LOCAL EXPOSURE AND EFFICACY OF A RESERVOIR-BASED DRUG DELIVERY DEVICE

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ABSTRACT

Prognoses for primary or metastatic brain tumor patients have been poor, despite developments in treatment over the last twenty years. 14,000 people die each year from glioblastoma multiforme (GBM). 200,000 new cases of breast cancer are diagnosed each year, with 15% of those patients experiencing multiple metastases into the brain. The primary cause of mortality is tumor recurrence, often centimeters away from the original lesion site. Current treatments involve systemic radiotherapy or chemotherapy, and improve median survival time by only a few months. Efforts to develop local treatment modules are motivated by the fact that patients experience systemic toxicities during conventional treatment. Implantable Gliadel® BCNU wafers were approved by the FDA, but patients still experienced side effects such as edema, and median survival time was improved only by 2 months. Convection-enhanced delivery (the infusion of chemotherapeutics via catheters) may achieve further distribution on the scale of centimeters, but there is a tendency for preferential flow along paths of least resistance.

An implantable, biocompatible microcapsule for localized delivery of chemotherapeutics in the brain was developed in the Cima Lab. In vitro experiments confirmed linear initial rates of release of temozolomide, an alkylating agent, and doxorubicin, a topoisomerase inhibitor, from the microcapsules. In vivo survival studies were conducted to compare the efficacies of these microcapsules against 9L rat gliosarcoma and CRL1666 rat mammary adenocarcinoma tumors. Local delivery of temozolomide via implanted microcapsules was efficacious against both tumor types and comparable to or better than systemic delivery of temozolomide via oral gavage. Local delivery of doxorubicin was not efficacious against either tumor type, and not significantly distinguishable from control groups. Exposure data revealed much higher levels of retained temozolomide across a larger area of brain tissue than doxorubicin after microcapsule delivery. Thus, successful local delivery of chemotherapeutics in the brain depends on the achievement of sufficient exposures over sufficient (cm-length) distances away from the implant. Microcapsules developed for this work could potentially be implanted at different locations in the brain, with each achieving mm-distance exposure. The overlapping exposures would add together to help treat excess tumor cells post-resection and prevent tumor recurrence.

Thesis Committee: Michael J. Cima (Advisor), Robert S. Langer, Darrell J. Irvine
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CHAPTER 1: BACKGROUND AND MOTIVATION

1.1 Malignant Primary Gliomas and Glioblastoma Multiforme

The annual incidence of new cases of malignant primary brain tumors, which include anaplastic astrocytomas and glioblastoma, is between 5-8 in every 100,000 persons [1,20]. Mortality rates for such tumors in the US are 5.6 per 100,000 males and 3.7 per 100,000 females. Peak incidences of malignant gliomas occur for two different age groups: 0-8 years and 50 to 70 years. Patients suffering from malignant glioma experience headaches, nausea, vomiting, speech, hearing, vision or balance problems, changes in mood or personality, convulsions or seizures, and memory problems, depending on where the glioma is located in the brain [23].

Glioblastoma multiforme (GBM) is the most common type of CNS primary tumor. It has very poor prognosis: the median survival time is between 12 and 18 months and the survival rate by five years is less than 5% [10,33,49,73]. Mangiola et al reported that from a population of selected patients with GBM who underwent gross total resection of their tumor, recurrence was observed near the resection margin to areas within 2-3 cm away from the margin [49]. The leading cause for treatment failure and mortality for glioma patients is the recurrence of the tumor, often distal to the original tumor site, some time after the patients undergo resection surgery [36,49].

Glioblastomas are infiltrative, highly vascularized anaplastic astrocytomas that consist of different types of cancer cells as well as necrotic cells. The diverse cells that make up an astrocytoma are at different stages of differentiation and progression. Astrocytes that make up these masses have a star-like shape, and the tumor mass typically grows into surrounding healthy tissue. Complete resection of the tumor is not possible, making it necessary to treat the remaining
tumor cells. Recurrence of the lesion is also likely and can take place distal to the resected lesion site due to the infiltrative nature of the glioma cells [1,33,75].

It has been suggested that brain cancer propagating cells, the cells that require the most targeting, are often resistant to chemotherapy and/or radiotherapy. It is also generally thought that recurrent tumor cells come from the progression of glioma stem cells. Glioma cells that express CD133 (Prominin) survive ionizing radiation more than tumor cells that do not express CD133. CD133+ GBM stem cells were found to be resistant to Temozolomide, which has had demonstrated efficacy in other studies against tumor cell lines [33]. These brain cancer propagating cells are usually not fully mature, differentiated cells. Bone morphogenetic proteins may be used in some cases to induce the differentiation of these cancer stem cells. This would reduce the propagation of these cells into tumor cells and make them more resistant to carcinogenesis. Disruption of the vascular network that supports cancer propagating cells is another therapy that can be implemented. The ability of CD133-expressing GBM cells to form tumors in vivo has been shown to be adversely affected when treated with Bevacizumab, an anti-VEGF antibody [33,36].

A histological brain slice provided by Dr. Costas Hadjipanayis at Emory University School of Medicine showed a region of glioblastoma tumor in the brain tissue, the boundaries of which are marked with red arrows. Histological analysis revealed the presence of infiltrated cells some distance away from the boundaries of the tumor, marked with red stars or asterisks. This image shows how, upon resection of a bulk tumor, infiltrated cells can remain in the healthy tissue and propagate into another tumor mass.
Infiltrated cells give rise to recurrent tumors, as seen in a study conducted by Huang et al of glioma stem cells obtained from a 52-year old patient diagnosed with malignant glioma. The mix of anaplastic ependymoma and astrocytoma, both WHO grade III tumors, was visible in the right temporal lobe (panel A). This particular patient underwent a resection surgery, followed by one dose of radiation and three doses of chemotherapy. MRI images show the initial tumor (A), the space left behind upon resection (B), and recurrent tumors that progressed to the WHO grade IV glioblastoma multiforme, in situ (C) as well as in the ipsilateral frontal lobe (D). Recurrence of the lesion took place six months after the original resection surgery. This case outlines the difficulty of treating malignant gliomas due to recurrence, further progression into a more malignant tumor, and recurrence distal to the original location of the lesion [36]. Treatment of such tumors must therefore take into consideration the fact that recurrence may take place a certain distance, often centimeters, away from the original lesion site [66].
Figure 2. MRI scans showing a grade III tumor (panel A) which was resected (panel B). Six months later, patient returned with recurrent tumor that had progressed to a stage IV GBM (panel C), and additional tumor in the ipsilateral frontal lobe (panel D) [36]

1.2 Metastatic Breast Cancer

Breast Cancer is the second most prevalent cause of cancer death in female patients, after lung cancer. Metastases of the cancer are the primary cause of mortality in one-third of breast cancer patients, and has been designated an “unmet medical need” by the US Food and Drug Administration [31]. Fifteen percent of metastatic breast cancer patients were traditionally diagnosed with metastases into the brain [83]. This number has increased in recent years to between 20% and 40% [31,57]. Metastases into the brain or CNS from breast cancer outnumber primary brain tumors ten to one—they pose a significant challenge for treatment due to their dispersive and recurrent nature. Breast cancer patients with metastases into the brain usually experience them after having already undergone multiple rounds of chemotherapy. The figure given below shows multiple metastases to the brain, illustrating the dispersive nature of breast metastases. It makes sense for the current mainstays of treatment for breast metastases to be systemic in nature given the locations of the lesions.
Multiple metastases to the brain are especially associated with poor prognosis for the patients, despite any surgical excisions that are performed [83]. Patients with brain metastases experience symptoms that include impaired sensory and motor functions, nausea, vomiting, and seizures [31]. Most patients experiencing metastases into the brain are diagnosed late in their disease, and palliative care undertaken in response. There is no real cure for metastatic breast cancer. Treatments that are attempted depend on the progression of the disease and the location(s) of the metastases [57,83]. Most breast cancer patients with metastases to the brain present with multiple lesions, leading to a 40% mortality rate. The median survival time for these patients depends on whether or not they undergo some form of treatment: one month for those untreated, two months for those given corticosteroids, three to six months for those given radiotherapy in the CNS. If a patient had a single lesion that could be surgically resected, their median survival goes up to between 10 and 16 months.

Detection of metastatic breast cancer is routinely done by bone scans, CT (computed tomography) scans, and tumor marker biopsies. Systemic chemotherapeutic and whole-brain radiotherapy treatments are the mainstay therapies for this disease [57,83]. Lesions that arise in the brain as a result of metastatic breast cancer are often found in the leptomeninges (the two
innermost layers of meninges in the brain, surrounded by cerebrospinal fluid). They line the brain as well as the spinal cord. The increased incidence of metastatic breast cancer, as pointed out by Gril et al, may be attributed in recent years to improvements in breast cancer treatments. These treatments allow patients to live longer with their disease, and the CNS or brain may provide a space for tumor cells to migrate. Improvements and increased vigilance in screening for evidence of disease in the brain may also have helped identify these cases where they may not have been before [31].

Aggressive breast cancers often have elevated levels of the human epidermal growth factor receptor 2 (HER2) protein. HER2-positive breast cancer patients are at higher risk for experiencing metastasis into the brain. Chemotherapeutics used for targeting HER2 include trastuzumab and lapatinib, while adriamycin (doxorubicin) has also been used to treat breast cancer without specifically targeting HER2 [31,62].

1.3 The Human Brain, Cerebrospinal Fluid, and Blood Brain Barrier

Figure 4. Anatomy of the human brain [89]
The blood brain barrier (BBB) is comprised mostly of endothelial cells and changes over time depending on different phenomena that may be happening to the brain environment, such as angiogenesis [76]. Transport of molecules from the blood stream into the brain environment is prevented by the tight intercellular junctions of the blood brain barrier [21,22]. This is especially true of larger molecules, which are restricted to the cerebral vasculature. Generally lipophilic or hydrophobic molecules have a better chance of crossing the BBB, but there is some chance that they would be extruded back to the bloodstream [76].

Cerebrospinal fluid produced by the carotid and vertebral systems is secreted by the choroid plexus tissues in the four ventricles of the brain. The human brain, which would normally weigh 1400 g, weighs only 45 g when suspended in CSF. CSF composition depends on water, ion, and protein exchanges with systemic circulation. It is made up of mostly water (99%), and has a specific gravity of 1.007. Changes in blood volume, including CO$_2$-induced vasodilation or vasoconstriction of the cerebrovascular bed, result in CSF pressure changes. The primary role of CSF is to transport micronutrients such as vitamin C and B$_6$, folates, and peptides into the brain. It also serves as a buffer medium to accommodate changes to the brain environment and in turn keep the brain extracellular ion composition relatively constant. Transport of molecules away from the CSF is done through the choroid plexus, while the CSF itself is reabsorbed or cycled out into the bloodstream through the subarachnoid villi. Drugs delivered to the brain are often cleared by organic solute transporters such as p-glycoprotein or MDR1 and the low density lipophilic receptor-related protein LRP-1 [14].

The flow of CSF in adult humans goes from the choroid plexus (origin), through the ventricles of the brain, through the sylvian aqueduct (between the third and fourth ventricles), and the cisterns of the subarachnoid space. The SAS, or subarachnoid space, has granulations or
pore-like structures that facilitate fluid flow, and plays an important role in the reabsorption of CSF. The SAS lies between the arachnoid mater and pia mater, forming a cover over the convexities of the cerebral hemisphere, and a sleeve around the spinal cord. Pia mater is a covering of the contours of nervous tissue while the arachnoid membrane, or mater, bridges the sulci of the brain and spinal cord. Large pockets of SAS exist at the base of the brain, and these are referred to as cisterns. One of the largest cisterns is the cisterna magna are located between the inferior surface of the cerebellum and the medulla. CSF samples may be sampled from this cistern, which is accessible at the foramen magnum [14].

Total CSF volume in an adult patient is 140 ml, 110 ml of which is in the SAS of the brain and spinal cord, and 30 ml of which surrounds the spinal cord. The largest CSF compartment cisterns in the SAS contain 80 ml of CSF. Contents of CSF in mammals are low in protein and organic substrates. Glucose or urea content is 60-70% less than that of plasma, while amino acid content is between 10% and 20% of plasma. CSF pH value is 7.35. Composition of the cisterna magna CSF is 158 mmol Na/L H₂O, 144 mmol Cl/L H₂O, 25 mmol HCO₃/L H₂O, 2.69 mmol K/L H₂O, 0.75 mmol Ca/L H₂O, and 0.67 mmol Mg/L H₂O [14].

CSF is separated from the outside surface of the brain tissue by a single layer of cells. The highly vascularized choroid plexus is separated from ventricular CSF by choroidal epithelium. Hydrophilic molecules and ions are occluded from the choroidal plexus epithelium, but the epithelium has numerous microvilli at the CSF interface. The ependymal lining is permeable to most ions and molecules so drugs carried in the CSF can easily penetrate the ependyma to reach neurons and glia. The pia/glial membranes allow bidirectional exchange of molecules between the SAS CSF and the subpial space [14].
Rate of CSF secretion is around 0.5 ml/min*g CP (choroidal plexus), and depends on the vascular perfusion of the choroidal plexus. CSF is replenished three times daily, and production of CSF increases at night. A twenty-four hour period sees a total of 500 ml of new CSF produced in adult humans, at a rate of 0.35 ml/min. Pharmacologic manipulation of the CSF formation rate is possible: acetazolamides have been shown to inhibit carbonic anhydrase production and reduce CSF formation rate by 50-60%. Its use is limited by systemic acidosis. Formation of CSF in the choroidal plexus comes with an initial hydrostatic pressure of about 11 mm Hg. The pulsation of blood in choroidal vessels also assists in CSF movement. CSF turnover in humans is reduced with aging, chronic hydrocephalus, and Alzheimer’s disease, which may in turn result in increased drug residence time in the CNS (or in other words, increased pharmotoxicity) [14].

1.4 Chemotherapeutics

1.4.1 Temozolomide

A prodrug that has been used in the treatment of gliomas and melanomas is temozolomide (8-carbamoyl-3-methylimidazo-[5,1,d]-1,2,3,5-tetrazin-4(3H)). Temozolomide has been demonstrated to show activity against high-grade glioma and melanoma tumors in phase I and II trials, as discussed by Baker et al at The Johns Hopkins Oncology Center [6]. Decomposition of temozolomide by the opening of a hydrolytic ring produces the active DNA alkylating agent MTIC (5-(3-methyltriazen-1-yl)imidazole-4-carboxamide). This ring opening and decomposition mechanism takes place at neutral pH, while the prodrug is stable at acidic pH values [81].

