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# Positron emission tomography (PET) guided glioblastoma targeting by a fullerene-based nanoplatform with fast renal clearance

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ABSTRACT

Various carbonaceous nanomaterials, including fullerene, carbon nanotube, graphene, and carbon dots, have attracted increasing attention during past decades for their potential applications in biological imaging and therapy. In this study, we have developed a fullerene-based tumor-targeted positron emission tomography (PET) imaging probe. Water-soluble functionalized $C_{60}$ conjugates were radio-labeled with $^{64}Cu$ and modified with cyclo (Arg-Gly-Asp) peptides (cRGD) for targeting of integrin $\alpha_v\beta_3$ in glioblastoma. The specificity of fluorescein-labeled $C_{60}$ conjugates against cellular integrin $\alpha_v\beta_3$ was evaluated in U87 MG (integrin $\alpha_v\beta_3$ positive) and MCF-7 cells (integrin $\alpha_v\beta_3$ negative) by confocal fluorescence microscopy and flow cytometry. Our results indicated that cRGD-conjugated $C_{60}$ derivatives showed better cellular internalization compared with $C_{60}$ derivatives without the cRGD attachment. Moreover, an interesting finding on intra-nuclei transportation of cRGD-conjugated $C_{60}$ derivatives was observed in U87 MG cells. *In vivo* serial PET studies showed preferential accumulation of cRGD-conjugated $C_{60}$ derivatives at in U87 MG tumors. In addition, the pharmacokinetic profiles of these fullerene-based nanoparticles conjugated with cRGD and 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA) fit well with the three compartment model. The renal clearance of $C_{60}$-based nanoparticles is remarkably fast, which makes this material very promising for safer cancer theranostic applications.

**Statement of significance**

Safety is one of the major concerns for nanomedicine and nanomaterials with fast clearance profile are highly desirable. Fullerene is a distinct type of zero-dimensional carbon nanomaterial with ultrasmall size, uniform dispersity, and versatile reactivity. Here we have developed a fullerene-based tumor-targeted positron emission tomography imaging probe using water-soluble functionalized $C_{60}$ conjugates radio-labeled with $^{64}Cu$ and modified with cyclo (Arg-Gly-Asp) peptides (cRGD) for glioblastoma targeting. The improved tumor targeting property along with fast renal clearance behavior of $C_{60}$-based nanoparticles makes this material very promising for future safer cancer theranostic applications.



## 1. Introduction

Since the discovery of the first fullerene $C_{60}$ in 1985 [1], carbonaceous nanomaterials have attracted much attention during the past decades for various potential applications due to their structural uniqueness [2–5]. Recently, great progress has been made in utilizing carbonaceous nanomaterials (e.g., carbon nanotubes, graphene derivatives and carbon dots etc.) for biomedical imaging and therapeutic cargo delivery [6–8]. Among these many carbonaceous nanomaterials, fullerene is a distinct type of zero-dimensional carbon nanomaterial with ultrasmall size, uniform dispersity, and versatile reactivity [9]. Due to its attractive physicochemical properties, numerous researchers have contributed to the development of fullerene-based nanoconjugates for various biological applications, for example as antioxidant agents, photodynamic therapy mediators, drug or gene delivery carriers and theranostic agents [10–13].

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Safety concerns are rising during the development of inorganic nanomaterials for biological applications. Particularly, according to the U.S. Food and Drug Administration (FDA), all of the injectable nanomaterials should be cleared in a reasonable time frame to avoid the potential toxicity from long-term retention [14,15]. In the literature, it has been demonstrated that the size, shape and surface properties of nanoparticles will significantly affect their *in vivo* pharmacokinetic behavior including organ distribution, clearance pathway, and metabolic speed [16–20]. For instance, functionalized silica-based nanoparticles smaller than 5.5 nm can effectively escape the capture of the mononuclear phagocytic system (MPS) and be cleared through the renal system after administration [15]. In contrast to carbon nanotubes and graphenes, $C_{60}$ molecule is well defined as a highly symmetrical sphere with the van der Waals diameter of 0.95–1.04 nm, which is expected to exhibit different pharmacokinetic profile in biological systems [21]. Integrin $\alpha_v\beta_3$ is chosen as the cancer-targeting receptor here due to its abundant expression in both tumor cells and vasculatures from different cancer types [22]. Integrin $\alpha_v\beta_3$ plays a critical role in both tumor growth and metastasis. It is over-expressed on most solid tumor types including glioblastoma, melanoma, breast, prostate, and ovarian cancer [23,24]. Integrin $\alpha_v\beta_3$ is also often up-regulated in tumors post-radiotherapy. The high-affinity interaction between arginine-glycine-asparate (RGD) motif-containing peptides (e.g., cyclo(RGDyK), cyclo(RGDfK), cyclo(RGDfC), *etc.*) and integrin $\alpha_v\beta_3$ has led to enormous interests in utilizing cRGD peptides for cancer theranostic applications [25]. In several previous reports, the strategy of either radiolabeling or fluorophore incorporation onto cRGD peptides has been successfully adopted for non-invasive tumor-targeted imaging [26–28].

In this study, our goal is to develop tumor-targeted $C_{60}$ conjugates via a bottom-up synthetic chemistry, which can be detectable by positron emission tomography (PET) imaging. Highly water dispersable carboxylfullerenes were covalently conjugated with eight-arm amine-terminal branched polyethylene glycol (PEG), which can be further attached by various functional entities (PET isotope chelator, active tumor targeting ligand, etc.). cRGD was covalently attached onto $C_{60}$ aggregates for selective identification of glioblastoma. To evaluate the targeting efficiency and pharmacokinetics of these $C_{60}$ conjugates, the distribution and clearance behaviors of $C_{60}$ conjugates in tumor-bearing mice were investigated by PET imaging at various time points, where $^{64}Cu$ ($t_{1/2}$: 12.7 h) was incorporated into the structure of the $C_{60}$ conjugates [26,27,29].

