International Journal of Pharmaceutics 395 (2010) 251–259

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Uptake and intracellular release kinetics of liposome formulations in glioma cells

Marc-André Bellavance[a], Marie-Belle Poirier[b], David Fortin[b,*]

[a] Department of Pharmacology, Universite de Sherbrooke, 3001, 12th Ave. North, Sherbrooke, Quebec, J1H 5N4 Canada
[b] Department of Surgery, Division of Neurosurgery and Neuro-oncology, Centre Hospitalier Universitaire de Sherbrooke, Sherbrooke (CHUS), Universite de Sherbrooke, 3001, 12th Ave. North, Sherbrooke, Quebec, J1H 5N4 Canada

ARTICLE INFO

Article history:
Received 11 March 2010
Received in revised form 7 May 2010
Accepted 12 May 2010
Available online 1 June 2010

Keywords:
Cationic liposomes
pH-responsive
PEG
Glioblastoma
Blood–brain barrier

ABSTRACT

Glioblastoma (GBM) is the most malignant primary brain tumor in adults, and its prognosis remains very limited despite decades of research. Enhanced drug delivery to GBM using liposomes represents a promising therapeutic strategy. In this study, we describe a novel cationic and pH-sensitive liposome formulation composed of DPPC:DC-Chol:DOPE:DHPE Oregon Green producing efficient internalization and intracellular delivery to F98 and U-118 GBM cells. With a series of derived modifications of the lipid composition, we investigated the impact of membrane fluidity, steric stabilization and loss of both cationic and pH-sensitive components on cellular uptake and intracellular release kinetics by flow cytometry and confocal microscopy, respectively. DPPC:DC-Chol:DOPE:DHPE Oregon Green liposomes were strongly internalized in both cell lines within 6 h. Following cellular uptake, liposomes traveled towards the nucleus (12 h) and gradually released their cargo in the cytosol (over 24 h). Modifications in liposomal composition of our original formulation had detrimental consequences on both the uptake and intracellular release kinetics in the two tested cell lines. Thus, we report a novel potent liposomal formulation for efficient cytosolic delivery of intracellular therapeutics such as chemotherapy agents and siRNAs to GBM cells.

1. Introduction

Glioblastoma (GBM) is the most common and malignant type of primary brain tumors in adults (DeAngelis, 2001; Vescovi et al., 2006; Bondy et al., 2008). The current standard treatment of newly diagnosed GBM consists of maximal surgical resection and a combination of radiotherapy and chemotherapy (temozolomide). The median survival of GBM patients is 12–14 months with optimal treatment (Stupp et al., 2005; Koo et al., 2006). This dismal prognosis has not improved significantly since decades despite active research and numerous technological advances in neuro-oncology (Rich and Bigner, 2004; Wen and Kesari, 2008). The therapeutic approaches to GBM remain challenging because of several factors, such as the highly proliferative and infiltrative behavior of malignant glial cells, their inherent or acquired resistance to chemoradiotherapy and the very limited drug delivery to the central nervous system (CNS) (Rich and Bigner, 2004; Nakada et al., 2007; Claes et al., 2007).

Systemic delivery of therapeutics to primary brain tumors is severely hindered by the blood–brain barrier (BBB). The BBB lies at the endothelium of CNS capillaries and prevents xenobiotics entry to the CNS by sealing the paracellular pathway with endothelial tight junctions (Wolburg and Lippoldt, 2002; Abbott et al., 2006; Cecchelli et al., 2007; Correale and Villa, 2009). Only lipophilic drugs displaying a molecular weight smaller than 400 Da have the ability to cross the BBB (Kemper et al., 2004; Deeken and Loscher, 2007; Pardridge, 2007). However, the very few drugs that possess such physicochemical properties are likely to be expelled from the CNS by BBB multi-drug efflux transporters, such as P-glycoprotein (P-gp) (Glavinas et al., 2004; Chen et al., 2004; Löscher and Potschka, 2005; Szakács et al., 2006). More so, glial tumor cells can also express these efflux transporters, thus creating a second layer of impediment to delivery (Henson et al., 1992; Löscher and Potschka, 2005; Calatozzolo et al., 2005). As a consequence, more than 98% of available drugs cannot reach malignant glial cells in therapeutic concentrations (Pardridge, 2005; de Boer and Gaillard, 2007). Many delivery strategies have thus been developed to circumvent the BBB in order to increase the CNS bioavailability of a wider therapeutic arsenal (Begley, 2004; Chen et al., 2004; Garcia-Garcia et al., 2005; Tiwari and Amiji, 2006; Brasnjevic et al., 2009). Liposomes stand among the most widely used technologies for brain delivery and their clinical use has already been approved in different indications (Merdan et al., 2002; Harrington et al., 2002; Garcia-Garcia et al., 2005; Huynh et al., 2006; Tiwari and Amiji, 2006). Considering the extensive angioarchitecture of the CNS, as well as the active vascular proliferation induced by GBM, liposomes represent one of the most promising systemic delivery strategies for GBM therapy (Kemper et al., 2004; Fenske and Cullis, 2008; Wen and Kesari, 2008).

* Corresponding author. Tel.: +1 819 820 6868x13119; fax: +1 819 564 5226.
E-mail address: David.Fortin@USherbrooke.ca (D. Fortin).

0378-5173/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2010.05.017

Liposomes are inert and polyvalent colloidal carriers made of concentric membranes bilayers containing biocompatible phospholipids. These particulate vehicles alter the pharmacokinetics and the biodistribution of entrapped compounds by masking their physicochemical properties, which provide many advantages over the free form of the equivalent agent. Liposomes increase drug bioavailability by protecting their therapeutic cargo from degradation by the immune system, enzymes and unfavorable conditions (Pinto-Alphandary et al., 2000; Brasnjevic et al., 2009). Due to their lipophilic nature, liposomes facilitate diffusion of entrapped drugs through the BBB and other biological membranes (Drummond et al., 1999; Langner and Kral, 1999; Mamot et al., 2003; Waterhouse et al., 2005; Sapra et al., 2005). Liposomes can also potentiate the efficacy of chemotherapy and other intracellular active drugs by bypassing multi-drug resistance pumps, thus achieving intracellular delivery to targeted cells (Mamot et al., 2003; Torchilin, 2005; Brasnjevic et al., 2009). To fulfill the aforementioned functions in malignant glial tumor therapy, liposomes must first be properly designed to release their cargo in malignant glial cells; it is well recognized that the liposomes interactions with distinct cell types is greatly dictated by their lipid composition, and thus, a liposomal formulation cannot be universal in terms of its application (Fenske and Cullis, 2008).

With this caveat in mind, we have undertaken the design and optimization of a liposomal formulation specifically intended for therapeutics delivery to malignant glial tumor cells. We opted for a mean liposome diameter of approximately 100 nm, since it provides a convenient carrying capacity while maximizing blood residence times and CNS biodistribution across the BBB (Uchiyama et al., 1995; Drummond et al., 1999; Koo et al., 2006; Fenske and Cullis, 2008). We also elected to work with a cationic liposomal charge as it promotes spontaneous electrostatic interactions with highly anionic proteoglycans of eukaryotic cells, and thus yield a high internalization efficiency *via* absorptive endocytosis (Merdan et al., 2002; Medina-Kauwe et al., 2005; Salvati et al., 2006; Hoekstra et al., 2007). Likewise, positively charged liposomes may also benefit from a greater CNS bioavailability since cationization of numerous compounds readily increases their CNS entry *via* adsorptive-mediated endocytosis and subsequent transcytosis at the BBB (Abbott et al., 2006; Béduneau et al., 2007; Brasnjevic et al., 2009). Finally, cationic lipids may also contribute to the intracellular release of liposomal contents, as proposed by Xu and Szoka (1996).

The following report details the design as well as the internalization and intracellular release efficacies of several liposomal formulations in two GBM cell lines (F98 and U-118 MG). Specifically, the impact of liposomal charge, membrane fluidity and incorporation of PEG-modified lipids on cellular uptake and intracellular release was studied. These experiments were conducted in the F98 cell line in the scope of upcoming *in vivo* studies using our syngeneic F98 Fisher rat model, which closely reproduces the human behavior of GBM (Mathieu et al., 2007; Barth and Kaur, 2009). The U-118 MG cell line was used as the human counterpart.