The following diagram shows a molecular schematic of the decomposition of temozolomide (TMZ) in aqueous solution to MTIC. Decomposition takes place via a base-catalyzed addition of water to the compound that leads to hydrolytic ring opening. NMR
measurements indicated the incorporation of deuterium into the methyl group of temozolomide during a reaction taking place at slightly acidic pH. The temozolomide molecule is stable at acidic pH values, with ring opening taking place at above neutral pH values. The MTIC molecule, on the other hand, is stable at basic pH values and unstable at acidic and neutral pH values. Decomposition of both temozolomide (ring-opening) and MTIC (release of methyl group) takes place in the physiological pH range. Half-life of temozolomide in the physiological environment (pH=7.4) is 1.81 hours, while the half-life of MTIC in the same environment is only 2 minutes. The decomposition of temozolomide into MTIC has an advantage over some other drugs (such as DTIC) in that it is a nonenzymatic degradation process that takes place at physiological pH [6,81].

![Figure 5. Temozolomide structure and degradation by hydrolysis into active drug MTIC [81]](image-url)

A schematic of the interaction of temozolomide and MTIC with DNA was also presented by Wheelock based on their NMR data and included below. They proposed that temozolomide would bind to a GC-rich sequence of DNA (in a GGG sequence groove) via hydrogen bonds. Base-catalyzed reaction of temozolomide takes place in a basic microenvironment that results from the presence of guanines, which have a negative electrostatic potential. The first sequence shows binding of temozolomide to DNA, followed by the second sequence showing the ring
opening reaction, and followed again by the third sequence showing the transfer of methyl group or degradation of the MTIC molecule into AIC (5-aminimidazole-4-carboxamide). The methyl group that transfers off, or the methyldiazonium ions, continue on to alkylate the DNA, causing damage to the DNA [81]. The structurally alkylating temozolomide decomposes into the active agent MTIC, which damages DNA by alkylating the O\(^6\) guanine and N\(^7\) positions.

Figure 6. Interaction between temozolomide and guanine group of DNA [81]
Single dose administrations of temozolomide in the concentration range of 200-1200 mg/m² were studied by Newlands et al and reported by Baker et al for their bioavailability. It was found that temozolomide has a 100% bioavailability. The exposure to temozolomide in the brain scaled linearly with dose—with the dose administered *per os* (orally) once a day for five days a week, in a total period of four weeks. Average temozolomide clearance rate in adult patients was 196 ml/min. This was quite high, given that the volume of CSF in an adult human is typically 140 ml (and the volume of ISF or interstitial fluid is typically 280 ml), with a typical renewal rate of three times per day [14]. Baker et al found that temozolomide was rapidly absorbed and eliminated in the body. The mean time to peak plasma concentration was one hour, with no drug accumulating in the plasma, and the mean terminal half-life of temozolomide was found to be 1.8 hours [6].

Studies done by Baker et al investigated the absorption, metabolization, and excretion of oral temozolomide in adult patients diagnosed with advanced cancer. Radiolabeled ¹⁴C-temozolomide was used to track the compound; a dose of 200 mg temozolomide with 70.2 µCi of ¹⁴C-temozolomide provided by Schering-Plough Research Institute was used alongside a controlled regimen of hydration and prophylactic anti-emetic medicine. Baker et al used a first-order, one compartment linear model to describe the metabolization of TMZ into MTIC and AIC. Rapid elimination of temozolomide in plasma was observed eight hours after treatment. Oral clearance of temozolomide in the adult patients was found to average 104 ml/min/m² [6]. With a body to mass index of 22 kg/m² [12,67], this translated to a clearance rate of 4.72 ml/min/kg.
1.4.2 Doxorubicin

One of the most widely used anthracycline chemotherapeutic agents is adriamycin, or doxorubicin [15,37]. It has been used to treat breast cancer, Kaposi’s sarcoma, and breast cancer. Doxorubicin is an anthracycline that interacts with topoisomerase II to prevent DNA ligation after strand translocation, thereby preventing tumor cells from replicating [80]. Intercalation of doxorubicin into DNA inhibits macromolecule synthesis and inhibits DNA binding and cross-linking [13]. The structure of doxorubicin is given below.

Doxorubicin is a poor drug candidate for systemic delivery past the blood-brain barrier because of its low lipophilicity and high molecular weight. Direct injection of doxorubicin into the tumor environment has been shown to produce 25 times higher drug concentration than systemic (intravenous) delivery [80].

The administration of adriamycin/doxorubicin is conventionally done through a bolus intravenous injection. Cardiotoxicity is its dose-limiting toxicity, at a lifetime total of 450-550
mg/m² [18]. Symptoms of cardiotoxicity include myopericarditis, supraventricular tachycardia, and premature heart beats. Acute cardiotoxicity takes place within the first 2-3 days after administration, and has an incidence rate of 11%, while chronic cardiotoxicity has an incidence of 1.7% and could be observed anywhere between a month to several years. Higher doses, as expected, produce a higher rate of adverse effects. Doses beyond 600 mg/m² result in a 36% cardiotoxicity rate. There is a 50% one-year mortality rate for patients who develop congestive heart failure. Histological evaluation of doxorubicin cardiomyopathy shows areas of interstitial fibrosis and vacuolated cardiomyocytes. Areas of healed myocarditis show evidence of fibroblast proliferation and histiocyte infiltration [13].

The use of doxorubicin to treat brain tumors involved modifications to the molecule to allow for better distribution across the blood brain barrier. Wohlfart et al explored the use of poloxamer 188-coated poly(lactic-co-glycolic-acid) (PLGA) nanoparticles to deliver doxorubicin across the BBB [82]. They added to the body of work in which doxorubicin-loaded nanoparticles were delivered intravenously to treat rodent brain tumor models. This modality of treatment involved the inclusion of drug into a polymer. The amount of drug that could potentially be delivered was limited based on the payload of drug that could be formulated with the polymer.

Liposomal forms of doxorubicin are used clinically under the registered name Doxil®. Doxil is designed to be administered intravenously to patients, and was initially approved for use in the US in 1995. Indications and usage for use of Doxil as a topoisomerase inhibitor include ovarian cancer (following and in case of failure in cisplatin based therapies), AIDS-related Kaposi’s sarcoma (following systemic therapy failure), and multiple myeloma (in combination with bortezomib, and again for patients who have already gone through another treatment).
Administration of Doxil takes place at an initial rate of 1 mg/min, and the doses vary from 20 mg/m² to 50 mg/m² intravenously over a period of a few weeks to several months [16].

Treatment of rats with Doxil is found to yield results significantly better than treatment with free doxorubicin. The systemic doses given seven days post tumor implantation (in studies involving tumor lines) are 5.67 mg/kg. Maximally tolerated dose of doxorubicin administered in liposomal form and intravenously is 17 mg/kg [87]. Liposomes have the ability to extravasate through the leaky vasculature of tumors, leading to accumulation of the liposomes. Systemic circulation time is increased for these <100 nm liposomes, allowing the drug to have increased activity against the tumor. Plasma levels of liposomal doxorubicin (Doxil) in tumor bearing rats were found to be higher than plasma levels of free doxorubicin [70].

![Figure 8. Liposomal formulation of doxorubicin produced higher plasma levels than free doxorubicin [70]](image)

### 1.5 Treatments

Brain gliomas are initially diagnosed through magnetic resonance imaging (MRI) and computed tomography (CT) scans. A biopsy usually follows, either on its own (for tumors deemed inoperable) or as part of surgery to resect a majority of the tumor mass. Analysis of the tumor mass is done to confirm the diagnosis as well as to determine the best next course of treatment [1].
The prognosis for glioblastoma patients has improved in the last few years because of new standards of treatment. Improvements in radiotherapy include better targeting with the help of image guidance and dose management. These improvements, along with improvements in systemic and local delivery of chemotherapeutics, have contributed to an increase in the average survival time for patients in the past few years [9,75]. A combination of local and systemic chemotherapy along with radiotherapy may be attractive in targeting diverse sets of cancer cells and reducing the dosage for any one of the treatment modalities [33]. The standard of treatment for glioblastoma includes resection surgery followed by radiotherapy and/or chemotherapy [9,33,55].

Nishikawa et al reviewed the results of a randomized phase III trial for GBM patients over 70 years old, an age range not as often covered in clinical trials. Median survival time increased from 16.9 weeks to 29.1 weeks for patients who received radiotherapy in additional to supportive care post-resection surgery [55]. Radiation therapy does not come without challenges and adverse effects. Multiple treatments may result in necrosis of the tissue. The dose and volume of radiation therapy is ideally minimized, taking into consideration factors such as the patient’s KPS (Karnovsky Performance Status), recurrent lesion size, proximity of recurrent lesion to primary lesion (and whether the primary lesion site has gone through a high amount of radiotherapy), and the time that has passed since the last radioactive dosing. Small-sized recurrent tumors (<4 cm) may require resection using stereotaxic radiosurgery. This type of radiosurgery was not found to produce a significant benefit in newly diagnosed patients, but does produce a positive response in patients with recurrent malignant gliomas [75].

The significant resistance of malignant gliomas especially in newly diagnosed patients to radiotherapy, and recurrence in sites where high doses have been applied, add to the challenges
of treating the tumor using radiotherapy. Certain chemotherapeutic agents, such as temozolomide, have been shown to produce radiosensitivity in glioma cells. Improvements in survival time have thus been observed for patient groups that receive concomitant temozolomide along with radiotherapy (followed by adjuvant temozolomide at a higher dose) [75].

Antiproliferative drugs such as carmustine (BCNU) and lomustine, as well as alkylating drugs such as temozolomide, have been shown to go through the blood brain barrier [66]. Additionally, modifications have been attempted in order to increase its permeability and make it easier for chemotherapeutics to go through. These include modification of the drug with hydrophobic side groups, conjugating drugs with ligands such as transferrin that make it easier to carry them across the BBB, and encapsulating drugs in liposomal formulations [66].

A study published in 2005 by the National Cancer Institute and European Organization for the Research and Treatment of Cancer resulted in an updated standard therapy for GBM patients, and is referred to as the Stupp Regimen [73]. The protocol for therapy following resection is six weeks of radiotherapy (1.8 Gy/day) with temozolomide chemotherapy (75 mg/m²/day), after which the patient undergoes six further cycles of adjuvant temozolomide (150-200 mg/m²) for five days every four weeks.

The combination of radiotherapy and chemotherapy produced a statistically significant improvement for the survival time, from 12.1 months for only radiotherapy to 14.6 months with the addition of adjuvant temozolomide (p < 0.001). Temozolomide causes some amount of radiosensitization, along with a reduction in MGMT (O⁶-methylguanine-deoxyribonucleic acid-methyltransferase) that in turn enhances the results of the radiotherapy. Other treatment regimes centered around administration of temozolomide include doses of 75-100 mg/m²/day during days 1-21 in a 28-day cycle, and a metronomic administration of 20 mg/day [55,73].
There are limitations to the treatment of brain tumors with chemotherapeutics administered systemically. Treatment regimes involving consistent administration of temozolomide from days 1 to 21 have been shown to cause infections such as lymphopenia and infection [55,73]. Studies examining dose escalation effects found that increasing systemic temozolomide dosing resulted in hematologic toxicity (including decreased counts of white blood cells and platelets), leucopenia (a decrease in leukocytes), and thrombocytopenia (appearance of a clot in one vessel that then goes on to block another blood vessel) [12]. These side effects and systemic toxicities contributed to the motivation to deliver temozolomide and other chemotherapeutic agents locally, where dosage could be increased without increasing the risk of these systemic toxicities. Increase in dosage and improved distribution of said dosage could improve prognosis and are the central focus of this work.

1.6 Animal Model for In Vivo and Ex Vivo Observations

Work on exploring the formation of fibrous capsules over passive drug delivery devices was performed on rat models to evaluate immune response. In vivo models for inflammatory responses to injury are important in determining the nature and details of the wound-healing process. The use of a rat model to study the biological response to biomedical implants is isomorphic (similar symptoms, different causes) and/or partial, but can still give valuable information on what may be expected in terms of immune response and fibrous encapsulation. Advantages of using a rat model include the ready availability of rats, low cost, and small sizes that allow for high number of samples and low amount of required space for housing. Their short gestation and life spans, as well as docile behavior, are also beneficial characteristics of rat models [14].
Human wound-healing is often modeled with rats. The Sprague-Dawley strain of rats serves as a good general purpose model for wound healing studies. The wound healing process in rats is accelerated, thereby allowing faster observation of standardized and controlled injuries that can be compared to each other based on a set of parameters, including size and time. The common location for wound healing studies is on the rat's dorsum, because these sites are not as reachable by the rats and therefore could be studied without interference [14].

Commonalities between rat and human skin include the presence of an epidermis, basement membrane, hair follicles, and dermis. There are differences as well, including skin adherence (tight in humans, loose in rats), lack of panniculus carnosus in humans, presence of apocrine and eccrine glands in humans, and possible scar formation in humans. The rat skin is loose and does not adhere strongly to the rest of the body, which allows for large subcutaneous pockets to be made. The subcutaneous panniculus carnosus muscle in rats plays a role in both contraction and synthesis of collagen. The muscle acts as a wound bed because it has a rich vascular supply, and therefore facilitates wound healing. The overall healing time for rats is low (fast healing) because contraction, and not endothelialization, is the primary mechanism [14].

Incisions that have been closed by sutures, or in the case of this work, autoclips, experience reepithelialization within 1-2 days, and a scab can be observed over the incision site. The incision itself results in the proliferation and migration of plasma and blood cells to the wound site, followed by formation of a clot that will eventually lead to the healing of the incision. Granulation tissue and wound fluid may be expected to be present in subcutaneous pockets once they are made. The exudate fluid contains metabolites, cytokines, growth factors, and nonadherent cells after 3-5 days [2,14].
The weights of rats correspond with their ages and therefore with the wound healing response. According to the Harlan Laboratory Animal Company, male Sprague-Dawley rats between 49-52 days old weigh between 200-224 g. Comparable general responses to young humans may be found in rats between 3 and 6 months old. A significant decline in the rate of wound healing was observed between juvenile (8 weeks) and adult (16 weeks) rats [14].

1.7 Thesis Objectives

The overarching goal of this thesis work is to develop a microcapsule device to intracranially deliver chemotherapeutics for effective treatment of brain tumors, and to understand the parameters that affect its successful use as a local drug delivery module. The first objective is to develop the microcapsule and characterize its ability to release two chemotherapeutic agents (temozolomide and doxorubicin hydrochloride) in vitro. The second objective is to evaluate the efficacy of these microcapsules in vivo against two tumor lines: 9L rat gliosarcoma and CRL1666 rat mammary adenocarcinoma. The third objective is to develop a protocol to quantify the biodistribution and exposure of temozolomide and doxorubicin in rat brain tissue after implantation of the microcapsules.
2.1 Polymeric Delivery Modules

Polymers have been used to deliver a variety of medications, including subcutaneous contraceptive systems (Norplant®) and glaucoma treatment systems to be used in the conjunctiva (Ocusert®). Different biodegradable as well as non-biodegradable (but all biocompatible) polymers have been utilized for the purpose of drug delivery. The primary mechanisms by which polymer delivery systems work are diffusion from non-biodegradable modules, and the degradation of the polymer matrix in addition to diffusion from biodegradable modules. The latter mechanism results in a burst release at the beginning, followed by a linear rate of release. Copolymer formulations may be used to tune release by taking advantage of the different rates of polymeric degradation [66].

