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Metallofullerene-Nanoplatfrom-Delivered Interstitial Brachytherapy Improved Survival in a Murine Model of Glioblastoma Multiforme

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Abstract

Fullerenes are used across scientific disciplines because of their diverse properties gained by altering encapsulated or surface bound components. In this study, the recently developed theranostic agent based on a radiolabeled functionalized metallofullerene (¹⁷⁷Lu-DOTA-f-Gd₃N@C₈₀) was synthesized with high radiochemical yield and purity. The efficacy of this agent was demonstrated in two orthotopic xenograft brain tumor models of glioblastoma multiforme (GBM). A dose-dependent improvement in survival was also shown. The *in vivo* stability of the agent was verified through dual label measurements of biological elimination from the tumor. Overall, these results provide evidence that nanomaterial platforms can be used to deliver effective interstitial brachytherapy.

Keywords

fullerene; nanomedicine; brachytherapy; glioblastoma; metallofullerene

Introduction

Glioblastoma multiforme (GBM) is a particularly difficult to treat and devastating disease. The prognosis for patients with newly diagnosed GBM carries a 1 year median survival, largely due to GBM resistance to current adjuvant therapies that often follow surgical resection, such as conventional radiation therapy and chemotherapy.^{1, 2} Residual cells at the margins of the resection frequently lead to tumor recurrence. Furthermore, a subpopulation of stem-like cells, known as GBM stem cells (GSCs), may be a major proponent of GBM recurrence due to higher chemo- and radio- resistance, as well as having a higher tumorigenic potential and the ability to differentiate into several cell types.^{3–8} These factors result in a high recurrence rate of these malignancies, and an inability to cure this difficult

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disease. Therefore, bringing novel nanomaterial based modalities to the clinician for treatment of high grade gliomas is of potential interest.

Nanomaterials offer promising solutions to the treatment of this serious disease because they present new strategies and have a size range capable of entering cells (1 to 1,000 nm), a substantial multi-functional capability, and an inherently large surface-to-volume ratio. These characteristics of nanomaterials are critical, as recent literature has reported that doxorubicin delivered to cells via gold nanoparticles increased the rate of cellular drug uptake and was 20-fold more cytotoxic.⁹ While the realm of nanomaterials is vast, the advancement and use of carbon based (carbonaceous) nanomaterials has continued to thrive since the discovery of the C₆₀ molecule in 1985 by Smalley, Kroto, Curl and coworkers (Nobel Prize 1996).¹⁰ Also known as “buckyballs” due to their resemblance to the geodesic domes of Richard Buckminster Fuller, fullerenes have shown potential for use in a multitude of medical fields due to their cage-like structure and diverse material properties. These multiple properties result from the encapsulation of various metals within the carbon cage^{11–13} or decoration of the fullerene surface^{14, 15} with targeting,^{12, 16} diagnostic^{17, 18} or bio-functional ligands^{19, 20}. The vision of nanomedicine has stimulated further fullerene advancements,^{21, 22} primarily due to the versatile functionality available in a single nanoplatform. There is increasing interest in the trimetallic nitride endohedral metallofullerenes A₃N@C_{2n} (n=34–50) because of their interesting electronic and structural properties.^{23–25} In comparison with empty-cage fullerenes such as C₆₀, C₇₀, and C₈₄, these endohedral metallofullerenes have higher stability corresponding to lower chemical reactivity. Nevertheless, they exhibit sufficient chemical reactivity to accommodate a variety of different surface functionalization methods.^{20, 26–31} In particular, tri-gadolinium nitride C₈₀ endohedral metallofullerenes (Gd₃N@C₈₀) have shown great potential for application in biomedicine, especially as MRI contrast agents.^{19, 20, 32–34}

