Dissertation Approved By

Date: 8/3/2018

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MULTIFUNCTIONAL LIPOSOMAL CARRIERS ENCAPSULATING TEMOZOLEMIDE, RG7388 AND ELACRIDAR FOR TARGETED GLIOBLASTOMA MULTIFORME THERAPY

By

TASNEEM ARSIWALA

A Thesis

Submitted to the faculty of the Graduate School of the Creighton University in Partial Fulfillment of the Requirements for the degree of Master of Science in the Department of Pharmaceutical Sciences

Omaha, NE

[July 22, 2018]
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ABSTRACT

Glioblastoma multiforme is a grade IV brain tumour that is highly malignant and has a very low patient survival rate. A hurdle in glioblastoma therapy is bypassing the blood brain barrier. Thus, this study was aimed at designing and comparing two multifunctional liposomal therapies for the delivery of multiple agents to address limitations in therapy like tumour resistance and efflux through the simultaneous delivery of temozolomide, RG7388 and elacridar in a single nano-carrier system. Two liposomal systems were characterized including a multilamellar system and nanoparticles in liposome system (PLL) were designed for this purpose.

The liposomes were surface modified by a folic acid conjugate for the tumour cell targeting. An HPLC technique was developed and validated for the simultaneous detection and quantification of temozolomide, elacridar and RG7388 and it was found to obey USP validation parameters for specificity, linearity, accuracy and precision. This method was applied for the determination of encapsulation efficiency, in vitro release, cell uptake and permeability studies. The folic acid conjugate was successfully characterized for its purity with a high yield of 89.6 \pm 4.91\% and the folic acid content was 86.5 \pm 2.63\%w/w. All liposomal systems were found to be below 200 nm with a negative charge. The effect of freeze thaw cycles on liposome bilayer characteristics was tested and it was found that increasing freeze thaw cycles to unilamellar vesicles caused an increase in the liposomal bilayer. The encapsulation efficiency for the surface modified multilamellar liposomes was found to be was found to be approximately 12, 47 and 86\%w/w for RG7388, TMZ and elacridar respectively, whereas it was 33, 37
and 79\% w/w respectively for the surface modified PLL systems. Sustained release was observed from the PLL system as compared to the multilamellar liposomal system.

*In vitro* characterization for cellular toxicity in BBB and glioblastoma models revealed that the liposomes showed lower toxicity in MDCK cells, while the toxicity for U87 and GBM 108 cells for the liposomal systems was higher than that of the drug solutions. Higher uptake for the folate decorated liposomes was seen in the U87 and GBM108 cell lines. The permeability of the systems was affected with the TEER values for the MDCK monolayer and in most cases, higher permeability was observed for the folate decorated liposomes upon prolonged treatment exposure.

Thus, the liposomal systems offer a unique carrier system for brain delivery of drugs, specifically for targeting glioblastoma. Additionally, sustained release can be achieved using PLGA nanoparticle in liposomes. However, the *in vitro* results need to be investigated *in vivo*. 
PREFACE

Poster presented in regional/national/international conferences:


2. Arsiwala T, Dash A.K., ‘Design and characterization of an optimized liposomal delivery system for the treatment of glioblastoma multiforme’ St Albert’s Science Day, Creighton University, Omaha, NE, USA 2018

Poster submitted:


Presented in national/international conferences:

Liposomes: Bringing the fight to brain tumors. Regional finalist, 3 MT thesis, annual Midwest Association for Graduate Schools (MAGS), April 2018, Grand Rapids, MI
Dedicated to my parents Abbas and Dilshad Arsiwala and

my brother Aliasgar Arsiwalla
ACKNOWLEDGEMENT

I would like to express my gratitude to Dr. Alekha K Dash who has been my advisor and guide throughout my journey at Creighton University. His supportive mentoring has brought many remarkable opportunities for me and has helped me develop a critical research mindset that will help me achieve my future goals. I am grateful to Dr. Jeff North, Dr. Somnath Singh and Dr. Anthony Kincaid who have helped me mold my project with their invaluable suggestions and critiques. Their help has helped me gain a greater scientific research understanding. Our research would not be possible without the opportunities provided by School of Pharmacy and health professions at Creighton University.

I am thankful to the pharmacy sciences faculty and staff for their guidance. A special thanks to Dawn Trojanowski and Dan Munt who have always helped me with any difficulties throughout my time at Creighton, with great patience and understanding. I would also like to thank Dr. Shah from the university of Nebraska at Lincoln, Dr. Zhang and Dr. Chauhan for their help with the XRD studies. I appreciate all the help from Dr. Sarkaria from the Mayo clinic and Dr. Elmquist from the university of Minnesota for helping us understand glioblastoma and providing the primary cells. I would also like to thank Dr. Igor Meerovich, Dr. Subra, Dr. Pavan and Susmita for always helping me with inputs in the lab. My journey would be incomplete without the support of my friends specifically Amreen, Prajakta, Pooja, Aayushi, Rachna, Shambhavi and Deepal who have always been by my side.

I would like to thank my parents for providing me with this opportunity and constant support. This journey would not be possible without their encouragement and
love. Lastly, I cannot thank my brother enough for being a constant inspiration and his unshakeable belief in me.
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<td>GBM</td>
<td>Glioblastoma Multiforme</td>
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<td>BBB</td>
<td>Blood brain barrier</td>
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<td>TMZ</td>
<td>Temozolomide</td>
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<td>RG7388</td>
<td>Idasanutlin</td>
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<td>ACN</td>
<td>Acetonitrile</td>
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<tr>
<td>MLL</td>
<td>Multilamellar liposomes</td>
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<td>DMEM</td>
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<td>TEER</td>
<td>Transepithelial/transendothelial electrical resistance</td>
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<td>MDCK</td>
<td>Madin Darby Canine kidney cells</td>
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<td>MDM2</td>
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<td>P-gp</td>
<td>P-glycoprotein</td>
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<td>MDRP</td>
<td>Multi-drug resistant protein</td>
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<td>PLGA</td>
<td>Poly lactic-co-glycolic acid</td>
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<td>MRI</td>
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<td>PET</td>
<td>Positron emission tomography</td>
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<td>CT</td>
<td>Computed tomography</td>
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<td>BCNU</td>
<td>Carmustine</td>
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<tr>
<td>MDRP</td>
<td>Multi drug resistant protein</td>
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<tr>
<td>BCRP</td>
<td>Breast cancer resistant protein</td>
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<tr>
<td>BMEC</td>
<td>Brain microvasculature endothelial cells</td>
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<tr>
<td>CED</td>
<td>Convection enhanced delivery</td>
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<td>AML</td>
<td>Acute myeloid leukemia</td>
</tr>
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<td>RP</td>
<td>Reverse phase</td>
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<td>ICH</td>
<td>International Council for Harmonisation</td>
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<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
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<td>Ultraviolet</td>
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Chapter 1: Introduction
1.1 Glioblastoma Multiforme

Glioblastoma Multiforme (GBM) is a malignant grade IV brain tumor that arises from astrocytes or glial precursor cells. GBM is the most common malignancy of the CNS (46.6%) and it accounts for nearly 55% of all observed primary brain gliomas as seen in figure 1 (1). It is a primary tumor that originates in the brain and spinal cord and is inoperable and fatal in the majority of cases. Although extensive therapy is available, the aggressive pathogenesis, biological mechanisms and molecular profiles of these tumors have limited the efficacy of treatments.

![Figure 1: Distribution of primary brain CNS and brain tumors](image)

GBM may be further classified as per the World Health Organization (WHO) into primary and secondary tumors. This classification is based on the origin of the tumor, where primary GBM are those tumors that may develop de novo due to factors like genetic mutation rather than the advance of a malignant precursor, while secondary
GBM arises from the slow progression of low grade gliomas like astrocytoma. These tumors are distinct and have characteristic features such as high mitotic activity and proliferation of the vasculature with a wide variance of the morphology of the tumor cells. Despite surgery, radiotherapy and chemotherapy, the survival prognosis of a patient suffering from GBM is only about 12 to 15 months (2). The relatively ineffective treatments have promoted the development of novel therapeutics and unique techniques of drug delivery.

1.1.1 Epidemiology

GBM is a relatively rare tumor with an incidence of about 10 per 100,000 people in the United States (3). Primary GBM predominantly occurs in older patient populations while secondary GBM occurs mostly in the younger patient populations (5). In most cases, patients younger than 40 years have a significantly better prognosis than older ones (5).

The occurrence of GBM is usually sporadic and there are no known specific risk factors in large populations. The incidence of GBM usually peaks at the age of 75-84 years (4) and according to the 2017 issue of the Central Brain Tumor Registry of the United States (CBTRUS) report, the occurrence of GBM is significantly higher in men as compared to women with an incidence rate of 3.99 and 2.52 respectively (5). The highest incidence is observed in Caucasian population; being twice as high as the incidence in the African American population (5). The relative rate of survival of a patient suffering is extremely low, with only 5.5% patients surviving 5 years once diagnosed with GBM (5).
1.1.2 Characteristic features of GBM

GBM tumors are heterogenous and usually consist of irregularly shaped cells, with polymorphic nuclei, increased mitotic activity and necrosis of the bordering cells (6). The cells of GBM may vary in their appearance from region to region, however they rarely metastasize. GBM tumors present as an anaplastic astrocytoma with small spindle shaped or polygonal cells that may not have distinct cell borders (7). A typical three-dimension model of GBM is shown in figure 2.

Figure 2: Three-layer model of cells of glioblastoma (8)

A characteristic feature of GBM is extensive angiogenesis which occurs via the hypoxia dependent formation of new branched capillaries from existing vessels (9). Necrosis observed in GBM is unique and often seen in two forms. Necrotic foci may be present as localized large areas seen centrally in the tumor mass, or irregularly
shaped extensions of dying tissue radially surrounded by living tissue (10). While the former type of necrosis is seen mostly in primary GBM, the latter is observed in both primary and secondary GBM (9).

1.1.3 Pathogenesis of the primary and secondary GBM

The development of both primary and secondary GBM follow sequential genetic pathways shown in figure 3 (7). Following the loss of heterozygosity on chromosome 10 (LOH 10q) from glial progenitor cells, primary glioblastoma commonly shows amplification of an epidermal growth factor receptor (EGFR) that promotes tumorigenic cell proliferation and prevents apoptosis. This is followed by mutation of the phosphatase and tensin homolog (PTEN) which is the tumor suppressor gene (11). In secondary GBM TP53 (tumor suppressor protein), a sequence specific transcription protein, undergoes early mutation in the development of the tumor. Once activated by stress, it in turn activates genes responsible for alternative sequences for cell apoptosis, cell cycle control and senescence.

The activity of the TP53 may be impaired by the MDM2 gene (mouse double minute 2). The binding of MDM2 with TP53 at a DNA binding site leads to a complex formation which prevents its pro-apoptotic activity. Additionally, wild-type TP53 which is the form present in most tumors, induces the transcription of MDM2 (11). Autoregulation causes the increase in TP53 to induce MDM2 expression, leading to anti-TP53 action. While MDM2 amplification is a regular occurrence for primary GBM, TP53 gene inactivation is typical for secondary GBM. The detection of these alterations in genes and understanding their role in the malignancy transformation is essential to understand the migration and invasion of the gliomas into the surrounding
tissues. Secondary GBM show over-expression of platelet derived growth factor, which is a mitogen for the glial cells consisting of two cell surface receptors type, PDGFR-a and PDGFR-b. The mutation of the p53 system that occurs in the secondary GBM makes them much more difficult to treat.

Figure 3: Difference between the pathogenesis of a primary and secondary GBM.

1.1.4 In vitro GBM models

The use of cell culture as in vitro models for cancer research has not only led to a better understanding of tumor environment and biology, but also allowed for the investigation of therapeutic action through high-throughput screening (12). Certain cell lines like the U87MG, U251, T98G and C6 glioma models have been used as GBM in vitro models for over 3 decades. However, these models suffer from certain limitations including serum media-induced changes in genomics and transcriptomes which depletes tumor stem cells (13), failure of development of specific GBM features like microvascular proliferation, and diffuse infiltration and a lack of clinical
characterization of the formed tumors for patient parameter correlation (14). To overcome the shortcomings, patient-derived primary xenografts have been established as shown in figure 4. Genetic and phenotypic features of primary and secondary GBM-like EGFR amplification and MGMT promoter status are retained in these lines by sequential transplantation of these cells in mice (15).

Figure 4: Orthotopic model of GBM cell implantation from patient derived cells

1.1.5 Detection and diagnosis

GBM is usually diagnosed as the disease progresses and the tumor exhibits its more aggressive state and one or more of its characteristic features. The spread of necrosis is characteristically utilized as a tool for GBM diagnosis since it is directly related to the extent of aggressiveness of tumor cells. The diagnosis of GBM depends on the exact location and size of the tumor mass and its integration into the surrounding brain structures. For example, presence of unilaterally focused headaches, behavioural changes and papilledema may indicate a relatively large tumor present near the frontal lobe (16). Traditional methods of tumor detection include magnetic resonance imaging (MRI) and computed tomography scans (CT) and positron emission tomography (PET). GBM appears as an irregular mass with a necrotic hypointense core of necrotic tissue
on most MRI’s (17). More advanced MRI techniques like the diffusion-weighted imaging (DWI), diffusion-tensor imaging (DTI), magnetic spectroscopy imaging (MRS) and intra-operative MRI can further define the location, size and invasiveness of the tumor. However, most of these techniques require significant tumor progression for detection of specific characteristic GBM-defining features. A more recent trend in GBM diagnosis is the use of bio-fluids to identify markers from the cerebrospinal fluid and serum, which may allow for the early detection of the tumor (18).

1.2 Traditional treatment

The aggressive nature of the tumors and the ability of cells to mutate into different tumorigenic lineages make GBM extremely difficult to treat. The standard care for a patient newly diagnosed with GBM includes surgical resection, which is usually followed by radiation therapy and chemotherapy. Considerations in treatment include the size and location of the tumor and the heterogenous nature of the cells. Current progress in therapy of GBM has improved quality of life to some extent but does not stop the disease. Moreover, therapeutic management of the tumors needs to be accompanied by supportive therapy for the management of seizures, neurological disorders, cognitive impairment and venous thromboembolism that often accompany GBM (3).

1.2.1 Surgery

Aggressive surgical resection of the tumor is usually the first step in the treatment of GBM since most patients are diagnosed only after there is significant tumor development (19). Multiple studies correlate surgery with somewhat longer patient survival and enhanced quality of life (19) (20). A more recent surgical approach is the use of tumor-treating fields (TTF) that emit electromagnetic energy in low intensity
electrical fields that inhibit the mitosis of tumorigenic cells (21). Another innovative approach is to insert wafers containing chemotherapeutic agents into the surgical cavity during surgery. Surgery can improve the patient’s quality of life by reducing the tumor size, controlling or reducing seizures, minimizing neurological effects and allowing for the incorporation of locally applied chemotherapeutic treatment (3).

1.2.2 Radiotherapy

Surgery is usually followed by radiation therapy to treat the remaining tumor cells. Targeted field radiotherapy reduces the side-effects and tissue damage to the brain compared to standard whole brain radiation (22). A standard dose of radiation is 60 Gy administered as approximately 2 Gy fractions for five days a week, for six weeks (23). However, Stupp et al. later showed that a combined therapy of radiation with the first line of chemotherapeutic treatment temozolomide (TMZ) increased the median survival by 3 months compared to radiotherapy alone (23). Additional newer techniques like iodine-125 brachytherapy, stereotactic radiosurgery and hyper-fractionated radiotherapy have been employed to reduce necrosis and neuronal damage caused by radiation, however these have not shown any advantage over traditional radiotherapy (22). Other treatment techniques like boron neutron capture are still under investigation for reduced toxicity and enhanced chemo-radiotherapeutic treatment (24).

Figure 5: Standard radiotherapy and temozolomide adjuvant therapy suggested by Stupp et al.
1.2.3 Chemotherapy

A patient who has undergone complete surgical resection typically waits four weeks before starting chemotherapy (25). Alkylating agents like TMZ are the standard regimen for chemotherapy. Other chemotherapeutic agents used for GBM treatment include carmustine, lomustine, bis-chloroethylnitrosourea (BCNU), cetuximab, erlotinib and bevacizumab (26). Since drug loss due to efflux mechanisms at the blood brain barrier (BBB) reduces drug efficacy, efflux inhibitors can be co-administered to prevent elimination. The efficacy of efflux inhibitors like elacridar, valspodar and zosuquidar was tested by Bihorel et al who found that efflux inhibition of the P-glycoprotein (P-gp) 1 substrate led to a nearly 9-fold increase in imatinib drug concentration in the brain (27). New curative therapies like immunotherapy, gene therapy, and the latest Cas9 or CRISPR technology are being developed for promotion of cancer cell death and gene mutation rectification. (28).

Multiple studies have focused on the concurrent administration of these chemotherapeutic agents for better GBM treatment response. The biggest difficulty with administration of drug therapy for GBM is bypassing the BBB that physically limits the concentration of the chemotherapy in the brain thus reducing chemotherapeutic efficacy.

1.3 Temozolomide

TMZ is generally administered orally as Temodal, Temodar® and is approved as the first line of treatment for newly diagnosed GBM and recurrent anaplastic astrocytoma (29). It is administered for six weeks at a daily dose of 75 mg/m², followed by a one-month break until radiotherapy is complete (26). Post the rest period, TMZ is
continued for maintenance at a daily dose of 150-200 mg/m² for 5 days every 28 days (26). TMZ has been shown to improve not only overall survival but also survival without progression, thus improving the quality of life in some patients (30).

Table 1: Physicochemical properties of temozolomide (31).

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<th>Property</th>
<th>Value</th>
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<td>IUPAC Name</td>
<td>3-methyl-4-oxoimidazo[5,1-d] [1,2,3,5] tetrazine-8-carboxamide</td>
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**1.3.1 Mechanism of action**

Temozolomide is a prodrug that generates an intermediate triazene 5-(3-methyltriazen-1-yl) imidazole-4-carboxamide (MTIC) by hydrolysis and decarboxylation at a pH above 7.0 (figure 6). MTIC undergoes further cleavage to form the highly active methyl diazonium ion which can interact with nucleophilic species like DNA. This ion causes methylation of the guanine moiety on the DNA at O6. MTIC itself is cytotoxic and can methylate the guanine at the O6 position (32). This leads to apoptosis by blocking the cell cycle due to a lack of a complementary base to the newly-
alkylated guanine. The mechanism of TMZ action is dependent on the DNA damage by the inability of the DNA mismatch repair system to identify the complementary base for the methylated DNA. Methyl Guanine Methyl Transferase (MGMT) is a DNA repair enzyme that may be present and is responsible for the removal of alkyl groups attached to the guanine (33). The ability of MGMT to remove methyl groups is much higher than other alkyl groups, which may cause the opposite effect to the damage induced in the DNA by the TMZ methylation. Studies have shown that the concentrations of MGMT is varied in the different types of GBM and that these concentrations are directly proportional to the concentrations of the MGMT promoter for methylation (32).

Figure 6: Metabolism of TMZ.

1.3.2 TMZ nanomedicine and clinical use
Due to the high number of MGMT mediated TMZ resistance cases, secondary options like co-therapy and MGMT gene silencing are being investigated (30) (28). Currently there are multiple nanoparticulate systems delivering TMZ itself or with other therapeutic agents (34) (35). A system for the concurrent delivery of TMZ and an siRNA molecule was targeted for gliomas by folate decoration of the nanoparticles (35). The polymeric nanoparticles showed prolongation of survival of the glioma bearing rats, but also synergistically inhibited the glioma. The pharmacokinetic evaluation of a novel PEG functionalized liposomal system co-encapsulating TMZ and quercetin for enhanced sensitization of glioma cells that are drug resistant revealed significant brain accumulation of the nanoparticles and a high uptake by U87MG cells (36).

1.4 Elacridar

Efflux mechanisms at the BBB play a vital role in determining the drug concentration at the tumor site. To maintain the drug concentration at the tumor site, the efflux systems can be inhibited by the concurrent administration of a more potent substrate for these receptors. Elacridar (GF120918,) is a third generation highly potent and non-competitive P-gp inhibitor that is relatively less toxic and has no cytochrome P450 interaction as compared to most other P-gp inhibitors (37).
Table 2: Physicochemical properties of elacridar (31).

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1.4.1 Mechanism of action

While the exact mechanism behind the P-gp inhibition by elacridar is not understood, it is known that its action is dependent on the manipulation of ATPase activity which leads to inhibition of the ATP hydrolysis. P-gp belongs to the ABC-transporters and is an energy dependent efflux pump that depends on ATP. A broad variety of substances can act as substrates for the P-gp pump and concurrent administration of elacridar can prevent the reduced bioavailability of such substrates by pump inhibition. However, it is known that while elacridar can increase the uptake of co-administered particles, there are other mechanism through which the concurrently
administered agents could face resistance. (38). The mechanism of action is shown in figure 7.

![Figure 7: Mechanism of competitive efflux inhibition](image)

1.5.2 Elacridar nanomedicine and clinical use

Multiple studies have attempted to deliver elacridar with a pharmacologically active moiety to alter drug concentration due to P-gp efflux. A major advantage of the use of elacridar is the non-competitive inhibition of both P-gp and MDRP. A study involving lorlatinib, which is an anaplastic lymphoma kinase inhibitor, successfully demonstrated a 4-fold increase in the brain accumulation of orally administered lorlatinib with elacridar co-administration verses lorlatinib alone. This may be due to inhibition of a MDRP efflux pump at the blood brain barrier which reduced lorlatinib concentrations (39). Owing to the low aqueous solubility and high lipophilicity, multiple studies have focused at alteration of route and formulation of elacridar. Studies by Sane et al. found that a polymer suspension of elacridar showed optimum brain distribution when administered orally as compared to intravenous and intraperitoneal
administration in wild-type mice. A study by Wong et al. demonstrated an increase in the cellular internalization of doxorubicin to achieve therapeutic levels when co-administered with elacridar in polymer-lipid hybrid nanoparticles (40) (41). A recent study by Gooijer et al. has compared the increase in temozolomide brain penetration with elacridar co-administration with deleted MDR efflux pumps as control. It was observed that a 1.5-fold increase in temozolomide brain concentrations occurred with elacridar co-administration and in knockout wild type mice, verses when TMZ was alone (42).

