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Abstract

Aim: To synthesize cRGDfK peptide conjugated poly(γ-glutamic acid)-phenylalanine nanoparticles to improve the therapeutic efficacy of camptothecin (CPT) against glioblastoma multiforme.

Methods: Peptide-conjugated, drug-loaded nanoparticles (cRGDfK-conjugated camptothecin-loaded PGA-PA nanoparticles [RCPN]) were prepared and physico-chemically characterized using different techniques. Nanoparticles were evaluated for in vitro anticancer activity, cellular uptake, induction of apoptosis and wound healing cell migration against U87MG human glioblastoma cells.

Results: RCPN, with a particle size of < 100 nm and 65% CPT encapsulation efficiency, exhibited a dose-and time-dependent cytotoxicity to glioblastoma cells. Compared with native CPT or unconjugated nanoparticles, RCPN induced apoptosis, increased reactive oxygen species generation and inhibited U87MG cell migration.

Conclusion: cRGDfK-mediated and amphiphilic copolymer-based nanomedicines represent a new approach for improved delivery of anticancer drugs to and treatment of glioblastoma multiforme.

Disciplines

Medicine and Health Sciences

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Peptide grafted and self-assembled poly(γ-glutamic acid)-phenylalanine nanoparticles targeting camptothecin to glioma

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Abstract

Aim: To synthesize cRGDfK peptide conjugated poly(γ-glutamic acid)-phenylalanine nanoparticles to improve the therapeutic efficacy of camptothecin (CPT) against glioblastoma multiforme (GBM). **Methods:** Peptide-conjugated, drug-loaded nanoparticles (RCPN) were prepared and physico-chemically characterized using different techniques. Nanoparticles were evaluated for *in vitro* anticancer activity, cellular uptake, induction of apoptosis and wound healing cell migration against U87MG human glioblastoma cells. **Results:** RCPN, with a particle size of <100 nm and 65% CPT encapsulation efficiency, exhibited a dose- and time-dependent cytotoxicity to glioblastoma cells. Compared to native CPT or unconjugated nanoparticles, RCPN induced apoptosis, increased reactive oxygen species generation and inhibited U87MG cell migration. **Conclusion:** cRGDfK-mediated and amphiphilic copolymer based nanomedicines represents a new approach for improved delivery of anticancer drugs to and treatment of GBM.

Keywords: Glioblastoma multiforme; camptothecin; poly(γ-glutamic acid); phenylalanine; cRGDfK; anti-cancer activity

***Abbreviations*:** Carboxy-DCFDA: 5(6)-Carboxy-2′,7′-dichlorofluorescein diacetate; CPN: Camptothecin loaded PGA-PA nanoparticles; CPT: Camptothecin; cRGDfK: Cyclo(Arg-Gly-Asp-D-Phe-Lys); DCFDA: Carboxy-2', 7'-dichlorofluorescein diacetate; DMEM: Dulbecco's modified eagle medium; DMSO: Dimethyl sulfoxide; EDC: 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide; FBS: Fetal bovine serum; GBM: Glioblastoma

multiforme; IC_{50}: half-maximal inhibitory concentration; MTT, 3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide; NHS: N-hydroxysuccinimide; PA: Phenyl alanine; PAE: Phenyl alanine ester; γ-PGA: Poly(γ-glutamic) acid; PGA-PA: Poly(γ-glutamic acid)-phenyl alanine conjugate; RCPN: cRGDfK conjugated and Camptothecin loaded PGA-PA nanoparticles; ROS: Reactive oxygen species; RPN: Rhodamine-b loaded PGA-PA nanoparticles; RRPN: cRGDfK peptide conjugated RPN; UPLC: Ultra performance liquid chromatography; VEGF: Vascular endothelial growth factor.

Introduction

Glioblastoma multiforme (GBM) is the most common type of brain tumor, accounting for 29% of primary brain cancers.[1] Among all types of cancer, GBM is the most difficult to treat as it localizes in brain that has limited capacity to repair itself. In addition, GBM tumors show inherent resistance to conventional therapy and have poor blood supply; thus drug delivery is inhibited.[2] Tumor infiltration into brain tissue makes it very difficult to treat GBM with conventional surgical removal. Because of this diffusive invasion, recurrence from residual tumors is also common.[3] Therefore, multiple therapies are needed to treat GBM. Currently, the best approach for the management of GBM is initial surgery followed by radiation and adjuvant chemotherapy.[4, 5] Chemotherapy plays an important role in the treatment of GBM especially in recurrent GB, however, the blood brain barrier, drug resistance and systemic toxicity of anticancer drugs decrease the therapeutic efficacy of available agents.[6]

In recent years, ligand-mediated, targeted drug delivery has shown promise in effective delivery of drugs to brain tumors.[7, 8] In ligand-mediated drug delivery, nanoparticle surfaces are modified with a ligand that selectively binds to specific receptors on cancer cells. Receptors for molecules such as glucose,[9] folate,[10] human epidermal growth factor,[11] lectins,[12, 13] lactoferrin,[14] transferrin[15, 16] and integrin have been studied as a potential targets for chemotherapy to treat brain cancer.[17-24] Peptides, such as TAT peptide, that can penetrate cells have also been used to target brain tumor.[25, 26] However, these cell-penetrating peptides may also enhance the penetration of nanocarriers into normal cells resulting in toxicity. $\alpha_v\beta_3$ integrins are over-expressed on endothelial and tumor cells in both primary and recurrent GBM.[27-29] RGD-based peptides have high affinity towards $\alpha_v\beta_3$ integrins and cyclic RGD peptides have been used successfully for in vivo imaging, magnetic resonance imaging and drug delivery to GBM.[30-32] Kim et al. used RGD-conjugated peptide and radioactive iodine-labelled gold nanoparticles for imaging of tumor sites.[33] Jin et al. synthesised cRGDyK

conjugated paramagnetic nanoprobes for the imaging of $\alpha_v\beta_3$ overexpressing U87MG tumor xenografts.[34] In a previous study, we demonstrated the targeting of cRGDfK-grafted nanomicelles to DU145 prostate cancer cells and to SKOV-3 ovarian cancer cells that overexpress integrin $\alpha_v\beta_3$.[35, 36]

