Hybridization of tumor homing and mitochondria-targeting peptide domains to design novel dual-imaging self-assembled peptide nanoparticles for theranostic applications

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Abstract
A novel hybridized dual-targeting peptide-based nanoprobe was successfully designed by using the cyclic heptapeptide. This peptide has Arg-Gly-Asp-Lys-Leu-Ala-Lys sequence, in which the RGD homing motif and KALK mitochondria-targeting motif were linked via amide bond. The designed peptide probe was further modified through covalent linkage to induce dual-imaging functionality, and self-assembled to form spherical nanoparticles. The novel Cy5.5-SAPD-99mTc nanoparticles were tested for in vitro cytotoxicity, cellular uptake, and apoptosis-inducing functionalities. The cellular internalization, enhanced cytotoxicity and selective receptor binding capabilities against U87MG cells, excellent dual-imaging potential, improved apoptosis-inducing feature by damaging mitochondria, and in vivo preclinical investigations suggested that our newly designed novel hybridized peptide-based dual-imaging nanoparticles may serve as an admirable theranostic probe to treat brain tumor glioblastoma multiforme.

Keywords Hybridized peptides · Dual-imaging · Mitochondria-targeting motif · Theranostic agent · Glioblastoma multiforme

Introduction
The incidence of malignant cells in the circulatory system is increasingly assisted by the growth of new blood vessels, which utilizes oxygen and nutrients to promote the development of tumor angiogenesis. Self-regulation of angiogenesis causes the progression of diseases like the proliferation of cancer cells, glioblastoma multiforme (GBM), myocardial infarction, and atherosclerosis [1]. To cure cancer, anti-angiogenic therapy in combination with anticancer therapy has been emerging as a unique strategy to halt the tumor cell growth by discontinuing their nutrient oxygen supply. Multidomain-targeted drug delivery serves as a guided missile to efficiently target the cancer cell vasculature. These fabricated drugs have been developed using a combination of peptides/proteins, integrin-receptor ligands, antibodies, and aptamers which possessed recognition domains and effector domains [2]. There are many cognate receptors and pro-angiogenic factors involved to promote the vessel formation in tumors such as fibroblast growth factor-2 (FGF-2), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) [3]. These growth factors possessed up-regulated expressions of integrins such as α1β1, α2β1, α4β1, α5β1, and αvβ3-integrins on blood and lymphatic vessels. Several investigations implicated these integrins as a key regulator of tumor angiogenesis which are also regulating endothelial cell survival and migration and mediate cell–cell adhesion [4, 5]. Basic clinical studies revealed that angiogenesis could be blocked by inhibiting the angiogenic signaling pathways, ultimately resulting in tumor dormancy and metastasis [6, 7]. Integrin-mediated signaling pathways play an important role in tissue development and homeostasis, while its deregulation causes multiple brain diseases [8]. Interestingly, integrins are crucial glycoproteins essential for many
physiological processes such as proliferation, cell migration, hemostasis, and oncogenic transformation [9, 10]. They are also important for cell–cell and cell-extracellular matrix (ECM) interactions and comprised of nineteen α- and eight β-subunits [11]. Among these αβ-subunits, α5β1, αvβ3, αvβ5, αvβ6, αvβ1, and αmβ3-integrins have been studied extensively for their active role as an excellent candidate for cancer theranostic [12]. More specifically, αvβ3-integrin serves as a receptor for ECM proteins such as vitronectin, fibronectin, laminin, and osteopontin with exposed arginine-glycine-aspartic acid (RGD) sequence [13]. It is expressed on mature epithelial cells at low levels, while, highly expressed on the surface of many tumors including carcinomas, melanomas, glioblastomas, and osteosarcomas [14–16]. Hence, αvβ3-integrin is considered a molecular target of interest for the early diagnosis of cancer and selective cancer therapy [17].

