



## Masking and triggered unmasking of targeting ligands on nanocarriers to improve drug delivery to brain tumors

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### ABSTRACT

Long-circulating nanocarriers have been extensively studied to deliver chemotherapeutics; however, the inclusion of targeting agents compromises circulation times thereby offsetting the benefits of active targeting. Here, we formulated cysteine-cleavable phospholipid–polyethylene glycol (PEG) to ‘mask’ nanocarrier bound targeting ligands from RES clearance and prolong circulation times of liposomes to allow passive targeting to tumors. This detachable polymer coating can be removed after nanocarrier extravasation to tumor is achieved to expose targeting ligands and promote active targeting to tumor cells. *In vivo* studies on folate receptor-targeted liposomes demonstrated our ability to prolong circulation in the bloodstream using this system thereby verifying the ‘masking’ capacity of cleavable phospholipid–PEG<sub>5000</sub>. Controlled modulation of uptake and cytotoxicity of targeted nanocarriers using cleavable phospholipid–PEG was demonstrated through *in vitro* studies. Finally, studies analyzing uptake by tumor cells *in vivo* confirmed enhanced intracellular delivery when tumor-inoculated animals received targeted liposomes containing cleavable phospholipid–PEG<sub>5000</sub> followed by a cysteine infusion to expose folate after liposomes had extravasated to tumor. These results indicate that cleavable phospholipid–PEG can be used in nanocarrier formulations for controlled exposure of targeting ligands to ensure that circulation times remain uncompromised by the inclusion of targeting agents while enabling active targeting to tumors after removal of the polymer coating.

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### 1. Introduction

The ability to specifically target systemically delivered chemotherapeutics to tumors offers potential advantages over conventional non-targeted chemotherapy, most notably a reduction in toxic drug side effects due to decreased delivery to non-target organs [1–3]. Receptor-targeted nanocarriers can be used to package drugs thereby facilitating delivery of high drug payloads to tumors. At the same time, nanocarriers can serve to shield non-target healthy organs from the toxic drug effects and also limit premature degradation of encapsulated drugs by the body [4,5]. Long-circulating nanocarriers, in particular, have been studied extensively as delivery vehicles for chemotherapeutics due to the inherent ability to preferentially accumulate in solid tumors by passive convective transport through leaky endothelium, a process

termed extravasation [6,7]. The long blood residence time and repeated passage through the microvascular bed results in high intratumoral concentrations. The efficacy of these passively targeted nanocarriers is dependent on the extent of their extravasation to tumors, and the degree of passive accumulation, in turn, is dependent on the nanocarrier circulation time [8].

While passive targeting of nanocarriers results in accumulation of drug at the target site, *in vitro* studies have shown that uptake by cells is limited unless a targeting agent is utilized to promote active targeting to cells [9–13]. Unfortunately, we have recently demonstrated that there is an inherent optimization problem as the properties that confer prolonged nanocarrier circulation times, such as the sphere of hydration made possible by incorporation of polyethylene glycol (PEG), are compromised by the presence of receptor-targeting ligands on the nanocarrier surface [14]. As a result of the incorporation of targeting moieties into nanocarriers, reduced circulation times substantially decrease passive dosing of tumors [14–21]. This consequence partially accounts for the limited success of receptor-targeted nanocarriers *in vivo* despite the promising results of *in vitro* experiments [17,22]. Others have investigated methods to achieve a balance between *in vivo* circulation times and active targeting to tumors by modulating the number of targeting ligands and PEG

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chain lengths; however, RES evasion comparable to that obtained with non-targeted, PEG-coated liposomes was not demonstrated with any of the targeted formulations tested [15]. In this study, we have developed an alternative method which allows for prolonged circulation times without requiring a reduction in the number of targeting ligands utilized to ensure that maximum binding and uptake by target cells may be achieved. To accomplish this goal, we have developed an elegant system utilizing cysteine-cleavable phospholipid-PEG conjugates that mask targeting ligands while in circulation, and unmask ligands after extravasation to the tumors solving the optimization conundrum (Fig. 1).

Here, we report a novel multifunctional nanocarrier system using a cleavable PEG-lipid conjugate to allow for precise control over ligand access beyond the stealth PEG layer on the nanocarrier surface. In general, there are currently no means to control the presentation of ligands on targeted nanocarrier drug delivery systems after *in vivo* administration to enable RES evasion. PEG chains are known to be capable of creating a hydrophilic barrier around liposomes inhibiting binding of opsonins by steric hindrance and thereby preventing recognition by the reticuloendothelial system (RES), which consists of phagocytic cells responsible for clearance of nanoparticles from circulation [23–25]. Therefore, binding of opsonins responsible for RES clearance should be prevented when targeting ligands are attached to nanocarriers using PEG chains which are shorter than adjacent PEG chains incorporated to confer the hydrophilic, stealth coating on nanocarriers.

The cleavable conjugate reported consists of a phospholipid bound to PEG<sub>5000</sub> via a disulfide bridge and is incorporated into the bilayer of receptor-targeted liposomes. While present, the cleavable PEG<sub>5000</sub> conjugates conceal the targeting ligands (folate), which are conjugated to PEG<sub>2000</sub>, from the RES allowing for prolonged circulation times and passive targeting to tumor. Once the majority of the nanocarriers have exited the bloodstream and passive extravasation to tumor is achieved, active targeting may be promoted in a controlled manner at the tumor site through exogenous administration of a safe cleaving agent responsible for detachment of PEG<sub>5000</sub> from the liposomes. Targeting ligands are then exposed to promote active targeting and uptake by tumor cells. In this manner, cleavable phospholipid-PEG conjugates confer the ability to control the time and location of ligand presentation *in vivo* allowing for maximization of both passive and active targeting while reducing the detrimental effects of targeting ligand incorporation.

Many strategies have been employed to develop conjugates for triggered drug delivery systems cleavable by mild acidic pH [26–28], thiols [26–33] or matrix metalloproteinase [34,35]. Thiol reducible cross-linkers offer the advantage of precise control over cleavage since they require an externally delivered reducing agent such as cysteine, which is only present in the unbound, reduced

form at low concentrations in the body ( $\sim 10 \mu\text{M}$  in blood), to sever the linkage. In addition, cysteine is innocuous to the body at the doses administered for cleavage ( $\sim 1 \text{ mmol/kg}$ ). For these reasons, a cysteine-cleavable phospholipid-PEG conjugate was selected as the ideal candidate for this ‘triggerable’ receptor-targeting liposomal system. As a test system, we chose folate as the targeting ligand, targeting folate receptors on a rat glioblastoma model. Folate is a versatile tumor targeting ligand, and the folate receptor has been shown to be over-expressed in tumors of the ovary, lung, colon, endometrium, brain, breast, and kidney [36].

