Research Article

Design and Testing of Dual-Targeted Gd₃N@C80-Containing Glioblastoma Theranostics

Getachew Tedla¹,¹ Jesse Plotkin,¹ Anthony Dellinger,^{1,2} and Christopher Kepley¹

¹The University of North Carolina at Greensboro (UNCG) and Joint School of Nanoscience & Nanoengineering (JSNN), 2907 E Gate City Blvd, Greensboro, NC 27401, USA

²Kepley BioSystems, Inc. 2901 E Gate City Blvd, Suite 2400, Greensboro, NC 27401, USA

Correspondence should be addressed to Getachew Tedla; g_tedla@uncg.edu

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The majority of the malignant brain tumors are gliomas with glioblastoma being the most common and aggressive type. Due to its extreme infiltrative nature and higher occurrence frequency, finding new glioblastoma therapeutics and diagnostics is a high priority. In line with this, we previously synthesized a glioblastoma-targeting theranostic that images and delivers a therapeutic payload of doxorubicin (DXR) to human xenograft tumors. Herein, a dual-targeted theranostic is synthesized on the hypothesis that targeting multiple receptors would enhance delivery precision. Doxorubicin and endo-fullerene (Gd₃N@C80) were encapsulated within liposomes and conjugated with transferrin (Tf) and lactoferrin (Lf). The hydrodynamic size, zeta potential, encapsulation efficiency, and tagging with proteins of the Gd₃N@C80- and DXR-loaded liposomes were characterized using dynamic light scattering (DLS), inductively coupled plasma (ICP) mass spectrometer, Fourier-transform infrared spectroscopy (FTIR), and ultraviolet-visible (UV-Vis) spectrophotometry analysis. As verification of efficacy, the Tf and Lf dual-tagged theranostic liposomes were able to significantly induce cell death compared to Lf monotagged theranostics.

1. Introduction

Close to 80,000 new glioma cell-involved primary central nervous system (CNS) tumors are diagnosed each year in the United States with roughly half being malignant [1, 2]. The prognosis of patients with malignant gliomas remains extremely poor, with a median survival of less than 15 months [3]. Hence, finding new therapeutics and diagnostics for gliomas is a high priority and improvements in drug development and measurement of tumor response to therapy may allow advancement of these efforts for other types of brain tumors [4].

Among all gliomas, glioblastoma multiforme (GBM), a World Health Organization grade IV glioma, is the most common and lethal primary malignancy of the central nervous system [5]. Targeted delivery of therapeutics in GBMs mainly relies on single biomarkers, and the chemotherapeutics suffer from the lack of specificity causing significant healthy cells' death. Another major obstacle for any

GBM diagnostic or therapy is transporting it across the blood-brain barrier (BBB).

Using Lf as a targeting ligand for GBM is attractive as it is able to cross the BBB and the transferrin receptor (TfR) is expressed on glioma cells and glioma stem cells [6, 7]. Indeed, Lf has been extensively used for targeted delivery of several drugs to the brain [8, 9] and was used for a magnetic resonance imaging (MRI) agent delivery to the brain [10]. Furthermore, phase I and phase II clinical trial results demonstrated the transferrin receptor as a viable target for tumor reductions in patients with GBM [11]. Similarly, Tf is considered a viable ligand for targeted delivery in gliomas [12, 13].

Tf as well as Lf is often used separately in crossing the BBB and targeting glioma cells [14, 15]. However, it was previously reported that Tf- or Lf-tagged nanoparticles have some limitations. For instance, Tf-tagged nanoparticles were reported to be deposited in major organs such as the heart, kidney, spleen, and lung [16]. On the other hand, Lf-tagged nanoparticles were reported to be identified by macrophages

TABLE 1: Forward and reverse primer sequences used for the real-time polymerase chain reactions.

Protein	Forward primer sequence	Reverse primer sequence
TFRC	AGGACGCGCTAGTGTCTTCTG+	CATCTACTTGCCGAGCCAGG-
DAG	CCCAGTACGTCAAGACCCTG+	GCACACAGAGCATTAGAAGCC-
EGFR	TTGCCGCAAAGTGTGTAACG+	CGATGGACGGGATCTTAGGC-
FABP7	AGAAACTGTAAGTCTGTTGTAGCC+	TCTGCCCAGAACTTGTGTAAC-
IL13RA2	GCGGGGAGAGAGGCAATATC+	GATAGACTGGCGGCAAAGGT-
LTF	GTGCCATTGCAACCCTTGTC+	GAGTTCGTGGCTGTCTTTTCG-