1.5 RG7388/ Idasanutlin

The TP53 system plays a critical role in suppressing tumor development. MDM2 directly inhibits the action of p53, thus promoting tumor growth. A novel approach to treating GBM involves the inhibition of MDM2, thus preventing its inactivation of the wild type-p53 and indirectly promoting tumorigenic cell apoptosis and cell cycle control. Derived through examination of the MDM2 structure itself, nutlin drugs were one of the most potent and highly selective inhibitors of MDM2 (43). The difficulty with nutlin class of drugs arises due to its highly unfavourable physicochemical properties. RG7112 which was derived from the nutlin3-a small molecules was the first MDM2 inhibitor to undergo clinical evaluation. RG7388, also known as idasanutlin, is under extensive review through pre-clinical and clinical trials for MDM2 inhibition for different indications including neoplasms, acute myeloid leukemia, non-Hodgkin’s lymphoma and others (44). These molecules associate themselves to MDM2 by binding in the p53 binding pocket.

Table 3: Physicochemical properties of RG7388 (44).


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1.5.1 RG7388 nanomedicine and clinical use

RG7388 is being extensively studied for single and combinational therapy. RG7117 and nutin-32 which are precursors of RG7388 have also been evaluated for anti-tumor efficacy. A preclinical study evaluating the effect of RG7388 for neuroblastoma treatment determined that for wild type p53, the RG7388 showed a 200-fold difference between the GI50 (concentration required for 50% death) of the p53 wild type and mutant cell lines (45). The same study evaluated the effect of co-administration of RG7388 with some commonly administered chemotherapeutics like
cisplatin, temozolomide, doxorubicin, busulfan and topotecan. It was found that while RG7388 itself was efficacious, co-administration with temozolomide and busulfan showed enhanced apoptotic activity. Although RG7388 shows a significantly lower toxicity profile as compared to RG7112, the systemic delivery would be hindered due to its potency and very high hydrophobicity. Thus, nanocarriers would be an ideal system for their delivery. A study comparing nutlin-3 loaded into liposomes, PLGA nanoparticles and antibody conjugated PLGA nanoparticle for their antineoplastic activity found that while the antibody conjugated nanoparticles showed the most pronounced biological activity in an \textit{in vitro} lymphoma cell line, among the unmodified nanoparticles, PLGA nanoparticles better encapsulated the nutlin due to the intercalation of nutlin between the phospholipids and subsequent breaking of liposomes (46).

1.6 The Blood Brain Barrier

The blood brain barrier (BBB) controls the passage of materials into the brain. The endothelial cells that form the BBB differ from other endothelial cells found in the body as seen in figure 8. The endothelial cells of the BBB are connected to each other by tight junctions that prevent the passage of material between cells and they lack fenestrations which prevents passage of material through the cells. (47). BBB endothelial cells have a high volume of mitochondria to account for the higher metabolism, high electrical resistance at the tight junctions and a lower number of endocytic vesicles which reduce the overall molecular flux (48).
The efflux transporters are systems which transfer the substances which have crossed over to the abluminal side of the BBB back to the luminal side into the systemic circulation. The major efflux transporters found on the surface of the brain include P-gp transporter, multidrug resistant proteins (MRPs) efflux transporter, as well as the breast cancer resistance protein (BCRP) transporter which swiftly clears any substances that have crossed into the luminal side of the brain back to the systemic circulation as seen in figure 9 (49).

Receptor mediated transcytosis takes place through the transporters present on the abluminal side of the BBB which are highly specific and are responsible for moving most large molecules and high molecular weight substances like proteins, transferrin and insulin across the BBB, while carrier mediated transporters are usually present on both sides of the BBB and utilize energy dependent processes to exchange substrates between the brain and the systemic circulation (48). Thus, the structure of the BBB inhibits the diffusion of drugs and molecules not only physically, but also through a chemical environment that is non-conducive to the delivery of therapeutics into the
brain. The microvasculature of the brain can be modified by a change in the integral constituents including the basal lamina especially in conditions like stroke and traumatic brain injury (50). This is true for conditions such as inflammation and certain angiopathies, where the vessels in the brain may become extremely leaky and brain edema can occur due to extravasation (51).

The phenomenon of enhanced permeability and retention (EPR), where leaky vasculature in the presence of tumors results in drugs and small molecules passively diffusing into them and being retained due to low drainage by the lymphatic system (52). Some studies consider this to be true for brain tumors as well since BBB impermeable contrast agents used for MRI’s, are suspected to pass through such leaky vasculature to indicate the location of the tumors (47). However, many studies believe that the role of the EPR effect for brain tumors is insignificant since the microvasculature for these tumors is differently permeable throughout the tumor mass. Despite the BBB being slightly compromised at the core of the tumor mass, it remains relatively intact at the tumor margins thus preventing therapeutics from reaching the tumor core. Additionally, the dense matrix of the brain creates increased interstitial pressure inside the tumor bulk which prevents the natural diffusion of molecules into the tumor, largely reducing the EPR effect (52).
1.6.1 *In vitro* BBB models

The diffusion of therapeutics and molecules into the brain requires the establishment of a BBB cell culture model for pharmaceutical research. Currently, a standard BBB model does not exist, however, there are several brain capillary endothelial cell-based and non-cerebral cell-based models that are well established and resemble the BBB in structure and function. Some well-defined primary endothelial cell-based models include porcine brain microvessel endothelial-cells (PBMEC) or bovine brain capillary endothelial (t-BBEC), mouse (bEnd5), or rat (RBE4) brain capillary endothelial and human (hCMEC/D3) brain microvessel endothelial (54). BBB models can be tested for leakiness or transepithelial electric resistance (TEER) for barrier tightness and the presence of efflux transporters like P-gp to test their resemblance to the BBB. The cell culture models with TEER values greater than 1500 $\Omega$ cm$^2$ are considered to have good electric impedance to molecules and tight junctions resembling the BBB (55). Current trends for BBB modelling involve co-culturing with astrocytes and/or pericytes or alternatively co-culturing with cell-culture resembling diseases states like glioma to better replicate the *in vivo* environment and

Figure 9: Enhanced retention and permeability effect observed for tumors (53)
simultaneously the material interaction with the BBB and tumor cells as seen in figure 10 (56) (57).

Figure 10: Single and co-culture of cells

1.7 Bypassing the BBB

The concentration of the drug available in the tumor environment is dependent on the amount of drug that passes across the BBB. The reduced availability of drugs due to the BBB has caused several therapeutic agents to have low drug effectiveness and thus failure. Several new techniques have emerged to bypass the BBB and increase the availability of the administered drug therapy to the brain. These methods involve the temporary disruption of the BBB either physically or chemically, alternative routes of drug administration or the pharmacological or structural modification of the therapeutic itself.

1.7.1 Disruption of the BBB

The temporary disruption of the BBB to increase its permeability to therapeutics can be brought about physically or chemically by the administration of osmotic agents, ultrasound or chemical agents as shown in figure 11. Early reports by Rapoport et al. showed that administration of mannitol or hypertonic arabinose caused the brain endothelial cells to lose water and shrink and cause subsequent stress on the tight
junctions which temporarily disrupts the barrier for up to 40 minutes; this increases the apparent permeability of small molecules by nearly 10-fold (58) (59) (60).

Figure 11: Methods of bypassing the BBB (61).

A less invasive alternative to osmotic agents is the use of MRI to guide and focus high intensity ultrasound with intravenously applied microbubbles or contrast agents to temporarily open the BBB without causing significant tissue damage (62). The blood brain barrier diffusion of molecules can be chemically enhanced using vasoactive agents like bradykinin, leukotriene and alkylglycerols which cause inflammation (63) (64). However, studies using chemicals to disrupt the BBB have yet to produce successful results and some have even shown metabolic changes in the barrier properties (65).
1.7.2 Alternative routes of drug administration

The biggest difficulty with intravenous drug administration is that dependency of BBB drug permeability on the drug concentration at the tumor site. This correlation can be voided by altering the route of drug administration. Multiple alternative routes have been investigated including intrathecal, intraventricular, intranasal, interstitial or most recently intradermal administration of therapeutics. Intrathecal and intraventricular delivery involves the direct administration of the drug molecules or nanoparticulate system into the subarachnoid lumbar space and ventricular cavities respectively. These techniques have been widely studied for the delivery of large molecules.

The intranasal route has been proposed as a route to bypass the BBB. It has advantage over other routes due to the non-invasive manner of delivering the drug which enter the CNS through the olfactory pathway or extracellularly through the subarachnoid space into the brain (66).

Interstitial delivery of drugs into the brain is the most direct method of application of drugs to the brain by applying the chemotherapeutics directly into the brain by means of catheters or biodegradable wafers. While both these techniques employ surgical means, biodegradable wafers are often implanted post tumor resection into the tumor cavity and incorporate drugs or macromolecules into polymeric chips or disks which slowly release the drug over time. Catheter-based convection enhanced delivery (CED) employs the stereotactic placement of infusion catheters to deliver drugs to the tumor site using positive hydrostatic pressure gradient to cause convective flow. The most common clinically applied polymeric wafer is the Gliadel wafer
approved by the FDA for newly diagnosed GBM and recurrent malignant gliomas by delivering infused BCNU (59) (67). Current studies focus at the concurrent therapy of Gliadel with systemic administration of presently approved chemotherapeutics like TMZ for malignant tumors like GBM (68). Multiple preclinical and clinical studies are currently focused at evaluating the delivery of chemotherapeutic loaded nanoparticles for GBM therapy (69) (70).

Newer techniques to alter the route of administration are still being investigated. A study by Yu et al. investigated the transport of Evans blue to the brain through the trigeminal nerves by an intradermal application (71).

1.7.3 Modification of the administered agent

Clinical application of these alternative routes is limited due to their cost, invasive nature and difficulty of administration. An alternative to these techniques involves modification of the administered agent itself to confer properties that increase their inherent BBB permeability. This can be brought about by refinement of the structure using medicinal chemistry to synthesize molecules that closely resemble substrates for the receptors present on the BBB or by delivery of the chemotherapeutics in systems that can passively diffuse across the BBB. Another advantage of using molecules that resemble normal substrates is the ability to alter the chemotherapeutic or nanoparticles such that they evade the efflux systems that may reduce drug concentration on the luminal side of the BBB. Nanoparticles are the subject of much research and offer multiple advantages including potential for varied sizes, materials, release characteristics, site specific targeting and added functionality like multiple drug delivery. Multiple classes of nanoparticles have been investigated in this regard and
certain classes like polymeric, inorganic and lipid-based nanoparticles have shown the most promise for brain delivery (72) (73).

1.7.3.1 Inorganic nanoparticles

Inorganic or metallic nanoparticles are typically derived from gold, zinc, silver or their allotropes and are typically smaller than other nanoparticles, ranging between 1-100nm (59). The dense nature of these nanoparticles allows for conjugation to drugs or macromolecules like antibodies, DNA/RNA fragments and peptides (74). Gold nanoparticles have been used for both diagnosis and therapeutic purposes since they can be conjugated to therapeutic agents and show strong fluorescence quenching, which can be approximately 100 times more that of traditional quenching agents (75).

1.7.3.2 Polymeric nanoparticles

These are generally referred to as soft biomaterials that can be manufactured to possess desired properties by organic synthesis depending on the intended use. Small and large molecules including drugs, proteins, peptides can be encapsulated into polymeric nanoparticles by using a combination of one or polymers like chitosan, polyethylene glycol (PEG), dextran, propylene glycol, poly (lactic-co-glycolic) acid (PLGA), polyacrylates or even dendrimers (76). Polymeric nanoparticles can self-assemble in the form of a core-surrounding shell (nanocapsule) or a matrix (nanosphere) depending on the properties and ratio of polymers used. Most nanoparticles are stabilized by coating with polysorbate 80 or PEG to evade the reticuloendothelial removal of these particles and prolong their systemic exposure (76). These biodegradable nanoparticles have been explored for pre-clinical and clinical studies and are suspected to cross the BBB mostly through receptor mediated...
transcytosis (77). PLGA is a co-polymer that is composed of polylactic acid and polyglycolic acid as observed in figure 12 and is approved by the FDA for human use (78).

Figure 12: structure of PLGA; x represents poly lactic acid and y represents polyglycolic acid

1.7.3.2.1 PLGA nanoparticles

Through alteration of PLGA synthesis conditions and quantity of individual constituents, the molecular weight of the polymer can be altered from 4 to 240 kDA. Inside the body, PLGA can be metabolized by hydrolysis and the metabolic products can be broken into carbon dioxide and water and eliminated from the body. The commonly available forms include PLGA 50:50, 65:35, 75:25 and 85:15. Release of the encapsulated drug takes place upon hydrolysis and the most commonly employed type is 50:50 due to the fast release. These nanoparticles have been used to deliver macromolecules (e.g. proteins, peptides, genes, vaccines, antibodies, growth factors) as well as chemotherapeutics (e.g. docetaxel, irinotecan and temozolomide) or a combination of the two (79) (80) (81). Non-surface modified PLGA nanoparticles are often acidic in nature, may undergo rapid hydrolysis or aggregation and have relatively
low blood circulation time; however, modification and stabilization of these nanoparticles utilizing polymeric coating like chitosan or PEG, alteration to the polymersome or nanosphere hybrid form, or decoration of the surface with groups like bisphosphonate, lectin, biotin, folate, transferrin, cell-penetrating peptides, nucleotides or others significantly prolongs the systemic exposure and action of these nanoparticles (78) (82). Drugs can be incorporated by multiple means into these polymeric nanoparticles by entrapment, encapsulation, dissolution into the matrix or simple attachment to the particle surface by different techniques like solvent evaporation, nanoprecipitation, emulsion diffusion and one step microfluidic techniques.

1.7.3.2.2 Solvent evaporation

One of the most commonly employed techniques for PLGA nanoparticle production is the solvent evaporation technique. Using this technique hydrophobic or hydrophilic drugs may be incorporated by preparation of single or multiple emulsions respectively. The key parameters that control the size and stability of the formulation synthesized through this technique is the type and concentration of organic solvent and surfactant used. During fabrication, hardening of the formed nanoparticles take place when the solvent diffuses through the oil phase into the continuous water phase (83). In the single emulsion technique, the polymer and hydrophobic drug are first dissolved in a partly water miscible organic solvent, which is then emulsified by homogenization or sonication with the external aqueous phase containing a surfactant, while the double emulsion technique involves the pre-emulsification of the polymer with an internal hydrophilic drug and surfactant containing aqueous phase before emulsification with the external phase. The organic solvent which is most often dichloromethane, ethyl acetate or acetone is then evaporated by stirring or pressure to produce the
nanoparticulate suspension. The nanoparticles may be stabilized by the addition of polyvinyl alcohol (PVA), polysorbate 80 or D-tocopheryl polyethylene glycol to the aqueous phase (83).

1.7.3.2.3 Salting out

In this technique, the polymer is dissolved in a water miscible organic phase like tetrahydrofuran (THF), which is then mixed with the aqueous phase, containing salts insoluble in the organic phase and emulsifying agents. Nanospheres are formed by the migration of added water due to reduction in the ionic strength (82).

1.7.3.2.4 Nanoprecipitation

Nanoprecipitation is a low-energy technique that is commonly used for hydrophobic drug encapsulation. It is like the solvent evaporation technique differing only in the type of organic solvent and mechanical stress applied (76).

In all formulation techniques, the properties and type of PLGA used and the concentration and type of surfactant used may impact the particle size, charge, rigidity and pharmacokinetics. A study evaluating the effects of difference in stabilizer, PLGA and surfactant concentration and sonication time was carried out by Feczkó et al. It was found that changes in these variables showed pronounced variation in particle size and entrapment efficiency with sonication time and PLGA concentration being the most influential (84). All preparation techniques are limited by the time required for solvent evaporation and low hydrophilic drug entrapment. However, newer techniques aim to resolve these by use of microfluidics and membrane emulsification to increase hydrophilic drug encapsulation (85).
1.7.3.3 Lipid based systems

The BBB is highly restrictive of drug movement due to the bilayer formed by the phospholipid component of the endothelial cells that form the barrier. Therefore lipid-based nano-therapy has gained popularity amongst other nanoparticles for brain drug delivery. Solid-lipid nanoparticles and liposomes are two of the most favoured forms of lipid-based brain therapy due to their biocompatibility, ease of formulation and ability for multifunctionality. Initially sought as an alternative to polymeric nanoparticles, these 20-1000 nm sized lipid nanoparticles are well established for applications like targeted drug, macromolecule, gene and biomolecule delivery as well as for diagnostic purposes (72). Lipid nanoparticle are often composed of natural (soy/egg phosphatidylcholine) or synthetic phospholipids (1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE)), cholesterol, surfactants, charge imparting agents and additional agents like triglycerides based on the characteristics and purpose sought.

1.7.3.3.1 Solid Lipid Nanoparticles (SLN)

These nanosized colloidal nanocarriers are also called lipospheres or lipid nanospheres. At physiological temperature, lipids remain in their solid form and can be manufactured from multiple components like diverse types of glycerides, waxes, surfactants and fatty acids (86). However, a major limiting factor in the use of SLN’s is the inferior drug loading of non-hydrophobic substances. Drug loading and encapsulation efficiency are key evaluation parameters for drug delivery systems that reflect the quantity of drug available through these systems. While drug loading refers to the percentage of nanoparticle that is composed of the drug, encapsulation efficiency refers to the quantity of drug entirely encapsulated into the nanoparticles as a percentage.
of the original drug added (87). Protein, peptides and biomolecules that are often required to be administered at small doses have been administered through SLN’s (88) (89).

1.7.3.3.2 Liposomes

Liposomes have been studied extensively as the nanoparticles of choice for the delivery of therapeutics to the brain. Liposomes are spherical lipid vesicles that comprise of one or more lipid bilayers shells surrounding an internal aqueous core as shown in figure 13. The properties of the lipid bilayer may be modified through the incorporation of materials such as cholesterol and triglycerides. Sterols like cholesterol and β-sitosterol are often used in the phospholipid bilayers of liposomes to control the phospholipid bilayer characteristics. The influence of these materials on membrane fluidity, permeability and stability is well documented (90) (91). Cholesterol is typically incorporated between the bilayers and enhances the lipid tail orientation of the bilayer which in turn changes the lipid exchange in the liquid-like phase and ultimately increasing the density of packing, fluidity and permeability (92). Other materials that can modify the bilayer properties involves experimental material like glucocorticoids, lipo-polymers and lipo-peptides (93) (94).

Liposomes offer the distinct advantage of simultaneous encapsulation of hydrophilic substances in the aqueous core and hydrophobic materials in the phospholipid bilayer. The capacity to attach targeting moiety functionalization to the liposomal surface makes them an attractive system for brain delivery. Multiple studies have evaluated liposomes for dual drug delivery including a study by Zhang et al. wherein liposomes co-loaded with TMZ and vincristine were evaluated for efficiency
for GBM therapy by functionalization with lactoferrin and arginine-glycine-aspartic acid (95). It was observed that not only did the system show good encapsulation of the drugs, but high in vivo and in vitro uptake and toxicity to the tumor cells by sustained release of the drug along with low systemic toxicity. There are multiple ongoing clinical studies using liposomes for chemotherapeutic delivery in the brain, with some formulations even attaining FDA approval (96).

The use of doxorubicin (Dox) for brain tumors was initially limited due to severe cardiomyopathy experienced by most patients. The approval of non-pegylated Dox (Myocet®, metastatic brain tumors, 2000) and pegylated Dox (Doxil®, recurrent brain tumor 1995) radically changed the therapeutic delivery landscape as a proof-of-concept to prove the utility of liposomes as biocompatible, non-toxic carriers (96) (97). In 2017 the FDA approved a daunorubicin and cytarabine combination therapy encapsulated into bi-lamellar liposomes (Vyxeos®) for newly diagnosed acute myeloid leukemia (AML) (98). The liposomal formulation with a 5:1 molar ration of cytarabine and daunorubicin has shown promise for multi-therapeutic delivery as proof that the synergistic action and efficacy of dual drug delivery can be preserved when through liposomal nanomedicine.
1.7.3.3.2.1 Liposome structure and formation

The phospholipids that form the liposomes consist of a hydrophilic head and two hydrophobic tails. When these phospholipids are dispersed in water, depending on the kinetic energy in the hydrophobic tail, the lipids may self-assemble such that the tails of two phospholipids will assemble to form bilayers to form a spherical vesicle with lower energy (99). The energy required for the bilayer to bend into the sphere shape is related to the radius of the sphere formed. Variable sized liposomes may form by the rearrangement and change in energy as more phospholipids and bilayers are added to molecule with simultaneous fragmentation by hydrodynamic forces (99). Phospholipids have a natural tendency to self-arrange into a bilayer stack upon suspension on a substrate and subsequent removal of the organic phase (100). When hydrated, these bilayers separate due to the low solubility and merge slowly to form multilamellar (multiple bilayers) liposome (MLV). Application of high energy or reducing hydrodynamic flow and pressure can reduce the number of bilayers per
structure to form unilamellar (single bilayer) liposomes (101). The liposomes may also form bi-lamellar (bilayer within bilayer), oligolamellar (multivesicular) structures as seen in figure 14. Liposomes can be formulated by multiple techniques with thin-film hydration being the most widely accepted technique.

Figure 14: Types of liposomes

1.7.3.3.2.2 Thin film hydration

Thin film hydration technique was originally developed by Bangham et al. and involves the dissolution of lipids and hydrophobic drugs in an organic solvent like chloroform (102). This is followed by the evaporation of the organic solvent to form a thin lipid film or cake by stacking of the phospholipids. Subsequent hydration of the lipid film by the aqueous phase leads to the formation of liposomes that are mostly multilamellar. A small population of unilamellar and multivesicular vesicles may also be observed (103). The liposomal size can be tailored by subjecting the raw liposomal suspension to stress in the form of probe sonication, membrane extrusion, ultracentrifugation or freeze-thawing. The liposomes can be then collected by freeze-drying or spray-drying the suspension. A recent study by Huang et al. evaluated the extent of encapsulation and payload of different small molecules, macromolecules like proteins, and hydrophilic molecules like dextran prepared by thin-film hydration. It was found that the small molecules showed maximal encapsulation, while larger molecules
like proteins had a relatively weaker payload, requiring alteration in liposomal composition for a higher encapsulation. It was seen that while hydrophilic polymer encapsulation was lower than that of proteins, the loaded nanoparticles showed good drug release upon external thermal stimulation (104).

1.7.3.3.2.3 Reverse phase evaporation

The technique involves the formation of a water in oil emulsion by first dissolution of the lipid components in an organic phase, followed by mechanical mixing of the organic phase with the aqueous phase and subsequent evaporation of the organic solvent to form the MLV.