Here, we synthesized cRGDfK conjugated poly(γ-glutamic) acid nanoparticles for the selective delivery of camptothecin (CPT) to GBM. γ-PGA is a microbially produced and non-toxic biopolymer. The degraded product of γ-PGA is a natural amino acid i.e. glutamic acid. Due to excellent biocompatibility and biodegradation, it has been explored for a wide range of biomedical applications including drug delivery.[37] High drug loading capacity, stability in biological fluids and controlled release properties are specific advantages of polymeric nanoparticles.[38] However, native γ-PGA is not suitable as drug delivery carrier because of aqueous solubility. Therefore, it was conjugated with hydrophobic phenyl alanine to produce an amphiphilic copolymer which spontaneously forms nanoparticles in aqueous solution. CPT is a natural quinone alkaloid that has high efficacy against malignant gliomas.[39-41] CPT binds to the topoisomerase I-DNA complex during cell division, stabilizing the complex and therefore preventing the replication processes.[42] Moreover, CPT acts only in the S-phase of cell cycle. As cancer cells have longer S-phase compared to normal healthy cells, S-phase selectivity makes CPT a suitable drug candidate in the development of a targeted drug delivery system for GBM.[43] Poor penetration across the blood brain barrier, dose-limiting toxicity and rapid hydrolysis at physiological pH are the major inherent problems with native CPT.[44] Therefore, encapsulation of CPT in cRGDfK conjugated poly(γ-glutamic) acid nanoparticles may overcome these drawbacks of CPT.

Experimental section

Materials

cRGDfK was purchased from Peptide International (Kentucky, USA). CPT, Poly(γ-glutamic) acid (γ-PGA) molecular weight ~50,000-100,000 Da, phenylalanine (PA), 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), N-hydroxysuccinimide (NHS), Dulbecco's modified eagle medium (DMEM), trypsin–EDTA, penicillin, streptomycin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and propidium iodide (PI) were purchased from Sigma Aldrich (AUS). Fetal bovine serum (FBS) was purchased from Gibco, USA. Carboxy-2',7'-dichlorofluorescein diacetate (DCFDA) and Hoechst 33342 were purchased from Life Technologies Australia Pvt Ltd. (AUS).

Synthesis and characterization of phenylalanine ester (PAE)

Thionyl chloride (0.87 mL, 12.1 mM) was added to a solution of phenylalanine (1 g, 6.05 mM) in methanol. The reaction was continued for 30 min in an ice-bath and then stirred for 24 h at room temperature. After completion of the reaction (as monitored by thin layer chromatography), an excess of thionyl chloride was removed under reduced pressure using a rota-evaporator. Phenylalanine ester (PAE) was obtained as a white solid powder and characterized by ^{1}H NMR and FTIR analysis.

Synthesis and characterization of poly(γ-glutamic acid)-phenylalanine (PGA-PA) conjugate

PGA-PA conjugate was synthesized as described previously.[45] Briefly, 200 mg γ-PGA was dissolved in 30 mL sodium bicarbonate solution (50 mM) and placed in an ice-bath on a magnetic stirrer. EDC (300 mg) was then added and stirred for 1 h. Thereafter, PAE (310 mg) was added to the reaction mixture and stirred overnight. PGA-PA conjugate was purified by dialysis against deionized water for three days, freeze-dried and stored. The conjugate was

characterized by proton nuclear magnetic resonance (^{1}HNMR) and Fourier transform infrared (FTIR) analysis.

Preparation of CPT loaded PGA-PA nanoparticles (CPN)

For the preparation of CPT loaded PGA-PA nanoparticles (CPN), CPT and PGA-PA were dissolved in DMSO and mixed with surfactant solution (1.5% w/v Tween 80). The dispersion was dialyzed against deionized water for 24 h and then centrifuged for 30 min at 15,000 rpm. The nanoparticle pellet was redispersed in distilled water and again centrifuged to remove free or non-encapsulated drug. Drug loaded nanoparticles were freeze-dried and stored at 4 °C. The amount of drug in the supernatant was determined using an Ultra performance liquid chromatography (UPLC) system (Waters Acquity UPLC, Aus). A reversed-phase column (Acquity C18, 1.7 μm, 2.5 × 50 mm) was used at 30 °C. The mobile phase was composed of methanol and water (45:55). The flow rate was set at 0.45 mL/min and the detection wavelength was 256 nm.

Characterization of nanoparticles

Particle size and zeta potential were determined by photon correlation spectroscopy using a Zetasizer Nano-ZS (Malvern instrument Ltd., Malvern, UK). The surface morphology of nanoparticles was studied by transmission electron microscopy (TEM). Differential scanning calorimetry of native CPT, PGA-PA, a physical mixture of CPT and PGA-PA, and CPN were carried out on aDSC-Q100 (TA Instruments, USA). The samples were scanned from 25 to 300 °C at a speed of 10 °C/min under nitrogen environment. Powder X-ray diffraction patterns of CPT and CPN were obtained in the range of 5–65º using an X-ray Diffractometer (D8 Advance, Bruker, Germany), equipped with a graphite crystal monochromator (CuKa). The instrument was set at a voltage 40 KV and a current of 40 mA.