2. Materials and methods

2.1. Materials

1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), cholesterol (Chol), 3β-[N-(N′,N′-dimethylaminoethane)-carbamoyl]cholesterol hydrochloride (DC-Chol), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DPPE-PEG_{2000}) were purchased from Avanti Polar Lipids (Alabaster, AL). The fluorescent phospholipid lipid analog Oregon Green 488 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (DHPE Oregon Green) and the membrane impermeant dye, propidium iodide (PI), were acquired from Invitrogen (Burlington, Ontario, Canada). HEPES and NaCl were obtained from Sigma–Aldrich (Oakville, Ontario, Canada) while chloroform was purchased at Fisher Scientific (Ottawa, Ontario, Canada).

2.2. Cell lines and culture

Rat F98 and human U-118 MG cell lines were obtained from American Type Culture Collection (Rockville, MD) and were both grown in Dulbecco's modified Eagle essential medium (DMEM) supplemented with 10% FBS (v/v), penicillin (100 IU/ml) and streptomycin (100 μg/ml) kindly provided by Wisent Bioproducts (St-Jean-de-Baptiste, Québec, Canada). Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Liposome preparation

Liposomes were formed by the lipid film method initially developed by Bangham et al. (1965). Different lipid mixtures were designed by sequential modifications of a basic liposomal formulation that is very similar to clinically approved DaunoXome liposomes, namely the formulation A (Table 1) (Lian and Ho, 2001; O'Byrne et al., 2002). Of these formulations, one interesting cationic (DC-Chol) pH-sensitive (DOPE) candidate was retained (formulation C, Table 1). We also generated a series of derived lipid modifications to identify key components and properties essential for delivery of liposomes content in glial cells (Table 1). More specifically, we addressed the impact of decreased liposomes membrane fluidity (formulation B), the addition of PEG-modified lipids (formulations D and E) and the abrogation of both the cationic charge and the pH-sensitivity (formulation A) on the cellular uptake and the intracellular release mediated by formulation C were studied with derived formulations (Table 1). To allow for fluorescence microscopy and flow cytometry studies, 0.01 mol% of the fluorescent membrane marker DHPE Oregon Green was included in every formulation.

Lipids were dissolved in chloroform and a lipid film was obtained following the removal of the solvent by rotary evaporation (Buchi Corporation, New Caslte, USA) during 1 h. Dried lipids were then re-hydrated with 4 ml of sterile HEPES buffer (20 mM HEPES, 150 mM NaCl, pH 7.4) containing 1.5 mM of PI. The lipid suspensions were vigorously shaken for 1 h in a water bath set at 10 °C higher than the phase transition temperature (T_c) of the phosphatidylcholine it contained (either DSPC or DPPC). This step was followed by 5 freeze–thawed cycles using liquid nitrogen and a heated water bath (T_c + 10 °C). Liposomes were extruded 10 times through two stacked polycarbonate filters of 200 (top) and 100 nm (bottom) (Millipore, Billerica, USA) with a Lipex Extruder (Northern Lipids, Vancouver, Canada) equipped with a thermobarrel set at T_c + 10 °C. The removal of non-entrapped PI was performed by size exclusion chromatography using a pre-packed PD-10 column of Sephadex G-25 M (GE Healthcare, Oakville, Canada). Liposomes were then stored at 4 °C in sealed glass vials and used within 7 days.

Liposomes concentration was determined by choline enzymatic quantification with the Phospholipid C kit (Wako Diagnostic, Richmond, VA). The protocol provided by the manufacturer was followed as specified, except that bichromatic wavelength readings were carried at 30, 40 and 50 min after liposomes were added to pre-warmed (37 °C) reagent buffer. The determination of each liposome formulation concentration was performed in triplicate.

2.4. Size and morphological analysis by transmission electron microscopy (TEM)

Liposome size and morphology were investigated by transmission electron microscopy (Hitachi H-7500, Japan) following a

Table 1
Lipid composition and mean size of the five liposomal formations (Form.) evaluated in this study. Corresponding letters are use throughout the text to identify the liposomal formulations.

Form.	Lipid composition	Molar ratio	Mean diameter ± SD [nm]
A	DSPC:Chol:DHPE-OG	(2.3:1:0.0003)	113 ± 29
B	DSPC:DC-Chol:DOPE:DHPE-OG	(1.3:1:1:0.0003)	100 ± 31
C	DPPC:DC-Chol:DOPE:DHPE-OG	(1.3:1:1:0.0003)	80 ± 19
D	DPPC:DC-Chol:DOPE:DPPE-PEG_{2000}:DHPE-OG	(1.3:1:1:0.033:0.0003)	111 ± 41
E	DPPC:DC-Chol:DOPE:DPPE-PEG_{2000}:DHPE-OG	(1.3:1:1:0.18:0.0003)	86 ± 18

negative staining protocol. Briefly, liposomes were stained with 2% ammonium molybdate and placed on a 400 mesh Formvar copper grid (Electron Microscopy Sciences, Hatfield, PA). After an incubation period of 20–30 min at ambient conditions, the excess staining was removed and grids were allowed to dry in a Petri box. Microscopic analyses were carried at a magnification of 60,000×.

The mean diameter of liposomes was measured with the acquisition software (AMT, Danvers, USA) calibrated with MAGICAL grid (Norrox Scientific Ltd., Ottawa, Canada). Diameter of at least 60 individual liposomes was recorded for each liposomal formulation generated. Results are expressed as mean diameter in nm ± SD.

2.5. Flow cytometry

Time course analyses of liposome uptake and intracellular delivery were performed by flow cytometry. F98 (18×10^4 cells/well) and U-118 MG (20×10^4 cells/well) cells were seeded in 6-well plates and let to rest for 48 h. The culture medium was then replaced by 2 ml of fresh supplemented medium containing 0.1 mM (200 nmoles) of liposomes or 0.5 μM of free PI and incubated for 1, 2, 4, 6, 12 and 24 h. Thereafter, cells were washed twice with warmed (37 °C) Dulbecco's phosphate-buffered saline (D-PBS) and dissociated with 0.05% trypsin/EDTA. After a centrifugation step at 1000 rpm for 5 min, the supernatant was discarded and the cell pellets were washed twice with 2 ml of ice-cold D-PBS. Finally, cells were resuspended in 300 μL of ice-cold D-PBS and kept on ice in the dark. The same protocol was followed to investigate liposomes toxicity, with the only difference that cells were exposed to liposomes (0.1 mM) carrying only buffer. PI (7.5 μM) was added to cell suspensions just prior to interrogation by flow cytometry.

Flow cytometry analyses were completed in the next hour using a FACS scan cytometer (Becton Dickinson, Mountain View, CA) equipped with a 15 mW argon ion laser tuned at 488 nm. The fluorescence emitted by DHPE Oregon Green and PI was collected on a logarithmic scale in FL1 (530 ± 15 nm) and FL3 (>650 nm) channels respectively. Fluorescence emitted by the liposomal membrane marker DHPE Oregon Green and PI were simultaneously acquired to evaluate the cellular uptake of liposomes as well as determine their intracellular delivery capacities. As PI is membrane impermeable, it thus remains confined to the internal aqueous phase of liposomes until the integrity of their lipid bilayer is compromised. Moreover, this intercalating agent exhibits a strong fluorescence signal only when bound to nucleic acids. The autofluorescence of each cell line was determined with untreated cells, which were used as a control. A total of 20,000 events were recorded for each samples and single cells were gated. Fluorescence intensity distribution was analyzed with BD CellQuest software (Becton Dickinson, Mountain View, CA) and expressed as geometric mean fluorescence intensity (GMFI). Data represent the mean ± SD of three independent experiments performed in triplicates.

2.6. Confocal microscopy

The intracellular fate of liposomes was monitored by confocal microscopy. F98 and U-118 MG cells were seeded on sterile glass coverslips for 48 h. Next, the complete culture medium was renewed and cells were incubated with 0.1 mM of liposomes (200 nmoles) for 3, 6, 12 and 24 h. After the incubation intervals, samples were washed twice with warm D-PBS and fixed with 4% PFA (Electron Microscopy Sciences, Hatlfield, PA) for 20 min at 4 °C. Coverslips were then rinsed twice with ice-cold D-PBS and mounted on glass slides with VectaShield anti-fade medium (Vector Laboratories, Burlington, Canada).

