 50 µL sample solution was placed on carbon-coated copper grids separately for each sample and dried at room temperature under normal atmospheric pressure before the TEM study. The images were acquired on TEM operating at 200 kV accelerating voltage.

***In vitro* Cell Study**

Tumor Cell Culture

Human brain tumor glioblastoma cell line U87MG ($\alpha_v\beta_3$, $\alpha_v\beta_5$-integrins positive) and human embryonic kidney cell line HEK-293 ($\alpha_v\beta_1$-integrin positive) were purchased from Cell Bank of Chinese Academy of Sciences (CBCAS) and cultured in Iscove's Modified Dulbecco's Medium (IMDM) and RMPI-1640, respectively. Both media were supplemented with 10% FBS and 1% antibiotics (50 U/ mL penicillin and 50 µg/ mL streptomycin). The cells were cultured in humidified incubator having 5% CO_2 and 37°C temperature.

Cytotoxicity assay

In vitro cytotoxicity study of Cy5.5@SAPD and Cy5.5@SAPD-$^{99m}Tc(CO)_3$ nanoparticles was assessed by MTT assay. The cancerous cells were first cultured with a density of 5×10^3 cells per well in a 96-well plate using respective growth mediums in a 5% CO_2 incubator at 37°C for 24 h before treatment. Nearly 90% of confluent cells were washed with phosphate buffer saline (PBS; pH 7.4) twice and added different concentrations (0 – 50 nM/ mL) of test samples supplemented with the serum-free medium in six replica columns and incubated for another 24 h. The next day, the samples containing medium were removed, washing twice with pre-heated (at 37°C) PBS and 10 µL MTT solution (5 mg/ mL in serum-free medium) was added in each well with incubation for further 4 h. After incubation, the medium was decanted completely, cells were dissolved in 200 µL DMSO and incubated for a further 10 min. Then, finally, the treated plates were scanned at 490 nm absorbance using micro-plate reader SPARK 10M (TECAN, Switzerland). The same data was used to calculate the half-maximal effective concentration (EC_{50}) values for both tested samples. All experiments were carried out in triplicate to measure ± standard deviation (SD) values.

Confocal Laser Scanning Microscopy (CLSM)

The cancer cells were seeded as described above with a density of 1×10^5 cells per well in a 6-well plate containing a 35 mm (Mat-Tek) glass-bottom cell culture dish and allowed to incubate for 36-48 h to achieve 90 – 95% confluency. The next day, the cells were treated with a 10 μM concentration of Cy5.5@SAPD nanoparticles and incubated in a 5% CO_2 incubator at 37°C. After 30 min incubation, the cells were washed thrice with ice-cold PBS to halt the internalization of peptide samples. To observe the mitochondria targeting capabilities of newly designed nanoparticles, the cells were further incubated with Mito-Tracker (green; 1 μM) for 20 min and washed thrice with ice-cold PBS to observe the co-localization of samples with mitochondria. Furthermore, for the nuclei staining study, the same cells were treated with Hoechst-33258 and incubated for another 20 min followed by washing thrice with PBS. Next, the activity of Caspase-3 was assessed using a Caspase-3 activity staining kit (Solarbio® Co., Ltd. Beijing, China). The cells were stained with a 5 μM Casp-3 kit (Ac-DEVD-pNA) for another 30 min, washed twice with ice-cold PBS. This assay is based on the detection of chromophore *p*-nitroanilide (pNA). All the cells were imaged with a confocal laser scanning microscope (CLSM). All experiments were repeated in triplicate (n = 3).