Over the last two decades, both linear and cyclic RGD peptide analogs have been discovered, radiolabeled (99mTc, 177Lu, 68Ga, and 18F), and evaluated as radiotracers for tumor diagnosis using single-photon emission computed tomography (SPECT) or positron emission tomography (PET) imaging [18]. The cyclic RGD peptides were observed to be highly stable towards proteases and increased affinity to αvβ3-integrin receptors and have reduced structural flexibility compared with their linear construct [19]. Furthermore, many researchers have studied hybrid peptide probes combining the RGD tripeptide with other anticancer peptides including RGD,111In-DTPA-octreotate [20, 21], 18F-bombesin-RGD [22], 99mTc-RGD-Bombesin [23], 64Cu-RGD5-PG12-bombesin heterodimer [24], 5FU-loaded SF-cRGD/DK-Ce6 [25], and RG301-MTX peptide [26] for targeted drug delivery and improved diagnostic as well as therapeutic efficacy.

In the light of the above studies, the aims of our study were, firstly, the designing of a small head-to-tail cyclic peptide sequence using RGD homing motif (αvβ3-integrin receptor binding) along with KLAK (mitochondria-targeting) motif. Furthermore, eliminating the unnecessary addition of linking amino acids that can improve the integrin receptor targeting and mitochondrial damaging potentials for therapeutic applications while minimizing the proteolytic degradation and structural flexibility. Secondly, the modification of this cyclic peptide by incorporation of Near-Infrared fluorescent (NIRF) dye for optical imaging as well as radiolabeling with βγ-emitting radionuclides for SPECT imaging, to introduce novel dual-imaging agent as SPECT/NIRF probe.

To achieve this goal, firstly, we modified the ε-amino group of terminal lysine residue by coupling with a deprotected free β-carboxylic group of diethylenetriamine pentaacetic acid (DTPA) via covalent linkage as a bifunctional chelating agent (BFC). This BFC is a useful tool for selective radiolabeling of γ-emitting radionuclide Technetium-99 m (half-life 6 h; Er = 140 keV) as SPECT imaging probe. The reaction was successfully accomplished with a satisfactory yield of ~78% as presented by Step I in Scheme 1. The Boc protecting group at the amino group of middle lysine residue was removed using 95:5% TFA:H2O to obtain the corresponding active amine group suitable for conjugation with a NIRF dye [27]. Secondly, this peptide-DTPA complex was intrinsically converted to self-assembled nanoparticles via co-assembly with Cyanine 5.5 N, N-succinimidyl ester (Cy5.5 NHS) as NIRF dye. The covalent bond was formed between the free amino group of an intermediate lysine residue and NHS ester site of NIRF dye to introduce optical imaging features. A simple and facile synthesis approach was used to design novel dual-targeting self-assembled cyclic peptide-DTPA (SAPD) nanoparticles with high chemical yield.

The purity of the cyclic peptide-DTPA (cPD) complex was confirmed by HPLC analysis indicating a single peak with ≥ 98% purity at retention time \( R_t = 3.780 \text{ min} \) (Fig. S1). Furthermore, the LC–MS analysis showed molecular mass peaks for calculated for \( C_{47}H_{81}N_{15}O_{18} \) with \( m/z \) found \( [M + H]^+ = 1143.01 \text{ a.m.u} \) and \( [M + 2H]^+ = 1144.01 \text{ a.m.u} \) (Fig. S2). Moreover, the FTIR-ATR analysis showed superimposed spectrum of cDTPA (black line), cyclic peptide (red line), and cPD complex (blue line) having peaks at 3265.1 cm\(^{-1}\) and 1640.2 cm\(^{-1}\) assigned for stretching vibrations of \(-\text{NH}_2\) and \(-\text{NC} = \text{O}\) group, respectively, and peaks at 2109.7 cm\(^{-1}\) designated to bend vibrations of \(-\text{COOH}\) group (Fig. S3).

