POLYMERIC BIOMATERIAL DRUG DELIVERY SYSTEMS FOR GLIOBLASTOMA THERAPY AND VACCINES

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ABSTRACT

Kathryn Margaret Moore: Polymeric Biomaterial Drug Delivery Systems for Glioblastoma Therapy and Vaccines (Under the direction of Kristy M. Ainslie)

Polymeric biomaterial drug delivery systems have been explored to improve therapies for a wide range of diseases, including cancer and infectious diseases, by providing control over delivery of therapeutic cargo. Scaffolds composed of polymeric biomaterials have been used to increase efficacy of cell therapies by promoting cell implantation and viability thereafter. Both scaffolds and particles can be loaded with small molecules and biological agents to enhance delivery to target cells. This dissertation aims to explore the use of polymeric biomaterials to improve both glioblastoma therapy and vaccines.

In the case of glioblastoma, the most common primary brain tumor, local drug delivery is a beneficial therapeutic strategy because its bypasses the blood brain barrier and allows for direct access to the site of tumor recurrence. Herein, scaffolds were fabricated by the process of electrospinning and characterized for the delivery of tumoricidal agent producing stem cells and chemotherapeutics into the glioblastoma surgical resection cavity. This work places a strong emphasis on the impact of scaffold degradation on the local glioblastoma therapies by utilizing the tunable polymer, acetalated dextran.

Vaccine formulations often require adjuvants to stimulate the immune system to generate a protective immune response. Polymeric biomaterials have been employed to deliver antigen and adjuvant more efficiently to antigen presenting cells, as well as create an immunostimulatory
depot. In this dissertation, electrospun acetalated dextran ribbon-like particles called microconfetti, generated by fragmenting scaffolds, were investigated as a new vaccine platform.
ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to all of those who have been with me through this challenging and rewarding journey – through the failures and the successes and everything in between.

Thank you to my advisor and mentor, Dr. Kristy Ainslie, for guiding me through this process. You have challenged me to work hard and to do my best. I will never forget the enormous amount of time and energy that you have devoted towards my success and your steadfast confidence in me throughout these five years. Thank you to Dr. Eric Bachelder and my committee for helping me shape my projects and assisting in my professional development.

Thank you to Dr. Elizabeth Graham-Gurysh for being an incredible mentor, role model, and friend. Words cannot express my appreciation for the abundant wisdom and encouragement with which you have generously gifted me, inside and outside of the lab. I would not be the scientist or person that I am today without you.

To all the members of the Ainslie Lab, current and past, working with you is my favorite part of doing science. I cannot overstate how grateful I am to have known every single one of you and the immense joy that you have brought me. You have made lab a fun place to do hard work and have inspired me with your curiosity and intellect. I will forever treasure your friendship and diverse perspectives. I could not have done the work in this dissertation without you and sincerely thank you.
And finally, thank you to my family – to my parents, brother, and grandparents. I am so fortunate to be loved and supported by you all. I could not have accomplished this without you.
This dissertation is composed of 4 chapters and 2 appendices. The chapters comprise my first author publications and discussion of that work, and the appendices are publications of which I was a major contributing second author. Chapter 1 is a review article published in ACS Biomaterials Science & Engineering summarizing the development of polymeric biomaterial scaffolds for tumoricidal stem cell therapy. Chapter 2 is published in Materials Science & Engineering C and describes our work evaluating the impact of scaffold degradation on neural stem cell persistence in the brain after implantation into the surgical resection cavity for glioblastoma therapy through the development of composite acetalated dextran scaffolds. Chapter 3 has been submitted to ACS Applied Materials & Interfaces and encompasses our work characterizing high aspect ratio ribbon-like particles called microconfetti, derived from acetalated dextran scaffolds, as a vaccine platform. The contents of Chapters 1-3 are summarized, and future directions are discussed in Chapter 4. None of the work in Chapters 1-3 would have been possible without the help from the members of the Ainslie Lab. Appendix 1 and 2 are publications centered around local drug delivery via electrospun acetalated dextran scaffolds for glioblastoma therapy spearheaded by Dr. Elizabeth Graham-Gurysh.
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<th>Description</th>
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<tbody>
<tr>
<td>Ace-DEX</td>
<td>Acetalated Dextran</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>AUC</td>
<td>Area under the curve</td>
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<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
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<tr>
<td>BHK</td>
<td>Baby hamster kidney</td>
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<tr>
<td>BLI</td>
<td>Bioluminescent imaging</td>
</tr>
<tr>
<td>BMDC</td>
<td>Bone marrow derived dendritic cells</td>
</tr>
<tr>
<td>CAC</td>
<td>Cyclic acetal coverage</td>
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<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>DHT</td>
<td>Dehydrothermal</td>
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<tr>
<td>DI</td>
<td>Direct injection</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
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<tr>
<td>Fluc</td>
<td>Firefly luciferase</td>
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<tr>
<td>GBM</td>
<td>Glioblastoma</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>HA</td>
<td>Hyaluronic acid</td>
</tr>
<tr>
<td>HEK 293</td>
<td>Human embryonic kidney</td>
</tr>
<tr>
<td>ICG</td>
<td>Indocyanine green</td>
</tr>
<tr>
<td>MC</td>
<td>Microconfetti</td>
</tr>
<tr>
<td>mCh</td>
<td>mCherry</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>MP</td>
<td>Microparticle</td>
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<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cells</td>
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<tr>
<td>MSR</td>
<td>Mesoporous silica rods</td>
</tr>
<tr>
<td>MTT</td>
<td>Thiazolyl blue tetrazolium bromide</td>
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<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<tr>
<td>NSC</td>
<td>Neural stem cells</td>
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<tr>
<td>OVA</td>
<td>Ovalbumin</td>
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<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular patterns</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PLA</td>
<td>Poly (L-lactic acid)</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly lactic-co-glycolic acid</td>
</tr>
<tr>
<td>SC</td>
<td>Stem cell</td>
</tr>
<tr>
<td>SCI</td>
<td>Spinal cord injury</td>
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<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>TBI</td>
<td>Traumatic brain injury</td>
</tr>
<tr>
<td>TCPS</td>
<td>Tissue culture treated polystyrene</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
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<tr>
<td>TEA</td>
<td>Triethylamine</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptors</td>
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<tr>
<td>TRAIL</td>
<td>Tumor necrosis factor (TNF)-related apoptosis inducing ligand</td>
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CHAPTER 1: REVIEW OF BIOPOLYMERIC SCAFFOLDS FOR TUMORICIDAL STEM CELL GLIOBLASTOMA THERAPY 1

1.1 Introduction

1.1.1 Glioblastoma and Treatment Strategies

Glioblastoma (GBM) is a devastating disease and accounts for 47.7% of all primary brain malignancies, making it the most common tumor in this category (1). The standard of care includes surgical resection, radiation, and oral chemotherapy with an alkylating agent such as temozolomide (2). Besides temozolomide, lomustine or bevacizumab (Avastin®) can be systemically administered for GBM therapy. Lomustine is a nitrosurea alkylating agent and bevacizumab is an anti-angiogenic monoclonal antibody. However, studies have indicated that neither lomustine nor bevacizumab has shown improvement in overall survival compared to the standard of care with temozolomide. In either newly diagnosed GBM patients or those with newly recurrent GBM the standard of care with temozolomide rendered the best outcome (3, 4). Even with these interventions, the median survival is only 15-18 months and the five-year survival is only 5.6% (1, 2). Development of more effective GBM therapies is a difficult task, complicated by several contributing factors. First, GBM is diffuse and infiltrative, making complete tumor resection impossible (5, 6). Second, GBM is a highly heterogenous tumor and is prone to developing drug resistance (7). To highlight the heterogeneity of GBM, recent retrospective genetic profiling of tumors has identified specific mutations, indicating that a more personalized

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1 This chapter previously appeared as an article in ACS Biomaterials Science and Engineering. The original citation is as follows: "Polymeric Biomaterial Scaffolds for Tumoricidal Stem Cell Glioblastoma Therapy," ACS. Biomater. Sci. Eng. (June 2020).
medicine approach using different chemotherapy regimens may lead to better outcomes (2). However, many systemically delivered drugs are poorly bioavailable in the central nervous system (CNS), dramatically reducing systemic chemotherapeutic options for GBM. This is primarily caused by the blood-brain barrier (BBB), which is a protective barrier formed by a number of cell types lining the blood vessels in the brain (8). Among the drugs that can potentially cross the BBB, systemic toxicity becomes a concern because large doses of drug are often required to achieve therapeutic levels in the brain (9).

Local drug delivery directly to the brain is one strategy to bypass the BBB, whereby drugs can be administered into the brain by implants, direct injection, or convection-enhanced delivery (10). This delivers high concentration of drug directly to the brain parenchyma, mitigating the need to deliver high systemic doses thereby reducing toxicity (11, 12). Local drug delivery in the tumor resection site can be of critical importance since up to 95% of resected GBM tumors recur within 2 cm of the tumor resection site (13). An FDA-approved local drug delivery method for interstitial therapy in the brain is the carmustine loaded polymeric wafer, Gliadel. Gliadel wafers are fabricated by compression molding spray dried microparticles composed of a copolymer blend of 1,3-bis-(p-carboxyphenoxy)propane and sebacic acid (14). In patients with newly diagnosed GBM, Gliadel has been found to improve mean overall survival by 2 months (11). The modest survival benefit from Gliadel highlights the difficulty of drug delivery to the brain even after the BBB has been bypassed. Complications such as edema, seizures, wound healing abnormalities, and surgical site infection have been associated with Gliadel, but the frequency at which these complications occur is varied across clinical trials (10, 11, 15). Moreover, even with this local delivery of therapy, short half-life and poor drug distribution throughout the brain tissue remain major challenges (16-19). One emerging therapy called tumoricidal stem cell (SC) therapy has the
potential to overcome limitations for current local and systemic chemotherapy strategies, in which SCs act as vehicles to deliver therapeutics for GBM therapy (20).

1.1.2 Tumoricidal Stem Cell Therapy for Glioblastoma

Employing genetically engineered cells that secrete therapeutic agents, such as prodrug enzymes, oncolytic viruses, tumoricidal proteins, angiogenesis inhibitors, and immunotherapeutic cytokines, have been explored to combat primary and recurrent GBM (21-23). In the context of post-surgical disease, implanting therapeutic cells after surgical resection can provide a local and sustained drug concentration to combat recurrence driven by residual peri-cavity tumor cells (24). This could be advantageous for delivery of drugs that have a shorter half-life. Moreover, therapeutic cells can offer advantages over other common local drug delivery modalities. These include polymer implants that contain a limited amount of drug and strategies employing repeated dosing of soluble drug through a catheter which is associated with a high rate of complications (e.g., incorrect catheter placement, infection, or blockage) (25, 26). By contrast, tumoricidal SCs could deliver a therapeutic dose of drug for longer periods without the need for additional invasive procedures, depending on the lifespan of the cells.

A number of different cell lines have been used as drug delivery platforms in pre-clinical testing, including NIH 3T3 fibroblasts (24), Chinese hamster ovary (CHO) cells (27), HEK 293 cells (24, 28-30), baby hamster kidney (BHK) cells (31, 32), mesenchymal stem cells (MSCs) (33-36), neural stem cells (NSCs) (37-40), and cells generated using reprogramming technology (iPSC derivatives, iNSCs) (41, 42). Among the cell lines studied, stem cells (SCs) offer a unique advantage over other cells because they can be tumor-tropic, meaning that they chemotactically migrate or “home” to tumor cells by a number of different mechanisms (43, 44). Once at the tumor, SCs can kill invasive tumor cells that could not be surgically resected (20, 22). One of the most
commonly studied migratory axis is the CXCR4/SDF-1 signaling pathway, where SCs expressing the CXCR4 receptor are recruited to GBM cells secreting SDF-1 (43, 44). Harnessing this migratory capacity suggests that drug penetration into the brain could be improve when SCs are used as drug delivery vehicle.

Both MSCs and NSCs have been explored for tumoricidal SC therapy for GBM (22, 23, 45). Both SC types are characterized by their ability to self-renew and differentiate into distinct cell lineages. MSCs are easily isolated from patients and can be found in many different tissue types. Yet, MSCs are typically derived from the bone marrow and these cells can differentiate into osteoblasts, chondrocytes, and adipocytes (23). The use of MSCs for GBM therapy remains controversial due to conflicting reports regarding the effect of MSCs on tumor growth. Some reports have shown that MSCs enhance tumor growth, angiogenesis, and invasion into the surrounding tissue in a number of different cancer types, including GBM, while others have found the opposite (46-48). However, studies evaluating tumoricidal SC therapy using MSCs have found that orthotopic GBM tumor growth is unaffected by infusion of non-therapeutic MSCs (49). NSCs are found in the brain, spinal cord, and the retina and have the ability to differentiate into neurons, astrocytes, and oligodendrocytes (50). NSC lines are either isolated from donated fetal tissue or derived from embryonic SCs (50, 51). This makes NSCs difficult to isolate directly from patients, and therefore allogenic NSCs are used for therapeutic applications. More recently, induced NSCs (iNSCs), have also been investigated preclinically as delivery vehicles for anti-GBM agents (41, 42). iNSCs are generated by transdifferentiation, a process that directly reprograms somatic cells, such as fibroblasts, into another cell lineage, in this case NSCs. Transdifferentiation is similar to the process for generating induced pluripotent SCs, but the pluripotent state is bypassed entirely (52). Using transdifferentiation would allow for autologous patient-derived therapeutic SC
generation and help evade immune rejection of SCs, thereby prolonging tumoricidal SC persistence in the brain (42).

Recently, a first-in-human phase I clinical trial was concluded where tumoricidal NSCs were administered into the brain of GBM patients at the time of tumor resection. The therapeutic cells were an allogenic human NSC line, HB1.F3, engineered to carry the enzyme cytosine deaminase. 5-fluorocytosine is administered to patients and converted into the chemotherapy 5-fluorouracil in the presence of cytosine deaminase to induce tumor killing. As such, five days after patients the NSCs were infused, the oral prodrug 5-fluorocytosine was given for seven days. The results of the study concluded that NSC therapy for GBM was safe and resulted in local production of chemotherapy in the brain (53). Currently, two other phase I trials are underway investigating safety of NSCs expressing an oncolytic virus (NCT03072134) and prodrug enzymes (NCT02192359). In these trials, NSCs were injected into the wall of the cavity in a cell suspension at the time of GBM surgical resection. However, pre-clinical studies have found that SCs have a limited lifespan in the brain after direct injection into the GBM surgical resection cavity compared to infusion into the intact brain parenchyma (36-38).