Bioconjugation of cRGDfK to CPN (RCPN)

Nanoparticles (50 mg) were dispersed in 5 mL phosphate buffer saline and incubated with EDC (35 mg) and NHS (21 mg). After 30 min, cRGDfK was added and stirred for 4 h at room temperature. cRGDfK conjugated nanoparticles were obtained by centrifugation and washed three times with PBS. The supernatant was used to determine the amount of unconjugated cRGDfK.

***In vitro* drug release studies**

The release of CPT from nanoparticles was studied by modified dialysis method. Phosphate buffer saline pH 7.4 and sodium acetate buffer pH 5 were used as release media. Nanoparticles, equivalent to 0.5 mg CPT, were dispersed in 1 mL of release media and placed in a dialysis bag. This dialysis bag was further placed in a beaker containing 50 mL of release media maintained at 37 °C and stirred at 100 rpm. At the end of each time point, 1 mL DMSO was added to the dialysis bag and drug content was measured. The % drug release (DR) was determined as follows: $\% DR = \{(D_I - D_F)/D_I\} \times 100$

where DI = Initial amount of drug present in nanoparticles added in the dialysis bag, and DF = Final amount of drug present in the nanoparticles after a time interval.

Cell culture

Mycoplasma-free U87MG human glioblastoma cells were obtained from Peter MacCallum Cancer Institute, Australia. Cells were grown in DMEM media supplemented with 10% FBS, 2 mM glutamine, penicillin (100 U/mL), and streptomycin (0.1 mg/mL). Cells were cultivated under standard conditions at 37 °C in a humidified atmosphere (85% relative humidity) containing 5% CO_2.

In vitro cytotoxicity assay

The cytotoxicity of the pure CPT and drug-loaded nanoparticles was evaluated by the MTT assay. U87MG cells (5×10^3) were seeded in 96-well plates and incubated for 24 h. Cells were then treated with CPT, CPN or RCPN (1-400 ng/mL) for 24 and 48 h at 37 °C. Untreated cells served as controls. After incubation, 100 µL DMEM medium containing MTT reagent (0.5 mg/mL) was added to each well. The plate was then incubated at 37 °C for 4 h. The violet-colour formazan crystals were dissolved in 150 µL DMSO. Absorbance was measured at 570 nm using a microplate reader and the percentage viability of cells was calculated as the ratio of absorbance of sample to control wells.

Cellular uptake of nanoparticles in cells

Comparative uptake of unconjugated (RPN) and cRGDfK-conjugated nanoparticles (RRPN) was studied using Rhodamine-b loaded nanoparticles, which were prepared in manners similar to those for CPN and RCPN, except that instead of CPT drug, Rhodamine-b was loaded in these nanoparticles. Cells were incubated with RPN and RRPN for 2 h at 37 °C. Cells were washed twice with PBS and incubated with 5 µg/mL Hoechst 33342 to stain nuclei. After 30 min incubation, cells were washed with PBS and observed by fluorescence microscopy. To confirm the receptor-dependent binding of RRPN, cells were incubated with an excess of cRGDfK peptide (100 µg/mL) 1 h prior to incubation with targeted nanoparticles RRPN.

Estimation of apoptosis

Apoptosis in cells treated with CPT, CPN and RCPN was estimated by an Annexin V PE Cys5.5 and PI assays. Cells were seeded in 6-well plates at 1×10^6 cells/well and incubated for

24 h to allow adherence to the plate surface. Cells were treated with 20 ng/mL CPT, CPN or RCPN for 24 h. The cells were then collected, washed with PBS, stained according to the manufacturer's instructions and analyzed by BD FACS Canto™ II.

Nuclear staining with Hoechst 33242

Nuclear morphology of CPT, CPN or RCPN treated cells was studied by Hoechst 33342 staining. Hoechst 33342 is a DNA specific fluorescent dye used to study nuclear morphology. U87MG cells (1×10^5) were seeded in 24-well microplates and incubated overnight. Cells were then incubated with free drug or nanoparticles (CPT concentration, 20 ng/mL). After washing with PBS, cells were stained with Hoechst 33342 (5 µg/mL) at 37 °C for 30 min in the dark. Cells were washed again with PBS and observed under afluorescence microscope. % Apoptosis was determined as follows:

% Apoptosis = (Number of apoptotic cells/Total number of cells) × 100

Measurement of reactive oxygen species (ROS)

U87MG cells were seeded at a density of 1×10^5 cells per well in 24-well plates and incubated for 24 h at 37 °C. Cells were then treated with CPT formulations equivalent to 20 ng/mL CPT for 24 h, washed twice with PBS and fixed with 4% paraformaldehyde for 10 min. After washing two times with PBS, cells were incubated with DCFDA (10 µM) for 30 min in the dark. Cells were finally washed with PBS and observed using a fluorescence microscope (Nikon, Japan).

For quantitative estimation of ROS, cells were harvested using 0.25% trypsin-EDTA and washed with PBS. Cell counts were adjusted for all formulations. Cells were incubated with DCFDA (10 µM) for 30 min in the dark and fluorescence intensity was measured at

excitation and emission wavelengths of 495 and 520 nm, respectively, using a Hitachi F-3010 spectrofluorometer.

Wound healing scratch assay

The effect of CPT formulations on wound healing or the migration of cells was analyzed by the scratch-wound healing assay, with slight modification as reported previously.[46] U87MG cells were cultured to form an approximate80% confluent monolayer. The monolayers were scratched with a sterile 200-μL pipette tip to form a "wound". Cells were washed twice with PBS to remove cellular debris. Cells were then incubated with CPT, CPN or RCPN in serum-free media for 24 h. Images were captured at 0 and 24 h to monitor the migration of cells into the wound area using a photomicroscope (Nikon, Japan). The % of wound closure was determined by measuring the wound area using Image J analysis software.