Cells apoptosis/ necrosis assay

The apoptosis-inducing potential of Cy5.5@SAPD nanoparticles against U87MG cells was further investigated by fluorescence-activated cell sorting (FACS) technique using fluorescein-annexin V (V-FITC) and propidium iodide (PI) double staining after treatment with newly synthesized nanoparticles. Briefly, the cells were seeded as described above and treated with a 20 μM concentration of Cy5.5@SAPD for 24 h. Further, the cells were trypsinized using 0.05% Trypsin-EDTA and collected by centrifugation at 2000 rpm for 5 min. The supernatant was decanted, cells were washed and resuspended in 100 μL PBS. Finally, cells were stained with annexin V-FITC/PI as per protocol and incubated for 20 min in dark at room temperature before FACS analysis. The percentage of apoptotic and necrotic cells were calculated by BD FACSCanto™ flow cytometer (USA) interconnected with FACSDiva version 6.1.2.

Bio-TEM Imaging

To observe the mitochondrial effect of newly designed nanoparticles, U87MG cells were treated with Cy5.5@SAPD nanoparticles overnight. The next day, the cells were washed with PBS three times to remove excess nanoparticles and fixed with 2.5 % glutaraldehyde in 1 mL of 0.1 M PBS

solution at 4°C for 24 h. Later on, the cells were trypsinized, collected the pellet, dehydrated with graded ethanol, embedded in epoxy resin, and sliced using a glass knife with a thickness of 40 – 60 nm. Finally, the cell sections were stained using 5% uranyl acetate followed by 2% lead acetate for 20 min and observed in TECAN Bio-TEM.

Supplementary Figures

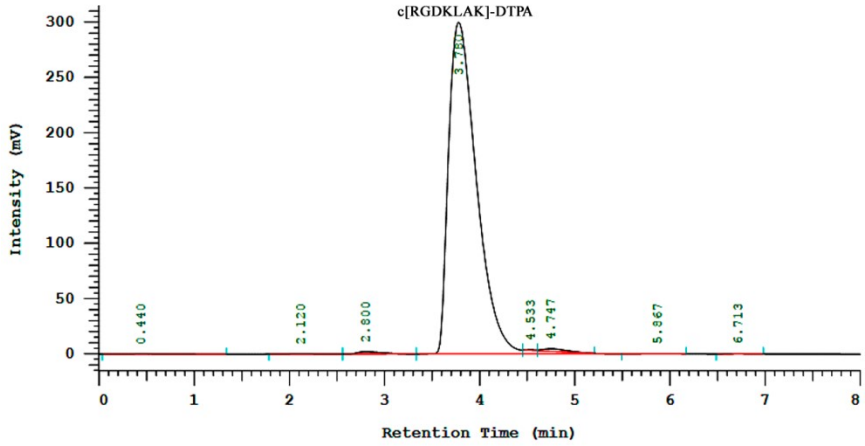

Figure S1: High-Pressure Liquid Chromatography (HPLC) analysis of cyclic peptide-DTPA (cPD) complex indicating single peak at retention time R_t = 3.780 min.

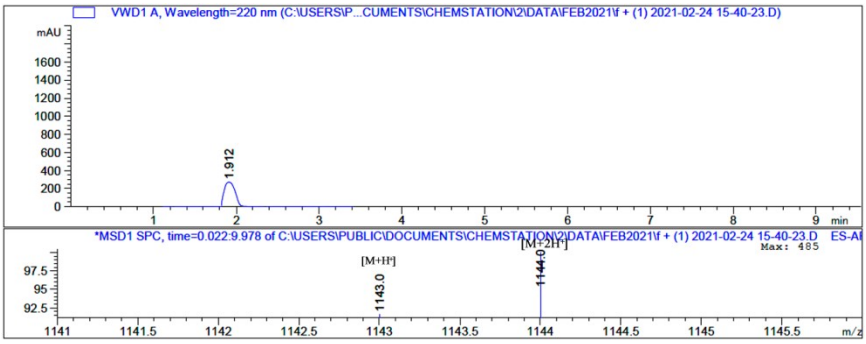

Figure S2: High resolution mass spectrometry (HR-MS) analysis of cyclic peptide-DTPA (cPD) complex showing molar mass in addition with proton [M+H^+] = 1143 a.m.u