Polyethylene-co-vinyl acetate, a copolymer of ethylene and vinyl acetate, is a common polymer used for delivery of drugs. It is a biocompatible, non-biodegradable polymer that has been used for controlled release of chemotherapeutics. Clinical use is limited by persistence of the polymer, but oftentimes when compared with the alternatives (high systemic toxicities, poor prognoses) the use of non-biodegradable modules may be far preferable as long as they demonstrate efficacy against their target disease. Biodegradable polymer formulations provide an additional advantage of clearing out of the body, mostly by hydrolysis. Carboxyphenoxypropane: sebacic acid copolymer systems are the most commonly used biodegradable formulation for intracranial use. CPP:SA polymer formulations have been used with, among others, mitoxantrone, BCNU (carmustine), 4-hydroperoxycyclophosphamide, paclitaxel, carboplatin, and doxorubicin (adriamycin). Release from wafers with polymer co-formulations may be affected by the hydrophobicity of the drug or its affinity for the polymer material [66].
Polylactic acid (PLA), polyglycolic acid (PGA), and polylactic-co-glycolic acid (PLGA) have been used as biopolymer materials for several decades. Electrospun PLGA matrices mixed with paclitaxel have been demonstrated to be effective in vitro against C6 glioma cells. BCNU-containing PLGA nanoparticles showed similar performance to BCNU-containing CPP:SA wafers [66].

Passive resorbable devices made of compression-molded PLLA (poly(L-lactic) acid) reservoirs with poly(lactide-co-glycolide) (PLGA) membranes have been developed [25-30,72]. The multiple-reservoir devices are 11.9 mm in diameter and 480-560 μm thick. These polymers degrade once implanted, releasing the drug over time. PLLA has a long biodegradation time, which allows the drugs and other biological compounds tested (heparin, human growth hormone, and dextran) to be released through the membrane before the entire device degrades in the implanted area. Release through the membranes takes place when water is absorbed into the polymer and swelling occurs. Higher molecular weight polymers take longer to rupture, so pulsatile release can actually be accomplished using this passive device by changing the molecular weights of the membrane polymers [25-30].

The controlled release of drugs from polymer formulations has an associated limited distribution or penetration. Diffusion of a predetermined payload of drug out of a polymer wafer may result in limited distribution across the tissue space. This is especially true in the case of high clearance or elimination rates, and the distribution is often limited to the millimeter scale. This may be even more limited if drug diffusivity and transport across tumor tissue is lower than it would be in normal tissue. Drug concentrations in these cases are often confined to a distance of 3 mm or less from the wafer site, whereas infiltrative tumor cells may migrate as far as 2-3 cm away from the site of a resected lesion [66].
2.2 Gliadel® Wafers: BCNU (Carmustine)

Biodegradable polymers have been used for commercially-available passive drug delivery systems. Polymer passive implants placed in the body near targeted (e.g. tumor) sites have been demonstrated to locally deliver drugs and decrease overall systemic toxicity. BCNU (carmustine) wafers are approved for use as local/interstitial chemotherapy upon resection of a brain tumor bulk. These Gliadel® wafers, approved by the FDA in 1996 and implanted in more than 20000 procedures since, were shown in a meta-analysis of two randomized phase III trials to improve average patient survival from 10.9 months to 13.1 months (p = 0.03) [9]. Gliadel® wafers are 14 mm in diameter and 1 mm thick; the payload is 7.7 mg or 3.85% of carmustine (BCNU, 1,3-bis-chlorethyl-1-nitrosourea) [66]. The use of Gliadel® wafers over a period of two to three weeks to treat remaining brain tumor tissues post tumor resection increased the rate of survival from 6% of patients after two years to 31 %. Passive devices release the drugs contained in their reservoirs slowly, as the biodegradable polymer dissolves within the body [39,43,50,53].

![Resection cavity in the brain, packed with Gliadel(R) wafers](image)

**Figure 9. Resection cavity in the brain, packed with Gliadel(R) wafers [45]**

Complications and adverse side effects resulting from the implantation of Gliadel® wafers have been observed. These include cerebral edema, hydrocephalus, wound infection, CSF leakage, seizures, and cyst formation [9,33]. Bock et al analyzed data from 44 patients at seven
neurosurgical institutions who were newly diagnosed with glioblastoma. These patients were given BCNU wafers, six weeks of daily radiotherapy (1.8 Gy/day) along with systemic temozolomide (75 mg/m²/day), followed by adjuvant temozolomide (150-200 mg/m²/day) every two to four weeks.

Three years after the study began, 64% of the patients had died. Karnofsky performance scores (KPS) improved for 14% of the patients, did not alter for 66% of the patients, and worsened for the rest. 23% of the patients experienced further progression of the GBM and had to go back into surgery. More than half of the adult patients (52%) experienced some form of adverse effects, including surgery-related effects, CSF leakage, and infection [9].

2.3 Convection Enhanced Delivery

A mode of drug delivery that has been extensively explored and used clinically is convection enhanced delivery (CED). This method utilizes a continuously applied pressure gradient to pump fluid into the brain’s interstitial space. A small catheter connected to a pump is implanted under image guidance and convectively delivers a liquid formulation of drug into the target area [64]. CED distribution and efficacy have been shown to be better than the distribution and efficacy of bolus injections. Long infusion times are possible with the use of a fluid drug reservoir. Raghavan et al studied the distribution of agents injected into the brain parenchyma that show improved distribution using CED methods over diffusion-dependent methods. A schematic showing this improvement in distribution is given below. Despite the potential improvement of distribution of drug fluid in the parenchyma, Raghavan et al found that measured spatial distributions were unpredictable between patients, unpredictable in general, and may not reach the intended target area [64].
Protocols utilized in the treatment of recurrent gliomas involve either infusion of the drug directly into the tumor bed, or infusion of the drug into the resection cavity following surgical resection. Patient response and adverse event recurrence are dose-dependent. Larger particles are especially good candidates for CED delivery, due to their restrictive diffusion. Even then, smaller particles (20 nm in diameter) are found to distribute further than larger particles. Coating particles with polyethylene glycol (PEG) or bovine serum albumin (BSA) improves the distribution volume—surface characteristics of the particles affect how they are distributed in tissue [66].

Infusion in humans using catheters usually takes place at a rate of less than 0.3 ml/hour. Catheterization treatment is done over a period of several days due to this slow rate of infusion. Factors that affect the efficacy of infusion via catheter into the brain parenchyma include pump flow rates and infusion duration, interstitial pathways in the brain, lipophilicity, and metabolism. The primary mechanism of distribution for large drug molecules is by fluid flow in the poroelastic environment in the brain. Interstitial pathways may allow large molecules to also pass through via convection (as opposed to diffusion, which is limited for larger molecules).
Hydrodynamic dispersion, or the tortuosity of drug dispersion, determines the distribution of drug delivered convectively. The limit of longer infusion times may see diffusion through the extracellular matrix, capillary loss, and metabolism also taking place in affecting drug distribution [64].

Challenges that CED modalities face include the previously mentioned unpredictability in intraparenchymal distribution. There is a possible backflow from the pressure used to drive this convective flow, which is around or up to 70 mm Hg. Application of such pressures may also disrupt the target tissue in a continuous manner and/or produce air bubbles in the brain, further contributing to the occurrence of backflow. Even with the absence of tissue damage or air bubble formation, intrinsic backflow may still take place. Depending on the depth of the catheter and its target location, the infused drug may be easily cleared away. White matter areas of the brain, for instance, are areas of high fluid conductivity [64].

Figure 11. Schematic showing preferential flow of drug infusion in white matter (panel a) and MRI showing an irregular infusion pattern following white matter tracts (panel b) [64]

If drug flow were to follow white matter tracts, this may adversely affect distribution of that drug into targeted areas if those targeted areas do not happen to be in the same tract or pathway.
Infusion rates that are too high or too fast (for a 23-gauge needle, more than a few microliters per minute) may result in leakage of the infusion solution out of the CED catheter [8].

### Table 1. List of phenomena relevant to convection-enhanced delivery [64]

<table>
<thead>
<tr>
<th>Phenomenon</th>
<th>Determining Parameters</th>
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<tbody>
<tr>
<td>tissue damage on catheter insertion</td>
<td>obviated by catheter design &amp; insertion procedure</td>
</tr>
<tr>
<td>air bubbles</td>
<td>obviated by stylet &amp; catheter design &amp; insertion protocol</td>
</tr>
<tr>
<td>backflow along catheter walls</td>
<td>poroelastic parameters near catheter: elastic moduli, extracellular volume, &amp; hydraulic conductivity of tissue</td>
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<tr>
<td>fluid flow in extracellular brain tissue</td>
<td>hydraulic conductivity of tissue &amp; induced variation of excess pressure</td>
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<tr>
<td>efflux rate of water from brain tissue</td>
<td>capillary hydraulic conductivity</td>
</tr>
<tr>
<td>drug transport</td>
<td>diffusion tensor of drug &amp; convective velocity</td>
</tr>
<tr>
<td>drug efflux from tissue</td>
<td>capillary molecular permeability—surface area product</td>
</tr>
<tr>
<td>drug metabolism, binding, &amp; other effects</td>
<td>reaction rates</td>
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A catheter with 0.85 mm diameter was used to infuse a solution into a region 14 mm inside the cortical surface of a pig brain at a rate of 5 µl/min. MR imaging showed that 32 minutes post-infusion, backflow of the catheter resulted in leakage into the subarachnoid space and contours of the cortex. The amount of backflow was also dependent on the fluid conductivity of the tissue; more backflow was observed in tissue areas that are less conductive or permeable. Images from this study are given below.
Significant unpredictability and variability in the convection distances across CED patients proves to be a challenge for the procedure [51]. Unpredictable flows resulting from extracellular matrix behavior as well as the infusing pump itself may result in drug flowing to and collecting in perivascular spaces, in wound tracks, or below the scalp. Flow of infusion in brain tissue depends on both the direction of the directed flow and the location in the brain in which the catheter is implanted. Increased pressure from unpredictable flows may lead to increased intracranial pressures, edema, and possibly wound dehiscence (unintentional reopening of the closed wound) [66]. Presence of edema may result in increased fraction of extracellular space, which may in turn result in clearance of the infused agent before it could have any effect on the targeted tissue [64].

Clarity of path in the catheter of a CED system is also a requirement for its successful use. Any occlusions would result in failure to appropriately deliver the drug payload. The type and range of therapeutics that may be delivered using CED are limited by their solubility and stability in solution. Encapsulation procedures may need to be undertaken in order to increase solubility of compounds and keep them from degrading. The inherent invasiveness of
Mardor et al utilized diffusion-weighted MRI as a way to assess the propagation in brain tissue following convective delivery of Paclitaxel in rat brain tumors. They found that distribution of the drug may be increased by increasing the viscosity of the drug solution—which may be yet another challenge, if drug solubilities and chemistries were difficult to begin with. T1-weighted MRI images were used to calculate the volume of carboplatin, taxol, and cremaphore infusate. Several of the rats were treated with infusates of higher viscosity. Evans blue dye was used along with the infusate in order to better visualize the extent of infusion. The figures below show their illustration for what poor CED distribution looks like compared to moderate and efficient CED distribution. They found that using higher infusate viscosities produce better CED distribution [51].

![Figure 13. Poor, moderate, and efficient CED infusions may depend on the infusate viscosity [51]](image)
Brains with tumors possess different characteristics than healthy brains that may affect the way drug delivery modalities work. These include elevated interstitial pressure, a more heterogeneous distribution of blood vessels than those found in normal tissue, decreased vascular surface area, increased intercapillary distances, and the presence of peritumoral edema. A disrupted blood brain barrier (BBB) due to tumor presence, as well as elevated interstitial pressures in the tumor region, results in a counter convection effect that in turn restricts infusion of drug into the tumor region and contributes to its efflux away from the tumor. Concentrations of drug in rat tumor models after CED have been found to be extremely variable. Although CED may provide distributions across a larger span, the unevenness and unreliable nature of the infusions suggest that improvements on multiple, local drug delivery modules that can each deliver drugs across mm-distances may be preferable to a convection-based module [64].

Kikuchi et al studied the release and distribution of polyethylene glycol-coated doxorubicin via CED in a rodent brain model with intracranial 251MG and U-87MG xenograft models. Fluorescence microscopy showed presence of the liposomal doxorubicin in 1 mm-interval rodent brain slices. They found, as others have, that liposomal doxorubicin distribution was superior to the distribution of non-liposomal doxorubicin [39]. Indeed, intravenous delivery of liposomal doxorubicin resulted in higher retention of the drug in the brain than intravenous delivery of free doxorubicin. The retention of doxorubicin remained high until 14 days after the administration of CED and gradually decreased. It was barely detected 60 days after administration [39,87].

Coating liposomal doxorubicin with polyethylene glycol (PEG) helped protect the liposomal drug from degradation or immune responses by monocytes and macrophages during systemic delivery. PEG-coated liposomal doxorubicin had longer circulation time in the
bloodstream, allowing more time for perfusion into the brain tissue. These liposomes also had an increased accumulation in the tissue and reduced systemic effects because they were not absorbed systemically the way non-liposomal doxorubicin is absorbed. That is, free doxorubicin would be absorbed in the nuclei of cells, while PEG-coated liposomal doxorubicin circulated in intercellular space. Free doxorubicin was also observed to cause more tissue necroses than liposomal doxorubicin. However, all variations of the CED procedure did lead to some amount of local tissue damage [39,87].
CHAPTER 3: *IN VITRO* RELEASE OF TEMOZOLOMIDE AND DOXORUBICIN

3.1 Introduction

Liquid crystal polymer microcapsules for delivery of drugs into the brain were developed by Alex Scott of the Cima Lab at MIT [67]. Vectra MT 1300 liquid crystal polymer microcapsules were produced via injection molding by Matrix/microPEP (East Providence, RI). These microcapsules had an opening diameter of 890 µm on top, and four 403 µm-diameter openings on the side were laser drilled by TeoSys Engineering LLC (Crofton, MD). *In vitro* release performance of microcapsules containing temozolomide has been discussed in previously published work [67].