Statistical analysis

All studies were performed in triplicate and results are expressed as Mean ± SD (standard deviation). Statistical significance was determined using the student t-test for two groups and one-way ANOVA for multiple groups. Statistical significance was assumed at $p < 0.05$.

Results

Synthesis and characterization of PGA-PA conjugates

For the synthesis of poly(γ-glutamic acid)-phenylalanine conjugate (PGA-PA) firstly, phenylalanine (PA) was converted to phenylalanine ester (PAE). Esterified PA was conjugated to γ-PGA through a carbodiimide reaction (Figure S1) and conjugation efficiency was determined by NMR analysis (Figure S2). The peaks corresponding to the methylene protons (δ 2.1) of γ-PGA and the aromatic protons of the benzene ring (δ 7.3) of PA were

compared. The conjugation efficiency was 52.9%. The PGA-PA conjugate was also confirmed by FTIR analysis (Figure S3). The FTIR spectrum of PA showed two characteristic N-H stretching vibration bands at 3078 cm^{-1} and 3030 cm^{-1}. Bands corresponding to asymmetric and symmetric stretching vibrations of the carboxylic group (COO^-) were observed at 1557 cm^{-1} and 1409 cm^{-1}, respectively. The stretching vibration band of benzene appeared at 745 cm^{-1}. After esterification of the carboxylic group of PA, a sharp band was observed at 1736 cm^{-1} indicative of C=O stretching of the ester bond. The FTIR spectrum of γ-PGA showed bands at 3263 cm^{-1} (NH stretching), 1644 cm^{-1} (amide I) and 1551 cm^{-1} (amide II). γ-PGA was conjugated to esterified PA through amide coupling between the amine group of PA and amine group of γ-PGA. The FTIR spectrum of the PGA-PA conjugate showed a shift in the γ-PGA peaks (3263 to 3278, 1644 to 1648 and 1551 to 1544 cm^{-1}). A new peak at 746 cm^{-1} corresponding to the benzene ring of PA confirmed successful conjugation of PA to PGA.

Characterization of nanoparticles

BPN (blank nanoparticles without the drug) and CPN were synthesized by the dialysis method. Physicochemical parameters of different nanoparticles are presented in Table 1. BPN showed particle size of 66 nm with very low polydispersity of 0.08 and high negative zeta potential of −30.9 mV. After CPT loading, the nanoparticle size was increased slightly to 81.6 nm without a significant change in polydispersity (0.09) and zeta potential (−29.7 mV). cRGDfK grafted nanoparticles were of 89.7 nm in size and −16.7 mV zeta potential (Table 1). A decrease in negative zeta potential of RCPN confirmed the conjugation of cRGDfK on the surface of CPN. Morphology of nanoparticles was studied using transmission electron microscopy (TEM) analysis (Figure S3). The size of the nanoparticles after drug loading and cRGDfK conjugation was less than 100 nm (Figure 1a); an ideal size for a nanocarrier system

to cross the blood-brain barrier.[8] RCPN were also characterized by FTIR analysis (Figure 1b). % encapsulation efficiency for CPN and RCPN was 68.4% and 65.2%, respectively.

Drug loaded nanoparticles were also characterized for differential scanning calorimetry (DSC) and X-ray diffraction (XRD) analysis. DSC spectra of CPT and CPN are presented in Figure 1c. The DSC spectra of CPT showed a sharp endothermic peak at 268 °C corresponding to the melting point. However, this peak was not observed in CPT-loaded nanoparticles (CPN). The absence of a CPT peak may be due to its amorphous or disordered crystalline state or to solid solution in polymeric matrix of nanoparticles.

Figure 1d demonstrates the XRD patterns of pure CPT, PGA-PA conjugate, a physical mixture of CPT and PGA-PA, and CPN. The XRD pattern of raw CPT showed characteristic high-intensity diffraction peaks at diffraction angles of 2θ 6.2°, 8.5°, 11.5°, 12.1°, 13.6°, 17.8° and 25° suggesting the crystalline nature of the drug.[47] Due to its amorphous nature, the XRD patterns of PGA-PA did not show any sharp peaks. We observed all the characteristic peaks of CPT in the XRD pattern of the physical mixture of CPT and PGA-PA, demonstrating a lack of chemical interaction between CPT and PGA-PA. However, owing to transformation of CPT from a crystalline to an amorphous phase during nanoparticle preparation, the peaks representing CPT were absent in the CPN XRD pattern.

The release of CPT from nanoparticles was determined by modified dialysis method. As CPT is unstable at physiological pH, drug release was indirectly determined by measuring the drug present in the nanoparticles. Figure S4 shows that the release of CPT from nanoparticles was pH-dependent. After 24, 48 and 72 h, the release of CPT from nanoparticles at pH 7.4 was 26%, 33% and 40%, respectively, whereas for the same time points, at pH 5 CPT was released 37%, 43% and 54%, respectively. The slow release of drug from nanoparticles at physiological pH would help in improved intracellular delivery.

Further, comparatively faster release at acidic pH indicates that encapsulated drug will release rapidly after reaching the cell organelles (lysosomes and endosomes) where the pH of drug remains below 5.5.[48] It was further observed that CPT was released in a biphasic pattern. Initially there was fast release of drug up to 12 h which was sustained up to 72 h. Approximately 21% and 29% of encapsulated drug was released from nanoparticles at pH 7.4 and pH 5, respectively, after 12 h of dialysis. This initial rapid release of drug can be explained by the release of drug present in the periphery of the nanoparticles.