In regards to cancers of the central nervous system (CNS), such as GBM, there is difficulty of treatment using conventional routes of administration due to the blood brain barrier (BBB). Advancements in clinical procedures have led to a method of drug delivery that circumvents the BBB and limits systemic toxicity known as convection-enhanced delivery (CED).^{35, 36} Also referred to as direct intraparenchymal infusion, CED is a procedural option that is less invasive than further tumor resection. It is often employed in cases when a patient has a region of tumor recurrence that is inoperable, or the physical burden of the surgery and recovery would be unwarranted or more detrimental to the patient’s overall quality of life. In these and other situations, CED provides beneficial adjuvant therapy by the strategic placement of cannulae or catheters directly into target tissues through stereotactic methods and delivery of a therapeutic bolus. A positive pressure gradient slowly drives the agent through the interstitial space providing a more uniform and reproducible volume of distribution compared to intratumoral injection. This also allows for the delivery of large molecules and nanoparticles (<100 nm) that due to their size would not cross the BBB or diffuse effectively upon simple injection. Although CED is efficient for locally administering a high therapeutic dose with uniform distribution, other issues such as relatively rapid diffusion of therapeutic agents out of the brain has incited investigations using liposomes and nanomaterials to slow the biological decay of these agents from the tumor.^{32, 37}

Interstitial delivery of radioactive isotopes into tumors for therapeutic purposes (brachytherapy) has a long history of development and use for a variety of cancers. Such approaches are common for treatment of prostate and breast cancer, and there is a substantial history of use of a variety of isotopes for the treatment of malignant primary brain tumors.^{38, 39} Most techniques have involved stereotactic placement of catheters to be after-loaded with radioactive seeds for short-term high dose rate brachytherapy (after which the

catheters are removed), or implantation of permanent seeds at the time of surgical resection of the tumor for low dose rate brachytherapy. A number of studies demonstrating promising results have been reported, although two prospective randomized trials using ^{125}I as the isotope in patients with newly diagnosed malignant gliomas did not demonstrate a benefit over conventional therapy with fractionated regional radiation treatments.^{40, 41} Nevertheless, significant interest in brachytherapy techniques remains for the treatment of primary brain tumors. These highly malignant tumors invariably recur after initial treatment with surgery, external beam radiation therapy and chemotherapy. The recurrences are often identified on follow up MR imaging while they are relatively discrete and small, making them excellent targets for therapy directed by image guidance. In some instances, the initial presentation of GBM is with a focal area of brain abnormality that is not amenable to surgical resection, making the possibility of needle biopsy followed by infusion of a brachytherapeutic isotope attractive. The ability to deliver an isotope by CED with high stereotactic precision and using ever more powerful neuro-imaging techniques promises improved targeting and coverage of the tumor cells. The possibility of using ^{177}Lu as the brachytherapy isotope for its advantageous path-length and half-life characteristics and the possibility of targeting tumor cells with affinity ligand-labeled radioactive nanoparticles¹⁶ makes investigation of the efficacy of ^{177}Lu -DOTA-f-Gd₃N@C₈₀ on model human gliomas of great interest for advancing this approach to treating brain tumors.

In the presented study, the recently reported³² theranostic agent ^{177}Lu -DOTA-f-Gd₃N@C₈₀ was infused by CED intratumorally to provide brachytherapy for orthotopic xenograft tumor-bearing mice. Since the imaging capabilities of this agent have previously been shown,³² this work focused on demonstrating a therapeutic dose response and efficacy with *in vivo* models using two glioblastoma cell lines, U87MG and GBM12. Furthermore, modifications to previous synthetic methods are described that resulted in a higher yield and a more radiochemically pure material. Finally, two analytical methods were employed in determining the biological decay from the tumor and intracranial stability of the agent.

