# Investigating a Novel Drug Delivery Method to Address Treatment of Glioblastoma Multiforme

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## Introduction

Glioblastoma multiforme (GBM) is the most prevalent type of malignant brain tumor. It develops from astrocytes — star-shaped glial cells that support the nerve cells primarily found in the cerebral hemispheres[1]. GBM is classified as a grade IV astrocytoma; these are the most invasive type of glial tumors, rapidly growing and commonly spreading to nearby brain tissue. GBM is a devastating brain cancer with a 100% mortality rate that typically results in death within the first 14 months after diagnosis[2]. The National Cancer Institute estimates that 22,910 adults (12,630 men and 10,280 women) will be diagnosed with brain and other nervous system tumors in 2012. It also estimates that in 2012, 13,700 of these diagnoses will result in death [2].

GBMs are biologically aggressive tumors that present unique challenges for conventional treatments. The standard treatment of GBM so far is a combination of surgery, radiation therapy and chemotherapy which has been shown to prolong survival in patients with GBM from 12 months to 14 months[2]. GBM tumors generally re-occur up to 2 cm from the resection site a median of 6.9 months after standard surgical treatment[3]. The high rate of recurrence of GBM is due to a zone of migrating, infiltrating tumor cells. These cells surround the tumor and will invade surrounding healthy tissues as the cancer proliferates. Radiotherapy can induce a temporary remission of these malignant cells, but the response is short-lived and the tumor typically recurs within 1 year, resulting in further clinical deterioration[3].

The most effective treatment thus far is Gliadel, a polyanhydride polymeric implant containing chemotherapeutic drug, which is placed in the surgical cavity following resection to allow for chemotherapeutic treatment into the surrounding brain tissue to prevent the migration and proliferation malignant cells [4]. Gliadel enables localized drug delivery without crossing the blood brain barrier, eliminating systemic toxicity and limiting degradation of drug molecules. However, Gliadel wafers demonstrate limited efficacy, since in vivo experiments show therapeutic concentrations of BCNU drug reach only 1 cm away from the resection cavity with Gliadel use, rather than the desired 2 cm penetration distance within which the tumors usually recur.

To further decrease the rate of GBM recurrence, we would like to investigate the use of alternate biomaterials and drug delivery strategies to extend the penetration distance of therapeutic BCNU concentrations to decrease tumor recurrence frequency.

## Research Design

Gliadel is currently the state-of-the-art treatment for GBM, so the proposed product uses Glaidel's design as a basis. Gliadel is composed primarily of the copolymer poly[bis(p-carboxyphenoxy) propane: sebacic acid] (pCPP-SA) with a molar ratio of 20:80. The bioactive drug is 1,3-bis (2-chloroethyl)-1-nitrosourea (also known as BCNU or carmustine). Each Gliadel wafer is a flat disc of diameter 14.5 mm and thickness 1 mm, composed of 192.3 mg of pCPP-SA and 7.7 mg of carmustine distributed homogeneously throughout the wafer[4]. The wafer degrades through surface erosion and releases the drug over approximately 1 week; a therapeutic dose is delivered to a maximum radius of approximately 1 cm from the implant site.[3]

The major limitation of Gliadel is its inability to provide adequate drug penetration into the brain. To improve drug penetration to the desired 2 cm, we propose the incorporation of drug-loaded PLA-PEG stealth nanoparticles. These particles are expected to be capable of carrying the drug further from the implant site than passive diffusion and convection, which are the only mechanisms of drug transport in Gliadel. The extension in penetration distance is due to protection afforded by the nanoparticle coating, which will prevent degradation and elimination. Additionally, carmustine has poor solubility in water (Pubchem), so encapsulation in a micellar nanoparticle will likely improve penetration due to the improved stability of the drug in an aqueous environment.

The material selected for the nanoparticles is a copolymer of poly(D,L-lactic acid) (PLA) and poly(ethylene glycol) (PEG). The preparation of nanoparticles from this material has been extensively described[5], and the stealth properties of PEG-grafted nanoparticles have also been studied[6]. Nanoparticles produced from PLA-PEG using an aqueous continuous phase will naturally form with a PLA core and PEG shell[5]; this will stabilize the hydrophobic BCNU within the core of the nanoparticle. Also, nanoparticles with a dense surface coating of PEG exhibit excellent transport properties through brain tissue[6]. Varying the PLA:PEG ratio will alter the size, loading capacity, degradation rate, and diffusivity of the particles in vivo, providing us a versatile parameter we can tune to better control the system. A particle size of <100 nm will be necessary to ensure extensive penetration; at least 25% of pores in the brain are larger than 100 nm[6]. Minimizing the size of the nanoparticles will also aid in improving penetration, so particle size is another mechanism by which the drug release profile can be controlled. PLA is hydrophobic and generally degrades hydrolytically via acid catalyzed bulk degradation. If the nanoparticles are small enough, each nanoparticle will have approximately burst release. Depending on the size distribution of the particles, the cumulative release (the sum of many small bursts) will result in a continuous release profile.