1.1.3 Animal Models of Glioblastoma

Many GBM therapies are commonly tested in subcutaneous models or non-resection orthotopic (intracranial) models, where either xenograft or allograft GBM tumors are established in mice or rats. Subcutaneous models allow the tumor to establish underneath the skin after injecting the tumor cell suspension subcutaneously in the flank. Orthotopic models of GBM without surgical resection are usually established by infusing a tumor cell suspension into the brain parenchyma. While a subcutaneous model often can be more easily accessed and monitored than an orthotopic tumors, the advantage of testing GBM therapies with tumors established in the brain
over the subcutaneous space is clear. The brain is the organ in which GBM develops and has tissue properties unique from other organs.

The standard of care for GBM includes surgical resection to remove the bulk of the tumor. This relieves neurological symptoms due to intracranial pressure, provides tissue samples for histologic diagnosis and molecular studies, and has been linked to both improved overall and progression-free GBM patient survival (54-56). However, there are mechanical and immunological changes in the brain resulting from GBM surgical resection which are certain to play a role in the outcome of tumoricidal SC therapy (57). Studies have shown that tumor resection greatly affects the tumor microenvironment, finding that recurrent GBM is more aggressive with increased infiltration, vasculature, and growth (58-60). Resection also results in increased fluid production that can influence the transport of locally delivered small molecule drugs, activate resident immune cells and recruit leukocytes to the brain (61-63). Testing in animal models that capture the complexity of the current standard of care are crucial in translating emerging GBM therapies to the clinic. For this reason, recent studies have begun exploring tumoricidal SC therapy in orthotopic models of surgical resection and recurrence.

Several models of orthotopic GBM resection and recurrence have been developed (5, 38, 64-68). To the best of our knowledge, Emerich et al. were the first to report develop a surgical resection model of orthotopic GBM in 2000 (64). Orthotopic tumors were established by injecting rat glioma cells in the right frontal lobe and were later surgically removed by aspiration (64). Bello et al. closely followed by establishing orthotopic xenografts using human GBM cells in nude mice (65). In 2009, Akbar et al., implanted GBM tumors expressing the fluorescent reporter, green fluorescent protein (GFP), to help guide surgical resection of the tumor using a fluorescent dissecting microscope (69). Later, both Denbo et al. (5) and Kauer et al. (38) further developed
this model to include real-time quantitative bioluminescent imaging (BLI) of tumor recurrence by implanting firefly luciferase (Fluc) expressing GBM cells. Hingtgen et al. validated the use of fluorescent guidance for resection and BLI of orthotopic GBM against 5-aminolevulinic acid (5-ALA) and magnetic resonance imaging (MRI), two methods currently used clinically (66). They found that fluorescent images captured throughout the procedure correlate tightly with the 5-ALA tumor marker and the percent of tumor resected was consistent between BLI and MRI measurements, reinforcing the clinical relevance of using fluorescent and bioluminescent measure in GBM surgical models (66). In 2017, Bianco et al. developed a protocol that did not require the specialized fluorescent microscope for the fluorescence guided resection and instead utilized a biopsy punch to generate positive tumor margins and a standardized cavity for implantation of experimental interstitial therapies (67, 68). A JOVE protocol was recently published providing a detailed list of materials and step by step instructions for fluorescence guided orthotopic tumor resection, implantation of therapeutic SC loaded scaffolds into the resection cavity, and post-operative imaging of tumor recurrence by BLI (70). It is notable that many of these models have utilized immunocompromised (nude/SCID) mice or rats, which allows for testing against human GBM lines (5, 38, 65-68, 71). However, immunocompetent models have also been explored to allow for testing in the presence of a fully active immune system (60, 64, 71). Importantly, these models of partial GBM resection and subsequent tumor recurrence mentioned above recapitulate multiple aspects of disease progression observed in the clinic, allowing emerging local therapies to be evaluated in a more clinically relevant model.

1.1.4 Role of Biopolymeric Scaffolds in Tumoricidal Stem Cell Therapy

The success of tumoricidal SC therapy depends on the viability and persistence of the cells in the brain after administration, in order to allow them to carry out their tumoricidal function. In
pre-clinical models, direct injection of SCs into the walls of the resection cavity resulted in a reduction of SC persistence in the brain compared to an intratumoral injection without resection (38). However surgical resection remains the standard of care for GBM, so lack of tumor resection is not an option. Implantation of tumoricidal SCs supported by a polymeric biomaterial scaffold into the surgical resection cavity has been found to improve SC persistence compared to a direct injection of SCs in pre-clinical models of GBM and overall therapeutic efficacy was improved as a result (36-38). Moreover, the use of biomaterial scaffolds to administer SCs improved survival of SCs and therapeutic outcomes for a number of different regenerative medicine applications in the CNS, such as traumatic brain injury (TBI), stroke, and spinal cord injury (SCI) (72-76).

Recently, specific scaffold properties have begun to be investigated for their impact on SC persistence in the brain after implantation (39, 40). It has been hypothesized that scaffolds enhance SC persistence by providing a substrate for attachment during implantation, preventing mechanical wash-out from the resection cavity by cerebrospinal fluid and blood, thereby increasing the number of SCs delivered in the resection cavity (37, 40, 77). Others have found that scaffolds that enhance SC growth in vitro also extend SC persistence in the brain, suggesting that the scaffold provides a supportive environment in the brain for the cells (39). However, the influence of other scaffold properties on tumoricidal SCs in the brain remain to be determined.

In this chapter, polymeric biomaterial scaffolds utilized for tumoricidal SC therapy will be discussed in two categories based on scaffold type: hydrogels and electrospun scaffolds. Proposed advantages and disadvantages based on scaffold type are outlined in Table 1. One class of polymeric biomaterial scaffolds used for tumoricidal SC therapy is hydrogels, which are employed by either encapsulating SCs within microcapsules or embedding them in bulk hydrogels. Microcapsules are spherical, micrometer sized hydrogel capsules that immobilize SCs and shield
On the other hand, with bulk hydrogels, SCs are embedded throughout the hydrogel and applied in the resection cavity as a singular system. Another class of polymeric biomaterial scaffolds explored for tumoricidal SC therapy is electrospun scaffolds, where tumoricidal SCs are seeded on the top-most layer of the thin fibrous mat. Electrospun scaffolds are administered by lining the walls of the resection cavity. Bulk hydrogels and electrospun scaffolds allow for SC migration out/off the scaffold towards remaining GBM cells beyond the resection cavity in response to chemotactic signals. This provides a means to killing more diffuse or distant GBM cells that escape surgical resection and conventional local drug delivery in the resection cavity (21). Figure 1 illustrates how tumoricidal SCs are delivered by these different polymeric biomaterial scaffolds in an orthotopic murine model of GBM resection and recurrence, compared to the clinical standard, direct injection.

A number of different polymeric biomaterial scaffolds have been explored to enhance therapeutic efficacy of tumoricidal SCs for GBM therapy and are summarized in Table 2 (33-40). Both natural and synthetic polymeric biomaterials have been explored to fabricate scaffolds for tumoricidal SC delivery. Among the hydrogels that have been published for tumoricidal SC therapy only naturally derived biopolymers have been explored. These include collagen (37), gelatin (hydrolyzed collagen) (38, 39), hyaluronic acid (HA) (38), fibrin (35), and alginate (33, 34). Naturally derived biopolymers are advantageous for cell therapies because they can mimic endogenous tissue and can be biocompatible (79). Among these naturally derived polymers are components of the extracellular matrix (ECM), such as collagen, gelatin, and HA, which contain ligands that favor cell attachment. Collagen is a the most common component of the ECM and has a fibrous, helical protein structure (80). Both collagen and its less immunogenic hydrolyzed form,
gelatin, are well-characterized and have been used in a wide variety of biomaterial scaffolds for other applications (81). HA is a linear polysaccharide and a prominent component of the brain ECM. (82, 83) Fibrin has also been explored to deliver tumoricidal SCs, likely due to its natural occurrence in the body and its ability to polymerize into a fibrous network upon fibrinogen cleavage by thrombin at sites of injury during coagulation (84). For microcapsule formation, alginate is exclusively used because it is well-characterized, nontoxic, inexpensive, and highly compatible with the microenvironment of the brain (85). Alginate is a naturally occurring anionic polymer typically derived from brown algae, composed of β-D-mannuronic acid and α-L-guluronic acid monomers, and easily complexes with divalent cations (85, 86). Synthetic polymers such as poly(L-lactic acid) (PLA) (36, 39) and acetalated dextran (Ace-DEX) (40) have been used to create electrospun scaffolds for tumoricidal SC therapy. Use of a biodegradable, synthetic polymer is advantageous because it allows for tight control over chemical composition (87). Gelatin has also been combined with synthetic polymers to enhance tumoricidal SC loading onto electrospun scaffolds (39, 40).

1.2 Hydrogels

Hydrogels are characterized as 3D networks composed of hydrophilic polymers that are capable of holding a substantial amount of water, lending to their name (88). Macromolecules are able to diffuse throughout hydrogels due to their swelling properties (88). Because hydrogels can closely resemble natural tissue, they are favorable scaffolds for sustaining cell viability (88) and as such they have been used for cell delivery devices for a variety of applications in the CNS, such as tissue regeneration and repair after TBI, stroke, and SCI (72, 74, 89, 90). They have also been applied at the site of CNS injury in the absence of any additional therapeutic agent to reduce
inflammation and regenerate tissue (89). For GBM and other therapies, hydrogels have also been employed for local drug delivery of small molecules (69).

Hydrogels can be formed by chemical or physical crosslinking of polymers through a variety of methods (Figure 2A). Chemical crosslinking forms covalent bonds whereas physical crosslinking results in non-covalent interactions between the polymer chains including ionic and hydrophobic interactions, and hydrogen bonding (Figure 2A-B) (91). SCs can be loaded during crosslinking or after, depending on the severity of crosslinking conditions to maintain cell viability (Figure 2B). Importantly, hydrogels can be fabricated to be either permissible to cell migration or not. As such, there are two categories of hydrogels that have been explored for tumoricidal SC therapy: bulk hydrogels and microcapsules. In the case of bulk hydrogels, hydrogels are often fabricated to allow for release of their cargo at the site of implantation. For tumoricidal SC therapy, this is advantageous because tumor-homing SCs are permitted to migrate out of the scaffold and into the surrounding brain parenchyma in response to GBM chemotactic signaling (35, 37, 38). Yet hydrogels can also be formed into micrometer sized, spherical hydrogel particles called microcapsules which immobilize cells by creating a physical barrier that serves to protect the cells from hostile extrinsic factors. Immobilized cells within microcapsules are able to survive due to exchange of nutrients and waste through the biocompatible, semipermeable membrane (92). By encapsulating genetically engineered SCs in microcapsules, SCs are able to produce therapeutic agents locally for extended periods of time (78).

1.2.1 Bulk Hydrogels for Tumoricidal Stem Cell Therapy

Bulk hydrogels have been shown to enhance persistence of SCs in the brain for other tissue engineering applications in the CNS (73-75, 93). One major advantage to bulk hydrogels is their high cell loading capacity, attributed to their 3D nature. Depending on the inertness of crosslinking
method, cells can be distributed throughout the hydrogel components prior to crosslinking (94). Hydrogels are highly tunable with respect to degradation, stiffness, porosity and response to stimuli (e.g. temperature), which can be varied through their material composition and crosslinking (79). Hydrogel degradation can be tuned to control the release of SCs over time (95), which could provide greater control over tumoricidal SC migration and ultimate GBM killing. Furthermore, some hydrogels are thermosensitive, meaning that they transition from solution to gel under physiological temperature, so that the resulting hydrogel can coat irregular spaces such as the GBM surgical resection cavity (79).

To our knowledge, Hansen et al. was the first reported delivery of migratory SCs into the GBM resection cavity using a polymeric biomaterial scaffold composed of collagen for tumoricidal SC therapy (37). Here they compared the direct injection of a cell suspension to SCs embedded in a 3D collagen scaffold mimetic of the ECM (37). They found that the scaffolds enhanced implantation efficiency of murine NSCs into the resection cavity of immunocompetent orthotopic GBM-bearing mice compared to direct injection. The NSCs were still able to exit the scaffold and migrate towards the tumor in the contralateral hemisphere. One week after implantation, a significantly higher number of NSCs were distributed throughout the recurrent tumor in the hydrogel group compared to directly injected group (37). The hydrogel was able to efficiently deliver SCs to the resection cavity and allowed SCs to maintain their tumor-tropism.

Kauer et al. used a commercially available hydrogel (HyStem-C, Advanced BioMatrix) composed of HA and gelatin as a synthetic ECM scaffold to implant therapeutic mouse NSCs into the GBM resection cavity in nude mice (Figure 3) (38). The hydrogel was fabricated by crosslinking thiol-modified HA and thiol-modified gelatin with a thiol-reactive crosslinker, polyethylene glycol diacrylate. NSCs were mixed in with the hydrogel components and gelation
took place over 20 minutes. Monitoring NSCs fate by BLI, they found that hydrogel supported NSCs had significantly increased retention immediately following implantation in the surgical resection cavity. The hydrogel extended NSC viability in vivo compared to direct injection of a cell suspension by at least two weeks (Figure 3A). In an orthotopic model of GBM resection, hydrogels loaded with NSCs secreting the tumoricidal protein, tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) extended survival in 100% of the mice up to day 63, whereas all mice administered with NSCs in suspension succumbed to tumor burden by day 42 (Figure 3B) (38).

Other studies evaluating human MSCs loaded with oncolytic viruses have reported extended murine survival and enhanced intratumoral distribution of oncolytic virus using the same hydrogel (HyStem-C) compared to direct viral injection into the GBM resection cavity as well (96, 97). Kauer et al. hypothesized that the benefit afforded by the hydrogel was due to decreased programmed cell death, mechanical wash-out by cerebrospinal fluid, as well as the hydrogel shielding the cells from the immune system. These claims are supported in part by studies in adjacent fields of regenerative medicine in which hydrogels are used to enhance SC therapies in the injured CNS (73-75, 93).

Fibrin hydrogels have also been explored for delivering human MSCs for tumoricidal SC therapy. Bagó et al. embedded MSCs into a commercially available fibrin sealant (TISSEEL, Baxter) used for hemostasis in the clinic. TISSEEL is composed of fibrinogen and thrombin derived from human plasma suspended in solutions of calcium chloride and aprotinin, a fibrinolysis inhibitor included to slow the breakdown of fibrin (98). Rapid gelation time, ability to be molded into different geometries, and hemostatic properties are proposed as advantages of this platform over a hydrogel composed of HA and gelatin utilized in previous studies for tumoricidal
SC therapy (35). Bagó et al. found that the hydrogel increased the initial retention and prolonged the persistence of the MSCs in the GBM resection cavity (35). These hydrogels remained permissive to tumor-tropic migration in vitro, where MSCs had an increased displacement in the direction GBM cells. MSCs expressing TRAIL combined with the fibrin scaffold delayed postsurgical GBM recurrence and prolonged survival in mice compared to non-therapeutic MSCs (35).