Experimental Procedures

^{177}Lu -DOTA-f-Gd₃N@C₈₀ theranostic agent synthesis

The Gd₃N@C₈₀ metallofullerenes were produced and surface functionalized for aqueous solubility and amide bond conjugation methods as previously reported to obtain f-Gd₃N@C₈₀.²⁰ Prior to activating the carboxyl groups on the f-Gd₃N@C₈₀ surface, 500 μL of f-Gd₃N@C₈₀ solution (0.27 mM - Gd₃N@C₈₀) was diluted with 500 μL of MES (2-[N-morpholino]ethanesulfonic acid; Sigma-Aldrich) buffer adjusted to pH 6.4 with Optima grade ammonium hydroxide (Fisher Scientific). The carboxyl groups were then activated for coupling reactions by addition of 100 μL of 1M EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide; Thermo Scientific) and 100 μL of 1M Sulfo-NHS (N-hydroxysulfosuccinimide; Thermo Scientific) and reacted at room temperature for 10 minutes. Immediately at 10 minutes, 2 μL of 2-mercaptoethanol (Sigma-Aldrich) was added to react excess EDC. Then 2mg of bifunctional *p*-NH₂-Bn-DOTA (DOTA) (2-(4-aminobenzyl)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; Macrocyclics) was immediately combined with the activated f-Gd₃N@C₈₀ and reacted at room temperature for 2 hours to couple the f-Gd₃N@C₈₀ to the primary amine on the bi-functional DOTA. Then, 100 μL of 0.275 M hydroxylamine was added to quench the reaction and convert non-reacted activated groups back to carboxyl groups. Purification at this step was carried out by elution through a PD-10 Sephadex desalting column (GE Healthcare) using 0.25 mM ammonium acetate in Optima grade water (DI) (Fisher Scientific) as eluent.

For radioactive labeling, $^{177}\text{LuCl}_3$ was used as received from Perkin Elmer (0.74 GBq, 15 IL) with the addition of DI water making a total volume of 50 μL . The 50 μL of $^{177}\text{Lu}^{3+}$

solution was then transferred into the purified DOTA labeled product (DOTA-f-Gd₃N@C₈₀) followed by the addition of 20 μ L of Optima grade ammonium hydroxide. This shifted the pH to the basic buffering region of the ammonium acetate, which is more favorable for the ¹⁷⁷Lu-DOTA chelate formation. The reaction tube was then sealed and placed in a 40 °C oil bath for 12 hours. Removal of free/unchelated ¹⁷⁷Lu³⁺ was accomplished using a cation exchange cartridge (Oasis MCX, 3 mL/60 mg, 60 μ m; Waters). The cartridge was pre-wet with 3 mL of methanol (Fisher Scientific) and conditioned with DI water. Following this, the reaction solution was loaded onto the cartridge (gravity fed). Then 1 mL of DI water was added to the cartridge and slowly extruded with a plunger to collect 1 mL of volume. Previous testing showed this methodology removed free ¹⁷⁷Lu³⁺ and eluted the fullerene nanoparticles.³² Purification to obtain pure ¹⁷⁷Lu-DOTA-f-Gd₃N@C₈₀ for infusions was carried out by elution through a second PD-10 desalting column using sterile PBS as eluent. Samples were analyzed by high-performance liquid chromatography (HPLC) (Waters–isocratic, 90% 50 mM ammonium acetate/10% methanol) with UV-vis absorption and radioactive detectors (Bioscan) for radiopurity.

Dynamic light scattering (DLS) characterization (Malvern Instruments, Nano-ZS) was carried out on the functionalized metallofullerenes alone, f-Gd₃N@C₈₀, and a non-radiolabeled analogue of the theranostic agent, Lu-DOTA-f-Gd₃N@C₈₀. This “cold” form of the theranostic was synthesized by methods identical to those described above with the exception of replacing the ¹⁷⁷LuCl₃ solution with a LuCl₃ solution prepared to have a matching total Lu concentration.

Cell culture

Human U87MG glioblastoma cells (American Type Culture Collection) and GBM12 cells (provided by Dr. C. David James - University of California, San Francisco, Department of Neurological Surgery) were grown in high-glucose DMEM supplemented with 10% FBS, 100 units/mL penicillin, and 100 units/mL of streptomycin (BioWhittaker Penicillin/Streptomycin, Cambrex Bio Science Walkersville, Inc.) at 37°C in a 5% CO₂/95% air atmosphere. GBM12 cells were used from short-term cultures established from excised flank xenografts as previously described to promote invasive behavior.⁴² To prepare cell inocula, cells were harvested from log phase cultures and resuspended in phosphate-buffered saline at a concentration of 5×10^4 cells/ μ L.