![Schematic and picture of LCP microcapsule devices](image)

Figure 14. Schematic and picture of LCP microcapsule devices [Alex Scott, MIT]

LCP microcapsules were demonstrated to release temozolomide at linear initial release rates, whether all orifices were open or only the large cap orifice was open. *In vitro* release profiles for temozolomide single-hole and multiple hole microcapsules are given below. Leak devices were completely sealed to demonstrate the effectiveness of epoxy sealing methods.
3.2 Materials and Methods

3.2.1 Microcapsules

Vectra MT 1300 liquid crystal polymer microcapsules were produced via injection molding by Matrix/microPEP (East Providence, RI). Microcapsules used for release of doxorubicin hydrochloride had an opening diameter of 180 µm on top, as opposed to the 890 µm opening for temozolomide devices. One of the four 403 µm-diameter openings on the side was also left open, the rest being sealed using a Dymax 1161-M UV-cured epoxy. Microcapsules used for release of temozolomide were described above.
3.2.2 Drugs

Temozolomide was initially donated to the Cima Lab by the National Cancer Institute (NCI), but the amount of temozolomide available from NCI was very limited and thus this drug was used in survival studies only. Early *in vitro* release experiments done by Alex Scott and Byron Masi (Cima Lab, MIT) were done on NCI temozolomide. Later experiments used temozolomide obtained from Sigma Aldrich, as well as $^{13}$C-temozolomide ($^{13}$C stable isotope labeling on the methane group of the temozolomide compound) from Cambridge Isotope Laboratories. Doxorubicin hydrochloride was obtained from Sigma Aldrich, and liposomal Doxil® was donated by Johnson & Johnson.

3.2.3 Drug Measurements

Temozolomide measurements were conducted using reverse-phase high pressure liquid chromatography (HPLC) and isotope-ratio mass spectrometry (IR-MS). The HPLC measurements were done with an in-lab Agilent 1200 Series HPLC. The method involved an Eprogen SCD-100, 5 µm, 100 Å spherical silica column of dimension 150 x 4.6 mm inner diameter for samples dissolved in HPLC-grade water. Samples that potentially had more protein and salt content, such as those from brain extracts/homogenates, were first run through an Eprogen FSCD-100, 5 µm, 100 Å guard column of dimension 50 x 4.6 mm inner diameter prior to being put through the analytical column. 0.01 M ammonium acetate (aqueous):acetonitrile (92:8) solution served as mobile phase for all experiments involving reading temozolomide signals at 316 and 360 nm in HPLC.

Fluorescence assays measured doxorubicin concentrations using a Synergy 4 plate reader from BioTek Instruments, Inc. The programmed internal temperature of the reader remained at
37°C. An excitation wavelength of 470 nm was used, with the fluorescence reading done at an emission wavelength of 590 nm. Standard calibrations of a range of doxorubicin concentrations (between 0 and 400 µg/ml) were obtained for comparison with measured values. Black 96-well plates (Costar) and samples were placed in every other well to prevent cross-reading of fluorescence signals.

Dissolved doxorubicin exhibited a pink/red color that increased in intensity with increasing concentration. There was an upper limit to the calibration measurements, beyond which distinctions between different concentrations could no longer be reliably made. Doxorubicin samples were uniformly diluted to bring them to the appropriate concentration range, so that their concentrations may be quantified by comparison with the established calibration standard.

3.2.4 Microcapsule Drug Loading and Preparation

Liquid crystal polymer (LCP) microcapsules were loaded with a given weight of drug and sealed with a UV-cured epoxy (Dymax 1161-M) around the edges. The microcapsules, once loaded with drug, were anchored down to a small piece of microscope glass slide using the same epoxy. This ensemble was then placed into a 7-ml scintillation vial, to which 2 ml of HPLC-grade water was added. A vacuuming step may be used to draw out air from the microcapsules prior to in vitro release or in vivo implantation.

3.2.5 In Vitro Release

Work by Alex Scott and Byron Masi (MIT, 2010) covered the in vitro release of temozolomide from five-hole liquid crystal polymer (LCP) microcapsules. LCP microcapsules
containing approximately 10-11 mg of temozolomide (Sigma Aldrich) were sealed, immersed in 2 ml HPLC-grade water, and vacuumed to remove any trapped air within the microcapsules. Each time point involved removing the entire volume of the vial, taking a 100-200 µl aliquot from it and running it through an HPLC column (Eprogen). Complete replacement of the bath allowed for sink conditions to persist for the duration of the release experiment. Peak intensities were measured at 316 nm and compared to a previously determined calibration standard. Time points were taken approximately every two hours for the first two days, and with more time in between for the remaining release period.

LCP microcapsules containing 2, 3.5, and 5 mg doxorubicin hydrochloride (Sigma-Aldrich) were sealed and immersed in 2 ml artificial CSF (Invitrogen), and vacuumed to remove any trapped air (n=3). The second set of experiments had LCP microcapsules with two, three, and five holes (n=2) containing 5 mg doxorubicin hydrochloride each. Each time point involved removing the entire volume of the bath and replacing it, allowing for sink conditions to persist. Dilution of the volume removed was often necessary to prepare the sample for the fluorescent plate reader (Biotek Instruments, Inc.). Dilutions were made using cold (stored in 4ºC) artificial CSF and vortexed to achieve good mixing. Aliquots of 100 µl were read for each time point (n=3 for each reading). Intensity values were compared to a previously determined calibration standard. Time points were taken approximately every two hours for the first two days.

3.2.6 Drug Calibration, Solubility, and Stability

Several sets of calibration curves were obtained for temozolomide, because different versions of the drug were used in this thesis work. Calibration curves were made for temozolomide in water (to measure drug remaining in resected microcapsules) as well as in brain
homogenates (to measure drug retained in tissue). Serial dilutions ranging from 800 µg/ml to 0.05 µg/ml were made of temozolomide in the various solvents (water, artificial-CSF, and brain homogenate or extract). Signals read at 330 nm were stronger than those read at 316 nm, so for samples with lower concentrations, readings were done at 330 nm to maximize the limit of detection, which was 1 µg temozolomide in 1 ml brain homogenate.

Calibration curves of doxorubicin in solution were made as a standard with which experimental readings were compared. Serial dilutions of doxorubicin were made and measured using the BioTek fluorescence plate reader. There was a saturated intensity beyond which concentrations cannot be clearly distinguished, so any samples of doxorubicin that read above 4000 on the intensity scale were diluted down and re-run. The limit of detection was 0.5 µg/ml of doxorubicin in a-CSF, below which the readings were baseline and could not be significantly distinguished from the intensity of a blank a-CSF sample.

Solubility values were determined by oversaturating a small volume of solution (0.5 ml-1 ml), vortexing for at least five minutes, and then centrifuging down to obtain a saturated supernatant. Stability values were determined by maintaining samples of temozolomide or doxorubicin in solution at physiological temperature (37ºC) and taking intermittent measurements. HPLC measurements were done for temozolomide solution, and fluorescence plate reader measurements were done for doxorubicin solution. HPLC plate and column temperatures were maintained at 37ºC, while doxorubicin samples were kept in an incubator at that temperature in between fluorescence plate reader measurements.
3.3 Characterization of Chemotherapeutics

3.3.1 Calibration

The following graphs show different typical calibration curves for temozolomide. Multiple data series for $^{13}$C-temozolomide are given in some of the graphs to show repeatability. The graphs below show calibration curves for Sigma temozolomide in brain homogenate, $^{13}$C (CIL) temozolomide in water, $^{13}$C-temozolomide in brain homogenate at 316 nm, and $^{13}$C-temozolomide in brain homogenate at 330 nm. Calibration equations are given along with their correspondent graphs.

![Graphs showing calibration curves for temozolomide in different media](image)

Figure 17. Calibration curves for temozolomide in different media

The following graphs show calibration curves for doxorubicin in artificial CSF and brain homogenate/extract. Limit of detection in both cases was found to be approximately 0.5 µg/ml.
3.3.2 Solubility

The solubility of doxorubicin was determined to be 22 mg/ml in both water and artificial CSF. The solubility of temozolomide was determined by Alex Scott and Byron Masi (both of MIT) at neutral pH to be 8.7 mg/ml in water and artificial CSF.

3.3.3 Stability

The half-life of temozolomide in water at 37°C was previously found by Alex Scott and Byron Masi to be approximately 40 hours. The half-life of \(^{13}\text{C}\)-temozolomide in brain homogenate was found to be approximately 3 hours at 37°C. The half-life of doxorubicin in artificial CSF at 37°C was determined to be approximately 25 hours. Preparation time for both doxorubicin and temozolomide samples in brain tissue was about half an hour to forty minutes, with the samples being at 4°C for twenty to thirty minutes. Given the easy readings of doxorubicin in the microplate reader (and full recovery from spiked brain sample), and the short preparation times for both doxorubicin and temozolomide, sample analyses were obtained within a reasonable time frame.
3.4 In Vitro Release of Doxorubicin from Microcapsules

Two primary experiments were done to investigate whether the microcapsule performed as expected and whether zeroth order release was achieved. The first set of in vitro experiments explored the release of three different payloads from a two-hole microcapsule: 2 mg, 3.5 mg, and 5 mg of doxorubicin. The second set explored the release of 5 mg payloads from microcapsules with different numbers of holes (two, three, and five) open on the microcapsule. Mass flow rates were compared with theoretical numbers to test whether drug release out of the microcapsule was diffusion limited.

3.4.1 In Vitro Release of Different Payloads

Microcapsules with five holes, four on the side of the cylinder and one on the cap—were filled with varying amounts of doxorubicin hydrochloride (DOX) and three of the side holes covered up. Theoretical calculations for diffusion-limited rate of release were done based on Fick’s first law: \( J = -D \frac{\Delta c}{\Delta x} \), where \( J \) is the flux, in mg/cm\(^2\)/s; \( D \) is the diffusivity, in cm\(^2\)/s; \( c \) is concentration, in mg/cm\(^3\), and \( x \) is distance, in cm. Diffusivity was estimated to be \( 2.96 \times 10^{-5} \) cm\(^2\)/s for small molecules. Assuming sink conditions, concentration \( c \) changes from 0 outside of the microcapsule to the solubility limit inside the microcapsule. The solubility value of
doxorubicin in water and artificial CSF was determined to be 22.8 mg/ml. The thickness of the microcapsule cap as per fabrication specs was 0.04 cm, and this is the distance x across which transport took place.

The mass release rate is $\dot{m} = J \cdot A$. The release rate of doxorubicin out of a microcapsule with one hole in the cap open and one hole in the side open (total two holes open), with the rest closed using UV-cured epoxy was computed. The total area of the openings, based on radii of 180 µm (cap) and 403 µm (side) was 0.00153 cm$^2$. Flux was calculated to be 0.016872 mg/cm$^2$s, giving a mass flow rate of 2.23 mg/day. Similar calculations were then done with three holes open (one cap, two sides) and five holes open (all holes open). The mass flow rates were found to be 4.09 mg/day and 7.81 mg/day, respectively.

The figure below shows *in vitro* release profiles over 200 hours for a two-hole microcapsule. The release rates were different for each payload; that is, the amount of drug inside the microcapsule affected how fast the drug was released over time.

![In Vitro Release, 2-hole device](image)

**Figure 20.** *In vitro* release profiles for 2-hole LCP microcapsules showing payload-dependent release rates. Surface-area limited release was found for payloads less than 5 mg.
Figure 21. Linear region of in vitro release profiles. Payloads greater than 5 mg were found to have release rates closer to theoretical rates calculated by Fick's First Law (diffusion-limited).

Closer examination of the release profiles’ linear region showed that the 5 mg payload release rate came close to the expected/theoretical release rate. This meant that a primarily surface-area limited regime (with concentration gradient inside the microcapsule) existed for payloads below 5 mg. Release of payloads above 5 mg are limited by diffusion, assuming the solubility limit of concentration on the inside and sink conditions on the outside of the microcapsule. Inside the microcapsule, a concentration gradient ranged 22.8 mg/ml right at the interface of the drug bed to 0 mg/ml right outside of the microcapsule. The calculated linear release rates based on the data obtained were 0.0225 mg/hour (0.54 mg/day), 0.0421 mg/hour (1.01 mg/day), and 0.0719 mg/hour (1.73 mg/day) for 2 mg, 3.5 mg, and 5 mg payloads, respectively.

The rates of release translated to concentrations at the point of release (cap hole) of 5.52 mg/ml, 10.33 mg/ml, and 17.64 mg/ml for the 2 mg, 3.5 mg, and 5 mg payloads, respectively. It was assumed that the primary concentration gradient is between the drug inside the microcapsule
and the cap orifice. Doxorubicin turned into a gel at supersaturated concentrations, and this gel abutted the side hole of the microcapsule. Thus it was not expected that release from the side hole was limited by an inside concentration gradient. The limitation for payloads that did not pack the microcapsule was a concentration gradient inside the microcapsule.

3.4.2 Release Rates from Different Number of Microcapsule Openings

A second *in vitro* series was conducted using a 5-mg doxorubicin payload released from a microcapsule with different numbers of holes open. The following graph shows the release profiles (n=3) for each of the groups. Error bars signify ± one standard deviation from the average. It was expected that the release rates would differ for each type of microcapsule, given the total area of flux open.

![Graph showing release profiles](image.png)

Figure 22. *In vitro* release profiles for 5 mg payloads of doxorubicin from devices with different number of holes open

The theoretical release rates/mass transport rates for microcapsules with two, three, and five holes open were calculated to be 2.23 mg/day for two holes, 4.09 mg/day for three holes,
and 7.81 mg/day for five holes. The following graph shows the \textit{in vitro} release experiment for the two-hole microcapsule configuration. A line signifying the theoretical release rate is also given in the graph.

Figure 23. \textit{In vitro} release profile for 5 mg of doxorubicin from a 2-hole LCP microcapsule. The blue line signifies the theoretical release rate.

The general linear release rate is close to the theoretical release rate for a 5 mg payload of doxorubicin out of a two-hole microcapsule. Discrepancies were observed, however: after a certain time the release rate was not as high as its theoretical rate. This was attributed to non-sink conditions; despite removal of the entire bath at every time point, the doxorubicin that did get released and was around for even the small amount of time between time points made it so that the concentration gradient was not equal to the solubility limit. Some coloration or staining of the microcapsule and insides of the glass vial containing the microcapsule was also observed, contributing to this decrease in concentration gradient between the inside and outside of the microcapsule.
CHAPTER 4: *IN VIVO* RELEASE AND EFFICACY OF MICROCAPSULES

4.1 Introduction

Polymer passive implants placed in the body near targeted (e.g. tumor) sites have been demonstrated to locally deliver drugs and decrease overall systemic toxicity. Biodegradable polymers have been used for commercially-available passive drug delivery systems. Passive devices release the drugs contained in their reservoirs slowly, as the biodegradable polymer dissolves within the body.