Sheets et al. tested a gelatin hydrogel that is clinically approved as a hemostatic agent (39). Scanning electron micrographs show high porosity, allowing for NSC attachment. Here, the same human NSC line previously used in clinical trials for tumoricidal SC therapy, HB1.F3, was used. Importantly, NSCs seeded onto the gelatin hydrogels after 10 days in vitro were still found to express nestin, a SC marker, indicating that they had not differentiated. NSCs implanted in the GBM resection cavity co-localized with GBM cells 1.5 mm from the resection cavity 11 days after scaffold implantation. NSCs expressing enzyme thymidine kinase administered into the GBM resection cavity extended median survival 15 days when the prodrug, ganciclovir, was administered (39). Taken together, these studies illustrate that bulk biomaterial hydrogels can enhance implantation of SCs while remaining permissive to chemotactic migration towards tumors in the brain, highlighting their potential for enhancing tumoricidal SC therapy for GBM.

1.2.2 Alginate Hydrogel Microcapsules for Tumoricidal Stem Cell Therapy

The most commonly used polymeric biomaterial for microencapsulation is alginate. Microcapsules are formed by suspending cells within a sodium alginate solution, and adding them dropwise into a salt solution containing divalent cations which crosslinks the anionic alginate into hydrogel microcapsules containing the cells (Figure 4) (92, 99). Alginate can be fabricated into hydrogels by crosslinking with divalent cations, Ca$^{2+}$ or Ba$^{2+}$, generating a 3D network that allows
for cell delivery and controlled release of therapeutic agents such as small molecule drugs and proteins (85).

Unlike bulk hydrogels, encapsulation of SCs into microcapsules typically immobilizes SC within the hydrogel. Here, microcapsules serve to insulate SCs from interaction with the host immune system, extending persistence in the brain and allowing for sustained local delivery of therapeutics to treat GBM (78). Human embryonic kidney (HEK 293) cells encapsulated in alginate microcapsules have been reported to survive for 4 months after implantation into the brain of immunocompetent rats (24). Considering that almost all of the drug, carmustine, has been released from the Gliadel wafer approximately 5 days after implantation into the brain, this could be a significant improvement for local drug delivery to GBM over polymeric implants (14).

There have been two studies investigating alginate encapsulation of therapeutic SCs for GBM therapy thus far. First, Kleinschmidt et al. explored the therapeutic effect of implanting alginate microencapsulated human MSCs producing endostatin directly into the orthotopic GBM tumor bed of immunocompetent rats (34). Endostatin is an angiogenesis inhibitor and is rapidly cleared from the blood, limiting its use as a systemic chemotherapeutic. Sustained local delivery of endostatin by encapsulated MSCs reduced tumor vasculature area 8.48-fold. This led to a reduction in tumor volume, as measured by histology, however it was not statistically significant compared to no treatment. Goren et al. investigated the use of human MSCs to deliver a different anti-angiogenesis protein, hemopexin-like protein (Figure 5) (33). They compared encapsulation of human MSCs to well-established cell lines: mouse NIH 3T3s and human HEK 293s. MSCs encapsulated in alginate microcapsules coated in poly-L-lysine remained viable at or above 100% viability in vitro for > 70 days, compared to the NIH 3T3s which decreased in viability over the first 28 days, and then oscillated around 50% viability thereafter (Figure 5A-B). Furthermore,
MSCs were found to have around 3x less inflammatory cytokine expression in the inguinal lymph nodes than the HEK 293 cells after subcutaneous implantation in immunocompetent mice. While fibrotic capsular tissue was found surrounding the encapsulated HEK 293 cells four weeks after implantation (indicative of a foreign body response), the human MSC containing microcapsules had a limited cellular density surrounding it (Figure 5C) (33). Authors attributed this to the relatively low inherent immunogenicity of the MSCs. Encapsulated hemopexin-like protein producing MSCs injected adjacent to subcutaneous GBM human xenografts were found to control tumor growth over three weeks. A significant reduction in tumor vascularization and increase in tumor apoptosis was reported compared to non-therapeutic encapsulated MSCs and empty capsules. Overall, these studies highlight the advantages of using microencapsulation for delivery of SC for GBM therapy.

1.3 Electrospun Scaffolds

Electrospinning is a scalable scaffold fabrication process that generates a non-woven fibrous mat (100). A fiber jet forms when high voltage is applied to a droplet of polymer-solvent solution, breaking the surface tension. As the fiber jet travels to a grounded collecting plate, the solvent evaporates, resulting in dry fibers that range from nanometers to micrometers in diameter (100). The electrospinning set up and a representative scanning electron micrograph of electrospun microfibers are illustrated in Figure 6A. The topography of electrospun scaffolds can mimic ECM and as such can enhance cell attachment. Similar to hydrogels, electrospun scaffolds have been used to deliver small molecules and cells to the CNS for repair and GBM therapy (101, 102).

There are several benefits to using electrospun scaffolds to deliver tumoricidal SCs for GBM therapy. First, electrospinning is a very versatile method that can generate scaffolds of different structural properties such as fiber diameter, and fiber alignment by changing process
parameters such as flow rate, solvent, and collection method (100). Additionally, synthetic and natural polymers can be used for electrospinning, offering a wide range of chemical and mechanical properties of the resulting scaffolds. These properties have been shown to influence SC adhesion, migration, and differentiation (103-107). One of the most compelling advantages for using electrospun scaffolds over other scaffold types is their effectively 2D quality on the macroscopic level. These scaffolds remain flexible enough to contour the surgical resection cavity, but their minimal volume reduces risk of increasing intracranial pressure after surgery, known as mass effects (36). Lastly, SCs seeded onto scaffolds primarily remain on the top layer of the scaffold due to limited porosity (Figure 6B) (108), and as a result SC loaded electrospun scaffolds implanted in the resection cavity are in direct contact with the brain parenchyma. This could allow for more rapid and direct migration off the scaffold in response to distant GBM foci.

1.3.1 Electrospun Scaffolds for Tumoricidal Stem Cell Therapy

Bagó et al. found that electrospun PLA scaffolds increased both the initial retention and long-term persistence of human MSCs in the resection cavity (36). The electrospun PLA scaffolds reported by Bagó et al. had a nanometer range fiber diameter. Their studies found that MSCs were able to migrate off the scaffold towards GBM cells in vitro. Persistence studies indicate that PLA scaffolds allowed for successful implantation of MSC into the resection cavity. Additionally, the scaffold significantly improved MSC persistence compared to direct injection. PLA scaffolds loaded with TRAIL expressing MSCs caused regression of solid GBM xenografts and also suppressed orthotopic post-surgical GBM recurrence, while extending overall survival compared to PLA scaffolds bearing non-therapeutic MSCs (36).

The effect of electrospun PLA scaffold parameters on human NSC persistence in the resection cavity were evaluated by Sheets et al. (39). HB1.F3 human NSCs were used to evaluate
an electrospun PLA scaffold with nanometer range fiber diameter similar to one used by Bagó et al. (36). Human NSCs were stained for the NSC marker, nestin, one week after seeding on PLA scaffolds in vitro and were still found to express nestin. These results suggest that NSC differentiation was not induced by the scaffold within this timeframe (39). Furthermore, human NSC persistence in the resection cavity was enhanced when seeded onto the nanofiber PLA scaffold compared to a direct injection. While 95% of human NSCs were cleared on day 3 when administered by direct injection, 95% of the NSCs seeded onto electrospun PLA scaffolds remained until day 8.

The study went further to modify PLA scaffold parameters to examine the impact of scaffold surface coating (without and with collagen), fiber diameter (nanometer vs micrometer), and morphology (2D vs 3D stacks) on human NSC persistence in vivo (39). Surface coating was assessed by incubating scaffolds in collagen for two hours prior to NSC seeding. Micrometer diameter fiber scaffolds were generated by meltblowing, a process by which the polymer is heated to a molten state instead of dissolved in a solvent and spun at a high velocity (109). 2D vs 3D morphology was assessed by comparing one versus three stacked scaffolds (39). Interestingly, none of these parameters resulted in significant extension of human NSC persistence in the resection cavity compared to the original nanofiber PLA scaffold. This work is an important first step into investigating the impact of scaffold parameters on tumoricidal SC therapy.

In the same study, electrospun PLA scaffolds were also compared to the gelatin hydrogel mentioned above (39). While human NSCs were found to remain viable on PLA scaffolds in vitro, they had a limited ability to proliferate on the scaffolds compared to the gelatin hydrogels. NSCs seeded on gelatin hydrogels had a 5-fold higher bioluminescent signal from NSCs compared to NSCs on electrospun PLA scaffolds 7 days after seeding in vitro. Moreover, the hydrogel had a
significantly longer persistence in vivo compared to the electrospun PLA scaffolds. The 3D nature of the hydrogels may be more permissive to growth due to enhanced surface area, compared to the primarily 2D electrospun scaffold morphology. Taken together, the enhanced persistence afforded by the gelatin hydrogel may be attributed the greater capacity to support NSC growth and increased NSC delivery into the resection cavity compared to electrospun scaffolds (39). This suggests that the ability of the SCs to grow on the scaffold or the number of SCs implanted in the brain influence their persistence.

1.4 Conclusions

Tumoricidal SC therapy has the potential to overcome some of the major challenges associated with current local and systemic chemotherapy strategies for GBM. Delivering therapeutic cells locally can bypass the BBB and provide high concentrations of tumoricidal agents locally to the site of the recurrent tumor. Furthermore, tumoricidal SCs can seek out more distant tumor cells while sparing healthy tissue. Herein we reviewed the use of polymeric biomaterial scaffolds made of hydrogels and electrospun scaffolds to increase implantation efficiency and persistence of SCs in the brain. Additionally, we gave an overview of clinically relevant orthotopic models of GBM surgical resection.

Polymeric biomaterial scaffolds have been found to enhance the persistence of tumoricidal SCs in brain after implantation in the GBM surgical resection cavity, providing a therapeutic benefit. One major observation is that scaffolds enhance SC persistence by increasing implant efficiency into the resection cavity. Recently, groups have begun to investigate the influence of specific scaffold properties on SC persistence. These preliminary studies suggest that scaffolds with a high capacity for SC proliferation (such as high surface area scaffolds) may support SC growth in the brain, extending persistence. Furthermore, an early study suggests that electrospun
scaffold degradation rate does not substantially influence NSC persistence in the brain. While the therapeutic advantage of using polymeric biomaterial scaffolds to deliver tumoricidal SCs in the surgical resection cavity is clear, continued investigation into the optimal scaffold parameters could greatly improve outcomes to optimize this therapy.
Table 1 Advantages and Disadvantages of Different Scaffold Types Used for Tumoricidal Stem Cell Therapy

SC: stem cell.

<table>
<thead>
<tr>
<th>Scaffold Type</th>
<th>Advantages</th>
<th>Disadvantages</th>
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<tbody>
<tr>
<td>Bulk hydrogels</td>
<td>• High SC loading capacity</td>
<td>• Increased intracranial pressure (mass effects) due to cavity filling</td>
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<tr>
<td></td>
<td>• Embedded SCs are more shielded from hostile resection microenvironment</td>
<td>• Potentially toxic degradation byproducts from chemical crosslinkers</td>
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<tr>
<td>Microcapsule hydrogels</td>
<td>• Long-term SC persistence as a result of shielding from immune system</td>
<td>• SC immobilization prevents utilization of tumor tropic migration; limited by</td>
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<td></td>
<td>• Potential for use of universal SC line</td>
<td>tumoricidal agent diffusion</td>
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<tr>
<td>Electrospun scaffolds</td>
<td>• Thin scaffold limits intracranial pressure</td>
<td>• Low SC loading capacity</td>
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<tr>
<td></td>
<td>• Contour resection cavity providing SCs with direct access to brain</td>
<td>• Surface seeding exposes SCs to hostile resection microenvironment</td>
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Table 2 Biopolymeric Scaffolds for Tumoricidal Stem Cell Therapy for Glioblastoma

SC: stem cell; PLL: poly-L-lysine; HA: hyaluronic acid; PLA: poly (L-lactic acid); NSC: neural stem cell; MSC: mesenchymal stem cell; TRAIL: tumor necrosis factor (TNF)-related apoptosis-inducing ligand; DI: direct inject; BLI: bioluminescent imaging; NT: no treatment; MST: median survival time.

<table>
<thead>
<tr>
<th>Scaffold-SC Therapy</th>
<th>Animal Model</th>
<th>SC Persistence</th>
<th>Therapeutic Efficacy</th>
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<td><strong>SC</strong></td>
<td><strong>Therapy</strong></td>
<td><strong>Animal</strong></td>
</tr>
<tr>
<td>Collagen hydrogel Mouse NSC N/A</td>
<td>GBM Line GL261</td>
<td>Scaffold: &gt; 35</td>
<td>Orthotopic with resection</td>
</tr>
<tr>
<td>HA and gelatin hydrogel Mouse NSC TRAIL</td>
<td>C57BL/6 mice</td>
<td>Scaffold: &gt; 28</td>
<td>Orthotopic with resection</td>
</tr>
<tr>
<td>Fibrin hydrogel Human MSC TRAIL</td>
<td>U87 Nude mice</td>
<td>Scaffold: &gt; 28</td>
<td>Orthotopic with resection</td>
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<tr>
<td>Gelatin hydrogel</td>
<td>Human NSC</td>
<td>Thymidine kinase</td>
<td>GBM8</td>
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<tr>
<td>Alginate-PLL hydrogel microcapsule</td>
<td>Human MSC</td>
<td>Hemopexin-like protein</td>
<td>U87</td>
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<tr>
<td>Alginate hydrogel microcapsule</td>
<td>Human MSC</td>
<td>Endostatin</td>
<td>BT4Ca</td>
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<tr>
<td>PLA electrospun scaffold</td>
<td>Human MSC</td>
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<td>Mouse NSC</td>
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</table>
**Figure 1 Schematic of Tumoricidal Stem Cell Delivery Methods in an Orthotopic Model of GBM Resection and Recurrence**

(A) GBM cells are implanted in the right frontal lobe of mice. After a solid tumor is established over time, the bulk of the tumor is surgically resected, leaving behind positive tumor margins. (B) Tumoricidal SCs are implanted into the resection cavity by either direct injection of the cell suspension or seeded onto polymeric biomaterial scaffolds. Left to right: hydrogel, microcapsules, and electrospun scaffold. Figure created using BioRender.
Figure 2 Schematic of Types of Hydrogels and Common Methods of Stem Cell Seeding

