Biointerface Research in Applied Chemistry
Platinum Open Access Journal (ISSN: 2069-5837)

Article
Volume 11, Issue 4, 2021, 11244 - 11255
https://doi.org/10.33263/BRIAC114.1124411255

^{99m}Tc-Anionic Linear Globular Dendrimer-G2-Phenylalanine Conjugate: Novel Brain Tumor SPECT Imaging

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Received: 6.11.2020; Revised: 4.12.2020; Accepted: 7.12.2020; Published: 10.12.2020

Abstract: The purpose of this study was the investigation of the targeting potential of ^{99m}Tc-Labeled dendrimer-phenylalanine conjugate for brain tumor SPECT imaging. L-Type amino acid transporters (LAT1) are highly expressed in the blood-brain barrier as well as in brain cancer cells; thus, targeting LAT1 using phenylalanine could improve the sensitivity and specificity of radiosynthesis nanocarrier. In this study, the dendrimer G2–phenylalanine conjugate was synthesized and characterized by Fourier transform infrared spectroscopy, atomic force microscopy, particle size, and zeta potential. MTT assay was done for cell viability measurement in different concentrations of nanoparticles (0.125, 0.25, 0.5 mg/ml) on C6 glioma cell lines; the uptake study was evaluated using fluorescence-activated cell sorter (FACS) instrument; finally, SPECT scintigraphy in glioma tumor-bearing Wistar rats was done. The dendrimer-phenylalanine conjugate particle size was found in the range of 74.14±2.2 to 109±3.1 nm, with a slightly negative surface charge. Also, phenylalanine present on the dendrimer's surface–phenylalanine conjugate enhanced the dendrimer's cellular uptake–phenylalanine conjugate on the C6 glioma cell line. Results of SPECT imaging and fluorescence studies revealed that dendrimer–phenylalanine conjugate accumulated into the brain tumor cells, and it can be suggested as a promising brain-targeting probe with no toxicity in brain tumor imaging.

Keywords: dendrimer G2; LAT1; phenylalanine; glioma; carrier-mediated transport; SPECT.

1. Introduction

Malignant glioma such as glioblastoma multiform (GBM) is one of the most significant challenges in front of cancerous patients worldwide. World Health Organization (WHO) has categorized gliomas based on histological characteristics as grades I-IV. A WHO grade IV glioma is glioblastoma. Glioblastoma (GBM) is the most malignant grade and most common

primary brain tumor [1-5]. The average patient's survival is about 1.5 years despite typical treatments, including surgery, chemotherapy with alkylating agents such as temozolomide, and ionizing radiation. However, early detection of cancer greatly increases the chance of successful treatment and survival. To diagnose cancer at the earliest stage, novel, and more effective targeted therapeutic and diagnostic agent delivery systems are needed [6,7]. Most therapeutic and contrast agents do not readily permeate the blood-brain barrier (BBB) [8,9]. BBB trafficking is the main limiting factor for delivering potential CNS drugs into the brain parenchyma, which makes it the interest of CNS drug designing. BBB is configured from capillary endothelial cells with tight intercellular junctions and surrounded by a basement membrane, astrocytes, and pericytes. BBB has a high electrical resistivity, 1500-2000(1300) Ωcm^2 (compared with other organs having an average resistance of 1.8 Ωcm^2). Tight junctions and basal membranes are responsible for the integrity and maintenance of the barrier [10-12](Figure 1). Some parameters should be considered for designing ideal carriers, including tight junction, electrical resistance, efflux proteins, and catalyzing enzymes [13,14]. For passive diffusion from BBB, hydrophobic, and hydrophilic agents' molecular weight must be less than 400-600 KDa with less than 8-10 hydrogen bands [15-18]. In addition, since diffusion depends on concentration, high concentration in capillary promotes diffusion. Due to these restrictions, 98% of small molecules and 100% of large molecules like antibodies, even engineering antibodies (around 15-150 KDa) do not pass BBB. These mentioned mechanisms are responsible for 98% of drugs that can not pass BBB [19-21]. It could be concluded that small lipophilic molecules with less protein binding (PB) and long serum half-life are good candidates to cross BBB. With this regard, lipophilicity, size, and ionization degree should be considered in designing BBB trafficable agents [22]. Carrier is conjugated to amino acids, glucose, nucleosides, or bigger molecules like insulin and transferrin to pass BBB [23,24]. Therapeutic or contrast agent delivery based on tumor cells' metabolism has shown significant success in recent years [25-33]. Amino acids are essential for cell growth and proliferation [34]. The L-type amino acid transporter-1 (LAT-1) can transport large, branched-chain and aromatic, neutral amino acids. Previous studies have shown a relationship between LAT1 level and tumor growth [35,36]. LAT1 expression is increased in several cancers [37-44]. Several studies have recently demonstrated the effectiveness of LAT-1 targeting for drug and contrast agent delivery in a wide range of cancers like breast cancer and glioma [25-33,45-53].