TFRC: transferrin receptor; DAG: dystroglycan; EGFR: epidermal growth factor receptor; FABP7: fatty acid-binding protein 7; IL13RA2: interleukin receptor subunit alpha 2; and LTF: lactotransferrin.

more easily compared to Tf-tagged nanoparticles [16]. Lf-tagged nanoparticles were found to get accumulated more in the liver compared to Tf-attached nanoparticles [16] and there are debating reports on the BBB crossing capabilities of Tf-tagged versus Lf-tagged nanoparticles [16, 17]. These debates could be due to differences on the expression level of TfR and LfR on the BBB as well as in cancer cells which may vary from patient to patient. Hence, our formulation is based on the premise that by modifying the theranostic liposomes with the two ligands, i.e., Tf and Lf, it is possible to minimize the drawbacks observed in using single ligands. The basis is that the dual-modified theranostics will be less identified compared to Lf-tagged particles by macrophages, will have lower effect on TfR expressing major normal tissues, and will have the potential to cross the BBB through TfR, LfR, and Tf and LfR depending on the level of expression of these receptors on a given patient.

On top of efficiently targeting the tumor cells, theranostics should incorporate excellent contrast agents. Water-soluble derivatives of gadolinium-containing metallofullerenes, $\text{Gd}_3\text{N}@C_{80}$, having $10.8\mu_B$ - $21\mu_B$ effective magnetic moment (μ_{eff}) values [18, 19] are excellent candidates for MRI. Further, magnetization curves of $\text{Gd}_3\text{N}@C_{80}$ at different temperatures showed no hysteresis indicating $\text{Gd}_3\text{N}@C_{80}$ as a completely paramagnetic molecule [20]. These molecules are also characterized by higher r_1 relaxivity, mainly, when designed to be water soluble. For example, hydrochalarone-6-functionalized $\text{Gd}_3\text{N}@C_{80}$ showed r_1 relaxivity of $205\text{mM}^{-1}\text{s}^{-1}$ ($68\text{mM}^{-1}\text{s}^{-1}$ Gd, compared with $3.8\text{mM}^{-1}\text{s}^{-1}$ for Magnevist [21]). $\text{Gd}@C_{82}(\text{OH})_{40}$, with just one Gd^{3+} , was reported to have significantly higher r_1 relaxivity ($67\text{mM}^{-1}\text{s}^{-1}$) than Gd-DTPA [22].

The purpose of this work was to develop a new theranostic that incorporates imaging and therapeutic entities within the same GBM-targeted molecule. A liposome-based carrier vehicle was used to incorporate the anticancer drug doxorubicin (DXR) and $\text{Gd}_3\text{N}@C_{80}$ which were proven to provide excellent MRI contrast in different studies [23–25]. The liposome was then dual tagged with Tf and Lf and tested for efficacy in vitro. This strategy may provide a better way to diagnose and treat GBM.

2. Materials and Methods

2.1. Materials and Reagents. Liposome components L- α -phosphatidylcholine (egg, chicken) (EGGPC), 1,2-dipalmitoyl-

sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (PEG200PE), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino (polyethylene glycol)-2000] (DSPE-PEG (2000)-NH₂), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol)-2000] (DSPE-PEG (2000)-COOH), and cholesterol were purchased from Avanti polar lipids. N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDCI) and N-hydroxysuccinimide (NHS) for protein-liposome conjugation, DXR-HCl (cancer drug), and Lf human and Tf for targeting liposomes were purchased from Sigma-Aldrich. Dialysis cassettes at 2000 and 3500 kDa molecular weight cut-off to remove unencapsulated materials, Vybrant® MTT Cell Proliferation Assay Kit for cell viability test, and Novex™ 8% Tris-Glycine Mini Gels for western blotting were purchased from Thermo Fisher Scientific. Gadolinium (Gd), a standard for induced coupling plasma spectrometer measurements, was purchased from Fisher Scientific. $\text{Gd}_3\text{N}@C_{80}$ -OH and $\text{Gd}_3\text{N}@C_{80}$ were purchased from Luna Innovations [21, 23]. The G1/221/12 mouse anti-human Tf receptor primary antibody was from the Developmental Studies Hybridoma Bank. Rabbit anti-mouse secondary antibodies were from Thermo Fisher.

2.2. Real-Time PCR. The expression of targets in U251-MG cells was verified using PCR [26]. Briefly, total cellular RNA was isolated using the one-step RNeasy mini-kit (Qiagen, Valencia, CA) according to manufacturer's procedure [27] and primers, whose sequences are shown in Table 1, are generated using Primer-BLAST as described in [28]. PCR was performed by setting a reverse transcription reaction temperature of 50°C (30 min) and an initial PCR activation temperature of 95°C (15 min). The 3-step cycling conditions were set to 94°C (45 secs), 54°C (30 secs), and 72°C (1 min) for denaturing, annealing, and extension, respectively (30 cycles). Finally, products were run in 2% gel.