Temozolomide was incorporated in pressed discs of a polyanhydride 1,3-bis-(p-carboxyphenoxy)propane:sebacic acid (CPP:SA) polymer and implanted against intracranial 9L gliomas. The proportion of temozolomide in the CPP:SA polymer formulation ranged from 10% to 20% to 50%. A single polymer loaded with 50% temozolomide was the equivalent of 5 mg temozolomide. The maximum amount of polymer that could be placed in the rat brain was two wafers, for a total of 10 mg temozolomide.

Administration of single implantations of the polymer wafers increased the percentage of long-term (>120 days) survivors to 25% from 0%. Rats given two wafers did not exhibit any neurological symptoms, so it was concluded that the maximally tolerated dose (MTD) of
temozolomide delivered via polymer wafer was not reached. Implanted wafers (especially two, payload 10 mg) were found to be efficacious against 9L gliosarcoma tumors.

Figure 25. Kaplan-Meier survival curves showing improved survivals for groups treated with TMZ polymers [12]

4.2 Materials and Methods

The work done in this chapter seeks to explore the in vivo efficacy of the microcapsules tested in Chapter 3. In vitro efficacy against the cell lines used to induce tumors in rat brain models was also performed in order to find the necessary amount of drug exposure. Similarly, ex vivo analysis was done to determine the implied amount of drug released from devices based on the amount of drug remaining in resected devices. Much of the methods described in this chapter will be applicable to both this and the next chapter, on drug distribution and exposure in the brain tissue. References to this section will thus be made in the next chapter’s materials and methods section.
4.2.1 Cell Lines: MTT Assay and Tumor Induction

MTT cell proliferation assays using doxorubicin were performed by collaborators at Johns Hopkins Medical Institute (JHMI) and Kevin Spencer of the Cima Lab at MIT. Survival experiments with rat tumor models were conducted with collaborators at JHMI, under the supervision of Prof. Betty Tyler. This writer participated in the experiments that took place at JHMI, helping with surgical and gavage procedures. 9L rat glioma and CRL1666 mammary adenocarcinoma cells lines were cultured in vitro. The cells were plated at a density of 3500 cells/well. Cell plating was followed 24 hours later with addition of doxorubicin hydrochloride diluted in Dulbecco’s Modified Eagle Medium (DMEM) culture media with 10% glucose (Invitrogen). The drug concentrations used ranged from 0.005 to 50 µg/ml, and blank media was used for control samples. Cells were counted 48 hours after the addition of doxorubicin (or blank media) to the wells. MTT assays were used to count cells in vitro.

Conversion of water soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into insoluble purple formazan in living cells was followed by solubilization of the formazan in SDS (sodium dodecyl sulfate) and HCl. This resulted in a colored solution whose intensity can be measured using a fluorescence plate reader at an absorbance wavelength of 570 nm. This MTT assay was used to count the number of living cells, and further to determine the effective in vitro concentration of drug against the cell lines.
Figure 26. 1 mm$^3$ pieces of 9L gliosarcoma tumor resected from rat flank and implanted intracranially for survival studies [JHMI]

$10^6$-$10^7$ 9L cells in 0.3-0.4 ml saline were injected into the flank of a female Fischer 344 rat using 18-20 gauge needles. Once the tumor grew to have a diameter of ~2 cm, the rat was sacrificed. Excision of the flank tumor was followed by cutting up of the mass into 1 mm$^3$ pieces that were maintained in 0.9% saline solution. The 1 mm$^3$ pieces of flank tumor were then inducted intracranially in Fischer 344 rats.

CRL1666 (ATCC) rat mammary adenocarcinoma cells were prepared for stereotactic injection into the brain for survival studies. They were plated and maintained in McCoy’s Media with 10% fetal bovine serum (FBS, Gemini BioProducts) and 0.2% Plasmocin (InvivoGen). The culture was maintained in 5% CO$_2$ atmosphere in a 37°C incubator. Monolayers of cultured CRL1666 cells were then harvested with 0.025% trypsin (Invitrogen), counted, and resuspended in medium. The drug concentrations that were used ranged from 0.001 to 400 µg/ml, and blank media was used for control samples. Cells were counted 48 hours after the addition of doxorubicin (or blank media) to the wells. Tumor induction involved stereotactic injection of 75,000 metastatic breast cancer cells intracranially via 2.5 µl media, at a depth of 3.5 mm, and over the course of 2 minutes.
4.2.2 Oral Gavage: Systemic Administration of Temodar®

Oral temozolomide was administered via gavage from the contents of Temodar® capsules, obtained from collaborators at the Brem Lab at JHMI, suspended in a small amount of water or saline solution. Previous work done by Brem et al and Zhou et al found that oral gavage concentrations of 50 mg/kg/day for five days reached the maximally tolerated dose [12,86]. Temodar® powder in that concentration was suspended in 0.5 ml of phosphate buffered saline or water, and delivered using a gavage feeding needle. Systemically treated rats were not placed under anesthesia. Administration of oral gavage was repeated once a day over a five day period, beginning with the same day that tumor was induced or implanted in the rat brain.

Figure 27. Drawing depicting oral gavage procedure for rats [12]
4.2.3 Intravenous Tail Vein Injections: Systemic Administration of Doxil®

![Intravenous tail vein injection](image)

**Figure 28. Intravenous tail vein injection [BU Animal Care]**

Systemic delivery of doxorubicin was done via intravenous injection of the liposomal formulation Doxil® into the rat tail vein at a dose of 5.67 mg/kg, repeated once a week for three weeks. Rat tail veins were warmed on a warming pad, and injection into the tail vein was done with rats under anesthesia (using continuous administration of isoflurane or intraperitoneal injection of ketamine/xylazine). Total volume of injection was between 0.5 and 1 ml, delivered over a period of fifteen to twenty minutes. Dosages were given on days 0, 6, and 13, with day 0 being the first experiment day in which tumor cells/tumor pieces were intracranially implanted.

4.2.4 Craniotomies

An intracranial rat model was used for all of the *in vivo* work done for this thesis. Procedures were conducted in accordance with federal guidelines and MIT Committee on Animal Care policies. Microcapsules were implanted in the intracranial space of 150-220 g Sprague-Dawley (at MIT) or Fisher 344 (at JHMI) female rats. Rats for JHMI experiments were obtained from Harlan Sprague Dawley, and rats for MIT experiments were obtained from
Charles River. Rats were anesthetized with continuous isoflurane gas (1 lpm O₂ with 2-2.5% isoflurane) for the work done at MIT, and intraperitoneal injection of ~3 ml/kg solution of ketamine hydrochloride (25 mg/ml), xylazine (2.5 mg/ml), and ethanol (14.25%) diluted in sterile saline (0.9%) for the work done at JHMI. Anesthesia was monitored using dorsal foot pad reflex and maintained throughout surgery. Buprenorphine, 0.05 mg/kg, was delivered subcutaneously prior to surgery and every 8 to 12 hours following surgery up to 48 hours.

Preparation for intracranial surgery included shaving the head and applying alternating betadine and alcohol scrubs. Intracranial implantation procedures were conducted under a stereoscope. A midline incision of approximately 2 cm in length was made, followed by opening up the membrane covering the skull using sideways movement of the scalpel’s blunt edge. The location of the burr hole for Dox microcapsules was in the left cortex, 2 mm away from the midline suture and 1-2 mm away from the lambda. The location of the burr hole for TMZ microcapsules was in the right cortex, 2 mm away from the midline suture and 1-2 mm away from the bregma. The latter location should be used in future surgeries, as implantation is done in the non-dominant side of the rat brain. Results reported in this thesis were unaffected because coronal sectioning was used across both hemispheres of the resected brain. A motorized power tip with a stainless steel drill bit (0.9 mm diameter, Aseptico) was used to drill a hole of up to 3.5 mm in diameter without drilling through the dura. Care was taken to drill the hole without hitting the cranial sutures to minimize bleeding. Jeweler’s forceps and scissors were used to cut away and remove the dura, exposing the brain matter underneath.
Jeweler’s forceps were used to gently indent a spot on the brain to help ease the microcapsule as it was implanted, cap opening facing down. If a 9L tumor was also induced, a ~1-2 mm³ piece of the flank tumor was implanted first, followed by the microcapsule. If a CRL1666 tumor was induced, the cells (75,000 in 2.5 µl) were injected prior to implanting the microcapsule. Post implantation, subcutaneous/subcuticular sutures and tissue glue were used to close up the incision. Post-surgical care included monitoring for signs of morbidity (hunched over, unresponsiveness to touch) and administration of analgesics (Buprenorphine, 0.05 mg/kg).

Euthanasia of the animals was done at different times during the course of distribution experiments (which will be covered in the next chapter). Survival surgery rats were sacrificed
upon showing signs of morbidity, or at 120 days, whichever is longest. *Ex vivo* exposure study rats were sacrificed on different days: those with implanted temozolomide microcapsules were sacrificed between days 1 and 20, while those with implanted doxorubicin microcapsules were sacrificed between days 1 and 12. Fatal-Plus solution, which contains sodium pentobarbital with minor amounts of propylene glycol, ethyl alcohol, and benzyl alcohol preservative as well as coloring for identification, was administered via tail vein injection or cardiac stick as one method of euthanasia (0.5-1 ml total volume). Carbon dioxide overdose was also used, especially in cases where blood samples had to be obtained. Euthanasia was followed by resection of the brain, and in some cases also liver and kidneys, as well as drawing of blood through cardiac puncture.

4.2.5 Efficacy Studies

Survival experiments to test efficacy of microcapsules in rat tumor models were conducted at the JHMI Neurosurgery lab under the supervision of Prof. Prof. Betty Tyler. The surgical procedures were described in the previous section. 1 mm³ pieces of 9L gliosarcoma that were maintained in the flanks of female Fisher 344 (F344 rats) were kept in ice-cold sterile 0.9% saline solution until it was time to implant them.

Distribution studies required only the implantation of the microcapsules in the cortex after making a small indentation to make space for the microcapsule in the brain tissue. Efficacy studies, however, required a small amount of cortex to be resected (up until white matter was visible under the microscope) in order to ensure proper implantation of the tumor. A piece of tumor would then be placed in the depths of resection, followed by a filled or blank microcapsule or simply closed up for control and systemic treatment groups. The number of rats per group was
Some cases had rats going through microcapsule implantation at day 5, after the tumor has had some time to attach and begin growing. This was a way to simulate treatments in which drug was not given until presence of tumor was observed. However, there was a difference in that in real patients, the tumor bulk was removed prior to implanting any drug-eluting compound (such as Gliadel). Tumor bulk was not removed prior to implanting the microcapsule in rats, in order to avoid excessive and further trauma to the rat.

Experiments that used CRL-1666 rat mammary adenocarcinoma instead of 9L gliosarcoma did not involve implantation of tumor pieces, but instead involved the injection of cells as mentioned in the craniotomy section. Cells were maintained in culture as described previously. 75,000 cells in 2.5 µl were injected at a depth of 3.5 mm over two minutes, without needing to use light suction to remove the cortex as in the case of the 9L tumor pieces. Rats were monitored post-operatively for signs of neurotoxicity, including ataxia and hemiparesis. Standard protocols for euthanasia in the event of ongoing symptoms of pain and distress were followed.

All efficacy studies were terminated at Day 120. Any animals that remained alive at that point were termed “long-term survivors” (LTS). Kaplan-Meier graphs were made using GraphPad Prism 5.1. Log-rank tests (Mantel-Cox) were conducted to determine statistically significant differences between experimental groups. Two-sided p-values were also determined, and p < 0.05 was considered to be statistically significant.

4.3 In Vitro Efficacy of Doxorubicin against 9L and CRL1666 Cells

In vitro efficacy work for doxorubicin against 9L and CRL1666 cell lines was conducted by collaborators at JHMI and by Kevin Spencer of the Cima Lab at MIT. The required in vitro exposure was calculated based on the IC$_{50}$ value for each assay. IC$_{50}$ is the half maximal
inhibitory concentration, or the concentration of drug that results in 50% inhibition (in this case, cell death). The given IC$_{50}$ value from each assay was multiplied by two, because the assays were done two days after addition of doxorubicin to the plated cells. This yields the exposure value, in units of µg/ml*day.

The graphs below show MTT assay results for the work done at JHMI and MIT. The first set of assay results showed IC$_{50}$ values of 0.075 µg/ml for 9L cells and 200 µg/ml for CRL1666 cells. The exposure over a two-day period was calculated to be 0.15 µg/ml*day for 9L cells and 400 µg/ml*day for CRL1666 cells.

Figure 30. MTT assay results for efficacy of doxorubicin against 9L and CRL1666 tumor cells [B. Tyler, JHMI]

The second set of assay results showed IC$_{50}$ values of 0.23 µg/ml for 9L cells and 0.52 µg/ml for CRL1666 cells. The exposure values over two days were calculated to be 0.46 µg/ml*day and 1.04 µg/ml*day for 9L and CRL1666 cells, respectively. The discrepancies between required exposures for both cell lines may be due to the number of passages for the cells.
4.4 Implied In Vivo Release and Maximally Tolerated Local Dosage

In vivo release rates were deduced from plots of the amount of temozolomide or doxorubicin remaining in resected microcapsules. Microcapsules were resected at different time points (days) along with the rat brains that they were implanted in, and opened in a water bath. Vortexing allowed for any remaining drug in the microcapsule to be fully dissolved.
The release rates for both doxorubicin and temozolomide were slower *in vivo* than *in vitro*. Temozolomide was mostly released from the microcapsule after twenty days; given its half-life of 1.8 hours, there was very likely a significant amount of degradation while the microcapsule was implanted. Doxorubicin was completely released from the microcapsule after six days. The implied *in vivo* peak release rates were 0.6 mg/day for temozolomide, compared to 2.16 mg/day theoretically, and 1.1 mg/day for doxorubicin, compared to 2.25 mg/day theoretically. Several factors affect the slower release rates *in vivo* than *in vitro*. The amount of fluid that would go inside the microcapsule and that flowed outside the microcapsule was much smaller than an *in vitro* diffusion bath. CSF volume in a rat brain is approximately 90 µl, whereas the diffusion bath volume was 2 ml. The implied *in vivo* release rate for temozolomide was slower than the implied release rate for doxorubicin; solubility of doxorubicin in artificial CSF was previously found to be more than double the solubility of temozolomide in artificial CSF at 37°C.