Recent studies have shown that the expression of LAT1 increases in glioma. Phenylalanine is an essential amino acid required for protein synthesis and is one of the LAT1 substrates. Therefore, targeting glioma cancer cells with the Phenylalanine ligand is suitable for targeting cancer cells [33,54-56].

Today, nanoparticles in disease diagnosis and therapy are an interesting field for researchers [27,57-71]. Nanoparticles can enhance drug dissolution; because of their small size, they can cross the membrane barrier. Another advantage is the possibility of particles targeted in a particular organ. Dendrimers are a family of compact spherical polymers at the nanoscale. They have numerous and diverse applications, including the biomedical field (i.e., drug delivery, cancer diagnostics). In this study, anionic G2 dendrimer (polyethylene glycol-citric acid) was synthesized using a simple method without the use of toxic and harmful agents.

Several modalities such as SPECT, PET, CT, and MRI have been introduced in metabolic imaging [72-76]. The purpose of this study was the investigation of ^{99m} Tc-labeled dendrimer–phenylalanine conjugate potential for brain tumor SPECT imaging. L-Type amino acid transporters (LAT1) are highly expressed on the blood-brain barrier as well as on many

brain cancer cells, thus targeting LAT1 using phenylalanine improved sensitivity and specificity of radiosynthesis nanocarrier

In the present research work, an L-type amino acid transport system was utilized to cross BBB. The phenylalanine was used as a targeting ligand, which was conjugated on the dendrimer's surface labeled with ^{99m}Tc as shown in Figure 2.

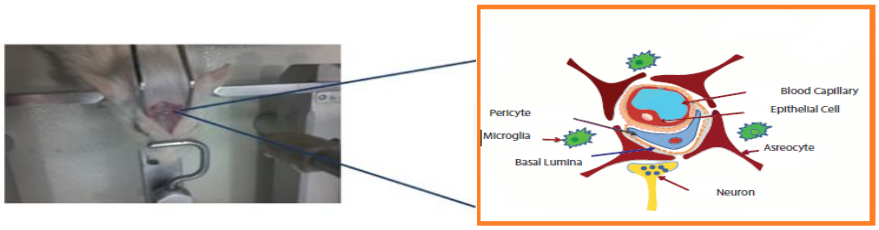

Figure 1. Schematic structure of BBB.

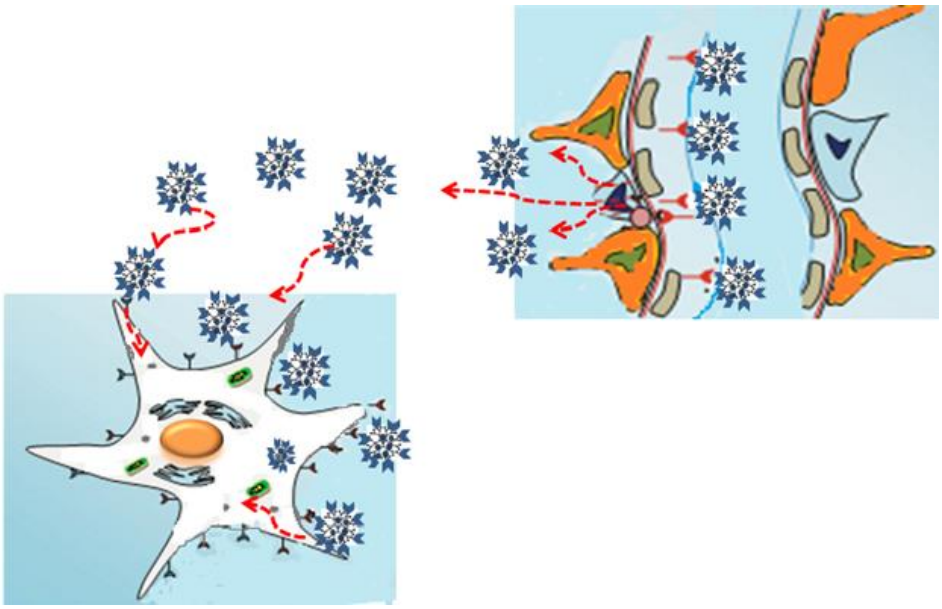

Figure 2. Schematic illustrates the transportation of Dendrimer-Phenylalanin across BBB and uptake by the brain tumor cell.