(A) Chemical crosslinking typically involves generating covalent bonds by introducing initiators, or heat to a polymeric solution; physical crosslinking results in non-covalent, transient interaction within or between polymer chains that can result from ionic bonds, hydrophobic interactions, or hydrogen bonding. (B) Stem cells can be encapsulated during or after the crosslinking process. Figure created using BioRender.
Figure 3 Example of Bulk Hydrogel Used to Enhance Tumoricidal Stem Cell Therapy

(A) Normalized BLI signal over time for Fluc expressing mNSCs implanted in the resection cavity either in suspension or the hydrogel scaffold (sECM). Dashed lines: Representative BLI on day 7 after implantation (*P < 0.05 versus mNSCs in hydrogel scaffold). (B) Kaplan-Meier survival curves of bearing orthotopic human U87 GBM tumors. mNSCs expressing either TRAIL (therapy) or Rluc (control) implanted in the resection cavity either in suspension or in the hydrogel scaffold. P < 0.05, resected + mNSC-S-TRAIL cells in sECM versus resected + mNSC-S-TRAIL cells in suspension. Data are mean ± SEM. mNSC: murine neural stem cell; sECM: synthetic extracellular matrix (i.e. hyaluronic acid and gelatin hydrogel, HyStem-C); Fluc: firefly luciferase; Rluc: renilla luciferase; TRAIL: tumor necrosis factor (TNF)-related apoptosis inducing factor; SEM: standard error of the mean. Adapted with permission from ref 38. Copyright 2011 Springer Nature.
Figure 4 Schematic of Stem Cell Encapsulation within Alginate Microcapsules

Stem cells in sodium alginate solution loaded into a syringe pump are added dropwise into a salt solution containing divalent cations. Alginate chains are ionically complexed together by the cations, forming stem cell loaded microcapsules within the droplets (purple lines: alginate; red dots: divalent cation). Figure created using BioRender.
Figure 5 Example of Alginate Microcapsules Used to Enhance Tumoricidal Stem Cell Therapy

(A) Bright-field images (top panels) and fluorescence images (bottom panels) of alginate microcapsules containing hMSCs 28 and 70 days after encapsulation, where viable cells are fluorescent. Scale bars are 100 μm. (B) Cell viability of hMSCs and NIH 3T3 cells encapsulated in microcapsules in vitro. (C) Quantification of fibrotic cell density surrounding microcapsules containing hMSCs or HEK 293 cells determined by H&E staining after 1, 2, 4, and 8 weeks after subcutaneous implantation in mice. Data represented as mean ± standard deviation. hMSC: human mesenchymal stem cells; PEX: hemopexin-like protein. Adapted with permission from ref 33. Copyright 2009 John Wiley & Sons.
Figure 6 Schematic of Electrospun Scaffold Fabrication and Stem Cell Seeding

(A) Polymer dissolved in a volatile solvent is loaded onto a syringe pump. High voltage is applied at the tip of the syringe needle and a metal collection plate. A fiber jet is formed as the polymer solution is expelled from the needle, and solvent evaporates as the fiber travels to the collection plate, forming a non-woven fibrous mat. Representative scanning electron micrograph of an electrospun acetalated-dextran scaffold. (B) Stem cell seeding results in cell attachment primarily on the top layer of electrospun scaffolds. Figure created using BioRender.
CHAPTER 2: IMPACT OF COMPOSITE SCAFFOLD DEGRADATION RATE ON NEURAL STEM CELL PERSISTENCE IN GliOBLASTOMA RESECTION CAVITY

2.1 Introduction

Glioblastoma (GBM) is the most common primary brain tumor and is classified by the World Health Organization (WHO) as Grade IV, the most malignant type of glioma (110). The current standard of care (surgical resection, oral chemotherapy with temozolomide, and radiation) typically fails to eliminate the tumor, leading to a dismal median patient survival of 15-18 months (2). There are several contributing factors that limit the efficacy of current GBM therapies, including high tumor infiltration into surrounding tissue, poor drug diffusion in and across the brain, and drug resistance. Moreover, GBM is diffuse and presents invasive projections that prevent gross total surgical resection. While small molecule drugs are limited by various barriers such as short diffusion distances in the tissue and across the blood-brain barrier, stem cells (SCs) may provide therapeutic benefit because they have been shown to migrate throughout the brain and seek out GBM cells in response to chemotactic signals (20, 111). As an emerging strategy to combat GBM, tumoricidal SC therapy employs genetically engineered cells to produce anti-tumor agents so they may seek out and kill remaining tumor cells after resection. SCs can be readily engineered to produce a wide array of therapeutics including tumoricidal proteins, prodrug enzymes, viral vectors, and immunomodulatory molecules (21, 112, 113). Phase I clinical trials

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have established the safety of injecting tumoricidal SCs in the brain, and Phase II clinical trials are currently underway (53).

Despite their promise, there are considerable challenges remaining for tumoricidal SC therapy for GBM; chiefly, effective and stable implantation of SCs into the surgical resection cavity. Preclinical studies have shown that SCs injected into the GBM surgical resection cavity are cleared rapidly from the brain between 4 and 14 days, whereas SCs seeded on a hyaluronic acid scaffold persist in the brain greater than 28 days (38). The increased SC persistence in the brain correlated with enhanced tumor killing and overall survival in mice (38). The benefit of scaffolds for enhancing persistence of SC after implantation in the resection cavity has been demonstrated using a number of other scaffold types, including those composed of hyaluronic acid (HA), fibrin hydrogels, electrospun poly(L-lactic) acid (PLA), and alginate gels (35, 36, 38, 77, 114, 115).

While these results show potential, these prior studies did not characterize the degradation profiles of the scaffolds and as a result, the role of scaffold degradation rate on persistence has yet to be elucidated. This study is aimed at determining the effect of the scaffold degradation profile on neural stem cell (NSC) persistence in the brain, utilizing the novel biomaterial, acetalated dextran (Ace-DEX). Ace-DEX has been used because of its tunable degradation properties to rationally design drug delivery platforms for a variety of therapies (102, 116-118). By simply changing reaction time, the ratio of cyclic to acyclic acetal coverage (CAC) is altered, which controls the polymer degradation rate that can range from hours to months (119). Ace-DEX is easily fabricated into scaffolds by electrospinning, a scalable process that generates flexible, fibrous scaffolds (118). We have previously demonstrated the safety of implanting drug loaded and blank electrospun Ace-DEX scaffolds in the brain (102). Using Ace-DEX, fast and slow
degrading electrospun scaffolds were fabricated, characterized, and evaluated in vitro and in vivo for their impact on NSC persistence in the brain.

2.2 Materials and Methods

All materials were purchased from Sigma (St. Louis, MO) and used as purchased, unless otherwise indicated.

2.2.1 Acetalated Dextran (Ace-DEX) Synthesis

Ace-DEX was synthesized as previously described (120). Briefly, lyophilized dextran and acid catalyst, pyridinium p-toluenesulfonate (>98%), were dissolved in anhydrous dimethyl sulfoxide (DMSO, >99.9%). Under anhydrous conditions, dextran (molecular weight 450-650 kDa) was reacted with 2-ethoxypropene (Matrix Scientific, Columbia, SC) in DMSO for ~16 minutes or 2 hours to generate pendant acetal groups. The reaction was quenched by the addition of triethylamine (TEA, >99%) and then the mixture was precipitated into basic water and lyophilized. Impurities were removed by dissolving the polymer in ethanol, centrifuging, and precipitating into basic water (0.04% TEA in water). The resulting polymer, Ace-DEX, was lyophilized and stored in -20°C until further use. Nuclear magnetic resonance (NMR, Varian Inova 400) was used to characterize the relative cyclic acetal coverage (CAC) as previously described (120).

2.2.2 Ace-DEX Gelatin Scaffold Fabrication

Ace-DEX (75% wt/wt) and gelatin (25% wt/wt) were dissolved in a tri-solvent consisting of hexafluoroisopropanol (>99%), 1-butanol (≥99.4%), and TEA at a ratio of 89%, 10%, 1% v/v, respectively at a concentration of 150 mg/mL. The polymer solution was pumped out of a glass syringe with a 21-gauge needle at a rate of 2 mL per hour. A voltage bias of 15 kV was applied from the tip of the needle to a collection plate 13 cm apart.
Scaffolds were crosslinked by dehydrothermal (DHT) crosslinking by wrapping in aluminum foil and placing in a vacuum oven at 130°C under -67.7 kPa for 0-100 hours. Then, scaffolds were stored in a vacuum desiccator at room temperature. ICG-loaded Ace-DEX gelatin scaffolds were fabricated as described above, with 0.5 % wt/wt ICG (VWR, Randor, PA) added to the polymer solution.

2.2.3 Scaffold Materials Characterization

Scaffold morphology was evaluated by scanning electron microscopy (SEM). Scaffolds were mounted onto aluminum stubs using carbon tape. Samples were coated with palladium using a sputter coater and imaged at 2 kV on the Hitachi S-4700 Cold Cathode Field Emission Scanning Electron Microscope. Gelatin loading was determined by fluorescamine assay adapted from a protocol described by Lorenzen et al. using a gelatin standard curve (121). Non-crosslinked scaffolds were degraded at a concentration of 4 mg/mL in hydrochloric acid (0.1 N), then neutralized by dilution to 1 mg/mL in 0.1 M borate buffer. A fluorescamine assay was performed by combining fluorescamine solution (3 mg/mL in DMSO) with samples at a 1:4 ratio and reading fluorescence in a plate reader (excitation: 390nm, emission: 460 nm) and comparing to a standard curve.

2.2.4 In Vitro Degradation and Release Studies

Scaffold degradation was measured by mass loss, where pre-weighed scaffolds were placed in individual microcentrifuge tubes containing 1X phosphate buffered saline (PBS, pH 7.4) at 1.5 mg/mL. Tubes were then kept at 37°C on a shaker plate at 150 RPM. At specific time points, the supernatant was removed and stored at -20°C for analysis of gelatin release. To determine degradation, scaffolds were washed with basic water (to remove salts from PBS), lyophilized and re-weighed. Mass loss was determined by subtracting final mass from initial mass and normalizing
to the initial mass at time 0. The supernatant was tested for gelatin content by a fluorescamine assay (121).

ICG-loaded scaffolds were characterized similarly, where degradation was determined by mass loss at various time points. To determine ICG release, after re-weighing for mass loss, scaffolds were dissolved in DMSO and water (50%:50% v/v). ICG remaining in the scaffolds was evaluated by fluorescence in a plate reader (excitation:788 nm, emission:813 nm) and compared to a standard curve.

**2.2.5 ICG Scaffold Degradation In Vivo**

Female athymic nude mice (6-8 weeks of age, University of North Carolina at Chapel Hill Animal Studies Core) were used for all in vivo experiments. All experimental protocols were approved by the Animal Care and Use Committees at The University of North Carolina at Chapel Hill.

Scaffold degradation in vivo was investigated using a surgical model of resection (36, 122). Mice were anesthetized by vapor isoflurane and secured on a three-point stereotaxic apparatus (Stoelting, Kiel, WA). A small circular window in the skull was made using a bone drill (Ideal Microdrill, Harvard Apparatus, Holliston, MA), exposing the right frontal lobe of the brain. A surgical resection cavity approximately 1 mm deep was made by removing a small portion of brain by aspiration. Once bleeding was controlled with the hemostatic agent, Surgicel (Ethicon, Somerville, NJ) and rinsed with cold saline, a single 3 mm ICG-loaded scaffold was implanted (fast, n = 2; slow, n = 2) and the incision was then closed with Vetbond Tissue Adhesive (3M, Maplewood, MN). For pain management, meloxicam (5 mg/kg) was administered subcutaneously prior to surgery and then daily for the next 3 days during recovery. Fluorescence from ICG was measured non-invasively in the brain over time using the Perkin Elmer IVIS Lumina In Vivo
Imaging System (Waltham, MA) at excitation 710-760 nm and emission 810-875 nm with 1-10 second exposure times. ICG remaining was quantified by normalizing total flux (ρ/s) from each mouse to the total flux from day 0.

2.2.6 Cell Lines

Immortalized C17.2 NSCs were used for initial in vitro cell viability experiments. C17.2s were cultured in Dulbecco's Modified Eagle Medium (DMEM, Corning, Corning, NY) supplemented with 1% penicillin-streptomycin (Hyclone, Pittsburg, PA) and 10% fetal bovine serum (Corning). Primary cortical NSCs derived from CD-1 mice (R&D Systems, Minneapolis, MN) were utilized for in vitro GBM killing, TNF-related apoptosis-inducing ligand (TRAIL) output analysis, and in vivo experiments. Patient-derived GBM cell line, GBM8, was a kind gift from Dr. Hiroaki Wakimoto (Massachusetts General Hospital, Boston, MA). Primary NSCs and GBM8 cells were cultured in suspension in neurobasal media (Gibco Laboratories, Gaithersburg, MD) supplemented with 2% B-27 supplement (Gibco Laboratories), 1.5% L-glutamine (Gibco Laboratories), 0.5% N-2 supplement (Gibco Laboratories), 0.5% antibiotic-antimycotic (Gibco Laboratories), 1 mg heparin (Gibco Laboratories), 10 µg fibroblast growth factor (FGF, Gemini Bio-Products, West Sacramento, CA), and 10 µg epidermal growth factor (EGF, Gemini Bio-Products).

Primary NSCs were transduced by lentivirus encoding either mCherry-firefly luciferase (mCh-FLUC) or GFP-TNF-related apoptosis-inducing ligand (GFP-TRAIL) vectors (Invitrogen, Carisbad, CA). GFP-TRAIL NSCs were isolated by fluorescence-activated cell sorting (FACS). GBM8 cells were transduced with mCh-FLUC, and puromycin selected.
2.2.7 In Vitro NSC Seeding and Viability on Scaffolds

Prior to seeding, 96-well plates were coated with agar (1% wt/v) dissolved in 50% DMEM and 50% PBS. After the agar solidified, scaffolds (punched into 3 mm diameter circles) were placed in the center of the well to hydrate. Slow degrading scaffolds were allowed to hydrate on agar coating overnight, and fast degrading scaffolds required < 0.5 hours. Silicone inserts with a 3 mm inner diameter and 5 mm outer diameter were placed on top of each scaffold. Cells were funneled onto scaffolds in 30 µL and allowed to attach for 4 hours (Figure 7). Scaffolds were transferred to 0.5 mL media in a 24-well plate 4 hours after seeding. In order to get the non-adherent primary NSCs to adhere to the scaffolds, NSCs were first treated with 2% FBS to make them adherent for 24 hours in a tissue culture dish before seeding onto scaffolds.