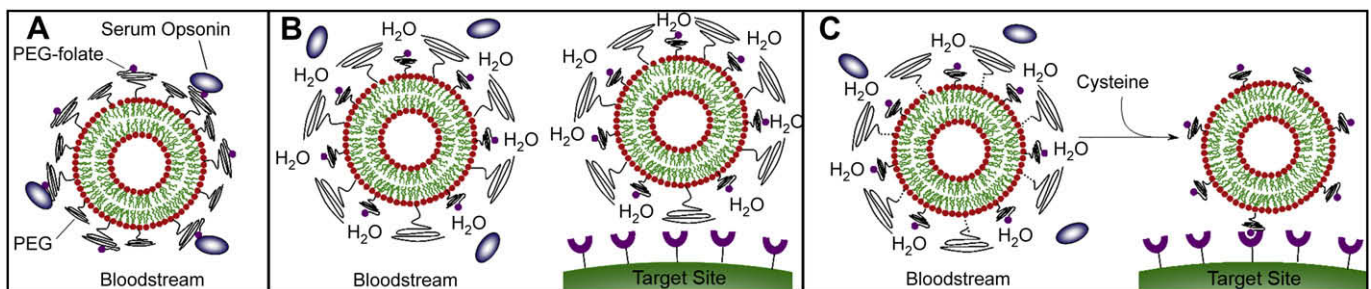
## 2. Materials and methods

### 2.1. Materials

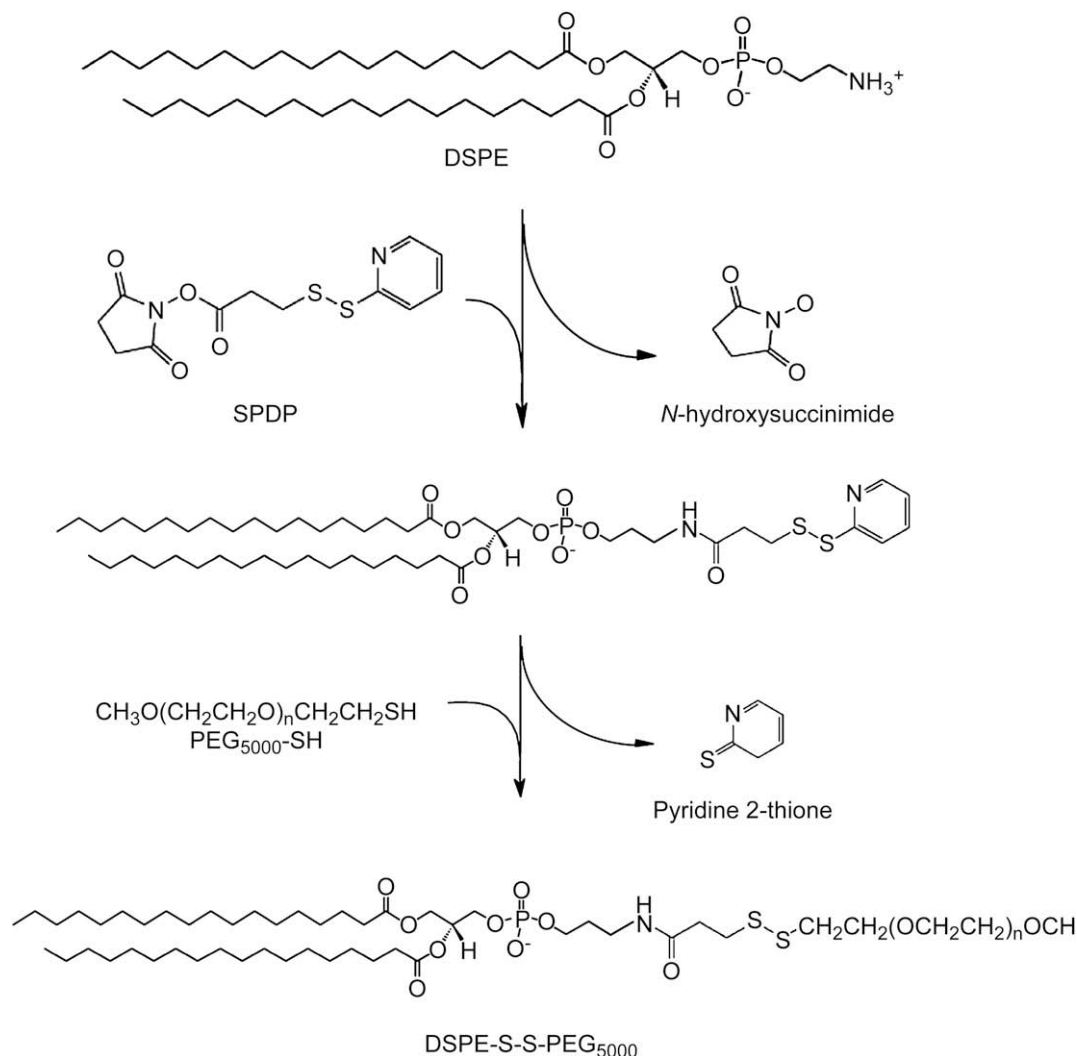
A 9L glioma cell line was received as a generous donation from the Neurosurgery Tissue Bank at UCSF, and a 9L glioma cell line transfected with the bacterial  $\beta$ -galactosidase encoding gene, lacZ, was purchased from American Type Culture Collection (Manassas, VA). Minimal essential medium containing Earle's balanced salt solution was purchased from Hyclone (Logan, UT). Gentamicin (50 mg/mL), fetal bovine serum, folate-free RPMI 1640 medium, Leibovitz's L-15 medium, 4', 6-diamidino-2-phenylindole, dihydrochloride (DAPI), a FluoReporter<sup>®</sup> lacZ flow cytometry kit, and a LIVE/DEAD<sup>®</sup> fixable red dead cell stain kit were obtained from Invitrogen (Carlsbad, CA). Trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA) in Hanks' balanced salt solution and Dulbecco's PBS containing calcium and magnesium were purchased from Mediatech (Herndon, VA). 1,2-Dipalmitoyl-*sn*-glycerophosphocholine (DPPC), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE), and DSPE-poly(ethylene glycol)<sub>2000</sub> (DSPE-PEG<sub>2000</sub>) were obtained from Genzyme (Cambridge, MA). DSPE-PEG<sub>2000</sub>-amine was purchased from Avanti Polar Lipids (Alabaster, AL). PEG<sub>5000</sub>-SH and DSPE-PEG<sub>5000</sub> were purchased from Laysan Bio (Arab, AL). *N*-Succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) was obtained from Pierce (Rockford, IL). Triethylamine, chloroform, dicyclohexyl carbodiimide, dichloromethane, dimethylsulfoxide, hydrochloric acid, methanol, and pyridine were obtained from Fisher Scientific (Pittsburgh, PA). Cholesterol, DL-cysteine, acetonitrile, folate, isopropanol, paraformaldehyde, and Triton X-100 were purchased from Sigma (St. Louis, MO). A water soluble laser dye, ADS645WS, was obtained from American Dye Source (Baie D'Urfé, Quebec). Isoflurane and doxorubicin were obtained from Henry Schein (Melville, NY). A cell viability test kit, CCK-8, was purchased from Dojindo Laboratories (Rockville, MD). Collagenase/dispase solution was purchased from Roche Diagnostics (Indianapolis, IN). Fisher 344 rats were purchased from Harlan (Indianapolis, IN) and maintained on a folic acid deficient diet (<0.05 ppm) containing 1% succinylsulfathiazole obtained from Purina TestDiet (Richmond, IN).

### 2.2. Synthesis of DSPE-S-S-PEG<sub>5000</sub>

A cysteine-cleavable PEG conjugate was synthesized using *N*-succinimidyl 3-[2-pyridyldithio]-propionamide (SPDP) as a crosslinker between 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE) and PEG<sub>5000</sub>-SH. Fig. 2 summarizes the reaction scheme and product. In brief, DSPE (790 mg, 1.05 mmol) was dissolved in chloroform (22 mL) with triethylamine (900  $\mu\text{L}$ ) at 55 °C. SPDP (263 mg, 0.844 mmol) was dissolved in 3 mL of chloroform and then added to the DSPE solution. The reaction mixture was stirred for 5 hours at room temperature. The reaction progress was monitored by thin-layer chromatography (TLC) which demonstrated the conversion of DSPE to a faster running product. PEG<sub>5000</sub>-SH (1.75 g, 0.351 mmol) was then dissolved in 9 mL of chloroform before being added to the solution of DSPE-PDP. The mixture was allowed to react overnight at room



**Fig. 1.** Schematic depicting FR-targeted nanocarrier options. (A) When attached to PEG chains longer than those incorporated for RES evasion, folate targeting ligands are readily recognized by the RES resulting in accelerated clearance and reduced extravasation to tumor. (B) Longer PEG chains conceal folate from the RES but hinder receptor-mediated uptake by target cells over-expressing the folate receptor. (C) Long cleavable PEG-phospholipid conjugates mask folate during circulation to enable passive targeting to tumor but may be detached at the target site. Resultant exposure of folate enables targeting to cells over-expressing the folate receptor.