In vitro cytotoxicity

In order to investigate the anti-proliferation activity of native CPT and CPT loaded formulations (CPN and RCPN), cell viability of U87MG human glioblastoma cells was determined using the MTT assay. Anticancer activities of pure drug as well as drug-loaded nanoparticles depend on both the dose of drug and time of incubation. Therefore, U87MG cells were incubated with free CPT, CPN or RCPN, equivalent to 1-400 ng/mL CPT, for 24 and 48 h. The cell viability of U87MG cells after 24 h and 48 h are shown in Figure 2a and b, respectively. All three formulations showed caused a significant decrease in cell viability. After 24 h, the cell viability was 99.2%, 77.3%, 63.4% and 34.5% after incubation with blank nanoparticles (BPN), CPT, CPN or RCPN, respectively, equivalent to 50 ng/mL CPT. As the incubation time was increased, cell viability further decreased. At the same drug concentration (50 ng/mL CPT), the observed cell viability was 98.1%, 45.8%, 33.4% and 20.7%, respectively, after 48 h incubation. Half-maximal inhibitory concentrations (IC_{50}) for the three formulations are presented in Table 2. After 24 h, targeted nanoparticles (RCPN, IC_{50} = 30.46 ng/mL) were more effective than non-targeted nanoparticles (CPN, IC_{50} = 168.29 ng/mL) and native CPT (IC_{50} = 552.21 ng/mL) ($p < 0.01$). A similar pattern was observed after 48 h incubation where the IC_{50} value of RCPN (11.34 ng/mL) was 2.3 and 5.4 times lower than CPN (27.05) and CPT (61.78 ng/mL), respectively. The results suggest that

RCPN not only inhibits cell growth at higher efficiency than native CPT ($p < 0.01$) but also lowers the minimum effective dose required for cytotoxicity.

Uptake of nanoparticles by U87MG cells

Internalization of nanoparticles was studied using Rho-b loaded nanoparticles. Nuclei were visualized by Hoechst 33342 staining. Figure 3 shows fluorescence microscopic images of U87MG cells after 2 h of incubation with nanoparticles. cRGDfK conjugated and Rho-b loaded γ-PGA nanoparticles (RRPN) showed higher intracellular accumulation of Rho-b than unconjugated nanoparticles (RPN). The increased uptake of targeted nanoparticles could be attributed to the presence of cRGDfK on the surface of nanoparticles. Peptide containing RGD moieties have been reported to be taken uptake by U87MG cells more efficiently than unconjugated nanoparticles.[49,50] Therefore, the enhanced cytotoxicity of RCPN formulation than CPN can be correlated to the higher uptake of RRPN. To further confirm uptake of RRPN through integrin receptors, competitive binding assay was performed. Integrin receptors expressed on the surface of U87MG cells were blocked by adding an excess of cRGDfK peptide before incubating with RRPN. The fluorescence intensity in these cells was significantly lower compared to cells incubated with RRPN alone which suggested that RRPN were taken by integrin receptors.

Analysis of apoptosis

Evasion of apoptosis by cancer cells is major barrier to cancer treatment.[51] Apoptosis induced by different CPT formulations was studied both qualitatively and quantitatively using Hoechst staining (Figure 4a, b). Cells treated with CPT, CPN or RCPN showed fragmented nuclei whilst control or untreated cells exhibited intact nuclei (Figure 4a). The percentage of apoptotic cells after treatment with RCPN (46.9%) was much higher than with CPN (35.6%) and CPT (26.9%) treatment (Figure 4b). Apoptosis was also analysed by annexin Cys5.5/PI

assay using a flow cytometer. As shown in Figure 4a, the percentage of live U87MG cells was decreased with the three CPT formulations. The percentage of live cells in control, CPT, CPN and RCPN was 98.4%, 71.6%, 62.4% and 52.5%, respectively. The ability of the different CPT formulations to induce cell death followed the order: RCPN>CPN>CPT (Figure 4a). Cells treated with RCPN were more apoptotic compared to those treated with native drug and CPN ($p < 0.01$).

Intracellular ROS generation

Anticancer drugs, including CPT, generate ROS.[52-54] Intracellular reactive oxygen species (ROS) generation can be measured using the DCFDA dye. Fluorescent intensity of DCFDA is directly related to intracellular ROS levels. In the present study, fluorescence intensity was measured both qualitatively and quantitatively (Figure 5a, b). Control, or untreated cells, showed less green fluorescence than drug treated cells. Among the three CPT formulations, RCPN showed 2.51 and 1.48 times higher fluorescence intensity than CPT and CPN, respectively. This may be explained by the enhanced intracellular delivery of CPT by targeted nanoparticles.

Wound-healing assay

Gliomas exhibit significant angiogenesis that is induced by vascular endothelial growth factor (VEGF). VEGF is responsible for the formation of new blood vessels required for tumor growth and cell migration.[55,56] To study the effect of targeted delivery of RCPN on the cell migration of U87MG, wound-healing or scratch assays were performed. Cells were observed by light microscopy. Figure 6a shows the migration distance observed after 0 and 24 h of wound creation. % Closure of wound area observed after 24 h of treatment was 86.3%, 63.8%, 47.4% and 9.6% for control, CPT, CPN and RCPN, respectively (Figure 6b).