## 2. Experimental section

### 2.1. Materials

Fullerene ($C_{60}$, purity >99.9%) and 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) were purchased from Aladdin (Shanghai, China). Diethyl bromomalonate and Sodium hydride (NaH) was acquired from J&K Scientific Ltd. (Beijing, China). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloide (EDCI) and Hydroxy-2,5-dioxopyrrolidine-3-sulfonicacid sodium salt (Sulfo-NHS) were purchased from Bide Pharmatech Ltd. (Shanghai, China). Eight-arm branched polyethylene glycol-amine (8-arm-PEG-$NH_2$, 10 kDa) was obtained from Changchun Institute of Applied Chemistry (Changchun, China). Fluorescein isothiocyanate (FITC) was purchased from Melone Pharmaceutical Corporation (Dalian, China). (*S*)-2-(4-isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid (*p*-SCN-Bn-NOTA, abbreviated as NOTA) was purchased from Macrocyclics, Inc. (Dallas, TX, USA). 4-Maleimidobutyricacid N-hydroxysuccimide ester (GMBS) and Tris(2-carboxyethyl) phosphine hydrochloride (TCEP·HCl) were provided by Energy Chemical (Shanghai, China). Cyclo (Arg-Gly-Asp-D-Phe-Cys) peptide (cRGDfC, abbreviated as cRGD) was purchased from Top-peptide Co., Ltd. (Shanghai, China). $^{64}CuCl_2$ was acquired from the cyclotron group at University of Wisconsin. Anti-integrin $\alpha_v\beta_3$ antibody (catalog number: ab78289) was purchased from Abcam (Cambridge, MA, USA). Chelex 100 resin (50–100 mesh) was acquired from Sigma-Aldrich (St. Louis, MO, USA) and PD-10 size exclusion columns were acquired from GE Healthcare (Piscataway, NJ, USA). Alexa Fluor 488-labeled rabbit anti-mouse IgGs were purchased from Lampire biological laboratories (Pipersville, PA, USA). Other reagents were purchased from Fisher Thermo Scientific. All chemicals, unless otherwise stated, were purchased from commercial sources and directly used as received without further purification. All buffers were prepared from Millipore-grade water with the pretreatment of Chelex 100 resin to minimize heavy metal contents in the aqueous solution.

### 2.2. Characterization

FT-IR spectra were recorded on a Nicolet iS10 spectrometer (Thermo) and $^1H$ NMR spectra were recorded on a Bruker 300 MHz instrument. Dynamic light scattering (DLS) (Zetasizer Nano ZS-90, Malvern Instrument, USA) was used for characterizing hydrodynamic (HD) size and size distribution, as well as ζ-potentials of $C_{60}$ conjugates at the concentration of 0.2 mg/ml (based on $C_{60}$). Atomic force microscope (AFM) measurement was carried out on Bruker Dimension ICON atomic force microscope (Bruker, Billerica, MA).

### 2.3. Preparation of $C_{60}$ conjugates

#### 2.3.1. Synthesis of carboxylfullerene $C_{60}[C(COOH)_2]_3$

The trimalonic acid modified $C_{60}$ derivative was synthesized following the procedures reported previously with minor modifications. $C_{60}$ was initially reacted with diethyl bromomalonate via Bingle cyclopropanation in the presence of DBU, then hydrolyzed by NaH to yield water-soluble fullerene derivatives [30,31].

Briefly, in a 100 ml dry three-necked flask equipped with a gas inlet, 50 mL dropping funnel, and magnetic stirrer, 253 mg $C_{60}$ (0.35 mmol) was dissolved in 50 mL of dry toluene. Subsequently, 200 μL diethyl bromomalonate (1 mmol) were added followed by the dropwise addition of 173 μL DBU (1 mmol) diluted in 10 mL of dry toluene. The reaction was carried out at room temperature with continuous stirring for 10 h under argon. The trimalonic ester modified $C_{60}$ was separated by silica gel chromatography and a rotary evaporator was used to remove the solvent.

The purified trimalonic ester modified $C_{60}$ (120 mg, 0.1 mmol) was re-dissolved in 50 mL of dry toluene followed by adding 90 mg NaH (2.0 mmol, 20 equiv.). The mixture was stirred and refluxed at 80 °C for 10 h. After the removal of the heating source, 5 mL of MeOH was added immediately to quench the reaction. Red precipitate was collected from centrifugation and repeatedly washed by toluene and hexane. The red solid was added into 4 M HCl and the resulting precipitate was repeatedly washed by 4 M HCl and deionized water. The precipitate was further dried under vacuum to yield the expected trimalonic acid modified $C_{60}$ derivative.

#### 2.3.2. Synthesis of PEGylated $C_{60}$ ($C_{60}$-PEG)

5 mg trimalonic acid modified $C_{60}$ derivative ($C_{60}[C(COOH)_2]_3$, 0.005 mmol) was dissolved in 2 mL of anhydrous DMSO. EDCI/Sulfo-NHS (0.015 mmol) as the coupling reagents was added into the DMSO solution. The solution was stirred for 15 min at room temperature to yield $C_{60}$ carboxylate active ester intermediate. Afterward, the above solution was added dropwise into 20 mL of 0.1 M phosphate buffered saline (PBS, pH 7.4) containing