1.7.3.3.2.4 Ethanolic injection technique

The use of a partly water miscible organic solvent like ethanol or ether to dissolve the phospholipids may govern the size and structure of the resultant. The slow addition of such an organic phase into the aqueous phase results in the formation of small unilamellar vesicles upon organic solvent dilution (105).

1.7.3.3.2.5 New alternative techniques

Microfluidic approaches are gaining popularity for the formulation of liposomes due to the one-step approach in preparation of nanoliter sized samples that can be tuned depending on the formulation purpose. The technique utilized a high-throughput process based on hydrodynamic flow and two separate flow and temperature-controlled fluid channels of the organic and aqueous phase to produce
uniform batches of liposomes that can be easily scaled up for large-scale manufacturing (106). An alternative technique involves the use of supercritical fluids like supercritical carbon-dioxide to prevent the use of organic solvents for liposome formulation. This method has been adapted for the encapsulation of biomolecules like melatonin (107).

17.3.3.3 Mixed lipid and polymeric systems

Current strategies at improving the brain delivery of chemotherapeutics are focused on designing a drug delivery system that is non-toxic, biocompatible and one that can deliver high loads of multiple chemotherapeutics for a prolonged period. While polymeric, inorganic and liposomal therapies come close to fulfilling these criteria, each individual system has their own shortcomings. An ideal system would be able to exploit the advantages of all these carriers and combine their properties into a single system. Incorporating the high drug loading capacity of liposomes and the ability to induce hyperthermia by iron-oxide nanoparticles, Babincová et al. formulated iron oxide nanoparticles containing liposomes or magneto-liposomes with Dox as a chemotherapeutic and thermosensitive superparamagnetic iron oxide nanoparticle (SPION) that had good anti-glioma activity and controlled release of Dox in C6 glioma cells (73). A more sought-after combination involves the formulation of lipid-polymer hybrid nanoparticles as seen in figure 15. Liposomes can inefficiently entrap hydrophilic molecules and macromolecules and lack in long-term stability; while PLGA nanoparticles are quick degrading and encounter difficulty in crossing biological membranes unmodified (108). To overcome these limitations, Ishak et al. attempted the brain delivery of Rutin through PLGA-lipid engineered nanoparticles that consisted of a polymeric PLGA core surrounded by a lipid shell which was coated with PEG to
evade clearance by the reticuloendothelial system (108). They demonstrated that this nanoparticulate system prepared through a one step-nanoprecipitation technique showed better bioavailability when administered in vivo as opposed to the drug solution in rats. A hydrophobic molecule was be encapsulated in the phospholipid bilayer of the liposome. However, this resulted in rapid release from the liposomes for some therapeutics like short-chain ceramides. To circumvent this issue, Zou et al. formulated a system wherein the ceramide was encapsulated in the aqueous core using PLGA nanoparticles inside the liposomal bilayer (109).

Figure 15: Lipid-polymer systems; left polymer lipid matrix, right polymer particles in lipid vesicle

1.7.3.5 Surface coating of nanoparticles

Although nanotechnology has substantially enhanced delivery of therapeutics to tumors, external factors like clearance by reticuloendothelial system (RES), efflux mechanisms and non-specific delivery of these carriers can still lead to lower than optimal efficacies. Contemporary trends in therapeutic delivery for glioblastoma
involves the functionalization of such nanoparticles with materials that help retain these particles longer in the systemic circulation and direct them towards a specific site of action. PEGylation or coating of the surface of nanoparticles with PEG is a technique that is commonly employed to increase the circulation time and reduce the immunogenicity of nanoparticles (110). Nanoparticles that circulate in the bloodstream may be recognized and adsorbed onto proteins called opsonins. This makes the nanoparticles more likely to be phagocytized or scavenged by the mononuclear phagocyte system which reduces their circulation time. Additionally, most nanoparticles like liposomes tend to aggregate during circulation by nanoparticle-nanoparticle or protein-nanoparticle interaction.

The clearance time for most unmodified nanoparticles from the blood circulation was found to be approximately 10 minutes (110). The hydrophilic nature of PEG present on particulate surface forms a large volume of hydration cloud around the nanoparticles that prevents direct interaction with blood constituents (110). It also provides conformational flexibility to the nanoparticle surface to make it more difficult to be recognized by opsonins. A study by Vanzë et al. showed that PEGylated TMZ liposomes showed an approximately 4-fold increase in the brain accumulation of TMZ as opposed to the drug solution (34). The aggregation of these nanoparticles was also investigated through increases in turbidity upon incubation with serum proteins and it was observed that the nanoparticles showed no aggregation.

Functionalization of nanoparticles to target them to specific site has also been tested as seen in figure 13. It has been observed that in the tumorigenic state certain receptors like folate, lactoferrin and transferrin may be significantly upregulated (111). The folate receptor traditionally captures folate and its derivatives into the cell cytosol and has been found to be overexpressed on both the glioma cells as well as the BBB,
but not in the normal brain cells (112). While folate is a water-soluble vitamin important for thymine synthesis; folic acid is a relatively small, non-immunogenic and inexpensive exogenous derivative of folate which can bind to the folate receptor upon drug conjugation or nanoparticle functionalization (113). Upon binding to the receptor, the folate conjugated nanoparticle can be internalized by endocytosis and as the pH of the endosome reduces, dissociation of the folate and receptor lead to internal drug release (113). Recent studies by Guo et al. demonstrated a significantly selective and higher betulinic acid uptake and cytotoxicity for HepG2 cells for folate functionalized liposomes when compared to non-targeted liposomes (114).

The BBB forms a formidable obstacle for the delivery of drug for GBM in the form of a physicochemical barrier. Additionally, efflux systems and tumor resistance and heterogenicity reduce the efficacy of the currently available chemotherapeutics. While multiple GBM therapy studies have attempted addressing some of these issues, the need for a better carrier system for drug therapy still exists. There is an urgent need for a delivery system that is non-toxic, biocompatible and that can deliver one or more chemotherapeutics for GBM therapy.

1.8 Objective, hypothesis and specific aims

The objective of this study was to prepare, characterize and compare two liposomal delivery systems loaded with temozolomide, elacridar and RG7388 intended to evaluate their in vitro brain distribution and cytotoxic effect for glioblastoma multiforme.
The underlying hypothesis of the study was:

A multifunctional PLGA encapsulated liposomal system of TMZ, elacridar and RG7388 will show enhanced transport across an in vitro BBB model and higher cytotoxic effect for GBM as compared to a multilamellar liposomal system.

The specific aims to investigate the hypothesis were:

1. Development and Validation of a simple and rapid HPLC technique for the simultaneous detection of TMZ, RG7388 and elacridar.

2. Preparation and characterization of Folate-PEG-cholesterol conjugate.

3. Preparation and characterization of multilamellar liposomes.

4. Preparation and characterization of PLGA loaded liposomes.

5. In vitro evaluation of the two drug-loaded liposomal systems.
Chapter 2:

Development and validation of an HPLC method for the simultaneous quantification of temozolomide, RG7388 and elacridar in a solution
2.1. Introduction

The novel liposomal formulations developed in this study is intended for the simultaneous delivery of TMZ, elacridar and RG7388. Thus, it was important to develop a simple and rapid analytical method that could quantify all three drugs concurrently.

Temozolomide is a DNA alkylating agent that shows good bioavailability on intravenous and oral administration, yet low CNS accumulation due to the BBB and efflux systems existing therein (115). TMZ is a pro-drug which undergoes metabolism at a pH above 7 to MTIC, which further lead to the formation of a methylated nucleophile that exerts a chemotherapeutic action as shown in figure 6 (32).

Elacridar is a non-specific third generation P-gp and MDRP efflux inhibitor. However, the clinical use of elacridar has been significantly limited due to its high hydrophobicity (log P 5.67) and molecular weight, and extremely poor aqueous solubility, which limits its bioavailability in the systemic circulation (40). While validated techniques for the analytical quantification of elacridar by HPLC and LCMS already exist, most of these techniques are developed for the simultaneous detection of elacridar with another therapeutic (116) (117).

RG7388 is an MDM2 inhibitor that targets an MDM2 pocket involved in p53 inhibition. This second generation highly potent (IC₅₀ value is 6-30 nM) MDM2 inhibitor is still undergoing pre-clinical and clinical development which is extremely hydrophobic and has negligible aqueous solubility (118). There are limited literature availability regarding its solubility and quantification by analytical methods.

It was experimentally determined that the most optimal conditions for the determination of all three drugs included the use of a reverse phase HPLC system using
a C-18 column and detection at 254 nm. The objective of this study was to develop and validate an HPLC method for the simultaneous quantification of TMZ, elacridar and RG7388 as per USP guidelines.

2.2. Materials

Temozolomide was purchased from SelleckChem (Houston, USA), elacridar was purchased from Toronto Research Chemicals (Ontario, Canada), RG7388 was purchased from Chemietek Organics (Indianapolis, USA), ammonium acetate, hydrochloric acid (HCl) and Optima grade water and acetonitrile were all purchased from Fischer Scientific (Fair Lawn, USA).

2.3. Methods

2.3.1. Chromatographic conditions

The chromatographic conditions are depicted in table 4. Isocratic elution of a mixture of 50:50 acetonitrile: 120mM ammonium acetate at pH 4.5. The stationary phase comprised of a Jupiter® 5 µm C18 300 Å, 150 x 4.6 mm column with a mobile phase flow rate 1ml/min. The detector employed was a photodiode array detector. The length of each run was determined to be 8.2 minutes.
Table 4: Chromatographic conditions for HPLC analytical determination

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>Jupiter® 5 µm C18 300 Å, 150 x 4.6 mm</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>Acetonitrile and 120 mM pH 4.5 ammonium acetate (50:50 v/v)</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1.0 ml/min</td>
</tr>
<tr>
<td>Injection volume</td>
<td>10 µL</td>
</tr>
<tr>
<td>Column temperature</td>
<td>40°C</td>
</tr>
<tr>
<td>Wavelength of detection</td>
<td>254 nm</td>
</tr>
<tr>
<td>Instrument</td>
<td>Shimadzu Prominence-i LC-2030</td>
</tr>
</tbody>
</table>

The developed technique was validated to comply with United States Pharmacopoeia (USP) and International Conference on Harmonization (ICH) guidelines.

### 2.3.2 Preparation of mobile phase solutions

The mobile phase used consisted of acetonitrile and 120 mM pH 4.5 ammonium acetate (50:50 v/v). The aqueous phase was prepared by dissolving 9.25 g of ammonium acetate in 700 mL of deionized water. The volume of the solution is then made up to 1 L by addition of deionized water and the pH was adjusted to 4.5 using 0.1 N HCl. Subsequently the aqueous phase was filtered through a 0.2 µm polycarbonate filter. The apparent pH of the mobile phase was maintained at 4.5.

### 2.3.3 Preparation of standard drug solutions
To determine the concentrations of the therapeutics a standard curve was prepared from solutions containing known amounts of all three drugs (TMZ, elacridar and RG7388). The standard solutions were prepared through serial dilution to the concentration range of 0.15-150 μg/mL for TMZ, and 0.05-50 for elacridar and RG7388 from a stock of 500 μg/mL TMZ and 250 μg/mL for both elacridar and RG7388. TMZ (5mg) and elacridar (2.5 mg) and RG7388 (2.5 mg) were weighed in a 10 mL volumetric flask and dissolved using the mobile phase consisting of aqueous ammonium acetate (120mM, pH 4.5): acetonitrile (50:50) to prepare the stock solution. Three standard curves were constructed representing each drug by plotting area under the peaks vs concentrations.

2.3.4. Determination of unknown drug concentration

The standard curve plot of signal (peak area) on the Y-coordinate against its known concentrations on the X-coordinate was graphed. The unknown concentration of each drug was calculated by linear regression using the calibration curve and the area obtained experimentally. Least-squares method was used to obtain parameters for the regression equation (y = mx +c).

2.4 Results and Discussion

2.4.1 Specificity

Specificity of an analytical technique is the ability of the method to selectively detect and determine an analyte while lacking any interference from impurities and other formulation components as per the USP and ICH guidelines (119). The current
method was tested for specificity in detecting and quantifying TMZ, elacridar and RG7388. Specificity was tested by chromatographic comparison of a mobile phase injection vs an injection of the drugs dissolved in the mobile phase under similar experimental conditions. The wavelength of detection in both cases were kept as 254 nm as it was found to be the wavelength of maximum detection for them in the UV visible spectrum. Figure 16 (a) and (b) depict a representative blank chromatogram and the drug solution chromatogram, respectively. It was observed that there was a chromatographic resolution of one or more minutes between the three compounds with TMZ, elacridar and RG7388 eluting at approximately 2, 4.5, and 7.8 minutes, respectively. The identity of an individual compound was confirmed by comparison against individual reference for each drug. The blank media showed no defined peaks depicting that there was no interference by the media due to lack of any component specific absorption at 254nm. Thus, the HPLC technique was found to be specific for the detection of TMZ, elacridar and RG7388 as per the USP.
Figure 16 (a): Representative chromatogram of the blank containing the HPLC media only. (b): Representative chromatogram of TMZ, elacridar and RG7388 in the HPLC media eluting at 1.99, 4.51 and 7.83 minutes respectively.

### 2.4.2 Linearity

Linearity of an analytical technique is the concentration range that produces results directly proportional to concentration of the analyte present in a sample (119). The current HPLC technique was evaluated for linearity by testing the proportionality between the known drug concentrations and the obtained peak area. The deviation from each obtained data value is also plotted on the regression line. The drugs were tested for linearity over the concentration ranges 0.150- 150 μg/mL for TMZ and 0.05- 50 μg/mL for elacridar and RG7388 as shown in table 5. The calibration curve for each drug is shown in figure 17 (a), (b) and (c), respectively. The linearity data are present in table 5. Thus, the protocol was linear as per the parameters set by the USP which state that the test product should be 80% to 120% of the true value.
A  Peak Area v/s concentration (Temozolomide)

$y = 4.019x + 3.9316$

$R^2 = 1$

B  Peak Area v/s concentration (Elacridar)

$y = 0.3302x + 0.6834$

$R^2 = 0.9999$
Figure 17(a): Standard curve for temozolomide (0.15-150 μg/mL) (b): Standard curve for elacridar (0.05-50 μg/mL) (c): Standard curve for RG7388 (0.05-50 μg/mL)

Table 5: Linearity for TMZ, elacridar and RG7388.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration range (μg/mL)</th>
<th>Equation of regression line</th>
<th>Residual sum of squares ($r^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temozolomide</td>
<td>0.15-150</td>
<td>$y = 4.019x + 3.9316$</td>
<td>1</td>
</tr>
<tr>
<td>Elacridar</td>
<td>0.05-50</td>
<td>$y = 0.3302x + 0.6834$</td>
<td>0.999</td>
</tr>
<tr>
<td>RG7388</td>
<td>0.05-50</td>
<td>$y = 0.1367x + 0.4611$</td>
<td>0.999</td>
</tr>
</tbody>
</table>

### 2.4.3 Precision

Precision of a procedure is the extent of agreement among the acquired results from tests that are evaluated through multiple evaluations of an individual sample (120). The precision is determined by evaluation of a series of measurements under the same operating conditions and determination of the standard deviation or the relative standard deviation.
deviation (RSD), which is also called the coefficient of variation. The degree of precision increases with a relatively smaller standard deviation. Intermediate (inter-day) precision is the precision determined on different days within the same environment, whereas repeatability (intra-day) is the precision determined for different sample sets of the same component on the same day. In the HPLC technique developed, both inter-day and intra-day studies were carried out. The percent RSD was determined by plotting the signal obtained (peak area) against the concentration. Table 6 (a, b and c) shows the inter-day and intra-day results for all samples. The percent RSD for all standards was within 10% which was within the acceptable limits as per the USP and ICH guidelines and thus the method was precise.

Table 6 (a): Intra-day and inter-day precision found in quantification of temozolomide (b): Intra-day and inter-day precision found in quantification of elacridar (c): Intra-day and inter-day precision found in quantification of RG7388

<table>
<thead>
<tr>
<th>Theoretical concentration TMZ (μg/mL)</th>
<th>Inter-day Precision (n=3)</th>
<th>Intra-day Precision (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Measured concentration</td>
<td>% RSD</td>
</tr>
<tr>
<td></td>
<td>(μg/mL)</td>
<td>(%)</td>
</tr>
<tr>
<td>0.148</td>
<td>0.15 ± 0.0021</td>
<td>1.45</td>
</tr>
<tr>
<td>0.591</td>
<td>0.545 ± 0.028</td>
<td>5.31</td>
</tr>
<tr>
<td>2.35</td>
<td>2.43 ± 0.058</td>
<td>2.43</td>
</tr>
<tr>
<td>9.38</td>
<td>8.69 ± 0.58</td>
<td>5.54</td>
</tr>
<tr>
<td>37.5</td>
<td>35.15 ± 1.66</td>
<td>4.74</td>
</tr>
<tr>
<td>125</td>
<td>120.70 ± 3.03</td>
<td>2.52</td>
</tr>
<tr>
<td>Theoretical concentration elacridar (μg/mL)</td>
<td>Inter-day Precision (n=3)</td>
<td>Intra-day Precision (n=3)</td>
</tr>
<tr>
<td>------------------------------------------</td>
<td>---------------------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>Measured concentration (μg/mL)</td>
<td>% RSD</td>
<td>Measured concentration (μg/mL)</td>
</tr>
<tr>
<td>0.04</td>
<td>0.044 ± 0.01</td>
<td>8.57</td>
</tr>
<tr>
<td>0.2</td>
<td>0.19 ± 0.01</td>
<td>8.87</td>
</tr>
<tr>
<td>0.78</td>
<td>0.69 ± 0.06</td>
<td>9.85</td>
</tr>
<tr>
<td>3.12</td>
<td>2.78 ± 0.22</td>
<td>9.66</td>
</tr>
<tr>
<td>12.5</td>
<td>13.65 ± 0.81</td>
<td>6.98</td>
</tr>
<tr>
<td>25</td>
<td>22.96 ±1.44</td>
<td>8.25</td>
</tr>
<tr>
<td>50</td>
<td>45.64 ± 3.07</td>
<td>1.17</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Theoretical concentration RG7388 (μg/mL)</th>
<th>Inter-day Precision (n=3)</th>
<th>Intra-day Precision (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measured concentration (μg/mL)</td>
<td>% RSD</td>
<td>Measured concentration (μg/mL)</td>
</tr>
<tr>
<td>0.048</td>
<td>0.04 ± 0.002</td>
<td>6.11</td>
</tr>
<tr>
<td>0.195</td>
<td>0.193 ± 0.002</td>
<td>1.08</td>
</tr>
<tr>
<td>0.78</td>
<td>0.66 ± 0.09</td>
<td>8.96</td>
</tr>
<tr>
<td>3.12</td>
<td>2.59 ± 0.76</td>
<td>8.21</td>
</tr>
<tr>
<td>12.5</td>
<td>11.75 ± 0.24</td>
<td>5.99</td>
</tr>
<tr>
<td>25</td>
<td>23.21 ±2.19</td>
<td>6.26</td>
</tr>
</tbody>
</table>
2.4.4. Accuracy

Accuracy of an analytical procedure is the equivalence between an experimentally determined value to the true value (120). Accuracy of the current HPLC technique was confirmed by comparing the results from injecting three quality control samples of known concentrations within the tested range against their experimentally determined values as required by the USP. The concentrations tested were 0.5, 25 and 100 μg/mL for TMZ and 0.625, 1.5 and 30 μg/mL for elacridar and RG7388. The percent accuracy was determined by comparing the experimental and theoretical values using the formulae:

\[
\text{Percent Accuracy} = \frac{\text{Measured concentration}}{\text{Theoretical concentration}} \times 100
\]

Table 7 represents the accuracy data for all sample sets. The percent accuracy for all samples was within 10% which was within the acceptable limits as per the USP and ICH and thus the method was accurate.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Theoretical concentration (μg/mL)</th>
<th>Measured concentration (μg/mL)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temozolomide</td>
<td>100</td>
<td>98.78 ± 5.80</td>
<td>98.78 ± 5.7</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>24.475 ± 0.936</td>
<td>97.9 ± 3.2</td>
</tr>
<tr>
<td></td>
<td>Concentration</td>
<td>ELAC</td>
<td>Drug Load</td>
</tr>
<tr>
<td>------------</td>
<td>---------------</td>
<td>------</td>
<td>-------------</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.49 ± 0.037</td>
<td>97.29 ± 4.56</td>
</tr>
<tr>
<td>Elacridar</td>
<td>30</td>
<td>28.24 ± 1.2</td>
<td>94.152 ± 7.3</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>1.48 ± 0.141</td>
<td>98.66 ± 1.95</td>
</tr>
<tr>
<td></td>
<td>0.625</td>
<td>0.57 ± 0.036</td>
<td>91.64 ± 1.32</td>
</tr>
<tr>
<td>RG7388</td>
<td>30</td>
<td>28.15 ± 1.30</td>
<td>93.83 ± 5.23</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>1.44 ± 0.425</td>
<td>95.99 ± 5.69</td>
</tr>
<tr>
<td></td>
<td>0.625</td>
<td>0.57 ± 0.04</td>
<td>90.88 ± 3.63</td>
</tr>
</tbody>
</table>

2.5. Applications

The HPLC-based quantification method was developed and validated for the simultaneous determination of TMZ, elacridar and RG7388, which was further used in the determination of drug load for studies such as encapsulation efficiency, *in vitro* release studies, nanoparticle uptake studies and *in vitro* permeability studies. The results for these tests are depicted in chapters 4, 5 and 6.

2.6. Summary of specific aim 1

1. A rapid and sensitive HPLC technique was successfully developed and validated as per USP guidelines for the simultaneous detection and determination of TMZ, elacridar and RG7388 in aqueous solutions.
2. The rapid reverse phase technique allowed TMZ, elacridar and RG7388 to elute at 2, 4.5, and 7.8 minutes, respectively showing good separation and resolution.
3. The technique was validated for specificity, linearity, precision and accuracy as per the USP and ICH guidelines.
Chapter 3:

Synthesis and characterization of a Folate-PEG$_{3400}$-cholesterol conjugate for surface modification of nanoparticles
3.1 Introduction

The attachment specific functional groups to nanoparticulate surface has been found to be an effective technique to target drug molecules to specific sites. The passage of xenobiotics as well as drug therapies is primarily limited by the BBB. Rapid clearance from the systemic circulation also reduces effective drug concentration. Drugs administered as chemotherapy for GBM are generally small molecules that are unable to bypass the BBB and most likely serves as substrates for its efflux mechanism (3). Therapeutics that do cross the BBB may not effectively reach or identify the tumor cells. Surface functionalization is the attachment of agents that modify the properties of the nanoparticulate surface for specific purposes such as targeting tumor cells, systemic clearance evasion, circulation prolongation or specific release. These properties can be attained through multiple means, for example targeting can be achieved through folic acid, lactoferrin or transferrin attachment which may direct the system more towards the tumors that upregulate their specific receptors. Attachment of long chain polymers like PEG can help serve multiple purposes like prolonging of circulation time and as spacers for targeting moiety attachment (121) (110) (34). These modifications can aid in site specific drug delivery while retaining their plasma concentrations which may contribute to increased drug amounts crossing the BBB.

