DEVELOPING A MINIMALLY INVASIVE SUSTAINED RELEASE SYSTEM FOR GLIOMA THERAPY

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DEVELOPING A MINIMALLY INVASIVE SUSTAINED RELEASE SYSTEM FOR GLIOMA THERAPY

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SUMMARY

Malignant brain tumor is one of the most lethal forms of cancers. In the United States alone, approximately 20,500 new cases of primary malignant brain and central nervous system tumors are expected to be diagnosed in 2007 with 12,740 deaths estimated. Treatment of malignant brain tumor remains a major challenge despite recent advances in surgery and other adjuvant therapies, such as chemotherapy. The failure of potential effective chemotherapeutics for brain tumor treatment is usually not due to the lack of potency of the drug, but rather can be attributed to lack of therapeutic strategies capable of overcoming blood brain barrier for effective delivery of drug to the brain tumor.

In this thesis, we developed a minimally invasive sustained release system for glioma therapy. The present study was initiated in an effort to incorporate Doxorubicin (DOX) loaded PLGA particle into an agarose gel, which can provide a continuous release of DOX locally to the tumor site. DOX, a toposiomearase II inhibitor, is not currently used clinically for brain tumor treatment because when delivered systemically it does not cross BBB. Our hydrogel particle system can overcome this shortcoming of DOX. The results from this study demonstrate that the DOX/PLGA particle gel system can maintain the bioactivity of DOX and sustained release DOX for at least 15 day in vitro. The result of in vivo study showed the DOX/PLGA particle gel treated group had significantly extended the medium survival of 9L glioma bearing rat from 21 days to 29 days. Therefore, the success experience of this local and sustained delivery device might benefit the development of future glioma therapy strategy.

CHAPTER 1

INTRODUCTION

1.1 STATEMENT OF PROBLEM

Malignant brain tumor is one of the most lethal forms of cancers. Although the incidence rate of primary malignant brain tumor is low (7.4 cases per 100,000 person-years) (CBTRUS 2006), death rate is still very high. In the United States alone, approximately 20,500 new cases of primary malignant brain and central nervous system tumors are expected to be diagnosed in 2007 with 12,740 deaths estimated. (Jemal et al. 2007) The need for treating this devastating disease has intensified the research in drug discovery and drug delivery to brain tumor. However, prognosis for patients with malignant brain tumor remains poor.

The traditional treatment of malignant brain tumor is surgical resection, followed by radiation therapy or chemotherapy; however, they are only capable of prolonging survival rather than eradicating the disease. With high-grade gliomas such as glioblastoma multiforme (GBM), the median survival time after surgical resection is six months and the addition of radiation therapy extends survival to nine months due to the recurrence of the tumor (Barker et al 1998). Currently, chemotherapy is not the primary treatment of choice for malignant brain tumor because most therapeutic agents for brain tumor cannot reach therapeutic doses within the brain due to the blood brain barrier (BBB). Only small (< 500 Dalton), lipid soluble molecules can passively cross the barrier (Lesniak and Brem 2004). To achieve the therapeutic level of drug in brain tumor, the drug is administered systemically, which leads to serious side effects. One

possible way to overcome the BBB is to administer the drug directly at the site of brain tumor. However, the risk of infections will increase with administration frequency. Thus, it will be beneficial to brain tumor treatment to develop a minimally invasive sustained drug delivery system that can continuously release therapeutic agents into the brain (Pardridge 2003).

One approach for intracranial drug delivery is to surgically implant a polymer system that is capable of sustained release of drug at the tumor site. Currently, Gliadel® Wafer is the only marketed therapy for recurrent tumor of GBM, in conjunction with radiation therapy and surgery (Kleinberg et al. 2004). It continuously releases Carmustine (BCNU), a chemotherapeutic agent, in the tumor resection site through the degradation of the polymer wafer. However, the agent is delivered from biodegradable wafers, which may or may not completely cover the surface after resection. Most studies showed majority of BCNU can only penetrate within 1-5 mm from the implantation site (Fleming and Saltzman. 2002), thus not reaching all of the tumor cells. Similar problems were also observed in other delivery systems (rods, sheets, and microparticles). Therefore, there is a great need to improve the current drug delivery system.

1.2 HYPOTHESIS

For successful treatment of brain tumor with an intracranial implant, the therapeutic agents must be released over several weeks from the delivery system and penetrate through the tissue surrounding the implant to reach cancer cells. Thus, a conformal coating implant will enhance the efficiency of the drug delivery by maximizing the contact area between implant and cancer cells. Our central hypothesis

is: *local and sustained release of chemotherapeutic agent will increase the efficacy of chemotherapy in the treatment of gliomas while minimizing the systemic side effects*. For sustained delivery, biodegradable polymeric particles poly(lactic-co-glycolic acid) were used for the slow release of therapeutic agent doxorubicin (DOX). For local delivery, hydrogel was used as a scaffold for the particles and allowed DOX to diffuse to the brain tumor cells.

1.3 OBJECTIVES

The overall goal of this thesis research is to develop a minimally invasive hydrogel-particle composite system capable of local and sustained delivery of DOX to treat glioma. NTo test our hypothesis and accomplish our goal, the following objectives were set:

1. Develop the DOX encapsulated hydrogel-particle composite and characterize its in vitro performance.
2. Evaluate the performance of the DOX encapsulated hydrogel-particle composite in a 9L glioma rodent model.

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CHAPTER 2

LITERATURE REVIEW

Treatment of malignant brain tumor remains a major challenge despite recent advances in surgery and other adjuvant therapy. Chemotherapy of brain tumor has been particularly inefficient, due to the special barriers in the central nervous system (CNS). The failure of potential effective chemotherapeutics for brain tumor treatment is usually not due to the lack of potency of the drug, but rather can be attributed to lack of therapeutic strategies capable of overcoming CNS barriers for effective delivery of drug to the brain tumor. Thus, in this chapter, we will first review the barriers in brain tumor therapy, and then discuss current strategies to overcome the barriers. Particularly, we will focus more on applying the local implant strategy to enhance the effectiveness of drug delivery in brain tumor therapy.

2.1 BARRIERS FOR DRUG DELIVERY TO BRAIN TUMOR

The failure of systemically delivered therapeutic agents to effectively treat brain tumor is mainly due to the presence of the highly impermeable blood brain barrier (BBB) (Pardridge 2003). The BBB, which composed of cerebral capillary endothelial cells with tight junctions, prevents the passive uptake of hydrophilic and large molecules into the brain parenchyma (Goldstein & Bezt. 1986). Only small molecular weight, uncharged, and lipophilic molecules can passively cross the barrier, and not all of them have therapeutic effect for treating malignant brain tumor (Habgood et al 2000). Moreover, the endothelial cells of brain capillaries have fewer pinocytotic vesicles than

those of capillaries elsewhere in the body (Pardridge, 2000). The transport of molecules, which depends on the cellular transcytosis, is therefore severely compromised. Finally, a number of transport proteins located in the luminal membranes of cerebro capillary endothelium are known to be involved in the influx and efflux of endogenous and exogenous molecules across the BBB (Kusuhara & Sugiyama, 2001a). Efflux transport proteins such as P-glycoprotein and multidrug resistance associated protein actively remove a wide range of chemotherpeutic agents before they cross into the brain parenchyma, thus, effectively restrict drugs entering CNS (Kusuhara & Sugiyama, 2001b; Lee G et al, 2001; Taylor 2002).

The second barrier for therapeutic drug delivery to the CNS and brain tumor is blood-cerebrospinal fluid barrier (BCB). This barrier is formed by the specialized tight junctions of endothelial cells in the choroids plexus. The BCB closely regulates the exchange of molecules between blood and cerebrospinal fluid (CSF), thus it also regulates the drug entry into brain parenchyma. However, the BCB is not as a major barrier as the BBB. The surface area of BCB is approximately 1000 fold less than that of the BBB (Pardridge 1997).

The abnormal microvasculature of brain tumor contributes to the third barrier for therapeutic drug delivery to brain tumor, the blood-tumor-barrier (BTB). The tumor microvessles are abnormal; e.g. distended capillary with leaky wall and sluggish flow, leading to inconsistent drug delivery. The leaky tumor vasculature leads to accumulation of interstitial fluid, which causes an increase of the interstitial pressure in brain tumor and creates a net flow of fluid from the center to the periphery of the tumor

and the surrounding tissue. This further limits the flow and tissue penetration of therapeutic agents from the blood stream to the tumor parenchyma (Jain RK 1994).

These three barriers together compose a very delicate control system to maintain homeostasis of the CNS. However, the same control system that protects the brain from foreign substances also restricts the entry of many potential therapeutic agents. There is a great need to find an effective drug delivery system that can overcome these barriers. This thesis is focused on the drug delivery to overcome the BBB, the major barrier for chemotherapeutic agents to reach to the brain tumor.