Total drug loading will need to be increased relative to Gliadel in order to sustain a therapeutic concentration at an increased distance. In order to prevent toxicity, however, this drug must be released far from the implant site so the concentration at any point does not exceed the toxic concentration. The nanoparticle formulations will need to be optimized to ensure that drug release is sufficiently delayed or displaced from the implant site to prevent toxicity.

The proposed design will retain the effective elements of Gliadel's design while incorporating novel elements to address Gliadel's shortcomings. The proposed wafers will still contain non-encapsulated BCNU within the matrix to permit fast, controlled release identical to Gliadel. Additional drug will also be incorporated within nanoparticles, resulting in the presence of both PLA-PEG-encapsulated and non-encapsulated drug within the pCPP-SA matrix. Drug release in Gliadel occurs over the course of 1 week, with the entire matrix degrading within 2-3 weeks. Our target release profile will extend cumulative drug release to approximately 2 weeks, though the pCPP-SA scaffold will still fully degrade within 2-3 weeks (after 1 week, drug release will be only from the nanoparticles). Together, the two encapsulation designs will extend the penetration distance of the BCNU, which will lead to better therapeutic efficacy and fewer tumor recurrences(figure 1).

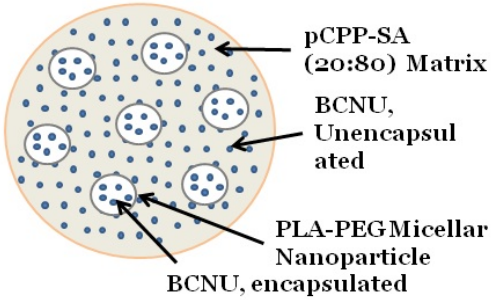



Figure 1 Modification of Gliadel with Nanoparticle.

## Product Characterization and Expected Result

### *Synthesis and Characterization of BCNU-Loaded Nanoparticles*

Diblock copolymers of PLA-PEG with varying molar ratios will be purchased from Polysciences, Inc.(Warrington, PA). A solution of the copolymers and the BCNU drug will be prepared in an appropriate organic solvent such as ethyl acetate. This solution will be added dropwise under vigorous stirring or sonication to water (or an aqueous solution containing an emulsifier such as sodium cholate, if necessary). This will form an emulsion which will be stirred under reduced pressure to evaporate the organic solvent. The aqueous solution will be filtered using a membrane with 1.2 µm pore size and the product lyophilized to produce a powder.[7] Control samples will be prepared using an identical procedure, except no BCNU will be added to the organic solvent.

The resulting powder will be visualized under field-emission SEM to determine the particle size and morphology. The procedure described should produce spherical nanoparticles, though the particle size will vary depending on the copolymer molar ratio, polymer molecular weights, and polymer and emulsifier concentrations. The required particle size will be below 100 nm, but the most desirable particle size will be determined during in vitro release profile testing. The prepared powder will then be suspended in water and the particle size and polydispersity index (PDI) will be measured using dynamic light scattering (DLS). The DLS-measured particle size should agree with the SEM particle size. A low PDI will indicate that our process is capable of producing particles

within a narrow size distribution; PDI values of <0.1 have been reported for similar loaded nanoparticles (Gomez-Gaete, 2006). Drug loading should not have a significant impact on particle size, though it has been shown to reduce PDI[7]. Drug release, however, will be strongly dependent on the particle size distribution; an extremely low PDI will result in burst release of all encapsulated drug after the delay period, while a high PDI will result in a continuous release.

Measurement of drug loading and the release profile (in PBS and cerebrospinal fluid) will be performed using UV-Vis spectroscopy, with quantitation based on BCNU's absorbance at 230 nm. The cumulative release of drug will be considered the total loading. Extraction of released BCNU into a suitable organic solvent (such as octanol) may be necessary to overcome the high absorbance of water near 230 nm. The partition coefficient between octanol and water for BCNU is known to be 34, so determining the exact concentration in aqueous solution should be straightforward even if partitioning is necessary (Pubchem). Finally, particle size and distribution over time will be measured using DLS.

Depending on the organic solvent that is used in the emulsion, it may be necessary to perform residual solvent testing by measuring the mass of any volatile condensates. If the solvent used is ethyl acetate, however, trace exposure is not likely to cause irritation in vivo.

***Preparation and Characterization of Drug-Loaded Wafers***

The matrix polymer pCPP-SA in solid powder form will be combined with powdered BCNU and powdered BCNU-loaded PLA-PEG nanoparticles (BCNU-NPs). These three powders will be mixed and then compression molded into a disc shape using an automated laboratory press. The entire procedure must be done under nitrogen to prevent hydrolytic degradation of the pCPP-SA polymer[8].

To ensure the compression molding process does not alter the nanoparticle size or morphology, the wafers will be immediately crushed and visualized under SEM, and then dissolved in water for particle size and PDI measurement using DLS. Ideally, the size, morphology, and distribution of the particles will be unchanged from the starting materials. Next, the in vitro release profile of the drug from the wafers will be determined in PBS using UV-Vis absorbance as previously described. Controls for these experiments will include: (1) commercial Gliadel wafers, (2) wafers made in-house with non-encapsulated BCNU only, and (3) wafers made in-house with BCNU-NPs only. Results from (1) and (2) should be nearly identical, as the preparation procedure described is similar to the manufacturing process for Gliadel. If the nanoparticles behave as expected, the drug release profile results from (3) should show delayed and prolonged release.