The utilization of folic acid to target GBM tumors and the BBB and PEG to evade the RES has already been explored (110) (113). While folic acid is used to target the tumors and to a small extent the BBB, it has not been investigated for evasion of the macrophages, which are responsible for the clearance of nanoparticles from the system. In this study we explored the functionalization of the liposomal surface with
DSPE-PEG$_{2000}$ as well as folic acid. Cholesterol is a biocompatible, systemically available component that can be used to alter bilayer properties of liposomes and aid in nanoparticulate movement across membranes (122). In this study, a Folate-PEG-Cholesterol (Fol-PEG-Chol) conjugate was synthesized in a two-step reaction by the formation of amide linkages by first reacting PEG$_{3400}$-bisamine with folic acid followed by reaction of the intermediate with cholesteryl chloroformate the structures of which are shown in figure 18.

![Figure 18: Structures of (a) folic acid (b) PEG Bisamine and (c) cholesteryl chloroformate.](image)

3.2 Materials

Folic acid, poly (ethylene glycol)$_{3400}$ bis(amine) and cholesteryl chloroformate were purchased from Sigma Aldridge (St. Louis, USA), (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) (EDCl) and methanol were purchased from Fischer Scientific (Fair Lawn, USA), N-hydroxysuccinimide (NHS) was purchased from Acros Organics (New Jersey, USA), dimethyl sulfoxide (DMSO), diethyl ether, chloroform and ACS reagent sodium hydroxide (NaOH) pellets was purchased from Sigma Aldrich.
3.3 Methods

3.3.1 Synthesis of Folate-PEG amine intermediate (STEP 1)

The folate conjugate (Fol-PEG-Chol) was synthesized according to a previously reported procedure by Guo et al with some modifications as shown in figure 19 (123). Folic acid (441.4 mg) was dissolved in DMSO to a final concentration of 25 mg/ml. To that, NHS and EDC (molar ratio 1:1) were added and stirred at room temperature overnight in the dark. The urea by-product was extracted into the water layer after mixing with a 1:1 mixture of chloroform and water. The chloroform layer was separated and evaporated under reduced pressure to obtain an activated folate (Fol-NHS) stock which was refrigerated at -20°C.

Fol-PEG amine was synthesized by first dissolving 500 mg PEG bisamine into DMSO (2 mL). To that, 2 mL of the Fol-NHS stock was added and stirred for 20 hours at room temperature in darkness. Low molecular weight by-products, folic acid and DMSO were removed by gel-filtration over a Sephadex G-25 gel filtration column equilibrated with 0.4 micron filtered deionized water. The elution products were collected in parts as ammonium acetate solutions which was later extracted in chloroform and washed three times with water. The chloroform layer was separated, dried over sodium sulphate and concentrated under reduced pressure. The intermediate was obtained as a yellow powder (931.56mg) with yield 98.78%. The conjugation of Fol-NHS and PEG bisamine was confirmed by $^1$H NMR in DMSO-d$_6$ (Brooker Avance III HD 400 MHz spectrometer) using DMSO as a solvent.

3.3.2 Synthesis of Folate-PEG-Chol (STEP 2)
The final conjugate was synthesized through the coupling of the Fol-PEG amine to cholesteryl chloroformate. Cholesteryl chloroformate was added to 931.56mg of the prepared Fol-PEG (1:1.2 molar ratio) dissolved in chloroform (10 mL) and the reaction was stirred for 20 hours in the dark at room temperature. The progression of this reaction was monitored by the disappearance of free amine through the ninhydrin assay. The reaction mixture was then concentrated under reduced pressure and the crude product was purified by multiple washings with diethyl ether. The final product was dried under vacuum to remove any residual ether and stored at 4°C. The formation of the product was confirmed by silica gel thin layer chromatography (TLC) using a 1:1 chloroform/methanol system with a small amount of acetic acid. The final product was characterized for its yield and purity by NMR, DSC, FTIR and analysis of folic acid concentration using an Ultraviolet (UV) Spectrophotometer (Synergy H1 Hybrid Reader).
The structure of folic acid, PEG bisamine and cholesteryl chloroformate is shown in figure 18. The folic acid was attached to the surface of the liposome using a stable PEG linker and cholesterol as its anchor. PEG was selected as the linker due to its extensive use in approved biopharmaceutical formulations, tuneable properties, low toxicity and low steric hinderance for binding to the receptor (124).

3.3.3 Determination of Folate content

The study aimed at the preparation of a folic acid conjugate which would aid in directing the nanoparticles to the glioma cells. The synthesized conjugate was tested for the concentration of folic acid by comparison against a standard curve of pure folic acid.

3.3.3.1 Preparation of solvents for standard curve
2N NaOH was prepared by dissolving 80 mg NaOH in 7 mL water. The mixture was vortexed for 2 minutes, followed by sonication in a water bath for 5 minutes. Volume of the solution was then made up to 10 mL. The solvent for standard curve and sample was prepared by mixing 10 mL of the prepared 2N NaOH solution with 10 mL methanol.

3.3.3.2 Preparation of standard solutions

A stock solution of folic acid was prepared by dissolving 2 g of folic acid in 2 mL of the 2N NaOH: methanol (1:1) system. The stock was then serially diluted to a concentration range of 15-1000 mg/ mL. Three samples concentrations (n=3) were prepared by dissolving 100, 50 and 10 mg of the prepared conjugate in 1 mL of the solvent system of 2N NaOH: methanol (1:1). Two hundred μl of the samples were added to wells in a 96 well plate and read at 285 nm wavelength on a Synergy H1 hybrid UV Spectrophotometer.

3.4 Results and discussion

The Fol-PEG-Chol conjugate was synthesized by a simple two-step reaction. The first reaction involved the formation of Fol-PEG amine intermediate from folic acid and PEG bisamine under esterification conditions of EDC and NHS. The intermediate was further chemically reacted with cholesteryl chloroformate to form the final Fol-PEG-Chol conjugate. The completion of reaction and purity of the formed conjugate was characterized by multiple means.

3.4.1 Conjugate Yield and TLC evaluation
The yield of a chemical reaction is the amount of desired product formed through synthesis. It can be reported as actual yield and percent yield which may provide insight towards the efficiency of the chemical synthetic reaction. Percent yield is calculated as the ratio of the weight of compound obtained through the reaction to the theoretical weight of the compound expected by measure of its initial reactants. The percent yield in this case was calculated as:

\[
\text{Percent yield (\%)} = \frac{\text{Mass of compound obtained}}{\text{Theoretical weight of compound}} \times 100
\]

The reaction yield for the Fol-PEG-Chol was found to be 89.6%.

A \( R_f \) value on the TLC plate was used to determine the presence of Fol-PEG-Chol. Through comparison of \( R_f \) values, the identity of a compound can be confirmed. Additionally, these values may also indicate the polarity of the synthesized compounds. The \( R_f \) value of the conjugate was found to be 0.84 in a 1:1 methanol/chloroform system.

3.4.2 Nuclear Magnetic Resonance (NMR)

The NMR was used to confirm the presence of the characteristic groups of the components that have been utilized to synthesize the Fol-PEG-Chol with DMSO-\( d_6 \) as the solvent. NMR has been typically used for the identification of compounds based on their chemical shifts (\( \delta \)) which are often expressed in parts per million (ppm). All samples were prepared by dissolving 3-4 mg of the sample in DMSO-\( d_6 \) and utilizing the Brooker Avance III HD 400 MHz spectrometer. The NMR spectra for folic acid, PEG bisamine, cholesteryl chloroformate, Fol-NHS and Fol-PEG-Chol has been shown
in figure 20. The signature proton peaks for the folic acid, PEG bisamine and cholesteryl chloroformate were confirmed in the spectra for the pure compounds and these were further utilized to characterize the formation of the conjugate. The small peaks observed in figure 20 (a) and (e) at 6.6, 7.6 and 8.6 ppm represent the protons on the aromatic ring of folic acid. An important change in the conjugate are the additional amide protons observed between 4.8 and 6.8 ppm which confirms the formation of the conjugate between the folic acid and PEG bisamine as well as PEG bisamine and cholesteryl chloroformate.

Figure 20: NMR for Fol-PEG-Chol, Fol-PEG amine, Cholesteryl chloroformate, Folic acid and PEG Bisamine.
Figure 20: NMR for Fol-PEG-Chol, Fol-PEG amine, Cholesteryl chloroformate, Folic acid and PEG Bisamine. Below: NMR focused on folic acid specific region (a) Fol-PEG-Chol (b) Fol-PEG amine intermediate (c) Folic acid

3.4.3 Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry is an analytical technique that can identify certain characteristic features of compounds such as melting point, crystallization and glass transition temperatures when the substance is subjected to controlled heating. In this study, the DSC was used to detect and confirm the presence of the reactants such as folic acid, PEG bisamine and cholesteryl chloroformate in the final conjugate and determine the physical state of the conjugate using Shimadzu DSC-60, Kyoto, Japan instrument and Shimadzu TA-60WS, Kyoto, Japan as its operating system. Samples of the reactant (folic acid, PEG bisamine and cholesteryl chloroformate) and the product
(Fol-PEG-Chol) were each individually weighed to 4-7 mg in an aluminium pan. A reference aluminium pan without any sample was also crimped. The samples were heated from a temperature range of 25 - 300°C, at a heating rate of 10°C/ min with nitrogen purge at flow rate of 20 mL/min.

Figure 21 shows the DSC thermograms for all samples. The melting point peak was observed for pure cholesteryl chloroformate peak at 118°C, while no defined peak was observed for the conjugate. A peak at 110°C was observed in the conjugate which demonstrated a change in the cholesterol moiety which altered its composition, possibly due to conjugation with PEG. A similar trend was observed for the PEG bisamine where peaks were observed at 158°C to 140°C for the pure compound and conjugate respectively. The peak shift is indicative of a change during the synthetic conjugation process. However, no specific melting peaks from 25-300°C were observed in the pure folic acid sample or the conjugate. Two endothermic events were observed in the case of folic acid sample at 136°C and 207°C which may represent the loss of glutamic acid moiety and pterin plus PABA respectively (125). While the glutamic acid loss may be observed as the broadening of the peak at 110°C, the loss of the pterin moiety may be responsible for the broad 218°C peak. To investigate the melting point of folic acid and its presence in the conjugate, the folic acid sample and conjugate runs were extended to 500°C as seen in figure 21. The extended run demonstrates that no sharp or defined melting peak is observed in the conjugate 490°C as opposed to the pure folic acid, which demonstrates a change in the original folic acid to form a different compound upon reaction with the PEG bisamine and cholesterol.
Figure 21: Above: DSC thermogram for folic acid, PEG bisamine, Cholesteryl chloroformate and Fol-PEG-Chol, below: extended folic acid and Fol-PEG-Chol thermogram

3.4.4 Fourier-transform infrared (FTIR) spectroscopy
Fourier-transform infrared spectroscopy is an analytical technique where infrared radiation is passed through a sample and the residual radiation is received at the detector in the form of a spectrum which represents the fingerprint of the chemical structure of the investigated sample. In this study, FTIR spectroscopy was used to identify characteristic peaks belonging to the folic acid, PEG bisamine and cholesteryl chloroformate and the changes in peaks in the conjugate to confirm the completion of synthesis and formation of the product through chemical bond investigation by using the Shimadzu IR prestige 21 (Shimadzu, Kyoto, Japan).

The first step involved the activation of folic acid by NHS and EDC and its conjugation to the primary amine of PEG bisamine. The characteristic folic acid peaks identified through FTIR spectroscopy as seen in figure 22 (a) include the C=C stretching vibrations of the folic acid aromatic backbone was observed at 1465 cm$^{-1}$ and 1734 cm$^{-1}$ in both the folic acid as well as the conjugate.

The most noteworthy step in the synthesis of Fol-PEG-Chol is the formation of the amide linkage (CONH) for which bands of the carbonyl group (C=O) and amine (NH) were observed at 1653 cm$^{-1}$ and 1571 cm$^{-1}$ respectively as observed in figure 22 (b).

The second step involved the synthesis of the Fol-PEG-Chol conjugate through the conjugation of Fol-PEG amine and cholesteryl chloroformate. Formation of the conjugate involves the formation of an ester linkage between the primary amine of Fol-PEG amine and acyl chloride of cholesteryl chloroformate which is represented as the peak at 1748 cm$^{-1}$ for the stretching vibrations of the carbonyl ester formed as seen in
figure 22 (c). The final product peaks were characterized for the amine (NH) at 1601 cm\(^{-1}\), 3481 cm\(^{-1}\), CH\(_3\) at 2881 cm\(^{-1}\), CH at 1881 cm\(^{-1}\) and carbonyl at 1721 cm\(^{-1}\).

![FTIR spectra](image)

Figure 22: FTIR spectra (A): Comparison of folic acid and Fol-PEG-Chol (B) Comparison of PEG-Chol (C) all reactants and final conjugate.

3.4.5 Determination of Folate content

The synthesized conjugate was tested for the concentration of folic acid by comparison against a standard curve of pure folic acid as seen in figure 23.
3.4.5.1 Standard curve results

A standard curve was constructed by plotting the absorbance vs concentration of the standard solution as shown in figure 23. The folate content was extrapolated from this standard curve.

Figure 23: Standard curve for pure folic acid

The folate content in the conjugate was found through extrapolation of the sample absorption to determine the folic acid content, which was found to be 86.5 ± 2.63%. Thus, a high folic amount of the originally added folic acid was retained in the formed conjugate. The increased folic acid concentration would allow more attachment of the nanoparticles to the folic acid receptors and thus higher particle uptake.

3.5 Discussion

Nanoparticles are often delivered to the desired site of action by the attachment of receptor specific or environment specific targeting moieties on the surface of the carrier systems. Folic acid receptors that are overexpressed in multiple tumors like
gliomas have been widely utilized to target and deliver chemotherapy. In this study, a folic acid targeting attachment was prepared by the synthesis of Fol-PEG-Chol conjugate where the folic acid was attached to the nanoparticle anchoring cholesterol through a PEG spacer molecule. A simple two-step chemical reaction led to the formation of a dark-yellow coloured folic acid targeting compound with a high yield of 89.6%.

The chemically synthesized conjugate was characterized qualitatively and quantitatively. The presence of the amide linkage formation in the 1HNMR of the final conjugate confirmed the formation of the conjugate. Additionally, the fingerprint peaks for the folic acid, PEG and cholesterol were compared to the pure compounds which confirmed the NMR presence of each in the final conjugate. Thermal analysis further depicted the formation of a crystalline conjugate with peaks not corresponding to the pure constituents which suggests a change in their original composition. The presence of the amide linkage was also detected through FTIR analysis confirmed the formation of new bonds of PEG bisamine with folic acid and cholesteryl chloroformate through the ester linkage and amide bonds. Finally, the extent of folic acid was determined by UV analysis to be 86.5%.

3.6 Summary of aim 2

1. A simple and rapid two step technique was utilized in the synthesis of a Fol-PEG-Chol derivative from folic acid, PEG Bisamine and cholesteryl chloroformate.
2. The conjugate was characterized for its purity, physical state and compound content by using NMR, DSC, and FTIR.
3. All analytical techniques confirmed the formation of the desired conjugate.

4. A high conjugate yield of 89% and high folic acid concentration of 86% in the conjugate was determined by UV analysis of conjugate.
Chapter 4:

Formulation and characterization of folate decorated temozolomide, elacridar and RG7388 loaded multilamellar liposomes
4.1 Introduction

Liposomes are nanocarrier vesicles that are composed of an aqueous core at the center surrounded by a phospholipid bilayer. Therefore, they encapsulate multiple therapeutics of different lipophilicity into a single carrier system. The properties of liposomes can be modified using different agents like cholesterol and triglycerides which can alter membrane properties and thereby affect drug encapsulation efficiency. The central objective of this study is to compare the ability of a multilamellar liposomal system with a PLGA nanoparticles in liposome system and their ability to carry temozolomide, elacridar and RG7388 across the blood brain barrier.

The BBB inhibits the entry of all kinds of chemicals, including drugs and compounds ingested from food sources, through its physical and chemical inhibition as well as the efflux systems present on its luminal and abluminal regions. Additionally, tumors prevent the passage of drugs within its cores due to the presence of blood tumor barriers. TMZ has been widely employed in GBM therapy, however most delivery mechanisms fail to achieve TMZ concentration required at the tumor site to provide effective action (30). Nanoparticles such as liposomes have been widely employed in this regard, however single drug therapy has major limitations including resistance and lack of tumor directed therapy. Additionally, recent efforts at inhibiting the BBB efflux system include co-administration of therapeutics such as elacridar, a P-gp inhibitor. Currently there is a need for the development of a system for the delivery of multiple agents to the tumor site without the systemic loss of drugs due to lack of site-specific targeting. Liposomes have high potential in this regard due to their ability to achieve surface modification, gaining effective ability to direct drugs to specific sites.
This study focusses at the formulation and characterization of a three-drug loaded multilamellar liposomal system surface modified with DSPE-PEG\textsubscript{2000} and Fol-PEG-Chol containing three drugs with different lipophilicity. While DSPE-PEG\textsubscript{2000} has been employed widely to increase the circulation of liposomes, the Fol-PEG-Chol would direct the nanoparticles towards the folate receptor overexpressing glioma cells (110) (123). L α-phosphatidylcholine and cholesterol were used as the lipid and Captex-300 was used as the triglyceride. The liposomes were prepared by thin film hydration technique followed by membrane extrusion and freeze thawing. Freeze thawing has been widely employed in multiple studies as a means of altering liposome bilayer characteristics as well as increasing drug loading into the liposomes (126). Studies have shown that remotely loaded liposomes had an increased encapsulation and drug load when they were subjected to minimum two-freeze thaw cycles (126) (127). This is because upon freezing, the liposomes may undergo fusion of the bilayers and upon thawing, the bilayers undergo rearrangement and fragmentation which may alter the number of lamellae present in the liposomes. Frohlich et al have studied the effects of freeze thawing a raw liposomal dispersion and shifts in the peak associated by the addition of a shift reagent (128). Using phosphorous (\textsuperscript{31}P) NMR, Frohlich et al have evaluated the phosphorous environment surrounding the liposomes and they found that unilamellar liposomes have even phosphorous environments surrounding them thus showing a single large peak on the NMR, while increasing the number of bilayers surrounding the liposomes creating unique phosphorous environments leading to a broader \textsuperscript{31}P peak. This represents a liposomal population which has multiple phosphorous environments due to the possible presence of multiple phospholipid layers in a single liposome.
However, most studies have focused on the effects of freeze thawing of multilamellar vesicles which may lead to the formation of unilamellar vesicles through destabilization of the lipid bilayer after fusion during the anneal stage and fragmentation during the thaw stage (129) (130). There is limited data on the effect of freeze thawing of a unilamellar dispersion of liposomes. In this study, we investigated the unique phenomenon of change in liposome structure upon freeze thawing post extrusion. The freeze thawing led to the formation of liposomes whose lamellarity was influenced by the number of freeze-thaw cycles.

The order of addition of the surface modifications can not only have an impact on the drugs loaded into the nanoparticles but also affect system stability and systemic interaction (131) (132) (133). The effect of different loading techniques on drug loading was also evaluated in this study. It is well known that polysorbate 80 can bind to the serum apolipoproteins and thus aid in non-specific blood brain barrier permeation by transcytosis using the apolipoprotein receptors (134) (135). Polysorbate 80 was used as a surfactant to increase the stability of the liposomes in this study.

4.2 Materials

L-α-phosphatidylcholine, Chloroform, Polysorbate 80, Sodium acetate anhydrous, lactose, trehalose was purchased from Sigma Aldridge (St. Louis, USA), dimethyl sulfoxide was purchased from Alpha Aesar (MA, USA), Captex 300 was purchased from ABITECH (Columbus, USA), Dicetyl phosphate (DCP) was purchased from ChemCruz Biochemicals (Dallas, USA), DSPE-PEG<sub>2000</sub> was purchased from Avanti Polar Lipids (Alabaster, USA), Temozolomide was purchased
from SelleckChem (Houston, USA), Elacridar was purchased from Toronto Research Chemicals (Ontario, Canada), RG7388 was purchased from Chemietek Organics (Indianapolis, USA), Ammonium acetate, Acetic acid, sucrose and Optima grade Acetonitrile was purchased from Fischer Scientific (Fair Lawn, USA) and maltrin 100 was purchased from grain processing corporation (Muscatine, USA).

4.3 Methods

4.3.1 Preparation of 0.1 M acetate buffer pH 5.0

Anhydrous sodium acetate (7.71 g) and 0.35 g of acetic acid were dissolved in 700 mL deionized (DI) water under magnetic stirring. The volume of the solution was made up to 1000 mL with DI water. The acetate buffer was filtered through a 0.4 μm polycarbonate filter and adjusted to pH 5 by the addition of 0.1 N NaOH.

4.3.2 Formulation of a multilamellar liposomal system

The multilamellar liposomes were prepared by a thin film hydration technique as shown in figure 24. The organic phase was composed of L-α-phosphatidylcholine (soy phosphatidylcholine), cholesterol and Captex 300 (70:20:10 molar ratio) and 3.2 mg DCP, while the aqueous phase constituted 1% polysorbate 80 (w/v) in acetate buffer. The organic phase was dissolved in 2 mL chloroform using a culture tube and subsequently the chloroform was evaporated over a nitrogen stream along the walls of the tube to form a thin lipid film. The film was hydrated with the aqueous phase slowly with vortexing to make the raw liposomal dispersion.
Schematically, the raw liposomal dispersion was subjected to sonication in water bath for 5 minutes followed by extrusion through a 0.1 μm polycarbonate membrane (Whatman Nuclepore Polycarbonate filters, GE healthcare life science, Pittsburgh, USA) using a mini extruder (Avanti Polar Lipids, Inc.) for 9 cycles. The unilamellar liposomal vesicles (ULV’s) formed were then subjected to either 0, 3 or 11 cycles of freeze thaw process by sequential freezing in liquid nitrogen at -196°C for 2.5 minutes followed by thawing in a water at 50°C for 3 minutes to form the multilamellar/ multivesicular liposomes. The prepared liposomes were then dialyzed in cellulose membranes with 12–14 kDa (Fischer Scientific, Pittsburgh, USA) molecular weight cut-off (MWCO) for a day. Post-dialysis, the liposomes were freeze dried using Millrock Technology LD85 freeze dryer (Kingston, NY) to obtain dried liposomes. Different cryoprotectants including lactose, maltrin 100, sucrose and trehalose (1:5 w/w lipid to cryoprotectant) were tested in their ability to prevent increase in particle size of the liposomes during the freeze-drying process.