**Fig. 2.** Schematic of synthesis of DSPE-S-S-PEG<sub>5000</sub>. The compound was synthesized using *N*-Succinimidyl 3-[2-pyridylthio]propionamide (SPDP) as a crosslinker between 1,3-bis(sn-3'-phosphatidyl)-sn-glycerol (DSPE) and PEG<sub>5000</sub>-SH. The synthesis was confirmed by <sup>1</sup>H NMR, mass spectroscopy, and thin-layer chromatography.

temperature. The reaction progress was monitored by the UV absorbance at 343 nm of the pyridyl-2-thione byproduct released from the DSPE-PDP intermediate once the disulfide bridge between the lipid and the PEG was formed. TLC was also utilized to monitor the reaction progress. Following evaporation of the organic solvents, excess DSPE was then removed by precipitation in acetonitrile and centrifugation. The supernatant was recovered, and acetonitrile was then removed by rotary evaporation. The residue was dissolved in dichloromethane and applied to a silica gel column. The column was washed with 200 mL of each of the following concentrations of methanol in dichloromethane: 4%, 6%, 9%, 12%, and 15%. During chromatography, 4 mL fractions were collected, and those determined by TLC to contain product were pooled and lyophilized. The product (DSPE-S-S-PEG<sub>5000</sub>) was characterized by matrix assisted laser desorption ionization time-of-flight mass spectroscopy, high performance liquid chromatography (HPLC), and TLC.

### 2.3. Thiolytic cleavability of DSPE-S-S-PEG

The thiolytic cleavability of the conjugate was confirmed by treating 1 mM micellar conjugate with DL-cysteine of 10 mM concentration in PBS for 30 min at 37 °C. The degree of cleavage was monitored by TLC and quantified via normal-phase HPLC analysis.

### 2.4. Non-targeted and folate receptor-targeted liposome preparations

Liposomes were composed from a 65:35 molar ratio of DPPC:cholesterol. Where non-cleavable or cleavable DSPE-PEG conjugates were utilized to confer stealth characteristics, a reduction in the percentage of DPPC corresponding to the percentage of PEG incorporated was utilized. 100 nm liposomes were formulated following previously described methods [14]. In brief, lipids were dissolved in ethanol at 60 °C before hydrating with 400 mM ammonium sulfate buffer. The

solution was extruded to 100 nm and dialyzed against 0.9% sodium chloride buffer to ensure that the external phase of the liposome formulations was isotonic to blood.

Targeted formulations received 0.15 mol% of DSPE-PEG<sub>2000</sub>-folate for insertion following established procedures [9,14]. To form the folate conjugate, DSPE-PEG<sub>2000</sub>-amine was mixed with folate at a molar ratio of 1:1.6 and dissolved in dimethylsulfoxide. This mixture was reacted with pyridine and dicyclohexyl carbodiimide and then rotary evaporated and rehydrated in water. Insoluble by-products were removed by centrifugation at 10,000g. The final product was lyophilized and then analyzed by thin-layer chromatography, <sup>1</sup>H NMR, and mass spectroscopy. Rf = 0.49 in 1.48 N ammonium hydroxide. <sup>1</sup>H NMR (CDCl<sub>3</sub> solvent): DPPE (0.84 ppm (t), 1.2, 1.5(d), 2.25(d), 2.9(t), 3.1(t), 5.04(m)), PEG (3.3 ppm), and folic acid (1.91, 2.03, 2.3(t), 4.33(m), 4.48(d), 6.5(d), 6.93(t), 7.64(d), 8.12(d), 8.6(s)). MW = 3144 Da. Conjugates were inserted into the liposomal bilayer after rehydrating with water to form micelles. Liposomes were mixed with DSPE-PEG<sub>2000</sub>-folate micelles at 60 °C for 1 hour to allow insertion. Unincorporated folate conjugates were removed by dialysis. To verify adequate incorporation, the folate content in the liposomal formulation was analyzed by measuring the UV absorbance at 285 nm after lysing the liposomal nanocarriers with 10% SDS.

Doxorubicin was remotely loaded into liposomes (120 mg doxorubicin/mmol lipid) according to the previously described methods [14,37]. Final doxorubicin content was assessed by lysing the liposomal nanocarriers with 5% Triton X-100 at 60 °C and measuring the absorbance at 480 nm.

For flow cytometric studies, liposomes were loaded with a water soluble fluorophore, ADS645WS, in lieu of doxorubicin. Lipids were dissolved in ethanol, and encapsulation of dye was carried out by hydrating with 10 mg/mL ADS645WS in 0.9% NaCl. Formulations were extruded to 100 nm and then loaded onto a Sepharose CL-4B chromatography column to remove unencapsulated dye. Insertion of targeting ligands was then conducted as described above. Prior to *in vitro* and *in vivo* experiments, treatments were sterilized by passing through a 0.2 μm filter.

## 2.5. *In vivo* circulation studies

Plasma clearance studies were conducted under a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at Georgia Institute of Technology. Adult, male Fisher 344 rats were given an i.v. injection of liposomal doxorubicin (10 mg/kg doxorubicin; ~60 mg/kg lipid). Each group received either non-targeted liposomal doxorubicin with 3% DSPE-PEG<sub>2000</sub> (n = 3) or folate receptor-targeted (FRT) liposomal doxorubicin with 2%, 6%, or 8% DSPE-S-S-PEG<sub>5000</sub> (n = 3 for each level of cleavable PEG incorporation). For these studies, 3% DSPE-PEG<sub>2000</sub> was utilized to formulate non-targeted liposomes to ensure consistency with previous experiments where we utilized 3% DSPE-PEG<sub>2000</sub> in FRT liposomal formulations since we have observed that higher levels of DSPE-PEG<sub>2000</sub> tend to hinder insertion of DSPE-PEG-folate into preformed liposomes. We have tested various levels of PEG chain incorporation in liposomal nanocarriers and have verified that the circulation kinetics in rats remains unchanged for liposomes bearing as little as 1.5% DSPE-PEG<sub>2000</sub> [38]. After i.v. administration of liposomes in rats, blood was collected from the orbital sinus at 1.5, 7, 18.5, 25, 43, 53.5, and 74.5 hours. Plasma was isolated by centrifugation, diluted 1:4 with deionized water, and doxorubicin was extracted, according to previously described methods [14,39], by treating in a solution of 0.5% Triton X-100 and 75% acidified isopropanol (0.75 N HCl) overnight at -20 °C. The solution was then warmed to room temperature, vortexed, and centrifuged. Fluorescence of supernatants was analyzed to determine doxorubicin content ( $\lambda_{ex}$  = 485,  $\lambda_{em}$  = 590). Plasma samples obtained prior to injection were used to correct for background fluorescence.

## 2.6. Cell culture

9L glioma cells and lacZ transfected 9L glioma cells were maintained in minimal essential medium containing Earle's balanced salt solution supplemented with 10% fetal bovine serum and 0.05 mg/mL gentamicin. Cells were passaged by trypsinization and washed with growth medium.

## 2.7. *In vitro* uptake studies

Liposomal doxorubicin was prepared as described above using 0% DSPE-PEG (conventional), 3% DSPE-PEG<sub>2000</sub> (Stealth), 8% DSPE-PEG<sub>5000</sub> (non-cleavable), or 8% DSPE-S-S-PEG<sub>5000</sub> (cleavable). Folate receptor-targeted formulations received 0.15% DSPE-PEG<sub>2000</sub>-folate for insertion. Prior to applying to cells, formulations were split in half and mixed with either cysteine (10:1 molar ratio of cysteine:lipid) or an equivalent volume of saline for 30 minutes at 37 °C and then dialyzed. 9L glioma cells were washed with folate-free RPMI medium, and then treatments were applied (10  $\mu$ M doxorubicin) in folate-free RPMI medium for 2 hours at 37 °C. Cells were then washed three times with ice cold PBS containing calcium and magnesium before fixing with a mixture of 4% paraformaldehyde and 1.5% methanol in PBS for 20 minutes. Cells were washed with PBS and stained with DAPI for 10 minutes before a final PBS wash. Images of the treated cells were obtained on a confocal laser scanning microscope (Zeiss LSM 510) equipped with argon lasers providing 364 nm and 488 nm excitation lines. Acquired images were analyzed with Carl Zeiss AIM software.