Characterization of the wafers also requires ensuring the activity of the drug once it is released from the matrix and nanoparticles. Released drug activity will be measured using a modified ELISA protocol using the aldehyde dehydrogenase enzyme (PubChem). Since the expected manufacturing parameters should not be any more damaging than the current Gliadel manufacturing process, no change in drug activity is expected.

***Finite Element Analysis: COMSOL Model of BCNU Drug Release***

Finite element analysis is an extremely useful tool to simulate drug delivery in future human studies using the input parameters obtained from the in vitro studies and in vivo animal studies. We will determine how the input parameters affect the overall drug release profile to ensure that the effective concentration of drug in the target kill region is maximized while minimizing the elimination of healthy cells. To confirm that the nanoencapsulation approach described above has merit in extending the reach of the BCNU into the surrounding tumor tissue, a simulation will be performed using COMSOL software. The Gliadel-like wafer implant, the remaining tumor region, and healthy brain region will be modeled in COMSOL as a compound hemispherical region in a 2D axisymmetric geometry[9]. This model will consider three transient diffusion species in the presence of convective flow: the release of nanoparticles from the wafer implant, the release of the free BCNU drug from the wafer implant, and the release of BCNU drug from the nanoparticle. The implant (1.0 mm thick), the tumor region (2.0 mm thick), and healthy region (semi-infinite, 2.5 cm thick) will replicate *in vivo* release conditions as the scaffold and nanoparticle degrade to release the encapsulated drug particles[9].

The COMSOL simulation will be conducted over a 14-day period to obtain a release profiles for both the PLA-PEG nanoparticle and the BCNU drug from the implant and into the surrounding tissue(figure 2). This model will provide the diffusive distance from the implant site at which a therapeutically effective concentration of BCNU is

present. To examine the efficacy of this approach, the resulting BCNU drug release profile and therapeutic distance will be compared to a normal Gliadel model to see if the proposed approach has increased therapeutic potential. The COMSOL model will also be used to perform sensitivity analysis by tuning the model's input parameters (nanoparticle size, drug loading, time of release from nanoparticle/degradation rate) to achieve the desired BCNU drug release profile and to inform design recommendations for then *in vivo* higher-order species and human testing.

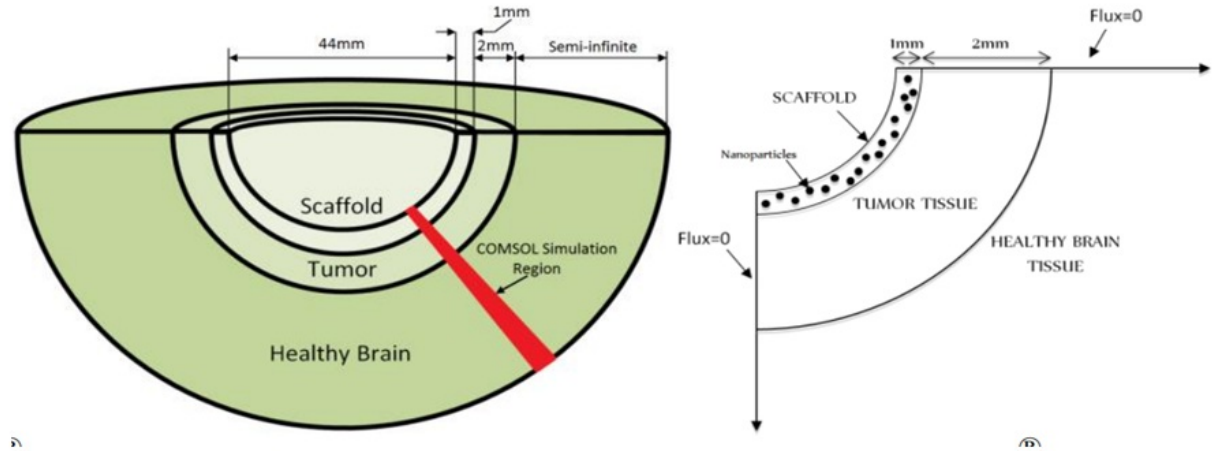



Figure 2 in vivo COMSOL model of PLA-PEG nanoparticle and the BCNU drug from the implant and into the surrounding tissue. The drug release profile is 14 days period.