Temozolomide microcapsules had five holes open and the slower release rate was found to result in significant survival improvements in rats with tumors, significant drug exposures (as will be discussed in a later section), and no observed signs of toxicity in the rats. Doxorubicin microcapsules had two holes open, one on the cap and one on the side, but the high solubility of the drug resulted in its relatively rapid release rate *in vivo*. Non-tumor rats with implanted doxorubicin microcapsules exhibited signs of toxicity after 14 days, including pigmentation on head fur and more aggressive-than-normal responses. Previous work done with collaborators at JHMI did not find 5 mg payloads to produce signs of toxicity, but that work was done using microcapsules that have only the cap hole open. It was discovered then, that release of the majority of a 5 mg doxorubicin payload over a period of four to six days resulted in signs of
toxicity after 14 days. The maximally tolerated dose (MTD) of intracranially implanted doxorubicin in rats was determined to be 5 mg in six days. The maximally tolerated dose of intracranially implanted temozolomide in rats was not reached.

4.5 Survival Studies: In Vivo Microcapsule Efficacy

Survival studies were conducted with collaborators at the Johns Hopkins Medical Institution, under the supervision of Prof. Betty Tyler. Fisher 344 rats were split up into groups including control, blank microcapsule, drug-filled microcapsule (TMZ or DOX), and systemic TMZ and/or DOX. 9L gliosarcoma and CRL-1666 mammary adenocarcinoma cells were used to test the efficacy of the microcapsules against glioma and breast metastasis tumors. The choice was made not to simulate real surgical resections by first resecting a grown tumor before implanting microcapsules or beginning systemic treatments. It was decided that the multiple procedures would cause too much trauma to the rats, and in turn confound the survival study results. Kaplan-Meier curves were obtained for the different experiments to evaluate the efficacy of local and/or systemic treatments as compared to control and blank microchip groups.

4.5.1 9L Gliosarcoma Tumors

Kaplan-Meier curves were obtained for rats with implanted 9L gliosarcoma tumors, previously grown in the rat flank. Administration of temozolomide was evaluated by having groups of control rats, oral temozolomide (Temodar®), blank LCP microcapsules/devices, and temozolomide-loaded microcapsules (each n=8). The payload for temozolomide microcapsules was 10-11 mg per microcapsule. Survival was the primary endpoint for these analyses—animals still alive after 120 days were sacrificed and deemed long-term survivors (LTS). Statistical
significance analyses were performed using a log-rank (Mantel-Cox) double sided test. P-values of less than 0.05 were considered to be significant.

Kaplan-Meier survival curves for temozolomide against 9L gliosarcoma tumors were made using GraphPad Prism 5.1 and are depicted above. Median survival times were 14 days for control rats, 13.5 days for blank device rats, 26.5 days for rats with administered oral temozolomide, and 69.5 for rats with locally-delivered temozolomide (microcapsule temozolomide). P-values were not significant for control and blank microcapsule groups (0.2295), and significant for control vs. oral and local temozolomide groups (0.0128 and 0.0009, respectively). The local/intracranial administration of temozolomide was also significantly more efficacious than systemic/oral administration (p=0.0401). Results of these efficacy studies for local delivery of temozolomide were indeed very promising; the microcapsule module of
delivery was demonstrated to be more efficacious in delivering temozolomide than the systemic delivery method.

Kaplan-Meier curves were similarly obtained for delivery of doxorubicin, systemically and locally, against 9L gliosarcoma tumors. Groups included control, blank microcapsule, doxorubicin-loaded microcapsule (local), and systemic doxorubicin (Doxil® delivered intravenously, via tail vein). Intracranially (locally) administered doxorubicin had a payload of 5 mg per microcapsule, and this was repeated for all locally administered doxorubicin. Two graphs are given below to show the results of this study. The first graph shows the survival of rats with administered intravenous Doxil® as compared to control and blank microcapsule groups. The second graph shows the survival of rats with implanted doxorubicin microcapsules compared to control and blank microcapsule groups.

![Kaplan-Meier curves](image)

Figure 34. Kaplan-Meier curves showing efficacy of systemically delivered Doxil(R) against 9L tumor [JHMI]
The graphs above clearly showed that systemic delivery was superior to other experimental groups, and was significantly more efficacious against 9L tumors. Median survival times were 14 days for control rats, 11 days for rats with implanted blank microchips, 14 days for local/microcapsule doxorubicin, and 33.5 days for systemic/intravenous Doxil®. P-values were found to be significant only for comparisons involving systemic Doxil®: 0.0095 against local doxorubicin groups, and 0.0001 against control groups. P-values for local/microcapsule doxorubicin were 0.0569 against control groups and 0.1567 against blank microcapsule groups.

Blank and control groups were also found to not be significantly different from each other. Median survival time for rats with implanted blank microchips was slightly less than for control rats due to additional trauma caused by the implantation procedure. Intracranial delivery of doxorubicin did not prove to be efficacious against 9L tumors, unlike intracranial delivery of temozolomide via the same microcapsule module. Doxil®, which has already been proven to have activity against different tumors, was unsurprisingly efficacious against the 9L tumors and contributed to a significant increase in median survival time for the rats. Similar results would be
observed for CRL1666 tumors, with the main difference being the kill time—rats with injected CRL1666 tumor cells exhibited mortality much earlier than those given 9L tumor pieces.

4.5.2 CRL1666 Mammary Adenocarcinoma (Breast Met) Tumors

Similar sets of Kaplan-Meier survival curves were obtained for breast met tumors in the rat brain. The following curve shows survival curves with different combinations of treatment. The larger experiment that produced these curves had several groups, including control, blank microcapsule, systemic doxorubicin (intravenous Doxil®), systemic temozolomide (oral Temodar®), local doxorubicin (microcapsule), and local temozolomide (microcapsule). The first figure shows a head-to-head comparison of both local delivery modules (with control and blank device results also shown). The second figure shows a head-to-head comparison of local and systemic temozolomide delivery. The third figure shows a head-to-head comparison of local and systemic doxorubicin delivery.

Figure 36. Kaplan-Meier survival curves showing superior exposure by local microcapsule delivery of temozolomide over local delivery of doxorubicin [JHMI]
Figure 37. Kaplan-Meier survival curves showing efficacy of both systemically and locally delivered temozolomide against CRL1666 breast met tumors [JHMI].

Figure 38. Kaplan-Meier survival curves showing non-efficacious local delivery of doxorubicin, compared to efficacious delivery of systemic Doxil(R) against CRL1666 breast met tumors [JHMI].
Median survival time for control, blank microcapsule, and local/microcapsule doxorubicin delivery was 13 days—no difference between the groups was observed, and none of these groups had long-term survivors. The median survival times for systemic and local temozolomide were essentially the same, at 19.5 days and 20 days, respectively. The median survival time for systemic Doxil® was 19 days. Treatments that were found to have significant efficacy against the breast met tumor were systemic and local temozolomide, and systemic Doxil®. Local administration of doxorubicin was found to not be efficacious against breast met tumors. The table below gives p-values for the different groups calculated using a double-sided log-rank test.

Table 2. P-values for CRL1666 tumor survival groups comparing efficacies of each mode of delivery [JHMI]

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>Blank</th>
<th>IV DOX</th>
<th>Local DOX</th>
<th>Oral TMZ</th>
<th>Local TMZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>xx</td>
<td>0.2636</td>
<td>0.0001</td>
<td>0.1296</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td>Blank</td>
<td>xx</td>
<td>0.0001</td>
<td>0.3225</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td>IV DOX</td>
<td>xx</td>
<td>0.0714</td>
<td>0.4824</td>
<td>0.0454</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Local DOX</td>
<td>xx</td>
<td>0.0251</td>
<td>0.0018</td>
<td></td>
<td>0.1536</td>
<td></td>
</tr>
<tr>
<td>Oral TMZ</td>
<td>xx</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Local TMZ</td>
<td>xx</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Efficacy studies revealed a general pattern: that temozolomide, when locally administered, proved to be efficacious against both gliosarcoma and breast metastasis tumors, and that on the other hand, locally administered doxorubicin was not significantly efficacious against either gliosarcoma or breast met tumors. Systemic delivery modalities for both doxorubicin (intravenous) and temozolomide (oral gavage) were confirmed to be efficacious against both tumor types, and local delivery of temozolomide was found to also be efficacious or
even more efficacious than systemic delivery of temozolomide. The median survival times are
summarized below.

Table 3. Median survival times for local and systemic treatment groups. Local doxorubicin was the only
delivery module to not significantly improve rat survival.

<table>
<thead>
<tr>
<th></th>
<th>9L Tumors</th>
<th></th>
<th>CRL1666 Tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMZ</td>
<td>DOX</td>
<td>TMZ</td>
<td>DOX</td>
</tr>
<tr>
<td>Systemic</td>
<td>Local</td>
<td>Systemic</td>
<td>Local</td>
</tr>
<tr>
<td>26.5</td>
<td>69.5</td>
<td>19.5</td>
<td>20.0</td>
</tr>
<tr>
<td>33.5</td>
<td>14.0</td>
<td>19.0</td>
<td>13.0</td>
</tr>
</tbody>
</table>

The pattern of discrepancies between the two microcapsule performances (temozolomide better
than doxorubicin) is explained by examining the biodistributions of each drug in the rat brain
tissue. This makes up the heart of this thesis work: that distribution of drug in the brain tissue is
key to achieving successful activity of the released chemotherapeutic compounds against their
targeted tumors. The next chapter will discuss the strong relationship between efficacy of an
intracranially implanted microcapsule with the drug exposure that it provides.
CHAPTER 5: EXPOSURE & BIODISTRIBUTION OF DRUGS IN RAT BRAIN TISSUE

5.1 Introduction

Survival studies were done by Brem et al at JHMI utilizing implantable polymer wafers of temozolomide. \(^3\)H-labeled temozolomide in CPP:SA polymer-temozolomide formulations allowed the tracking of their biodistributions in the brain of rat models. Similarly, the distribution of oral temozolomide (Temodar) mixed with radiolabeled \(^3\)H-temozolomide was tracked. Oral gavage of Temodar was given at a dose of 50 mg/kg/day, due to discovery of transient leucopenia and thrombocytopenia after 3 days at that dose which resolved after 7 days. The MTD of temozolomide delivered orally was found to be 50 mg/kg/day, or for a 200 gram rat to be equal to 10 mg/day for a total of 50 mg delivered systemically in five days [12].

The biodistribution of temozolomide for rats with either implanted polymer-TMZ wafers or oral gavage temozolomide was measured using the liquid scintillation counter and counted against the total amount of radioactivity in the drug payload. Serum levels of temozolomide following intracranial delivery (via polymer) were consistently lower than those levels of temozolomide in the serum following oral gavage. This demonstrated that delivering drugs locally would lower systemic concentrations and in turn would reduce systemic side effects. Brem et al found peak serum levels of temozolomide to be 0.12 ng/ml for oral/systemic delivery, and 0.06 ng/ml for local delivery via polymers [12].

A distance of 2 mm away from the polymer implant saw 224 ng temozolomide/mg brain tissue, or 224 µg/g, after 4 hours. The concentration decreased after 28 hours to 163 µg/g, 62 µg/g after 76 hours, and 17 µg/g after 172 hours [12]. Computation of exposure or area under the curve (using the method used in the body of this thesis work) yielded exposure over 172 hours (7 days and 4 hours) to be 576.5 µg/g*days at a distance of 2 mm away from the polymer implant.
Biodistribution data was also given for a location ipsilateral to the polymer implant. Data given for a location contralateral to the implant (in symmetry to the location for the data given above) was as follows: 18 µg/g at 4 hours, 31.3 µg/g at 28 hours, and 11 µg/g at 73 hours [12]. This was computed by this author to yield an exposure of 64.21 µg/g*day contralateral to the polymer implant. The average exposure at 2 mm away from the polymer implant, averaged over both sides of the brain, was deduced to be 320.34 µg/g*day.

![Figure 39. Distribution of temozolomide in rat brain at a distance of 2.6 mm away from polymer wafer implant site. Data is given for ipsilateral distribution, contralateral distribution, and the calculated average distribution [based on Brem et al, 12].](image_url)

Brem et al also reported biodistribution concentrations for a rat brain exposed to temozolomide administered via oral gavage. These concentrations were measured based on amount of radioactivity present; the Temodar® used was mixed with tritium-tagged temozolomide. Average drug concentrations (assuming more or less uniform distribution) in the brain were 36 µg/g at four hours and 73 µg/g at 28 hours [12]. Over a period of 28 hours, this yielded an exposure of 54.5 µg/g*day.
5.2 Materials and Methods

Procedures for intracranial implantation, oral gavage, and intravenous injection into the tail vein were the same as those described in section 4.2. Other procedures are described in this section. Rats with intracranially implanted microcapsules were euthanized, as mentioned previously, at particular time points (days 2, 4, 6, 8, 10). Their brains were resected and stored in a -80°C freezer as soon as possible post resection (within half an hour to an hour) to preserve the integrity of the drugs that may be contained within the brain tissue.

5.2.1 Tissue Homogenization and Extraction

Tissues to be homogenized were taken out of the freezer and kept at room temperature to thaw while other materials needed for the protocol were prepared. A tissue extraction reagent from Invitrogen (FNN 0081, Invitrogen), stored at -20°C, was thawed in a 37°C incubator until there is only a small amount of ice left and then taken out to room temperature. NextAdvance air-cooled tissue bead homogenizer and ZrO beads were used to disrupt the various tissues. A temperature controlled centrifuge helped isolate tissue debris so that supernatants may be analyzed for drug concentration.

Extraction protocols for tissues exposed to doxorubicin began with slicing or cutting the resected brain, kidneys, or liver pieces into 0.1±0.02 g sections. Larger sections may be used for kidney or liver tissue, in which case everything else was scaled up accordingly. Each section was added to an amber-colored eppendorf tube (1.5 or 2 ml, VWR), to which 0.25 ml tissue extraction reagent and approximately 1 gram of ZrO beads were previously added. Centrifugation followed after homogenization of the tissue, at 13.2 krpm for 10 minutes at 4°C.
The supernatant was mixed in a 1:2 by volume ratio with methanol, vortexed, and centrifuged a second time under the same settings. The final supernatant was then measured, in aliquots of 100 µl, for fluorescence in the plate reader using Costar black 96-well plates. Each sample measurement signified the concentration of doxorubicin in a 1.25 mm slice of rat brain tissue.

Figure 40. Extraction procedure for measuring doxorubicin content in brain tissue

Homogenization of tissues used for isotope-ratio mass spectroscopy experiments was modified to reduce the carbon load from the detergent contained in the extraction reagent. One tenth the amount of extraction agent was used for the same amount of tissue. The rest of the volume was made up by adding distilled water. This extraction protocol was used for all consequent measurements for temozolomide concentration in brain tissue. A stock solution was prepared using 1 ml tissue homogenizer, 2 ml methanol, and 27 ml water, mixed gently to avoid frothing. This stock solution would ideally be kept ice-cold without freezing it, and thus kept in a freezer for a short amount of time while other samples were prepared. Brain tissue was cut into
0.1 ± 0.02 g pieces, and placed into an amber eppendorf tube with 1 gram of zirconium oxide beads. Added to each 0.1 g piece of tissue was 0.75 ml of the homogenizer stock solution, newly thawed and ice-cold. Three to four minutes of homogenization was followed by centrifuging for 10-15 minutes at 4ºC, after which the supernatant could be stored at -20ºC or run directly in the HPLC for analysis. Each sample measurement made by the HPLC signified concentration of temozolomide in a 1.3 mm slice of rat brain tissue.