## 2.8. *In vitro* cytotoxicity studies

For cytotoxicity studies, cells were treated as described above with liposomal formulations (10  $\mu$ M doxorubicin) in RPMI medium containing either 0 or 2 mM folate. Afterwards, cells were washed three times with fresh growth medium and then incubated at 37 °C for 5 days in growth medium. Viability was then determined using a formazan based cell counting kit. Untreated cells served as live controls for normalization of the data. The liposomal doxorubicin concentration leading to 50% cell death (LC<sub>50</sub>) was determined in a similar manner with cells exposed to increasing concentrations of liposomal doxorubicin (1.56, 3.13, 6.25, 12.5, 25, 50  $\mu$ M). The best fit line of at least 3 points in the linear range of cell viability was then used to determine LC<sub>50</sub>.

## 2.9. Tumor inoculation

A rat glioma model was established by orthotopic inoculation of  $2 \times 10^6$  9L/lacZ glioma cells following established methods [14]. Animals were fed a folate-free diet containing 1% succinylsulfathiazole for approximately 18 days prior to surgery to eliminate competitive inhibition of FRT liposome uptake and prevent down-regulation of folate receptors *in vivo*.

## 2.10. Flow cytometric analysis of liposome uptake by tumor cells *in vivo*

Quantitative analysis of liposome internalization by tumor cells *in vivo* was evaluated in a manner similar to previously described methods [40]. Animals were allowed to recover from surgery, and 21 days later, saline sham, Stealth NT (3% DSPE-PEG<sub>2000</sub>), or cleavable FRT (8% cleavable DSPE-PEG<sub>5000</sub>) liposomal ADS645WS (1.77 mg/kg ADS645WS; ~70 mg/kg lipid) treatments were administered i.v. After 28.5 hours, either 0.9% NaCl or a solution of 60 mg/mL cysteine in 0.9% NaCl was infused i.v. over 15 minutes at a dose of 2 mL/kg. Animals were anesthetized with 5%

isoflurane 1.5 hours later and decapitated immediately after obtaining a cardiac blood sample. Tumors were dissected from explanted brains, mechanically fragmented on ice, and then treated for 60 minutes at 37 °C with a solution of collagenase (0.1 U/mL PBS) and dispase (0.8 U/mL PBS) to dissociate cells. Cells were kept on ice thereafter to minimize uptake of liposomes by cells *ex vivo*. The cell solution was resuspended in 4% fetal bovine serum in PBS and treated with a *FluoReporter*<sup>®</sup> lacZ flow cytometry kit. Tumor cells expressing the lacZ reporter gene product, beta-galactosidase, hydrolyzed the fluorogenic beta-galactosidase substrate allowing fluorescent detection of expression to distinguish tumor cells from non-tumor cells. A LIVE/DEAD<sup>®</sup> fixable red dead cell stain kit was also used separately to identify dead cell populations during flow cytometric analysis. Flow cytometry was conducted using a Becton-Dickinson DLSR digital flow cytometer equipped with 488 nm and 633 nm excitation lasers using the APC channel for detection of liposomal ADS645WS, the FITC channel for tumor cell (lacZ) detection, and the Texas Red channel to identify dead cell populations. Liposome uptake by lacZ<sup>+</sup> (tumor) cells and lacZ<sup>-</sup> (non-tumor) cells was then quantified. Tumor cells obtained from saline treated animals served as a negative control for ADS645WS while non-transfected 9L glioma cells stained with the *FluoReporter*<sup>®</sup> lacZ flow cytometry kit were utilized as a negative control for  $\beta$ -gal production. Blood samples were analyzed for liposome content after centrifugation to isolate plasma and treatment with 10% SDS to lyse liposomes. Fluorescent signal of samples was then quantified to determine liposome concentration.

## 2.11. Statistical analysis

Means were determined for each variable in this study and the resulting values from each experiment were subjected to an analysis of variance (ANOVA) with Tukey post-hoc pairwise comparisons. Significance was determined using a 95% confidence level. Normality of each data set was confirmed using the Ryan-Joiner test.

## 3. Results

### 3.1. Synthesis of DSPE-S-S-PEG<sub>5000</sub>

To test this liposomal system, we first synthesized a cysteine-cleavable conjugate by linking a lipid, DSPE, to a PEG<sub>5000</sub> via a disulfide bridge using methods similar to those described elsewhere [31,33,41,42]. TLC confirmed the presence of the final product (R<sub>f</sub> = 0.25 in CHCl<sub>3</sub>:MeOH = 85:15). NMR and mass spectroscopies verified the structure of the final conjugate, DSPE-S-S-PEG<sub>5000</sub>, which had a purity of 85% with the remaining being inert compounds. Mass spectroscopy resulted in a bell-shaped spectra verifying the expected molecular weight of ~6210 Da with lines spaced at 44 Da. <sup>1</sup>H NMR (dimethylsulfoxide-*d*<sub>6</sub>, solvent):  $\delta$  0.83 (t, CH<sub>3</sub>, 6H), 1.2 (s, CH<sub>2</sub>, 56H), 1.6 (br, CH<sub>2</sub> CH<sub>2</sub>C=O, 56H), 2.24 (2xt, CH<sub>2</sub>C=O, 4H), 2.5 (2xt, S=CH<sub>2</sub> CH<sub>2</sub>CON, 4H), 2.85 (t, CH<sub>2</sub>CONHDSPE, 4H), 3.22 (s, CH<sub>3</sub>O, 3H), 3.5 (s, PEG, ~456H), 3.7 (t, NCH<sub>2</sub>CH<sub>2</sub>OP, 2H), 5.0 (m, OCH<sub>2</sub>CHCH<sub>2</sub>O, 1H).

### 3.2. Thiolytic cleavability of DSPE-S-S-PEG<sub>5000</sub>

The thiolytic cleavability of the conjugate was confirmed by treating micellar conjugate with DL-cysteine. Upon exposure to cysteine, the parent spot on thin-layer chromatography (R<sub>f</sub> = 0.25) disappeared while the native lipid (R<sub>f</sub> = 0.05) and PEG (R<sub>f</sub> = 0.5) appeared. The degree of cleavage was quantified via normal-phase HPLC analysis which confirmed that treatment with a 10-fold excess of cysteine resulted in fragmentation of 86% of the conjugate (data not shown).