2. Materials and Methods

2.1. Chemical materials.

Phenylalanine (PA), Sephadex G-50, and 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC), sulfo-N-hydrosuccinimide (sulfo-NHS), triethylamine, dimethylformamide, MTT, and stannous chloride dihydrate were purchased from Sigma Chemicals (St Louis, MO, USA). Polyethylene glycol, citric acid anhydrous, sodium bicarbonate, methanol were purchased from Merck, Solvents, and chemical reagents were analytical grade from Merck.

2.2. Cell culture.

C6 cell line was supplied by the Pasteur Institute of Iran and used for cell cytotoxicity and imaging studies. The cells were cultured in complete RPMI-1640 medium, supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C with 5% CO_2.

2.3. Animal models.

All animal experiments were done according to the guideline of the INMAS animal ethics committee. Miura *et al*. and Shahmabadi *et al.* protocols were used for tumor formation, 50.000 cells of C6 cells in 10 µl of PBS were injected into the frontal lobe of the skull of a male rat (200-250g, n=5) [9,77].

2.4. Preparation of dendrimer G2-phenylalanine conjugate dendrimer (G2) synthesis.

Dendrimer was synthesized using the method reported previously [26-28,59,61,62]. Briefly, polyethylene glycol (PEG) with two carboxyl groups on the sides formed the core, and citric acid formed the dendrimer's shell. PEG600, engineered Polyethylene glycol, (5.65 mmoles) was added to dried Dimethyl Sulfoxide (DMSO), and $Cacl_2$ then mixed for 10 minutes. N, N'- Dicyclohexylcarbodiimide (DCC) (2mM) was added and mixed for 15 minutes. 0.5gr citric acid was added to the reaction container and stirred at 400 rpm for 1h. In the next step, to synthesize the second generation, DCC (3 mM) was added. Then citric acid (5.2mM) was added to the reaction and stirred at 400 rpm for 7 days. The color change to a dark brown color verified the synthesis of the G_2 dendrimer. Filter paper, chromatography against Cephadex, and lyophilization were used to purify dendrimer (G2) purification.

2.5. Conjugation phenylalanine on the surface of dendrimer G2.

The amine group of phenylalanine was covalently coupled to the carboxyl group of dendrimer (G2), phenylalanine was decorated on the surface of the dendrimer. Briefly, the phenylalanine's free amine functional group reacts to carboxyl groups of the G_2 dendrimers via an amide bond. In the final step, the BODIPY (fluorescent dye) was loaded into the synthesized G2 dendrimer- phenylalanine (Figure 3). To remove the unentrapped Bodipy, the nanoparticle was dialyzed against DDW under sink conditions for 4h.