Discussion

GBM is a grade IV astrocytoma arising from glial cells. These tumors show cellular heterogeneity and can rapidly invade surrounding tissues. Despite efforts, and multimodal aggressive treatments, the median survival time is only about 12 months.[57] The most common problem in the treatment of GBM is ineffective delivery of anticancer drugs to the tumor cells. Only low molecular weight (<500 Da) molecules with high lipophilicity can enter the central nervous system.[58] Nanoparticles have potential to the enter central nervous system and represent as a promising vehicle for the thernostic applications in cancer treatment. However, due to complex particle internalization processes in brain cells, a simple nanoparticle formulation is not suitable to treat GBM. Therefore, use of targeting ligands with specificity for particular receptors is an ideal approach for the delivery of nanoparticles in GBM. Apart from better uptake and enhanced intracellular delivery of encapsulated drug molecules, targeted and specific delivery also overcomes the inherent problems of anticancer drugs such as non-specific and systemic toxicity and drug resistance.[59] In this study, we synthesized amphiphilic poly(γ-glutamic acid)-phenylalanine conjugate and used this conjugate as a biomaterial for the preparation of CPT loaded nanoparticles. For the preparation of targeted nanoparticles, cRGDfK was conjugated via the amine group of lysine moieties to free carboxylic groups on the surface of drug loaded nanoparticles using a carbodiimide reaction. This nanocarrier system was evaluated for anticancer efficacy, cellular uptake and induction of apoptosis using U87MG human glioblastoma cells.

γ-PGA is a water soluble, biodegradable, naturally occurring poly(amino acid) composed of glutamic acid molecules linked through the amide bonds between α-amino and γ-carboxylic acid groups.[37, 60] The structure of PGA-PA nanoparticles is a shell type with a hydrophobic PA core and an outer hydrophilic cortex of PGA. Drug loaded nanoparticles were prepared by a dialysis method and an optimized nanoparticle formulation was

conjugated with cRGDfK peptide to prepare targeted nanoparticles. The size of the nanoparticles after drug loading and cRGDfK conjugation was less than 100 nm; an ideal size for a nanocarrier system to across the blood-brain barrier.[8]

The aim was to develop receptor-specific nanoparticles to enhance the specificity and cellular toxicity of CPT. Cellular uptake of nanoparticles in cancer cells was determined by fluorescence microscopy. After 2 h of incubation, peptide conjugated nanoparticles are taken up more by the cells and showed higher fluorescence intensity than unconjugated nanoparticles. The competitive binding assay confirmed that uptake of targeted nanoparticles is through integrin receptors. Enhanced cellular internalization of peptide conjugated nanoparticles results in higher cytotoxicity (more than 5 times) of encapsulated CPT than plain CPT.

Induction of apoptosis and generation of ROS are two common mechanisms of action for many anticancer drugs.[61-64] Chromatin condensation and nuclear fragmentation are morphological hallmarks of apoptosis.[61] Induction of apoptosis by the different CPT formulations was evaluated by Annexin-V Cys 5.5 and Hoechst nucleus staining assays. In both assays, RCPN induces more apoptosis than CPN or non-encapsulated CPT; most likely due to enhanced intracellular delivery of drug. Similarly, RCPN treatment results in more ROS generation than other formulations. ROS generation causes oxidative stress and kills tumor cells through induction of apoptosis.[64]

In wound-healing scratch assays, control or untreated U87MG cells exhibit self-healing and migration. RCPN significantly diminishes cell migration, whereas CPN and CPT show slight inhibitory activity. The greater inhibition of cell migration by RCPN can be attributed to higher anti-angiogenic activity via $\alpha_v\beta_3$ integrin receptors that are up regulated on vascular endothelial cells in tumors.[64]

Conclusions

In this study, self-assembled poly(amino acid) based nanoparticles were successfully prepared for effective delivery of cytotoxic drug against glioma cells. These nanoparticles contain a hydrophobic phenylalanine core and a hydrophilic surface corona of poly(γ-glutamic acid). Antitumor effects of RCPN are shown by anti-proliferation activity, cell morphology and induction of apoptosis. Targeted nanoparticles show higher cellular uptake, induced apoptosis through ROS generation and better control over cell migration than non-targeted nanoparticles. Future research will focus on pharmacokinetic and in vivo tumor activity of the prepared formulation using animal models.

Notes

- The authors declare no competing financial interest.

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Executive summary

- Glioblastoma multiforme (GBM) or grade IV astrocytoma is the most common and most aggressive tumor of the glial tumors.
- Poor physiochemical properties and systemic toxicities limit the efficacy of the anticancer drugs against GBM.
- Poly(γ-glutamic acid)-phenyl alalnine conjugate was synthesized to encapsulate camptothecin (CPT) at nanoscale level.

- To improve the specificity towards GBM, nanoparticles were conjugated to cRGDfK peptide.
- Targeted nanoparticles showed significantly higher cytotoxicity against human glioblastoma cells than pure CPT.
- Targeted nanoparticles had time and concentration dependent cytotoxicity.
- Cellular uptake studies revealed that higher anticancer activity of targeted nanoparticles was due to cRGDfK mediated enhanced uptake of nanoparticles.
- The improved delivery of CPT resulted in reactive oxygen species generation, induction of apoptosis and greater control over the migration of glioblastoma cells.

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Figure legends

Figure 1. Characterization of nanoparticles: (**a**) Hydrodynamic diameter of blank poly(γ-glutamic acid)-phenylalanine nanoparticles (BPN), camptothecin (CPT) loaded poly(γ-glutamic acid)-phenylalanine nanoparticles (CPN) and cRGDfK conjugated CPN (RCPN). (**b**) FTIR spectra of CPT, CPN, cRGDfK peptide and RCPN. (**c**) Differential scanning calorimetry spectra of CPT and CPN. (**d**) X-ray diffraction pattern of CPT, PGA-PA, physical mixture of CPT and PGA-PA (PM) and CPN.

Figure 2. % Cell viability of U87MG human glioblastoma cells treated with camptothecin (CPT), CPT loaded PGA-PA nanoparticles (CPN) or cRGDfK conjugated loaded CPN (RCPN), equivalent to 1-400 ng/mL CPT, for (**a**) 24 h and (**b**) 48 h. The results are expressed as mean ± standard deviation, n = 3.