The pH-sensitive method was used for successful self-assembly of cPD complex with Cy5.5 NHS via covalent interactions to form uniform nanoparticles with well-defined spherical shape and “Always ON” NIR-fluorescence property as presented in Step II (a) of Scheme I [28, 29]. It will be fruitful in improving the pharmacokinetics, diagnostic, and therapeutic efficiencies of dual-imaging peptide nanoparticles, while maintained intrinsic biocompatibility and biodegradability [30]. The effect of self-assembly on change in fluorescence intensity was assessed by a fluorescence spectrophotometer. The spectrograms presented in Fig. 1a showed...
peaks with $\lambda_{\text{ex}} = 650 \text{ nm}$, while $\lambda_{\text{em}} = 702 \text{ nm}$ in aggregation state. It was observed to be nearly consistent with the parent NIRF dye (Cy5.5 NHS; $\lambda_{\text{ex}}/\lambda_{\text{em}} = 650/700 \text{ nm}$) with a slight increase in $\lambda_{\text{em}}$ [31]. The freshly synthesized Cy5.5@SAPD nanoparticles were further radiolabeled with Technetium-99 m by using $\text{fac-}\left[\text{99mTc-(CO)}_3(\text{H}_2\text{O})_3\right]^+$ core formation strategy for SPECT imaging.

The radiosynthon introduced by Alberto et al. [32] has been used widely for preferential labeling of peptides/proteins to achieve relatively high specificity keeping retained biological property of bioactive molecules. The radiosynthon was successfully prepared with a high percentage of radiochemical purity (% RCP) of $\geq 97\%$ giving a single peak with high intensity at retention time $R_t = 4.727 \text{ min}$ as indicated by radio-HPLC analysis (Fig. S4). The inset figure showed TLC-SG results depicted that $\text{fac-}\left[\text{99mTc-(CO)}_3(\text{H}_2\text{O})_3\right]^+$ moves with the solvent front at $R_f = 0.90$, while free $\text{99mTcO}_4^-$ remained at origin ($R_f = 0.00$). The active nitrogen and oxygen atoms (as electron donor) of DTPA participate in radiolabeling of $\gamma$-emitting radionuclides in the presence of a suitable reducing agent and optimum pH value as shown in Step II (b) of Scheme 1. Furthermore, the radiolabelled nanoparticles were observed to remain stable at room temperature over a 4-h incubation period and no change in radiolabeling efficiency was observed by increasing the concentration of Cy5.5@SAPD-$\text{99mTc}$ nanoparticles having % RCP $\geq 96\%$ (Fig. 1b). The same results were calculated from the TLC-SG technique, as the inset image presented in Fig. S4 showed $\sim 97\%$ yield at $R_t = 0.65$, while $<3\%$ impurities were found in saline [33].

Additionally, transmission electron microscope (TEM) images presented in Fig. 1c, d showed spherical morphology of Cy5.5@SAPD and Cy5.5@SAPD-$\text{99mTc}$ nanoparticles, respectively, with uniform dispersion in an aqueous medium. The size of Cy5.5@SAPD nanoparticles was observed to be between 30 and 40 nm as indicated in inset Fig. 1c’ acquired by high-resolution TEM. However, for Cy5.5@SAPD-$\text{99mTc}$ nanoparticles, the size was reduced to 20–25 nm as shown by inset Fig. 1d. The clear consecutive bright and dark lattice fringes with interplanar lattice fringe distance of 0.294 nm seen in Fig. 1d’ indicate the tight interface among all three ingredients. This could facilitate in balancing the charge on the surface of nanoparticles as well as demonstrated the crystallinity in novel designed nanoparticles [34]. The dynamic light scattering (DLS) measurements revealed the hydrodynamic size of Cy5.5@SAPD nanoparticles with a diameter of less than 50 ± 8 nm, as depicted in Fig. S5, enabling the capabilities of novel designed nanoparticles in vitro and in vivo [35].

Upon successful modification of hybridized cyclic peptide and development of self-assembled peptide-DTPA (SAPD) nanoparticles, the specificity and effectiveness were evaluated. To assess the dual-targeting capabilities of SAPD nanoparticles, $\alpha_v\beta_3$-integrin positive cancer cells (U87MG) and $\alpha_v\beta_3$-integrin-negative cells (HEK-293) were used. Both cancer cells were separately treated with Cy5.5@SAPD and Cy5.5@SAPD-$\text{99mTc}$ nanoparticles to estimate the in vitro cytotoxicity potential. The results presented in Fig. 2a showed that Cy5.5@SAPD-$\text{99mTc}$ nanoparticles possess a comparatively higher cytotoxicity effect than Cy5.5@SAPD nanoparticles against U87MG cells, with $EC_{50}$ values of 20 µM and 25 µM, respectively. On the contrary, the MTT assay showed that both nanoprobes were weakly cytotoxic towards HEK-293 cancer cells as $\geq 80\%$ viable cells were found in 96-well plates as presented in Fig. 2b. The significantly higher cytotoxicity of Cy5.5@SAPD-$\text{99mTc}$ nanoparticles was attributed to the $\alpha_v\beta_3$-integrin targeting property of Cy5.5@SAPD and the radiolabeling with $\text{99mTc}$.