### 3.3. *In vivo* circulation studies

*In vivo* plasma clearance studies were performed to determine the minimal percentage of DSPE-S-S-PEG<sub>5000</sub> required to adequately mask DSPE-PEG<sub>2000</sub>-folate on the surface of the liposomes and achieve circulation times comparable to those obtained with non-targeted 'Stealth' formulations. Table 1 contains details regarding formulation components for all treatments used in these studies. The cleavable FRT formulation containing 8 mol%

DSPE–S–S–PEG<sub>5000</sub> demonstrated optimal *in vivo* circulation performance compared to other percentages of DSPE–S–S–PEG tested as shown in Fig. 3 which displays the percentage of initial doxorubicin in plasma over time for animals receiving either non-targeted liposomes (Stealth NT) or FRT liposomes containing 6% or 8% DSPE–S–S–PEG<sub>5000</sub> (Cleavable FRT). This plot indicates that 8% DSPE–S–S–PEG<sub>5000</sub> is capable of concealing DSPE–PEG<sub>2000</sub>–folate from the RES since the plasma half-life achieved with this formulation is comparable to that demonstrated by Stealth NT liposomal doxorubicin. In fact, the circulation profile of this ‘masked’ FRT formulation actually outperforms that of the Stealth NT nanocarriers for the first 25 hours after injection, demonstrates an area under the curve (AUC) that is comparable during this timeframe, and performs significantly better than our previous FRT liposomal control formulation bearing 0.15% DSPE–PEG<sub>3350</sub>–folate and 3% DSPE–PEG<sub>2000</sub> [14]. After 25 hours in circulation, cleavable FRT liposomes exhibited a slow acceleration in plasma clearance resulting in an overall AUC which was significantly lower than that of Stealth NT formulations. Folate receptor-targeted formulations composed with 6% DSPE–S–S–PEG<sub>5000</sub> demonstrated AUCs that were comparable to those exhibited by Stealth NT and 8% cleavable FRT nanocarriers for the first 25 hours in circulation, however, there was a significant reduction in plasma half-life determined for the 6% cleavable FRT formulation compared to both Stealth NT and 8% cleavable FRT liposomes.

### 3.4. *In vitro* uptake studies

*In vitro* studies were performed to evaluate our ability to precisely control ligand presentation and cellular uptake and cytotoxicity using 8% cysteine-cleavable DSPE–S–S–PEG<sub>5000</sub>. 9L glioma cells were exposed to doxorubicin encapsulated in Stealth NT liposomes, conventional FRT liposomes, non-cleavable FRT liposomes, or cleavable FRT liposomes (see Table 1 for formulation details). Treatments were either applied directly to cells or after pre-treatment with cysteine. In addition, excess folate was added to a subset of treatments before being applied to cells. Images of cells were obtained through confocal microscopy (Fig. 4). Cells treated with Stealth NT formulations demonstrated negligible uptake with or without cysteine pre-treatment, while those treated with conventional FRT liposomes exhibited substantial uptake of liposomes resulting in nuclear localization of doxorubicin under both conditions. When applied without cysteine pre-treatment, ‘masked’ FRT formulations containing either non-cleavable or cleavable DSPE–PEG<sub>5000</sub> were not internalized by cells. Pre-treatment with cysteine, however, considerably enhanced cellular uptake of cleavable FRT liposomes. When treatments were applied in the presence of excess folate, uptake of conventional FRT and cysteine-treated cleavable FRT liposomes was considerably reduced (data not shown).

**Table 1**  
Phospholipid–PEG components (mol%) for each nanocarrier formulation.

Formulation	DSPE–PEG <sub>2000</sub>	DSPE–PEG <sub>5000</sub>	DSPE–S–S–PEG <sub>5000</sub>	DSPE–PEG <sub>2000</sub> –folate
Conventional NT	0	0	0	0
Conventional FRT	0	0	0	0.15
Non-cleavable NT	0	8	0	0
Non-cleavable FRT	0	8	0	0.15
Cleavable NT	0	0	8	0
6% Cleavable FRT	0	0	6	0.15
8% Cleavable FRT	0	0	8	0.15
Stealth NT	3	0	0	0

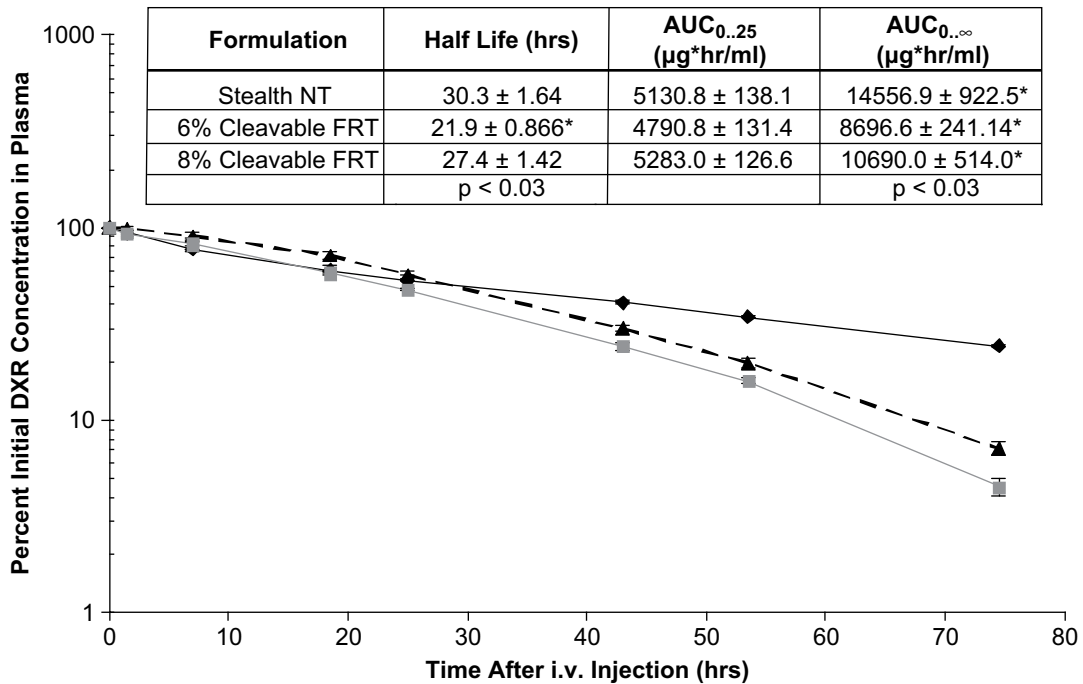
### 3.5. *In vitro* cytotoxicity studies

Cytotoxicity of each formulation was determined by evaluating cell viability after treatment exposure to ensure that not only targeted uptake was achieved but that the extent of doxorubicin uptake was cytotoxic (Fig. 5). Cells exposed to Stealth NT liposomal doxorubicin remained largely unaffected by treatment application and exhibited approximately 100% viability under all four conditions (Fig. 5A). Conventional FRT liposomal doxorubicin demonstrated a significant ( $p < 0.0001$ ) increase in cytotoxicity exhibited by a dramatic reduction in cell viability. The addition of excess folate significantly ( $p < 0.0001$ ) reduced the cytotoxicity verifying that uptake occurred via the folate receptor. Cysteine had no effect on cytotoxicity, and the results obtained from cells receiving cysteine-treated conventional FRT liposomes in the presence of excess folate were comparable to those resulting from treatment application (without cysteine pre-treatment) in the presence of excess folate. ‘Masked’ formulations (both cleavable and non-cleavable FRT) did not demonstrate any cytotoxic effects with or without excess folate, and cysteine had no effect on the cytotoxicity of non-cleavable FRT liposomes. Pre-treatment with cysteine, however, did significantly ( $p < 0.0001$ ) decrease viability of cells treated with cleavable FRT liposomes verifying that the application of cysteine allows for controlled release of PEG<sub>5000</sub> and exposure of folate to facilitate cellular uptake of drug. When excess folate was applied to cells exposed to cysteine treated-cleavable FRT liposomal doxorubicin, cytotoxicity was significantly ( $p < 0.0001$ ) decreased. Cleavable NT formulations were also investigated and did not demonstrate any effect on cellular viability with or without cysteine pre-treatment (data not shown).

Results from studies conducted to determine the lethal concentration that kills 50% of the cells (LC<sub>50</sub>) of select formulations corroborate the outcome of the cytotoxicity studies (Fig. 5B). The LC<sub>50</sub> of free doxorubicin on 9L glioma cells was very low (1.6  $\mu\text{M}$ ) as was the LC<sub>50</sub> of conventional FRT liposomal doxorubicin (4.2  $\mu\text{M}$ ). Masked formulations, however, were not cytotoxic at any of the concentrations tested (0–60  $\mu\text{M}$ ). Upon the addition of cysteine, cleavable FRT liposomal doxorubicin exhibited a substantial reduction in LC<sub>50</sub> (9.2  $\mu\text{M}$ ).