Figure 3. Cellular uptake of rhodamine-b loaded γ-PGA nanoparticles (RPN) and cRGDfK conjugated RPN (RRPN) by U87MG human glioblastoma cells after 2 h of incubation. Cellular uptake of RRPN was also studied in the presence of 100 µg/mL cRGDfK.

Figure 4. Apoptosis studies. Apoptosis induced to U87MG human glioblastoma cell after 24 h treatment with camptothecin (CPT), CPT loaded PGA-PA nanoparticles (CPN) and cRGDfK conjugated loaded CPN (RCPN), equivalent to 20 ng/mL CPT, was determined by two methods, Hoechst 33342 staining and Annexin-V Cy5.5/PI staining. (**a**) Fluorescent microscopic images of nucleus of U87MG cells stained with Hoechst 33342 (5 µg/mL) after treatment with CPT, CPN or RCPN. (**b**) Quantitative analysis of the fluorescent microscope images (Mean ± SD, n = 3). (**c**) Percentage of live and apoptotic cells measured using a flow cytometer after annexin-V Cy5.5/PI staining (Mean ± SD, n = 3).

* represents comparison with control group. (*$p < 0.05$, **$p < 0.01$)

represents comparison with CPT. (#$p < 0.05$, ## $p < 0.01$)

Figure 5. (**a**) Qualitative and (**b**) quantitative determination of reactive oxygen species (ROS) generation in U87MG human glioblastoma cell after treatment with camptothecin (CPT), CPT loaded PGA-PA nanoparticles (CPN) and cRGDfK conjugated loaded CPN (RCPN). Data are presented as Mean ± SD, n = 3.

* represents comparison with control group. (*$p < 0.05$, **$p < 0.01$)

represents comparison with CPT. (#$p < 0.05$, ## $p < 0.01$)

Figure 6. Wound healing scratch assay. (**a**) The wound area in U87MG cells after 0 and 24 h of treatment with different formulations. Camptothecin formulations showed inhibition of wound healing by decreasing the migration of the cells. (**b**) Bar graph of wound closure obtained with different camptothecin formulations. Inhibition of cell migration was significantly greater in cRGDfK peptide conjugated nanoparticles (RCPN; $p < 0.01$) than plain CPT and unconjugated nanoparticles (CPN). Control or untreated cells showed almost complete healing of wound after 24 h. Quantitative analysis is an average of six field views.

* represents comparison with control group. (*$p < 0.05$, **$p < 0.01$)

represents comparison with CPT. (#$p < 0.05$, ## $p < 0.01$)

Supplementary Information

Peptide grafted and self-assembled poly(γ-glutamic acid)-phenylalanine nanoparticles targeting camptothecin to glioma

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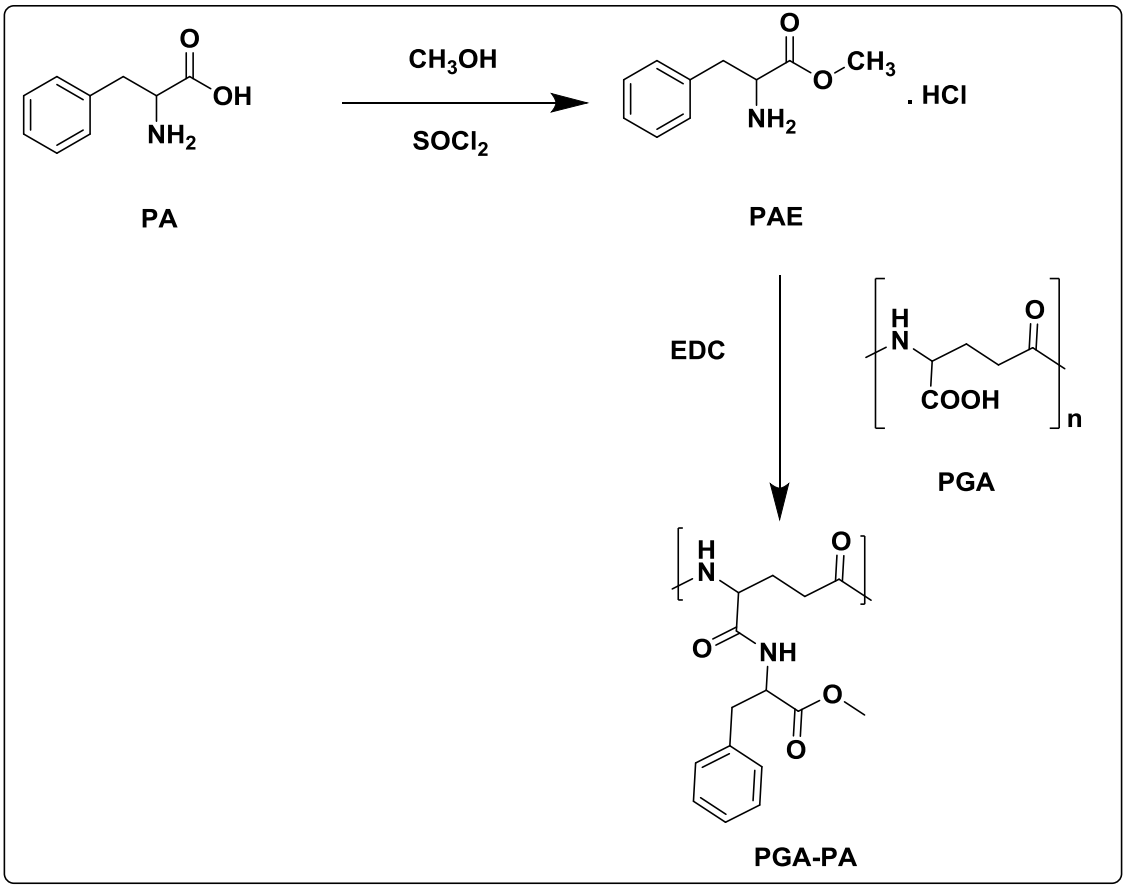


Figure S1. Chemical synthesis of phenyl alanine ester from phenyl alanine (PA) and then conjugation of PAE to poly(γ-glutamic) acid (PGA) to form PGA-PA conjugate.