Cells were examined with a FluoView FV1000 Confocal Scanning Laser Microscope (Olympus, Tokyo, Japan) coupled to an inverted microscope with a 63× oil immersion objective. DHPE Oregon Green was excited at 488 nm with a 40 mW argon laser whereas PI was excited at 543 nm with a helium–neon laser. In order to avoid spectral overlap, DHPE Oregon Green and PI fluorescence were collected sequentially. For illustration purposes, images were contrast enhanced, pseudocolored (DHPE Oregon Green in green, PI in red) and merged with the FluoView Software (Olympus, Tokyo, Japan).

2.7. Statistical analysis

Results are presented as mean ± SD and statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA). The normality of our samples was first evaluated with the d'Agostino–Pearson test. Statistical differences between treatments were analyzed using one-way ANOVA followed by a Tukey post-test and were considered significant when the p-value was < 0.05.

3. Results

3.1. Liposomes characterization by TEM

The size and morphology of liposomal formulations were determined by TEM. The mean diameter of liposomal formulations ranged from 80 ± 19 to 113 ± 29 nm (Table 1). The size distribution was relatively homogeneous and all formulations presented a similar spherical morphology (Fig. 1). Interestingly, the neutral formulation (A) and pegylated formulations (D and E) were less prone to generate cluster-like structures than unpegylated cationic formulations (B and C).

3.2. Liposomes uptake and intracellular delivery

F98 and U-118 MG glioblastoma cell lines were exposed to 0.1 mM of the five different liposomal formulations for 1, 2, 4, 6, 12 and 24 h. Cellular uptake of liposomes and cytoplasmic release of their cargo were determined by flow cytometry. For intracellular delivery analyses, both cell lines were also treated with 0.5 μM of free PI as a control.

3.2.1. Time course of liposome uptake

Fig. 2A depicts the accumulation of the liposomal formulations in F98 cells over 24 h. Unpegylated cationic formulations C and B presented the greatest uptake by F98 cells. Mean fluorescence produced by formulation C rose steeper than formulation B, and maximum values of 145.4 ± 16.7 and 94.2 ± 12.5 were respectively observed at 6 h. Prolonged (>6 h) exposure did not further increased

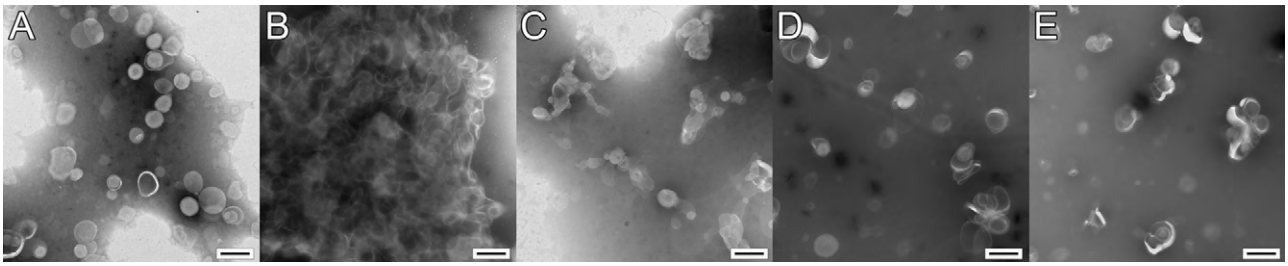

Fig. 1. Electron micrographs of liposomes. Letters correspond to the liposomal formulations detailed in Table 1. The black scale bar represents 200 nm.

the mean fluorescence of the liposomal membrane marker, rather remaining stable over 24 h for formulation C, while formulation B significantly declined from 6 to 24 h. The addition of 1 mol% (formulation D) or 5 mol% (formulation E) of DPPE-PEG_{2000} to the lipid composition of formulation C hindered cellular uptake of liposomes, more so for the formulation E (55.1 ± 18.3 vs. 14.4 ± 0.5). Interestingly, the unpegylated neutral formulation A interacted only marginally with F98 cells yielding a peak value of 7.8 ± 0.7 after 6 h.

Divergent uptake kinetics were observed with the U-118 MG cells (Fig. 2B). Formulation C still generated the highest mean fluorescence value (266.3 ± 15.0) at 6 h, but was surprisingly outperformed by formulation B at 12 h (419.3 ± 79.7). However, after 24 h of incubation, both formulations ultimately exhibited similar values (B 339.5 ± 40.7 and C 347.2 ± 49.2). The addition of PEG polymers once again severely impeded cellular uptake of liposomes (formulations D and E). Formulation D was significantly uptaken by U-118 MG cells for the first 6 h yielding a peak value of 59.9 ± 20.3, while formulation E depicted a minimal but yet significative mean fluorescence value of 7.1 ± 0.5. Cellular interactions were even more limited with formulation A (neutral unpegylated), as a maximum of 3.8 ± 0.2 was recorded after 24 h of incubation.

3.2.2. Time course studies of intracellular delivery

The mean fluorescence of F98 cells treated with PI loaded liposomes or free PI is illustrated in Fig. 2C. Formulation C significantly delivered more PI than other formulations at any time points. Mean fluorescence values increased sustainably over 24 h and a maximum of 58.0 ± 3.8 was recorded after 24 h. In addition and interestingly, formulation C uptake and intracellular delivery fluorescence signals strongly correlated during the first 6 h (Fig. 3A). Even though formulation B presented a superior uptake at 6 h (shown in Fig. 2A and C), it did not generate significantly greater PI fluorescence than pegylated formulation D. The latter formulation also reached a plateau and both eventually led to nearly identical mean fluorescence values at 24 h (B 18.1 ± 1.9 and D 17.7 ± 2.9). PI fluorescence spawned by formulation E was significantly greater than formulations B and D at both 12 and 24 h. A plateau was reached at 12 h and a peak value of 22.0 ± 0.4 was obtained at 24 h with this lipid mixture. Formulation A lent insignificant PI signals, as did free PI.