Tumor implantation procedures

All experiments involving the use of mice were carried out in accordance with protocols approved by the institutional animal care and use committee. Female Athymic Nude-Foxn1nu mice (Harlan Laboratories) were anesthetized with isoflurane (3% knock-down, 2% maintenance in oxygen) and placed in a stereotactic frame (David Kopf Instruments). Following a midline incision, a 0.7-mm burr hole was drilled in the skull 0.5 mm anterior to the bregma and 2 mm laterally to the right. A 25- μ L Hamilton Syringe with a 28-gauge removable needle was used to inject 1×10^5 cells (U87MG or GBM12) in 2 μ L of PBS at a depth of 4.0 mm from the surface of the skull and at a flow rate of 0.2 μ L per minute using a microinjection pump (Bioanalytical Systems). The burr hole was sealed with sterile bone wax, the incision sutured, and animals were allowed to recover and subsequently placed in the vivarium.

Dose preparations and survival studies

The radioactivity of the final ¹⁷⁷Lu-DOTA-f-Gd₃N@C₈₀ product in PBS was determined by gamma well counting and converted to units of megabecquerel (MBq). Since the focus of these studies was to determine a therapeutic dose response and efficacy, the mice were not imaged during the experiment because stress from imaging and anesthesia can alter survival

outcome. As the survival studies would involve 48 mice requiring 3 consecutive days of 16 surgical procedures, each dose level was prepared in three separate vials. A combination of ^{177}Lu -DOTA-f-Gd₃N@C₈₀, f-Gd₃N@C₈₀, and PBS (all sterile or sterile filtered) were mixed in order to account for ^{177}Lu radioactive decay (half life = 6.7 days) over the 3 day period, while maintaining a constant Gd₃N@C₈₀ concentration. This was important in order to keep the infused volume constant (10 μL). Survival studies were designed to assess a dose response (3 doses plus control) for the U87MG model and a single dose efficacy for the GBM12 model (high dose plus control). Thus, 36 mice (12 per day) were implanted orthotopically with U87MG cells as described above and 12 mice (4 per day) were implanted with GBM12 cells. Eight days post tumor cell inoculation, U87MG tumor-bearing mice were randomized into 4 groups of 9 mice each: a control group to receive only f-Gd₃N@C₈₀ and 3 treatment groups receiving 0.25, 0.75, or 1.35 MBq of ^{177}Lu -DOTA-f-Gd₃N@C₈₀ product. The GBM12 tumor-bearing mice were also randomized into 2 groups of 6 mice each: a control group (only f-Gd₃N@C₈₀) and a treatment group to receive 1.35 MBq of ^{177}Lu -DOTA-f-Gd₃N@C₈₀. Animals were anesthetized as before, incision made, and bone wax removed to re-open the burr hole. Centered stereotactically on the burr hole, the agents were delivered to the center of the tumor (depth, 4 mm) via CED at a rate of 0.2 $\mu\text{L}/\text{min}$ for a total volume of 10 μL . The combination of the needle size (28-gauge), aqueous infusate medium, and slow infusion rate (0.2 $\mu\text{L}/\text{min}$) has been shown to provide efficient CED in rodent models (37). A corresponding set of 1.5 mL agarose gels (0.6%) were infused with 10 μL of each dose under identical stereotactic conditions following surgical procedures. Activities of the gels were measured by gamma well counting and back decayed to the time of infusion to obtain an accurate assessment of ^{177}Lu activity delivered. Animals were observed and weighed daily to follow the progression of disease. A 20% decrease in body weight relative to the treatment day was established as the primary criteria for euthanasia and the survival time was recorded as the number of days post tumor cell inoculation.