2.2 CURRENT STRATEGIES TO OVERCOME BLOOD BRAIN BARRER

Chemotherapy of brain tumor has been particularly inefficient, mainly due to the presence of the highly impermeable BBB. The understanding of BBB has been used in the rational design of new therapeutic strategies. There are a series of techniques that have been applied to improve drug delivery efficacy to brain tumor. These techniques can be divided into two main categories: non-invasive methods and invasive methods. They are discussed below.

2.2.1 Non-invasive methods

2.2.1.1 Enhance the lipophilicity of drug

As mentioned previously, only small molecular weight, uncharged, and lipophilic molecules can passively cross the BBB from systemic circulation (Habgood et al 2000). The first strategy to improve the passive drug-uptake into the brain is to increase the lipophilicity of drug by chemical alteration. Carmustine (BCNU), the

active component of Gliadel® wafer, is an alkylating agent used to treat malignant brain tumor. Although more than 20 lipophilic carmustine analogs were synthesized, clinical trials have not shown improved efficacy of these drugs over carmustine. (Kornblith & Walker. 1988) The efficacy of alkylating agent is inversely proportional to their lipophilicity (Pardrigre 1988). In addition, lipophilic drug tends to bind to plasma protein when administrated intravenously, which results in lower drug concentrations within the brain and brain tumor (Rautio and Chikhale. 2004).

The other method to enhance the lipophilicity of a drug is to encapsulate the drug within liposome, a lipid bilayer vesicle. In particular, the encapsulation of doxorubicin (DOX) within liposome has increased the delivery of the drug in experimental brain tumor model (Koukourakis MI et al. 2000). Furthermore, the advantage of using liposomal carriers is their ability to incorporate ligands on their surface directed to brain capillary receptors. For example, OX-26, a mouse monoclonal antibody (MAb) to the rat transferrin receptor, could attach to the endogenous transferrin receptor-mediated transcytosis mechanism to cross the abluminal membrane and deliver large molecules into the brain (Friden et al. 1991). Huwyler and colleagues showed that OX-26 conjugated liposome could cross BBB and deliver radioactive daunomycin into the brain, while PEGylated liposome (stealth liposome) could not pass the BBB (Huwyler 1996). This delivery system is important for chemotherapeutic drug delivery because it permits brain targeting of liposomally encapsulated drugs, and consequently reduce the side effects of systemic delivery.

2.2.1.2 Temporary disruption of BBB

Instead of modification of chemotherapeutic agents, temporary BBB disruption could be considered as a therapeutic strategy in conjunction with systemic administration of chemotherapeutic drug for brain tumor treatment. An ideal BBB disruption method should be temporary and reversible so that BBB does not permanently loose its protection function for the brain. The first approach to transient BBB disruption is to inject hypertonic agents, such as mannitol. Mannitol shrinks the endothelial cells by drawing fluid out of them, and opens the tight junctions for a few hours to allow therapeutic drugs entering to brain tumor. However, administration of mannitol has disadvantage of being limited to the vascular supply and exposing a large area of non-neoplastic brain to chemotherapeutic agents, which could possibly result in severe neurotoxic side effect (Millay et al. 1986). Other compounds, such as RMP-7, can increase the tight junction permeability by activating B2 receptor of the endothelial cell, thus enhancing the delivery of therapeutic drugs by directly opening of the BBB (Emerich et al. 2000). Although RMP-7 has better specificity to brain tumor capillary than normal brain capillary, similar neurotoxic side effect was observed as well (Inamura et al. 1994).

2.2.2 Invasive methods

2.2.2.1 Catheter with pump system

The simplest and most direct strategy to overcome the barriers to brain tumor is to administer therapeutic drug into the tumor via catheter systems. Currently, several implantable catheter pump systems are available for brain tumor therapy. For example, the Ommaya reservoir can intermittent bolus injections of therapeutic agents (including

DOX, BCNU, cisplatin, interleukin 2 and interferon-γ) to brain tumors (Lesniak and Brem 2004). Recently, more advanced implantable infusion pumps were tested in clinical trial, such as Infusaid pump, the MiniMed PIMS system and the Medtronic SynchorMed system (Rautio and Chikhale. 2004). These systems are able to deliver drug at a constant rate over a prolonged period of time at the brain tumor site through the outlet catheter, and they can be refilled by subcutaneous injection. Despite encouraging results, there are still a lot of flaws limiting the success of this type of drug delivery system, such as infection, catheter obstruction and discomfort to the patient.

2.2.2.2 Local implantation of sustained controlled release polymer

The objective of implantable polymer for brain tumor therapy is to provide a continuous drug delivery to the brain tumor using a matrix that also protects the drug from degradation. The first generation of controlled release polymer system is based on non-biodegradable polymer. Langer and Folkman first reported the sustained and controlled release of macromolecules from ethylene vinyl acetate copolymer (EVAc) (Langer and Folkman. 1976). The encapsulated drug is released by diffusion through the micropore of the polymer matrix. Although EVAcs have found applications in glaucoma, diabetes and asthma therapy, it is not specifically FDA approved for use in the brain (Lesniak and Brem 2004).

The major step for utilizing the controlled release polymer in brain tumor therapy is the development of biodegradable polymer system. Brem and coworkers have studied the treatment of recurrent malignant glioma with the anticancer drug carmustine via a biodegradable polymer as a polyanhydride wafer (Brem & Gabikian

2001). This biodegradable polymer, poly[bis(p-carboxyphenoxy)propane-sebacic acid](p(CCP-SA)), releases drug by both hydrophobic degradation and drug diffusion. They demonstrated that this polymer system is biocompatible and sustained release of carmustine in a preclinical study (Brem et al. 1991). After clinical trials, FDA finally approved carmustine loaded p(CCP-SA) wafer (Gliadel®) for the treatment of malignant glioma. Although it has been used clinically, one of the limitations of this p(CCP-SA) copolymer is that it is designed for delivery of hydrophobic molecules. To overcome this limitation, the fatty acid dimer-sebacic acid (FAD-SA) copolymer, another biodegradable polymer, was developed for delivery of hydrophilic molecules (Domb et al 1991). These two biodegradable polymers share the same mechanism of release and the possibility to vary the release kinetic by varying the ratio of the two monomers.

Among the few biodegradable polymers, poly(latide-co-glycolide) (PLGA) has been extensively studied as a drug transport vehicle in controlled release delivery system. An advantage of PLGA is that its degradation can be regulated by varying molecular weight and composition of the copolymer. In addition, it can encapsulate both hydrophilic and hydrophobic therapeutic agents, thus making it better than the previously mentioned polyanhyidide biodegradable polymers.

The general limitation for all the above polymeric implant systems is that drug release cannot be controlled once the system has been implanted, thus an increased risk of local neurotoxicity might occur at higher polymer or therapeutic agents concentration (Rautio and Chikhale. 2004).

2.3 CONCLUSIONS

Chemotherapy of brain tumor has been particularly inefficient, due to the special barriers in central nervous system (CNS). The failure of potential effective chemotherapeutics for brain tumor treatment is usually not due to the lack of potency of the drug, but rather can be attributed to lack of therapeutic strategies capable of overcoming CNS barriers for effective delivery of drug to the brain tumor. This chapter briefly summarizes invasive and non-invasive strategies to overcome the barriers. Particularly, we have focused more on applying the local implant strategy to enhance the effectiveness of drug delivery in brain tumor therapy. Most of the implantable polymer systems are capable of sustained release anti-cancer drug both *in vivo* and *in vitro*, however, the therapeutic agents might or might not cover the tumor resection area due to its geometry. Thus, developing a system that can combine both spatial and temporal control will benefit brain tumor therapy.

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CHAPTER 3

Conformally Coated Particle-Doxorubicin Therapy for Brain Tumor

3.1 INTRODUCTION

Chemotherapy of brain tumor has been particularly inefficient, due to the barriers in central nerve system (CNS), especially blood brain barrier (BBB). The failure of potential effective chemotherapeutics for brain tumor treatment is usually not due to the lack of potency of the drug, but rather can be attributed to lack of therapeutic strategies capable of overcoming BBB for effective delivery of drug to the brain tumor. Local delivery of chemotherapeutic agents has the advantage of bypassing the BBB, thereby delivering a higher drug concentration at the site of interest while minimizing the systemic side effects. Particularly, we have focused more on applying the local implant strategy to enhance the effectiveness of drug delivery in brain tumor therapy. Most of the implantable polymer systems are capable of sustained release anti-cancer drug both *in vivo* and *in vitro*, however, the therapeutic agents might or might not cover the tumor resection area due to its geometry. Thus, developing a system that can combine both spatial and temporal control will benefit brain tumor therapy.