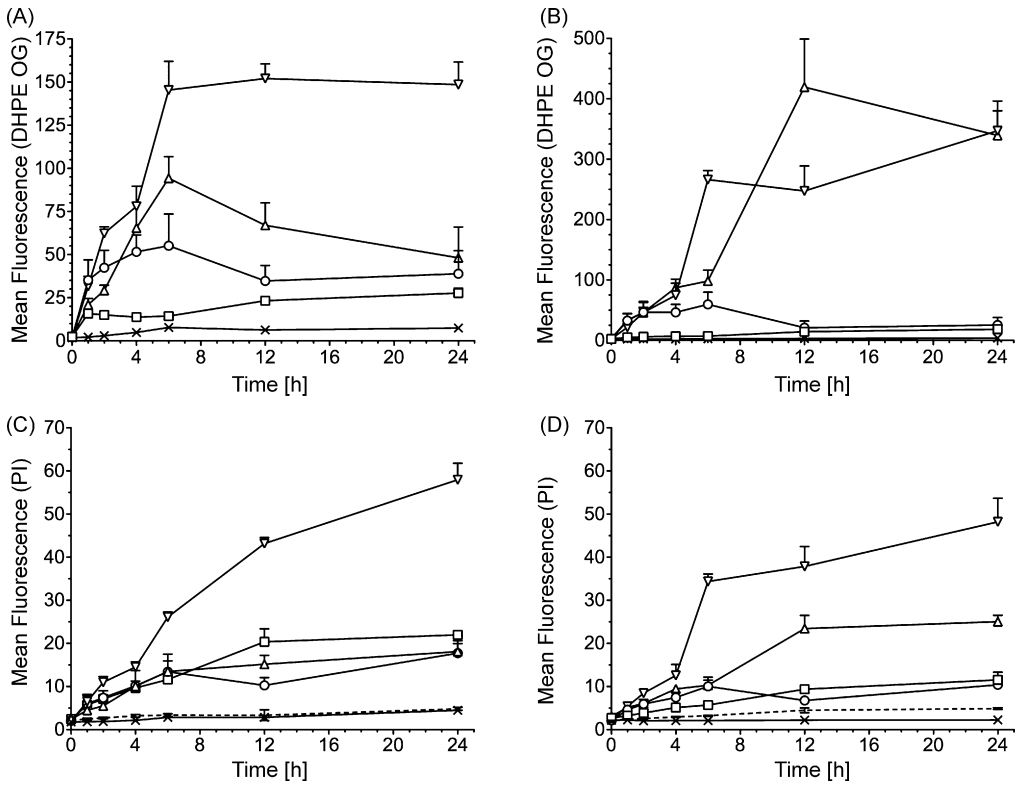

Fig. 2. Cellular uptake of fluorescent-labelled liposomes using the liposomal marker DHPE Oregon Green and consequent intracellular delivery of the intercalating dye PI in F98 (A and C) and U-118 MG (B and D) cells. Results are expressed as mean fluorescence values determined by FACS following 0, 1, 2, 4, 6, 12 and 24 h of continuous exposure to 0.1 mM of liposomal formulations A (×), B (△), C (▽), D (◯), E (□) or 0.5 μM of free PI (- - -).

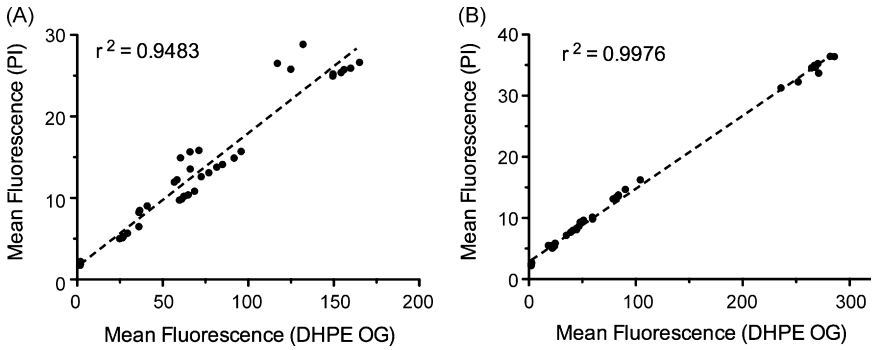

Fig. 3. Correlation between formulation C uptake and PI intracellular delivery in (A) F98 and (B) U-118 MG cells within the first 6 h of liposome exposure. Mean fluorescence values of liposomal marker DHPE Oregon Green (*x*-axis) are plotted against PI cargo values (*y*-axis).

The PI fluorescence measured in U-118 MG cells is shown in Fig. 2D. Formulation C again generated the highest PI fluorescence, reaching a mean fluorescence value of 48.2 ± 5.5 after 24 h. Likewise, cellular uptake and PI release associated fluorescence signals exhibited strong correlation over the 24 h course (first 6 h is shown in Fig. 3B). Formulation B also produced a significant PI delivery, generating a mean fluorescence of 25.1 ± 1.5 at 24 h. Formulation D depicted a gradual increase of PI signal during the first 6 h (10.0 ± 2.1), and then remained stable during the 24 h time course. The modest but constant increase of PI mean fluorescence generated by formulation E ultimately reached similar values than formulation D at both 12 (9.4 ± 0.8) and 24 h (11.5 ± 1.8). Intracellular delivery of PI by formulation A or free PI was not found significant.

3.2.3. Liposome toxicity

It is noteworthy that the liposomal formulation harboring the highest intracellular delivery rates generated very slight toxicity in F98 and U-118 MG cells, since the highest differences with controls were 3.4% and 1.3% respectively (Fig. 4). However, no cytostatic effect was observed during our experiments (data not shown).

3.3. Intracellular trafficking of liposomes

Cellular uptake and PI release of formulation C was confirmed by confocal microscopy. F98 and U-118 MG cells were exposed to 0.1 mM of formulation C for 3, 6, 12 and 24 h prior to confocal microscopic analyses. Influence of 1 mol% of the PEG-modified lipid DPPE-PEG_{2000} on intracellular release was also investigated by the same method, the only difference being that both cell lines were treated for 24 h with formulation D (0.1 mM).

Confocal photomicrographs of F98 cells exposed to formulation C are shown in Fig. 5. Both fluorescent markers colocalized near the cytoplasmic membrane at 3 h, possibly at the pericellular surface and/or in nascent endocytic or pinocytic vesicles. Liposomes were clearly internalized at 6 h (confirmed by 3D confocal image analysis, data not shown) as supported by the intracellular release of PI and by the complete lost of colocalization of both fluorescent markers. Interestingly, the liposomal tracer DHPE Oregon Green remained near the cytoplasmic membrane while PI dispersed throughout both the cytosol and the nucleus. At 12 h, DHPE Oregon Green was disposed around the nucleus in a punctate distribution while PI fluorescence was still scattered in whole cells. At the end of the 24 h incubation, both fluorescent markers generated a congregated pattern with clusters widely disseminated throughout the cytosol. Very similar patterns of fluorescence distribution were obtained with U-118 MG cells when exposed to the same concentration of liposomal formulation C (data not shown).

Fig. 6 depicts confocal micrographs of both cell lines following a 24 h incubation period with formulation D. Both fluorescent markers remained heavily colocalized at 24 h and no PI nuclear staining was observed, suggesting that 1 mol% of DPPE-PEG_{2000} completely and sustainably hindered the intracellular release from liposomes up to 24 h after initiation of exposure.

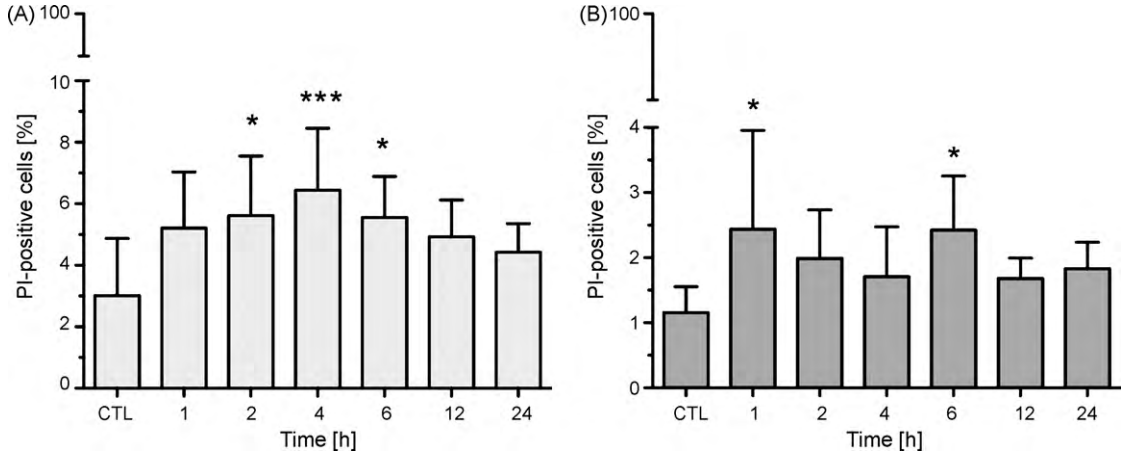

Fig. 4. Toxicity induced by liposomal formulation C (DPPC:DC-Chol:DOPE:DHPE Oregon Green). (A) F98 and (B) U-118 MG cells were exposed to formulation C for different periods and dead cells were stained with PI (7.5 μM) just prior to quantification by flow cytometry. Control (CTL) cells were not treated with formulation C. Data are represented as the mean ± SD from triplicates samples of three independent experiments. $^{*}p < 0.05$ and $^{***}p < 0.001$ versus control (CTL).