***<u>In vitro Biological Performance Testing</u>***

***Cell Cytotoxicity Testing for Drug Efficacy and Material Safety***

Cytotoxicity testing is fundamental to ensure that the biomaterials used in our product are safe and possess proper functionality. The brain is an incredibly exceptional organ; it is responsible for maintaining all bodily functions, as such it is extremely important that any drug devices implanted does not adversely interfere with the extraordinary role in plays in the quality and length of life. Maximizing the BCNU drug delivery to the tumor region, while minimizing the BCNU drug delivery to the healthy tissue region is the primary goal of the novel approach presented herein. Since it is expected that complete BCNU drug release (1 week) due to diffusion will occur faster than the complete degradation of the implant material components (2-3 weeks), it is important to ensure that the remaining material does not have significant negative effects on the healthy cells after the tumor cells have been eliminated (Dang et al, 1996). An *in vitro* cell culture system will be developed to assess the toxicity of the implant system on both a human glioblastoma cell line (A-172)[10] and on healthy human neuronal cell line (HCN-1) [11], as shown below in Figure 1. Cytotoxicity of each component of the implant system will be examined qualitatively using the agar diffusion assay and the MEM Elution assay [12]

To perform the agar diffusion assay(figure 3) to examine safety, empty PLA-PEG nanoparticles, empty pCPP:SA wafers, and empty pCPP:SA wafer with nanoparticles will be placed on the agar surface overlaid on a monolayer of A-172 glioblastoma cell line or healthy human neuronal cell line, HCN-1 for 24-72 hours[12]. Empty means the nanoparticle and wafer does not contain BCNU drug. This assay will be used to ensure that the individual components of the implant system will not be toxic to the healthy brain tissue as the wafer degrades. To guarantee that the BCNU drug released from the wafer and nanoparticles is able to kill glioblastoma cells, empty pCPP:SA wafers with nanoparticles (negative control), BCNU drug-loaded PLA-PEG nanoparticles, drug-loaded pCPP:SA wafers, and drug-loaded pCPP:SA wafers with drug-loaded nanoparticles, just BCNU drug (positive control), and latex (positive control) will be placed on the agar surface to perform additional agar diffusion assays for drug functionality upon release. Evidence of malformed, degenerated, or lysed cells below the agar visualized by light microscropy will be indicative of cytotoxicity[12]. Experiments with loaded BCNU drug will be compared to the experiments without drug to show that the cell death induced is due exclusively to the presence of

BCNU drug. We expect to see extensive cell death due to the presence of BCNU drug. Since pCPP:SA, PLA, and PEG are all biocompatible polymers that have been used in biological systems with minimal negative effects, we expect that the degree of cytotoxicity induced by these components of the implant will be little to none.

To examine the effects of the drug delivery implant leachables *in vitro*, MEM elution assays will be performed. These assay will show be used to confirm the idea that specific components of the drug delivery implant will not kill healthy brain cells. Extracts of empty pCPP:SA wafers and empty PLA-PEG nanoparticles will be generated by incubating in extracting media—saline or vegetable oil at 37 C[12] These extracts will be placed on a monolayer of A-172 and HCN-1, incubated, and examined using microscopy for cell malformation, cell degeneration, and cell lysis[12].

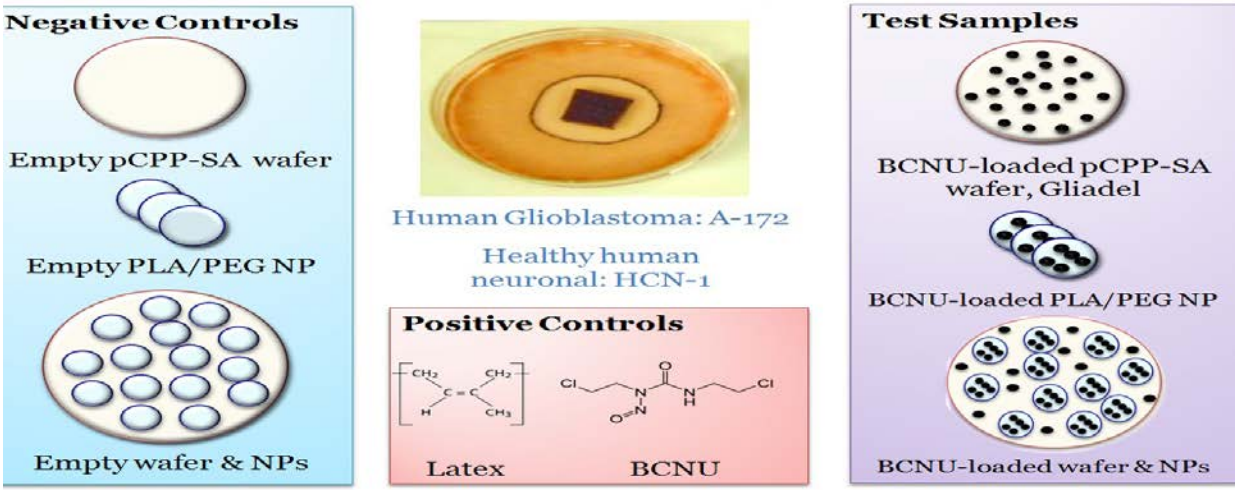



Figure 3 In vitro Cytotoxicity: Agar overlay diffusion assay. Include negative control and positive controls. This assay will be used to measure cell lysis levels for different controls, to verify that the tumoricidal properties of the proposed design are attributed to the presence of BCNU and not to the material components of the wafer and nanoparticle system