In the present study, we are pursuing the design and development of conformal coating technology so that the entire resected area is covered conformally with a hydrogel-particle composite that locally delivers doxorubicin (DOX). DOX, a topoisomerase II inhibitor, stabilizes the topoisomerase II complex after it has broken the DNA chain for replication, preventing the DNA double helix from being resealed and thereby stopping the process of replication (Momparler et al. 1976). It is currently

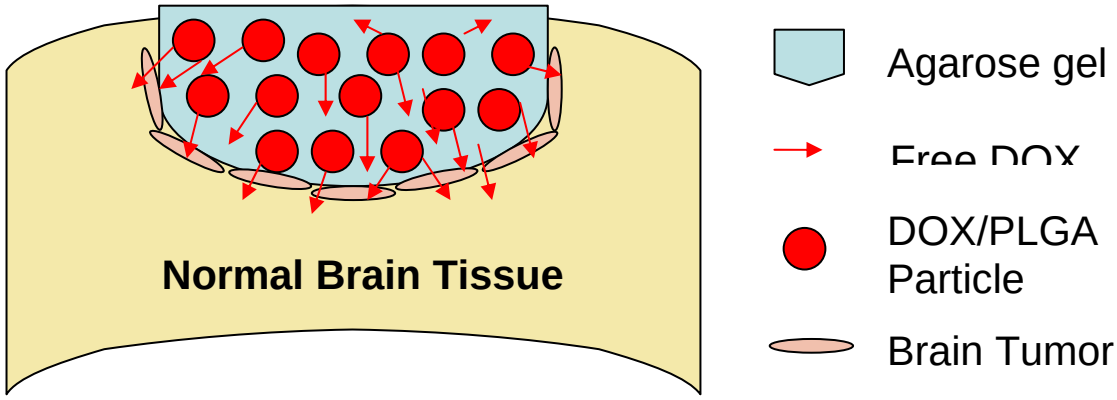
used in the treatment of leukemias, Hodgkin's lymphoma, as well as cancers of the bladder, breast, stomach, lung, ovaries, and others (Lesniak et al. 2005),however it is currently not used clinically for brain tumor treatment because when delivered systemically it does not cross the BBB. Our hydrogel-particle system will overcome this shortcoming of DOX.

For sustained delivery, biodegradable polymeric particles made from poly(lactide-co-glycolide) were used for the slow release of DOX. For local delivery, hydrogel was used as a scaffold for the particles and allowed DOX to diffuse to the brain tumor cells (See Figure 3.1.A). The thermoreversible property of this hydrogel allows it to be liquid at 38^0C, and gels at physiological temperature, thus making it possible for conformal coating of the soft tissue surfaces, and providing an effective treatment of the entire surface exposed after resection. In present study, we would develop the DOX encapsulated hydrogel-particle delivery system and characterize its in vitro performance. Followed by Evaluation of the performance of the DOX encapsulated hydrogel-particle composite in a 9L glioma rodent model.

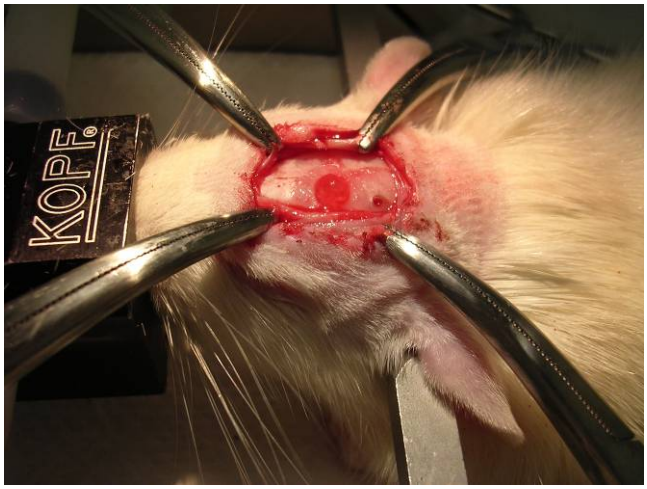
3.2 METHODS

3.2.1 Materials

A 9L glioma cell line was received as a generous donation from the Neurosurgery Tissue Bank at UCSF. Rat astrocyte was kindly provided by Dr. Kacy Cullen of Neurolab at Georgia Tech. Minimal essential medium containing Earle's balanced salt solution (MEM/EBSS) and fetal bovine serum (FBS) were purchased from Hyclone (Logan, UT). Gentamicin (50 mg/ml), and Leibovitz's L-15 medium were



A. Schematic of DOX/PLGA gel system



B. Images of implantation of DOX/PLGA particle gel system.

Figure 3.1. Local sustained release system. **A.** Schematic of DOX/PLGA gel system. DOX released from DOX/PLGA particle into the hydrogel, then diffused into the surrounding tissue. **B.** Image of implantation of DOX/PLGA particle gel system. The surgical incision used for inoculating the tumor was reopened 5 days later. The dental acrylate resin and hydrogel were carefully removed. Prior to polymer-gel implantation the dura was gently pierced and retracted with fine micro-forceps. The animals were then intracranially implanted with either a DOX encapsulated gel system (25 µL), or with DOX/PLGA particle encapsulated in gel system (25 µL). After fixing with dental acrylate resin, the skin was sutured closed.

obtained from Gibco (Carisbad,CA). DMEM/F12 50/50 mixture 1X with L-Glutamine and 15 mM HEPES, 0.05 % Trypsin-EDTA (0.05% trypsin, 0.53mM EDTA) and 0.25% Trypsin (0.25% trypsin, 2.21 mM EDTA), Penicillin-Streptomycin Solution (Pen-Strep) were purchased from Mediatech (Herndon, VA). Isoflorane and Doxorubicin (DOX) were obtained from Baxter Healthcare (Deerfield, IL). Ketamine (100 mg/ml) was purchased from Fort Dodge Laboratories (Madison, NJ). Marcaine (0.5%) was obtained from Abbott Laboratories (Abbott Park, IL). Flunixin meglumine was purchased from Phoenix Scientific (San Marcos,CA). Xylazine (100 mg/ml) was purchase from Bulter Company (Dublin, OH). Acetylpromise (10mg/ml) was obtained from Boehringer Inglheim (Ingelheim, Germany). Poly vinyl alcohol (PVA), 87-89 hydrolized, Mw 31000~50000) was purchased from Aldrich. Dichloromethane (DCM) reagent ACS 99.5% was purchased from ACROS (ACROS, New Jersy). Poly (dl-lactide/glycolide)75:25 was purchased from Polyscience Inc(Warrington, PA). SeaKem GTG Agarose was purchased from Cambrex(Cambrex, Rockland, ME).

3.2.2 Cell Cultures

A 9L glioma cell line was maintained in MEM/EBSS medium supplemented with 10% FBS and 0.05 mg/ml gentamicin. 9L glioma cells were passaged by trypsinization (0.05% Trypsin/EDTA) and washed with growth medium. Prior to implantation, cells were resuspended in serum-free Leibovitz's L-15 medium to a concentration of $2x10^8$ cells/mL.

Rat astrocytes were maintained in DMEM/F12 medium supplemented with 10% FBS and 1% Pen-Strep. Astrocytes were passaged by trypsinization (0.25% Trypsin/EDTA) and washed with growth medium.

3.2.3 *In vitro* cytotoxicity experiment

To determine effect of free DOX concentration and incubation time on cells, cytotoxicity studies were conducted by seeding 9L glioma cells and astrocytes,

respectively, at a density of 3000 cells per well on a 48-well plate twenty-four hours prior to incubation with drug. Cells were incubated with different concentrations (from 5-1000ng/mL) of free DOX for different incubation times (2, 24, 48, 72 hours) at 37^{o}C. Cells were then washed three times with fresh growth medium and re-incubated for 48 hours. The numbers of viable cells were determined with a water soluble formazan-based assay (CCK-8, Dojindo, Kumamoto, Japan) in a Synergy^{TM} HT microplate reader (Bio-Tek, Winooski, VT).

3.2.4 Preparation of PLGA particles

Saline and DOX loaded particles were prepared through a modified double emulsion method (Li et al. 2001). Briefly, DOX was reconstituted with 0.9% Saline to 10 mg/mL DOX solution, and 2% PLGA copolymer (75:25) was dissolved in 5 mL DCM. 250 µL of DOX solution was then added into 5mL PLGA solution. The mixture was then emulsified three times by homogenization at 5000 rpm (13 seconds/run). The resulting emulsion was then added into 50 mL of 0.4% PVA solution and homogenized three times at 8000 rpm (18 seconds/run). The resulting double emulsion was stabilized in 150 mL of 0.1% PVA solution. DCM was removed by evaporation in a vacuum chamber for 3 hours with moderate stirring. The resulting DOX/PLGA particles were centrifuged at 8500 g for 15 minutes (ThermoForma High Performance centrifuge), the supernatant was removed and the pelleted particles were resuspended with deionized water. This process of centrifugation and resuspension were repeated twice before lyophilization (LABCONCO Freeze Dry System/ Freezone 4.5). The lyophilized DOX/PLGA particle was stored at -20^{o}C. The size of the particles was measured by

Dynamic Light Scattering (DLS) (Brookhaven Instrument Corp). Saline encapsulated PLGA particles (Saline/PLGA particles) were fabricated as an experimental control. The fabrication procedure for Saline/PLGA particles was the exactly same as for the DOX/PLGA particles, except that saline was encapsulated instead of DOX. Encapsulation efficiency and loading was determined by measuring the amount of DOX in the supernatants generateed during the recovery steps of particle preparation. The DOX concentration was determined by measurement of the UV absorbance at 480 nm. (Bio-Tek Synergy^{TM} HT microplate reader, Winooski, VT).