![Diagram of extraction protocol](image)

**Figure 41. Extraction protocol for measurement of temozolomide concentration in brain tissue**

HPLC analysis was done using a guard column and at a column and analyzer temperature of 20ºC. Up to ten samples could be run at a time; samples waiting to be run were stored in the freezer to prevent them from degrading. Injection volume was 40 µl. Calibration curves using brain homogenates (processed the same way) spiked with temozolomide were made in order to quantify the results of the HPLC assays.
5.2.2 Isotope-Ratio Mass Spectroscopy

Isotope-Ratio Mass Spectroscopy (IRMS) experiments were conducted in collaboration with Prof. Ann Pearson at the Laboratory for Molecular Biogeochemistry and Organic Geochemistry at Harvard University. Through these carbon isotope ratio measurements, relative ratios of $^{13}$C to $^{12}$C in samples of homogenized brain tissue were obtained. $^{13}$C temozolomide has an isotopically-labeled carbon in its methyl group, which is present in the prodrug and active drug compounds but not present in the final degradation product of AIC.

A nickel spooling wire with a wire diameter of 0.25 mm was drawn through a moving wire interface at a rate of 0.8 cm/s. After going through a cleaning oven set at 950°C, the wire received analyte in the form of 0.5 µl droplets by automatic injection methods (for instance, an HPLC injector). Dispensing of solutions could also be done by hand when other injection methods are not readily available. Solvents evaporated under a heating element, leaving carbon-containing analytes to go through a combustion oven set at 750°C. A positive pressure of He gas was maintained in the CuO-containing combustion oven. This spooling wire microcombustion (SWiM) interface was based on a design by Brand and Dobberstein [11] and design modifications made by Sessions et al (2005) [68] and Nelson et al (2007) [54]. The mixture of gases coming out of the combustion oven (CO$_2$ from the analyte, He, and water vapor) were then passed through a Nafion membrane to remove any remaining water, leaving CO$_2$ and He to be analyzed against a standard CO$_2$ sample by a ThermoFinnigan DeltaPlus Advantage isotope-ratio mass spectrometer.
Analyses of IRMS temozolomide samples were done alongside analyses of internal amino acid standards with known concentrations and $^{13}$C/$^{12}$C isotope ratios: leucine, glycine, and histidine. Each sample analysis was done in quintuplicate and four samples were run one after another, with each group of four samples being separated by repeat runs of the amino acid standards.

Sample preparation for the first large set of experiments with IRMS involved cutting each resected rat brain in five sections of 0.3±0.03 g each. Five rat brains were used for each of the two experiments conducted; the brains were resected on days 2, 4, 6, 8 and 10 to match the resection times for brains used in both HPLC temozolomide and fluorescence reader doxorubicin readings.
Previously described extraction procedures for temozolomide in tissue were followed, with the exception that tissue/extract weight-to-volume ratios were multiplied by three to accommodate the larger mass (0.3 g) of each tissue slice. IRMS experiments yielded lower-than-expected concentration values, indicating that extracting drug from 0.3 g brain tissue pieces at one time was likely not as efficient as it could be. The second set of experiments still analyzed five regions of brain, but sample preparation was done for 0.1 g pieces instead of 0.3 g pieces. This was done to improve the extraction efficiency. Once extraction for 0.1 g pieces was done, three extracts were combined into one, vortexed, and measured in the IRMS. This process yielded higher measured temozolomide concentration values.

5.2.3. Computing Drug Concentrations and Biodistribution based on IRMS Data

IRMS measurements yielded ratios \( R = \frac{^{13}C}{^{12}C} \) for experimental samples, which were then compared to the ratio \( R_{VPDB} \) of a standard reference material, VPDB (Vienna Pee Dee Belemnite). The isotopic carbon ratio parameter \( \delta^{13}C \) was computed using the equation

\[
\delta^{13}C = \left( \frac{R}{R_{VPDB}} - 1 \right) \times 1000
\]

and presented in units of per mille (‰). Mass balance calculations were done taking into account the fractional abundance of the isotopes \( F = \frac{^{13}C}{(^{12}C+^{13}C)} \) or \( R/(1+R) \) [Hayes 1983]. The overall general mass balance equation for different samples, or different components of the same sample, is

\[
m_2F_2 + m_1F_1 + m_2F_2 + \ldots = m_{BrainExtr}F_{BrainExtr}
\]

Standard dilution curves of brain tissue homogenate, homogenizer solution, and temozolomide standards were used to determine temozolomide concentrations in experimental brain homogenate samples. “Clean” brain homogenates were analyzed to determine the extractable amount of carbon from brain tissue (\( m_{BrainExtr} \)) and the isotopic composition of that
extractable carbon ($F_{\text{Brain}}$). This required also knowing the amount of carbon in the homogenizer stock solution ($m_{\text{Homog}}$) and measuring the isotopic composition of the homogenizer stock ($F_{\text{Homog}}$). The measured amounts of the composite (brain tissue + homogenizer) and these other variables were related as follows: $m_{\Sigma} F_{\Sigma} = m_{\text{Homog}} F_{\text{Homog}} + m_{\text{BrainExtr}} F_{\text{Brain}}$. Measurements yielded the value of $F_{\text{Homog}}$ to be -31.2%. The ratio of brain mass $m_{\text{Brain}}$ per volume extract remained constant (initial undiluted concentration of 0.1 g brain tissue in 0.75 ml extract/homogenized volume). Spiked standards with known amounts of temozolomide (7, 14, and 28 µg/ml concentrations) were measured to complete the mass balance: $m_{\Sigma} F_{\Sigma} = m_{\text{Homog}} F_{\text{Homog}} + m_{\text{BrainExtr}} F_{\text{Brain}} + m_{\text{TMZ}} F_{\text{TMZ}}$. Calibrations made with spiked standards of known temozolomide amount were used to determine the concentrations of temozolomide in the experimental brain tissue extracts.

5.2.4 Computing Drug Exposure and Biodistribution based on Concentration Data

Biodistribution data was obtained for drug concentration at a given distance away from the location of the implanted microcapsule. The concentrations for a set number of time points (days 2, 4, 6, 8, and 10) could be integrated to obtain an overall exposure value for that location in the rat brain. Matlab calculations were done for the exposure as area under the curve for $x = [2, 4, 6, 8, 10 \text{ days}]$ and $y$ values corresponding to concentrations of drug at that location for those days. A Matlab 7.11.0 (R2010b, obtained through MIT IS&T) script was written to evaluate these integrals.

The number of samples for doxorubicin distribution was $n = 4$ rats for each time point. Data for temozolomide distribution in the rat brain had $n = 5$ rats for each time point. Both sets of data have the same time points (days 2, 4, 6, 8, 10). A Matlab script was written to evaluate
all the exposure values for $N_s^N_d$ permutations, where $N_s =$ number of samples per day and $N_d =$ number of days to find the average and spread of exposure for each drug. The script, written by Michiel Vanhoutte of MIT and modified as needed, is given below. Exposure data for doxorubicin and temozolomide were compiled and given error bars of one standard deviation based on the Matlab script output. Error bars given for the biodistribution data signify one standard deviation from the average.

```matlab
Nd = 5;  % number of days
Ns = 4;  % samples per day
Ns^Nd;
P = npermutek(1:Ns,Nd);
X=1:Nd;
T = xlsread('inputdata.xls');

for location= 1:length(T(:,1))
    for permutation = 1:Ns^Nd
        for day = 1:Nd
            sample=P(permutation,day);
            Y(day)=T(location,(day-1)*Ns+sample);
        end
        R=cumtrapz(X,Y);
        result=R(Nd);
        output(permutation,location)=result;
    end
end
xlswrite('outputdata.xls',output);
```

5.3 Validation of Tissue Extraction Process

A test to see whether temozolomide in the brain tissue is fully recovered through the extraction process confirmed its validity. 50 µg/ml and 100 µg/ml temozolomide were prepared and mixed thoroughly using a hand homogenizer. The brain tissue pieces were then put through the homogenization/extraction process, followed by analyses of supernatants in HPLC. The intensity of the supernatant corresponding to the 50 µg/ml sample translated to a 43.55 µg/ml
concentration when computed using calibration equations previously determined. The intensity of the supernatant corresponding to the 100 µg/ml sample translated to a 101.14 µg/ml concentration. Repeats using different variations of the extraction process (completed vs. partially completed) also showed similar results. That is, the extraction process was qualified to prepare samples for analysis of drug concentration in tissue.

5.4 Calibration of $^{13}$C-Temozolomide in IRMS

Calibrations for $^{13}$C-temozolomide measured using isotope ratio mass spectrometry (IR-MS) were done using spiked samples of brain extracts/homogenates. The mass balance equation

$$m_{\text{Homog}}F_{\text{Homog}} + m_{\text{BrainExtr}}F_{\text{Brain}} + m_{\text{TMZ}}F_{\text{TMZ}}$$

was used to determine a calibration curve for isotopically-labeled temozolomide concentrations in these spiked brain extracts. Additional details of isotope-ratio mass spectrometry will be discussed in a later section of this thesis.

Calibration curves were obtained for isotopically labeled $^{13}$C-temozolomide analyzed using isotope-ratio mass spectrometry (IR-MS). Error propagation yielded estimated uncertainties for all experiments of ± 0.7 µg/mL extract, or ±5µg/g brain, with a minimum detection limit of ~2 µg/g brain. Two experiments were conducted, the first involving extraction of a larger mass of brain tissue (0.3 g tissue/2.25 ml homogenate or extract solution) with all the requisite extraction components being scaled accordingly.

The second IR-MS experiment involved a finer extraction method for sample preparation that processed 0.1 g brain tissue/0.75 ml extract, with extract composition having one-tenth the volume of lysis buffer/homogenizing agent than normally used amounts. The actual amount of brain tissue $m_{\text{Brain}}$ between samples differed based on the total weight of each, but the brain tissue/extract ratio always remained constant. First IR-MS experiment brain samples were cut
into five pieces, while second IR-MS experiment brains were cut into fifteen pieces. The extractions from three pieces of brain tissue in the second experiment were then combined to make five total extraction samples. Given the differences between the two IR-MS experiments, the dilution series of temozolomide was normalized to the quantity of brain tissue in the extracted samples. The calibration curve of δ^{13}C values (in ‰) vs. amount of temozolomide (in µg) is given below.

![Calibration curve of δ^{13}C ratio and temozolomide](image)

Figure 43. Calibration curve of δ^{13}C ratio and temozolomide [A. Pearson, Harvard]

The calibration curve itself is represented by the diamond points (dilution series data), while the model for dilution series involving the mass balance equation is represented by square data points. Adjustments were made for the amount of brain tissue difference between the IR-MS experiments. An adjusted model for the first IR-MS experiment is represented by triangles. R^2 of the calibration equation, shown as a solid line on the graph, was 0.997. The calibration equation

\[ y = 0.0000229x + 0.0108523 \]

\[ R^2 = 0.997 \]
itself was $y = 0.0000229x + 0.0108523$. Dotted lines signify an adjusted calibration curve based on the different mass of brain tissues used in that first experiment.

5.5 Conversion of Concentration Data to Exposure Data

Exposure studies were undertaken to determine distribution of locally-administered doxorubicin and temozolomide, and systemically-administered Doxil® and Temodar® in resected rat brain tissue. Non-tumor rats were used for these studies in order to better control the integrity of the tissue, as well as the resection time points (otherwise animals may expire before the planned time points, and death may vary depending on how much of the tumor takes). Microcapsules containing doxorubicin (payload 5 mg) and temozolomide (payload 10 mg) were implanted in rat brains. Rats were sacrificed on days 2, 4, 6, 8, and 10, and their brains (cerebellum included) were resected, keeping the shape as intact as possible. Doxil® was administered at a concentration of 5.67 mg/kg, and rats were sacrificed on days 1, 2, and 3 post intravenous tail vein injection. Temodar® was administered per os at a concentration of 50 mg/kg/day for five days, and rats were sacrificed 4 hours, 28 hours, 76 hours, 7 days, and 9 days post first gavage. Rat brains were immediately frozen upon resection to prevent any drug from degradation, and homogenized following the protocols previously described.
The computations done to obtain exposure are as follows. First, a data set was obtained for n rats on each time point. The data set consisted of drug concentrations in µg drug per g brain tissue for each “location” in the brain. A location of “0” was defined to be the site of microcapsule implantation, or in cases of systemic delivery, the center of the brain. Length of rat brains were recorded and divided by the number of 0.1 g tissue slices cut from each brain. The average length of brain slices was 1.3-1.8 mm.

Table 4. Temozolomide concentration data for n=5 for each of five time points, across different locations in the brain tissue.

<table>
<thead>
<tr>
<th>Dist. (cm)</th>
<th>Day 1 conc. (µg drug/g tissue)</th>
<th>Day 2 conc. (µg drug/g tissue)</th>
<th>Day 4 conc. (µg drug/g tissue)</th>
<th>Day 6 conc. (µg drug/g tissue)</th>
<th>Day 8 conc. (µg drug/g tissue)</th>
<th>Day 10 conc. (µg drug/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-0.78</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
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<td>0.00</td>
</tr>
<tr>
<td>-0.65</td>
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</tr>
<tr>
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Once the data set was obtained, the average exposure for each location was obtained. This was done by selecting one concentration value out of each time point and computing the integral or area under curve. The Matlab script described previously was used to compute each possible
combination of values given that n=5 for each time point. One such example is given below, where the highlighted values are the input for area-under-curve calculations. The resulting value equals the exposure to temozolomide drug at a distance of 0.26 cm away from the implant location.

Table 5. Temozolomide concentration data for n=5 for each of five time points, across different locations in the brain tissue. Highlighted values show an example of a data set that would be integrated to obtain the area under curve or exposure value.

<table>
<thead>
<tr>
<th>Dist. (cm)</th>
<th>Day 2 conc. (µg drug/g tissue)</th>
<th>Day 4 conc. (µg drug/g tissue)</th>
<th>Day 6 conc. (µg drug/g tissue)</th>
<th>Day 8 conc. (µg drug/g tissue)</th>
<th>Day 10 conc. (µg drug/g tissue)</th>
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Each combination of areas under curve was then averaged through the output of the Matlab program, and an error bar obtained as ± one standard deviation from the average. The graph for temozolomide concentrations in tissue a distance of 0.26 cm away from the implanted microcapsule over a period of 8 days is given below. The AUC, or area under the curve, is obtained by taking the integral of the curve. This AUC value is equivalent to the temozolomide exposure 0.26 cm away from the implanted temozolomide microcapsule. The value in this case was computed to be 271 µg/g·day.
This procedure was repeated for each location or slice of brain tissue, building an exposure vs. time graph for each type of treatment: microcapsule temozolomide, microcapsule doxorubicin, systemic temozolomide, and systemic doxorubicin.