Figure 24: Scheme of multilamellar liposome formulation by thin film hydration
4.3.3 Drug loaded multilamellar liposomes

The drug loaded liposomes were prepared by passive loading of the drugs into the liposomes. Elacridar and RG7388 were added to the organic phase for incorporation into the lipid film. TMZ was added to the acetate buffer containing polysorbate 80 and incorporated into the liposomal aqueous core during liposome hydration. The effect of the initial drug added on encapsulation efficiency was tested by the addition of 2.5, 5 and 10 mg of each drug into the liposomes.

4.3.4 Surface Modification of the of liposomes with DSPE-PEG\textsubscript{2000} and Fol-PEG-Chol

Two methods of insertion of the targeting moieties DSPE-PEG\textsubscript{2000} and Fol-PEG-Chol were investigated. The pre-insertion technique included the addition of both polymeric modifications (5 mole percent each) into the organic phase for incorporation into the lipid film. Alternatively, in the post insertion technique 5 mole percent of each DSPE-PEG\textsubscript{2000} and Fol-PEG-Chol were added to the 2 mL aqueous phase (0.1 M acetate buffer containing 1% polysorbate 80) and the emulsion was vortexed for 5 minutes. The micelles were then added to preformed liposomes and the liposomal suspension was incubated overnight at 52°C on an orbital shaker.

4.4 Characterization of liposomes

4.4.1 Evaluation of particle size and zeta potential

The liposomal particles were tested for their particle size and zeta potential using the Brookhaven Zetameter (ZetaPlus, Brookhaven Instruments Corporation,
NY). Measurements were made for the blank liposomes using lactose, sucrose, maltrin 100, trehalose or no cryoprotectant before and after freeze drying. Additionally, the particle size of drug loaded samples were tested using trehalose as the cryoprotectant for freeze drying. The particle size and zeta potential were measured by dilution of 100 microliters of the liposomal sample with 10 mL of 0.45 μm filtered acetate buffer for the liposomal suspensions and dissolution of 1mg of the freeze-dried samples in 10 mL of the acetate buffer. Five measurements were carried out for each sample. All samples were tested in triplicates.

4.4.2 Nuclear Magnetic Resonance analysis

The samples were tested by Nuclear Magnetic Resonance as a preliminary determinant of the liposome lamellarity using the method previously demonstrated by Frohlich et al. (128). The phosphorous environment surrounding the liposomal structure was evaluated using $^{31}$P NMR analysis by Brooker Avance III HD operating at 161 400MHz. The liposomal sample was diluted to 50 mM lipid using 10 % D$_2$O as the solvent. Samples of the ULV’s were represented by the liposomes collected after extrusion through a 0.1 μm polycarbonate membrane while the MLV’s were represented by the liposomal dispersion obtained after 11 freeze thaw cycles. To evaluate the effect of number of freeze thaw cycles, samples were collected after 0, 3 and 11 cycles for $^{31}$P NMR analysis. The temperature was set to 25°C while the pulse length used was adjusted to 14 μs pulse with a relaxation delay of one second which overall corresponded to its flip angle of 63°. The experiment was set to 10,000 scans and $^1$H broad-band decoupling was applied to all measurements. The resultant peaks
were analysed using computer integration by the Bruker Topspin 3.5 software. All samples were tested in triplicate.

4.4.3: X-ray diffraction studies

Powder X-ray diffraction (XRD) studies and small angle XRD (SAXS) studies were performed on blank liposomal samples at the x-ray structural characterization facility, Nebraska centre for materials and nanoscience, University of Nebraska–Lincoln. The study was carried out under experimental conditions specified by Wang et al with minor modifications (130). The powder XRD studies were performed using the PANanalytical Empyrean diffractometer (PANalytical Inc., Westborough, MA, USA) using a 3 kW Cu Kα radiation source (1.5418 Å) at 45kV, 40 mA, setting and the Bragg-Brentano focusing geometry. The experiment was carried out in the 2θ range of 3–60° and the mask of 20 mm and a divergence slit of 1/8° was set on the incidence beam path. The liposome solutions (ULV, MLV 3 cycle and MLV 11 cycle) were drop casted on an acrylic sample holder having 20 mm x 20 mm x 0.2 mm pockets. Data of the diffraction patterns were collected in 0.052-degree steps by continuous scanning of the source through the PIXcel 3D detector at a scan rate of 0.067 °/s. The peaks suspected to originate from Cu-Kb radiation were removed by the application of a Ni-foil Kb filter on the detector.

The Small Angle X-ray Scattering (SAXS) experiments were carried out using Rigaku Smartlab diffractometer (Rigaku Corp., Tokyo, Japan) with Cu-Kα radiation (1.5418 Å) at 40 kV, 44 mA setting. A Cross Beam Optics (CBO) – SAXS slit – Soller slit combination was used to prepare the incident beam for the transmission SAXS geometry used in the current experiments. The liposomal suspension was then
transferred to a capillary tube (0.7 mm OD, 0.01 mm wall thickness) and the capillary was mounted horizontally to perform the experiments. A vacuum beam path was introduced in between the sample and the detector to reduce the air scattering contributions in the transmitted beam. Length limiting slit of 10 mm was used to restrict the beam size in the horizontal direction. Optics and sample alignment are performed automatically using Smartlab guidance software package. An incidence slit of 0.5 mm and receiving slits of 0.2 mm were used in the experiment. A scintillation counter detector was scanned at a speed of 0.24 deg/min collecting data in steps of 0.02° in the 2q range of 0.06 – 8°.

4.4.4: Electron microscopy

Cryogenic-Transmission electron microscopy (cryo-TEM) was performed on blank multilamellar liposomes at the Department of Diagnostic and Biological Sciences, University of Minnesota, Minnesota. The liposomal samples were diluted to 2-8 mg/mL in acetate buffer and the grids were glow discharged for 30s. A magnification 4700 times was used.

4.4.5: Folic Acid content determination

The folic acid concentration present on the liposomes after modification of the liposomal surface was evaluated by the method described in the previous section. The concentration of folic acid in the liposomes upon pre- or post-insertion was determined using a standard curve for pure folic acid solution. The description of standard curve preparation is provided in section 3.4.6. The concentration range for standard curve was 0.1-10 mg/mL of a NaOH: methanol solvent system (1:1). A
sample from the pre-insertion and post-insertion technique was prepared by dissolving 10 mg of the liposomal sample in 1 mL of the NaOH: methanol solvent system. Two hundred μl of the sample was then added to wells in a 96-well plate and read at 285 nm wavelength on a Synergy H1 hybrid UV Spectrophotometer for determination of the folic acid content.

4.4.6: Thermogravimetric analysis

The change in the weight of the sample on heating was evaluated using a thermogravimetric analyser (Shimadzu DSC-60, Kyoto, Japan). The freeze-dried sample (4-6 mg) was weighed in an aluminium pan and heated in a nitrogen environment (20 mL/minute) from 20°C to 300°C at a constant rate of 10°C per minute. The samples were tested in triplicate and the percent weight loss was evaluated by the thermal analysis operating system (Shimadzu TA-60WS, Kyoto, Japan).

4.4.7: Differential Scanning colorimetry

DSC was performed to determine the physical states of the drugs in the nanoparticulate carrier system using Shimadzu DSC-60, Kyoto, Japan instrument. About 4-6 mg of the samples of pure drugs, blank liposome and drug loaded liposomes were weighed in an aluminium pan and crimped. Characteristic sample properties were observed against a blank reference pan by heating both pans from room temperature to 300°C at a rate of 10°C per minute under a nitrogen environment of 20mL/minute purging. The samples were tested in triplicate and the thermal events
were evaluated by the thermal analysis operating system (Shimadzu TA-60WS, Kyoto, Japan).

4.4.8: Determination of Entrapment efficiency and drug loading:

The entrapment efficiency of the multilamellar liposomal system was evaluated by dissolving 10 mg of the drug loaded liposomal sample in the 2mL of HPLC mobile phase consisting of Ammonium acetate: Acetonitrile (50:50) by vortexing for 5 minutes followed by bath sonication for an additional 2 minutes. The obtained solution was then filtered through a 0.2 μm syringe filter and the quantity of temozolomide, elacridar and RG7388 was determined using the HPLC technique validated for the simultaneous detection of the three drugs as described earlier in chapter 2. The standard curve for the HPLC determination was prepared by dissolving 10mg of each temozolomide, elacridar and RG7388 in 10mL of the mobile phase. Entrapment efficiency is the percent of drug encapsulated into the liposome as a ratio of the initially added drug and is calculated as shown in formula:

\[
\text{Entrapment efficiency(%) } = \frac{\text{Amount of drug in the formulation}}{\text{Initial drug added to formulation}} \times 100
\]

The amount of drug loaded in the liposomes was determined as the ratio of the percent of drug in liposomes as a ratio of the total weight of the nanoparticles as shown in the formula below:

\[
\text{Drug loading (\% ) } = \frac{\text{Amount of drug in the formulation}}{\text{Total weight of the formulation}} \times 100
\]
4.4.9: *In vitro release study*

The *in vitro* release characteristics of drugs from the multilamellar liposomes were studied by dialysis using a cellulose membrane 12–14 kDa MWCO. The release medium included phosphate buffer pH 5.0 with 1% (w/v) polysorbate 80 since TMZ is a prodrug and is prone to hydrolysis at a pH above 7. About 10 mg of the multilamellar nanoparticles were dissolved in 2mL of the acetate buffer pH 7.4 and sealed in a dialysis bag which was placed in beaker containing 300 mL of the release medium. These were incubated at 37°C in an incubator (Max Q 4450, Thermo Scientific) with orbital shaking. A volume of sample (500 μL) was collected from the beaker at pre-determined time intervals (0, 0.08, 0.16, 0.33, 0.6, 1, 2, 4, 8, 12, 24, 48 hours) and filtered through a 0.2 μm syringe filter. The volume of sample was replaced with an equal volume of fresh release media at each time point. The sample collected was evaluated for amount of drug using HPLC.

4.4.10 Statistical analysis

All experiments were analysed for statistical significance by using the two tailed Student’s t-test and ANOVA. A statistically significant difference was defined as a p-value less than 0.05.

4.5: Results

4.5.1 Evaluation of particle size and zeta potential

The multilamellar blank and drug loaded liposomes were characterized for their size and zeta potential. The particle size of the samples tested is shown in figure 25.
Figure 25: Particle size of multilamellar liposomal system

Table 8: Zeta potential of multilamellar liposomes

<table>
<thead>
<tr>
<th>Liposome type</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank ULV (extruded)</td>
<td>-38.8 ± 1.8</td>
</tr>
<tr>
<td>Drug loaded ULV</td>
<td>-34.8 ± 2.3</td>
</tr>
<tr>
<td>Blank MLV (11 cycles freeze thaw)</td>
<td>-26.4 ± 1.7</td>
</tr>
<tr>
<td>Drug loaded MLV</td>
<td>-29.8 ± 3.9</td>
</tr>
<tr>
<td>Blank surface modified MLV (pre-insertion)</td>
<td>-12.6 ± 1.4</td>
</tr>
<tr>
<td>Blank surface modified MLV (post-insertion)</td>
<td>-9.3 ± 2.9</td>
</tr>
<tr>
<td>Drug loaded surface modified MLV</td>
<td>-10.1 ± 1.6</td>
</tr>
</tbody>
</table>
The particle size of the liposomes was found to increase sequentially from the ULV’s to the freeze thawed MLV’s. The increase in particle size may be due to the increased number of lamellae per liposome upon freeze thawing. Previous studies have shown that as opposed to this, when raw multilamellar liposomal dispersion are freeze thawed, there is a sharp increase in the trapped volume which may result in reduced number of lamellae and thus a much lower particle size. However, unilamellar vesicles (extruded liposomes) are assumed to have trapped volume equal to the theoretical volume which is the maximal volume encapsulated by a vesicle with a specific lipid ratio without fragmentation or increase in lipid concentration. Thus, the increase in particle size due to increased trapped volume can be ruled out in this case.

A slight decrease in particle size was observed upon the addition of the surface modification in the form of DSPE-PEG\textsubscript{2000} and Fol-PEG-Chol, which agrees with reported studies regarding folate and PEG attachment on liposomal surface (136). Reports have shown that the addition of a hydrophilic PEG may increase the trapped volume in the liposomes due to its facilitation of membrane fusion during the freezing process which has been shown to be proportional to the concentration of PEG in previous literature (137). This is reflected in the larger decrease in the particle size upon pre-insertion as opposed to the slight decrease upon post-insertion of the modifications.

All liposomal formulations were negatively charged which may be attributed to the presence of soy phosphatidylcholine and DCP. However, upon the addition of PEG, a decrease in the negative charge is observed as seen in table 8. PEG coating has
been known to cause a shielding effect in nanoparticles for PEG with molecular weight above 10 kDa through steric stabilization by the PEG corona (138).

Additionally, the lowering of the zeta potential may also be attributed to the presence of positively charged folic acid.

Figure 26: Effect of freeze thaw cycles on multilamellar liposomal size

To evaluate the effect of freeze thaw cycles on liposome lamellarity, the extruded liposomes were freeze thawed 0, 3, 5 and 11 times and the change in particle size with number of freeze thaw cycles was observed. As seen in figure 26, there was a proportional increase in particle size with an increase in freeze thaw cycles. The particle size remained unchanged after 11 cycles and all further studies were done with 11 cycles as the standard for freeze thawing.
Different cryoprotectants were evaluated for their ability to inhibit particle size increase upon freeze-drying. Lactose, sucrose, maltrin-100 and trehalose were added to the liposomal suspensions before freeze drying (5:1 cryoprotectant: lipid). The results obtained are shown in figure 27. A substantial increase in particle size was observed with the use of lactose and maltrin-100. While both sucrose and trehalose limited particle size increase as compared to the liposomal formulation without cryoprotectant, trehalose had a lower particle size as compared to all other formulations and was selected as the cryoprotectant for further studies.

![Effect of cryoprotectant on particle size](image)

Figure 27: Effect of cryoprotectant type on particle size

**4.5.2 Nuclear Magnetic Resonance analysis**

The liposomal lamellarity was evaluated using the $^{31}$P NMR analysis as a preliminary analytical measure. Previous studies by Frohlich et al have demonstrated the difference in the phosphorous environments between a unilamellar and multilamellar vesicle which may result in a narrow and sharp or a wide diffused peak.
respectively (128). Figure 26 demonstrates the change in the phosphorous environments surrounding the liposome when the raw liposomal dispersion is extruded and subsequently freeze thawed.

Figure 28: $^{31}$P NMR (Scale: ppm) of (A) raw multilamellar liposome (hydrated film dispersion), (B) unilamellar (extruded) and (C) freeze thawed liposome (11 cycles)

The raw liposomal dispersion displays a wide peak that most likely resembles a mixture of ULV, large ULV’s, MLV and multivesicular liposomes. During extrusion, the number of lamellae is reduced due to the physical force applied in passing the liposomes through the polycarbonate membrane and the liposomes are reduced to the size of the polycarbonate pore size of 0.1 μm. The reduction in lamellae makes the liposomal phosphorous environment more uniform leading to a sharp narrow peak as observed in figure 28 (B). Freeze thawing of these liposomes
leads to the re-arrangement of the phospholipids present in the bilayer through the movement of drug and the lipid materials. Additionally, the fusion, aggregation and fragmentation lead to alteration of the single bilayer to a more thermodynamically stable form leading to the formation of multilamellar vesicles. It should be noted that figure 28 (C) shows a wide diffuse main peak which can be characterized into two separate peaks. This may be due to the presence of two separate populations (multilamellar and multivesicular) or a representative of two different phospholipid environment in a single vesicle (two or three bilayers in one liposome). As stated by other studies, the $^{31}$P NMR cannot differentiate between multilamellar and multivesicular vesicles (128).

The change in liposome structure with freeze thaw cycles was also evaluated. Liposomes were subjected to 0, 3 or 11 freeze thaw cycles and the results of its $^{31}$P NMR are displayed in figure 29. A sequential broadening of the sharp and narrow ULV peak is observed upon increasing freeze thaw cycles. The start of peak broadening was observed at 3 freeze thaw cycles through the appearance of a shoulder to the sharp peak. This may be representative of the formation of a new population of liposomes that have an altered phosphorous environment and may be indicative of the presence of more than one bilayer. The shoulder increases on further freeze thaw cycles and reaches a maximum at 11 cycles after which no change was observed.
4.5.3 X-ray diffraction studies

Powder XRD patterns of the ULV, 3 cycles freeze thawed MLV, and 11 cycles freeze thawed MLV was evaluated as seen in figure 30. The extruded ULV sample which contains one phospholipid bilayer without any geometrically related stacking showed a single broad peak whereas sharp peaks of increasing intensity representing regular stacking of the bilayers are seen in the 3 cycle MLV and 11 cycle MLV respectively. The diffraction pattern of the both MLV’s show two distinct peaks. The larger peak angle is observed at d value (repeat spacing) of 3.98 Å and 3.26 Å for the 3 and 11 cycle MLV respectively and a smaller peak angle at d equal to 11.6 Å and 8.52 Å for the 3 and 11 cycle MLV respectively. A small indistinct peak is also observed for the MLV’s at 2.86 Å and 2.13 Å for the 3 and 11 cycle MLV respectively. The XRD data can be correlated to the NMR analysis since the peak intensity gradually increases from the ULV to the 3 cycle MLV and is strongest for the 11 cycle MLV which shows two sharp peaks reflecting the liposomal lamellae.
Figure 30: XRD diffraction patterns of liposomes of varying lamellarity

The determination of number of lamellae can be carried out using SAXS. Figure 31 represents the SAXS for the liposomal MLV’s. The diffraction pattern shows no obvious peaks for the MLV samples. This was attributed to the large particle size (>150 nm) of the MLV’s which was higher than the maximal length scale allowed by the instrument (<70 nm).
4.5.4: Cryogenic-Transmission electron microscopy (cryo-TEM)

Cryo-TEM was performed to confirm the preliminary lamellarity analysis by NMR and XRD as well as to analyse the structure and size of the nanoparticles. Structural analysis shows that all liposomal samples were found to be spherical as seen in figure 32 (a). The extruded samples (ULV) showed the lowest particle size of 100 nm, the 3 cycle MLV showed a particle size of approximately 140 nm whereas the largest particle size was observed for the 11 cycle MLV of about 185 nm which was slightly higher than that observed by the Brookhaven zetameter (approximately 170 nm). The analysis of lamellarity by NMR and XRD was confirmed by the TEM analysis. The extruded samples showed the presence of vesicles with a single bilayer (ULV), which upon freeze thawing showed an increase in the number of bilayers (bilamellar structures, 3 cycle MLV) that increased in phospholipid content upon freeze thawing (11 cycle MLV). Most multilamellar liposomes showed the presence
of about 3-5 lamellae as seen in figure 32 (B). However, it should be noted that the 11 cycle MLV also showed the presence of a large population of multivesicular structures as shown in figure 32 (B).

Figure 32 (A): Cryo-TEM analysis of liposomal structure, size and lamellarity left: ULV, middle: 3 cycle MLV, right: 11 cycle MLV (scale bar: 100nm)

Figure 32 (B): Multilamellar vesicles with 3-5 lamellae

The dark dense matter present in the images may be due to the breaking of the carbon films due to interaction with the sample. Additionally, in certain images the liposomes are seen to cluster together due to the gradient observed during experimentation due to lack of spreading of the liquid over the carbon film. The high-
density material may also be attributed to contamination by frozen ethane or degradation of sample under the high energy used for TEM.

**4.5.5: Folic Acid concentration determination**

Folic acid present in the liposomes was evaluated by the technique mentioned in the previous section (3.4.6). The standard curve for the determination of folic acid content is shown in figure 33. The folic acid concentration entrapped in the liposomes by the pre-modification technique was determined as 65.89 ± 1.03% while that for post-inserted technique was found to be 79.44 ± 4.437%

![Folic acid standard curve](image)

Figure 33: Standard curve for folic acid content determination

**4.5.6: Determination of Entrapment efficiency and drug loading**

The entrapment efficiency of the multilamellar liposomes was tested by the HPLC technique discussed in chapter 2. The relation between entrapment efficiency and order of addition of the surface modification (pre/post) and initial drug load was
investigated. The order of addition of the PEG and Folate moieties affects the amount of drug encapsulated as seen in figure 34 (a). When PEG and folate are added to the liposomes during their formation, a lower encapsulation of the drug was observed as opposed to when they are added to pre-formed liposomes. This can be attributed to the presence of their anchors in the phospholipid bilayer of liposomes. The DSPE for PEG\textsubscript{2000} and cholesterol for folate act as anchors and are responsible for the attachment of these molecules to the surface of the liposomes. However, the presence of these increases packing density inside the phospholipid bilayer reducing the amount of hydrophobic drug that could be encapsulated within the membrane.

Following the results, post-insertion technique was used for all further evaluation of the liposomes.

Figure 34 (a): Effect initial drug load on encapsulation efficiency
Initial drug load also affected the encapsulation efficiency. Change in encapsulation efficiency was tested with three initial drug loads (2.5, 5 and 10 mg) of each drug. The results are shown in figure 34 (b). It was observed that at 2.5 mg drug load each drugs showed low encapsulation into the liposomes. Upon increase in concentration to 5 mg, the encapsulation of all drugs increases sharply. However, RG7388 the encapsulation of RG7388 significantly (p<0.05) increases as compared to the 2.5 mg dose. Further increase in dose led to varying results for the three drugs. While the encapsulation efficiency remained approximately the same for temozolomide, it significantly (p<0.05) reduced to about 4% for RG7388 and increased slightly for elacridar. It was found that elacridar and RG7388 may have competitive insertion into the phospholipid bilayer with elacridar having a larger encapsulation as opposed to RG7388. The drug initial dose was selected as 5 mg for each drug to the significantly higher RG7388 encapsulation as well as relatively high encapsulation of TMZ and elacridar.