### 3.6. Flow cytometric analysis of liposome uptake by tumor cells *in vivo*

To determine intracellular uptake of systemically delivered nanocarriers by tumor cells *in vivo* and the ability to control uptake using cleavable PEG, we performed studies on brain tumor-bearing rats (9L/lacZ glioma model) receiving either Stealth NT liposomes or cleavable FRT liposomes encapsulating a fluorochrome (ADS645WS) and a subsequent infusion of either saline or cysteine. Animals were euthanized, tumors were dissociated and stained for  $\beta$ -gal production, and cells were analyzed through flow cytometry. Cytometric detection of  $\beta$ -gal in the 9L/lacZ glioma tumors delineated two populations of cells,  $\beta$ -gal(+) tumor cells and  $\beta$ -gal(–) non-tumor cells. The  $\beta$ -gal(–) populations exhibited similar, minimal ADS645WS staining intensity per cell regardless of treatment type (NT +/- cysteine, FRT +/- cysteine) indicating that the uptake of liposomes by non-tumor cells was nominal (Fig. 6). The  $\beta$ -gal(+) population of cells obtained from animals receiving cleavable FRT liposomes followed by a cysteine infusion, however, demonstrated a significant shift in liposome uptake indicated by an increase in ADS645WS signal detected in the APC channel (Fig. 6D).  $\beta$ -gal(+) cells obtained from the other treatment groups showed comparably low uptake of liposomally encapsulated dye with only a minimal shift in uptake compared to  $\beta$ -gal(–) cells (Fig. 6A–C). Fluorometric detection of liposomal ADS645WS in blood samples

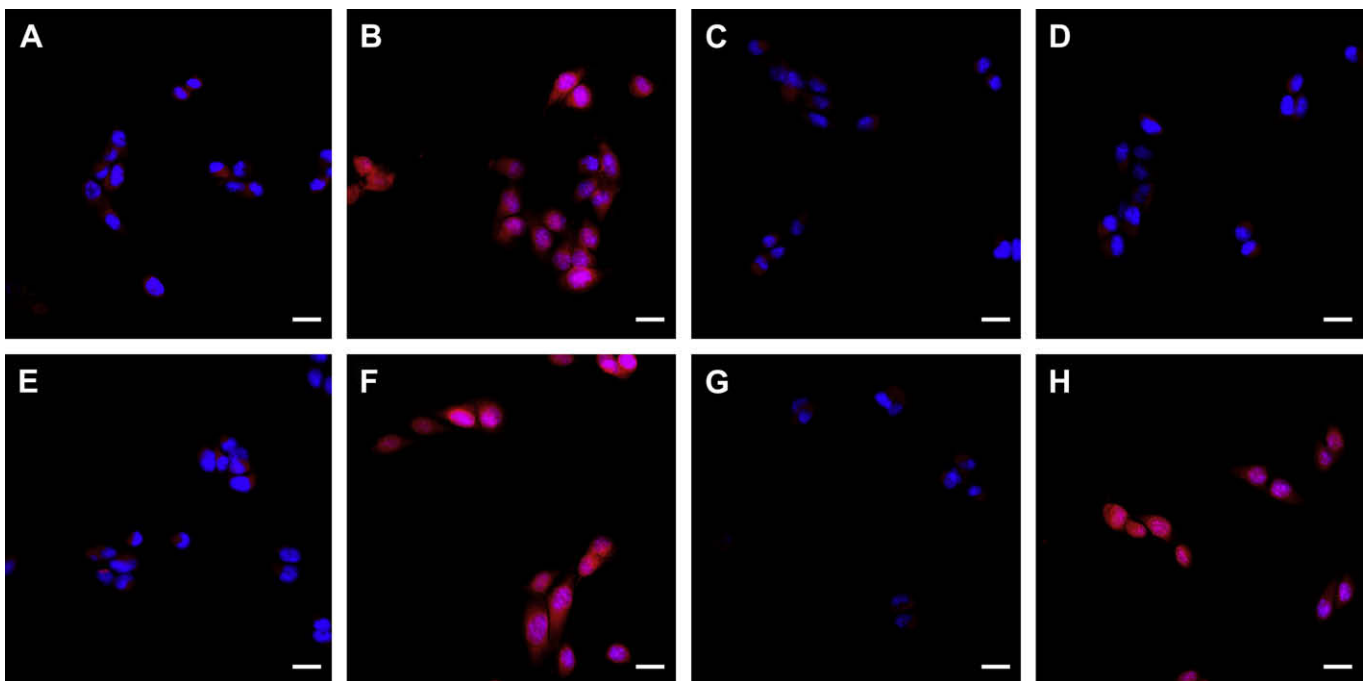


**Fig. 3.** Inclusion of 8% DSPE-S-S-PEG<sub>5000</sub> prolongs circulation of FR-targeted nanocarriers. Circulating levels of doxorubicin in the bloodstream, expressed as a percentage of initial doxorubicin concentration, over time in animals receiving an i.v. injection of Stealth NT (◆), 6% cleavable FRT (■), or 8% cleavable FRT (▲) liposomal doxorubicin. Data were fit to exponential curves to determine half-lives and AUCs. The cleavable FRT formulation containing 6% DSPE-S-S-PEG<sub>5000</sub> demonstrated a half-life in circulation which was significantly lower than the half-lives of the other formulations. AUCs from  $t = 0$ –25 hours were similar for all formulations, however, there was a significant difference in AUC calculated from  $t = 0$  to infinity between all formulations. Data represent mean ± SEM.

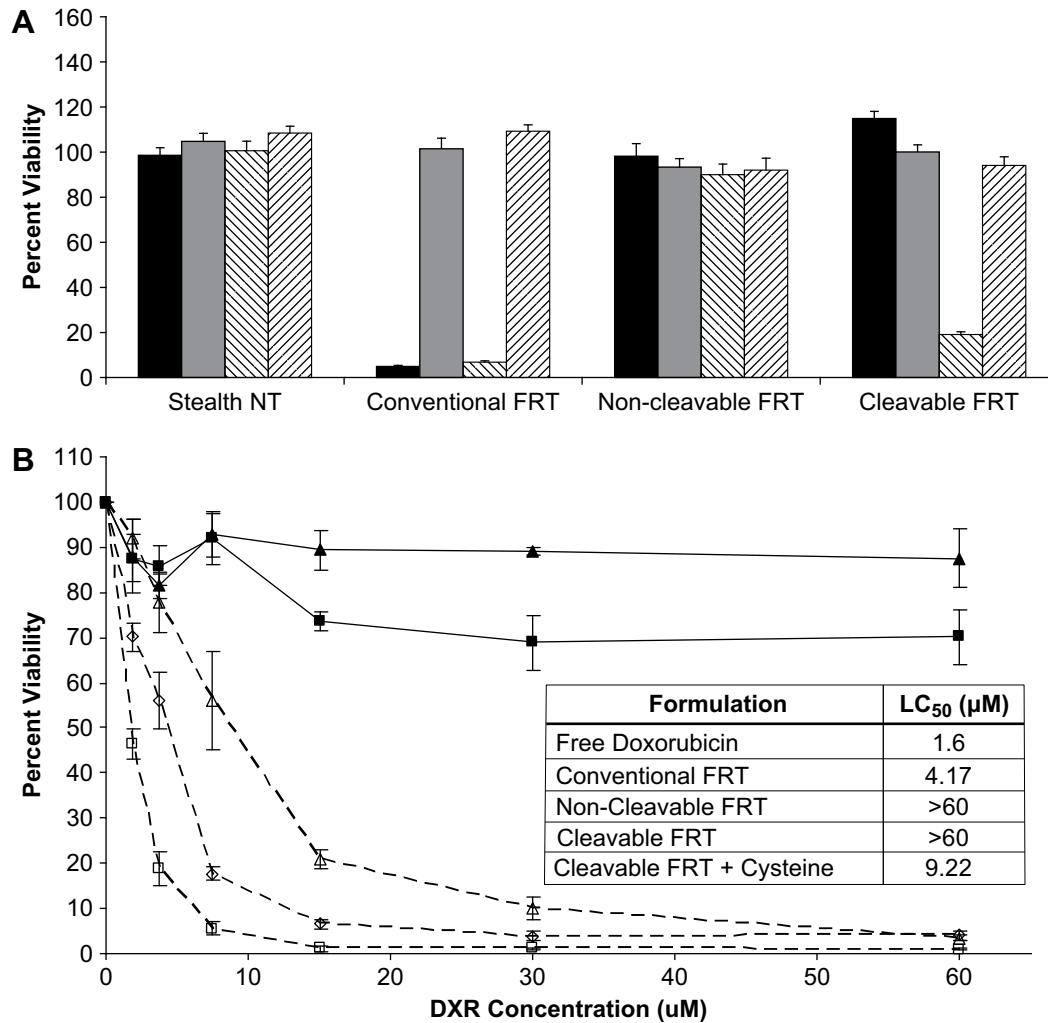
demonstrated a 25–30% reduction in liposome blood levels of animals receiving FRT liposomes and a cysteine infusion compared to other treatment groups.