Tumor/brain ^{177}Lu -DOTA-f-Gd₃N@C₈₀ retention studies

Inductively coupled plasma mass spectrometry (ICP-MS) (Varian 820-MS; Agilent Technologies) was used to determine the Gd concentration and radioactivity determined by gamma well counting in the tumor-bearing hemisphere over time. Twelve tumor-bearing mice were infused with 10 μL of ^{177}Lu -DOTA-f-Gd₃N@C₈₀ by CED as described earlier. The delivered ^{177}Lu activity was $0.571 \pm .001$ MBq and the total Gd delivered was 0.654 ± 0.045 Ig determined from analysis of 4 gels infused under the stereotactic conditions. The animals were randomized into 4 groups of 3 mice, each group being euthanized at 4 different time points (immediately and 24, 48, and 168 hours post infusion). Upon euthanasia, the brain of each mouse was excised and divided into the tumor-bearing hemisphere and the contralateral hemisphere. The two hemispheres were placed in 25 mL conical tubes, and frozen at -80°C until all samples were obtained at the experimental time points. The samples were then thawed and sonicated vigorously in 1 mL of DI water for 30 seconds (sufficient time for complete tissue disruption). Then 1 mL of Optima grade nitric acid (Fisher Scientific) was added to each sample, tubes subsequently sealed and incubated at 60°C in an oven for 12 hours (sufficient time for complete digestion of the tissue). The samples and a 1 mL wash with DI water were then filtered through a $0.45\text{-}\mu\text{m}$ polytetrafluoroethylene (PTFE) syringe filter (Whatman GD/XP, GE Healthcare, Princeton, NJ) into scintillation vials and incubated open at 80°C until dry. Next, 375 μL (tumor-bearing hemispheres) or 200 μL (contralateral hemisphere) of Optima grade nitric acid was used to re-dissolve each sample, with the addition of DI water to bring the total volume to 1.5 mL and 0.8 mL respectively. Then 700 μL aliquots of each sample were subjected to gamma counting (for ^{177}Lu content) and ICP-MS (for Gd content). The percent infused dose was calculated by dividing each sample's Gd content or ^{177}Lu activity by the total amount

of Gd or ^{177}Lu activity infused. Values were corrected for percent recovery from the digestion protocol as done previously.³²

Statistical Analysis

All measured values are presented as mean $\pm$ standard error. Biological decay experiments involved three mice at each time point studied. The ICP-MS measurement of Gd content or gamma count of ^{177}Lu activity was expressed as a percentage of the gadolinium or ^{177}Lu activity relative to the infused dose (percent infused dose). For survival studies, statistical software (IBM SPSS statistics 19.0) was used to compute the mean survival time ($\pm$ standard error) for the dose groups by using the Kaplan-Meier method. Survival curves were compared pairwise within each tumor model using the Mantel-Cox log rank test with $p < 0.05$ being considered significant.

Results

Improved synthetic yield and purity

The overall radioactive yield of the theranostic agent (^{177}Lu -DOTA-f-Gd₃N@C₈₀) from previous studies was reported to be $< 5\%$ with radiochemical purity of 80%.³² The starting Gd₃N@C₈₀ fullerene nanoparticle and ^{177}Lu -DOTA-f-Gd₃N@C₈₀ are illustrated in Figure 1 (top left and bottom respectively). Using the methods outlined above in this work, the overall radioactive yield was improved to $> 50\%$. This was accomplished primarily by switching the order of the synthesis to link the DOTA on the f-Gd₃N@C₈₀ first, with a subsequent additional purification step to remove un-conjugated DOTA. Analysis by HPLC with radioactive detection (Figure 1 – top right) showed the ^{177}Lu -DOTA-f-Gd₃N@C₈₀ product peak retention time around 20 min and the “free” or unbound ^{177}Lu -DOTA at 25 min. These two peaks integrate to give a value of 97% radiochemical purity. These results verify that the ^{177}Lu -DOTA-f-Gd₃N@C₈₀ can be produced with high yield and purity.

Further characterization by DLS was carried out to investigate the effect of the surface radiolabeling on functionalized metallofullerene cluster formation and size distribution. As seen in Figure 2, the f-Gd₃N@C₈₀ alone has a bimodal size distribution with peaks around 20.5 nm and 6.7 nm in terms of hydrodynamic diameter. The addition of the trivalent metal chelate, ^{177}Lu -DOTA, to the f-Gd₃N@C₈₀ surface resulted in a smaller size distribution centered at 1.4 nm. Therefore, the addition of the ^{177}Lu -DOTA disrupts the cluster formation and yields a hydrodynamic diameter more consistent with a single ^{177}Lu -DOTA-f-Gd₃N@C₈₀ particle.