***Protein Adhesion to Implant and Nanoparticle Surface***

Protein adsorption to surface of the wafer implant and to the nanoparticle surface can impede BCNU drug release from the wafer and limit nanoparticle diffusion and BCNU drug release into the surrounding brain tissue, as indicated in Figure 4. Since the PLA-PEG nanoparticles degrade by bulk degradation and the pCPP:SA wafers degrade by surface erosion, which are both hydrolytic processes, it is important that these materials have access to water to allow degradation to occur at the desired rate. Excessive protein adsorption will threaten the biological performance of the drug delivery system. This is particularly important for diffusion of the "stealth" PEG-coated nanoparticles through the surrounding brain tissue, as aggregation of proteins from the cerebrospinal fluid on the nanoparticles can impede migration. Furthermore, interactions between attached proteins and cells can exacerbate the inhibition of drug release and can activate the immune response. To assess protein adsorption, PLA-PEG nanoparticles and the pCPP:SA wafer will be incubated in pooled rat and human cerebrospinal fluid. CSF protein adsorption will be measured using Quartz Crystal Micro Balance with Dissipation(QCM-D) where the change in recorded resonant frequency correlates to the change in mass or the amount of proteins bound to the surface[13]. Since PEG has been shown to limit protein adsorption to biomaterial surfaces and the traditional Gliadel wafer has been examined for safety and efficacy, we expect to see limited

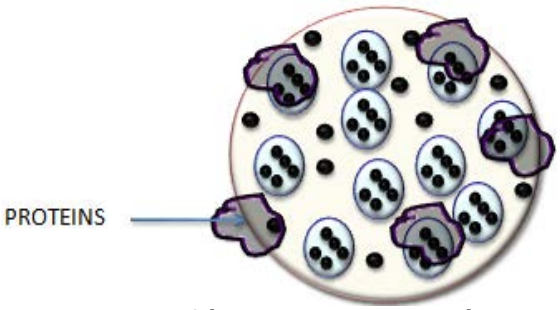


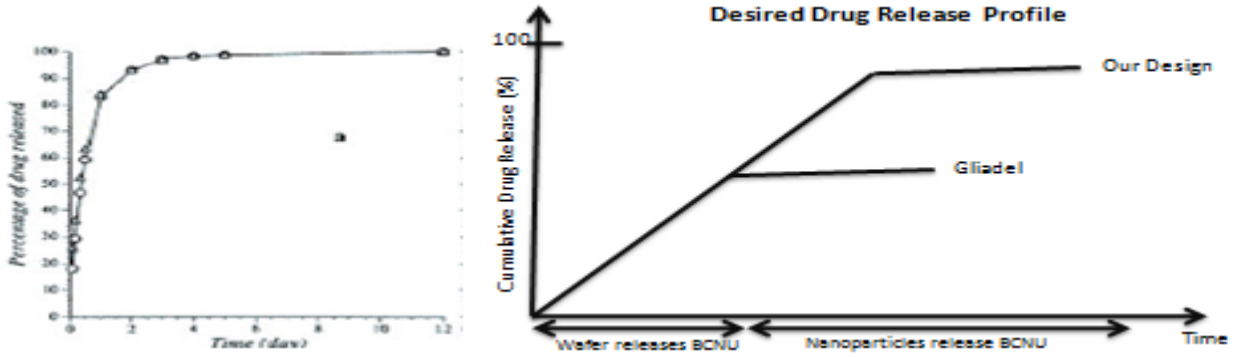

Figure 4 Protein Adsorption Limits Drug and Nanoparticle Diffusion. The PEG outer layer of the micelles is stealth and can resist protein adsorption. Additionally, the Gliadel wafer demonstrates previously established safety and efficacy, so we expect limited protein adsorption

protein adsorption to our wafer/nanoparticle system.

***In vitro diffusion model: Kinetics of BCNU Drug Release from Scaffold and from Nanoparticles***

To determine the drug release rate from the wafer and nanoparticles upon implantation, *in vitro* erosion of the BCNU drug-loaded wafer, the BCNU drug-loaded nanoparticles, and BCNU drug-load wafer/nanoparticle implant will be examined. The drug release rate is dictated by the diffusion of the drug through the wafer/nanoparticle and by the degradation of the wafer/nanoparticle by surface/bulk erosion. *In vivo*, the Gliadel wafer releases the BCNU drug contained in a one-week period; a similar result is expected from this Gliadel-like design[14] (Dang et al, 1996). To ensure that the drug concentration at the target regions (up to 2 cm from the implant site) is therapeutically effective and that healthy cell death is curbed, a drug delivery system that releases the drug over an increased time span is ideal.