2.3. Western Blots. The protein expression of TfR was verified using western blotting as described [29]. Briefly, U251-MG lysate proteins were separated by SDS-PAGE, transferred onto nitrocellulose, and probed with 0.3 $\mu\text{g}/\text{ml}$ mouse anti-human anti-transferrin receptor antibody. After washing, HRP-conjugated rabbit anti-mouse antibody (0.4 $\mu\text{g}/\text{ml}$) was added for an hour, washed, and bands visualized using clarity Max™ Western ECL Blotting Substrate mixture and an Amersham Imager 600 (Figure 1).

FIGURE 1: (a) RT-PCR analysis on agarose gel electrophoresis of total RNA extracted from U251-MG cells demonstrates the expression of DAG1, EGFR, FAB7, IL13RA2, LTF, and TFRC. (b) Western blots on 8% tris-glycine gel electrophoresis were probed with G1/221/12 mouse anti-human TF receptor primary antibody followed by HRP-tagged rabbit anti-mouse secondary antibody and imaged with an Amersham Imager 600.

FIGURE 2: A flow chart indicating the formulation and tagging of DXR- and Gd₃N@C80-OH-loaded liposomal theranostics.

2.4. Liposome Preparation and Characterization. Liposomes containing either Gd₃N@C80 or hydroxyl functionalized-Gd₃N@C80 (Gd₃N@C80-OH) and free of these particles were formulated as described in [30–33]. As the formulation of Gd₃N@C80-OH-containing liposomes represents all other formulation procedures, the process described below focuses on Gd₃N@C80-OH-containing theranostics.

Gd₃N@C80-OH-encapsulated PEGylated liposomes composed of EGGPC:CHOL:PEG2PE:DSPE-PEG2K-NH₂ (53:43:4:1) or EGGPC:CHOL:PEG2PE:DSPE-PEG2K-Amine:DSPE-PEG2K-COOH (53:43:4:0.5:0.5) were formulated as shown in Figure 2 [30–33]. Briefly, lipids dissolved in chloroform were evaporated using rotary evaporator to form a thin lipid film followed by hydration with 250 mM ammonium sulfate and Gd₃N@C80-OH (1.4 mg/ml) solution adjusted to a 5.4 pH (40°C) which results in multilamellar

vesicles (MLV). The liposomes were resized using an Avanti mini extruder passing seven times through 200 and 100 nm pore diameter polycarbonate filters, respectively.

Next, liposomes were conjugated with proteins using EDC/NHS chemistry. Briefly, 748 mg of EDCI and 1.13 mg of NHS were added to 3 different 1 ml carboxylic-functionalized liposomes and the mixture was gently stirred for 10 minutes at room temperature. Monotagged liposomes were formulated by adding an equal amount of Tf and Lf (5 mg) to the two 1 ml suspensions, respectively, and the mixture was kept for 3 h at room temperature. For a dual tagging process, an equal amount of protein (half each) was mixed with COOH-functionalized liposomes for the same period as in the case of monotagged liposomes. Unattached proteins were removed using dialysis cassette immersed in HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

FIGURE 3: Transmission electron microscope images and Brownian movement analysis support the formulation of stable liposomes. (a–d) TEM images of EGGPC+Chol liposomes, main lipid components of all liposomes, at (a) 4,000x, (b) 12,500x, (c) 20,000x, and (d) $\text{Gd}_3\text{N@C80}$ 160,000x. (e, f) Snapshots of a video capturing Brownian motion analyzed using nanoparticle tracking analysis corresponding to size distribution versus intensity of $\text{Gd}_3\text{N@C80}$ -loaded liposomes. A video file of the particles moving under Brownian motion is also provided as a supplementary data.

saline buffer overnight. The attachment of proteins to the liposomes was confirmed as described below. Lastly, 0.44 mg of DXR was added to 1 ml of each class of liposomes (for a lipid concentration of 6.54 mg/ml, the final DXR concentration was 0.76 mM). After thorough mixing, the suspensions were incubated at 40°C in a water bath and intermittently shaken for 20 min to produce DXR- and $\text{Gd}_3\text{N@C80}$ -encapsulated liposomes.