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**Scheme 1** Schematic representation of reaction’s mechanisms for the modification of cyclic peptide c[RGDKLAK] with cDTPA to design c[RGDKLAK]-DTPA complex (Step I). Furthermore, the complex was self-assembled to form nanoparticles in the presence of Cyanine5.5 NHS ester to form Cy5.5 coupled self-assembled cyclic peptide-DTPA (Cy5.5@SAPD) nanoparticles (Step IIa) followed by radiolabeling with $\text{99mTc}$-Tricarbonyl precursor (Step IIb).
nanoparticles might be due to the attachment of γ-emitting radionuclide compared with Cy5.5@SAPD nanoparticles [36]. So, further cell studies were performed using Cy5.5@SAPD nanoparticles to avoid the effect of radionuclides.

Next, the confocal laser scanning microscope (CLSM) images acquired after treatment with Cy5.5@SAPD nanoparticles showed weak red fluorescence intensity in HEK-293 cells (Fig. 2c); alternatively, images presented in Fig. 2d showed bright red fluorescence intensity in U87MG cells. The co-localization of DAPI staining in the nuclear region showed blue fluorescence, while the merged image showed localization of red fluorescence in the nuclear periphery region. These results highlight the specificity and efficacy of newly designed nanoparticles for αvβ3-integrin positive cancer cells due to RGD tripeptide and mitochondria-targeting effect due to the KLAK motif [11]. Furthermore, to investigate the appropriate cell apoptosis-inducing pathway, we also performed a CLSM imaging study by treating the U87MG cancer cells with Cy5.5@SAPD nanoparticles and co-stained with Mito-Tracker Green and Caspase-3 dyes. The CLSM images acquired with Cy5.5 filter after 30 min incubation time showed that Cy5.5@SAPD nanoparticles internalized

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**Fig. 1** Qualitative analysis of Cy5.5@SAPD and Cy5.5@SAPD-99mTc nanoparticles, (a) shows fluorescence intensity measurement for Cy5.5@SAPD nanoparticles, (b) shows percent radiochemical purity of Cy5.5@SAPD-99mTc nanoparticles via ultracentrifugation, (c, d) shows TEM images acquired using dried Cy5.5@SAPD and Cy5.5@SAPD-99mTc nanoparticles; respectively. The inset figures (c', d') show high-resolution TEM images of respective nanoparticles.
into cells via pinocytosis and enters into the mitochondria by disrupting the mitochondrial membrane as indicated in Fig. 2e. The merged images showed well-overlapped fluorescence to give yellowish-green color; scale bar 20 µm was set for all images. This ultimately produces reactive oxygen species (ROS) that causes mtDNA damage, while,

Fig. 2 In vitro cytotoxicity study of novel self-assembled peptide nanoparticles in HEK-293 and U87MG glioblastoma cancer cell lines. Cytotoxicity study was performed in (a) U87MG cancer cells and (b) HEK-293 cells using Cy5.5@SAPD and Cy5.5@SAPD-99mTc nanoparticles. CLSM images acquired after co-localization of Cy5.5@SAPD nanoparticles along with Hoechst-33258 staining dye for (c) U87MG and (d) HEK-293 cells. Further images indicate staining of U87MG cells with (e) Mito-Tracker green and (f) Caspase-3 dyes. FACS analysis shows apoptosis-inducing factor in U87MG cells (g; left) as control experiment and (right) for nanoparticle-treated study, while (h; left) for control and (right) nanoparticle-treated HEK-293 cancer cells. (i–j) shows Bio-TEM images acquired after treatment with Cy5.5@SAPD nanoparticles in U87MG cells indicate successful targeting and damage of mitochondria.
it releases cytochrome c to promote the formation of the apoptosome, causing activation of the Caspase-3 enzyme as shown in Fig. 2f [37]. The bright green fluorescence is due to the activation of the Caspase-3 enzyme, which is well-overlapped with red fluorescence as illustrated in the merged image showing bright yellow fluorescence in the nuclear periphery region [38, 39]. The merged CLSM images with blue-colored holes showed stained nuclear region (Fig. 2c, d), while blank holes showed unstained nuclear region (Fig. 2e, f) which indicates the improved specificity of Cy5.5@SAPD nanoparticles to selectively target mitochondria and induces cell apoptosis.