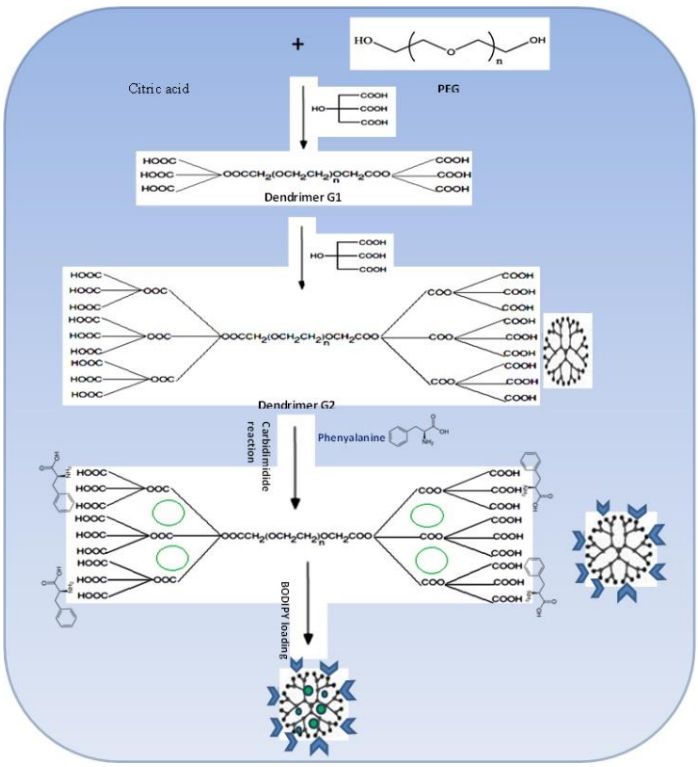

Figure 3. Schematic presentation of preparation of Dendrimer G2-Phenylalanine.

2.6. Characterization of nanoparticles.

Mean size, size distribution, and zeta potential of particles were determined by Zetasizer (Zetasizer Nano ZS; Malvern Instruments, Malvern, UK). The conjugation of phenylalanine on the surface of Dendrimer G2 is confirmed by the FTIR analyses (NEXUS 870; Thermo Fisher Scientific, Waltham, MA, USA).

2.7. ^{99m}Tc radio-labeling of dendrimer -phenylalanine conjugates.

Dendrimers (3.4 mg/ml) in a sterile water suspension were labeled with 99m Tc pertechnetate (^{99m}TcO42) at *pH 3.4*, stannous chloride ($SnCl_2.2H_2O$) has been used as a reducing agent.^{99m}TcO42 at 300–700 MBq/0.7 ml, eluted from a ^{99}Mo/^{99m}Tc generator (DRN 4329; Ultratechnekow FM), was injected into a sterile vacuum collection vial, following, 0.5 mg of $SnCl_2.2H_2O$ was dissolved in 0.5ml of distilled water, and the solution was stirred for a few seconds and passed through a 0.22-mm Millipore filter and was added into the vial containing the $^{99m}TcO_4$- nanoparticle, gently shaken for 30 s and incubated for 60 min at RT.

2.8. Radiochemical purity.

^{99m}Tc-dendrimer- Phenylalanine complex was analyzed with ITLC-SG using the method reported previously [26].

2.9. In vitro cytotoxicity of dendrimer -phenylalanine.

The *in vitro* cytotoxic effect of Dendrimer -Phenylalanine was examined using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, using C6 glioma cell line. The cells were seeded at a density of 2×10^4 cells per well in 96-well plates; then, the plates were incubated for 24 h in a humidified incubator with a CO_2 concentration of 5% to permit adherence of the cells. Then, the cells were incubated either 0.1ml of medium containing Dendrimer -Phenylalanine at different iron concentrations (0.125, 0.25, 0.5mg/ml) for 4h. After the incubation time, 10µl of 5mg/ml MTT solution was added. Incubation was continued for a further 4h. Purple formazan crystals were (indicating cell viability) were solubilized by adding 100µl DMSO per well. The absorbance was measured at 570nm by the Statfax-microplate reader (Statfax, Awareness Technology, Inc, USA). Experiments were performed in triplicate, and cell survival was determined as a percentage of viable cells in comparison with control wells. All assays were performed in triplicate for each concentration. Cell survival was determined as a percentage of viable cells compared to controls (cell line without ant treatment).

2.10. Cellular uptake study.

The uptake study was measured using a fluorescence-activated cell sorter (FACS) instrument (BD bioscience, FACSv Vantage California, USA).C6 glioma cell lines used in the study were cultured in the set of 12-well flat-bottom plates at a density of 3×10^5cells/well, supplemented with 10% FBS and1 % antibiotic and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 24h. Adhered cells were treated with Bodipy-loaded Dendrimer G2-Phenylalanine (300µg/well) and incubated for 1h. Cells were washed twice with phosphate saline buffer (PBS, pH 7.4); the following cells were harvested by adding 1ml PBS

(pH 7.4) sonicated five times to lysate the cells. Finally, cell lysate was centrifuged (10,000 rpm, 10min), the supernatant was used for fluorescence assay using FACS.