3.2.5 Preparation of hydrogel

DOX loaded 1% hydrogel was prepared by dissolving 100 mg SeaKem powder with 10 mL of various concentrations (0.015, 0.03, 0.1, 0.5 and 2.0 mg DOX/mL) of DOX solution. Saline loaded 1% SeaKem gel was also prepared as an experiment control. DOX/PLGA particle gel system was fabricated by encapsulating different amount of DOX/PLGA particles in 1% SeaKem gel. Saline-PLGA particle gel system was used as an experiment control.

3.2.6 In vitro release experiment

200 ul of DOX loaded gel or DOX/PLGA particle gel was added to a 2 ml microcentrifuge tube. After gelling, 1000 µL of saline was added and the tube was shaken at 60 rpm (IKA-VIBRAX-VXR electronic), 37^{o}C. At different time intervals, 800 µl of the supernatant was pipetted out, and the same volume of fresh saline was added to the tube. The concentration of DOX released in the supernatant was measured

(λex=485 nm,λem=590nm) by the fluorescence spectrometer(Bio-Tek Synergy™ HT microplate reader, Winooski, VT).

3.2.7 Bioactivity assay

Bioactivity of the released DOX was examined by the viability of 9L glioma cells and rat astrocytes. 9L glioma cells and rat astrocytes were seeded at a density of 10^4 cells per well on a 24-well plate twenty-four hours prior to incubation with delivery system. Cells were incubated with the Saline-PLGA particles, DOX/PLGA paticles, 1% SeaKem gel, Saline-PLGA particle in gel, and DOX/PLGA particle in gel for 24 hours at 37°C. Cells were then washed three times with fresh growth medium and re-incubated for 48 hours. The numbers of viable cells were determined with a water soluble formazan-based assay (CCK-8, Dojindo, Kumamoto, Japan) in a Synergy™ HT microplate reader (Bio-Tek, Winooski, VT).

3.2.8 Tumor Inoculation

A rat glioma model was established by surgically implanting $2x10^6$ 9L glioma cells into the frontal lobe of 11-12 week old male Fisher 344 rats. All procedures were conducted under a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at Georgia Institute of Technology. Adult male Fischer 344 rats were anesthetized with a mixture of 5% isoflurane and 1L/min O_2 prior to surgery. Each

animal was positioned into a stereotactic frame where anesthesia was maintained at 2-3% isoflurane during surgery (with 0.3 L/min O_2). The surgical site was shaven and then cleaned with isopropyl alcohol and chlorohexaderm. An ophthalmic ointment was applied on the eyes to prevent corneal abrasion during and after the surgery. A midline incision was made in the scalp to expose the skull. A dental drill was used to create a 3.2 mm hole at a position 3mm lateral and 1mm anterior to the bregma, with a custom trephine (24 tooth X 3mm O.D.). The bone plug was carefully removed. A 22 gauge needle was inserted into the frontal lobe at a depth of 3mm to inject 10µl of the glioma cell suspension ($2x10^6$ cells). The cells were slowly injected over a period of approximately 30 seconds. Following injection of the cell suspension, the needle was slowly retracted over a period of approximately one minute. The tumor injection site was first covered with 1% SeaKem gel followed by dental acrylate resin, after which the wound was closed with suture. Animals then received 5 mL of Lactated Ringer's solution through intraperitoneal (IP) injection. Flunixin meglumine (2.5mg/kg) was administered through an intramuscular (IM) injection to alleviate pain as needed.

3.2.9 Survival Studies

The surgical incision used for tumor inoculation was reopened 5 days later. The dental acrylate resin and hydrogel were carefully removed. Prior to polymer-gel implantation, the dura was gently pierced and retracted with fine micro-forceps. The animals were then intracranially implanted with either a DOX encapsulated gel system (25 µL), or with DOX/PLGA particle encapsulated in gel system (25 µL) (See Figure

3.1.B). After fixing with dental acrylate resin, the skin was suture closed. Animals received 5 mL of Lactated Ringer's solution through intraperitoneal (IP) injection. Flunixin meglumine (2.5mg/kg) was administered through an intramuscular (IM) injection to alleviate pain as needed. Tumor growth was allowed to progress until the animal showed signs of morbidity, at which point interventional euthanasia was administered. Time of death was determined to be the following date. The survival curve was statistically analyzed with Kaplan-Meier method.

3.2.10 Histological examination.

Nine Fisher 344 rats (control (n=3), DOX/PLGA gel (n=3), PBS-PLGA gel (n=3)) were used for histopathological examination. Animals were perfused at Day 9 (4 days after treatment). The brain was retrieved, and the tissue was fixed with 4% paraformaldehyde, blocked in OCT, coronally sectioned into 12μm samples, and stained with hematoxylin and eosin (H&E).

3.3 RESULTS.

3.3.1 *In vitro* cytotoxicity of DOX

The cytotoxicity of DOX on 9L glioma cells as measured by viability is shown for each DOX concentration and incubation time in Figure 3.2. The abscissa is the concentration of DOX while the ordinate is the viability relative to an untreated control (untreated control= 100%). As shown in Figure 3.2, at 2 hour incubation period, only

higher concentrations of DOX (> 200 ng/ml) had significant cytotoxic effects on 9L glioma cells. However, when DOX incubation time is longer than 24 hour, we observed the relative viability drop into 70% at 20 ng/mL DOX and observed even lower viability (<25%) as the DOX concentration increased. In general, the cytotoxicity is proportional to DOX concentration and incubation time for 9L glioma cells.

Comparison of the cytotoxic effect of various amount of DOX on 9L and rat astrocyte at different incubation time is shown in Figure 3.3. Rat astrocyte was used in this study as a control to represent normal healthy brain tissue. In general, the cytotoxicity of DOX is proportional to DOX concentration and incubation time for both 9L glioma cells and rat astrocytes. However, we didn't observed a significant decrease in viability of rat astrocyte when compare to 9L glioma cells.

At 2 hour incubation period, only higher concentrations of DOX (> 100 ng/ml) had significant cytotoxic effects on rat astrocyte as shown in Figure 3.3.(a). However, when incubation time is longer than 72 hour, we began to observe a decrease in relative viability even when DOX concentration was as low as 20 ng/mL (Figure 3.3.(d)).

The relative viability of 9L glioma and rat astrocytes became significantly different when the incubation time was longer than 24 hour and DOX concentration was

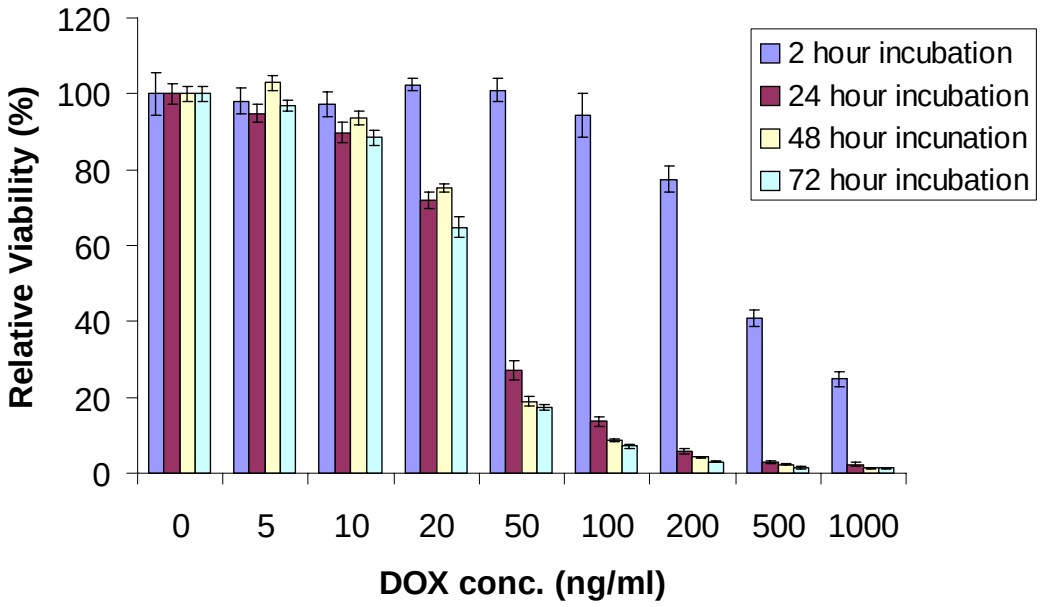


Figure 3.2. Cytotoxic effects of various amounts of DOX on 9L glioma at different incubation times. Cytotoxicity studies were conducted by seeding 9L glioma cells at a density of 3000 cells per well on a 48-well plate twenty-four hours prior to incubation with drug. Cells were incubated with the different concentrations (from 5-1000ng/mL) of free DOX for different incubation times (2, 24, 48, 72 hours) at 37°C. Cells were then washed three times with fresh growth medium and re-incubated for 48 hours. Relative viability is obtained by comparing each group with non treated group.