C17.2 cell viability and proliferation were determined by a thiazolyl blue tetrazolium bromide (MTT) assay alongside a cell standard curve in a 24-well plate with 0.5 mL of media. Briefly, cells were incubated with MTT dissolved in media at 0.5 mg/mL for 3 hours. Media was then removed, and isopropanol (>99.5%) was added to dissolve formazan crystals. Absorbance was measured at 560 nm using 670 nm as a reference. Cell seeding efficiency was determined by normalizing number of cells on scaffold at 6 hours to number of cells seeded. Cell carrying capacity was calculated by quantifying cell number on scaffolds at 24 hours after seeding. Cell viability was measured at 6, 24, and 48 hours.

2.2.8 Quantification of TRAIL Output from TRAIL-NSCs on Scaffolds

Scaffolds were seeded with TRAIL-NSCs at a density of 2.5 x 10^5 per well for 4 hours, and then were transferred into a 96 well plate with 200 µL of media. At the same time, a range of cell densities were also seeded on TCPS in a 96-well plate with 200 µL of media. Twenty-four hours after seeding, the media was replaced with fresh and allowed to be conditioned by the cells
in the incubator for 4 hours. Media was then collected and stored in -20°C for TRAIL quantification by human TRAIL ELISA kit (Invitrogen). Cells were dissociated from scaffolds or the 96-well plate and counted by hemocytometer. TRAIL output was normalized by cell number.

2.2.9 In Vitro GBM Killing

A co-culture experiment was performed in a 96-well plate by seeding a fixed number of mCherry-FLUC-GBM8 tumor cells (1 x 10^4 per well) along with a range of TRAIL-NSCs or NSCs (blank) (1.25 x 10^3 – 4 x 10^4 cells per well) in a total of 200 µL of media. After 72 hours of co-culture, GBM8 viability was determined by bioluminescence using a plate reader (BioTek Instruments, Winooski, VT) and normalized to untreated GBM8 cells.

TRAIL-conditioned media was generated after 6.5 hours of TRAIL-NSC (2.5 x 10^5 per well) incubation in a 96-well plate. TRAIL concentration in the conditioned media was quantified by ELISA. GBM8 tumor cells (2 x 10^4 per well) were treated for 48 hours with serially diluted TRAIL-conditioned media in a 96-well plate with a total of 200 µL of media. GBM8 viability was measured by MTT assay to calculate the half maximal inhibitory concentration (IC_{50}) of TRAIL secreted by NSCs.

2.2.10 NSC Persistence Model

The effect of scaffolds on NSC persistence was evaluated in the surgical model described above. 2.5 x 10^5 mCherry-FLUC-NSCs were implanted in the resection cavity either seeded onto a single 3 mm scaffold or by direct injection (DI) loaded into a Hamilton syringe in 4 µL PBS (fast, n = 5; slow, n = 4; DI = 5). NSC persistence was monitored by bioluminescent imaging (BLI) for the duration of the study (120 days). At each imaging time point, mice were anesthetized by inhaled isoflurane and imaged by the Perkin Elmer IVIS Lumina In Vivo Imaging System. Mice were given an intraperitoneal injection of D-luciferin (15 mg/kg in PBS; Perkin Elmer) and imaged
25 minutes later at a 5 minute exposure to monitor NSC persistence. Image exposure length and time after D-luciferin injection was optimized to maximize BLI signal. BLI signal (total flux, p/s) was normalized to background signal from a mouse containing no NSCs. NSC implant efficacy was determined by quantifying the BLI signal from each mouse on day 2 and normalizing it to the “maximum intended dose” which is the average BLI signal from the 5 mice with the highest signal, regardless of group. The time to NSC clearance was determined by fitting trendlines to the normalized BLI signal for each mouse and applying a threshold of 15% of “maximum intended dose” (Figure 8). The time to clearance was plotted as a Kaplan-Meier survival curve.

2.2.11 Statistical Analysis

Statistical analysis was performed using GraphPad Prism (La Jolla, CA). Cell seeding and growth comparisons were performed by Student t-test. Kaplan-Meier curves of NSC clearance in the brain was analyzed by Log-Rank (Mantel-Cox) test. All other comparisons done by one-way ANOVA with Tukey’s Multiple Comparisons post-test.

2.3 Results and Discussion

2.3.1 Fabricating Ace-DEX Gelatin Scaffolds by Electrospinning and Dehydrothermal Crosslinking

In tissue engineering, degradation has been regarded as an important scaffold parameter, where it is widely accepted that the rate of scaffold degradation should match the rate of tissue formation to allow for smooth integration into the surrounding tissue (123). However, in NSC-based cancer therapy, the goal is to stabilize transplanted cells post-surgery, then ultimately release the therapeutic cells into brain where they can track down residual cancer foci. By investigating the role of scaffold degradation, we could move towards a more optimal NSC therapy for GBM. Previously, scaffold degradation has been tuned by blending differently degrading polymers (107,
and changing fabrication parameters that include polymer concentration (89), solvent properties (125), and crosslinking (126). Here, we utilized the tunability of Ace-DEX to generate scaffolds with two different degradation times. Reacting Ace-DEX for approximately 16 minutes or 2 hours resulted in polymer with an average of 46.0 ± 0.7 or 61.3% ± 1.7 CAC, respectively (Figure 10A). These times were chosen based on previous studies to generate “fast” and “slow” degrading polymers (127). Initially, it was determined that NSCs would not attach to pure Ace-DEX electrospun scaffolds, likely due to the hydrophobicity of the polymer (data not shown). In order to facilitate NSC adherence, Ace-DEX polymer was blended with gelatin, a widely used, natural biopolymer derived from denatured collagen (81, 126, 128). Furthermore, a commercially available gelatin foam named Gelfoam® has been utilized for hemostasis during brain surgery, illustrating the safety of gelatin for use in the brain (129). With the addition of gelatin, we anticipated that NSCs would readily adhere to the scaffolds, consistent with previous reports in the literature (130-132).

Composite scaffolds were fabricated with a gelatin mass loading of 28.4% ± 1.97 and 29.0% ± 1.65 for fast and slow scaffolds, respectively (Figure 9A). Next, scaffolds were stabilized via dehydrothermal (DHT) crosslinking to prevent rapid release of hydrophilic gelatin when placed in aqueous solution. Under high heat and vacuum, amide bonds are generated by condensation reactions between adjacent amino acid residues resulting in crosslinked gelatin (133, 134). DHT crosslinking was utilized because it does not pose the potential risk of toxicity that can be seen with more commonly used chemical crosslinkers like glutaraldehyde (126). Scaffolds were crosslinked for times ranging from 0-112 hours and then evaluated for gelatin release (Figure 9A-B). Un-crosslinked scaffolds rapidly released gelatin upon incubation in PBS and had released almost all gelatin within 72 hours. However, with increasing DHT crosslinking time, the rate of
gelatin release decreased. Saturation of gelatin crosslinking was achieved by increasing DHT time until the gelatin release profile plateaued, occurring between 99 and 112 hours for both scaffolds (Figure 9A-B). For this reason, 100 hours was chosen as the crosslinking time for the remainder of the study. Scanning electron microscopy (SEM) micrographs demonstrate that scaffold morphology was not altered after 100 hours of DHT crosslinking (Figure 9C-F).

The effect of DHT time on crosslinking has been previous reported, however, with different results likely due to scaffold composition. Haugh et al. found that DHT times beyond 24 hours resulted in no further crosslinking of freeze dried collagen scaffolds (133). This is in line with our results which indicate a saturation of crosslinking occurs; however, these pure collagen scaffolds may achieve crosslinking saturation more rapidly compared to our composite scaffolds, where the presence of Ace-DEX may increase the distance between amino acid residues on gelatin.

2.3.2 Degradation of Ace-DEX Gelatin Scaffolds In Vitro and In Vivo

Despite blending with gelatin, the influence of Ace-DEX cyclic acetal coverage (CAC) remained the driver of scaffold degradation, in vitro. Ace-DEX gelatin scaffolds composed of polymer with 46.0% ± 0.7 CAC degraded rapidly, with only 25% mass remaining by day three (Figure 10A-B). Conversely, scaffolds composed of the 61.3% ± 1.7 CAC Ace-DEX had ~60% mass was remaining on day 56. After an initial burst release of gelatin within the first 24 hours, the remaining scaffold mass attributed to gelatin was relatively stable over time. This is likely due to saturation of gelatin crosslinking by DHT. SEM micrographs show that Ace-DEX CAC does not influence scaffold morphology (Figure 10D-E).

In order to investigate Ace-DEX gelatin scaffold degradation in vivo, indocyanine green (ICG), a near infrared fluorescent dye, was added to the composite scaffolds. ICG loaded composite scaffolds had the same degradation rate as un-loaded blank composite scaffolds (data
not shown). Because ICG is embedded in the scaffold fibers and not chemically conjugated, its release from the scaffolds is likely governed by a combination of diffusion and polymer degradation (135). While the ICG release from fast degrading scaffold tightly follows its degradation profile in vitro, the slow degrading scaffold releases ICG more rapidly than it degrades (Figure 11A). Yet, the fast and slow degrading scaffolds exhibit unique ICG release rates allowing us to approximate scaffold degradation in vivo. In vivo, ICG fluorescence was monitored non-invasively using serial fluorescent imaging after being implanted in the brain resection cavity. Interestingly, mice bearing fast degrading scaffolds had 2% of their original fluorescent signal after 7 days compared to 18% at the same timepoint in vitro, suggesting that their degradation profile in vivo is faster than in vitro (Figure 11B-C). On day 28, there was less than 0.15% of the initial fluorescent signal from the fast degrading scaffold group compared to the slow degrading scaffold group which had on average a signal 6.72x higher signal (Figure 11B). Consistent with our in vitro study, these results illustrate a difference in degradation between the fast and slow Ace-DEX gelatin scaffolds in vivo.

Scaffold degradation has been found to impact brain tissue regeneration after CNS injury (89, 136, 137). Ghuman et al. generated hydrogels that degraded in the brain at rates similar to Ace-DEX gelatin scaffolds by changing protein concentration (89). This work showed that degradation influenced neural tissue regeneration, with the rapidly degrading hydrogel leading to neovascularization and an increase in infiltrative cells such as neurons and oligodendrocytes in the stroke cavity. Considering the significant influence of scaffold degradation on the local microenvironment after CNS injury, we investigated how scaffold degradation would affect the persistence of NSCs implanted in the surgical resection cavity with Ace-DEX gelatin scaffolds of two distinct degradation rates.
2.3.3 Effect of Ace-DEX Gelatin Scaffold Degradation on NSCs In Vitro and In Vivo

For clinical translation, controlling the number of implanted cells is important for standardizing patient dose. Therefore, we evaluated the ability of the Ace-DEX gelatin scaffolds to hold a high capacity of NSC while maintaining viability. A series of in vitro studies were performed with an immortalized murine NSC line, C17.2, for initial scaffold testing. The NSC carrying capacity and seeding efficacy was measured on scaffolds scaled to fit in the surgical resection cavity of the murine brain (Figure 12A). With increasing number of cells, the seeding efficiency decreased slowly until the number of cells adhered to the scaffolds at 24 hours approached a saturation of 139,000 ± 15,700 cells and 114,000 ± 14,900 per a 3 mm scaffold for fast and slow degrading scaffolds, respectively, when 500,000 cells were seeded (Figure 12B). There was no statistical significance between fast and slow degrading scaffolds for cell carrying capacity or seeding efficiency (Figure 12C). Previous studies using electrospun scaffolds have only reported the cell number seeded, which may not be the accurate number of cells that adhere to the scaffolds subsequently implanted in the brain (36).

Moving forward to in vivo studies, a primary murine cortical NSC line was utilized, as the immortalized line C17.2 was found to proliferate rapidly when implanted in the brains of nude mice (data not shown). Primary NSCs were able to be seeded onto the scaffolds at a higher seeding efficiency than immortalized cells, where the maximum number of cells was 190,000 ± 69,800 cells on fast and 165,000 ± 40,300 cells on slow degrading scaffolds when seeding 250,000 cells (Figure 12C). We hypothesize this increase in seeding efficiency is because the primary NSC are smaller than the immortalized line. Similar to the immortalized NSC line, primary NSCs maintained the same level of viability after seeding onto scaffolds and supported proliferation for 48 hours (Figure 12D). Despite the fact that fast degrading Ace-DEX gelatin scaffolds had only
32% mass remaining by 48 hours, NSCs remained viable (Figure 10C, Figure 12D). This is in line with literature, where Hackett et al. found that 72 hours after seeding, around 90% of NSCs remained viable on electrospun composite polycaprolactone collagen scaffolds despite the scaffold degrading completely by 100 hours (138). These results support our finding that NSCs can maintain viability despite significant scaffold degradation.

In addition to the number of cells implanted, the amount of therapy released from the cells is vital to characterize. TRAIL was selected for its potency against GBM, which induces apoptosis via binding to the death receptor 5 on the surface of cancer cells, and because it is commonly used in tumoricidal SC studies (139-141). NSCs transduced with a lentiviral vector for TRAIL have been previously reported (141-143), and a similar method was used here. To investigate if Ace-DEX gelatin scaffolds would alter therapeutic efficacy, TRAIL output was measured from TRAIL-NSCs seeded onto scaffolds and TCPS. Our results indicate that TRAIL output per cell was the same across both fast (3.20 ± 1.20 fg/cell) and slow (3.23 ± 1.13 fg/cell) degrading scaffolds as well as TCPS (2.81 ± 0.665 fg/cell), suggesting that the nanofibrous structure does not alter TRAIL secretion (Figure 13A). In addition, we determined that TRAIL output was not affected by NSC density and correlated linearly with NSC number (Figure 14).

To evaluate the efficacy of secreted TRAIL, TRAIL-NSCs were cultured in the presence of a human derived GBM cell line, GBM8. TRAIL-NSCs showed potent killing compared to control unmodified NSCs (Figure 14B). In order to investigate the amount of TRAIL required to kill GBM cells in vitro, GBM cells were treated for 48 hours with supernatant conditioned by TRAIL-NSCs (Figure 14C). The half maximal inhibitory concentration (IC$_{50}$) of GBM8 cells was determined to be 8.63 ng TRAIL/mL. Taken together, these studies suggest that TRAIL-NSCs seeded onto Ace-DEX gelatin scaffolds should be highly effective in killing GBM in vivo.
In order to determine the impact of scaffold degradation on NSC persistence, a murine model of surgical resection was utilized to recapitulate implantation of the NSCs after debulking a GBM tumor, and thus maintain clinical relevance. Firefly luciferase expressing NSCs were seeded onto scaffolds for 24 hours and then implanted into a surgical resection cavity in the right frontal lobe of nude mice. NSCs on scaffolds were compared to a direct injection (DI) of an equivalent number of cells into the wall of the resection cavity, mimicking the current administration of therapeutic NSCs in the clinic (45). BLI was used to monitor NSC persistence over time (Figure 15A). Both fast and slow degrading Ace-DEX gelatin scaffolds increased the delivery efficacy of NSCs into the resection cavity 2.87 and 3.08-fold, respectively, (P-value = 0.0047, P-value = 0.0034) over DI (Figure 15B). Bagó et al. saw a similar increase in SC delivery efficacy by fibrin scaffolds compared to DI, reporting a 2.17-fold increase in human mesenchymal stem cell (MSC) BLI signal at 3 hours after implantation (35). Additionally, Hansen et al. reported increased NSC delivery efficacy using an ECM-based scaffold compared to DI as measured by number of NSC that migrated from the resection cavity to an established GBM tumor in the contralateral hemisphere 1 week after implantation (37). The prevailing hypothesis in the literature for scaffold enhanced transplant efficacy is that providing SCs with a substrate for attachment during the implantation process prevents mechanical wash-out from the resection cavity by cerebrospinal fluid and reduction in programmed cell death (37, 77).