2.11. SPECT scintigraphy in tumor-bearing Wistar rats.

The rats were divided into two groups. The first group was treated with ^{99m}Tc-unconjugated Dendrimer, and the second group with ^{99m}Tc -Dendrimer -phenylalanine. 1ml (3.7 MBq) of the labeled complex (0.1µM of each complex) was administered to each group through the tail vein**.** Rats were then placed in the planar gamma camera, and images were taken at 1h after injection.

3. Results and Discussion

3.1. Characterization of nanoparticles.

Zeta potential and size of dendrimer and dendrimer-phenylalanine were shown in table1. Significant change following the Phenylalanine conjugation was found (74.14±2.2 nm and -5.21±1.1 mv to 104.9±3.1 nm and -9.01±1.8mv respectively).

Table 1. Size and zeta potential distributions of the Dendrimer G2 and Dendrimer G2- Phenylalanine.

Agent	Size distribution, mean(nm)	Zeta potential (mv)
Dendrimer G2	74.14±2.2	-5.21±1.1
Dendrimer G2- Phenylalanine	104.9±3.1	-9.1±1.8

3.2. FT-IR result.

FT-IR results of Dendrimer G2 and Dendrimer Phenylalanine were compared and showed in Figure 4. The peaks between 3200-3600cm^{-1} are related to NH and OH groups. The peak 1590-1760 wavelength shows carbonyl groups of the compound, aliphatic carbon-hydrogen. 1738.4 cm^{-1} peak that exclusively depicts the dendrimer; this also is in dendrimer G2-Phenylalanine (Figure 4).

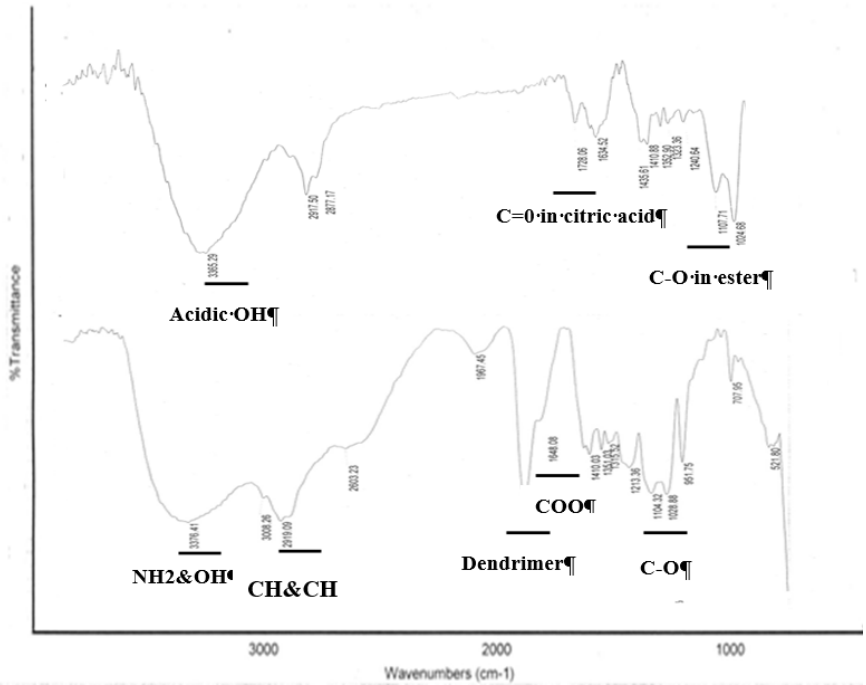

Figure 4. FT-IR spectrum of Dendrimer G2 and Dendrimer G2-Phenylalanine**,** respectively**.** Abbreviation: FTIR: Fourier transform infrared spectroscopy.

3.3. In vitro cytotoxicity of nanoparticles.

MTT assay was done on the C6 cell line to investigate the cytotoxic potential of nanoparticles. The results of the MTT assay exhibit no toxicity for Dendrimer G2-Phenylalanine **(**Figure 5).