Cytometric data allowed for quantitative analysis of the results obtained from treated animals (Table 2). The shift in fluorescent

intensity representing liposomal uptake per cell observed in the group receiving cleavable FRT nanocarriers and a cysteine infusion was significant compared to the remaining treatment groups. In addition, mean liposome associated fluorescence in tumor cells obtained from FRT liposome/cysteine-treated animals was about



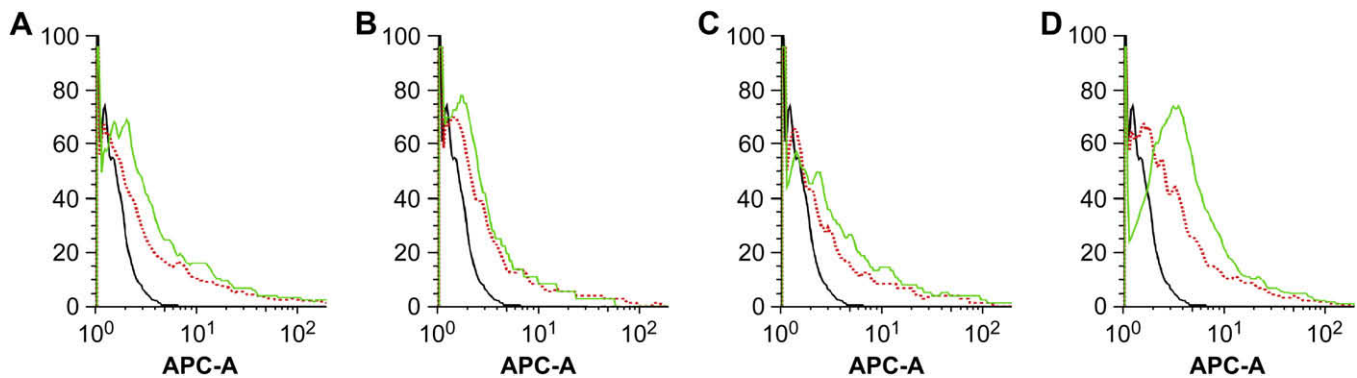
**Fig. 4.** DSPE-S-S-PEG<sub>5000</sub> enables triggered uptake of doxorubicin encapsulated within liposomal nanocarriers by glioma cells. Confocal images demonstrate doxorubicin uptake (red) by cells and delivery to DAPI-stained cell nuclei (blue). Cells received either unaltered formulations (A–D) or those pre-treated with cysteine (E–H). Treatments consisted of Stealth NT (A and E), conventional FRT (B and F), 8% non-cleavable FRT (C and G), or 8% cleavable FRT (D and H) liposomal DXR. Scale bars represent 20  $\mu\text{m}$ .



**Fig. 5.** Cytotoxicity of liposomal doxorubicin is controllably altered through the inclusion of DSPE-S-S-PEG<sub>5000</sub>. Percent viability of cells after treatment with liposomal doxorubicin formulations is shown (A). Treatments were applied alone (■), with excess folate (▨), cysteine treated (▧), or with excess folate and cysteine pre-treatment (▩). Cytotoxicity curves for the various liposomal DXR formulations tested provide evidence for modulation of LC<sub>50</sub> with DSPE-S-S-PEG<sub>5000</sub> (B). 9L glioma cells were treated with free DXR (□), conventional FRT (◇), non-cleavable FRT (■), cleavable FRT (▲), or cysteine-treated cleavable FRT (△) liposomal DXR at various concentrations and percent viability was assessed. LC<sub>50</sub> values (inset) were calculated from the best fit line of at least 3 points in the linear range of cell viability. Data represent mean ± SEM.

2.8 times greater than that of host cells verifying specificity for tumor cells. This tumor/host uptake ratio was significantly greater than that obtained from other treatment groups, which did not demonstrate selectivity for tumor cells. In addition, the percentage

of tumor cells that demonstrated liposome uptake (APC+/FITC+) was significantly greater from animals receiving cleavable FRT liposomes followed by a cysteine infusion than that of other treatment groups.



**Fig. 6.** *In vivo* cellular uptake of liposomes is enhanced when folate on FR-targeted nanocarriers is masked during circulation and ultimately exposed after extravasation into tumor. Uptake of liposomes represented by APC staining intensity, is shown for (A) Stealth NT/saline treated (n = 8), (B) Stealth NT/cysteine treated (n = 6), (C) cleavable FRT/saline (n = 6), or (D) cleavable FRT/cysteine treated (n = 6) rats after gating the non-tumor (–) and tumor (+) populations. Saline treated animals (n = 3) served as a negative control for APC staining (–).

**Table 2**

Flow cytometric analysis of liposome uptake by cells recovered from 9L/lacZ tumors in rats.

Formulation	APC median signal intensity $\pm$ SEM		% APC+/FITC+
	Tumor cells	Tumor/host ratio	
Stealth NT + saline	1.371 $\pm$ 0.099	1.355 $\pm$ 0.099	8.1 $\pm$ 0.9
Stealth NT + cysteine	1.425 $\pm$ 0.110	1.411 $\pm$ 0.109	7.7 $\pm$ 1.0
Cleavable FRT + saline	1.398 $\pm$ 0.102	1.384 $\pm$ 0.101	8.5 $\pm$ 1.0
Cleavable FRT + cysteine	2.845 $\pm$ 0.160*	2.715 $\pm$ 0.180*	21.7 $\pm$ 1.2*

\* $p < 0.0001$ .

#### 4. Discussion

We have previously demonstrated and discussed the importance of circulation time on the efficacy of therapeutics encapsulated within actively targeted nanocarriers [14]. With the knowledge that passive accumulation is critically dependent on circulation times [8], it is imperative that prolonged circulation of nanocarriers is uncompromised upon inclusion of targeting moieties. Here, we have shown that cleavable phospholipid-PEG conjugates can be used to enable precise control over ligand exposure, effectively concealing ligands to prolong liposomal circulation times and exposing targeting moieties at the desired time point upon administration of an *in vivo* safe cleaving agent such as cysteine. Utilizing this system, we have been able to prevent the accelerated clearance of targeted liposomal nanocarriers in an effort to enhance drug delivery to tumors.