2.4.1. DXR and Gadolinium Quantification. The amount of DXR inside the liposomes was measured using Biotech MX/Mono-based Microplate Reader. First, an absorbance standard curve was established for 12.5, 6.25, 3.125, and 1.5625 μg/ml DXR at 475 and 580 and 470/570 nm excitation

and emission wavelengths. The DXR concentration inside the protein-tagged liposomes was estimated by measuring the absorbance, after lysis with methanol, and using the standard curve equation. The encapsulation efficiency of the liposomes was calculated using the equation $\text{EE}\% = (W_{\text{inside}}/W_{\text{total}}) \times 100$, where W_{inside} is the inside liposome DXR concentration and W_{total} is the initial DXR concentration. Similarly, the amount of Gd was estimated via inductively coupled plasma optical emission spectrometry (Varian 710-ES ICP). Briefly, the $\text{Gd}_3\text{N@C80}$ -encapsulated liposomes were diluted to 3 ml (3% nitric acid) and the emission intensity of Gd in the sample was measured together with the emission intensity of standard Gd concentrations. The final amount of Gd ($\text{Gd}_3\text{N@C80}$) inside

the theranostics was estimated from the standard curve equation developed for known concentrations. The encapsulating efficiency was calculated using $EE\% = (W_{\text{inside}}/W_{\text{total}}) \times 100$, where W_{inside} is the concentration of Gd inside the theranostics and W_{total} is the total concentration of Gd in functionalized and nonfunctionalized stock $Gd_3N@C80$ solution used for hydration (formulation).

2.4.2. Characterization Using TEM, UV-Vis, and FTIR Spectra. The spherical assembly of the liposomes was examined using transmission electron microscopy (Carl Zeiss Libra 120 Plus TEM Microscope). Briefly, TEM grids were sputter coated using a Leica Ace Sputter coater. Then, the grids were immersed into the sample, air dried, and the particles were examined at several magnifications. UV-Vis spectroscopic absorbances were measured to assess the attachment of proteins to liposomes. Similarly, FTIR spectra were measured to confirm the targeting process and to evaluate the presence of functional groups associated to the liposomes and ligands. For this purpose, the symmetric/asymmetric stretch of CH_2 (around 2850 cm^{-1} , $2920\text{--}2960\text{ cm}^{-1}$, respectively), the carbonyl stretching mode $C=O$ ($1800\text{--}1680\text{ cm}^{-1}$), and the phosphate head group vibration regions ($1260\text{--}1000\text{ cm}^{-1}$) [34] are considered to monitor liposome components. The existence and modification of amine/carboxylic functional groups, before and after tagging the liposomes with proteins, were assessed by evaluating the N-H stretch, N-H bend, and C-N stretch occurring at $3300\text{--}3500$, 1600 , and $1080\text{--}1360\text{ cm}^{-1}$ wave numbers. Similarly, the presence of carboxylic acid was assessed in the $1725\text{--}1700\text{ cm}^{-1}$ region [34].

2.4.3. Size and Zeta Potential Measurements. The size and zeta potential of the liposomes were measured using Malvern Zetasizer, and Brownian motion of the liposomes was captured using NanoSight nanoparticle tracking analysis. For Zetasizer measurements, liposomes were diluted with water (at 0.1/0.9 ratio) and the refractive index, the viscosity, and dielectric constants of the dispersant were set to 1.33 (at 25°C), 0.87 cP , and 78.5 , respectively. As the average size and zeta potential values alone may not be informative enough, the quality control correlation graphs as well as the corresponding size and zeta potential values are presented. The particle size was also measured using NanoSight LM10 device mounted on an optical microscope at $20\times$ objective, and the Brownian motion of the liposomes was captured at room temperature and is provided as supplementary material (available here).

2.5. Cytotoxicity. The cytotoxic effect of the theranostic on U251-MG cells was examined using the MTT assay. U251-MG cells were seeded in triplicate for 72 hours in a 96-well plate at a density of 2.8×10^4 cells per well. After the cells reached confluence, the theranostic or controls were added ($12\text{ }\mu\text{g/ml}$ DXR in $100\text{ }\mu\text{l}$) in cell culture medium. The first groups of control cells were treated with equal volume of DXR-free liposomes. Positive control cells were also treated with an equal concentration of free DXR (no liposomes), $12\text{ }\mu\text{g/ml}$, and another group of wells were left untreated to

FIGURE 4: UV-Vis spectra support the formulation of protein-tagged theranostics. Tf-tagged liposomes are indicated in purple, Lf-tagged liposomes indicated in red, dual-tagged (Lf and Tf) liposomes indicated in brown, and nontagged liposomes indicated in green. The presence of absorbance amplitude change is evident on protein-tagged liposomes compared to that of nontagged liposomes.

serve as negative controls. After 72 hours of treatment, the cell viability was determined by MTT method as described in the Vybrant MTT cell proliferation assay kit according to manufacturer's protocol [35].