Furthermore, we also investigated the potential of apoptosis/necrosis induction in U87MG and HEK-293 cancer cells upon treatment with Cy5.5@SAPD nanoparticles. The results of U87MG cells presented in Fig. 2g (left) showed 95.6% live cells as control and Fig. 2g (right) showed 17.2% live cells as well as 26.8% early apoptotic and 52.7% late apoptotic cells, while only 1.3% necrotic cells. Moreover, the apoptosis study of HEK-293 cells presented in Fig. 2h (left) showed 91.8% live cells as control, while Fig. 2h (right) shows 66.7% live cells, 14.6% early apoptosis, and 17.9% late apoptosis with only 0.8% necrotic cells. These results give confirmation of our hypothesis that our novel designed nanoparticles have the potential to kill cancer cells significantly by inducing apoptosis in glioblastoma cancer cells compared with kidney cancer cells. This is due to the presence of high expression levels of αvβ3-integrin receptor and mitochondrial-targeting effect of the KLAK motif [40].

Additionally, we also performed the Bio-TEM imaging study using Cy5.5@SAPD nanoparticle–treated U87MG cancer cells. The TEM images showed pinocytic internalization of nanoparticles as shown in Fig. 2i. The image shows the accumulation of nanoparticles in the inner layers of the cell membrane and targets the mitochondria to induce apoptosis by damaging the mitochondrial membrane as illustrated by the HR-TEM image presented in Fig. 2j. The small white dots indicated the targeting of nanoparticles at the membrane of mitochondria and deformed mitochondrial shape indicates the disruption of membrane, which causes damage to mitochondria. The Bio-TEM images are in agreement with the CLSM study evidenced the induction of cancer cell apoptosis by damaging the mitochondria with high specificity and improved efficiency [35, 41, 42].