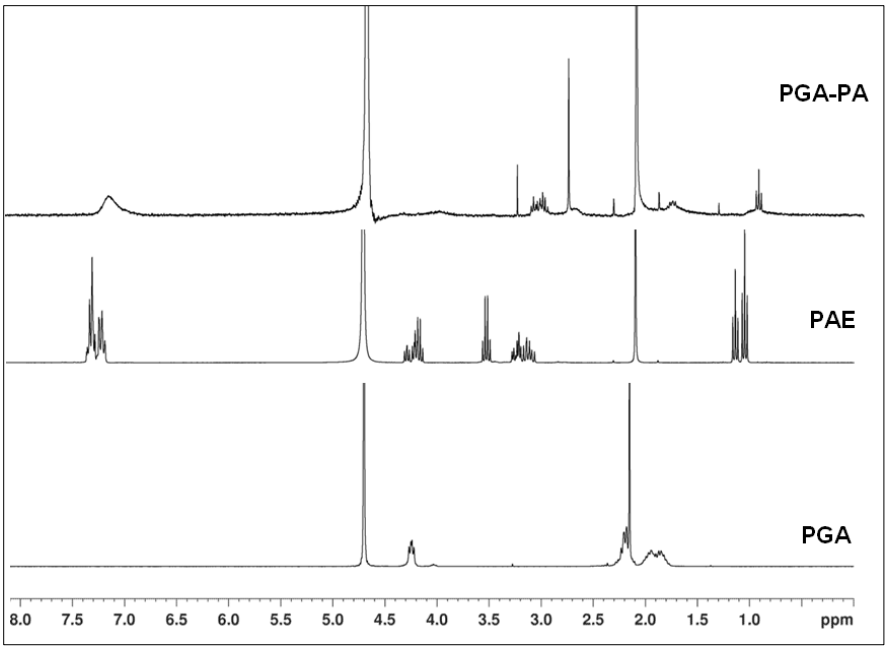
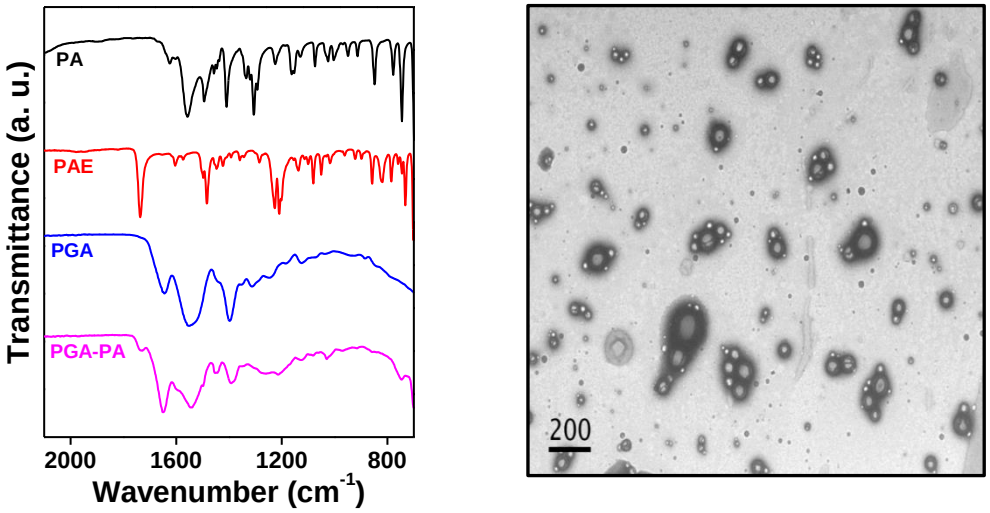


Figure S2. ^{1}H NMR spectra of phenylalanine ester (PAE), poly(γ-glutamic acid) (PGA) and poly(γ-glutamic acid)-phenylalanine conjugate (PGA-PA).



a) **b)**

Figure S3. a) FTIR spectra of phenyl alanine (PA), phenylalanine ester (PAE), poly(γ-glutamic acid) (PGA) and poly(γ-glutamic acid)-phenylalanine conjugate (PGA-PA). **b**) Transmission electron microscopic image of PGA-PA nanoparticles. The scale represents size in nanometers.

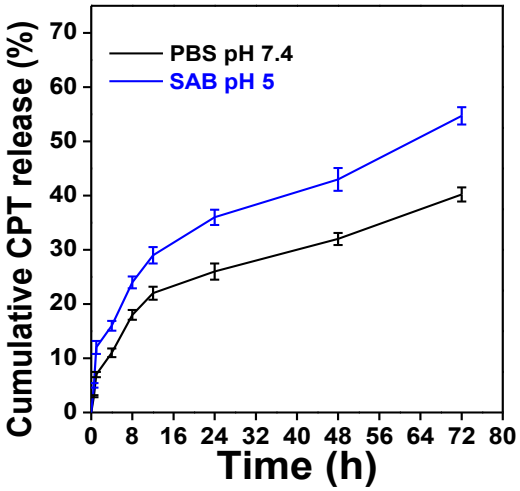


Figure S4. *In vitro* release of drug from cRGDfK conjugated, camptothecin loaded PGA-PA nanoparticles (RCPN) in phosphate buffer saline (PBS) pH 7.4 and sodium acetate buffer (SAB) pH 5.