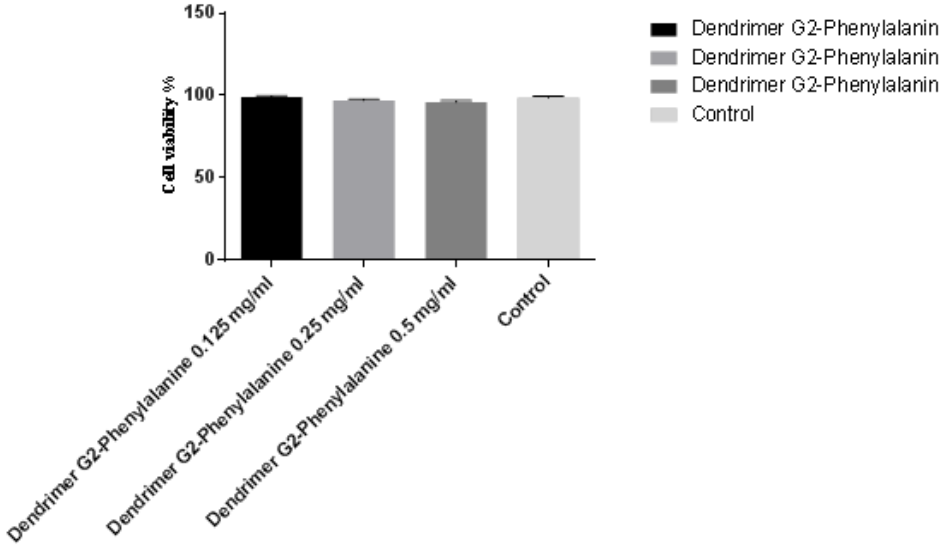

Figure 5. MTT cytotoxicity assay of Dendrimer G2-Phenylalanine measurement in different concentrations of nanoparticle (0.125, 0.25, 0.5 mg/ml). Abbreviation: MTT : 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide.

3.4. Cellular uptake using flow cytometry.

The flow cytometric analysis was utilized to verify the potential of G2-Phenylalanine to target LAT1 transporter that overexpressed on glioma cells. C6 cell line was incubated with G2-Phenylalanine-BODIPY nanoparticles (300 µg), and cellular uptake was measured using fluorescence-activated cell sorter (FACS) instrument (BD bioscience, FACS vintage California, USA). 95.13±0.6 % and 98.02%±0.8 uptakes were observed with Dendrimer G2-BODIPY and Dendrimer G2-Phenylalanine-BODIPY, respectively, after 1h (Figure 6).

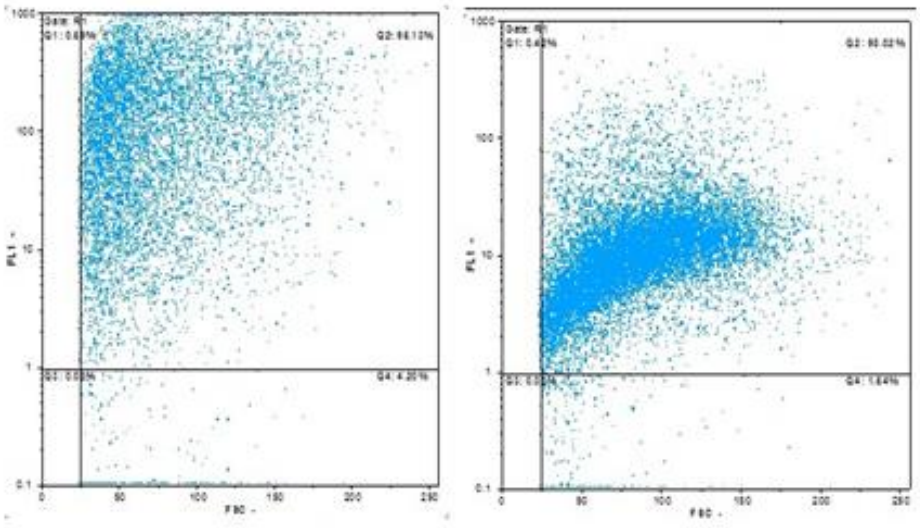

Figure 6. Flow cytometry studies for cellular uptake of Dendrimer G2-Phenylalanine-BODIPY and Dendrimer G2- loaded with BODIPY. Present PEG on the surface of Dendrimer promotes the uptake of nanoparticles (95.13±0.6 %). C6 cell line treated with G2-Phenylalanine-BODIPY exhibited strong green fluorescence (98.02±0.8 %).

3.5. SPECT scintigraphy in tumor-bearing Wistar rats.

The planar and SPECT projections were made with Siemens Healthcare Global. The uptake of radioactivity in the brain of rat bearing C6 glioma cells after 1h into the tail vein injection of Dendrimer G2 and Dendrimer G2-Phenylalanine was visualized on the images (Figure 7 and Table 2).