Treatment of murine orthotopic xenograft GBM tumors with ^{177}Lu -DOTA-f-Gd₃N@C₈₀ shows improved survival and dose response

Previous studies demonstrated therapeutic potential of this theranostic agent, providing prolonged survival above control out to 60 days at a single dose of 1.11 MBq.³² While these previous results were positive, they were limited in part due to the low overall radioactive yield. Now with the improved synthetic methods and yield reported above, it was possible to investigate the necessary dose response of this agent. The therapeutic dose response of ^{177}Lu -DOTA-f-Gd₃N@C₈₀ was tested using the U87MG mouse model. To demonstrate translatability to another more invasive brain tumor cell line, a single dose therapeutic efficacy was tested using the GBM12 mouse model. Measurement results of the actual infused doses for each treatment group are as follows: U87MG model – 0.25 ± 0.01 MBq, 0.75 ± 0.04 MBq, and 1.35 ± 0.05 MBq; GBM12 model – 1.35 ± 0.05 MBq. During the survival studies, two of the U87MG tumor-bearing mice were euthanized due to complications unrelated to the experiment, and thus were removed from the study and not included in the following results. As seen in Figure 3 (top), the mean survival times post

tumor cell inoculations for the U87MG tumor-bearing mice were 28.7 ± 1.2 days (20.7 days post treatment) for the control group and 48.7 ± 2.5 days (40.7 post treatment) for the 0.25 MBq group. Since the 0.75 MBq and 1.35 MBq groups both had censored events (mice still surviving at 100 days) only estimates of the mean survival could be made that are limited to the largest survival time. In the case of the 0.75 MBq group, the mean survival was 69.5 ± 8.3 days (61.5 post treatment) with 25% surviving at 100 days. In comparison, the 1.35 MBq group had 87.5% surviving at 100 days, euthanizing only 1 of 8 mice during the experimental period. These surviving mice were still thriving at day 100 as evidenced by their increasing/stable relative body weights (Figure 3 bottom). In regards to the GBM12 tumor-bearing mice, the control group survival was 38.3 ± 1.2 days (30.3 post treatment) while the 1.35 MBq group had 83.3% surviving at 100 days (euthanized 1 of 6 mice during experiment). Results of the pairwise comparisons between treatment groups within each tumor model revealed that all treatments were significantly different from their control ($p < 0.001$) and from each other ($p = 0.050$). These data verify efficacy of the ^{177}Lu -DOTA-f-Gd₃N@C₈₀ in two human GBM cell line models.

Biological decay of the ^{177}Lu -DOTA-f-Gd₃N@C₈₀ from the tumor shows *in vivo* stability

The biological decay of the ^{177}Lu -DOTA-f-Gd₃N@C₈₀ out of the tumor was determined by tracking both the Gd and ^{177}Lu components of the agent. Figure 4 shows the percent of the infused dose (% ID) for the ^{177}Lu -DOTA-f-Gd₃N@C₈₀ in the tumor-bearing hemisphere as measured by ICP-MS (Gd) and gamma counting (^{177}Lu). If the covalent linkage between the fullerene cage and ^{177}Lu -DOTA were cleaved, the free ^{177}Lu -DOTA would be more rapidly cleared from the brain than the fullerene nanoparticle as previously reported.³² Therefore, the agreement between the % ID measurements at each time point gives indication that the ^{177}Lu -DOTA-f-Gd₃N@C₈₀ is not being significantly degraded over this period of time in the brain. Results for the contralateral brain hemisphere in terms of ^{177}Lu are as follows: 0 hrs = 1.90 ± 0.46 % ID, 24 hrs = 0.54 ± 0.07 % ID, 48 hrs = 0.47 ± 0.16 % ID, and 168 hrs = 0.50 ± 0.05 % ID.