Fast and slow degrading scaffolds also had a significantly increased area under the curve (AUC) for BLI signal compared to DI (Figure 15C, P-value = 0.0107 and P-value = 0.0037, respectively). Furthermore, both fast and slow degrading Ace-DEX gelatin scaffolds significantly extend the length of time that NSCs persist in the brain compared to the DI, P-value = 0.0039 and P-value = 0.0472, respectively (Figure 15D). The time to NSC clearance below the 15% BLI
threshold is longer for the slow degrading scaffold group compared to fast, however not significantly (P-value = 0.0766), with a median persistence of 21 versus 16 days (Figure 15D). Our results demonstrate that scaffolds enhance SC persistence in the surgical resection cavity over DI which has been seen previously with other SC lines (35, 36, 144-146). Other previous studies with scaffolds compared persistence of SCs implanted into the brain surgical resection cavity with DI also via BLI signal, yet only monitor persistence until 28 days at the latest (35, 36, 38). Bagó et al. found that fibrin scaffolds extended persistence of human MSCs implanted into a resection cavity in the brain greater than 14 days compared to DI in the resection cavity (35). The BLI signal for SC implanted by DI, normalized to its initial signal on day 0, was non-existent by day 14, as well as representative images showing disappearance in pixels attributed to BLI signal between day 10 and 14 as well. In the same time frame, Kauer et al. reported that murine NSCs administered by DI cleared between 7 and 14 days (38). These results are in line with ours, where all mice that received NSCs administered by DI had cleared by day 11 determined by our threshold (Figure 15D). Hansen et al. observed a longer persistence of NSCs, reporting that NSCs were still detectable by histology at day 36 (37). However, the disparity in persistence seen there could be due to the difference in sensitivity of NSC detection method.

Interestingly, Ace-DEX gelatin scaffolds extend long-term survival of NSCs in the brain significantly compared to DI. NSC BLI signal still detected in both scaffold groups at day 120, despite in vitro degradation occurring within ~7 days and ICG having only 0.15% of its original fluorescent signal after 28 days (Figure 10C, Figure 11B, Figure 15A). Yan et al. utilized a composite scaffold, composed of the polysaccharide, chitosan, and collagen to transplant MSCs after traumatic brain injury. They found that MSCs seeded on the scaffolds incurred a greater cognitive improvement compared to MSCs administered by DI. The scaffolds had degraded by 1
month after implantation and MSCs transplanted on the scaffolds had differentiated into neurons or glial cells, suggesting that a relatively rapidly degrading scaffold is sufficient to enhance MSC persistence (146).

While there was a trend towards more NSCs remaining in the brain with slower scaffold degradation rates in our study, this was not statistically significant compared to fast scaffolds. One explanation for the minimal difference in NSC persistence may be that increasing NSC implant efficacy is the main factor influencing NSC survival in the brain. Moreover, the NSCs may also be remodeling the fast degrading scaffolds with ECM, allowing them to sustain viability in the cavity beyond the lifetime of the original scaffold (147). Overall, the results of this study support literature findings that the use of a scaffold increases short-term and long-term persistence of NSCs in the brain. As such, we conclude that scaffold degradation in the time range we explored has a limited impact on the persistence of NSCs in the resection cavity.

2.4 Conclusion

Tumoricidal SC therapy is a promising avenue for GBM therapy due to the ability to overcome conventional drug delivery barriers. While scaffolds have been found to improve SC implantation in the resection cavity, the role of scaffold degradation rate on SC persistence has not been investigated systematically. To this end, the tunability of the Ace-DEX polymer platform allowed us to generate composite scaffolds with two different degradation profiles in vitro and in vivo simply by changing the reaction time of Ace-DEX polymer synthesis. In vitro, fast degrading scaffolds degraded within 7 days while slow degrading scaffolds retained 60% of its mass out to 56 days. Scaffold degradation did not influence NSC seeding efficiency, short-term viability, or TRAIL output in vitro. The effect of scaffold degradation was evaluated in a clinically relevant orthotopic mouse model that mimics surgical GBM resection. Ace-DEX gelatin scaffolds
significantly increased NSC implantation efficacy and long-term persistence compared to DI. However, scaffold degradation profile had limited impact on NSC persistence. These results illustrate the importance of supporting NSCs during the process of implantation to enhance NSC persistence in the brain after surgical resection.
Figure 7 Schematic for Scaffold Cell Seeding Method

Scaffolds are placed on an agar coated 96-well plate where the agar hydrates them. Silicone tubing with an outer diameter of 5 mm and an inner diameter of 3 mm is placed in the well, used to funnel 30 µL of full media and cells onto the scaffold. Scaffolds are incubated for 4 hours to allow for cell attachment, and then are transferred to a new well with fresh media.
Figure 8 BLI Trendlines for NSC Clearance

Trendlines fit to BLI signal normalized to background over relevant timepoints for each mouse. NSC clearance was determined by solving for the value for 15% of the maximum intended dose of NSCs, illustrated by black dashed line.
Figure 9 Optimization of Gelatin Crosslinking in Composite Ace-DEX Gelatin Scaffolds

Effect of dehydrothermal (DHT) crosslinking on gelatin release from fast (A) and slow (B) degrading composite scaffolds. Data presented as mean ± standard deviation. Representative scanning electron microscopy (SEM) micrographs of scaffolds with (C,E) fast and (D,F) slow degrading scaffolds before crosslinking (C,D) and after 100 h DHT crosslinking (E,F). Scale bar is 10 µm.
Figure 10 Degradation Profiles of Composite Ace-DEX Gelatin Scaffolds

(A) Table of fast and slow scaffold parameters, including Ace-DEX % cyclic acetal coverage (CAC), gelatin loading (% w/w), and degradation half-life of resulting scaffolds. (B) Degradation profiles of fast and slow scaffolds after 100 hours of crosslinking. Percent of total scaffold mass (solid marker) and mass attributed to gelatin (open marker) are shown. (C) Fast degradation profile magnified. Data presented as mean ± standard deviation. Representative scanning electron microscopy (SEM) micrographs of scaffolds with (D) fast and (E) slow Ace-DEX gelatin scaffolds crosslinked for 100 hours. Scale bar is 10 µm.
Figure 11 In Vitro and Vivo Characterization of ICG-loaded Ace-DEX Gelatin Scaffolds

(A) In vitro characterization of ICG release from fast and slow Ace-DEX gelatin scaffolds. Data presented as mean ± standard deviation. (B) Quantified % remaining ICG at representative timepoints in vivo. Data presented as mean ± standard deviation. (C) Fluorescent images of ICG-loaded scaffolds implanted in the surgical resection cavity in vivo at representative time points. Fluorescence scale is in radiance (ρ/s/cm²/sr) with limits set at $2.00 \times 10^7$ to $2.00 \times 10^9$ chosen to best visualize both the high initial signal and the disappearing signal thereafter.
Figure 12 NSC Seeding and Viability on Ace-DEX Gelatin Scaffolds

(A) Image of 3 mm size scaffold scaled to fit in surgical resection cavity of a mouse. (B) Cell carrying capacity of scaffolds at 24 hours with C17.2 NSCs. (C) Cell seeding efficacy determined by normalizing number of cells on scaffold to number of cells seeded for immortalized and primary NSCs. (D) Growth of cells seeded on scaffolds at 48 hours, normalized to viability at 24 hours for immortalized and primary NSCs. Data presented as mean ± standard error of the mean.
Figure 13 Generation of Therapeutic TRAIL-NSCs and Seeding onto Scaffolds

(A) Correlation between number of TRAIL-NSCs adhered to TCPS with TRAIL output/cell. (B) Linear correlation of TRAIL output with number of TRAIL-NSCs adhered to TCPS.
Figure 14 Generation of Therapeutic NSCs and Seeding onto Scaffolds

(A) Quantification of TRAIL secreted from TRAIL-NSCs seeded onto fast and slow degrading scaffolds compared to TCPS. (B) Viability of GBM cells cultured with increasing ratios of unmodified NSCs or TRAIL-NSCs. (C) Viability of GBM cells treated with supernatant collected from NSCs containing secreted TRAIL. Data presented as mean ± standard deviation. ANOVA was done to determine statistical significance utilizing Tukey’s multiple comparisons post-test. **p < 0.005 and ****p < 0.0001 with respect to NSCs of the same ratio to GBM.
Figure 15 Persistence of NSC Seeded on Ace-DEX Gelatin Scaffolds in Resection Cavity

(A) BLI images showing NSCs at representative time points after implantation in surgical resection cavity. BLI scale is in radiance ($\rho$/s/cm$^2$/sr) with limits set at $2 \times 10^4$ to $2 \times 10^6$. (B) Efficacy of NSC implant method comparing DI to NSC seeded on fast degrading and slow degrading scaffolds. Data presented as mean ± standard deviation. Statistical significance measured by ANOVA with Tukey’s
multiple comparison post-test. *p < 0.05 and **p < 0.005 with respect to DI. (C) BLI signal of NSCs in the brain normalized to background over time. Data presented as mean ± standard error of the mean. An ANOVA was done to determine significance of the AUC. **p < 0.005 with respect to DI. (D) Kaplan-Meier curve of NSC clearance from the brain comparing DI to NSCs seeded on fast and slow degrading scaffolds. Clearance was defined as BLI signal dropping below a 15% threshold. Log-rank (Mantel-Cox) test was done to determine statistical significance. *p < 0.05, **p < 0.005 with respect to DI. (E) Average radiance of NSCs at day 120 showing NSC implant efficiency. Data presented as mean ± standard deviation. Statistical significance measured by ANOVA with Tukey’s multiple comparison post-test. *p < 0.05 with respect to DI.
CHAPTER 3: INJECTABLE, RIBBON-LIKE MICROCONFETTI BIOPOLYMER PLATFORM FOR VACCINE APPLICATIONS

3.1 Introduction

In response to the poor immunogenicity of proteins and peptides on their own, often an adjuvant is used in a vaccine formulation to stimulate a protective immune response. Examples of FDA-approved adjuvants include the aluminum salts (alum) and emulsion-based adjuvants like MF59 (squalene oil, Span 85, Tween 80, and citrate buffer) and AS03 (squalene and DL-α-tocopherol, a more bioavailable form of vitamin E) (148-150). Many of these adjuvants serve as depots that create a reservoir at the site of injection for extended stimulation of antigen presenting cells (APCs). This approach has led to an enhanced adaptive immune response compared to saline controls (149-151).

In addition to depot-like approaches, the use of pathogen-associated molecular patterns (PAMPs) as adjuvants has also been applied to FDA-approved vaccines. PAMPs bind to pattern recognition receptors, such as toll-like receptors (TLRs), to trigger potent innate signaling. Examples of PAMP-containing adjuvants are AS04, CpG and AS01B, which are formulated into a wide variety of vaccines. TLR agonists (e.g. CpG and monophosphoryl lipid A (MPL)) are viewed as promising vaccine adjuvants because they can enhance type 1 helper (Th1) cellular immunity against intracellular pathogens, whereas most depot-forming adjuvants primarily skew towards a type 2 helper (Th2) phenotype (152, 153). Additional TLR agonists have been investigated preclinically, such as resiquimod, the TLR 7/8 agonist imidazoquinoline that mimics
single-stranded viral RNA (154). Resiquimod (also known as R-848) was developed by 3M (St. Paul, MN) and has demonstrated 10-100x more potent adjuvant activity compared to its analog, Imiquimod (Aldera®), an FDA-approved topical adjuvant (155). Resiquimod has illustrated protection and potent Th1 responses pre-clinically as a vaccine adjuvant for a variety of vaccines including anthrax (156, 157), melioidosis (158), melanoma (159), and HIV (160).

Combining depot forming adjuvants with a TLR agonist has been a strategy to create a more balanced Th1/Th2 response. For example, AS04, the adjuvant used in the human papillomavirus vaccine Cervarix®, is a mixture of alum and MPL. Combining MPL with alum was found to extend the duration of cytokine production afforded by MPL and correlated with higher numbers of activated APCs in the lymph nodes (161). Cervarix® stimulated higher serum antibody titers and memory B cells compared to Gardasil®, another HPV vaccine that uses very similar antigens, but contains only alum as the adjuvant (162).

Biomaterial scaffolds have recently emerged as another method to enhance vaccine efficacy by serving as an immunostimulatory depot. In a seminal study, Ali et al. found that poly lactic-co-glycolic acid (PLGA) foams loaded with tumor lysate, a dendritic cell maturation agent (granulocyte-macrophage colony-stimulating factor, GM-CSF) and TLR agonist CpG provided a persistent, antigen-rich environment with immunostimulatory signals that generated an anti-tumor immune response through recruitment and stimulation of APCs and T cells (163). A number of other scaffolds have since been explored as vaccine adjuvant depot platforms, such as hydrogels composed of dextran (164), alginate (165), poly (L-valine) (166), poly(ethylene glycol)-PLGA (167), chitosan (168, 169) and poly(2-hydroxyethyl methacrylate) with poly(dimethylsiloxane) (170). Similarly, mesoporous silica rods (MSRs) have also been shown to self-assemble into a 3D immune depot capable of stimulating robust immune responses (171-173).
In many of these scaffold vaccines, antigen and adjuvant are loaded within the scaffold to be released in a soluble form. Release of these vaccine elements from a large scaffold provides less control over antigen delivery to APCs, limiting potential antigen presentation. Nano- and microparticles have frequently been used to efficiently deliver antigen and adjuvant in biomaterial vaccines because they can be internalized by APCs (174, 175). As such, several scaffolds have loaded nano- or microparticles within the scaffold to target APCs; however, this adds substantial complexity to the system. Development of a simplified platform that allows for more effective and controlled vaccine element delivery, while also maintaining long-term persistence of the vaccine depot, could be a beneficial next step in scaffold vaccine development.