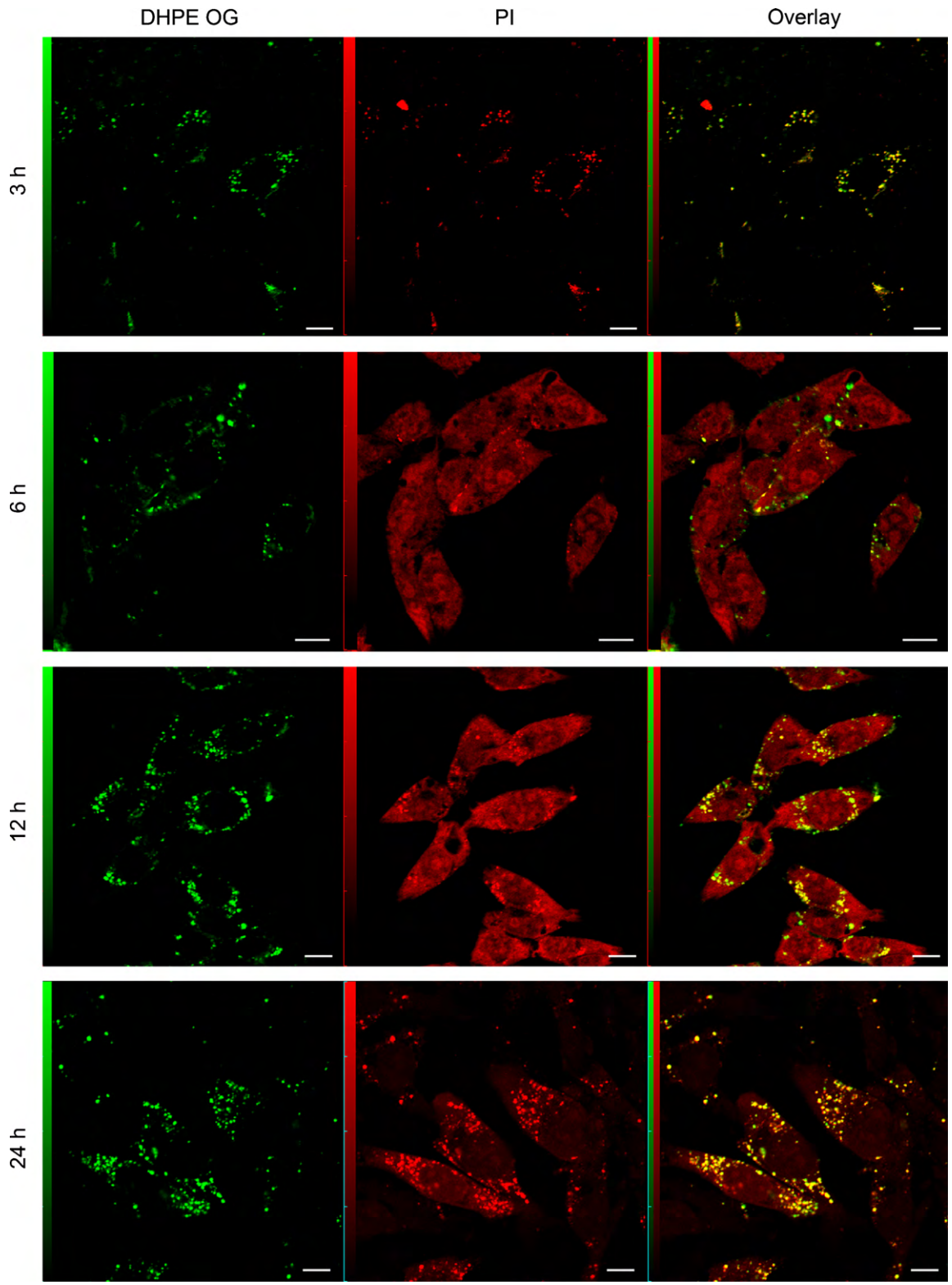

Fig. 5. Confocal photomicrographs of F98 cells exposed to formulation C liposomes for 3, 6, 12 and 24 h. At 24 h, the excitation of PI by the helium–neon laser was intentionally reduced to allow the visualization of the vesicles in which both fluorophores colocalized. Fluorescent images of liposomal membrane marker DHPE Oregon Green (DHPE OG) (left), PI cargo (middle) and overlay (right) are presented. The scale bar represents 10 μm.

4. Discussion

Liposomes represent one of the most promising systemic delivery strategies for brain tumor treatment, and have already been used for brain drug delivery as well as in various other indications (Merdan et al., 2002; Harrington et al., 2002; Garcia-Garcia et al., 2005; Huynh et al., 2006; Tiwari and Amiji, 2006). Liposomes enhance CNS bioavailability of drugs by masking their unfavorable physicochemical properties, thereby favoring BBB entry and bypassing multi-drug resistance pump systems *via* intracellular

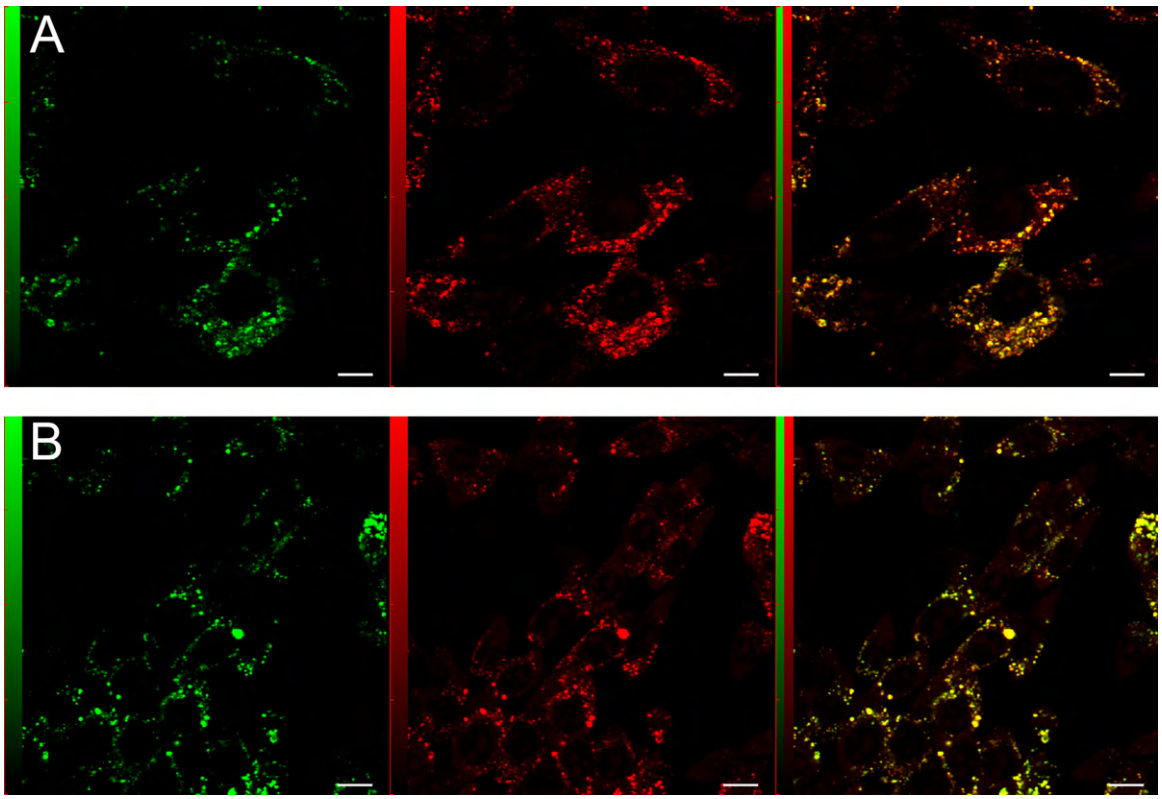

Fig. 6. Confocal photomicrographs of (A) F98 and (B) U-118 MG cells exposed to formulation D (1 mol%) for 24 h. Fluorescent images depict the intracellular distribution of the liposomal membrane marker DHPE Oregon Green (DHPE OG, left), PI cargo (middle) and overlay (right). The scale bar represents 10 μm.

release of their cargo (Drummond et al., 1999; Mamot et al., 2003; Torchilin, 2005; Sapra et al., 2005). In the scope of translational research on malignant glial tumor treatments, we aimed at developing a universal delivery vehicle to malignant brain tumor cells. We elected to work with liposomal formulations, and undertook the design of cationic formulations for this indication. Since liposomes interactions with targeted cell types is mostly dictated by the lipid composition, we investigated different lipid formulations, assessing the impact of membrane fluidity and incorporation of PEG-modified lipids on the extent of cellular capture and intracellular delivery. We retained and worked with the five liposomal formulations described in this paper (see Table 1).