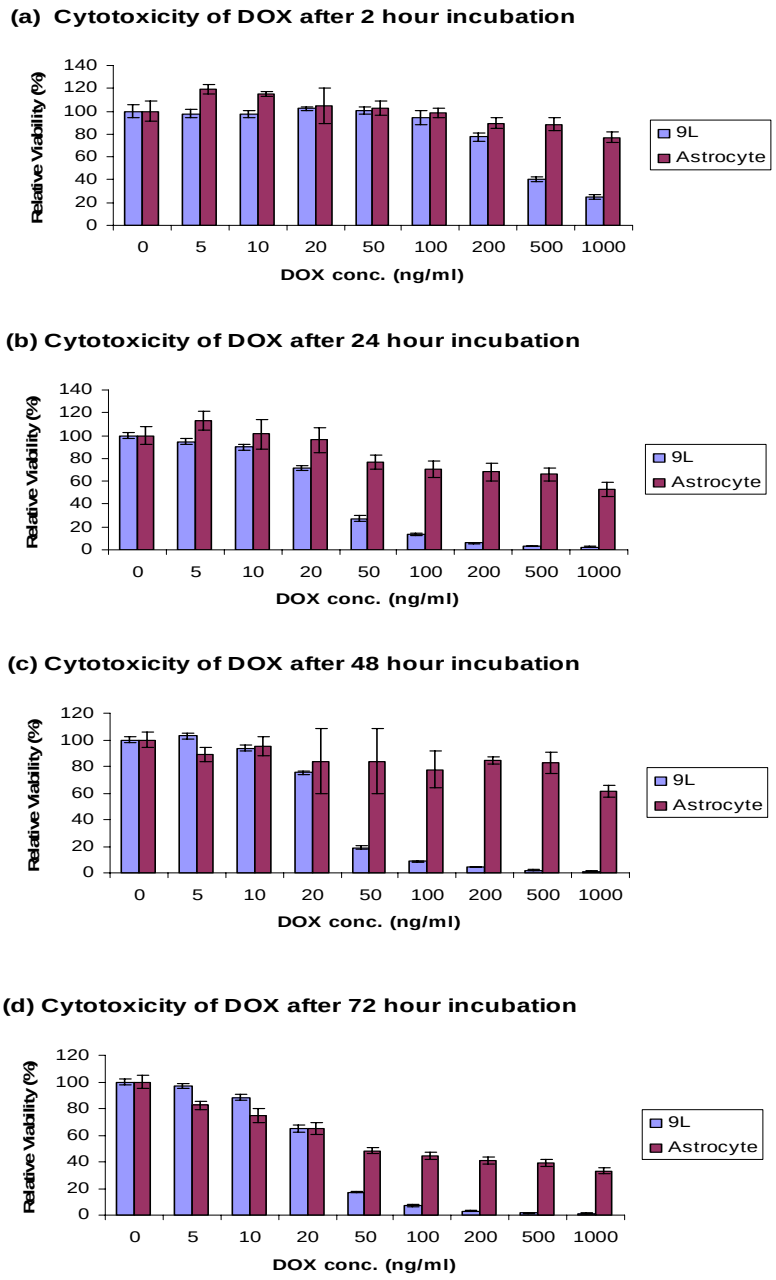


Figure 3.3. Comparison of cytotoxic effect of various amounts of DOX on 9L glioma and rat astrocyte at (a) 2 hour, (b) 24 hour, (c) 48 hour and (d)72 hour incubation. Cytotoxicity studies were conducted by seeding 9L glioma cells and rat astrocytes at a density of 3000 cells per well on a 48-well plate twenty-four hours prior to incubation with drug. Cells were incubated with the different concentrations (from 5-1000ng/mL) of free DOX at 37^{o}C. Cells were then washed three times with fresh growth medium and re-incubated for 48 hours. Relative viability is obtained by comparing each group with non treated group.

higher than 50 ng/mL. For example, when incubating 9L glioma with 50 ng/mL DOX for 24 hour period, the relative viability of 9L glioma dropped to 25%, while the rat astrocytes still showed 80% viability at the same condition. The difference of viability between 9L glioma and rat astrocytes became bigger as the DOX concentration become higher.

3.3.2 Particle characterization and loading efficiency

Biodegradable PLGA based particles were prepared by double emulsion method. The yield of the particle after lyophilization is 63.7%. The DOX loading efficiency was 10.1% (w/w%, DOX loaded /initial DOX amount), and the drug content of the particle was 3.85μg DOX per mg DOX/PLGA particle. The particles have a size range of 200-1000 nm and the mean size of the particle is 631.1± 363.4 nm.

3.3.3. *In vitro* release of DOX

3.3.3.1. *In vitro* release of DOX from 1% SeaKem gel.

The release profile of DOX from 1% SeaKem gel is shown in Figure 3.4. We observed a burst release with in first 6 hours. Approximately 80% of free DOX released in the first 12 hour for all concentrations, and no further release was observed after 24 hours. The results showed that initial loading concentration of DOX did not affect the release profile of DOX in hydrogels.

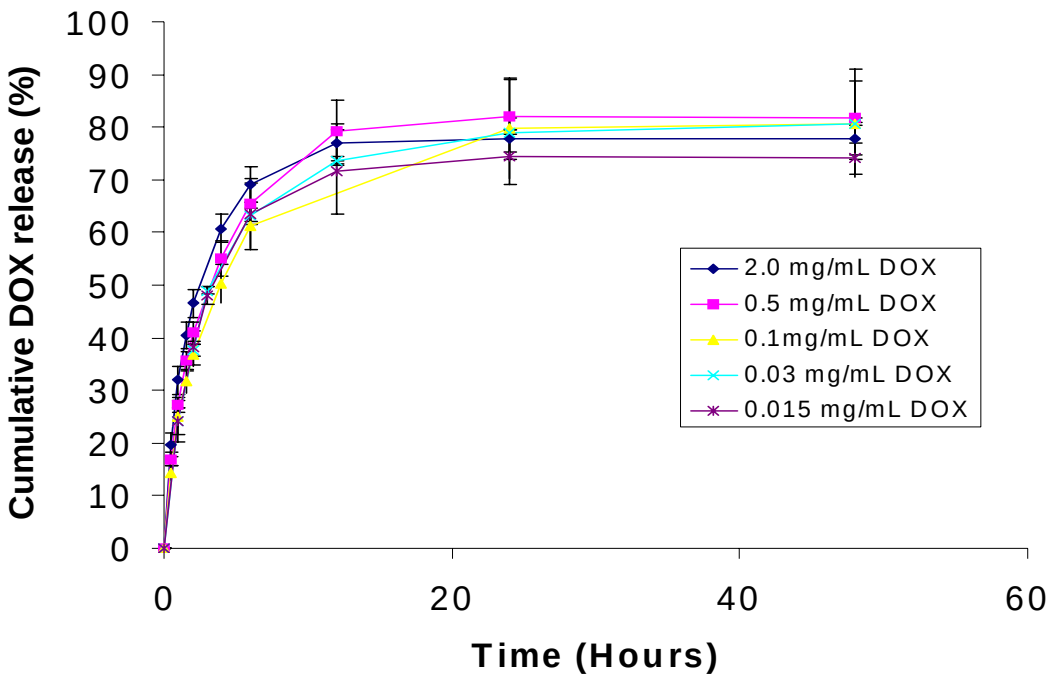


Figure 3.4. Cumulative release of DOX from 1.0 % SeaKem gels at different DOX concentrations. (2.0, 0.5, 0.1, 0.03 and 0.015 mg/mL) at 37°C. 200 ul of DOX loaded gel was added to a 2 ml microcentrifuge tube. After gelling, 1000 µL of saline was added to the centrifuge tube and shake at 60 rpm, 37°C. At different time intervals, 800 µl of the supernatant was pipetted out, and immediately after that the same volume of saline was added. The concentration of DOX released in the supernatant was measured (λex=485 nm,λem=590nm) by the fluorescence spectrometer(Bio-Tek Synergy™ HT microplate reader, Winooski, VT).

3.3.2 *In vitro* release of DOX from DOX/PLGA particle gel system

The cumulative release profile of DOX from DOX/PLGA particle gel system is shown in Figure 3.4 (A). Approximately 6 μg of DOX was released from DOX/PLGA particle gel (40 mg DOX/PLGA particle in 200 μL of 1% SeaKem) in 15 days period. 3.5 μg of DOX was released within the first day. After initial burst release in Day 1, DOX was continuously released at a rate of 0.5~0.75 μg per day between Day 2 and Day 5. Very small amount of DOX was released after Day 6.

To study the differences of DOX release mechanism between DOX gel and DOX/PLGA particle gel system, we conducted a similar release experiment. We prepared a 200 μL of DOX gel contained 7.5 μg DOX. We assumed a total release amount of 6 μg (80% of 7.5 μg) of DOX would be released from previous DOX gel release experiment as described in section 3.3.1. As shown in Figure 3.5.(B), the DOX/PLGA gel system has lower burst effect and has more constant release when compare to DOX gel.