*In vivo* circulation studies verified our ability to mask targeting ligands on intravenously delivered nanocarriers to evade the RES and prolong circulation times. Using cysteine-cleavable phospholipid-PEG chains on targeted liposomal formulations, we were able to achieve circulation half-lives comparable to those exhibited by Stealth liposomes. We did observe a slow acceleration in plasma clearance over time exhibited by the cleavable FRT formulation which is presumably due to the low levels ( $\sim 10 \mu\text{M}$ ) of reduced thiols naturally present in the bloodstream slowly cleaving some of the PEG chains from the liposomes and gradually exposing folate which accelerates RES clearance. The half-life of cleavable FRT formulations, however, matched the values achieved with Stealth NT liposomes despite this gradual cleavage of PEG. In addition, the initial AUC, measured from 0 to 25 hours, was comparable to that obtained with Stealth NT liposomes. In fact, the AUC of Stealth NT liposomes did not begin to surpass that of cleavable FRT liposomes until approximately 37.5 hours after administration, and the difference between the AUCs of both formulations after 48 hours in circulation was not statistically significant as determined by Student's t-test. We have previously shown, using the same tumor model described here, that nanocarrier drug levels at the tumor site do not increase substantially between 20 and 50 hours after administration [14], and others have demonstrated that passive accumulation of nanocarrier encapsulated drugs in various tumor models reaches a maximum in as little as 24–48 hours [17,39,40,43,44]. Based on these observations, prolonged circulation times achieved during this timeframe by masking targeting agents from the RES are adequate to enhance passive extravasation to tumor. In addition, the *in vivo* circulation performance of the masked FRT formulation relative to that of Stealth NT liposomes showed dramatic improvement over the previously reported relative performance of traditional FRT liposomes composed using 3% DSPE-PEG<sub>2000</sub> and 0.15% DSPE-PEG<sub>3350</sub>-folate, which demonstrated a 63% reduction in plasma half-life, a 42% reduction in AUC, and a 20% reduction in circulating drug levels within the first hour compared to Stealth NT liposomes [14]. These circulation data prove that DSPE-PEG<sub>2000</sub>-folate can be concealed by DSPE-S-S-

PEG<sub>5000</sub> *in vivo* enabling RES evasion and prolonged circulation times. Appropriate PEG lengths and percentages included in each liposomal formulation need to be considered for various applications (i.e. utilizing an alternative targeting moiety) as these parameters greatly affect the ability to conceal targeting agents and prolong circulation times. For example, we have demonstrated here the detrimental effect on plasma half-life when the amount of DSPE-S-S-PEG<sub>5000</sub> utilized in FRT liposomes is reduced from 8% to 6%. Others have investigated the use of cleavable DSPE-PEG conjugates to coat FRT liposomes but failed to exhibit stability in circulation [19]. There were fundamental differences, however, in this study compared to ours which account for the disparity in the reported results. Gabizon et al. utilized 4% cleavable DSPE-PEG<sub>2000</sub> in an attempt to mask DSPE-PEG<sub>2000</sub>-folate. Here, we have reported that even 6% of a longer cleavable PEG chain is incapable of preventing accelerated clearance of liposomes from the bloodstream. Not until 8% of a longer cleavable PEG conjugate was utilized did we see a significant improvement in the circulation profile. This indicates that 4% cleavable DSPE-PEG<sub>2000</sub> would be incapable of completely masking DSPE-PEG<sub>2000</sub>-folate which accounts for the rapid clearance of these formulations reported in mice by Gabizon et al. [19].

Through these studies, we have also verified that cleavable phospholipid-PEG conjugates can be utilized to promote active targeting in a controlled manner through *in vitro* studies where we demonstrated the ability to regulate uptake and cytotoxicity of targeted nanocarriers. *In vitro*, cleavage of PEG conjugates on FRT formulations was achieved with cysteine and promoted intracellular uptake of liposomes resulting in nuclear localization of drug payloads, a mandate for doxorubicin efficacy. Application of cysteine-treated cleavable FRT formulations to 9L glioma cells resulted in a significant enhancement of uptake and cytotoxicity, which approached values attained with conventional FRT liposomes, verifying successful removal of PEG<sub>5000</sub> and exposure of folate due to cysteine cleavage of disulfide bridges. These results demonstrated that cleavable phospholipid-PEG conjugates can be inserted into targeted nanocarriers to enable precise control over uptake and cytotoxicity. The addition of folate to cells exposed to cysteine-treated cleavable FRT liposomal DXR significantly decreased uptake and cytotoxicity due to competitive inhibition confirming that uptake of 'unmasked' FRT formulations occurred via the folate receptor. Negligible uptake and cytotoxicity of NT liposomal DXR observed *in vitro* clearly demonstrated the need for active targeting to facilitate uptake of liposomal therapeutics. These studies confirmed that removal of PEG<sub>5000</sub> chains is necessary to expose DSPE-PEG<sub>2000</sub>-folate and promote cellular uptake of FRT formulations and that the uptake and cytotoxicity of targeted liposomal doxorubicin can be manipulated through the inclusion of cleavable PEG conjugates that conceal targeting ligands.

While conventional FRT liposomes have been shown to outperform FRT liposomes with adjacent DSPE-PEG chains *in vitro* [45], until now, PEG chains have been present on FRT liposomes at the tumor site *in vivo* because they are mandatory to prolong circulation time. Removal of adjacent PEG chains *in vivo*, a feat made possible with this multifunctional liposomal system, should promote uptake of these formulations beyond that previously achieved, thereby maximizing active targeting of liposomal formulations. Through *in vivo* tumor uptake studies, we demonstrated a significant increase in intracellular cleavable FRT liposomes compared to Stealth NT liposomes both in the number of tumor cells positive for liposomes and the number of liposomes per cell when animals received cleavable FRT liposomes followed by a cysteine infusion. Increased uptake of cleavable FRT formulations by tumor cells obtained from animals receiving a cysteine infusion is indicative of PEG removal *in vivo*. Further verification of cleavage

was obtained through analysis of blood samples obtained from animals immediately prior to euthanasia. A 25–30% reduction in circulating liposomes was exhibited by animals receiving FRT liposomes and a cysteine infusion compared to other treatment groups confirming that cleavage of PEG, leading to RES recognition of exposed folate, was achieved *in vivo*. These *in vivo* studies verified that cleavage of DSPE–S–S–PEG<sub>5000</sub> conjugates on cleavable FRT nanocarriers is achievable at the tumor site *in vivo* using an i.v. cysteine infusion. Cleavage was sufficient to allow for increased binding and internalization of liposomes by tumor cells due to exposure of the targeting ligand, folate. Animals that received cleavable FRT liposomes that were ultimately ‘unmasked’ demonstrated a significantly higher uptake of liposomes per tumor cell as well as a significantly greater percentage of tumor cells internalizing liposomes. These results confirm that PEG<sub>5000</sub> coatings on nanoparticles are adequately removed at the tumor site through the intravenous administration of cysteine to expose targeting ligands thereby facilitating specific uptake by tumor cells.

## 5. Conclusion

Utilizing the system described here for FRT liposomal nanocarriers, we succeeded in satisfying the criteria for maximizing passive tumor accumulation of targeted liposomal formulations by ensuring that prolonged circulation is uncompromised by the inclusion of targeting agents. Using a detachable polymer coating on nanocarriers to shield targeting agents from the RES, we have enabled controlled exposure of targeting agents after intravenous administration to retain prolonged circulation in the bloodstream and enhance passive extravasation to tumors. The data presented here have confirmed that cysteine is capable of cleaving DSPE–S–S–PEG conjugates, exposing targeting ligands, and promoting uptake *in vivo*. In addition, these studies demonstrated that utilization of cleavable conjugates in targeted nanocarrier formulations reduces the detrimental effects of targeting ligand incorporation and enables true optimization of both passive and active targeting to tumors. These findings should allow for a significant enhancement in treatment efficacy of targeted nanocarrier chemotherapeutics *in vivo*.

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## Appendix

Figures with essential colour discrimination. Certain figures in this article, in particular parts of Figs. 1, 4 and 6, may be difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.biomaterials.2009.04.012.

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