We observed that cationic and unpegylated liposomes composed of DPPC:DCChol:DOPE:DHPE Oregon Green (formulation C, Table 1) were highly and rapidly internalized, and efficiently released their content in both F98 and U-118 MG glioblastoma cells. In addition, the negatively charged components of the serum contained in the culture medium (10% FBS) did not prevented effective uptake of these cationic liposomes by both cell lines, as suggested elsewhere (Nakanishi and Noguchi, 2001; Wasungu and Hoekstra, 2006). More so, insignificant toxicity was detected by flow cytometry (Fig. 4) and no cytostatic effect was observed.

In both tested GBM cell lines, our results demonstrated that cellular uptake of formulation C liposomes mostly occurred within 6 h of treatment and that PI was gradually released over 24 h (Figs. 2 and 4). Our observations were in keeping with preservation of the structural integrity of formulation C liposomes after 3 h; hence PI intracellular release was only initiated between 3 and 6 h of exposure. Likewise, a very strong correlation between uptake and intracellular release fluorescence signals prevailed, indicating that PI was efficiently released from internalized formulation C (Fig. 3). While a nearly perfect correlation was found with U-118 MG cells throughout 24 h (r^2 = 0.9902, data not shown), a strong correlation was only obtained within 6 h of treatment with F98 cells. Beyond 6 h, mean fluorescence values for DHPE Oregon Green stabilized (Fig. 2A) while those related to PI intracellular delivery still continued to increase. It is therefore tempting to speculate that liposomes uptake by F98 cells got saturated after 6 h and that subsequent intracellular release of PI from already internalized liposomes still progressively occurred over 24 h. Translated to the clinic, this result would suggest that formulation C liposomes must remain for 6 h in the GBM cells surroundings to produce a maximal uptake. Liposomal content is thereafter gradually released in the cytosol for at least 24 h which should considerably enhance the potency of chemotherapeutics agents, particularly those that are cell-cycle specific.

As PI is an intercalating agent not permeable to phospholipid membranes, it requires the destabilization of both liposomes and endosomes bilayer membrane to interact with nucleic acids in cells. Its intracellular release can thus be easily confirmed by confocal microscopy, as shown in Fig. 5. Confocal imaging also revealed that liposomes were internalized in punctate aggregates, which traveled from the cytoplasmic membrane to the perinuclear area within the first 12 h. However, after 24 h, this punctate distribution pattern was dispersed more evenly in the cytosol and a colocalization with PI was obtained. As endocytosis is the most characterized and common entry mechanism of liposomes in a variety of cells (Bartsch et al., 2005; Torchilin, 2005; Huth et al., 2006; Wasungu and Hoekstra, 2006), these dots might thus represent endosomes, supporting endocytosis as the internalization pathway for formulation C. On the other hand, this wide distribution of punctate aggregates may be the result of ongoing liposome uptake and/or the translocation of DHPE Oregon Green from liposomal to endosomal membranes and its ensuing distribution in organelles. As for the cargo-associated fluorescence, it was found particularly bright in punctate vesicles, colocalizing with DHPE Oregon Green, but also

throughout the cytosol and the nucleus. The former phenomenon might correspond to cells attempting to expel intracellular PI *via* the exocytic pathway. Experiments using endocytosis inhibitors are underway to characterize the specific pathways involved in formulation C uptake by GBM cells.

In this study, we also showed that the reduction of liposome membrane fluidity (formulation B), the addition of PEG-modified lipids (formulations D and E) and the withdrawal of the cationic charge and pH-sensitive lipid components (formulation A) by altering the lipid composition of formulation C increasingly impaired liposomes uptake and intracellular release of their cargo in the two GBM cell lines tested. It is well acknowledged that liposome stability and retention capacity are inversely related to bilayer membrane fluidity (Drummond et al., 1999; Lian and Ho, 2001; Waterhouse et al., 2005; Hart, 2005; Kobayashi et al., 2007). Therefore, the greater membrane stability of DSPC containing liposomes (formulation B) may have impaired the intracellular destabilization of vesicles, and thus, the capacity of the liposomal carriers to release their content. The membrane of formulation C liposomes was rigidified by the substitution of the main phospholipid component DPPC for DSPC since the longer acyl chains (18 vs. 16 carbons tails) of the latter strengthen membrane cohesion (formulation B) (Waterhouse et al., 2005; Hart, 2005). This lipid permutation had divergent effects on liposomes uptake by F98 and U-118 MG cells but clearly hindered PI release from liposomes in both cell lines ($p < 0.001$ at 2 h and beyond), as can be appreciated in Fig. 2. As a result, formulation B uptake was significantly reduced ($p < 0.001$ at 6 h and beyond) in F98 cells whereas it was only delayed in U-118 MG cells. As seen with the latter, the mean fluorescence peak was recorded at 12 h instead of 6 h and comparable fluorescence values were recorded at 24 h.

PEG-modified lipids are commonly incorporated in liposomes since they considerably extend the half-life of liposomes in the systemic circulation, virtually rendering them stealth (Sapra et al., 2005; Tiwari and Amiji, 2006; Wasungu and Hoekstra, 2006; Huwyler et al., 2008). The addition of either 1 or 5 mol% of DPPE-PEG_{2000} to formulation C lipid composition hampered liposomes uptake and intracellular PI release in both cell lines (formations D and E, Fig. 2). Our results thus support the increasing evidence that these stabilizing polymers interfere with both cellular uptake and inhibit endosomal escape of liposomes content in various cancer cell lines, such as glioma cell lines *in vitro* (Shi et al., 2002; Wasungu and Hoekstra, 2006; Kobayashi et al., 2007). As previously reported by Shi and colleagues (Shi et al., 2002), we observed that liposomes uptake was reduced in a PEG-dependent manner during the active uptake phase ($\leq$6 h, Fig. 2A and B). Formulation D (1 mol%) and E (5 mol%) however depicted similar PI fluorescence levels in both cell lines despite greater uptake of formulation D (mainly during the first 6 h, Fig. 2A and C). The addition of only 1 mol% of DPPE-PEG_{2000} drastically impaired the intracellular release of liposomes cargo over 24 h in both cell lines (Fig. 6). Although more PI was introduced in cells by formulation D than E, comparable collected fluorescence signals for these two pegylated formulations may thus imply that the addition of only 1 mol% of DPPE-PEG_{2000} is sufficient to induce a complete paralysis of intracellular liposomal delivery. In fact, the weak fluorescence emitted by unbound PI (still trapped in pegylated liposomes) fluctuates more subtly according to the amount introduced in treated cells. A significant difference in PI fluorescence levels between formulation D and E was observed at 12 ($p < 0.001$) and 24 h ($p < 0.001$) in F98 cells, with a higher PI signal maintained in formulation E. Although this unexpected result deserves further investigation, we hypothesize that, owing to its higher PEG–lipid content, formulation E might simply better resist cellular degradation than D over time, thus allowing less PI elimination at 12 and 24 h in both cell lines. Inhibitory effect of PEG-modified lipids on cellular uptake of liposomes and especially on intracellular release of their content might explain why the free antineoplastic drugs yield higher cytoxicity than pegylated liposomal forms in studies comparing the efficiency of different formulations (Sharma et al., 1997; Kobayashi et al., 2007; Charest et al., 2009).

Finally, the abrogation of cationic and pH-sensitive lipids was the most damaging manipulation as the unpegylated and neutral formulation A interacted very poorly with both GBM cell lines. Indeed, very little uptake occurred with F98 cells whereas no DHPE Oregon Green-related signal was detected in U-118 MG cells (Fig. 2). Likewise, the cellular uptake of neutral liposomes was so low that we barely could monitor the intracellular release of their PI cargo in treated cells (Fig. 2). This suggests that a cationic lipid component such as DC-Chol, coupled to the pH-sensitive lipid, DOPE, are beneficial components for efficient interaction with glioblastoma cells *in vitro*, which is consistent with the superior uptake of cationic liposomes containing DOPE in eukaryotic cells (Bailey and Cullis, 1997; Hart, 2005; Medina-Kauwe et al., 2005; Hoekstra et al., 2007). The mediocre performance of the neutral and pH-insenstive formulation A emphasizes the need to develop liposomal formulations specifically intended for GBM therapy.