![Figure 34 (b): Effect of conjugate addition to liposomes on encapsulation efficiency](image-url)
The drug loading for TMZ, elacridar and RG7388 was found to be 5.26 ± 1.1%, 14.97 ± 3.5 % and 1.83 ± 0.28 % respectively (n=3).

4.5.7: Differential scanning calorimetry

The encapsulation of three drugs into the multilamellar liposomes was tested by investigating the physical states of the drug through DSC analysis. The thermograms obtained are shown in figure 35. No peaks are seen in the thermogram of the blank liposomes. It was found that TMZ exhibits a sharp exothermic peak at 208.7°C, while elacridar and RG7388 exhibit endothermic peaks at 217.24°C and 283.41°C respectively. However, these peaks do not appear in the drug loaded Folate modified liposomes. The disappearance of the peaks may indicate that the drugs are present in molecularly dispersed state in the liposome, embedded inside the lipid matrix or core. Studies suggest that the presence of the drug in a dissolved state in nanoparticles may enhance their in vitro release characteristics.

Figure 35: DSC thermograms of TMZ, elacridar, RG7388, blank multilamellar liposome (MLL) and Fol modified MLL
4.5.8: Thermogravimetric analysis

The change in weight upon heating the nanoparticles was observed by thermogravimetric or TGA analysis. The results are shown in figure 36. It was observed that the highest weight loss was observed in the blank multilamellar liposomes (24.52%), followed by the drug loaded liposomes (20.83%). The least weight loss of about 6.19% was observed for the folate conjugated liposomes. The difference in the weight loss of the drug loaded liposomes and blank liposomes may be due to lower trapped volume in these particles due to the presence of the drugs with surfactant. PEG is a very large polymer that coats the particles and enhances particle hydrophilicity. Additionally, the presence of folic acid conjugate on nanoparticulate surface may reduce water adsorption onto the nanoparticles. These factors may account for the much lower weight loss observed in the surface modified liposomes.

Figure 36: TGA of blank, drug loaded, and folate modified multilamellar liposomes (MLL).

![TGA graph showing weight loss of different liposomes](image-url)
4.5.9: Drug release study

The release of TMZ, elacridar and RG7388 was examined from drug loaded multilamellar liposomes (DLL) and folate decorated drug loaded multilamellar liposomes (F-DLL) as shown in figure 37. It was observed that for all drugs, no significant difference was observed on the drug release when the nanoparticles were surface modified with folate. However, a significant difference (p<0.05) was observed in the release of elacridar and RG7388. The most rapid as well as highest release is seen for elacridar in both surface modified and non-modified particles. Elacridar is quickly released and reaches about 69% (w/w) and 80% (w/w) for the non-modified and modified particles respectively within 12 hours, after which the release continues to increase. At 72 hours, the release of elacridar was found to be 88% (w/w) and 93% (w/w) respectively for the DLL and F-DLL respectively which is significantly higher than the 12-hour release (p<0.05). The high release of elacridar may be due to its high concentration dependent release from the phospholipid barrier directly into the release medium. TMZ shows a much lower release than elacridar with a cumulative release of 76% (w/w) and 65% (w/w) for the DLL and F-DLL respectively. TMZ is a hydrophilic drug which is encapsulated into the liposomal aqueous core. TMZ would face an additional barrier in the form of the liposomal phospholipid bilayer to be released into the medium, which may be the reason for its lower release.

The lowest release was observed for RG7388 with approximately 49% (w/w) and 53% w/w release from the DLL and F-DLL respectively in 72 hours. The drug release of RG7388 may be inhibited by the competing high release of elacridar from the same phospholipid bilayer pores. The maximal release for RG7388 is observed within 12 hours after which the concentration of drug does not significantly increase (p<0.05) for both DLL and F-DLL systems.
Figure 37: Cumulative drug release of TMZ, elacridar and RG7388 from drug loaded and folate decorated drug loaded multilamellar liposomes.

4.6 Summary for specific aim 3

5. Multilamellar liposomes were prepared by the thin film hydration technique

6. All liposomes (blank, drug loaded and surface modified) were found to be negatively charged and their particle sizes were under 200nm

7. The liposomes were found to have a high folic acid concentration of approximately 79%

8. Liposomal lamellarity was determined by $^{31}$P NMR and powder XRD and confirmed by cryo-TEM
9. The number of freeze thaw cycles was found to affect liposomal structure and 11 cycles were found to be optimal for the formulation of multilamellar liposomes

10. DSC and TGA analysis confirm encapsulation of drugs in liposomal system through change in physical state of drug

11. The encapsulation efficiency was altered by initial drug load as well as the order of insertion of the surface modification

12. Five mg drug load and post-insertion of PEG and folate were found to optimize the encapsulation of all three drugs

13. The percent cumulative release of drugs from the conjugated multilamellar systems was 93%w/w, 65%w/w and 53%w/w for elacridar, TMZ and RG7388 respectively

14. No significant differences in release characteristics of RG7388, elacridar and TMZ upon folate modification of drug loaded multilamellar liposomes
Chapter 5:

Formulation and characterization of folate decorated PLGA nanoparticles in liposomes
5.1 Introduction

Polymers have been widely used as carrier systems for drug delivery to the brain due to their biocompatibility and ability be modified to attain purpose specific physicochemical properties. Additionally, FDA approved polymers like PLGA have been employed as nanocapsules or nanospheres for passage across the blood brain barrier since they can be formulated into ultra-nano sized delivery systems. However, their limitation arises from the quick systemic degradation and low capability to encapsulate hydrophilic drugs. Novel emerging studies have now focused on the ability to formulate a hybrid system of a lipid and polymer that can encapsulate one or more drugs for brain specific delivery.

In this study we have formulated a vesicle in vesicle system by encapsulating PLGA nanoparticles inside a liposomal system. Therapeutics administered in nanoparticulate systems often show early release of the drugs into the systemic circulation thereby reducing the drug that reaches the target sites like the lung and brain. Moreover, nanoparticulate and drug elimination by the RES may further reduce drug efficacy due to reduced concentration in the systemic circulation. Certain diseases like gliomas may requires prolonged drug exposure for therapeutic efficacy which may not be attained by traditional systemic administration. The combination of a lipid and polymeric nanoparticles may help overcome these difficulties. This study aimed at designing a dual-purpose liposomal carrier system that would encapsulate multiple drugs at their therapeutic concentrations as well as sustain the release of drugs while remaining non-toxic and biocompatible.
Although both hydrophilic and hydrophobic drugs have been encapsulated into the polymeric nanocapsule formed by the PLGA nanoparticles, in this study we have selected to encapsulate RG7388 into the PLGA nanoparticles. RG7388 belongs to the nutlin class of MDM2 inhibitors. Although most nutlin inhibitors have very low IC$_{50}$ values, their loading into nanoparticulate systems is often low due to the very high hydrophobicity (45). Additionally, the release of elacridar is required before or simultaneously with the chemotherapeutics (TMZ and RG7388). Due to these factors, RG7388 was selected as the compound to be encapsulated into the polymeric nanoparticle which would be further entrapped within the liposomal core so that the prolonged release of RG7388 would allow efficient therapy for GBM with TMZ.

The PLGA nanoparticles were formulated by a simple (o/w) emulsification through evaporation of the organic solvent. Pluronic F-127 was used as a surfactant in the PLGA nanoparticle formulation. The drug encapsulated nanoparticles formed were first freeze dried and then reconstituted in the aqueous liposomal hydration buffer for incorporation into the liposomes. Studies have shown that the size of PLGA nanoparticles can greatly affect the release of encapsulated material as well as its movement through membranes like the blood tumor barrier. Thus, a key goal for the study was to optimize particle size to achieve maximal drug load with minimal particle size. It is known that process parameters like time of sonication, amount and concentration of the constituents and surfactants used can greatly affect the particle size, thus these parameters were optimized for this study.
5.2 Materials

Poly (lactic-co-glycolic acid) (PLGA) 50:50 (0.17 dL/g), Pluronic F-127, Polyvinyl alcohol (PVA), Polysorbate 80 were purchased from Sigma Aldridge (St. Louis, USA), Dichloromethane, Ethyl acetate and Acetone (optima grade) were purchased from Fischer Scientific (Fair Lawn, USA), Dicetyl phosphate (DCP) was purchased from ChemCruz Biochemicals (Dallas, USA), DSPE-PEG<sub>2000</sub> was purchased from Avanti Polar Lipids (Alabaster, USA), Temozolomide was purchased from SelleckChem (Houston, USA), Elacridar was purchased from Toronto Research Chemicals (Ontario, Canada), RG7388 was purchased from Chemietek Organics (Indianapolis, USA).

5.3 Methods

5.3.1 Preparation of PLGA nanoparticles

The PLGA nanoparticles were prepared by an (o/w) emulsification technique followed by solvent evaporation as seen in figure 38. A known quantity of PLGA 50:50 (0.17 dL/g) was weighed in a 20 mL scintillation vial along with the surfactant Pluronic P-127. The materials were dissolved in 2 mL of an organic solvent (DCM, DMF, acetone or ethyl acetate). The aqueous phase consisted of a solution of polyvinyl alcohol and 0.1% tween 80. The organic phase was added dropwise in the aqueous phase through a 28-gauge syringe (BD U-100 Micro-Fine IV Syringe, 1cc) over 5 minutes under magnetic stirring. The emulsion was immediately placed over ice and sonicated by ultrasonication (Misonix Sonicator 3000, Farmingdale, NY) for a specified amount of time (2, 4 or 8 minutes) at 40 amplitude. The resultant nanoparticulate suspension was then transferred to a 15mL Thermo centrifuge tube.
and centrifuged at 4000 rpm for 10 minutes at 4°C to remove any debris from the ultrasonication. The suspension was then diluted with 0.5% PVA for solidification and stabilization of the nanoparticles. The organic phase was evaporated by overnight magnetic stirring. The last step included the addition of trehalose dihydrate (1% w/w) followed by freeze drying the PLGA nanoparticles by Millrock Technology LD85 freeze dryer (Kingston, NY).

Figure 38: Scheme of preparation of PLGA nanoparticles

Drug loaded PLGA nanoparticles were prepared by the dissolution of 15 mg RG7388 into the organic solvent with PLGA and Pluronic P-127. The freeze-dried nanoparticles were refrigerated at 4°C until further incorporation into the liposomes. The amount of RG7388 encapsulated in the PLGA nanoparticles was determined by the procedure discussed in section 3.4.8. The PLGA particle size of the PLGA nanoparticles was optimized by the adjustment of:

- Type of organic phase used
- Type and concentration of oil phase surfactant
- Type and concentration of PLGA
- Concentration of PVA
- Sonication time

### 5.3.2 Incorporation of the PLGA nanoparticles in the liposomes

The blank and drug loaded PLGA nanoparticles were incorporated into the liposomes by the addition of the nanoparticles into the hydration buffer of the liposomes as shown in figure 39. A quantity of the freeze dried PLGA nanoparticles equivalent to 5 mg of the drug was weighed into a 15 mL Thermo centrifuge tube and reconstituted in the liposome aqueous phase of 1% polysorbate 80 containing acetate buffer (pH 5.0). Five mg of TMZ was weighed into the centrifuge tube. The suspension was vortexed for 2 minutes and sonicated in a water bath for one minute. This drug containing aqueous phase was used to hydrate a lipid film formed of SPC: Chol: Captex 300 (70:20:10) with 3.2 mg DCP and 5 mg elacridar. The raw liposomal dispersion formed was vortexed for 5 minutes followed by sonication in a water bath for 45 minutes.

PEG and folate modification was carried out by the addition of the surface modification moieties as mentioned in section 3.3.2. Here, for the pre-insertion technique the modifications were added to the lipid mixture during thin film formation, whereas post modification was carried out after the bath sonication of pre-formed PLGA in liposome system. The liposomes thus formed were filled in cellulose dialysis bags of 12–14 kDa MWCO and dialyzed against acetate buffer containing 5% (w/w) dextrose for 12 hours. The resultant suspension was then freeze dried to obtain the folate and PEG modified PLGA in liposome (PLL) nanoparticles.
5.4 Characterization of PLL nanoparticles

5.4.1 Evaluation of particle size and zeta potential

The nanoparticles were tested for their particle size and zeta potential using the Brookhaven Zetameter (ZetaPlus, Brookhaven Instruments Corporation, NY). The particle size and zeta potential were measured by dilution of 100 microliters of the PLGA nanoparticles or PLL samples with 10 mL of 0.45 μm filtered acetate buffer. The relationship between particle size and organic phase, sonication time and concentration and type of surfactant, PVA or PLGA used was evaluated. Five measurements were carried out for each sample. All samples were tested in triplicates.

5.4.2 Nuclear Magnetic Resonance analysis

The PLL sample was tested by Nuclear Magnetic Resonance for determination of nanoparticle environment and structure by $^{31}$P NMR analyses using the technique
discussed previously in section 4.4.2. The PLL sample was diluted to 50 mM lipid using 10 % D$_2$O as the solvent. All samples were tested in triplicate.

**5.4.3: Electron microscopy**

Cryogenic-Transmission electron microscopy (cryo-TEM) was performed on the blank PLL samples to determine particle structure as discussed in chapter 4. The PLL samples were diluted to 3 mg/mL in acetate buffer and the grids were glow discharged for 30s. A magnification 4700 times was used.

**5.4.4: Folic Acid concentration determination**

The folic acid concentration present on the PLL surface was determined after modification by the pre-insertion and post-insertion techniques against a standard curve of folic acid. The procedure for standard curve preparation has been discussed in chapter 3. The concentration range for standard curve was 0.1-10 mg/mL. The samples obtained from the pre-insertion and post insertion technique was prepared by dissolving 10 mg of the freeze dried PLL sample in 1 mL of the NaOH: methanol solvent system. Two hundred µL of this stock was then added to wells in a 96 well plate and read at 285 nm wavelength on a Synergy H1 hybrid UV Spectrophotometer for determination of the folic acid content.

**5.4.5: Thermogravimetric analysis**

The change in the weight of the sample on heating was evaluated using a thermogravimetric analyzer (Shimadzu DSC-60, Kyoto, Japan). The freeze-dried
sample (7-16 mg) was weighed in an aluminium pan and heated in a nitrogen environment (20 mL/ minute) from 20°C to 300°C at a constant rate of 10°C per minute. The samples were tested in triplicate and the percent weight loss was evaluated.

5.4.6: Differential Scanning colorimetry

DSC was performed to determine the physical states of the drugs in the nanoparticulate carrier system. About 4-6 mg of the freeze-dried samples of pure drugs, blank PLL and drug loaded PLL were weighed in an aluminium pan and crimped. Characteristic sample properties were observed against a blank reference pan by heating both pans from room temperature to 300°C at a rate of 10°C per minute under a nitrogen environment of 20mL/ minute. The samples were tested in triplicate.

5.4.7: Determination of Entrapment efficiency and drug loading

The entrapment efficiency of the PLL liposomal system was evaluated by dissolving 10mg of the drug sample in the 2mL of HPLC mobile phase consisting of Ammonium acetate: Acetonitrile (50:50) by vortexing for 5 minutes followed by bath sonication for an additional 2 minutes. The samples were then filtered through a 0.2 μm syringe filter and the quantity of temozolomide, elacridar and RG7388 was determined by HPLC. The standard curve for the three drugs was prepared as per the procedure mentioned in section 2.3.3. Ten mg of each temozolomide, elacridar and RG7388 was dissolved in 10mL of the mobile phase for the standard curve. Entrapment efficiency was calculated as shown in formulae below:
The amount of drug loaded in the liposomes was determined by the formula below:

\[
\text{Entrapment efficiency(\%)} = \frac{\text{Amount of drug in the formulation}}{\text{Initial drug added to formulation}} \times 100
\]

The effect of order of surface modification (Pre/ Post insertion) on the encapsulation efficiency of the three drugs was evaluated in this study.

\[
\text{Drug loading (\%)} = \frac{\text{Amount of drug in the formulation}}{\text{Total weight of the formulation}} \times 100
\]

5.4.8: \textit{In vitro release study}

The aim of the study is to design a nanoparticulate carrier system that would simultaneously encapsulate multiple drugs and prolong their release kinetics. In this study, we aimed at prolonging the release of RG7388 for glioblastoma therapy. The release of the three drugs was determined by the \textit{in vitro} technique described in section 4.4.9. Ten mg of the freeze dried PLL was dissolved in acetate buffer and the sample was filled in cellulose dialysis bags with MWCO of 12–14 kDa and placed in the release medium comprising of phosphate buffer pH 5.0 with 1% (w/v) polysorbate 80 as the release medium at 37°C. A volume of sample (500 μL) was collected from the beaker at pre-determined time intervals (0, 0.08, 0.16, 0.33, 0.6, 1, 2, 4, 8, 12, 24, 48 hours) and filtered through a 0.2 μm syringe filter. The volume of sample was replaced by an aliquot of fresh release media at each time point. The sample collected was evaluated for amount of drug using HPLC.
5.4.9 Statistical analysis

All experiments were analysed for statistical significance by using the two tailed Student’s t-test and ANOVA (analysis of variance). A statistically significant difference was defined as a p-value less than 0.05.

5.5: Results and Discussion

5.5.1 Evaluation of particle size and zeta potential

The results of particle size and zeta potential for the PLL blank and drug loaded liposomes are shown in figure 40. The influence of different parameters including type and concentration of surfactant and PLGA, sonication time and organic phase on particle size was evaluated. It was observed the particle size observed when Pluronic F-127 was used as the surfactant present in the organic phase was significantly lower than when poloxamer 68 or span 60 were used as the surfactant (p <0.05) as seen in figure 40 (a). Thus, for all further studies Pluronic F-127 was selected as the surfactant.

There is a large dispute amongst studies investigating the formulation of PLGA nanoparticles for the polymer to surfactant ratio. The role of the surfactant is to stabilize the particles and, in some studies, suggest that the surfactant exert these effects by increasing the compactness of the polymer. To investigate this, two surfactant concentration of polymer to surfactant ratio of 2:1 and 1:1 was investigated for all surfactants. It was found that there was a significant difference in the particle size on changing the surfactant concentration for Span 60 and P-127 (p<0.05), but not
For P-68. For all further studies, the concentration of the surfactant Pluronic-127 was fixed to a 2:1 polymer to surfactant concentration.

Figure 40 (a): Effect of surfactant type and concentration on particle size of the PLL nanoparticulate system (n=3)

PLGA with different lactide: glycolide ratios were tested in varying concentrations to attain nanosized particles. The results are displayed in figure 40 (b). The lowest particle size was observed for PLGA 50:50 (0.17 dL/g) which was significantly lower than the two other grades of PLGA i.e. 25:75 and 75:25 used (p <0.05). Thus, PLGA 50:50 at an inherent viscosity of 0.17 dL/g was selected for all further studies. The analysis for effect of PLGA concentration revealed that there was no significant difference in the effect on particle size when 60 or 100 mg of PLGA 50:50 was used. Due to the more uniform results obtained by using 100 mg PLGA, it was selected for all further studies.
Figure 40 (b): Effect of PLGA type and concentration on particle size of the PLL nanoparticulate system (n=3)

The effect of different organic solvents on PLGA nanoparticle size was evaluated. The organic solvents commonly employed for PLGA nanoparticle formulation were selected as seen in figure 40 (c). The solvents included dimethylformamide (DMF), acetone, dichloromethane (DCM) and ethyl acetate. The lowest particle size was obtained with the use of ethyl acetate as the organic solvent which was significantly lower than all the other solvents tested (p<0.05).
Additionally, the effect of PVA concentration on particle size was tested as seen in figure 40 (d) and it was observed that there was a non-significant decrease in the particle size from 1 to 2.5 to 5% w/v of PVA. Despite the insignificant change in particle size, we selected 5% w/v PVA as the stabilizer for the PLGA nanoparticles due to the smaller particle size.
Lastly, the relationship between sonication time and PLL particle size was explored. The results are shown in figure 40 (e). It is known that long sonication times can lead to excess energy production in the sample causing its degradation preventing long term use (139) (140). The sonication times tested included 2, 4 and 8 minutes. It was found that the particle size decreased as the sonication time was increased from 2 to 4 minutes and further sonication led to the non-significant increase in particle size. Thus 4 minutes was selected as the optimal sonication time to reduce thermal stress in the PLGA nanoparticles.
Figure 40 (e): Effect of sonication time on particle size of the PLL nanoparticulate system (n=3)

Table 9: Particle size and zeta potential

<table>
<thead>
<tr>
<th>Nanoparticle</th>
<th>Particle size (nm)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLGA nanoparticle</td>
<td>67.4 ± 8.9</td>
<td>-8.1 ± 2.6</td>
</tr>
<tr>
<td>RG7388 loaded PLGA nanoparticle</td>
<td>85.9 ± 4.3</td>
<td>-6.2 ± 1.3</td>
</tr>
<tr>
<td>Blank PLL</td>
<td>127.8 ± 8.2</td>
<td>-18.8 ± 2.8</td>
</tr>
<tr>
<td>Drug loaded PLL</td>
<td>178.6 ± 7.9</td>
<td>-9.1 ± 1.9</td>
</tr>
<tr>
<td>Blank surface modified PLL</td>
<td>138.1 ± 1.8</td>
<td>-11.4 ± 1.1</td>
</tr>
</tbody>
</table>
Drug loaded surface modified PLL | 198.4 ± 1.7 | -5.7 ± 0.3

A non-significant increase in particle size was observed between the blank and drug loaded nanoparticles and the blank and surface modified nanoparticles (p>0.05). A significant increase (p<0.05) was observed in the particle size when the PLGA nanoparticles were formulated into drug loaded and surface modified PLL. The zeta potential for all particles showed the presence of negatively charged particles. Like the results obtained in the previous section, the addition of PEG and folate caused a non-significant decrease in the negative charge as observed in table 9 due to possible shielding effect of the large PEG at the liposomal surface.

5.5.2 Nuclear Magnetic Resonance analysis

The phosphorous environment of PLL system was evaluated using $^{31}$P NMR analysis. The PLL system consists of PLGA nanoparticles surrounded by a lipid vesicle. Since the liposomes are only treated with bath sonication, it was expected that there may be both unilamellar and multilamellar liposomes encapsulating the nanosized PLGA particles. While the NMR does show the presence of some multilamellar, it is unable to distinguish between systems that are multilamellar and multivesicular as discussed in previous studies by Frohlich et al and section 3.5.2 (128). The peak observed in figure 41 (a) is similar to the peak observed for multilamellar liposomes as seen in figure 41 (b). However, the NMR confirms that the phosphorous environment surrounding the final liposomes bilayer is not uniform and
that some changes in the bilayer characteristics of liposome causes the formation of a short, wider peak in figure 41.