2.6. Statistical Analysis. Statistical analyses were performed using Excel. The variances between the samples were analyzed and significance levels were calculated using Student's *t*-test ($*p < 0.05$, $**p < 0.01$, ns $p > 0.05$) and data are presented as mean $\pm$ standard deviation.

3. Results and Discussions

3.1. Liposome Development and Characterization

3.1.1. Characterization Using TEM, UV-Vis, and FTIR. TEM images of the liposomes at different magnifications confirmed the spherical assembly of the liposomes (Figure 3). The UV-Vis and FTIR spectra measurements support the successful conjugation of proteins to the liposomes as depicted by a clear difference in the absorbance signatures of those protein-tagged liposomes compared to nontagged counterparts.

Proteins absorb UV light at 280 and 200 nm , due to the absorption of aromatic amino acids and the absorption of peptide bonds, respectively. Several authors have reported UV-Vis signatures of Tf [36] and Lf (Figure 1 A of [37]) and both ligands have similar relatively higher absorption at 280 nm . As Tf and Lf are proteins of the same family with similar shape, similar absorbance signatures were anticipated around 280 nm in Tf-tagged and Lf-tagged as well as Tf+Lf-tagged formulations. As expected, a clearly evident

FIGURE 5: FTIR spectra support the tagging and PEGylating of theranostic liposomes. The FTIR absorption spectra of protein-tagged liposomes compared to nontagged are indicated after subtracting the buffer spectra. FTIR scanning parameters were set to 4 scans at 4 cm^{-1} resolution covering $400\text{--}6,000\text{ cm}^{-1}$ wave number range (for a detailed explanation of the absorbance, see Section 2.4). The smoothed version of the original figure (a) is given (b).

higher absorption, at nearly 290 nm wavelength, on the UV-Vis spectra in protein-tagged liposomes is observed compared to the absorbance signature of the nontagged liposome (Figure 4). A high absorption around 230 nm wavelength is in agreement with previously reported UV-Vis liposome signatures [36, 38] and a relatively amplified absorption around 280 nm is consistent with an extinction measurement of Tf-attached liposomes reported in [36].

The presence of PEG in the PEGylated liposomes is supported by FTIR absorbance around 2880 cm^{-1} due to a C-H stretch of PEG (Figure 5) [39]. CH_2 asymmetric stretching near 2925 cm^{-1} and symmetric stretch near 2850 cm^{-1} and CH_2 scissor and bend at 1460 and 1420 cm^{-1} , respectively, are in agreement with previous EGGPC signatures [40]. PO_2 asymmetric stretching near 1220 cm^{-1} and symmetric stretch at 1088 cm^{-1} (Figure 5) are also in agreement with previously reported signatures of EGGPC or EGGPC/cholesterol liposomes [40]. Those signatures support unaffected and nonmodified EGGPC in the liposome particles. As in the case of the UV-Vis spectra, the FTIR signatures also support the tagging process. The peak near 1650 cm^{-1} in the

FTIR signature of protein-tagged liposomes could be related to amide I band associated with a C=O stretching vibrations of peptide bonds. The peaks near 1540 cm^{-1} (N-H bending vibration/C-N stretching vibration) and 1240 cm^{-1} (C-N stretching vibration/N-H bending vibration) could be due to the amide II band and amide III bands, respectively (Figure 5). The peak near 3300 cm^{-1} can also be N-H bending vibration, and the peak near 1400 cm^{-1} from protein side chain of COO^- is indicated maximum in the protein-tagged liposomes. The highest amplitude on $3300\text{--}2500\text{ cm}^{-1}$ depicted on nontagged liposomes compared to protein-tagged liposomes may show a modification of the carboxylic functional group, in the latter, during the conjugation process (Figure 5). The higher amplitudes between 1320 and 1210 cm^{-1} , a typical wave number range for C-O stretch, in nontagged liposomes than on protein-tagged ones may indicate the modification of carboxylic functional group. The NH stretch at 3288 cm^{-1} clearly visible on Lf-, Tf-, and Lf- and Tf-tagged liposomes compared to nontagged counterparts is likely from secondary amines (Figure 5). These results, in general, support the conjugation of the liposomes with the proteins.

TABLE 2: Zeta potential, average hydrodynamic diameter, and polydispersity index of single-, dual-, and nontagged liposomes loaded with Gd₃N@C80-OH and DXR. Gd₃N@C80-loaded represents devoid of DXR and DXR+Gd₃N@C80-loaded contains both.