A linear, continuous drug release profile, with respect to time, is desired for optimal drug delivery, as shown in Figure 5. Since the wafer degrades via surface erosion, the non-encapsulated drug and nanoparticles will be released at a continuous rate, as has previously been described for Gliadel wafers [14]. Due to the small size and bulk erosion properties of individual nanoparticles, drug will be released from each particle in a near single burst. Depending on the size distribution of the nanoparticles, the cumulative release from all of the nanoparticles can be tuned, ranging from a burst release (if the nanoparticles are all of identical size) to a continuous release (if the size distribution of the nanoparticles is flat)[6]. The release rates obtained will further tune the wafer and nanoparticle specifications and the input parameters for the COMSOL model to achieve the desired release profile for future *in vivo* studies. Drug and nanoparticle release from the wafer and drug release from the nanoparticle over time will be quantified in PBS, rat CSF, and human CSF. Measurements made in rat and human CSF are included because future *in vivo* studies will be carried out in rats and ultimately in humans. It is important to know if the degradation of the wafer and therefore the release of drug is dependent on the medium used *in vitro* to induce degradation. Drug release kinetics will measured by placing the test sample (wafer, nanoparticle, or combination) into PBS, rat CSF, and human CSF and transferring to fresh PBS and CSF at 2 hours, 4 hours, 6 hours, 8 hours and every 24 hours for 7 days. The BCNU released into the PBS and CSF from which the test sample was extracted will be quantified using UV-Vis spectroscopy set at 230nm [6]. From this, a cumulative drug release profile over time will be generated to verify that the release rate is continuous and matches that of the Gliadel wafers.




A) B)

Figure 5 In vitro drug release profile. A). Amount of BCNU released per time for Gliadel. B.) Though individual micelles burst-release, there is overall drug release increase over time in the micelle-modified device

***<u>In vivo Biological Performance Testing</u>***

We will implant our modified device and Gliadel Wafer into the brains of GBM affected rats and conduct a sequence of testings to characterize implant safety and efficacy.

There is a negative control where only the pCPP:SA wafer and empty nanoparticles are implanted into the resection site. The negative control is aimed to test if our PEG-PLA micellar nanoparticles are safe modifications for Gliadel. In addition, Gliadel wafer serves as a positive control in our experiments. The purpose of the positive control is to show whether or not our improvement demonstrates more efficacy than Gliadel.

At different time after the implant, the body has different types of immune responses.

Therefore, to better characterize the immune response, the devices will be explanted at different time points in a 28-day period. Specifically, the device will be implanted at day. At day 1, samples of the brain tissue surrounding the implant are excised to test for acute inflammatory response. Similarly, explants are obtained on day 4 and day 7 to test for adaptive inflammatory response. Explants are obtained on day 10, day 14 and day 21 to test for chronic inflammatory response. At day 21 and day 28, explants are examined for fibrotic encapsulation.

At day 14, the autoradiography will be conducted in explant brain tissues of rats to characterize the diffusion distance of BCNU drug. Two-week is an appropriate time to do autoradiography because it allows enough time for BCNU drugs to be released and diffuse out while maintaining low systemic clearance of drugs. Live/Dead assay will be performed on the cells in the surrounding tissues of the implant at day 28; this long-time period allows the apoptosis pathways of the tumor cells to be carried out.

***Acute Inflammatory Response***

This first stage of the immune-response is innate immune response which is also called acute inflammatory response. The central features in the brain tissue of this stage are spontaneous phagocytosis of implant by neutrophils and macrophages.

Activated macrophages secrete many chemical mediators, such as cytokines interleukin(IL)-1, tumor necrosis factor (TNF)-α,interleukin(IL)-6[15]. Both IL-1 and TNF-α act to promote cell migration by increasing the expression of adhesion molecules. In addition, TNF-α can directly activate both neutrophils and macrophages. IL-6 and IL-1 also provide communications with the acquired immune response because they are found to promote migrations and proliferations of lymphocytes. Previous researches shows that cytokine signaling not only participate in immune functions of astrocytes, but overexpression of those cytokines also lead to progressive neuron degeneration.[16] Kolbe and et al suggested that TNF-α, IL-6, IL-1β, can bind to specific dopaminergic neuron cell surface receptors and activate apoptotic pathways of neurons.

Therefore the significance of control over acute inflammatory response manifests itself in avoiding activation of adaptive immunoresponse in which more lymphocytes will be generated to attack the implant, and preventing apoptosis of healthy neurons in brain tissues. As stated previously, cytokines TNF-α, IL-6, IL-1β, which involved in these pathways can be used to characterize acute inflammatory response.

After 1 day of implantation of the device into the rats, the brain tissues surrounding the device will be excised. Following the protocols of TNF-α Rat ELISA Kit®, Abcam (L)-1β Rat ELISA Kit®, and invitrogen IL6 ELISA kit, the amount of TNF alpha, IL-1 beta, and IL-6 present in the rats can be quantitatively detected. We expect to see similar amount of proinflammatory cytokines expressed in our device and Gliadel.

***Adaptive Immune Response***

The second stage of the immune response is adaptive immune response, also called specific immune response. The specificity of acquired of acquired immunity comes from the fact that lymphocytes (B cell and T cells) are activated in response to antigens. When the body recognized an antigen as foreign, they produce of T cells, B-cells, nature killer cells and cytokines in response to antigens. CD4+cells (helper T-cells) and CD8+ (cytotoxic T-cells) take an important role in this stages. CD4+ cells initiate the body's response to infection. Antigen presentation activates CD4+ cells, and then they secrete cytokines to aid the binding of antigens to cytotoxic T-cells and B-cells. CD8+ cells (cytotoxic T-cells) can kill the target by secretion of cytotoxin or by activation of apoptosis pathway of the foreign virus or cells[17].