In this study, we characterize a novel injectable vaccine platform composed of the biodegradable polymer acetalated dextran (Ace-DEX) formulated as high aspect ratio, ribbon-like particles termed microconfetti (MC). We hypothesize that MC create a 3D depot in the subcutaneous space upon injection and offer an advantage over scaffold-based vaccines that require surgical implantation. MC were first developed for long-acting delivery of antiretroviral drugs to treat HIV, to deliver sustained drug release to reduce frequency of drug administration (116). MC were fabricated by mechanically fragmenting electrospun scaffolds, and were found to retain the ability of electrospun scaffolds to provide sustained drug release (102, 116, 176). In this way, MC combine the favorable characteristics of both microparticle and scaffold-based vaccines. Ace-DEX was chosen as the polymer platform based on its compatibility with a wide range of electrospinning solvents, acid sensitivity, pH neutral byproducts, and tunable degradation (177). Notably, the tunable degradation of Ace-DEX has been shown to enhance antigen presentation by APCs and drug release profiles from microparticles and scaffolds, making it a desirable foundation for immunomodulatory platforms (176, 178, 179). Furthermore, among the other common
polymers (PLGA and polycaprolactone) evaluated for MC, Ace-DEX was the only polymer that did not melt during the process of fragmenting the fibers due to the polymer’s high glass transition temperature (116).

Herein, MC were fabricated with and without resiquimod in three unique sizes by tuning electrospinning parameters. APC internalization of formulated MC, as well as the resulting cytokine release and antigen presentation, were evaluated in vitro. In vivo, the effect of formulated MC on humoral immune responses was assessed against a model antigen.

3.2 Materials and Methods

All materials were purchased from Sigma (St. Louis, MO) and used as purchased, unless otherwise indicated.

3.2.1 Acetalated Dextran (Ace-DEX) Synthesis

Ace-DEX was synthesized as previously described (120). Briefly, lyophilized dextran (MW 450-650 kDa) and pyridinium p-toluenesulfonate (acid catalyst) were dissolved in anhydrous dimethyl sulfoxide (DMSO). The dextran was reacted with 2-ethoxypropene (Matrix Scientific, Columbia, SC) in DMSO for 2 hours under anhydrous conditions, after which the reaction was quenched with the addition of triethylamine (TEA). The mixture was precipitated dropwise into basic water (0.04% v/v TEA in water) and lyophilized overnight. Next, the polymer was dissolved in ethanol, centrifuged to remove impurities, re-precipitated into basic water, and then lyophilized. The final product, Ace-DEX, was stored at -20°C until further use.

3.2.2 Microconfetti Fabrication

Electrospun Ace-DEX scaffolds were fabricated as previously described (116). Briefly, Ace-DEX polymer was dissolved in a tri-solvent of hexafluoroisopropanol, 1-butanol, and TEA at a ratio of 59%, 40%, 1% v/v, respectively. Electrospun scaffolds were fabricated with a range of
fiber diameters by changing polymer concentration and flow rates to create scaffolds with three unique widths (Figure 16). Polymer concentrations of 225, 300, and 400 mg/mL were chosen for small, medium, and large MC, respectively. The polymer solution was pumped out of a glass syringe fitted with a 21-gauge blunt needle 16 cm away from a metal collection plate with a voltage bias of 15 kV. Three different flow rates were used depending on fiber diameter size: 0.25, 1, and 4 mL/hour for small, medium, and large, respectively. Resiquimod-loaded and BODIPY-loaded scaffolds were fabricated in the same manner, with the addition of 1 w/w% resiquimod (Enzo Life Sciences, Inc., Farmingdale, NY) or 0.25 w/w% BODIPY 493-503 (Thermo Fisher Scientific) to the polymer solution, respectively. Electrospun scaffolds were collected from the collection plate and weighed.

To make the MC, scaffolds were submerged in basic water at 20 mg/mL and homogenized for 30 seconds at 21,000 RPM using an IKA® T25 digital ULTRA-TURRAX homogenizer. The resulting MC was lyophilized and stored at -20°C until further use. Resiquimod loading was determined by dissolving samples in dimethylsulfoxide (DMSO) and reading fluorescence in a plate reader (excitation: 260 nm, emission: 360 nm) alongside a standard curve. The following equations were used to determine resiquimod loading and encapsulation efficiency:  

\[
\text{Resiquimod Loading} = \frac{\text{Mass of Resiquimod}}{\text{Mass of Microconfetti}} \times 100\%.
\]

\[
\text{Resiquimod Encapsulation Efficiency} = \frac{\text{Actual Resiquimod Loading}}{\text{Theoretical Resiquimod Loading}} \times 100\%.
\]

Table 3 contains information of MC made for in vitro and in vivo studies.

### 3.2.3 Microconfetti Size Characterization

Scaffold and MC morphology were evaluated by scanning electron microscopy (SEM, Hitachi S-4700 Cold Cathode Field Emission). Scaffolds were mounted onto aluminum stubs using carbon tape and microconfetti were suspended in water and dried onto stubs. Samples were coated
with palladium using a sputter coater and imaged at 2 kV. Images of MC obtained on the SEM at various magnification were analyzed by ImageJ for dimensions. Dimension measurements were compiled from two different batches of MC with three unique SEM images per batch. Representative images of measurements taken for each size can be seen in Figure 17.

3.2.4 Microconfetti Injectability

The ability to deliver a full dose of MC through a 26 G needle was determined by quantifying the amount of polymer expelled from the needle and comparing to a pipetted control. Small, medium, and large MC were suspended in PBS at a similar concentration to the vaccine formulation and 0.2 mL volumes were either injected through the needle or pipetted into microcentrifuge tubes. Samples were degraded into dextran by acid hydrolysis and bicinchoninic acid assay was run to determine the percent intended dose of MC, defined as the signal from samples injected through the needle, normalized to the pipetted sample.

3.2.5 Detection of Endotoxin

MC samples were suspended in endotoxin-free water at 1 mg/mL and incubated at 4°C overnight. Samples were assessed for presence of detectable endotoxin using the Pierce LAL Chromogenic Endotoxin Quantitation Kit (Thermo Fisher, Scientific, Waltham, MA) in accordance with manufacturer instructions. All samples had undetectable levels of endotoxin (< 0.1 EU/mg).

3.2.6 Characterization of Resiquimod Release Profile

MC were suspended in microcentrifuge tubes containing 1X phosphate buffered saline (PBS, pH 7.4) at 1 mg/mL and incubated at 37°C on a shaker plate at 150 RPM. At specific time points, samples were centrifuged for 20 minutes and the supernatant was separated from the pellet. Both samples were stored at -20°C until the end of the study. Resiquimod release was quantified
by reading fluorescence of the supernatant diluted in DMSO on a plate reader (excitation: 260 nm, emission: 360 nm) and normalized to fully degraded by incubation at 80°C for several days.

3.2.7 Characterization of Ovalbumin Adsorption by Flow Cytometry

BODIPY-MC was incubated at 5 mg MC/mL in a solution of ovalbumin (OVA) conjugated to TexasRed dye (Thermo Fisher Scientific) at 50 µg/mL in PBS for 20 minutes at room temperature. Samples were washed three times with PBS to remove any un-adsorbed OVA and then ran on the flow cytometer (Attune NxT, Thermo Fisher Scientific) at a concentration of 0.1 mg/mL with respect to MC.

3.2.8 Isolation and Culture of Bone Marrow Derived Dendritic Cells

Bone marrow derived dendritic cells (BMDCs) were obtained from C57BL/6J mice or B6CBAF1/J mice and cultured 8-10 days prior to using in Dulbecco's Modified Eagle Medium (DMEM, Corning, Corning, NY) supplemented with 1% penicillin-streptomycin (Hyclone, Pittsburgh, PA), 10% fetal bovine serum (Corning), and 10 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF, Thermo Fisher Scientific). Fresh media was added every other day until use.

3.2.9 Cell Lines and Cell Culture

B3Z (CD8+) and KZO (CD4+) T cell hybridoma lines with β-galactosidase reporters for IL-2 were a kind gift from Dr. Nilabh Shastri (Johns Hopkins University). B3Z/KZO cells were cultured in RPMI 1640 media (VWR, Radnor, PA) supplemented with L-glutamine (2.05 mM), 1 mM sodium pyruvate (Gibco, Thermo Fisher Scientific), 1% penicillin-streptomycin, 10% fetal bovine serum, and 50 µM 2-mercaptoethanol. The immortalized dendritic cell line, DC2.4, was used for major histocompatibility complex (MHC) I presentation assay (B3Z). DC2.4 cells were obtained from Sigma (St. Louis, MO) and cultured in RPMI 1640 supplemented with L-glutamine
(2.05 mM), HEPES (25 mM), 1% nonessential amino acids (Gibco), 1% penicillin-streptomycin, 10% fetal bovine serum, and 50 µM 2-mercaptoethanol. BMDCs derived from B6CBAF1/J mice were used for the MHC II presentation assay (KZO). The immortalized macrophage cell line, RAW 264.7 (ATCC, Manassas, VA), was cultured in DMEM supplemented with 1% penicillin-streptomycin, 10% fetal bovine serum. All cells were cultured at 37°C with 5% CO2 and 100% relative humidity.

**3.2.10 Characterization of Bone Marrow Derived Dendritic Cell Cytotoxicity**

BMDCs from C57BL/6J mice were plated in 96-well plates overnight at 50,000 cells per well in 0.1 mL of media containing 10 ng/mL GM-CSF. The next day, MC at a range of concentrations (500 – 15.6 µg/mL) in 0.1 mL was added to the cells and incubated for 24 hours. Blank and resiquimod-loaded MC (1% w/w) were evaluated. Cytotoxicity was measured by the Pierce LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific) in accordance with manufacturer instructions. Percent cytotoxicity was reported by normalizing signal to the control of cells treated with lysis buffer (100% death). Supernatant was collected and stored at -20°C for cytokine analysis.

**3.2.11 Detection of Cytokine Secretion by ELISA**

Cytokine secretion was measured in the supernatant of BMDCs treated with MC for 24 hours by enzyme-linked immunosorbent assay (ELISA). TNF-α, IL-1β, and IL-6 were measured in accordance with manufacturer instructions (eBioscience, Thermo Fisher Scientific).

**3.2.12 Detection of MHC Presentation of Ovalbumin Peptide**

DC2.4 or BMDCs derived from B6CBAF1/J (Jackson Labs) were plated in 96-well plates at 50,000 cells per well in 0.1 mL overnight for MHC I and MHC II presentation, respectively. The next day, cells were treated with 10 µg/mL OVA (EndoFit, InvivoGen, San Diego, CA) and
MC (500 – 15.6 µg/mL) suspended in media. 0.1 mL containing 100,000 B3Z/KZO (MHC I/II) cells were introduced immediately after treating cells and co-incubated for 24 hours. Presentation was determined by β-galactosidase activity, indicative of IL-2 production. Cell culture media was replaced with 0.1 mL of solution containing 0.155 mM chlorophenol red β-D-galactopyranoside, 0.125% Nonidet P-40 Alternative, and 9 mM MgCl₂ in PBS. Plates were sealed and incubated at room temperature for 14 hours and then 75 µL supernatant was transferred to a new plate and absorbance was read on a plate reader (570 nm).

3.2.13 Microconfetti Internalization by Confocal Microscopy

Chambered coverglass slides (Thermo Fisher Scientific) with a surface area of 0.7 cm² per well were coated with poly-D-lysine in water (75 µg/mL) for at least 4 hours, and then washed 3x with water. BMDCs from C57BL/6J mice were seeded into chambers overnight at 200,000 cells/well. Cells were treated with 12.5 µg of BODIPY-MC for 24 hours. Cells were stained with CellTracker Blue CMAC dye (Thermo Fisher Scientific) and LysoTracker Deep Red dye (Thermo Fisher Scientific) in accordance with manufacturer instructions and were then live imaged on a confocal microscope (Zeiss LSM 710 Spectral Confocal Laser Scanning Microscope). Tile scans were taken at the center of the well and then z-stacks were obtained in the tiles along the diagonals of the tile scans to reduce sample bias. Uptake was confirmed by z-stacks, where MC were labeled as internalized when CellTracker Blue CMAC dye surrounded the entire circumference of the MC. The percent of cells with internalized MC was calculated by normalizing number of cells with one or more MC internalized to the total number of cells within a z-stack frame. Number of medium and large MC internalized per cell was also counted in z-stacks. Z-stacks were converted into maximum intensity projections and 3-rendered images using the Imaris microscopy image analysis software (Oxford Instruments, Concord, MA). The same method was repeated with the
cell lines, DC2.4 and RAW 264.7, which were seeded at 35,000 and 50,000 cells per well, respectively, to account for different cell growth rates.

3.2.14 Preparation of Microconfetti Vaccine and Immunization of Mice

C57BL/6J mice (6-8 weeks old) were obtained from Jackson Labs. Mice were immunized subcutaneously in the flank with 0.2 mL using a 26-gauge needle. All mice in groups with OVA received 10 µg OVA per dose. MC were suspended in sterile PBS at a concentration 2x of the final injection concentration and sonicated for 10 minutes. MC were then mixed 1:1 with 2x OVA solution (0.1 mg/mL) in PBS and incubated at room temperature for 20 minutes prior to injection. Alum vaccine was prepared by incubating Alhydrogel (InvivoGen, San Diego, CA) at 2.5 mg/mL with 2x OVA solution (0.1 mg/mL) for 20 minutes prior to injection. To test the effect of resiquimod dose on the humoral response, vaccine groups (n = 4 per group) included PBS, OVA, Alum, soluble resiquimod at various doses (0.02, 0.1, 1, or 10 µg), and resiquimod loaded MC to match the soluble resiquimod doses. MC resiquimod loading was targeted to maintain ~ 1 mg of MC per dose for each resiquimod dose. Depending on the encapsulation efficiency of resiquimod loading, the ultimate MC dose was then adjusted to maintain 0.02, 0.1, 1, or 10 µg resiquimod (Table 3). Mice were immunized on day 0 and received a boost on day 21. To evaluate the effect of MC size on the humoral and cellular immune responses, mice were immunized on day 0, and received a boost on days 21 and 35. Vaccine groups (n = 5 per group) included PBS, OVA, Alum, resiquimod-loaded MC (small, medium, and large), and blank MC (small, medium, and large). The dose of resiquimod-loaded MC was adjusted to maintain 10 µg resiquimod, depending on the resiquimod encapsulation efficiency. Blank MC were delivered at the same dose as their resiquimod-loaded MC counterpart (Table 3).
3.2.15 Quantification of Ovalbumin Antibody Titers

Serum was collected on Days -7 (prior to initial Day 0 vaccination), 28, and 42. Blood was collected by a submandibular bleed and samples were centrifuged at 4,000 x g for 10 min at 4°C to separate the serum from the clot. Serum was then collected and stored at -80°C until further analysis. Anti-OVA IgG and IgG2c antibody titers were quantified by ELISA. High-affinity 96-well plates were coated with 5 μg/mL OVA and incubated overnight at 4°C. Plates were then washed three times with wash buffer (0.05% Tween 20 in PBS), and then blocked with blocking buffer (5% non-fat milk in PBS) for two hours at room temperature. After three more rounds of washing, serum serially diluted in blocking buffer was added to plates and incubated overnight at 4°C. Plates were washed three times and then incubated with HRP-conjugated anti-IgG or anti-IgG2c secondary antibodies (Southern Biotech, Birmingham, AL) for 1 hour at room temperature. Plates were washed five times to remove the secondary antibody and developed with the addition of 3,3′,5,5′-tetramethylbenzidine (TMB) solution for 15 or 30 minutes for IgG and IgG2c, respectively. After the addition of 1 M sulfuric acid to terminate development, absorbance was measured on a plate reader (450 nm). Trendlines were applied to antibody dilutions for each mouse and endpoint antibody titers were solved for as the dilution to reach an absorbance optical density (OD) of 1. Endpoint titers were reported log transformed.