Liposome type	Zeta potential (mean $\pm$ SD mV)	Average size (mean)	PDI
Tf-tagged (Gd ₃ N@C80-loaded)	-14.7 $\pm$ 1.07	148.3	0.215
Lf-tagged (Gd ₃ N@C80-loaded)	-16.5 $\pm$ 1.04	142.2	0.257
Tf+Lf-tagged (Gd ₃ N@C80-loaded)	-17.1 $\pm$ 1.27	148.7	0.218
Nontagged (Gd ₃ N@C80-loaded)	-19.8 $\pm$ 1.56	146.3	0.220
Tf-tagged (DXR+Gd ₃ N@C80-loaded)	-19.9 $\pm$ 0.81	139.4	0.204
Lf-tagged (DXR+Gd ₃ N@C80-loaded)	-17.6 $\pm$ 1.31	142.4	0.184
Lf+Tf-tagged (DXR+Gd ₃ N@C80-loaded)	-15.5 $\pm$ 0.61	149.3	0.212

FIGURE 6: DLS size measurements and size distribution analysis of Gd₃N@C80+DXR-loaded-targeted liposomes versus Gd₃N@C80-loaded-targeted liposomes support the formulation of stable theranostics. (a) Example of correlation coefficient graph of Tf+Lf-tagged Gd₃N@C80- and DXR-loaded liposomes with (b) corresponding size distributions. (c) An overlay of size distribution versus intensity of Lf-tagged Gd₃N@C80- and DXR-loaded (blue), Lf+Tf-tagged Gd₃N@C80- and DXR-loaded (black), Tf-tagged Gd₃N@C80- and DXR-loaded (green), and nontagged liposomes (red). (d) An overlay of size distribution versus intensity of Lf-tagged Gd₃N@C80-loaded (green), Lf+Tf-tagged Gd₃N@C80-loaded (blue), Tf-tagged Gd₃N@C80-loaded liposomes (red), and nontagged liposomes (black).

3.1.2. Encapsulation Efficiency. A scatter plot and correlation-based encapsulation efficiency predictions confirmed successful loading of the liposomes with DXR as well as with Gd₃N@C80 particles (Supplementary information 2). DXR encapsulation efficiency of nonfunctionalized Gd₃N@C80-containing liposomes formulated using the ammonium

phosphate hydration process was 94% and accords with the encapsulation efficiency reported in [33]. However, the nonfunctionalized Gd₃N@C80 encapsulation efficiency was low (2%). In an attempt to increase Gd₃N@C80 encapsulation efficiency, OH-functionalized Gd₃N@C80 dissolved in ammonium sulfate solution was used for the

FIGURE 7: The size distribution of liposomes by their number confirms that most nanoparticles possess <100 nm hydrodynamic diameter. (a) Lf+Tf-tagged, Gd₃N@C80- and DXR-loaded, (b) Tf-tagged, Gd₃N@C80- and DXR-loaded, and (c) Lf-tagged, Gd₃N@C80- and DXR-loaded liposomes.

hydration process. In the latter approach, Gd₃N@C80 encapsulation efficiency of the liposomes was increased to 13%. After DXR loading and targeting these functionalized Gd₃N@C80-containing liposomes, the DXR encapsulation efficiencies were 45%, 57%, and 46%, respectively, for Tf+Lf-, Tf-, and Lf-tagged liposomes.

3.1.3. Size and Zeta Potential Measurements. The average hydrodynamic size of nontagged liposomes was 146 nm (Table 2) and after the tagging process it was 148, 142, and 148 for Tf-, Lf-, and Tf+Lf-tagged liposomes, respectively (Table 2, Figure 6). After the liposomes are loaded with DXR, the average hydrodynamic sizes were 139, 142, and 149 nm for Tf-, Lf-, and Tf+Lf-tagged theranostics, respectively (Table 2). The size differences observed after loading with DXR could be due to additional processes such as the remote loading and dialysis. The polydispersity index (PDI) values for most of the formulations are within 0.2 suggesting a narrow size distribution range. Approximately 70% of the liposomes are 60–80 nm in size (Figure 7). Such smaller-size liposomal formulations depicted by single- as well as dual-tagged and PEGylated particles are predicted to have less interaction with plasma proteins, i.e., their smaller sizes will help evade a capture by the reticuloendothelial system (RES), may ensure longer half-life in the blood, and have a potential to accumulate at the tumor site [41]. These size distributions are also in accord with the size of nanoparticles reported to cross the BBB and got accumulated in the brain

[42, 43]. The average zeta potential of Gd₃N@C80-loaded nontagged liposomes at 7.4 pH was -19 mV and after targeting it was -14, -16, and -17 mV for Tf-, Lf-, and Tf+Lf-tagged liposomes, respectively (Table 2, Figure 8). After the drug loading process, the average zeta potential was -19, -17, and -15 mV, respectively, for Tf-, Lf-, and Tf+Lf-tagged liposomes at the same pH (Table 2, Figure 8). The zeta potential values in this study are in accord with the zeta potential values reported being effective for brain delivery [43–45].