Therefore, CD4+ and CD8+ are the good indicator of present of T-cell. Follow the protocol of BD FastImmune™ CD8 and CD4 Cytokine Detection Kits, CD4+ and CD8+ can be detected. This kit contains cytokine specific, multicolor antibody reagents, a matching multicolor isotope control, and sample processing reagents to measure antigen-specific T-cell responses. Combining the kit and the flow cytometer with laser excitation at 488nm and 635nm, and BD FACSComp software(BD Bioscience, n.d.), we will be able to obtain the quantitative and qualitative measurement of active T-cell and immune cells from the rat's extract. We expect to observe very similar result on our device and Gliadel.

***Chronic Inflammatory Response***

The third stage of the inflammatory response is chronic inflammatory response. This process happens in a prolonged duration (weeks or months) in which active inflammation, tissue destruction, and attempts at repair are proceeding simultaneously. CD68 is a 110-kD transmembrane glycoprotein that is highly expressed by human monocytes and tissue macrophages, and also expressed in murin macrosialin. It is a member of the lysosomal/endosomal-associated membrane

glycoprotein (LAMP) family. The protein primarily localizes to lysosomes and endosomes with a smaller fraction circulating to the cell surface. It is a type I integral membrane protein with a heavily glycosylated extracellular domain and binds to tissue- and organ-specific lectins or selectins. The protein is also a member of the scavenger receptor family. Scavenger receptors typically function to clear cellular debris, promote phagocytosis, and mediate the recruitment and activation of macrophages[18].

Immunostaining of CD68 is conducted to determine macrophage infiltration. At day 10, day 14 and day 28 of the implantation of the drug delivery devices, the rats will be euthanized via CO2 asphyxiation. The implants and surrounding tissue will be excised from the brain of the rats. The explants are deparaffinized and autoclaved in pH 6.0 citrate buffer (121 oC, 15 min) for antigen retrieval. Endogenous peroxidase activity is blocked by peroxidase-blocking reagent (DAKO).The primary CD68 monoclonal antibody (MCA341GA; Serotec Inc.) is used at a dilution of 1:50 and incubated with the sections at room temperature for 30 min. A biotinylated rabbit anti-rat secondary antibody (DAKO) and LSAB Streptavidin-HRP (DAKO) are then used. The slides were lightly counterstained with haematoxylin to assist in tissue visualization, and the macrophages and monocytes in the brain tissues of each rat could be identified under scanning electron microscopy (SEM). Since these material are known to be biocompatible, we would expect to see low immunogenicity for our implant.

***Fibrotic Encapsulation***

The last stage of the normal would healing cascade is foreign body response, which describes the non-specific immune response to implanted foreign device. This stage involves granulation tissue maturation, which is marked by the presence of larger blood vessels and alignment of collagen fibers and a dense layer of fibrotic connective tissue.[19] This fibrotic capsule shields the foreign body from the immune system and surrounding tissues.

The development of a fibrotic capsule serves as a protective mechanism for our body to limit exposure to toxic or allergenic materials, but it also presents a problem for our drug delivery device. As a result of the isolation, BCNU drugs are not able to deliver into the brain tissue and nanoparticles cannot diffuse which leads to device failure. What may become even worse situation is that the presence fibrotic capsule is likely to cause further malfunctions of the brain which results in unexpected death of patients.

Fibrous encapsulation compromises the efficiency of the device and frequently leads to device failure.[19] Therefore, it is important to characterize the severity fibrotic encapsulations before any clinical trial. In our study, fibrotic encapsulation will be characterized by H&E histology staining because it allows one to evaluate the tissue structure of a capsule.

After 21 days and 28 days of the implantation of the drug delivery devices, the rats will be euthanized via CO2 asphyxiation. The implants and surrounding tissue will be excised from the brain of the rats. The tissues will then be fixed in 10% formalin, embedded in paraffin, cut into 5 µm sections, and stained using hematoxylin and eosin (H&E) for histological analysis under Scanning Electron Microscopy (SEM).

We expected to see very little capsule formed surrounding the devices, since PLA-PEG is biodegradable materials which can be hydrolyzed by water in the body fluid. Once PLA-PEG is hydrolyzed, the structure of the polymer mixture becomes porous. This porous structure will lower macrophage interaction with the device and further resist fibrotic encapsulations[20]. Also, due to the small size (1-100nm) of our nanoparticles and high surface to volume ratio, the fibrotic encapsulation is also very unlikely to occur[20].

***Autoradiography***

The use of Autoradiography is common for the prospect of detecting the effectiveness and range of a specific drug. Due to the difficulty of imaging the brain specifically, autoradiography is desireable due to the methods in which this technique constructs its images - the detection of the decay in emmision of specific markers (tritium ($^{3}H$) or $^{125}I$). To detect tissue localization of a radioactive substance, the markers either (1) binds to a receptor, (2) detects via a metabolic pathway or enzyme, or (3) hybridizes to a nucleix acid. This technique can be used either in vivo or in vitro, but we will conduct our experiments with autoradiogrpahy in vivo. We will be using a similar process as the one utilized in the cited paper of detecting drug dffectiveness of treating alzheimers in the brain.[21] 3H-BCNU autoradiography binding will be carried out through implantation and after 28 days, enough time for the drug to fully diffuse, explants will be sectioned and examined. The explanted sections from each animal will then be washed with PBS, and non-specific binding will be determined by incubating adjacent sections with 1 mM unlabeled BCNU with an exposure time of 3 days. The plates were processed with a phosphorimager and binding densities will be analyzed. The relative optical density calculated from the tritium

standards was used to calculate binding values in the tissue. Using this technique, we will be able to quantify the tissue localization of our BCNU drug. The expected result is showed in figure 6. For the negative control, we expect to see no drug localized on the image. For the positive control (Gliadel), we expect to see a certain degree of drug diffusion. Finally, for our implant, we expect to see the drug penetration distance to be increased.