3.3.4 Bioactivity of DOX/PLGA particle gel system

The bioactivity of the released DOX was examined by the viability of 9L glioma cells and rat astrocytes. As shown in Figure 3.6. DOX/PLGA gel system reduced 9L glioma viability to 38% after 24 hour incubation, while the rat astrocytes still showed 80% viability under the same condition. Saline/PLGA gel was used as our negative control in this bioactivity experiment. We observed that there is no reduction of viability in 9L glioma and rat astrocyte when treated with Saline/PLGA gel.

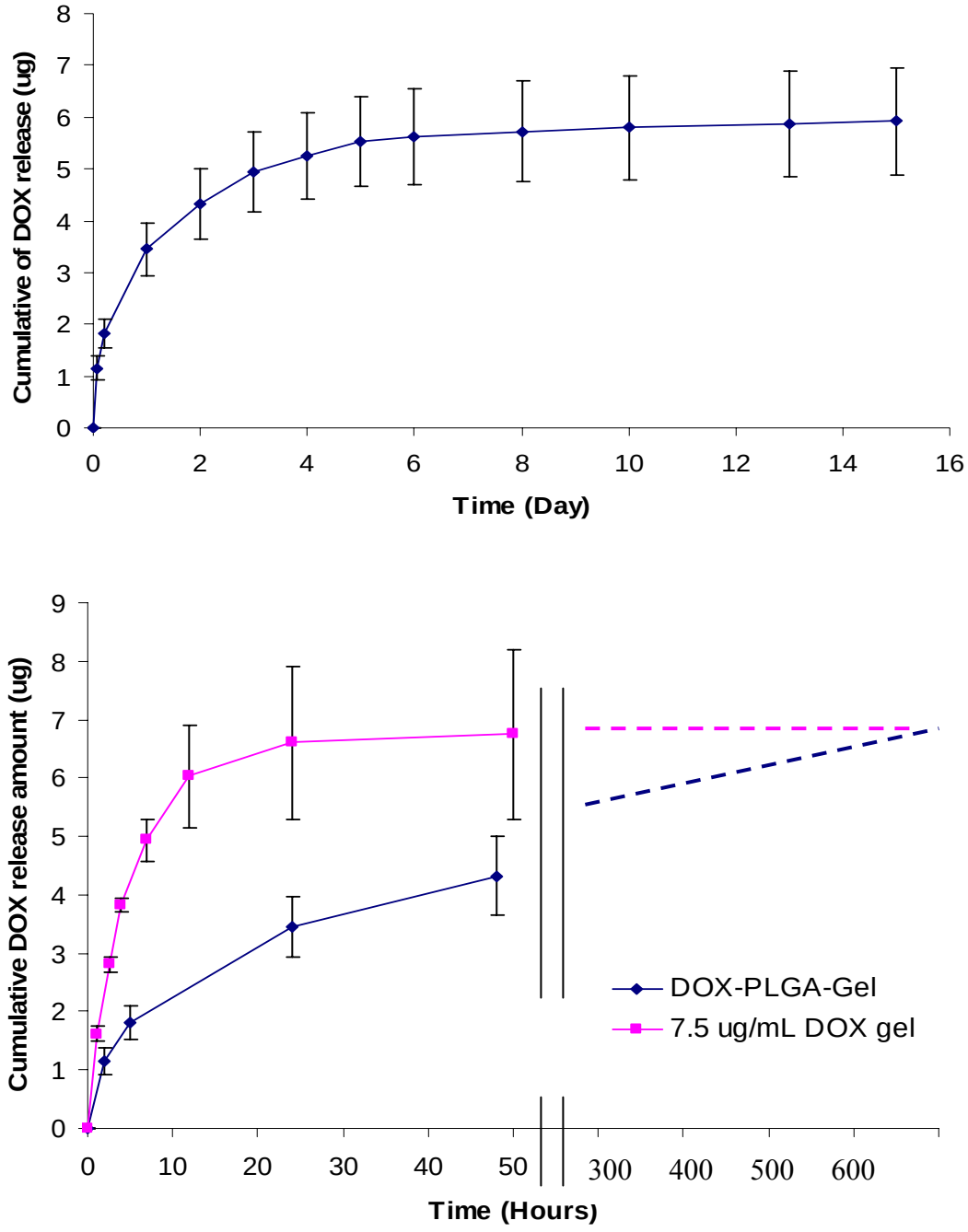


Figure 3.5. *In vitro* release of DOX. **A.** *In vitro* release assay of DOX over the first 15 day from DOX/PLGA gel (contain 40 mg of DOX/PLGA particle). The graph shows that an initial burst released in the first 4 days. **B**. *In vitro* release assay of DOX over the first 48 hours from DOX/PLGA gel (contains 40 mg of DOX/PLGA particle) and DOX gel (contains 7.5 µg DOX). The graph shows DOX-gel released 6.5 µg DOX in the first 24 hours, while only 3.3 µg of DOX was released from DOX/PLGA particle gel.

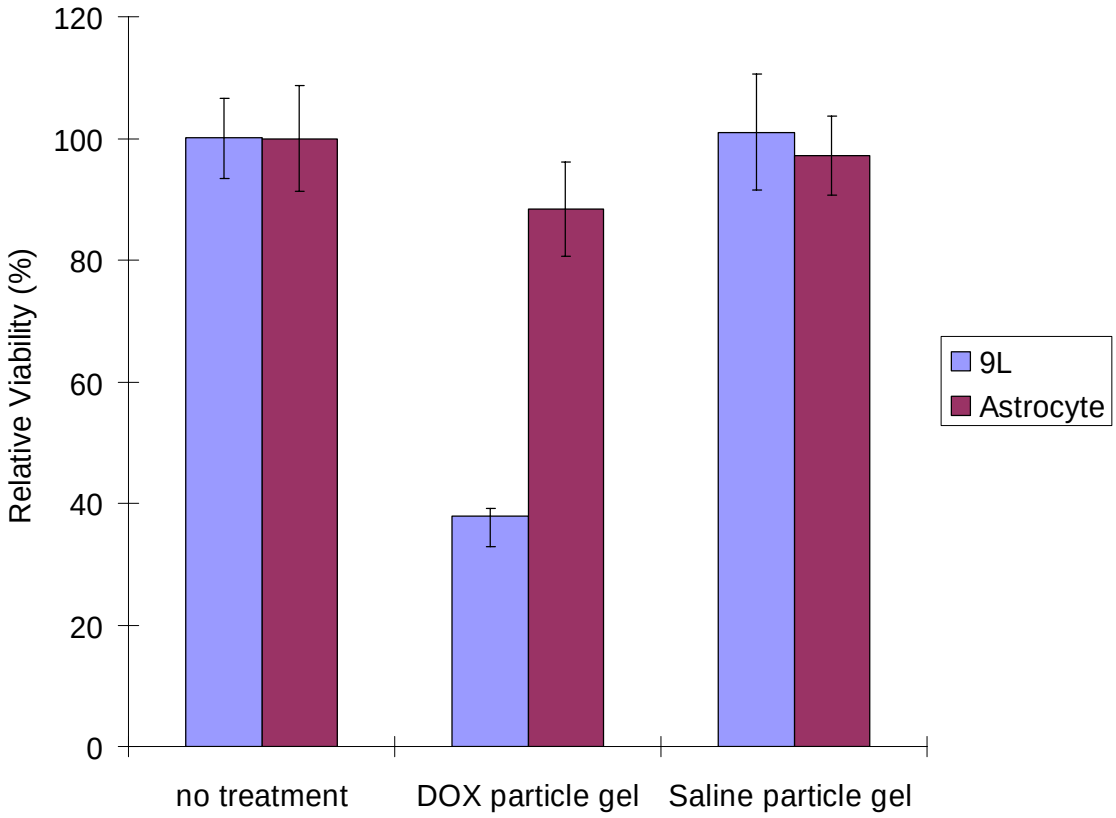


Figure 3.6. Bioactivity assay for DOX particle gel system. The bioactivity of the released DOX was examined by the viability of 9L glioma cells and rat astrocytes. 9L glioma cells and rat astrocytes were seeded at a density of 10^4 cells per well on a 24-well plate twenty-four hours prior to incubation with delivery system. Cells were incubated with the 300 ug of Saline/PLGA particle in gel and 300 ug of DOX/PLGA particle in gel for 24 hours at 37°C. Cells were then washed three times with fresh growth medium and re-incubated for 48 hours. The numbers of viable cells were determined with a water soluble formazan-based assay (CCK-8, Dojindo, Kumamoto, Japan) in a Synergy™ HT microplate reader (Bio-Tek, Winooski, VT). The data shows that DOX released from DOX/PLGA particle gel system is bioactive even after 24 hour incubation.

3.3.5 Survival Studies

The therapeutic effect of DOX gel and DOX/PLGA particle gel treatment was determined by comparing the respective survival times in response to treatment type (Figure 3.7.) Treatments were intracranial implantation of 25 uL of DOX gel or DOX/PLGA gel at a DOX dosage of 100 ug/kg five days after tumor inoculation. Equivalent volume of PLGA particle gel was implanted serving as negative control. As shown in Figure 3.7., a statistically significant increase in survival time was observed for both DOX gel ($p<0.05$) and DOX/PLGA particle gel treatments ($p<0.01$), in comparison to non-treated animals. However, no significant difference was observed in survival between DOX gel and DOX/PLGA particle gel treatments ($p=0.69$).