3.2. In Vitro Evaluation. Tf and Lf are quite similar in sequence and structure and coordinate iron in the same manner, but they differ in their affinities for iron as well as their receptor-binding properties [46]. While Tf is known to couple with Tf receptor, Lf is suggested to couple with its own receptor (Lf receptor) [47] but also with low-density lipoprotein receptor-related protein 1 (LRP1) and LRP2 [48]. U251-MG cells express TfR to a greater extent compared to their LRP1 or LRP2 expression and U87-MG expresses LRP1 and TfR (the protein atlas and [49, 50]). As the difference in the magnitude of TfR and LRP1 expression in U87-MG is not clear, U251-MG was selected for the *in vitro* evaluation. As shown in Figure 9(a), the viability assay is in line with the expression level of the receptors in U251-MG. Cells treated with Tf-tagged and Tf+Lf-tagged theranostics showed significant reductions in metabolic activity (Figure 9). Tf-tagged theranostics are more toxic, mainly, because Tf+Lf is formulated in such a way that

FIGURE 8: Zeta potential measurements support the formulation of stable, at physiological pH, drug and imaging agent containing liposomes. (a) Example phase plot of Tf+Lf-tagged, Gd₃N@C80- and DXR-loaded liposomes and (b) corresponding apparent zeta potential. (c) An overlay of zeta potential distribution of Gd₃N@C80+DXR-loaded liposomes where nontagged liposomes are portrayed in red, Lf-tagged liposomes portrayed in black, Lf+Tf-tagged liposomes portrayed in blue, and Tf-tagged liposomes portrayed in green. (d) An overlay of zeta potential distribution of Gd₃N@C80-loaded liposomes. Nontagged liposomes are represented in black, Lf-tagged liposomes represented in green, Lf+Tf-tagged represented in blue, and Tf-tagged liposomes represented in red.

the number of Tf is half of what was used for the Tf-attached theranostics. However, it is critical to notice that Tf+Lf-tagged theranostics also significantly reduced the cell viability.

Blank (DXR-free) liposome-treated cells never showed significant metabolic activity difference compared to untreated controls. From visual inspection, it may seem that the cell viability in blank liposome-treated cells is higher than that in untreated controls. However, the difference

is not statistically significant. The relatively higher viability in blank liposome-treated cells is associated with a higher standard deviation. Hence, as DXR-free liposomes never induce a significant negative effect on the metabolic activity of the cells, viability reductions observed in Tf-tagged and Tf+Lf-tagged theranostic-treated cells are most likely due to DXR and these results accord with the viability reductions observed in free DXR-treated cells (Figure 9). One possible reason for the difference in toxicity (i.e., for the

FIGURE 9: MTT cell viability assay confirms the cytotoxic effect of single- (Tf) and dual-tagged (Tf+Lf) theranostics on U251-MG cells. The cell viability is indicated in percent after 72 h of incubation of U251-MG cells with Tf- and Lf-tagged (Tf+Lf), Tf-tagged (Tf), and Lf-tagged (Lf) liposomes all loaded with $Gd_3N@C80$ and DXR; blank liposomes (loaded with $Gd_3N@C80$ but devoid of DXR) and free DXR (nonencapsulated DXR). The untreated cells were set to 100% (controls) cellular viability. These results are representatives of three independent experiments and the data values are expressed as the mean $\pm$ standard deviation (SD), $n = 3$. (b) A comparison of U251-MG cells treated with DXR-free liposomes with respect to those treated with DXR-loaded liposomes supported the cytotoxic effect of the theranostics on glioma cells. Cells treated with targeted liposomes loaded with DXR and those treated with free DXR showed low viability as depicted with light microscope visualizations of the cells after 72 h of the treatment. The images from right to left represent U251-MG cells treated with TF-tagged ($Gd_3N@C80$ - and DXR-loaded), LF-tagged ($Gd_3N@C80$ - and DXR-loaded), and TF+LF-tagged ($Gd_3N@C80$ - and DXR-loaded) liposomes, free DXR, blank liposomes ($Gd_3N@C80$ -loaded but DXR-free), and untreated cells.

reductions in metabolic activity of the cells) in those targeted theranostic-treated cells is the degree of expression of the receptors targeted by the ligands. The expression of LRP1 and intelectin1 (ITLN1) is quite low and no LRP2 expression is observed on U251-MG cells (the protein atlas, [49]).