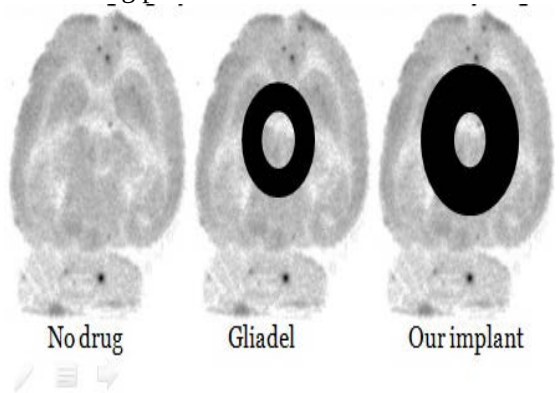



Figure 6 Autoradiography of resection site after 14-days explants. A sample autoradiograph of an explant after 14 days. [$^{3}$H] BCNU is observed as black regions on the images.

***Live/Dead Cell Assay***

We will also assess for improved longevity of our BCNU drug by performing a live/dead assay, and quantifying levels of cell death at varying diffusion distances at different time points. Since ubiquitous intracellular esterase activity can be used to distinguish live cells, such activity are able to convert the virtually nonfluorescent cell-permeant calcein AM to the intensely fluorescent calcein. Such calcein dye is present within the live cells. Under the microscopy, green fluorescence is observe in live cells. If Ethidium Homodimer, another dye, gets into cells with damaged membranes, it will produc a bright red fluorescence in dead cells. Based on these two dyes, live cells and dead cells can be distinguished. Thick sections of brain tissue, on the order of 300 um, will be taken for each explants. By following the protocol of Live/Dead Viability/Cytotoxicity Kit from Molecular ProbesTM Invitrogen Detection Technologies[22]. The color changing showed under microscopy can be observed. The amount of dead cells can be measured at different time points and different control group. The result will indicate the diffussion distance of BCNU. After using the modification design of nanoparticle, the result should show the increased diffusion distance of BCNU. A sample image of the staining after 14 days that would demonstrate improved longevity BCNU activity is observed in Figure 7. For the negative control, we expect to see primarily live cells. For the positive control (Gliadel), we expect to see significant cell death due to BCNU activity. For our implant, we expect to see highest levels of cell death. We can subsequently stain for glioblastoma multiforme tumor cell-surface markers to determine relative level of tumor cell death compared to healthy cell death. Finally, we expect that once the BCNU drug reaches an inactive state, no more cell death will be observed, so we should see saturating levels of cell viability starting at time points that are approximately past the 2-3 week mark.

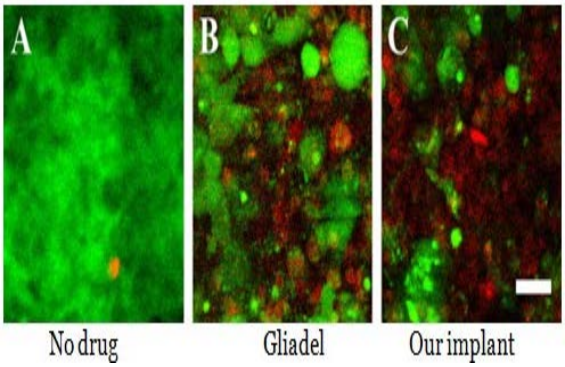



Figure 7 Live/dead assay. A sample Calcein AM/Ethidium Homodimer staining assay of an explant after 14 days. Green cells denote viable cells, while red cells denote dead cells

**Discussion**

With so many tests to ensure the viability and reliability of our improved drug design, our modifications to Gliadel seems like a viable solution. However, there still exists room for improvement and further modifications to our design. Firstly, after we determine a lead candidate with optimized parameters for our design, we must consider that BCNU non-specifically targets cells. That is, the drug is not limited to cancer cells and causes cell death on any cells it reaches. Therefore, it would be more desirable to have a drug that specifically target cancer cells in the brain and not harming the healthy brain cells. This may prove difficult due to the fact that finding a solution to this problem calls for a cure to cancer. However, we may be able to add additional factors to our drug to make BCNU more cancer cell specific.

Furthermore, our in vivo studies we conducted with rats. The fundamental limitation to this was that we would have to scale down our drug penetration accordingly and predict our results. The next step would be to characterize our design on higher-order species, such as primates, which have brain sizes more closely to humans in order to better understand the efficacy of our proposed design.

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