3.3.6 Histology Evaluation

As shown in Figure 3.8 (A) the tumor cells were confined in the injection area in the untreated animal. For DOX/PLGA particle gel (Figure 3.8 (B)) and DOX gel (Figure 3.8 (C)) treated group, the tumor cells were less than the untreated group. For Saline/PLGA particle gel treated group, the tumor cells migrate all the way from injection site to the surface of PLGA gel implantation site. We also observed damage near the surface of implanting site in these three treated groups (Figure 3.8. (B), (C), and (D)). The damage might have been due to the removal of dura prior implanting the delivery device.

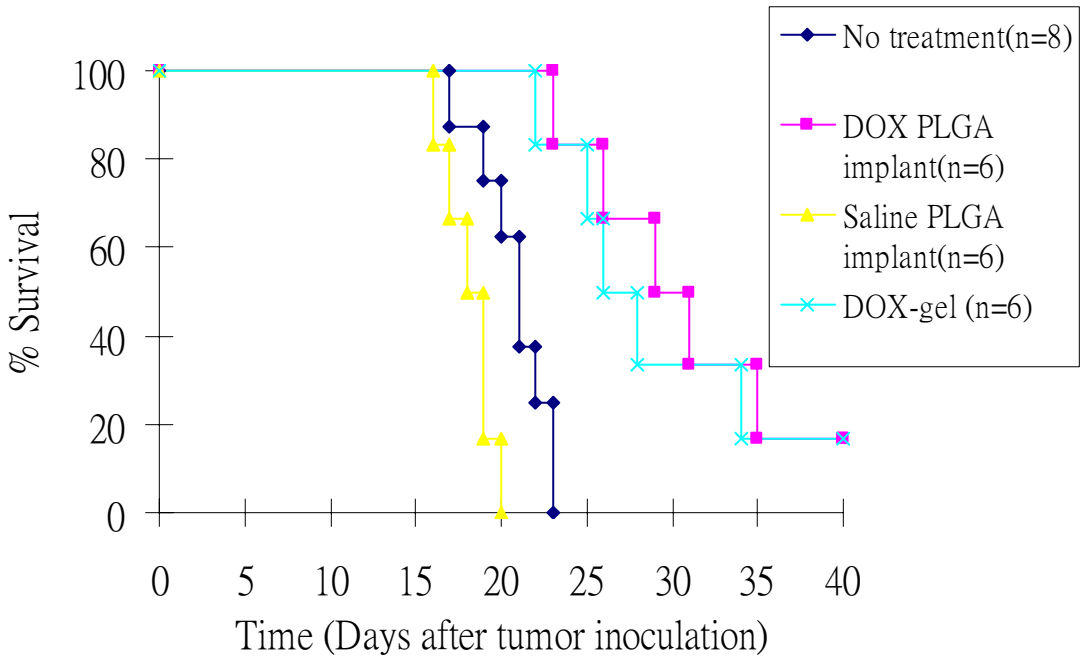
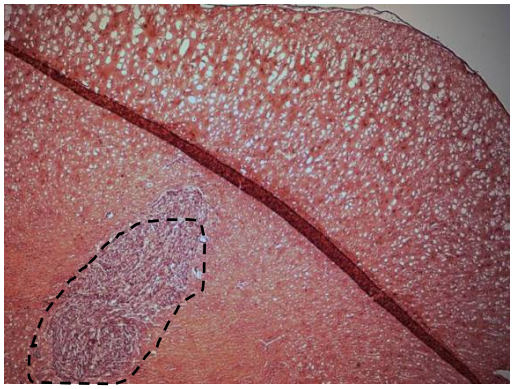
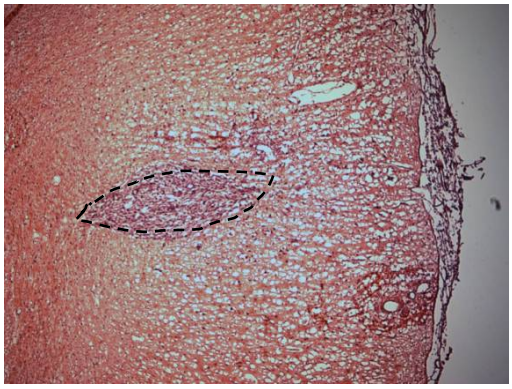


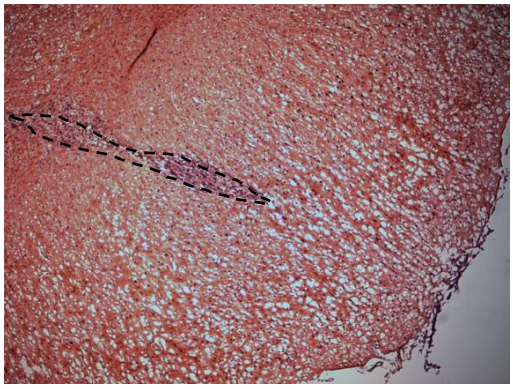
Figure 3.7. Survival study. The therapeutic effect of DOX gel and DOX/PLGA particle gel treatment was determined by comparing the respective survival times in response to treatment type. Treatments were intracranially implanted 25 uL of DOX gel or DOX/PLGA gel at a DOX dosage of 100 ug/kg five days after tumor inoculation. Equivalent volume of PLGA particle gel was implanted as a negative control group. The data show a statistically significant increase in survival times for both DOX gel ($p<0.05$) and DOX/PLGA particle gel ($p< 0.01$) treatments when compared to non-treated or saline/PLGA treated animals. There is no significant difference between DOX/PLGA particle gel treated group and DOX gel treated group ($p=0.69$)



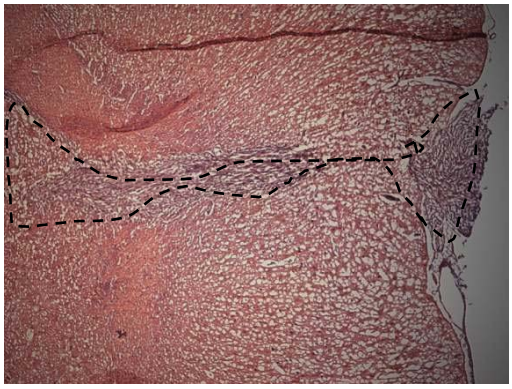
A. Untreated



B. DOX/PLGA particle gel treated



C. DOX gel treated



D. Saline/PLGA particle gel treated

Figure 3.8. Haematoxylin and eosin stain (H&E)-stained coronal sections (12μm) of rat brains 9 days after tumor inoculation. (A) Untreated, (B)Treated with DOX/PLGA particle gel, (C) Treated with DOX gel, and (D) Treated with saline/PLGA particle gel at Day 5 after tumor inoculation. The pink area represents normal brain tissue and the purple area (within dash line) represents brain tumor. (original magnitude 40X)

3.4 DISCUSSIONS

Implantable biodegradable polymer system has been used for brain tumor therapy to provide a continuous drug delivery to the brain tumor and protect the drug from degradation. The present study was initiated in an effort to incorporate drug loaded biodegradable particles into an agarose gel, which can provide a continuous release of therapeutic agent locally to the tumor site. This is of particular interest and importance because it incorporates the distinct benefits of these two families of materials into a single delivery system. The only limitation in using this delivery system is that the therapeutic drug must be hydrophilic so that it can be released from the PLGA particles into the hydrogel and subsequently diffuses to the surrounding tissue.

DOX, a hydrophilic chemotherapeutic agent, stabilizes the topoisomerase II complex after it has broken the DNA chain for replication, preventing the DNA double helix from being resealed and thereby stopping the process of replication. Tumor cells replicate faster than normal cells, making them more susceptible to the presence of DOX. DOX is used to treat leukemia, Hodgkin's lymphoma, as well as cancers of the bladder, breast, stomach, lung, ovaries, and others. However, it is not currently used for brain tumor treatment since it cannot cross the blood brain barrier due to its hydrophilicity.

Initially, in vitro cytotoxicity experiments were performed to test how tumor cells and normal cell respond to the DOX. The cytotoxic effect of DOX is proportional to DOX concentration and incubation time for both 9L glioma cells and rat astrocytes (representing normal brain tissue). However, the results also suggest that DOX has a higher cytotoxic effect on 9L glioma cells. Since DOX interferes with the process of

cell division and protein synthesis, when incubated with the same amount of DOX, the growth of fast dividing cells (9L glioma) would be easily suppressed than that of normal cells. This assumption was supported by the experimental comparison of cytotoxic effect of DOX on 9L cells and rat astrocytes (Figure 3.3).