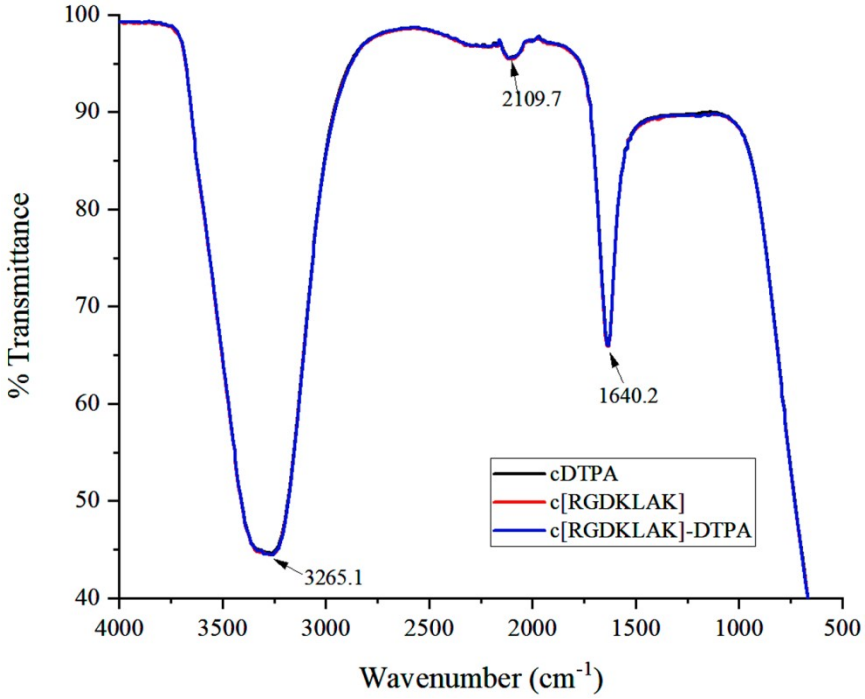

Figure S3: Fourier-Transform Infrared Spectroscopy with Attenuated Total Reflection (FTIR-ATR) analysis of cDTPA (black line), cyclic peptide (red line), and cyclic peptide-DTPA (cPD) complex (blue line) indicates superimposed spectrum

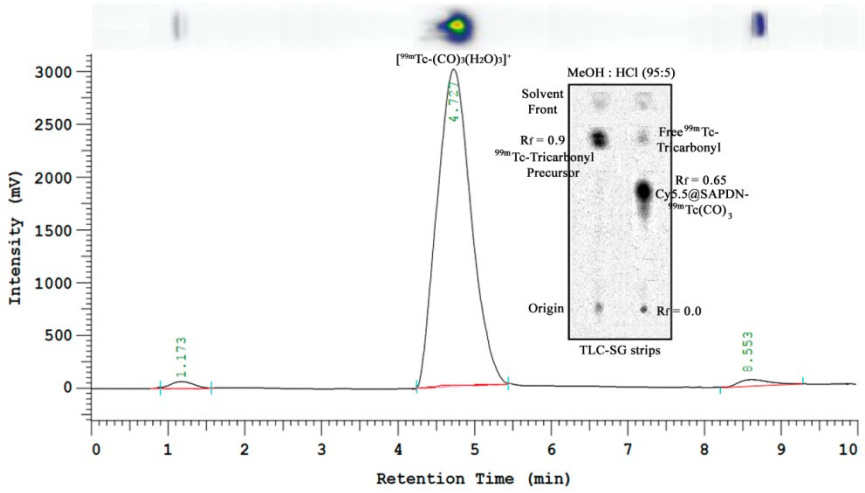

Figure S4: Ultra-High Performance Liquid Chromatography (UHPLC) analysis of *fac*-[^{99m}Tc-$(CO)_3(H_2O)_3]^+$ core complex (^{99m}Tc-Tricarbonyl precursor) indicating ≥ 97% purity, inset figure shows TLC-SG analysis of ^{99m}Tc-Tricarbonyl precursor (left) and Cy5.5-SAPD-^{99m}Tc nanoparticles (right).