Additionally, we also investigated the dual-imaging potentials of Cy5.5@SAPD-99mTc nanoparticles in brain tumor glioblastoma (using U87MG cells) and human embryonic kidney (HEK-293) tumor-induced (5 × 10⁷ cells/mice subcutaneously) female Balb/c mice models. Firstly, the nanoparticles with a concentration of 20 μg/200 μL (~74 MBq) saline were injected via tail vein, and images were acquired using a dynamic SPECT/CT camera (NM/CT 670 Pro Discovery). The images presented in Fig. 3a indicate SPECT/CT images acquired after 30 min post-injection (p.i) with coronal, sagittal, and transaxial directions. The hybrid images showed the occurrence of brain tumor glioblastoma and kidney tumor with an excellent accumulation of proposed nanoparticles as tumor-to-background contrast showed at the left side of Balb/c mice [43]. The results presented in Fig. 3b depicted planar SPCET images acquired at 30 min p.i before the therapeutic dose treatment, and showed high internalization of radiotracer in brain tumor glioblastoma with 4.7 ± 0.8% ID/g as compared to kidney tumor (2.1 ± 0.9% ID/g). The results of the planar SPECT imaging study are comparable with that biodistribution study. After the therapeutic dose (30 mg/kg b.w) treatment within a very short period of nearly 1 week, Fig. 3c showed very less or negligible accumulation of radiotracer at the site of a brain tumor, while prominent uptake can be seen at the site of kidney tumor. The uptake in kidney tumor is because of the binding potential of the RGD motif to αvβ3-integrin overexpressed on HEK-293 cells [9]. The sharp decrease in brain tumor size indicated the insightful excellent therapeutic potential of Cy5.5@SAPD-99mTc nanoparticles for GBM. Furthermore, the brain tumor–bearing female balb/c mice models were subjected to fluorescence imaging study (IN VIVO FX Pro Carestream Camera) and the image presented in Fig. 3d showed prominent bright red fluorescence intensity at the brain tumor site in a live animal model. Moreover, Fig. 3e indicated the ex vivo images presenting pharmacokinetic of Cy5.5@SAPD-99mTc nanoparticles before and Fig. 3f after the therapeutic dose treatment. The main accumulation of radiotracer was found in the brain, liver, lungs, and kidneys, and nonspecific uptake was observed in the heart, stomach, and spleen. Similarly, the results presented in Fig. 3g, h showed ex vivo images before and after the therapeutic dose treatment, respectively. The tumor uptake was observed to be highly prominent than normal brain tissues and other body organs pointed out the enhanced efficacy, specificity, and effectiveness of our newly designed novel Cy5.5@SAPD nanoparticles as compared to previously reported nanoparticles [44–47]. Furthermore, we have also calculated the tumor weight and tumor volume of the dissected tumor tissues to estimate the changes occurring as a result of treatment with our novel designed nanoparticles. The tumor weights of nanoparticles treated groups were significantly twofold lower than that of the control group as presented in Fig. 3i. Similarly, the tumor volumes were also observed to be decreased continuously because of the therapeutic effect of dual-targeting nanoparticles, while the opposite trend was observed for the control group as presented in Fig. 3j. The very slight change in tumor volumes of mice models treated with Cy5.5@SAPD and Cy5.5@SAPD-99mTc can be seen in Fig. 3j that might be due to the ɣ-radiations emitted from radionuclide 99mTc.
Fig. 3  In vivo SPECT/CT and fluorescence imaging studies in tumor-bearing animal models. (a) shows SPECT/CT study of Cy5.5@SAPD-99mTc nanoparticles in tumor-bearing mice models, (b) shows in vivo planar SPECT images after 30 min post-injection (p.i) before therapeutic dose treatment, and (c) shows SPECT images at 30 min p.i after treatment. While, (e, f) shows ex vivo SPCE images indicating pharmacokinetic study before (left) and after (right) treatment, and (g, h) shows the ex vivo fluorescence images before (left) and after (right) treatment, (i) indicates the tumor weight and (j) shows tumor volume calculated from ex vivo images in tumor-bearing mice models.
Conclusion

In light of the abovementioned results, firstly, we designed a novel hybridized peptide sequence consisting of RGD motif for targeting αvβ3-integrin and KLAK pro-apoptotic motif for targeting mitochondria, which ultimately induces cancer cell apoptosis by producing ROS and the activation of the Caspase-3 enzyme. This dual-targeting peptide probe was further modified with DTPA for radiolabeling with γ-emitting radionuclide and serves as SPECT/CT imaging agent. Next, the peptide-DTPA complex was self-assembled with Cy5.5 NHS to form uniform spherical-shaped nanoparticles for molecular optical imaging study as the novel dual-imaging probe. Consequently, these novel dual-imaging dual-targeting self-assembled cyclic peptide nanoparticles were successfully designed to possess improved diagnostic and therapeutic capabilities with enhanced specifically and efficiently for GBM. The in vitro cytotoxicity assay, apoptosis assay, and CLSM imaging studies illustrated that these newly synthesized Cy5.5@SAPD nanoparticles have the potential to internalize specifically and efficiently into U87MG brain tumor cells as compared to HEK-293 kidney tumor cells for diagnosis of GBM. SPECT/NIRF theranostic studies in tumor-bearing female Balb/c mice models shows the excellent potential of our novel designed Cy5.5@SAPD-99mTc nanoparticles to diagnose brain tumor more prominently as well as showed excellent therapeutic effectiveness within 1 week of treatment. These outcomes suggested that our novel designed theranostic nanoparticles (Cy5.5@SAPD-99mTc) may serve efficiently, and specifically as potential SPECT/NIRF nanoprobes for future preclinical and clinical studies against brain tumor glioblastoma multiform.