Figure 41: $^{31}$P NMR (Scale: ppm) of (a) PLL system, (b) freeze thawed multilamellar liposomes (11 cycles)

The size, shape and structure of the PLL system was evaluated and the results of the cryo-TEM are shown in figure 42. The PLGA nanoparticles appear as dark matter scattered within and outside the liposomes. The liposomes are mostly unilamellar with some bilamellar structures as well. The presence of the dark matter in the multilamellar liposomes (section 4.5.4) as well may suggest that either the liposomes or PLGA may be undergoing transition under the high energy required for TEM or that there are contaminants present. Additionally, since the dark matter vesicles could also potentially be smaller liposomes or lipid vesicles in vesicles the presence of PLGA inside the liposomes, the TEM cannot ascertain that the PLGA
nanoparticles are encapsulated inside the liposomes or present as matter outside the liposomal vesicles.

![Figure 42: Cryogenic TEM image of PLGA nanoparticles in liposomes](image)

**5.5.4: Folic Acid concentration determination**

Folic acid present in the liposomes was evaluated by the technique mentioned in the previous section (4.4.5). The standard curve for the determination of folic acid content is shown in figure 43. The folic acid concentration entrapped in the liposomes by the pre-modification technique was determined as 81.32 ± 6.91% while that for post-inserted technique was found to be 80.81 ± 2.64%
There was no significant difference between the pre and post surface modification technique for the PLL. Additionally, the difference in amount of folic acid concentration in the multilamellar liposomes and PLL was found to non-significant for both, the post-insertion and pre-insertion techniques. A possible explanation for this might be due to the presence of the hydrophobic drug/s, cholesterol and triglyceride in the bilayer which govern the area in the phospholipid membrane available for the loading of the conjugate.

5.5.6: Determination of Entrapment efficiency and drug loading

The entrapment efficiency of the PLL was tested by the HPLC technique discussed in chapter 2. The relation between entrapment efficiency and order of addition of the surface modification (pre/post) was investigated. The order of addition
of the PEG and Folate moieties affects the amount of drug encapsulated as seen in figure 44.

![Graph]

Figure 44 (a): Encapsulation efficiency of TMZ, elacridar and RG7388 in PLL by pre-modification and post-modification techniques compared to the encapsulation of the drugs in multilamellar liposomes (MLL)

The order of addition of the surface modification has no significant impact on the encapsulation of elacridar, RG7388 or TMZ. However, compared to post-modified multilamellar liposomes, the encapsulation of RG7388 significantly increases from $12.72 \pm 1.489\%$ to $33.10 \pm 4.16\%$. There is no significant change in the encapsulation efficiency of TMZ or elacridar between the post modified PLL and
multilamellar samples. The increase in RG7388 can be attributed to its encapsulation in the polymer matrix due to which there is limited competitive encapsulation of RG7388 with elacridar.

The drug loading for TMZ, elacridar and RG7388 was found to be $4.66 \pm 0.91\%$, $17.25 \pm 1.9\%$ and $4.91 \pm 2.61\%$ respectively ($n=3$). As compared to the multilamellar liposomes (post-modification), there was no significant difference in the drug loading of elacridar and TMZ, however the drug loading for RG7388 increased significantly by nearly 2.5 times. Thus, the post-modification technique was selected for the preparation of surface modified PLL systems.

5.5.7: Thermogravimetric analysis

The weight loss between the blank and drug loaded PLL was measured by thermogravimetric analysis, which also provided an insight to the possible water content present in the nanoparticles. The results for the TGA are shown in figure 45. It was observed that the weight loss in the PLGA nanoparticle was very high as compared to all other formulations (approximately 37.688%). This could in part be due to the very hygroscopic nature of the PLGA polymer as well as the low polymer to PVA solution ratio (organic to water phase) which may increase the aqueous volume entrapped in the polymer matrix. When the polymer in the form the PLGA nanoparticle is encapsulated into the liposome, it was observed that the weight loss reduces greatly, which reinforces the assumption that the PLGA loss can be attributed as water loss. The change in weight from the blank PLL to the drug loaded and folate modified drug loaded liposomes follows the same trend as the multilamellar
liposomes. The total weight loss in the folate modified liposomes is the least (1.249%).

Figure 45: TGA thermograph of blank PLGA nanoparticles and blank, drug-loaded and folate modified drug loaded PLL

5.5.8: Differential scanning calorimetry

The physical states of the drugs in the PLL system was evaluated by comparison of the DSC thermograms of the drug, blank and drug loaded folate modified PLL systems as shown in figure 46. The DSC thermogram demonstrates that TMZ shows its characteristic sharp exothermic peak at 208.7°C, while RG7388 and elacridar show melting endothermic peaks at 283.41°C and 217.24°C respectively. It was observed that blank PLL liposomes show no specific peaks while the folate modified PLL systems show a similar thermogram with quickly changing smaller
peaks with small uncharacteristic peaks at 148.3, 159.4, 205.7, 218.2, 237.9 and 285.8 degrees Celsius. However, since no fingerprint drug peaks are observed, it may be concluded that the drugs are embedded in the liposomes in a dissolved state. The results are in close agreement with that of the multilamellar liposomal system.

![DSC thermograph of blank PLGA nanoparticles and blank, drug-loaded and folate modified drug loaded PLL](image)

Figure 46: DSC thermograph of blank PLGA nanoparticles and blank, drug-loaded and folate modified drug loaded PLL

### 5.5.9: Drug release study

The *in vitro* release characteristics of the drugs were studied by investigating the release from the drug loaded PLGA nanoparticles in liposomes (DL PLL) and Folate modified DL PLL (DL F-PLL) as shown in figure 47. The results of comparison between the DL PLL and DL F-PLL are in agreement with those observed with the multilamellar systems. However, it was found that the release
characteristics were significantly different in the PLGA liposomes as compared to the multilamellar liposomes for elacridar and RG7388. It was found that in comparison to the multilamellar system where there is a significant increase (p<0.05) in the concentration released from 12 to 72 hours, the PLL systems show that the release of elacridar reaches a high at 12 hours (approximately 85%w/w) after which the release does not increase and remains at 88%w/w at 72 hours. This may be due to the lone presence of elacridar (without RG7388) in the phospholipid bilayer of the liposomes.

Although the extent of release for RG7388 remained like that observed by the multilamellar liposomes, the release characteristics were different. The multilamellar system shows a faster release of the drug in the initial 12 hours of about 45%(w/w) which plateaus over until the 72 hours; the PLL system shows a much slower release of 28%(w/w) at 12 hours which increases significantly to 54%w/w over 72 hours, thus showing sustained release. The encapsulation of RG7388 inside the PLGA nanoparticles possibly adds an additional barrier to the exposure of the drug to the release media, slowing its release. There was significant difference in the release of RG7388 from the multilamellar system at 12 hours as compared to the PLL system. The slower release from the PLL system demonstrates a more sustained system as compared to the MLL system. The highest release is observed for elacridar, RG7388 and TMZ which also follows its drug loading.

There was no significant difference in the release of TMZ between the multilamellar and PLL systems or the surface modified and unmodified systems. Approximately 63%(w/w) and 68%(w/w) of the drug was released from the non-
surface modified and modified PLL systems respectively. The drug encapsulation of TMZ in both systems was similar, which can explain the similar release between the two systems.

Figure 47: Cumulative drug release vs time for the PLL system

5.6 Summary

1. Multilamellar liposomes were prepared by the thin film hydration technique
2. All liposomes (blank, drug loaded and surface modified) were found to be negatively charged and below 200 nm
3. The liposomes were found to have a high folic acid concentration of approximately 79%
4. Liposomal lamellarity was determined by $^{31}$P NMR and powder XRD and confirmed by cryo-TEM

5. The number of freeze thaw cycles was found to affect liposomal structure and 11 cycles were found to be optimal for the formulation of multilamellar liposomes

6. The DSC and TGA studies suggest that the drug is present in the dissolved state in inside the liposomes

7. The encapsulation efficiency was altered by initial drug load as well as the order of insertion of the surface modification

8. Five mg drug load and post-insertion of PEG and folate were found to optimize the encapsulation of all three drugs

9. The drug release for RG7388 and elacridar in the PLL is significantly different than the release shown by the multilamellar liposomes, TMZ release remains similar to the multilamellar liposomal release

10. The surface modification does not significantly affect the in vitro release of the drugs from the liposome
Chapter 6:

*In vitro* testing of cytotoxicity, uptake and permeability in GBM and BBB cell culture models
6.1 Introduction

Investigation of the \textit{in vitro} behaviour of pharmaceutical formulations in cell culture models is often employed as a preliminary determinant of its behaviour \textit{in vivo}. Cell culture models that are used for these tests often represent the disease or condition for which the treatment is investigated or a target site at which the therapeutic action is desired. The tests performed assess the safety of the pharmaceutical formulation as well as the mechanism in which it acts and efficacy of treatment. We have formulated two novel liposomal systems encapsulating multiple therapeutics as therapy for GBM. A major hurdle for GBM therapy is the delivery across the stoic BBB which limits drug passage.

The evaluation of cytotoxicity was done by using the MTT cytotoxicity assay. MTT (3-(4, 5- Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H tetrazolium bromide) is an agent that reveals the metabolic activity shown by living cells. Living cells possess certain mitochondrial NAD(P)H-dependent cellular oxidoreductase enzymes that are not present in non-viable cells. When the MTT dye is added to cells, the viable cells containing these enzymes can metabolize the dye to form purple coloured insoluble formazan crystals. The quantity of formazan crystals can be determined through colorimetric determination using UV analysis. The extent of reduction would be proportional to the number of viable cells thus reflecting the toxicity encountered by cells due to any xenobiotic exposure.

Current research trends have promoted the formulation of site specific or targeted nanoparticle therapy for increasing drug efficacy. The true ability of these
targeted materials to reach the target is evaluated by in vitro cellular uptake studies. In these studies, in vitro cell cultures representing the target site are exposed to the nano-systems for a pre-determined incubation period. The extent of nanoparticles taken up by the cells is then evaluated by analytical techniques for quantitative (e.g. HPLC, UPLC) or qualitative (confocal or flow cytometry) measurement.

Two in vitro cell culture models representing GBM for the evaluation of cytotoxicity and uptake were used. The cell lines used include the U87MG (U87), which is the most widely used in vitro cell model for glioblastoma and clinical isolate, GBM108. The use of two cell culture models for GBM ensured a more rounded reflection of the invasive nature of the disease as cell models like U87 may not be as aggressively invasive as the disease itself.

Nanoparticle transport across the BBB can be evaluated in vivo or in vitro in cell culture models. In this study, we evaluated the permeability of the both liposomal formulations in an in vitro BBB model by the analyses of the diffusion of nanoparticles across a monolayer of cells. The cell monolayer was grown on a Transwell insert, following which it was placed between the donor and receptor compartments of a diffusion chamber. The nanoparticle permeability was determined by measuring concentrations between the donor and receiver compartments at pre-determined time points.

Madin-Darby Canine Kidney (MDCK) cells were used as the in vitro model for the BBB due to its tight junctions and barrier properties. The integrity of cell monolayers
for its cellular barrier properties is often tested by measurement of its trans-endothelial/epithelial electrical resistance (TEER). It is a non-invasive and reliable technique that measures the paracellular ionic conductance of the cell monolayers. High TEER value reflect tighter cellular junctions.

The objectives of this study were to evaluate:

A. The cytotoxicity of these nanocarriers in U87, GBM108 and MDCK cell cultures.
B. Cellular uptake of these nanocarriers in U87, GBM108 and MDCK cell cultures.
C. Measurement of the transport of nanoparticle through MDCK cell culture BBB model.

6.2 Materials

MDCK II BBB cell line was purchased from American Type Culture Collection (ATCC) (Manassas, VA), while the U87 and GBM108 were a kindly provided by the Mayo Clinic Brain Tumor Patient-Derived Xenograft National Resource through Dr. Jann Sarkaria. The Dulbecco’s Eagle’s Minimum Essential Medium (DMEM), penicillin-streptomycin, L-glutamine and sodium pyruvate were purchased from CellGro® (Manassas, USA). MTT was purchased from Acros Organics (New Jersey, USA), DMF and DMEM/F-12 was purchased from Fisher Scientifics (Fair Lawn, USA), Gibco® 0.25%, Trypsin-EDTA, FGF, EGF and Sodium Dodecyl Sulphate (SDS) was purchased from Thermo Fisher Scientific, Fetal Bovine Serum (FBS) was purchased from Atlanta Biologicals (Flowery Branch, GA).
6.3: *In vitro* cell culture

MDCK cells and the U87 cells were cultured and sub-cultured in DMEM medium supplemented with sterile 10% FBS and 1% Penicillin/Streptomycin (Pen/Strep), sodium pyruvate and L-glutamine (DMEM cell media) and incubated in a humidified chamber at 37°C and 5% CO₂ after seeding in Nunc™ EasyFlask™ 75cm² Cell Culture Flasks (Thermo Scientific). Upon confluency, the MDCK cells were sub-cultured at a ratio of 1:30 while the U87 were sub-cultured at a ratio of 1:5.

The GBM primary patient-derived cell line GBM108 was cultured and sub-cultured in DMEM/F-12 medium supplemental with 10% FBS and 1% Pen/Strep, sodium pyruvate and L-glutamine, 10 µg Fibroblast Growth Factor (FGF) Basic Recombinant Human and 10 µg of Epidermal Growth Factor EGF Recombinant Human (DMEM/F-12 cell media) and incubated in a humidified chamber at 37°C and 5% CO₂ after seeding in Nunc™ EasYFlask™ 75cm² Cell Culture Flasks. After plating, the cells were allowed to adhere for 1-4 days after which the cell culture media was changed every 2 days until confluency. The cells were sub-cultured at a ratio of 1:1.

6.4: Cell viability evaluation

6.4.1 Preparation of samples

The drug samples were prepared by diluting a freshly prepared DMSO stock solution of TMZ, elacridar and RG7388 to a final total drug concentration of 1200 µM in DMEM for MDCK and U87 cells and in DMEM/F-12 for GBM108 cells. The final DMSO concentration in all solutions was less than 0.1%(v/v). These solutions were then diluted to six concentrations with a range of 0.001-1200 µM. The drug
loaded liposomal solutions were diluted with the cell specific medium to equivalent total drug concentrations.

6.4.2 MTT cytotoxicity method

Cytotoxicity of the blank and drug loaded liposomal formulations with and without surface modification was determined by the tetrazolium dye (MTT) colorimetric assay. A solution of the MTT was prepared by dissolving the MTT reagent in sterile phosphate buffered solution (PBS) pH 7.4 as a 5 mg/mL solution. The MDCK, U87 and GBM cells were plated in 96-well plates as shown in table 10. The cells were incubated overnight in a humidified chamber at 37°C and 5% CO₂. Following the overnight incubation, the cells were treated with 100 μL of specified concentrations of TMZ, elacridar or RG7388 solutions or the blank and drug loaded liposomal formulations with and without surface modification for 24, 48 and 72 hours (n=3). All formulations were prepared in PBS. A control consisting of 3 wells with cells not exposed to any treatment and supplemented with cell medium with 0.1% DMSO and a double control with cells without any treatment or DMSO was maintained in each plate.

Table 10: Seeding density and media for MTT assay of different cell lines

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Seeding density (cells per well)</th>
<th>Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDCK</td>
<td>1.0 X 10⁴</td>
<td>DMEM cell media</td>
</tr>
<tr>
<td>U87</td>
<td>3.0 X 10⁵</td>
<td>DMEM cell media</td>
</tr>
<tr>
<td>GBM108</td>
<td>3.0 X 10⁵</td>
<td>DMEM/F-12 cell media</td>
</tr>
</tbody>
</table>
At the end of treatment, the drug or nanoparticle containing media was aspirated from the wells and all cells were washed with sterile PBS. A solution of MTT (30 μL) was added to all wells and the cells were returned to incubation for 4 hours, after which the MTT solution was removed and the cells were treated with a solution of 20% (w/v) SDS: DMF. The cells were then placed on an orbital shaker (MaxQ 4450, Thermo Scientific) at 37°C for an hour. The amount of formazan crystals was then evaluated by spectroscopic analysis at 540nm.

6.5 Evaluation of cellular uptake

The cellular uptake of both liposomal nanoparticles with or without surface modification was investigated against the uptake of TMZ, elacridar and RG7388 solutions in MDCK, U87 and GBM108 cell lines. Individual cell lines were plated in Nunc™ 6-well tissue culture plates (Thermo Scientific) at a seeding density of 4.5 X 10^6 cells/well. The plates were incubated in a humidified chamber at 37°C and 5% CO2 until the cells reached confluence.

A solution of TMZ, elacridar and RG7388 was prepared by the dilution from a freshly prepared stock solution made by the procedure stated in section 6.4.1. All liposomes were diluted and adjusted to equivalent drug concentrations. Both the drug stock solution and liposomal dilutions were carried out with DMEM for MDCK and U87 cell lines and DMEM/F-12 for the GBM 108 cell line. The cells were treated with 2 mL of these drug or liposomal solutions upon confluency into a monolayer. The treatment was removed at 0.5, 1, 2, 4 and 6 hours by aspiration of the well after which the treated cells were washed three times with ice-cold PBS to remove residual non-
adherent particles like drug crystals or nanoparticles. A cell lysis solution of 1%(v/v) Triton X-100 was added to the wells and the solution was mixed by light mechanical scraping.

A portion of the cell lysate was collected in micro-centrifuge tubes and centrifuged for 5 minutes at 4oC at 24,000g. The supernatant was analysed for TMZ, elacridar and RG7388 content by HPLC analysis by the method described in chapter 2. BCA assay (Pierce, Rockford, IL) was performed on the remaining lysate (20μL) for determination of total cellular protein content. The results for cellular uptake were reported as the concentration of drug (μg) per total protein concentration (mg). All samples were evaluated in triplicates.

6.6 Permeability across MDCK cells

The extent of transport of the drug solutions and liposomal formulations was tested in an in vitro BBB model comprised of MDCK cells. The Permegear side-by-side (Permegear Inc. Hellertown, USA) assembly was used for this study.

6.6.1 Preparation of drug and liposomal solutions

A solution of TMZ, elacridar and RG7388 was prepared by the dilution from a freshly prepared stock solution prepared by the procedure stated in section 6.4.1. Both multilamellar and PLL liposomes were diluted and adjusted to equivalent drug concentrations. All dilutions were done in Dulbecco’s PBS (DPBS, pH 5.0) containing 1% tween 80. The stock preparation and dilutions were done on the day of the experiment to prevent any drug degradation in aqueous media.
6.6.2 Method of permeability study

MDCK cells were seeded on the Transwell inserts (BD Falcon Transwell 6 well plates, Franklin Lakes, NJ), having a 24.5mm polycarbonate filter bottom; at a seeding density of 4.5 X 10^6 cells per well using DMEM. The lower chamber of Transwell plate was filled with 1.5mL DMEM as well. These plates were incubated in a humidified chamber at 37°C with 5% CO₂ until a uniform monolayer was formed. Integrity of the monolayer was tested by evaluating its TEER values. The permeability was tested using the PermeGear Inc. side-bi-side H3 diffusion chambers (Hellertown, PA). The diffusion chamber was fixed by mounting the MDCK cell monolayer containing Transwell inserts between the donor and receptor compartments. The cells were equilibrated by the addition of 3 mL DPBS (pH 5.0) in both donor and receptor compartments and incubation for 15 minutes. Post equilibration, the DPBS from the donor compartment was removed and 3 mL of drug solutions or liposomal samples were added to the donor chamber. At pre-determined time points 200 µL of the sample was removed from the receptor compartment and the replaced with an equivalent volume of DPBS to maintain sink condition. The sample was filtered through a 0.2µm filter and analysed for drug content using HPLC analysis.

It was found that prolonged incubation of cells (greater than 3 weeks) led to sharp increase in TEER values from 350-450 Ωcm² to 1400-1800 Ωcm². The two effect of increase in TEER on the permeability of nanoparticle diffusion was also studied (n=3). The apparent permeability was calculated as per a previous study and is shown in equation 1 (141).
\[ P_{\text{app}} = \left( \frac{dQ/dt}{A \times C_0} \right) \]  

Eq. 1

Where Papp is the apparent permeability coefficient, dQ/dT is the rate of permeation, A is the Transwell insert surface area and Co is the initial concentration.

6.7 Results

6.7.1 MTT cytotoxicity results

Cell viability was analysed in MDCK, U87 and GBM108 cell lines. The samples tested included blank nanoparticles and a RG7388, TMZ and elacridar containing solution and MLL and PLL samples for both surface modified and non-modified samples.

6.7.1.1 MDCK cells

The results for the 24 and 72-hour cell viability is shown in figure 48. It was observed that all formulations show similar toxicity for the 24-hour study. However, there is a significant difference observed on the viability of cells treated with the drug solution and the pegylated drug loaded Fol-PEG-Chol multilamellar liposomes (F-DL MLL) and the drug loaded multilamellar liposomes (DL MLL) between the 24-hour treated and 72 hour treated samples (p<0.05). The lowest viability for the MDCK cells at 72 hours was observed for the drug solution which showed an LD50 of about 1.68 µM and the highest viability was observed for the blank liposomes which showed limited toxicity. There was no significant difference in the cytotoxicity between the pegylated plus PEG-Fol-Chol modified liposomes and non-surface modified liposomes.
Figure 48: Cell viability v/s concentration at (A) 24 hours and (B) 72 hours for the multilamellar liposomes
It was observed that the cytotoxic profile of the PLGA in liposomes (PLL) systems matched that of the 24 and 72-hour viability of the MLL. A significant decrease in the cell viability was observed between 24 and 72 hours for the drug solution, drug loaded PLL and surface modified PLL. No significant difference was observed between the surface modified and unmodified samples. All samples for the PLL showed very low cell death when tested at 24 hours.
Figure 49: Cell viability v/s concentration at (Above) 24 hours and (Below) 72 hours for the PLGA in liposome nanoparticles

At 72 hours, the highest toxicity is observed for the F-DL MLL. There is no significant difference in the toxicity profile observed between the DL PLL, F-DL PLL and the drug solution as seen in figure 49 A and B. In most cases, a slightly lower or similar toxicity is observed between these groups. A possible explanation may be due to the elacridar induced false positive observed for MDCK cells. MDCK cells express P-gp, multidrug resistant protein (MDR) in massive quantities. Elacridar is an efflux inhibitor that can act as a substrate for a wide variety of efflux systems. Thus, elacridar may affect the drug toxicity through interference with these systems and the MTT reagent, which is in agreement with previous literature (Vellonen, Honkakoski, & Urtti, 2004).
6.7.1.2 U87 cells

The results for the 24 and 72-hour cell viability for multilamellar liposomes is shown in figure 50. It was observed that the blank liposomal formulations (MLL and PLL) were non-toxic at 24 hours. The toxicity observed for the F-DL PLL is significantly higher than the drug solutions (p<0.05). Although the toxicity observed for the drug loaded liposomes was higher than the drug solutions, there was no significant difference between them. The toxicity observed was for the blank MLL, while lowest viability was observed for the F-DL MLL (6.1 µM LD50) at 24 hours.