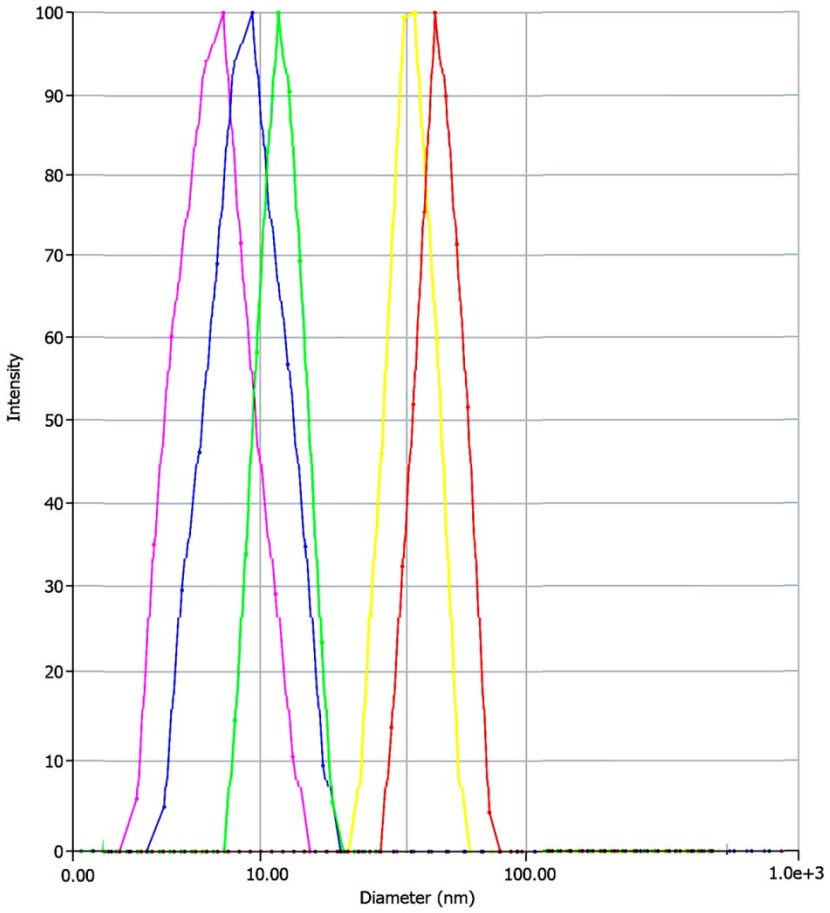

Figure S5: Dynamic Light Scattering (DLS) analysis of Cy5.5 coupled self-assembled cyclic peptide-DTPA (Cy5.5@SAPD) nanoparticles shows the hydrodynamic size of nanoparticles

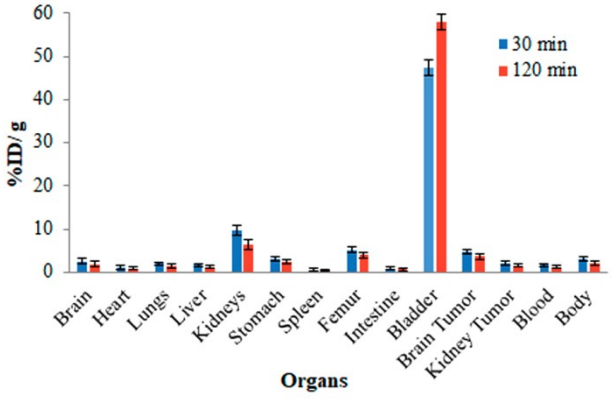

Figure S6: Biodistribution study of radiotracer Cy5.5@SAPD-^{99m}Tc in tumor induced Balb/c mice models at various post-administration time interval characterized by dual-imaging technique