Materials and methods

Chemistry: synthesis of cyclic peptide-DTPA complex

A simple condensation reaction of DTPA dianhydride for coupling with cyclic peptide was carried out using the protocol reported by Shi et al. [48] with fewer modifications. Briefly, DTPA dianhydride (1.5 mg, 0.0042 mmol) was dissolved in 1:3 volume of DMF (50 µL) and DMSO (150 µL). The deprotected free carboxylic group was activated by treating with HATU (1.59 mg, 0.0042 mmol) dissolved in DMF (50 µL) and stirred for 30 min at room temperature. Then, the white crystals of cyclic peptide c[RGD(Boc)KLAK] (3.65 mg, 0.0042 mmol) were dissolved in Milli-Q water (200 µL) and were added dropwise over 30 min in cDTPA mixture and stirred for another 30 min. Later on, the coupling agent DIPEA (20 µL, 0.115 mmol) dissolved in DMF (50 µL) and stirred for 30 min at room temperature in darkness under N2-purging. On the next day, the solution was stirred at three different speeds till 48 h while the presence of additional functional groups was confirmed by FTIR-ATR analysis for the cPD complex.

Synthesis of fluorescent functionalized self-assembled peptide-DTPA nanoparticles

A facile self-assembling strategy was adopted for the synthesis of cyclic peptide nanoparticles as reported in literature [28]. For this purpose, the cPD complex (1 mg, 0.87 mmol) was dissolved in 1.8 mL of Milli-Q water. Additionally, 0.2 mL of 0.1 M sodium borate solution (Na2B4O7, pH 8.3) was added dropwise in cPD solution and stirred for 2 h at 4 °C. The intrinsic radiolabeling of fluorescently coupled self-assembled cyclic peptide-DTPA (Cy5.5@SAPD) nanoparticles, followed by washing with deionized (DI) water twice to remove the acidic water. Next, the sonication was applied for 2 h to get dispersed SAPD nanoparticles in the DI water and characterized by a fluorescence spectrometer, dynamic light scattering (DLS), and transmission electron microscope (TEM).

Radiosynthesis of Cy5.5@SAPD nanoparticles

The intrinsic radiolabeling of fluorescently coupled self-assembled cyclic peptide-DTPA (Cy5.5@SAPD) nanoparticles with a γ-emitting radionuclide (99mTc) was carried out by using sodium borohydride as reducing agent via...
Cultured in humidified incubator having 5% CO₂ and 37 °C. The cells were supplemented with 10% FBS and 1% antibiotics (50 U/mL penicillin and 50 µg/mL streptomycin). Both media were the Cell Bank of Chinese Academy of Sciences (CBCAS) containing medium were removed, washed twice with deionized water (0.5 mL), vortex for 30 s followed by purging with N₂-gas for 5 min to remove bubbles in the solution. Then, ~ 185 MBq of ⁹⁹mTc was added to the solution vial and allowed to incubate for 30 min at 95 °C. After successful complexation, the solution was cooled at room temperature and pH 7 of the complex was adjusted using 0.1 M HCl solution. The radiochemical purity of the resulting ⁹⁹mTc-tricarbonyl complex was assessed by using TLC plates coated with silica gel as stationary phase and by using a mixture of methanol:HCl (95:5% v/v) as mobile phase (Rf for ⁹⁹mTc-tricarbonyl = 0.85–0.95; Rf for Cy5.5@SAPD-⁹⁹mTc(CO)₃ = 0.55–0.75) as well as radio-HPLC method. Approximately 98% pure ⁹⁹mTc-tricarbonyl complex (185 MBq/0.5 mL saline) was filtered through 0.22 µm Milipore and added into Eppendorf tubes containing Cy5.5@SAPD nanoparticles (as ligand; 20 µg/100 µL Na-PBS, pH 6.5). The reaction mixture was allowed to incubate for 20 min at 55 °C. After successful radiolabeling, the newly developed radiotracer (Cy5.5@SAPD-⁹⁹mTc(CO)₃ nanoparticles) was cooled at room temperature, and quality control analysis was performed using the TLC-SG method followed by ultracentrifugation methods.