Keeping the differences in the degree of expression of these receptors on U251-MG cells, a higher toxicity of Tf-tagged theranostics compared to Lf-tagged theranostics supports the notion that the mechanism of delivery is indeed receptor mediated. The cell viability reductions in Lf+Tf-

tagged theranostic-treated cells (73%) accord with the lower viability recorded in cells treated with Tf-tagged theranostics (41%) and in Lf-tagged theranostic-treated cells (92%). The viability assay, shown in Figure 9(a), is also in line with the light microscope visualization taken after the treatments (Figure 9(b)). Treatments with free DXR, Tf-tagged, and Tf+Lf-tagged theranostics resulted in lower cell proliferations compared to untreated controls. These results support the hypothesis that Tf+Lf-tagged theranostics have the potential to specifically kill Tf receptor-expressing cancer cells. In general, the superiority of the dual modification is that it

would be less toxic to the major organs which express TfR to a larger extent while killing the cancer cells which express TfR and LfR or those which express both TfR and LfR. This work is an extension of our previously synthesized similar liposome-based glioblastoma-targeting theranostic loaded with Gd₃N@C80 which enabled tumor regression monitoring in human xenograft tumors [23] and detected atherosclerotic plaque lesions [51]. Future work includes testing these particles in BBB models and *in vivo* studies to evaluate the BBB crossing and to compare the distributions of these particles in major organs [16] including cardiotoxicity studies [52, 53].

4. Conclusion

The search for new therapeutics and diagnostics for GBM, the most deadly brain tumor, has been a higher priority. In the present study, novel theranostic liposomes tagged with both Lf and Tf (1:1 ratio) are constructed for the first time to harness the potential of crossing the BBB and kill transferrin receptor expressing GBM and glioma stem cells. These dual-tagged theranostics contain DXR and a novel imaging agent (Gd₃N@C80-OH) and are PEGylated. Furthermore, >70% of these theranostic liposomes possess 60–80 nm diameter and have <0.2 polydispersity index which signifies narrow particle size distribution. Encapsulation efficiency, zeta potential, FTIR and UV-VIS measurements support the formulation of DXR & Gd₃N@C80-OH loaded, targeted and stable liposomes at physiological PH. These dual-tagged theranostics exhibited improved inhibitory effects in U251-MG cells compared to Lf monotagged counterparts. The results of this research demonstrated that DXR- and Gd₃N@C80-OH-loaded and Lf and Tf dual-tagged PEGylated liposomes might be potential drug and imaging agent delivery systems for GBM treatment.

Data Availability

The data used to support the findings of this study are included within the supplementary information file included within the article.

Conflicts of Interest

The authors declare no conflict of interest.

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Supplementary Materials

Supplementary information 1: supplementary movie which indicates the Brownian motion of liposomes captured using NanoSight nanoparticle tracking analysis. The file is associated with Figure 3. Supplementary information 2: standard curves to estimate DXR and Gd encapsulation efficiencies. (A) A linear standard curve used to predict Gd₃N@C80-OH encapsulation efficiency in liposomes. Emission intensity

of different concentrations of Gd standards dissolved in 3% HNO₃ solvent matrix measured using ICP and Gd₃N@C80 was predicted from the correlation equation (for the detailed quantification process, see Section 2.4). (B) A linear standard curve equation used to predict gadolinium concentration in Gd₃N@C80-OH solution. (C) Linear standard curve used to predict DXR encapsulation efficiency of targeted theranostic liposomes. DXR excitation and absorption were set to 470 and 570 nm wavelengths, respectively. Supplementary information 3: formulation of nonfunctionalized Gd₃N@C80-encapsulated liposomes: Gd₃N@C80-free as well as Gd₃N@C80-encapsulated liposomes were formulated by dissolving EGGPC+Chol (8.1:1.75), EGGPC+Chol+Gd₃N@C80 (8.1:1.75:0.5), EGGPC+Chol+PEG2PE+Gd₃N@C80 (7.1:1.75:1:0.5), and EGGPC+Chol+AmineDSPE+Gd₃N@C80 (7.1:1.75:1:0.5) mg ratios in 5 ml chloroform. After removing the chloroform, the dried lipid layer was hydrated using 300 mM (NH₄)₂HPO₄ solution at 7.4 pH, vortexed, and sonicated to completely dissolve the lipid layer in the hydrating medium. Resulting multilamellar vesicles (MLV) were extruded seven passes through 400–100 nm filters, respectively. Free molecules, those not part of the formulated liposomes, were removed using a 2 kDa molecular weight cut-off dialysis cassette immersed in HEPES saline solution (140 mM NaCl 10 mM HEPES buffer). DXR was loaded (0.25 mg/ml) remotely by incubating the liposomes and DXR in a 70°C saline HEPES buffer. (*Supplementary Materials*)

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