Discussion

Glioblastoma is highly malignant, usually occurring as a primary focus of tumor mass surrounded by a wide area of invasive tumor cells infiltrating the normal brain.⁴³ Peripheral metastases of GBM to other regions of the body are extremely rare, owing to the lack of predilection for metastasis of the cells and to the relatively short survival time of patients suffering from this disease. Most clinicians and investigators agree that improved control of locally invading tumor cells around the primary site of the tumor can provide significant improvements in the survival of these patients. Thus, GBM represents an ideal disease in which local delivery with wide distribution, such as is obtained with CED, can be expected to produce improvement in outcome. However, systemic delivery of targeted therapeutics brings with it additional considerations and concerns. For instance, even with small-molecule therapeutic agents that have good BBB penetration, the CNS delivery for a systemic dose is only in the 1% to 2% range. Particularly in the case of nanoparticle therapeutics and internal emitters for brachytherapy, effective therapeutic benefit in the brain from systemic delivery would require administration of a two orders of magnitude greater dose which poses significant concerns for peripheral toxicities. For these reasons, the investigations in this work were confined to local delivery by CED.

In regards to the improved survival and dose response, there are several factors that can be related to this positive outcome. First, the stability and retention of the ^{177}Lu -DOTA-f-Gd₃N@C₈₀ over the first 7 days is a critical parameter for delivering an absorbed radiation dose to the tumor. Also, the low levels of radioactivity found in the contralateral hemisphere as compared to the tumor-bearing hemisphere verify the benefits of local delivery by CED.

Especially relevant to ^{177}Lu , this 7 day period corresponds to 1 half-life, during which 50% of the possible cumulative dose is delivered. Furthermore, the stability over this period would allow for the MRI contrast characteristics of the theranostic platform to be used for dosimetric analysis, which is essential for determining the absorbed radiation dose from the delivered activity. It is important to note here that our use of the term “theranostic” is defined as the modification of a diagnostic agent to provide therapy. In this case, the imaging aspect can be used to provide important information relevant to afore mentioned dose calculations, monitoring for backflow during CED, and ensuring that the distribution of the agent adequately covers a pre-diagnosed treatment volume and area of interest. Concerning the biological decay, while a single C_{80} fullerene is < 1 nm in diameter, once functionalized they exhibit a dynamic aggregation/disaggregation behavior which in part accounts for the high MRI relaxivity and tumor retention (20, 32). Whereas the ^{177}Lu -DOTA-f- $\text{Gd}_3\text{N@C}_{80}$ platform showed $\sim 13\%$ remaining at 7 days (168 hrs), previous work with the f- $\text{Gd}_3\text{N@C}_{80}$ alone showed $\sim 45\%$ remaining.³² Reasoning for the differences in biological decay of the two platforms can be speculated using a simplistic model for escaping the brain/tumor. Figure 5 is an illustration of the possible routes the clustered or non-clustered fullerenes could be eliminated from the brain. While the BBB is complex in terms of its discrimination between compounds, it can be rationalized that the fullerene clusters that are closer to 20 nm in size are physically too large to cross the BBB or brain-cerebral spinal fluid-blood barrier (B-CSF-B). This then requires phagocytosis by immune responsive cells (e.g. macrophage, microglia, histiocyte, etc.) for clearance. A primary difference between the f- $\text{Gd}_3\text{N@C}_{80}$ and ^{177}Lu -DOTA-f- $\text{Gd}_3\text{N@C}_{80}$ platforms delivered intratumorally is that the ^{177}Lu -DOTA-f- $\text{Gd}_3\text{N@C}_{80}$ is inducing radiation damage and apoptosis to the tumor cells,³² thus resulting in a higher local immune response. This could partially account for the increased clearance rate. Also as shown in the dynamic light scattering results in Figure 2, the attachment of the chelate containing the trivalent $^{177}\text{Lu}^{3+}$ disrupted the aggregation properties, which are primarily due to hydrogen bonding. This shifted the dynamics more toward the smaller size, thus opening the physical possibility of crossing the disrupted and less discriminative BBB or B-CSF-B barriers due to the presence of the tumor. Regardless, the biological decay of ^{177}Lu -DOTA-f- $\text{Gd}_3\text{N@C}_{80}$ was gradual enough to provide effective brachytherapy, as evidenced by the outcome of the survival studies.