**Tumor cell culture**

Human brain tumor glioblastoma cell line U87MG (αvβ1,-integrin positive) and human embryonic kidney cell line HEK-293 (αvβ1,-integrin positive) were purchased from the Cell Bank of Chinese Academy of Sciences (CBCAS) and cultured in Iscove’s Modified Dulbecco’s Medium (IMDM) and RPMI-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% CO₂ and 37 °C temperature.

**Cytotoxicity assay**

In vitro cytotoxicity study of Cy5.5@SAPD and Cy5.5@SAPD-⁹⁹mTc(CO)₃ nanoparticles was assessed by MTT assay. The cancerous cells were first cultured with a density of 5 × 10⁵ cells per well in a 96-well plate using respective growth mediums in a 5% CO₂ 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 µM) 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, washed twice with preheated (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 10 M (TECAN, Switzerland). The same data was used to calculate the half-maximal effective concentration (EC₅₀) values for both tested samples. All experiments were carried out in triplicate to measure plus/minus standard deviation (± SD) values.

**Confoal laser scanning microscopy (CLSM)**

The cancer cells were seeded as described above with a density of 1 × 10⁵ 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% CO₂ 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 Caspase-3 kit (Ac-DEVD-pNA) for another 30 min, and 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 and HEK-293 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 30 µM concentration of Cy5.5@SAPD for 24 h. Furthermore, 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-targeting 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.

In vivo imaging, pharmacokinetics, and therapeutic study

For in vivo therapeutic study, female Balb/c mice (5 weeks old, 16–18 g, 20 mice in total) were purchased from the National Institute of Health (NIH), Islamabad, Pakistan. All animal studies were carried out as per guidelines issued by the animal ethical committee of the National Institute of Health (NIH) and National Regulation of China for Care and Use (NRCCU) of Laboratory Animals (Lanzhou University, Gansu, China). Each mouse model bearing glioblastoma and human embryonic kidney cancers was successfully established by injecting 5 × 10⁷ cells suspended in 100 µL PBS (pH 7.4) subcutaneously into the right flank area of each mouse and when tumor volume reaches 500–600 mm³ diameter. All mice were divided into two groups (n = 3 each group) randomly to receive normal saline (control), and Cy5.5@ SAPD (treated). To observe the in vivo tumor accumulation and therapeutic potential, a single therapeutic dose of Cy5.5@SAPD in saline (30 mg/kg body weight; b.w) was injected intravenously via the tail vein of each group. The body weight and tumor size of each mouse were monitored after a couple of days. On the sixth day of post-injection, mice were injected with 20 µg/200 µL of Cy5.5@SAPD-NaI(Tl)γ and images were acquired using SPECT camera. Later on, they were sacrificed after giving chloroform anesthesia; tumor mass, as well as other organs, was segregated and weighed and radioactivity was measured using NaI(Tl) γ-scintillation counter. At the same time, the images were also acquired using IN VIVO FX camera for optical imaging, and segregated organs were also observed for uptake of our newly designed nanoparticles by observing the fluorescence intensity in each organ. Furthermore, the tumor weight and tumor volume were also calculated from tissues obtained as a result of ex vivo studies.

Statistical analysis

All experiments reported in this study were performed in triplicate and results are given as ± standard deviation (± SD) of ‘n’ independent measurements. Statistical significance was calculated using Student’s t-test. The significance level was assigned as p < 0.05.

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Author contribution S.F.A.R.: conceptualization; methodology; validation; investigation; writing—original draft. S.S.: formal analysis, visualization, resources. S.M.: validation, visualization. H.Z.: validation; project administration; writing—review and editing; supervision.

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Declarations

Ethical approval All animal studies performed in this study were in accordance with the compliance of the animal ethical committee of the National Institute of Health and National Regulation of China for Care and Use of Laboratory Animals (Lanzhou University, Gansu, China).

Consent to participate Informed consent was obtained from the human participants of this study.

Consent for publication Consent for publication was obtained from the participants.

Conflict of interest The authors declare no competing interests.

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