A significant difference is observed between the 24 and 72-hour toxicity for the drug loaded MLL (surface modified and un-modified). There is no significant difference in cytotoxicity between the DL MLL and the drug solution cytotoxicity. There is a significant difference between the cytotoxicity between the drug solution and F-DL MLL samples at 72 hours.
Figure 50: Cell viability v/s concentration at (A) 24 hours and (B) 72 hours for the multilamellar liposomes
Figure 51: Cell viability v/s concentration at (A) 24 hours and (B) 72 hours for the PLGA loaded liposomes

The 24-hour MTT of the PLL systems is similar to the 24-hour cytotoxicity observed for the MLL system, however a significant difference was observed between
the viability behaviour of the DL PLL and F-PLL systems as compared to their multilamellar counterparts. Additionally, the difference was also observed with the 72-hour treatment study. The blank nanoparticles retain very low toxicity in both the 24 and 72 hours studies.

An interesting occurrence observed for both the multilamellar and PLL systems is that for the lower concentrations, the drug solutions show a sharp decrease in the cell viability after which the effect reduces significantly and the cell viability plateaus. In contrast, the cell viability reduces slowly for the lower concentrations in the nanoparticles and reduces sharply at approximately around 0.1 µM. This may be attributed to the nanoparticulate features like lipophilicity which govern the process of passive diffusion of the drug based on extent of concentration gradient between its internal and external environment. Additionally, the sharp increase in the toxicity at 72 hours is in agreement with the release trend observed previously for these particles. The toxicity for the non-drug loaded nanoparticles and drug solution remains similar for both exposure times, whereas it increases sharply for the sustained release formulations.

6.7.1.3 GBM108

The results for the 24 and 72-hour cell viability for multilamellar liposomes is shown in figure 52 A and B. The blank multilamellar formulations with and without surface modification showed some toxicity at high concentrations in the 72-hour study. The toxicity trend between the multilamellar drug loaded, multilamellar surface modified and drug loaded, and the drug solutions remains the same as that observed
with the U87, although the toxicity at 72 hours is much higher as compared to the
same formulation toxicity in the U87. A much higher cell death is observed in all
samples for the folate and PEG modified liposomes, which may be due to their
targeting of the cells through folate receptor binding. The highest LD50 was seen for
the F-DL MLL (approximately 0.21 µM), which was significantly higher than that
observed in the U87. Additionally, for the 72-hour study, a significant difference was
observed between the viability of the surface modified MLL and the drug solution, as
well as between the non-surface modified liposomes and the drug solution (p<0.001).
A significantly higher cell viability was observed in the multilamellar liposomes as
compared to the surface modified liposomes for the 72 hours study (p<0.05).
The results for the cell viability at 24 and 72-hours of the PLGA in liposomes systems is shown in figure 53 A and B. A much higher blank nanoparticle toxicity was observed at concentrations above 100 µM for both the 24 and 72 hours. The LD50 for the surface modified drug loaded liposomes was significantly higher than that of the blank liposomes (p<0.05). The cell toxicity increases in the order of drug solution< DL PLL < F- DL PLL and the difference between each is significant (p<0.05).
Figure 53: Cell viability v/s concentration at (A) 24 hours and (B) 72 hours for the PLGA loaded liposomes

6.7.1.4 Discussion
Overall among the three cell lines, the cell viability increased in the order of drug solution > DL PLL > F- DL PLL. The overall trend suggests that the toxicity is highest for the folate modified pegylated PLGA nanoparticles in liposome systems, although in some cases the difference between the surface modified and non-modified liposomes is insignificant. However, in most cases, all types of blank liposomes were found to be relatively non-toxic and showed cytotoxicity only at very high concentrations. For all formulations, the toxicity was found to be dose-dependent. More cell death was observed in all drug loaded liposomal types compared to the drugs in solution for the U87 and GBM108. In MDCK cells, the order of cell viability was reversed with the drug loaded liposomes being more viable. This may be a result of the false positive results observed with elacridar due to extreme P-gp inhibition and MTT reagent interaction (Vellonen, Honkakoski, & Urtti, 2004). The significant increase in drug toxicity for the drug loaded liposomes with increased treatment exposure concurs with the release data which suggests that the slow release of drug over time is a possible cause of increases in cell toxicity.

6.7.2 Results for cellular uptake

The uptake of nanoparticles was tested in MDCK cells and the results are shown in figure 54. The graph for temozolomide uptake in figure 54 (a) shows that the initial uptake from the drug solutions is significantly higher than all formulations (p<0.05) up to 2 hours of the treatment exposure. No significant difference in the uptake of the liposomal suspensions was observed for the length of the study except for at 6 hours where there is a significant difference between the uptake of the surface modified PLL systems as compared to all other formulations (p<0.001). The PLL
systems and multilamellar surface modified liposomes released the drugs slowly in a sequentially increasing order, while a high uptake at 2 and 4 hour is seen for the drug solution and DL MLL respectively after which their uptake decreases.

The uptake of RG7388 in the drug solution was found to be significantly higher than all other formulations at every time point tested for the MDCK cells. A plausible reason for high drug solution uptake may be due to the sustained release observed for the liposomal systems which lowers the inherent concentration available for uptake by the cells.

The difference in uptake of elacridar by all nanoparticles is non-significant for the first 4 hours. The uptake of the drug solution is significantly lower as compared to both the PLL systems (surface modified and non-modified) at 6 hours (p<0.05).
Figure 54: Cellular uptake by MDCK cells of (A) TMZ, (B) RG7388 and (C) Elacridar

The results for the uptake of the formulations by the U87 is shown in figure 55. The uptake of temozolomide by the drug solution, drug loaded multilamellar
liposome and surface modified multilamellar liposome increases sharply for the first few hours of treatment after the uptake is reduced. There is a significant difference between the uptake of the drug solution at 1 hour as compared to the PLGA nanoparticles loaded in liposome (DL PLL) for the same treatment duration (p<0.05). The PLGA loaded in liposomes show an increase in temozolomide uptake with time. At 6 hours, the uptake by both the PLGA in liposomal systems (DL PLL and F-DL PLL) is significantly higher than the drug solutions.

The uptake of RG7388 follows a similar trend as TMZ in U87 cells as seen in figure 55 (b). After 30 minutes of exposure, the uptake of the drug solution is significantly higher than that of the surface modified PLL systems. However, at 6 hours the uptake of the F-DL PLL is significantly higher than the drug solution, DL MLL and the F-DL MLL. A significant difference was also observed between the uptake of the drug solution and both the F-DL MLL and the DL PLL. However, it should be noted that, compared to the other two drugs, the highest uptake of RG7388 was approximately 288ng/mg protein which was significantly lower than the TMZ uptake (p<0.05). The disproportionate uptake of TMZ: RG7388 coincides with the synergistic concentration required for efficacious cell death of the U87 in the MTT analysis.

A low uptake of elacridar is observed in the U87 as seen in figure 55 (c). A significantly higher uptake is seen by the elacridar solution as compared to the nano-formulations for the first and second hours (p<0.05). At 6 hours the concentration of the F-DL PLL is higher than the drug solution, however the difference is not significant. An overall low uptake was observed with elacridar, which can be expected since it acts in the P-gp which does not act on the tumor cell itself.
Figure 55: Uptake by U87 cells of (A) TMZ, (B) RG7388 and (C) Elacridar

The results for the uptake of the formulations by the GBM 108 is shown in figure 56 (a, b and c). The uptake for TMZ differs from that observed in the U87. Up to one hour after treatment, the uptake shown by surface modified MLL is significantly higher than that observed for the drug solutions (p<0.05). At two hours there is no significant difference between any treatment groups, however for the 4 and 6 hour after treatment its was observed that PLGA in liposomes (Surface modified and unmodified) showed a significantly greater uptake than the drug solutions. Additionally, the surface modified PLGA liposomes show a significantly higher uptake than the non-modified liposomes at 6 hours (p<0.05). Although there is a difference in higher cellular uptake between the surface modified multilamellar liposomes at 0.5, 1 and 6 hours, the difference is not significant.
The trend for RG7388 uptake into the GBM108 cells is similar to that of the U87 as seen in figure 56 (b). The PLGA in liposomes, show a significantly higher uptake at 4 and 6 hours as compared to the drug solution and drug loaded multilamellar liposomes (p<0.01).

The uptake of elacridar is relatively lower than the observed uptake in the GBM108 cell line. A significant difference is observed at 0.5 hours for the uptake between surface modified multilamellar system and the PLGA in liposome system (p<0.05). The initial release of elacridar is very low for the first two data points. At 4 and 6 hours, the uptake of the PLGA loaded liposomes (surface modified and unmodified) is significantly higher than the multilamellar systems and drug solutions (p<0.05). However, no significant difference was observed between the folate surface modified multilamellar liposomes and non-modified liposomes.
Figure 5: Cellular uptake by GBM108 cells of (A) TMZ, (B) RG7388 and (C) Elacridar.
### 6.7.2.1 Cellular uptake discussion

The uptake for the drug solutions in all cell lines is quick and typically reaches a high at 1-2 hours, whereas there is a slow increase in the liposomal formulations which show their highest uptake typically at 2-6 hours. The increase in the uptake seen for the PLL systems in each cell line was sequential and increased with time which is consistent with its drug release. As more drug is released, the particles a proportional uptake by the cells. The highest uptake of TMZ was observed in GBM108 at 6 hours for the F-DL PLL system at a concentration of about 298 mcg/mg of protein, whereas the highest uptake for RG7388 was by the U87 at 6 hours at a concentration of 276ng/mg protein.

### 6.7.3 Results of the permeability study

The multilamellar and PLL systems were tested for their BBB permeability by using an *in vitro* model of MDCK cells. The results are shown in figure 57 and 58. Figure 57 represents the $P_{\text{app}}$ determination at a lower TEER of 400 Ω model was obtained for elacridar, followed by temozolomide. The lowest BBB permeability was found for RG7388. It should be noted that RG7388 is a highly hydrophobic compound which should be expected to have good permeability. The RG7388 permeability through the MDCK cells may be affected by uptake of the drug. The uptake for elacridar was significantly increased in surface modified liposomal systems for all tested times except 0.5 hours. At 6 hours, there was a significant increase in the TMZ and RG7388 permeability for the F-DL PLL sample when compared to the drug solution and non-modified samples ($p<0.05$).
When using the 2000 Ω TEER MDCK cell monolayer, it was observed that the permeability for all formulations was reduced greatly. However, this reduction was not significant. For RG7388, the drug was not detected in the samples for up to 2 hours which was significantly different from the 400 Ω TEER MDCK cell monolayer model. The much higher resistance arises from the formation of tighter gaps/junctions between the cells and thus prevention of the paracellular transport of small molecules. Thus, the higher TEER reduced the permeability of the drugs which can potentially affect the drug distribution into the brain.
Figure 57: Apparent permeability using MDCK cells (TEER 400 Ω) for Papp determination of (A) TMZ, (B) RG7388 and (C) Elacridar
Figure 58: Apparent permeability using MDCK cells (TEER 1800 Ω) for Papp determination of (a) TMZ, (b) RG7388 and (c) Elacridar

6.7 Summary of specific aim 5

1. The multilamellar liposomes and PLL system were evaluated for in vitro cell viability, uptake and BBB permeability by U87, GBM108 and MDCK cell lines.

2. It was observed that both the surface modified liposomal systems showed a higher toxicity than the unmodified liposomes in most cases.

3. There was no significant difference in the viability of cells between the multilamellar system and PLL with and without surface modification.

4. The surface modified PLL liposomes show enhanced uptake of RG7388, elacridar and TMZ.

5. All surface modified liposomes show better cellular uptake than their non-modified counterparts.

6. Blood brain barrier permeability for folate modified liposomes was significantly higher than that of non-modified liposomes.

7. The apparent permeability coefficient was found to be dependent on the TEER value of the MDCK monolayer used.
Chapter 7:

Summary and Future Directions
7.1 Discussion and summary:

Multilamellar liposomes and PLGA nanoparticles in liposomes were designed with a distinctive aim to resolve the limitations that are faced by drug therapy for GBM due to the BBB barrier properties and chemical efflux as well as the lack of a single carrier mediated multidrug therapy system. The particles also were aimed at addressing the issues of loss of drug due to premature elimination by RES recognition and lack of site specific delivery. Here, we formulated two novel liposomal systems which could encapsulate three drugs for GBM therapy. The nanoparticles decorated with folate and PEG encapsulated TMZ and RG7388 to treat GBM by two different mechanisms as well as elacridar to inhibit loss of drug efficacy due to drug removal by BBB efflux.

A simple and rapid HPLC technique was specifically developed and validated for the simultaneous quantification of TMZ, RG7388 and elacridar. Good chromatographic peak resolution was obtained by using reverse phase chromatographic conditions and 120mM and 4.5 pH ammonium acetate: acetonitrile (50:50) as the mobile phase with a 1mL/ min flow rate at 40°C and UV spectrophotometric analysis at 254 nm. The technique allowed the rapid determination of TMZ, elacridar and RG7388 which eluted at approximately 2, 4.5 and 7.8 minutes respectively. The technique was evaluated as per USP guidelines and was found to be specific, accurate and precise for the three drugs. The technique could detect very low drug concentrations up to 0.5 μg/mL and was successfully employed in drug content determination in in vitro release, uptake and permeability studies.
A surface conjugate of folate was synthesized for incorporation into the liposomes for tumor targeted drug delivery to the brain. The conjugate was prepared in a two-step procedure to attain a high product yield of approximately 89%. The folic acid was reacted with PEG bisamine through a simple esterification reaction, the product of which was further reacted with cholesteryl chloroformate to synthesize the Fol-PEG-Chol. The product formation was identified and characterized by TLC, NMR, DSC and FTIR. The change in NMR primary amine peaks confirm the completion of the conjugation reaction. It should be noted however, that the possible by product of Fol-PEG-Fol was not removed. The cholesterol in the Fol-PEG-Chol helped anchor the conjugate to the liposomes through the incorporation of the cholesterol into the phospholipid bilayer. Thus, it would be difficult for that by-product to attach to the liposomes and thus its effect would be negligible. The folate content in the conjugate was determined to be 86.5%.

Blank and drug loaded multilamellar and PLL liposomes with or without surface modification were successfully synthesized. The multilamellar liposomes were prepared by a novel freeze-thaw technique post extrusion of thin film hydrated liposomes. $^{31}$P NMR, X-ray diffraction and cryo-TEM showed the effect of freeze thaw cycles on nanoparticle shape and it was observed that the liposome lamellae were altered possibly due to the fusion and rearrangement during the freeze thaw cycle. The increase in lamellae was related to the number of freeze thaw cycles. The PLL were formulated by solvent evaporation to form PLGA nanoparticles which were then incorporated into the liposomes. The DSC and TGA data show incorporation of the drugs into the liposomes due to the disappearance of the endothermic and exothermic peaks of RG7388, elacridar and TMZ in the drug loaded multilamellar
liposomes and PLL. Additionally, TGA data provided vital information regarding possible loss of sample weight due to loss of trapped volume inside the liposomal core which differed in both the multilamellar and PLL systems.

*In vitro* drug release in acetate buffer was investigated to understand the release characteristics of the drugs from the nano-carriers. Drug release was highest for elacridar which showed about 88% (w/w) and 93% (w/w) release from the PLL and multilamellar systems respectively. Elacridar release in both systems differed in that the maximal drug release was observed in the PLL system were reached at 12 hours after which the release pattern did not change, however the opposite effect of maximal *in vitro* release at 72 hours was seen in the multilamellar system. TMZ showed moderate release with similar release characteristics in both the PLL and multilamellar liposomes. The biggest change was observed in the release of RG7388 which showed sustained release of the drug over 72 hours as opposed to the quick release and plateau of the drug concentrations post 12 hour that was observed in the multilamellar system. The post-modification of surface folate and PEG did not significantly change the drug release characteristics in any liposomal system. Thus, sustained the release of RG7388. Additionally, for delivery to the brain, it is required that elacridar be released before the chemotherapeutic agents to prevent its efflux from the site of release. Such release was observed from the multilamellar and specifically the PLL system.

The formulations were all tested *in vitro* in MDCK cells and U87MG and GBM108 cells which represented the BBB and GBM models, respectively. MTT
analysis revealed that the blank formulations were not cytotoxic for all liposomal formulations. There was a difference in the cell viability observed between the 24 hour and 72-hour treatments, with the cell viability reducing sharply for the 72-hour treatment. This effect can be attributed to the sustained release characteristics of the liposomes which release the internally trapped drug slowly. In most cases, there was a significant difference in the toxicity observed between the folate modified liposomes and the drug solutions and non-modified multilamellar liposomes. For MDCK cell, the formulation showed false positive viability probably due to interaction between elacridar, MTT and the drugs. In the U87 and GBM 1080 cell line, the folate modified PLL and multilamellar liposomes showed higher toxicity. Cell uptake experiments showed a concentration dependant uptake in the cells which increased with time for the folate modified multilamellar liposomes, PLL and the non-modified liposomes. These three formulations showed greater uptake in both the U87 and GBM108 cells after 4-6 hours before exposure. Upon 0.5-2 hours of exposure, in most cases the drug solutions shows a significantly higher uptake than all formulations. This data is in agreement with the release studies. Drug permeability studies showed that prolonged exposure (4-6 hours) leads to better uptake of the surface modified liposomes as opposed to the non-modified ones.

The *in vitro* release studies, cellular uptake, cell viability studies and BBB permeability studies show that while both multilamellar and PLL systems work efficiently for brain delivery, the best results were often seen for the pegylated PLL folate modified liposomes which serve as enhanced carriers for TMZ, elacridar and RG7388 for glioblastoma therapy.
**7.2 Future directions:**

The liposomal systems have been designed for brain targeted GBM therapy. The liposomes for both the surface modified multilamellar and PLL systems in this study have been found to lie between 150-200nm. Current studies for brain targeted therapy focused on passive nanoparticulate delivery across the BBB suggest an optimal size range between 70-250nm in vitro, however *in vivo* studies suggest a nanoparticulate size of 70-120nm for optimal BBB passage (142). Thus, the particle size of these nanoparticles can further be decreased by using synthetic lipids like DPPC and DOPC which have known phospholipid saturations and content as compared to natural ones. Additionally, the use of alternative surfactants to polysorbate 80 like poloxamers may led to tighter lipid surface interfaces, reducing particle size.

The effectiveness of both liposomal formulations to inhibit P-gp due to presence of elacridar can be evaluated by comparison against P-gp knockout cells. All liposomal formulation in this study were found to be negatively charged due to the presence of soy phosphatidylcholine and the steric stabilizer DCP. Most membranes in the body are negatively charged which would potentiate the attachment of positively charged carriers ultimately aiding the passage of materials through the membranes. Incorporation of positively charged lipids like DOTAP or positive charge imparting agents like stearyl amine may confer a positive charge to the liposomes. The effect of positive charges may thus lead an enhancement of brain delivery.

Recent studies have suggested that pre-exposure of the system to PEG may lead to the production of antibodies which may ultimately affect the efficacy of PEGylated
nanoparticles (143). To avert these issues, PEG is often employed as a spacer molecule (like the Fol-PEG-Chol) which reduces such effect. Alternative targeting moieties including transferrin can be employed which may increase the particle size and hydrophilicity while maintaining BBB targeting.

The encapsulation of RG7388 in the PLL system was carried out to sustain its release after administration. Traditionally PLGA nanoparticle are used for hydrophobic drug encapsulation, however with new techniques like w/o/w emulsion preparation, both hydrophilic and hydrophobic drug can be encapsulated in the PLGA nanoparticles which may be ultimately further be encapsulated into liposomes for prolongation of drug release.

Due to the large variation between in vitro and in vivo results, it is essential that the cytotoxicity, uptake and BBB permeability be tested in in vivo models. Traditionally rats or mice are orthotopically implanted with glioma cells (U87, GBM108, U251) for preparation of the in vivo model of gliomas for testing drugs and nanoparticle for GBM therapy.

7.3 Global impact

Glioblastoma is the most aggressive brain tumor that constitutes of 80% of all gliomas. Despite aggressive therapy with surgery, radiotherapy and chemotherapy, the current median survival of a GBM patient is only 15 months with a therapeutic aim of enhancement of quality of life rather than a cure. Although it is a relatively rare brain
tumor, the life expectancy of patients if less than 5%. Commonly observed in Europe, USA and Asia, the aggressive disease has only a few marketed therapeutic options. Additionally, high resistance to the currently available therapy has led to high regression of the disease. The first line of chemotherapeutic action of GBM, TMZ faces resistance in some tumors due to administration as monotherapy. The lack of efficacy for the majority of chemotherapy arises from multiple systemic factors like limited passage across the BBB, active efflux and elimination by the RES system. The nanoparticles designed in this study aim to overcome these limitations and treat GBM by the simultaneous administration of TMZ and RG7388 which act by separate mechanisms of DNA alkylation and MDM2 inhibition respectively, thus limiting the possibility of resistance. Co-administration of a P-gp inhibitor can also reduce efflux of the drugs from the brain. The nanoparticle in liposome ensures sustained drug release. These factors make this system a prime candidate for further in vivo and clinical analysis so as to possibly increase the patient survival and ultimately act as a cure for the disease.

Moreover, the ability to carry multiple therapeutics and cross membrane barrier imparts utility for the system to not only GBM, but most organ and metastatic tumors. Modification of the targeting property can additionally help site specific delivery which ultimately affects therapeutic efficacy for most agents administered for patient care. Thus, further development of this system can lead to a universal drug delivery technique to potentially cure tumors affecting patient outcomes globally.
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