Due to the positive dose response observed, it is plausible to relate the therapeutic benefit to the increasing amounts of ^{177}Lu activity delivered. The control comparison was specifically chosen to be the f- $\text{Gd}_3\text{N@C}_{80}$ alone, as reports have shown positive therapeutic effect linked to angiogenesis inhibition by clustering fullerenes.⁴⁴ Then by keeping the total $\text{Gd}_3\text{N@C}_{80}$ constant, these studies isolated the effect to the delivery of brachytherapy. Recently, ^{177}Lu has emerged as a potential radioisotope of choice, due to its favorable nuclear characteristics: 6.67 day half life, beta-emitter at 497 keV max, gamma-emitter at 208 keV (11%), 113 keV (6.4%).^{45–47} These low energies provide localized dose delivery to the immediate cellular environment (0.7 mm tissue penetration), and thus following molecular targeting or direct intratumoral infusion, they irradiate primarily the malignant cells. Although this work focused on ^{177}Lu -radiolabelled fullerenes, other radionuclides (e.g., ^{90}Y) are expected to have similar biodistribution, exhibit complimentary features, and can be used with the same methods presented. The β^- particles emitted by the ^{90}Y radionuclide have a path length of ~ 4 mm in tissue and a half-life of 2.67 days. This could be an important isotope to investigate in optimizing the radiation dose with respect to the size of the tumor for translation into the clinic. Further long-term studies will be required to investigate biodistribution and radiotoxicity to peripheral organs, as these effects may be dose limiting.

In summary, this work demonstrated a dose-dependent increase in survival in a human GBM orthotopic tumor-bearing mouse model (U87MG) treated with the radiolabeled metallofullerene, ^{177}Lu -DOTA-f-Gd₃N@C₈₀, administered by CED. Efficacy was also shown in a second, more invasive tumor model (GBM12) at a single dose. Biological elimination from the brain and tumor measured by two independent methods verified that the platform was stable *in vivo* over a 7 day period allowing for the delivery of an efficacious brachytherapy dose. In addition, this nanoplatform has the potential to be further manipulated to concomitantly deliver radiosensitizing and other therapeutic agents. These co-treating agents can be selected to increase the tumor specificity of the therapy, thereby reducing the possibility of normal tissue damage. Also, when delivered by CED, all components would have identical intratumoral distribution which could be an important factor for maximizing the synergistic interactions between treatment modalities. Along with its MR imaging attributes, the nanoplatform presented may offer advantages to help overcome GBM resistance to current therapies.

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Figure 1. Illustration of the metallofullerene based therapeutic agent and improved yield and purity. (*Top-Left*) - Structures of the starting endohedral metallofullerene and (*Bottom*) final radiolabeled theranostic agent. (*Top-Right*) - HPLC analysis with radioactive detection showing the retention profile of the final purified product.

Figure 2. Dynamic light scattering analysis of clustering and size distribution of the f- $\text{Gd}_3\text{NC}_{80}$ nanoplatform alone (green) and ^{177}Lu -DOTA-f- $\text{Gd}_3\text{N}@\text{C}_{80}$ (red).

Figure 3.

Survival studies with U87MG and GBM12 orthotopic tumor-bearing mice. (**Top**) - Survival curves for the U87MG (solid lines) and GBM12 (dashed lines) tumor-bearing mice receiving either f-Gd₃N@C₈₀ as control (black, U87MG: n = 9, GBM12: n = 6) or a treatment of ¹⁷⁷Lu-DOTA-f-Gd₃N@C₈₀ at activities of 0.25 MBq (yellow, U87MG: n = 9), 0.75 MBq (green, U87MG: n = 8), or 1.35 MBq (red, U87MG: n = 8, GBM12: n = 6). (**Bottom**) - Weights of the mice during the survival studies normalized to the day of treatment or control infusion (day 8).

Figure 4.

Tumor retention of ^{177}Lu -DOTA-f- $\text{Gd}_3\text{N@C}_{80}$. Measured values are plotted as the percent of the infused dose (%ID) determined by Gd measurement (dark grey) or ^{177}Lu measurement (light grey) at four time points of 0, 24, 48, and 168 hrs post infusion (n = 3 per time point).

Figure 5.

Illustration of biological elimination or decay from the brain/tumor. The dynamic clustering is illustrated (not to scale) with possible (green arrows) or improbable (red arrows) routes of elimination based on physical size alone.