The present work highlights the fact that lipid composition of liposomes must be carefully adjusted for optimal capture by glioma cells and the subsequent intracellular release of their content. A cationic and unpegylated liposomal formulation composed of DPPC:DCChol: DOPE:DHPE Oregon Green efficiently delivered its cargo to murine F98 and human U-118 MG glioblastoma cell lines *in vitro*. While incorporation of cationic lipids is beneficial, reduction of the membrane fluidity or incorporation of 1 or 5 mol% of PEG-modified lipids to this formulation rather impaired its cellular uptake and endosomal release in these glioma cell lines. However, the use of such a liposomal formulation may prove to be challenging *in vivo*. Because of their global positive charge, cationic liposomes are especially prone to unspecific interactions as well as destabilization by plasma proteins and capture by the phagocytic cells of the reticuloendothelial system (RES) (Nakanishi and Noguchi, 2001; Wasungu and Hoekstra, 2006). Hence, unpeguylated and cationic liposomes undergo prompt elimination rendering their short *in vivo* half-life less than optimal for cancer therapy. Thus the use of stabilizing PEG-modified lipids typically serves the purpose of extending the half-life of liposomes *in vivo*, unfortunately at the expense of endosomal escape.

We hereby propose a novel administration paradigm, adapted for malignant brain tumors, which might render PEG-modified lipids dispensable for cationic liposomes. While, the unspecific interactions as well as liposomes destabilization by plasma proteins did not prevent cellular uptake and internalization of our cationic unpegylated liposomes (10% FBS in culture media), the RES-dependent elimination still represents a major issue *in vivo*. Accordingly, we intend to administer formulation C liposomes *via* an intra-arterial cerebral infusion following the osmotic or pharmacological transient opening of the BBB. Unpegylated cationic liposomes would thereby reach malignant primary brain tumor vasculature first, prior to hepatic first-pass where most of the elimination by the RES occurs. Permeabilization of the BBB should maximize the extravasation of liposomes to the CNS, where they would be more likely to interact with malignant glial cells (hopefully >6 h) to delivery their antineoplastic cargo over a sustained period even if the BBB resume a normal function. *In vivo* studies are currently underway to characterize the opening of the BBB and assess the CNS bioavailability of such carriers in the tumor-bearing F98 Fisher model (Blanchette et al., 2009; Mathieu et al., 2007). We intend to deploy this treatment paradigm to further improve primary brain tumor therapy.

Acknowledgments

This work was supported by the National Bank research chair on the treatment of brain tumors and by a grant from the Canadian Institute of Health Research (CIHR application number 160075). We thank Wisent Bioproducts for generously providing all cell culture reagents. We are also grateful to Leonid Volkov for discussions and its excellent technical assistance for flow cytometry and confocal microscopy.