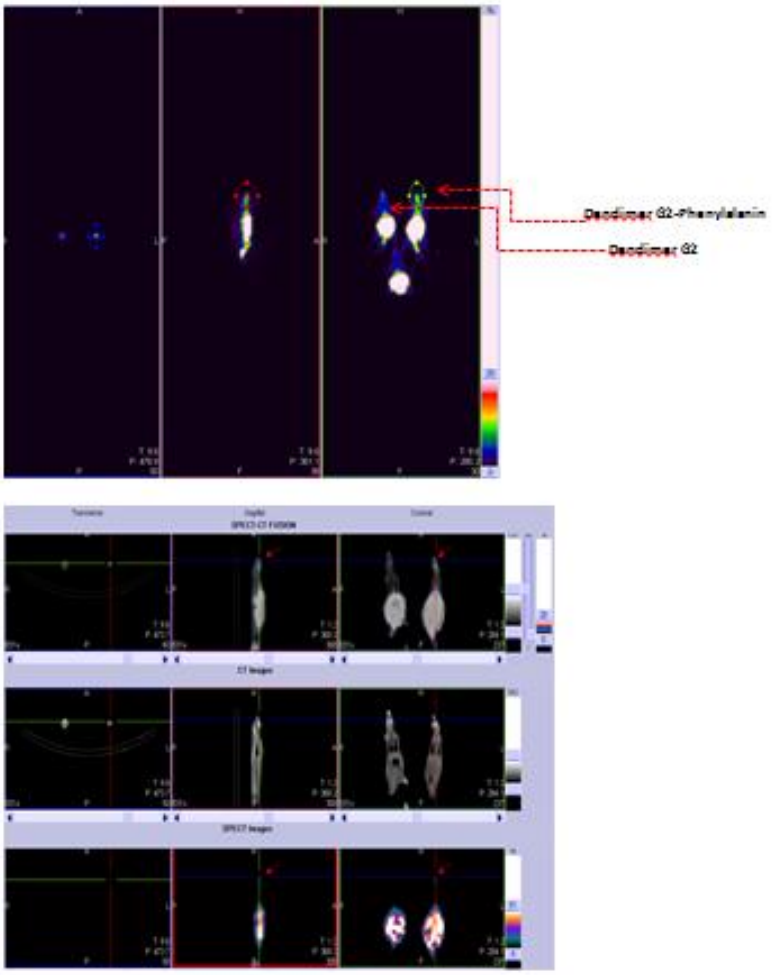

Figure 7. SPECT/CT images of rat bearing C6 glioma cells 1h after injection with ^{99m}Tc-Dendrimer G2 AND ^{99m}Tc-Dendrimer G2-Phenylalanine. Abbreviation: SPECT: Single Photon Emission Computed Tomography.

Table 2 exhibits ^{99m} Tc count in Dendrimer G2, Dendrimer G2-Phenylalanine, and Dendrimer G2-Phenylalanine / Dendrimer G2 ^{99m} Tc count ratio in the rat bearing C6 glioma cell's brain.

	Dendrimer G2-Phenylalanine	**Dendrimer G2**	**Dendrimer G2-Phenylalanine / Dendrimer G2 ratio**
Max	11053	6876	1.67
Min	5668	3544	1.59
Ave	7279.13	4594.83	1.58
Std. Dev	1851.29	1314.3	

4. Conclusions

The present study demonstrated the feasibility of labeling Dendrimer G2-Phenylalanine with ^{99m}Tc. Using *in vitro* and *in vivo* studies, we verified that the targeted Dendrimer G2 agent can deliver 99mTc through the BBB and preferentially target the glioma cells in a rat model of glioma. After the tail vein injection, the accumulation of ^{99m}TcDendrimer G2-Phenylalanine was verified by SPECT/CT detection in the rat's brain. Our results provide a promising avenue for future researches toward finding novel approaches to treat neurological diseases.

Funding

This work was supported by Students' Scientific Research Center, Tehran University of Medical Sciences, and Tehran University of Medical Sciences (Grant no. 94-03-61-30013) and International Campus, Tehran University of Medical Sciences (Grant no. 94-01-103-28528).

Acknowledgments

This research has no acknowledgment.

Conflicts of Interest

The authors declare no conflict of interest.

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