The cytotoxicity results also showed that when the cell incubation time with DOX was increased, the growth of 9L glioma was suppressed while the viability of normal cells was preserved, especially at certain DOX concentrations (50 ng/mL and 100 ng/mL). This suggests that a system with sustained release of DOX would benefit the glioma therapy with minimal toxic effect on healthy cells.

The use of biodegradable microspheres for the delivery of anticancer agents has generated considerable interest. Among the few biodegradable polymers, PLGA has been extensively studied as a drug carrier in control release delivery system. There are several advantages to using PLGA for drug delivery. One of them is that PLGA degradation can be regulated by varying its molecular weight and the composition of the copolymer (Anderson and Shive 1997). Secondly, both hydrophobic and hydrophilic drugs can be encapsulated into the polymer spheres through different emulsion methods (Barichello et al. 1999). In addition, it degrades into biocompatible byproducts which can be eliminated by the normal metabolic pathways (Bazile 1992). All these advantages make PLGA the ideal drug carrier for our system.

As we mentioned before, most of the implantable polymer systems are capable of sustained release of anti-cancer drug, however, the therapeutic agents might not cover the tumor resection area due to its geometry. To overcome this drawback, we utilize hydrogel as a scaffold for the particles which allows DOX to diffuse to the brain tumor

cells. The use of agarose gel in the pharmaceutical and biomedical field is very attractive since it is nontoxic, inexpensive materials and has a very high potential for use with a variety of medicinal agents. Our special interest in using SeaKem agarose gel as our gel system is that it gels at 37 degree without using any additional crosslinker. Such thermoreversible property allows it to be liquid at 38^0C, and gels at physiological temperature, thus making conformal coating of the soft tissue surfaces possible, and providing an effective treatment of the entire surface exposed after resection.

As we described in previous section, the in vitro release assay demonstrated that DOX was released from DOX/PLGA particle gel system for at least 15 days. When compared to DOX gel system, the DOX released from DOX/PLGA gel system showed more controlled release property and reduced the initial burst effect to 50% in the first 24 hour. Although there is only small amount (0.1 ug) of DOX released from our system from day 8 to day 15, such amount of DOX should still be high enough to exert therapeutic effect. The bioactivity assay also demonstrated that our DOX/PLGA particle gel system can preserve the potency of DOX after encapsulating in PLGA and agarose gel system. Based on the results from the in vitro study, we believe that the same system will demonstrate similar therapeutic effect *in vivo*.

Our in vivo efficacy study indicated that there was significant difference between animals treated with DOX/PLGA particle gel or DOX gel, and animals treated with Saline/PLGA particle gel or untreated animals. In particular, treatment of DOX/PLGA particle gel extended the medium survival time of 9L glioma bearing rats from 21 days to 29 days. It is worth mentioning that no significant difference was observed between DOX/PLGA particle gel and DOX gel treated animals. The results

were further supported by subsequent histology examination. Both DOX/PLGA particle gel and DOX gel treated animals had less severe tumor than Saline/PLGA particle gel treated and untreated animals. The lack of difference between DOX gel treatment and DOX/PLGA particle gel treatment is not yet accounted. A possible explaination could be that the burst release of DOX from DOX gel suppressed the growth of glioma cells in early-on, but the tumor growths back after the depletion of DOX. On the other hand, the sustained release of DOX from DOX/PLGA gel did not suppress as many tumor cells as DOX gel at the early time point. However, the sustained release of DOX from DOX/PLGA gel eventually suppressed the growth of glioma and had similar effect as DOX gel treated group.

The saline/PLGA treated animals had even shorter survival than untreated animals. It might be due to the hemorrhage caused by the removal of dura prior to implantation of the delivery device. Histology results showed that tumor cells have migrated to the surface of the brain, leading to spread proliferation and consequently caused the early death of the animal. We believe the same migration also occurred in DOX/PLGA gel treated and DOX gel treated groups, however, the released DOX from these two devices suppressed the migration eventually.

3.5 CONCLUSION

The present study was initiated in an effort to incorporated PLGA/DOX particle into a SeaKem agarose gel, which can provide a continuous release of DOX locally to the tumor site. This conformally coated DOX/PLGA gel system can deliver a therapeutic dosage of DOX in the tumor site while minimize systemic side-effect. The

results from this study demonstrated that the DOX/PLGA particle gel system can maintain the bioactivity of DOX and sustained release of DOX for at least 15 day *in vitro*. We have significantly prolonged the survival time of 9L glioma bearing rat with this local and sustained DOX delivery system.

3.6 ACKNOWLEGEMENTS

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CHAPTER 4

CONCLUSION & FUTURE PERSPECTIVES

Malignant brain tumor is one of the most lethal forms of cancers. Treatment of malignant brain tumors remains a major challenge despite recent advances in surgery and other adjuvant therapy. Chemotherapy of brain tumor has been particularly inefficient, due to the special barriers in CNS. In this thesis, we are pursuing the design and development of conformal coating technology to enhance the effectiveness of DOX delivery in brain tumor therapy. DOX is currently used in the treatment of leukemias, Hodgkin's lymphoma, as well as cancers of the bladder, breast, stomach, lung, ovaries, and others. However, it is currently not used clinically for brain tumor treatment because when delivered systemically it does not cross the BBB and causes severe toxicity in the body. These limitations further suggested that DOX might be best used when delivered locally as an adjunct to surgery resection.

The *in vitro* results from this study demonstrated that the DOX/PLGA particle gel system can maintain the bioactivity and sustained release of DOX for at least 15 days. For in vivo application, we have significantly prolonged the survival time of 9L glioma bearing rat with this local and sustained DOX delivery system. While these results are rather promising, there is still a long way to reach our ultimate goal, to eradicate the remaining tumors after resection. Thus, we will discuss future prospects for developing more efficient local implant drug delivery system.

4.1 OPTIMIZATION OF IN VIVO DOSAGE

In the in vitro cytotoxicity experiment in Chapter 3, We observed that cytotoxicity of DOX on 9L and rat astrocyte is proportional to the DOX concentration and incubation time. We observed that at certain concentration, DOX has more effect on 9L glioma, while the rat astrocytes were still viable under the same condition. However, only one concentration of DOX was delivered to the brain tumor site in our in vivo efficacy experiment. Therefore, delivering different dosages of DOX to determine the optimal dosage in vivo is needed for future work.

4.2 OPTIMIZATION OF DELIVERY VEHICLE

PLGA particle gel composite was used to deliver DOX in our in vivo study. We also demonstrated that the PLGA particle gel composite could release DOX for at least 15 days. However, most of the DOX was released in the first week. It will be benefitial to tumor therapy, if we could increase the duration of DOX release. The release of DOX from PLGA particle is mainly controlled by the degradation rate of PLGA. The advantage of using PLGA is that we could manipulate degradation rate of PLGA by using different ratios of the two monomers.

The other optimization of delivery vehicle is to enhance the DOX encapsulation efficiency by fabricating the particle using spray drying technology instead of double emulsion method. The problem of using double emulsion technique for DOX encapsulation is that DOX is easily lost in washing step. Unlike emulsion methods, spray drying technique does not involve the use of water. Thus, this technique is suitable for fabrication of DOX/PLGA particle. Lin et al. showed that using spray

drying technique, they are able to increase the DOX encapsulation efficiency to 73.8% (Lin et al. 2007).

4.3 USING BIODEGRADABLE HYDROGEL AS DELIVERY SCAFFOLD

Although we have proved the advantages of using agarose gel as delivery scaffold, there is one major limitation of this non-biodegradable hydrogel. Like all other non-biodegradable implant, once the drug diffuses and is completely released, the agarose gel remains in place permanently as a foreign body. One possible solution is to use biodegradable hydrogel. Konishi et al. have studied the release of adriamycin (DOX) and cisplatin from biodegradable gelatin hydrogel (Konishi et al. 2005). Thus, applying biodegradable hydrogel in our delivery system might benefit future in vivo work.

4.4 MULTI-FACTORIAL APPROACH

The other advantage of this particle gel composite system is that we can incorporate different anticancer drugs within one single device. As mentioned before, only hydrophilic agents are suitable for our delivery system. There are several hydrophilic anticancer drugs, such as cisplatin and 5-Fluorouracil (5-FU), which have been used to treat brain tumor in vitro and in vivo. These drugs have different anticancer properties compared to DOX. Cisplatin acts by crosslinking DNA in several different ways, making it impossible for rapidly dividing cells to duplicate their DNA for mitosis. Promising results have been shown in studies treating 9L glioma with cisplatin (Lillehei et al. 1996).

5-FU, a pyrimidine analogue, is transformed inside the cell into different cytotoxic metabolites which are then incorporated into DNA and RNA, finally inducing cell cycle arrest and apoptosis by inhibiting the cell's ability to synthesize DNA. Menei et al. has reported that 5-FU was delivered locally by PLGA and achieved a significant concentration in cerebrospinal fluid (Menei 1999).

A multi-factorial approach, rather than a unilateral one, can minimize the expression of drug-resistance in tumor cells. Since these hyrophilic anticancer drugs have different mechanisms of actions on brain tumor, we propose that we will prolong the medium survival by the synergetic effect of these drugs.

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