References

Abbott, N., Ronnback, L., Hansson, E., 2006. Astrocyte–endothelial interactions at the blood–brain barrier. Nat. Rev. Neurosci. 7, 41–53.
Bailey, A.L., Cullis, P.R., 1997. Membrane fusion with cationic liposomes: effects of target membrane lipid composition. Biochemistry 36, 1628–1634.
Bangham, A.D., Standish, M.M., Watkins, J.C., 1965. Diffusion of univalent ions across the lamellae of swollen phospholipids. J. Mol. Biol. 13, 238–252.
Barth, R.F., Kaur, B., 2009. Rat brain tumor models in experimental neuro-oncology: the C6, 9L, T9, RG2, F98, BT4C, RT-2 and CNS-1 gliomas. J. Neurooncol. 94, 299–312.
Bartsch, M., Weeke-Klimp, A.H., Meijer, D.K.F., Scherphof, G.L., Kamps, J.A.A.M., 2005. Cell-specific targeting of lipid-based carriers for ODN and DNA. J. Liposome Res. 15, 59–92.
Begley, D.J., 2004. Delivery of therapeutic agents to the central nervous system: the problems and the possibilities. Pharmacol. Ther. 104, 29–45.
Béduneau, A., Saulnier, P., Benoit, J.-P., 2007. Active targeting of brain tumors using nanocarriers. Biomaterials 28, 4947–4967.
Blanchette, M., Pellerin, M., Tremblay, L., Lepage, M., Fortin, D., 2009. Real-time monitoring of gadolinium diethylenetriamine penta-acetic acid during osmotic blood-brain barrier disruption using magnetic resonance imaging in normal wistar rats. Neurosurgery 65, 344–350.
de Boer, A., Gaillard, P., 2007. Drug targeting to the brain. Annu. Rev. Pharmacol. Toxicol. 47, 323–355.
Bondy, M.L., Scheurer, M.E., Malmer, B., Barnholtz-Sloan, J.S., Davis, F.G., Il'yasova, D., Kruchko, C., McCarthy, B.J., Rajaraman, P., Schwartzbaum, J.A., Sadetzki, S., Schlehofer, B., Tihan, T., Wiemels, J.L., Wrensch, M., Buffler, P.A., Consortium, B.T.E., 2008. Brain tumor epidemiology: consensus from the Brain Tumor Epidemiology Consortium. Cancer 113, 1953–1968.
Brasnjevic, I., Steinbusch, H.W.M., Schmitz, C., Martinez-Martinez, P., Initiative, E.N.R., 2009. Delivery of peptide and protein drugs over the blood–brain barrier. Prog. Neurobiol. 87, 212–251.
Calatozzolo, C., Gelati, M., Ciusani, E., Sciacca, F.L., Pollo, B., Cajola, L., Marras, C., Silvani, A., Vitellaro-Zuccarello, L., Croci, D., Boiardi, A., Salmaggi, A., 2005. Expression of drug resistance proteins Pgp, MRP1, MRP3, MRP5 and GST-pi in human glioma. J. Neurooncol. 74, 113–121.
Cecchelli, R., Berezowski, V., Lundquist, S., Culot, M., Renftel, M., Dehouck, M.-P., Fenart, L., 2007. Modelling of the blood–brain barrier in drug discovery and development. Nat. Rev. Drug Discov. 6, 650–661.
Charest, G., Paquette, B., Fortin, D., Mathieu, D., Sanche, L., 2009. Concomitant treatment of F98 glioma cells with new liposomal platinum compounds and ionizing radiation. J. Neurooncol. 87, 187–193.
Chen, Y., Dalwadi, G., Benson, H.A.E., 2004. Drug delivery across the blood–brain barrier. Curr. Drug Deliv. 1, 361–376.
Claes, A., Idema, A.J., Wesseling, P., 2007. Diffuse glioma growth: a guerilla war. Acta Neuropathol. 114, 443–458.
Correale, J., Villa, A., 2009. Cellular elements of the blood–brain barrier. Neurochem. Res. 34, 2067–2077.
DeAngelis, L.M., 2001. Brain tumors. N. Engl. J. Med. 344, 114–123.
Deeken, J., Loscher, W., 2007. The blood–brain barrier and cancer: transporters, treatment, and Trojan horses. Clin. Cancer Res. 13, 1663–1674.
Drummond, D.C., Meyer, O., Hong, K., Kirpotin, D.B., Papahadjopoulos, D., 1999. Optimizing liposomes for delivery of chemotherapeutic agents to solid tumors. Pharmacol. Rev. 51, 691–743.
Fenske, D.B., Cullis, P.R., 2008. Liposomal nanomedicines. Expert Opin. Drug Deliv. 5, 25–44.
Garcia-Garcia, E., Andrieux, K., Gil, S., Couvreur, P., 2005. Colloidal carriers and blood–brain barrier (BBB) translocation: a way to deliver drugs to the brain? Int. J. Pharm. 298, 274–292.
Glavinas, H., Krajcsi, P., Cserepes, J., Sarkadi, B., 2004. The role of ABC transporters in drug resistance, metabolism and toxicity. Curr. Drug Deliv. 1, 27–42.
Harrington, K.J., Syrigos, K.N., Vile, R.G., 2002. Liposomally targeted cytotoxic drugs for the treatment of cancer. J. Pharm. Pharmacol. 54, 1573–1600.
Hart, S.L., 2005. Lipid carriers for gene therapy. Curr. Drug Deliv. 2, 423–428.
Henson, J.W., Cordon-Cardo, C., Posner, J.B., 1992. P-glycoprotein expression in brain tumors. J. Neurooncol. 14, 37–43.
Hoekstra, D., Rejman, J., Wasungu, L., Shi, F., Zuhorn, I., 2007. Gene delivery by cationic lipids: in and out of an endosome. Biochem. Soc. Trans. 35, 68–71.
Huth, U.S., Schubert, R., Peschka-Süss, R., 2006. Investigating the uptake and intracellular fate of pH-sensitive liposomes by flow cytometry and spectral bio-imaging. J. Control. Release 110, 490–504.
Huwyler, J., Drewe, J., Krähenbühl, S., 2008. Tumor targeting using liposomal antineoplastic drugs. Int. J. Nanomed. 3, 21–29.
Huynh, G.H., Deen, D.F., Szoka, F.C., 2006. Barriers to carrier mediated drug and gene delivery to brain tumors. J. Control. Release 110, 236–259.
Kemper, E., Boogerd, W., Thuis, I., Beijnen, J., Tellingen, O.V., 2004. Modulation of the blood–brain barrier in oncology: therapeutic opportunities for the treatment of brain tumours? Cancer Treat. Rev. 30, 415–423.
Kobayashi, T., Ishida, T., Okada, Y., Ise, S., Harashima, H., Kiwada, H., 2007. Effect of transferrin receptor-targeted liposomal doxorubicin in P-glycoprotein-mediated drug resistant tumor cells. Int. J. Pharm. 329, 94–102.
Koo, Y.-E.L., Reddy, G.R., Bhojani, M., Schneider, R., Philbert, M.A., Rehemtulla, A., Ross, B.D., Kopelman, R., 2006. Brain cancer diagnosis and therapy with nanoplatforms. Adv. Drug Deliv. Rev. 58, 1556–1577.
Langner, M., Kral, T.E., 1999. Liposome-based drug delivery systems. Pol. J. Pharmacol. 51, 211–222.
Lian, T., Ho, R.J., 2001. Trends and developments in liposome drug delivery systems. J. Pharm. Sci. 90, 667–680.
Löscher, W., Potschka, H., 2005. Drug resistance in brain diseases and the role of drug efflux transporters. Nat. Rev. Neurosci. 6, 591–602.
Mamot, C., Drummond, D.C., Hong, K., Kirpotin, D.B., Park, J.W., 2003. Liposome-based approaches to overcome anticancer drug resistance. Drug Resist. Updat. 6, 271–279.
Mathieu, D., Lecomte, R., Tsanaclis, A.M., Larouche, A., Fortin, D., 2007. Standardization and detailed characterization of the syngeneic Fischer/F98 glioma model. Can. J. Neurol. Sci. 34, 296–306.
Medina-Kauwe, L.K., Xie, J., Hamm-Alvarez, S., 2005. Intracellular trafficking of non-viral vectors. Gene Ther. 12, 1734–1751.
Merdan, T., Kopecek, J., Kissel, T., 2002. Prospects for cationic polymers in gene and oligonucleotide therapy against cancer. Adv. Drug Deliv. Rev. 54, 715–758.
Nakada, M., Nakada, S., Demuth, T., Tran, N.L., Hoelzinger, D.B., Berens, M.E., 2007. Molecular targets of glioma invasion. Cell. Mol. Life Sci. 64, 458–478.
Nakanishi, M., Noguchi, A., 2001. Confocal and probe microscopy to study gene transfection mediated by cationic liposomes with a cationic cholesterol derivative. Adv. Drug Deliv. Rev. 52, 197–207.
O'Byrne, K.J., Thomas, A.L., Sharma, R.A., DeCatris, M., Shields, F., Beare, S., Steward, W.P., 2002. A phase I dose-escalating study of DaunoXome, liposomal daunorubicin, in metastatic breast cancer. Br. J. Cancer 87, 15–20.
Pardridge, W., 2005. The blood–brain barrier: bottleneck in brain drug development. NeuroRx 2, 3–14.
Pardridge, W.M., 2007. Drug targeting to the brain. Pharm. Res. 24, 1733–1744.
Pinto-Alphandary, H., Andremont, A., Couvreur, P., 2000. Targeted delivery of antibiotics using liposomes and nanoparticles: research and applications. Int. J. Antimicrob. Agents 13, 155–168.
Rich, J.N., Bigner, D.D., 2004. Development of novel targeted therapies in the treatment of malignant glioma. Nat. Rev. Drug Discov. 3, 430–446.
Salvati, A., Ciani, L., Ristori, S., Martini, G., Masi, A., Arcangeli, A., 2006. Physicochemical characterization and transfection efficacy of cationic liposomes containing the pEGFP plasmid. Biophys. Chem. 121, 21–29.
Sapra, P., Tyagi, P., Allen, T.M., 2005. Ligand-targeted liposomes for cancer treatment. Curr. Drug Deliv. 2, 369–381.
Sharma, U.S., Sharma, A., Chau, R.I., Straubinger, R.M., 1997. Liposome-mediated therapy of intracranial brain tumors in a rat model. Pharm. Res. 14, 992–998.
Shi, F., Wasungu, L., Nomden, A., Stuart, M.C., Polushkin, E., Engberts, J.B., Hoekstra, D., 2002. Interference of poly(ethylene glycol)–lipid analogues with cationic-lipid-mediated delivery of oligonucleotides; role of lipid exchangeability and non-lamellar transitions. Biochem. J. 366, 333–341.
Stupp, R., Mason, W.P., Bent, M.J.V.D., Weller, M., Fisher, B., Taphoorn, M.J.B., Belanger, K., Brandes, A.A., Marosi, C., Bogdahn, U., Curschmann, J., Janzer, R.C., Ludwin, S.K., Gorlia, T., Allgeier, A., Lacombe, D., Cairncross, J.G., Eisenhauer, E., Mirimanoff, R.O., European Organisation for Research and Treatment of Cancer Brain Tumor and Radiotherapy Groups, National Cancer Institute of Canada Clinical Trials Group, 2005. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N. Engl. J. Med. 352, 987–996.
Szakács, G., Paterson, J.K., Ludwig, J.A., Booth-Genthe, C., Gottesman, M.M., 2006. Targeting multidrug resistance in cancer. Nat. Rev. Drug Discov. 5, 219–234.
Tiwari, S.B., Amiji, M.M., 2006. A review of nanocarrier-based CNS delivery systems. Curr. Drug Deliv. 3, 219–232.
Torchilin, V.P., 2005. Recent advances with liposomes as pharmaceutical carriers. Nat. Rev. Drug Discov. 4, 145–160.
Uchiyama, K., Nagayasu, A., Yamagiwa, Y., Nishida, T., Harashima, H., Kiwada, H., 1995. Effects of the size and fluidity of liposomes on their accumulation in tumors: a presumption of their interaction with tumors. Int. J. Pharm. 121, 195–203.
Vescovi, A.L., Galli, R., Reynolds, B.A., 2006. Brain tumour stem cells. Nat. Rev. Cancer 6, 425–436.
Wasungu, L., Hoekstra, D., 2006. Cationic lipids, lipoplexes and intracellular delivery of genes. J. Control. Release 116, 255–264.
Waterhouse, D.N., Madden, T.D., Cullis, P.R., Bally, M.B., Mayer, L.D., Webb, M.S., 2005. Preparation, characterization, and biological analysis of liposomal formulations of vincristine. Methods Enzymol. 391, 40–57.
Wen, P.Y., Kesari, S., 2008. Malignant gliomas in adults. N. Engl. J. Med. 359, 492–507.
Wolburg, H., Lippoldt, A., 2002. Tight junctions of the blood–brain barrier: development, composition and regulation. Vascul. Pharmacol. 38, 323–337.
Xu, Y., Szoka, F.C., 1996. Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. Biochemistry 35, 5616–5623.