It was hypothesized based on the survival data that higher exposures to therapy yielded higher median survival times and higher percentages of long-term survivors. The local or microcapsule delivery of temozolomide was expected to have superior exposure to local delivery of doxorubicin based on their very disparate survival results. It was also hypothesized that exposure of temozolomide delivered locally was similar to or greater than exposure of systemically administered temozolomide (Temodar®). Exposure of locally administered doxorubicin was expected to be less than exposure of systemically administered doxorubicin (Doxil®).
5.6 Temozolomide and Doxorubicin Exposure in Brain Tissue

This section will discuss exposures of the two chemotherapeutic agents studied in this thesis. Sub-sections are framed in terms of hypotheses based on results of the efficacy work discussed in Chapter 4.

5.6.1 Local Temozolomide Exposure is Greater than Local Doxorubicin Exposure

The following graph confirmed the hypothesis that local temozolomide exposure was greater than local doxorubicin exposure.

![Graph showing exposure comparison between Temozolomide (TMZ) and Doxorubicin (DOX).]

Figure 46. Exposures from local delivery of temozolomide were higher and more extensive than exposures from local delivery of doxorubicin.

Not only was the value of exposure greater for temozolomide, it was consistently greater across the entire rat brain. Temozolomide exposure exceeded 100 μg/g*day for most of the brain. Doxorubicin exposure exceeded that same value for only the middle three slices—that is, beyond about 1.3 mm away from the implant site, doxorubicin exposure rapidly decreased.
Rat brain tumors tend to take up a significant amount of space given the already-small size of the rat brain. Indeed, tumors take up most of the brain mass by the time the rats succumb to them. It would then make sense that efficacious treatment of the tumors would require exposures that reach the entire span of brain tissue, and not just at or near the implant site. This head-to-head comparison of the local exposures explained the discrepancies in efficacy results for microcapsule deliveries of temozolomide and doxorubicin. Sufficient biodistribution and exposure are critical for successful activity against tumors.

5.6.2 Local Temozolomide Exposure is Greater than Systemic Temozolomide Exposure

A systemic temozolomide exposure value of 54.5 µg/g*day was obtained from Brem et al based on their work on implantable polymer-drug wafers for intracranial delivery in rat tumors [12]. Experiments repeated at MIT found the systemic temozolomide exposure value to average 12.59 µg/g*day, with a standard deviation of 5.3 µg/g*day. The average concentration of temozolomide (n=2) was found to be 1.93 µg/g after four hours and 23.25 µg/g after 28 hours. The exposure profile for oral Temodar® in the rat brain (n=4) over a total period of 28 hours (the period in which any temozolomide was detected by HPLC) is given below.
Very little accumulation of temozolomide occurred in this case, so even with repeated administrations, the exposure to systemic temozolomide was not very high. These experiments measured the concentration of temozolomide as an intact, retained compound in the rat brain tissue. This contrasts with the concentration of drug measured based on tritium concentration, which was the protocol for measurements conducted by Brem et al [12]. This may explain the discrepancy between the values—that only temozolomide was detected in this experiment, and any degradation products that may be present were not measured in the HPLC due to their very short half lives (MTIC in particular). The following graph compares overall local temozolomide exposure with the range of exposure values for systemic temozolomide (represented by a gray bar). Local temozolomide exposure was indeed higher than systemic temozolomide exposure.
Figure 48. Exposure from local delivery of temozolomide was higher than exposure from systemic (oral) delivery of temozolomide.

5.6.3 Local Doxorubicin Exposure is Less than Systemic Doxorubicin Exposure

Exposure curves were obtained for doxorubicin administered systemically through intravenous tail vein injections. Doxorubicin was administered in the liposomal, clinically-approved form of Doxil®. The distribution across a healthy rat brain ("0" being the center of the brain) is given below.

Figure 49. Exposure of doxorubicin in clean rat brain as a result of systemic delivery of doxorubicin was low.
This distribution of Doxil® was obtained for the first week of systemic administration. Given an administration schedule of three weeks, even if this exposure were to accumulate over the full three week period, the exposure in a healthy brain tissue would only be up to about 6 µg/g*day, which is orders of magnitude less than the higher ends of local exposure. However, as reported in the literature [70,87], the use of PEG-coated liposomal formulation of doxorubicin allowed the drug to go through the BBB, remain in circulation for a longer time, and have a higher retention of drug in the brain tissue. The graph below shows exposure of Doxil® in a rat brain that contains tumor.

![Graph showing exposure of brain tissue with tumor to systemically delivered doxorubicin](image)

**Figure 50.** Exposure of brain tissue with tumor to systemically delivered doxorubicin was higher than exposure to clean rat brain.

The exposure of liposomal doxorubicin in tumor brains was clearly greater than the exposure of liposomal doxorubicin in healthy brains. Compromised vasculatures of the tumor bed enabled the liposomes to extravasate through and be retained in the brain tissue for a longer time. These exposure values were uniform across the entire brain tissue, which conforms to what would be expected of a systemic method of delivery. Systemic Doxil® exposures to brain tissues with tumors were then compared to local doxorubicin exposures, as shown below.
Figure 51. Exposure of locally-delivered doxorubicin fell drastically 3 mm away from implant site. Exposure from systemic Doxil(R) was consistent across the entire span of brain tissue.

The exposure of brain tissue to doxorubicin delivered intracranially was higher than systemic exposures for only about a 5 mm distance away from the site of the implant. Coverage across the entire brain tissue was more consistent when Doxil® was delivered systemically. These results suggested that the exposures achieved by systemic administration were sufficient to achieve efficacy against tumors, but the limited extent of exposure achieved by local administration of doxorubicin was not efficacious.

Improvements to local delivery of doxorubicin from the microcapsules used in this work may be done in several ways. Modifications to the drug may be made; it is already known that liposomal forms of doxorubicin have improved circulation time, so different formulations of the drug may allow it to have higher retention in the brain tissue once delivered locally. Current and future work will explore delivery of modified forms of doxorubicin. The device itself may be further modified. It was found that in vivo release of a 5 mg payload of doxorubicin over four
days reached the limit of maximally tolerated dose (MTD). Rats did not exhibit signs of toxicity until 14 days post implantation, but the fact that they did at all showed that the MTD was reached. Modifications to the device could be made to further tune the rate of in vivo drug release, such that larger payloads could be released over a much slower time frame. This, combined with modifications to the drug itself, may well improve both exposure and efficacy results for locally administered doxorubicin.

5.6.4 Isotope Ratio Mass Spectroscopy Results

Five regions of brains resected on days 2, 4, 6, 8, and 10 were analyzed for their isotopically-labeled temozolomide content. Exposure data was obtained the same way that exposure data from HPLC and fluorescence plate reader was computed. Two experiments were conducted. The first experiment had larger slices of brain that were homogenized as is. It turned out that the extraction of these larger slices of brain was not as efficient as it could be. The analogy is of squeezing water out of a wet cloth: clumping up a large piece of cloth and squeezing it is much less efficient in getting water out than squeezing small pieces of the cloth at a time. The highest concentration of temozolomide measured in the first IRMS experiment was 8.9 µg/g, and the lowest detected concentration measured was 0.5 µg/g. Exposure data for brain tissue concentrations of temozolomide obtained from the first IRMS experiment is shown below. Exposure values above 10 µg/g*day was obtained consistently across the entire span of brain tissue. The exposure values, however, were clearly far below the measured magnitudes found through HPLC concentration data.
Figure 52. Temozolomide exposure computed from the first set of IRMS data. Exposure values were lower than expected due to inefficient extraction procedures.

The second IRMS experiment still measured concentration values of temozolomide for five slices of the brain (each measuring 3.9 mm thick). The difference in preparation, however, was in measuring concentrations for 0.1 g slices of brain tissue instead of 0.3 g slices, and then combining the extracts for sets of three brain tissue slices. Going with the same analogy, this was akin to squeezing the wet cloth little by little, from one end to the other.

This refined extraction method was indeed more efficient than the extraction method for the first experiment. The highest temozolomide concentration measured was 17.2 µg/g, while the lowest measured temozolomide concentration was 9.1 µg/g. Exposure data for the second IRMS experiment is given below.
Exposure across the entire span of brain tissue was greater than 90 µg/g*day, consistent with the order of magnitude of exposure values calculated from HPLC concentration data. Despite this, the actual exposure values of brain tissues measured with IRMS were lower than exposure values of brain tissues measured with HPLC. It may just be that the five brains (n=1 for each time point) that were measured happened to not have high concentrations of retained temozolomide. The spread of distribution data for temozolomide was indeed high due to limited detection levels (10-20 µg/g brain for HPLC, 2 µg/g brain for IRMS), so actual exposure values may be higher than what was detected by either measurement technique. The similarities in magnitude of HPLC exposure data and IRMS exposure data, however, did reconfirm that temozolomide was released far into the tissue away from the implant site.
5.6.5 Microcapsule Temozolomide vs. Polymer Wafer Temozolomide: A Comparison

Results for localized delivery of temozolomide showed great promise for intracranial delivery of temozolomide by LCP microcapsules developed in the Cima Lab. Comparisons were made with exposure data from Brem et al to see how the microcapsule compared to a temozolomide-polymer wafer developed at JHMI. Distribution data was reported in the literature for a distance of 2 mm away from wafer implants. Each CPP:SA polymer wafer contained a maximum of 50 wt% temozolomide, making each wafer payload 5 mg of temozolomide. Up to two wafers could be implanted in the rat cranium, which meant the maximum payload for treatment in rat brain with polymer wafers was 10 mg.

Based on the given distribution data for ipsilateral and contralateral sides of the brain 2 mm lateral to the wafer implant site, an average distribution profile was obtained. The average distribution profile for JHMI wafers at 2 mm away from the implant site was overlaid with the distribution profile for microcapsules at 2.6 mm away from the implant site. Exposure values were computed for each by finding the area-under-curve values.

![Figure 54. Comparison of temozolomide distribution at a distance of 2 mm and 2.6 mm away for MIT microcapsules and JHMI polymer wafers.](image-url)
Exposure at a distance of 2 mm away from JHMI wafers was calculated to be 332 µg/g*day, and exposure at a distance of 2.6 mm away from microcapsules was calculated to be 271 µg/g*day. These values were comparable, and both modules of delivery have been shown to be efficacious against intracranial rat tumors. Both modules had the same payload, about 10 mg of temozolomide. The microcapsule mode of delivery has a few advantages that could potentially make it even more efficacious compared to the wafer module. First, the payload of microcapsules can go up to 12 mg of temozolomide per microcapsule. Second, two microcapsules could be implanted in the rat brain, which means that up to a 24 mg payload could be used.
CHAPTER 6: SUMMARY AND FUTURE WORK

Challenges in current mainstays of brain cancer treatment revolve around the prevalence and mortality rate of recurrent tumors and multiple metastases. The median survival time for patients with recurrent or metastatic brain tumors is often on the order of ten months. Current treatments remain systemic in nature, including radiotherapy and chemotherapy, and improve prognoses only by a small amount. Systemic administration of chemotherapeutics results in systemic toxicities, including hematologic events such as decrease in white blood cell count and platelet count, cerebral hemorrhage, and pneumonia.

Localized chemotherapeutic delivery modules have the potential to improve prognoses while avoiding systemic toxicities. The currently approved local treatment module for patients with resected brain tumors, Gliadel® wafers, was shown to improve median survival times by about 2-3 months. Patients still experienced side effects including edema, hydrocephalus, and leukopenia, some caused by toxicities associated with the BCNU (carmustine) in the wafers. Researchers such as the Brem lab at Johns Hopkins Medical have developed CPP:SA polymers for localized diffusion-based release of temozolomide, a DNA alkylating agent. Survival experiments have shown these polymers to be efficacious against rat brain tumor models.

A biocompatible, implantable reservoir-based delivery microcapsule was developed for release of temozolomide and doxorubicin into the brain. This liquid crystal polymer microcapsule was demonstrated to work \textit{in vitro}, with rates of release following Fick’s First Law for the payloads that were used. Two rat brain tumor models were utilized to test the microcapsules’ \textit{in vivo} efficacy: a 9L rat gliosarcoma tumor model and a CRL1666 rat mammary adenocarcinoma tumor model. Microcapsules containing temozolomide were found to be efficacious against both tumor models, and in the case of 9L gliosarcoma, even more efficacious.
than systemic administration of temozolomide. Microcapsules containing doxorubicin did not perform as well and were found to not be significantly efficacious against either tumor model.

Biodistribution studies revealed the reason for this discrepancy in the in vivo performances of doxorubicin and temozolomide microcapsules. Exposure of locally-delivered temozolomide was high across the entire span of brain tissue, while exposure of locally-delivered doxorubicin fell drastically past a distance of 2-3 mm away from the implant site. Sufficient exposures over “long” distances in treated tissue were crucial for efficacious local treatment.

Future and current work will partly focus on improving the local delivery of doxorubicin in the brain. Doxorubicin has the potential to be a good candidate for localized delivery because of its high systemic toxicity. Liposomal forms of the drug have been shown to extravasate preferentially to tumor tissue through compromised vasculature. Improvements to the drug formulation, then, are being explored to increase retention time in tissue. Improvements to the microcapsule itself may contribute to a more consistent release profile. These may include the inclusion of membranes to slow down release, adding excipients to the loaded drug to modify in vivo release rates, and increasing payloads that would release over a slower period of time.

The maximally tolerated dose for locally delivered temozolomide has not been found, by this author or by other researchers. Increasing temozolomide payload could potentially increase efficacy in a larger tumor model (larger animal cranial tumor models) by improving accumulated exposure over larger distances. Each temozolomide microcapsule is capable of releasing an efficacious amount of drug up to a distance of 0.8 cm away in either direction. Implanting two microcapsules distanced 0.8 cm away from each other would double the exposure zone. Implanting multiple microcapsules distanced 0.8 cm away from each other in an area surrounding a tumor resection cavity in a patient could mean temozolomide coverage extensive
enough to reach distances where recurrent tumors often appear (2-3 cm away from the original lesion or resection site). Schematics for what this might look like clinically are given below.

Figure 55. Schematic for clinical use of biocompatible LCP microcapsules. The microcapsules may be implanted in the brain tissue using a large biopsy-needle apparatus.
REFERENCES


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