3.2.16 Statistical Analysis

Statistical analysis was performed using GraphPad Prism (La Jolla, CA). Group comparisons were made by ANOVA, followed by Tukey’s multiple comparisons post-test, to determine statistical significance.
3.3 Results and Discussion

3.3.1 Fabrication and Characterization of Microconfetti

In this study, MC were fabricated by homogenizing electrospun scaffolds in water, then subsequently lyophilizing to achieve a dry powder (Figure 18A). Changes in electrospun scaffold fiber diameter was found to be the main parameter for tuning MC dimensions (Figure 18A-D). The electrospun scaffold fiber diameter remained unchanged by homogenization. Interestingly, on average, scaffold fiber width indirectly affected the length of the MC after homogenization, where length was defined as the two ends fragmented by homogenization. Interestingly, on average, the MC length depended greatly on the electrospun scaffold fiber diameter, where wider fiber diameters resulted in longer MC fragments. By increasing both electrospinning polymer concentration and flow rate, longer MC fragments were achieved, while reducing those parameters led to thinner fiber diameters and shorter MC (Figure 18B-D). Variations in homogenization speed and length of time were not influential in changing the resulting MC length (data not shown). A panel of scaffolds were electrospun by tuning polymer concentration and flow rate (Table 3) to fabricate three MC populations of different sizes. The resulting three MC populations with distinct widths (Figure 18C, F) and lengths (Figure 18D, F) were defined as small (W: 0.67 µm ± 0.16, L: 10.2 µm ± 4.45), medium (W: 1.28 µm ± 0.28, L: 20.7 µm ± 8.61), and large (W: 5.67 µm ± 1.01, L: 90.2 µm ± 46.3). Average aspect ratios (length:width) were maintained across all three populations, around 15-16 (Figure 18F). Despite the magnitude of their length, MC of all three sizes were able to be injected through a 26 G needle, negating the need for surgical implantation, which is an advantage for immunization (Figure 18E). Lastly, the potent toll-like receptor (TLR) 7/8 agonist, resiquimod (R-848), was loaded into MC (Figure 18F, Table 3) to serve as an adjuvant.
3.3.2 In Vitro Bioactivity of Microconfetti

Release of resiquimod from small, medium, and large MC was assessed in vitro over 42 days (Figure 19A). Initial burst release of resiquimod in the first 24 hours was the highest for small MC (49.3% ± 0.87), followed by medium MC (21.1% ± 2.30), and then large MC (8.6% ± 0.06) (Figure 19A). A size dependent trend was also seen where decreasing MC size resulted in more rapid steady state release of resiquimod. It took 1.2 days for 50% resiquimod release from small MC, followed by 19.2 days by medium MC, and large MC released at the slowest rate, where less than 50% resiquimod was released by day 42 (Figure 19A, Table 4). This is likely due to changes in degradation or the surface area to volume ratio impacting drug diffusion. As MC size decreases the surface area to volume ratio increases leading to faster drug release. MC appeared to release resiquimod more slowly compared to previously fabricated spherical Ace-DEX microparticles (MPs), likely due to a combination of their size and shape. Duong et al. fabricated electrosprayed Ace-DEX MPs slightly smaller than small MC (1 – 5 µm) and at half the weight loading (0.54%) of resiquimod had released all resiquimod at pH 7.4 within two days (180). The slower and controlled release of adjuvant distinguishes MC from spherical particle systems and may provide more long-term immune stimulation in vivo as a vaccine depot.

Treatment of bone marrow derived dendritic cells (BMDCs) with MC resulted in dose dependent cytotoxicity in vitro after 24 hours (Figure 19B-C, Figure 20A-B). A size dependent trend in cytotoxicity was observed, where decreasing MC size resulted in increased cytotoxicity in BMDCs for both blank (Figure 19B) and resiquimod-loaded MC (Figure 19C). Because MC were unable to be fabricated with other commonly used biodegradable polymers such as PLGA, head to head comparisons with MC could not be performed. Although, previously, PLGA MPs were found to induce comparable cytotoxicity to Ace-DEX MPs (119). Small and medium blank
MC induced a level of cytotoxicity comparable to spherical Ace-DEX MPs (Figure 19B, Figure 20A). Taken together, these results suggested that neither the polymer, nor the ribbon-like shape of MC dramatically altered their cytotoxicity compared to spherical particle shapes.

Around 1% w/w loading of resiquimod did not enhance cytotoxicity of MC (Figure 19C) compared to blank MC (Figure 19B), which was expected considering that comparable doses of soluble resiquimod resulted in negligible cytotoxicity (Figure 20B). However, loading resiquimod in MC did stimulate inflammatory cytokine secretion. Notably, resiquimod loading increased TNF-α secretion 1-2 logs over MC blank counterparts. (Figure 19D-F). Resiquimod-loaded MC stimulated levels of TNF-α secretion within the same range as soluble resiquimod (Figure 20D), with the exception of lower doses of large MC. Secretion of the inflammatory cytokines IL-1β and IL-6 also increased significantly with resiquimod loading in MC (Figure 21).

### 3.3.3 Evaluation of Microconfetti Internalization by BMDCs

As APCs, dendritic cells (DCs) are a critical target for vaccines because they are responsible for processing protein antigens and presenting them to T cells to mount an immune response (181). For this reason, BMDCs were assessed for their ability to internalize MC by confocal microscopy (Figure 23). Small, medium, and large BODIPY-loaded MC were fabricated for fluorescent visualization. All three sizes of MC were found to be internalized by BMDCs, defined as complete surrounding of CellTracker dye, observed in a z-stack (Figure 24A, Figure 25, Figure 26). This is most surprising for the medium (L: 20.7 µm ± 8.61) and large (L: 90.2 µm ± 46.3) MC, which in many cases were found to be as long or longer than the BMDCs (approximately 20 µm). Furthermore, adsorption of LysoTracker dye to the surface of some internalized MC suggests that the MC eventually become exposed to the acidic microenvironment of the endosome/lysosome. This phenomenon was observed for all MC sizes and was most
dramatic in the small MC group, as seen in Figure 24A. Unlike the other sizes where most of the LysoTracker red dye localized in small, circular vesicles, in cells treated with small MC, the LysoTracker red dye depicted rod-like shapes that were the same size and geometry as the small MC themselves. While all three sizes of MC were found to be internalized by BMDCs (Figure 24A), unsurprisingly, smaller MC have a higher incidence of uptake compared to medium and large MC (Figure 24C). Additionally, there was a trend of fewer MC internalized as MC size increased. The average number of medium and large MC that were internalized per cell were 2.69 ± 0.22 and 0.18 ± 0.05, respectively (Figure 27A). Too many small MC were internalized to be quantified accurately. Among the cells that had at least one MC internalized, only 1.00 ± 0.00 large MC was internalized per cell and 3.47 ± 0.51 medium MC were internalized (Figure 27B). Similar trends were observed with other commonly used cells lines for MC internalization studies, RAW 264.7 macrophages (Figure 28, Figure 29), DC2.4 DCs (Figure 30).

While several studies have investigated the effect of size on internalization of high aspect ratio particles, the particle sizes typically ranged between 0.3 and 10 µm in length, which were either smaller than small MC or the same size (182-184). One exception is the stretched worm-like polystyrene particles investigated by Champion et al., which were around 20 – 25 µm in length, similar in average length to the medium size MC (185). These particles were internalized by rat macrophages (NR8383) at extremely low frequency compared to spherical particles and on average less than 0.5 worm-like particles were internalized per cell at 22 hours compared to 1.5 – 2 for spherical particles. Their hypothesis for this phenomenon is that the ability of a macrophage to form an actin ring around the particle at the point of contact to drive the membrane down the particle is necessary for the particle to be internalized (185, 186). Formation of this structure requires there to be less than 45° between the membrane normal and the point of contact on the
particle due to the high metabolic expenditure required. As such, they observed that their oblong shaped particles with high aspect ratios are only internalized when the macrophage comes in contact with either end of the particle, and not when approached along the middle (186).

Here, we observed that our high aspect ratio MC at a comparable length (medium MC) were internalized at a much higher frequency compared to the polystyrene worm-like particles. It is notable that Champion et al. treated cells with 30 particles per cell seeded, whereas we treated cells with a fixed mass of 12.5 µg MC which resulted in varied number of MC per cell seeded depending on the size. However, despite these differences in experimental design, BMDCs (Figure 23A) and RAW 264.7 (Figure 23B) macrophages can be seen wrapping around the middle of medium and large MC, the orientation that did not result in complete internalization for worm-like polystyrene particles in NR8383 cells. Furthermore, in many images acquired, the CellTracker dye appears to be spreading down the MC towards the ends to further engulf the MC. Perhaps the ribbon-like geometry of the MC, with a very short height relative to the width, creating an acute angle that may allow cell spreading and formation of an actin ring around the particles more easily than other cylindrical or rod shaped particles with wider and more rounded edges, such as the worms from Champion et al. (185). It is also possible that differences in particle material properties resulting in different stiffness (187) or surface chemistry (188, 189) are responsible for the enhanced internalization of MC. Nevertheless, the ability of APCs to internalize MC indicates that they can readily function as delivery vehicles for antigen and adjuvant in a vaccine.

3.3.4 Adsorption of Ovalbumin to the Surface of Microconfetti

The adsorption of the model protein antigen, ovalbumin (OVA), to the surface of MC was investigated by flow cytometry (Figure 30, Figure 31). Within 20 minutes of co-incubation in PBS at room temperature, OVA was found to adsorb to almost all medium and large MC (99.3% ±
0.08, 96.8% ± 0.91, respectively), and was detectable for around half of the small MC (52.4% ± 2.8) (Figure 30A). The extent of OVA adsorption was quantified by OVA median fluorescence intensity (MFI) comparing MC incubated with and without OVA (Figure 30B). There was a significant increase in OVA MFI for each MC size when incubated with OVA, as well as significantly higher OVA MFI for both medium and large MC compared to small MC when incubated with OVA (Figure 30B). MC size was distinguished by forward scatter (FSC, Figure 32A), and was used to evaluate the relationship between OVA adsorption (OVA MFI) and size (Figure 32B). There was a significant correlation between OVA adsorption and MC size, determined by Pearson’s correlation (r = 0.09, p-value = 0.001). The OVA likely adsorbs to the MC through either hydrophobic or electrostatic interactions. Hydrophobic regions within the OVA molecules may be interacting with the surface of the MC, as it is composed of the hydrophobic polymer, Ace-DEX. Furthermore, there may be electrostatic interactions between charged amino acid groups within OVA and the hydroxyl or acetal groups found on Ace-DEX. The ability of protein antigen to readily adsorb to the surface of MC circumvents the need for more complicated formulation methods such as encapsulation or covalent conjugation which can subject the antigen to denaturing conditions.

Furthermore, combining OVA with blank MC resulted in the functional outcome of enhanced OVA peptide presentation on major histocompatibility complex (MHC) I (Figure 30C) and II (Figure 30D) by DCs in vitro. In the presence of co-stimulatory signals and inflammatory cytokines, presentation of antigen on MHC I and II molecules by APCs (notably, DCs) to antigen specific CD8+ and CD4+ T cells, respectively, results in their activation (190, 191). Presentation assays were performed using B3Z (CD8+) and KZO (CD4+) T cell hybridoma lines and compared to soluble OVA alone. While maintaining a consistent 10 µg dose of OVA, MC treatment
concentration was varied to measure the role of MC in presentation. OVA peptide cross-presentation on MHC I was enhanced by all sizes of MC compared to soluble OVA (Figure 30C). Cross-presentation is a crucial process for eliciting effective immune responses against tumors and intracellular pathogens through the generation of CD8+ T cells (192). Interestingly, the medium MC was the optimal size for cross-presentation on MHC I (Figure 30C). This could be due to the higher level of OVA adsorption observed on medium sized MC compared to the small MC, combined with its relatively high incidence of internalization observed in Figure 23B. In contrast, while large MC had the highest OVA adsorption, fewer cells overall internalized the large MC (Figure 23C), which may explain the low overall MHC presentation. OVA adsorption to MSRs has been previously shown to enhance MHC I presentation by DCs (173).

Previous studies have shown that biodegradable particles encapsulating OVA enhance MHC I presentation compared to soluble OVA (120, 193). Rapidly degrading Ace-DEX MPs with encapsulated have been shown to exhibit superior cross-presentation of OVA peptide compared to MPs composed of slower degrading Ace-DEX, PLGA, and iron oxide, suggesting that the process of degradation enhances the escape of antigen into the cytosol for MHC I presentation (178). In this study, at all concentrations of MC, MHC I cross-presentation was higher for the medium MC compared to spherical electrosprayed MPs when OVA was adsorbed to the surface. This highlights the ability of MC to efficiently deliver surface adsorbed antigen to APCs, compared to a conventional spherical particle control.

For MHC II presentation, a size dependent trend emerged, where decreasing size was more effective at enhancing presentation, with small MC outperforming other MC sizes and MPs (Figure 4D). CD4+ T cells activated by MHC II presentation are responsible for promoting a more robust humoral response (192, 194). It is notable that loading resiquimod into medium MC did not
enhance the benefit of MHC I or II presentation provided by blank MC (Figure 33). Taken together, these results demonstrate the simplicity of the MC platform and its efficacy in facilitating both MHC I and II presentation of OVA peptide. Encouraged by these results, we evaluated the potential for MC to elicit a robust immune response in vivo.

3.3.5 Humoral Response to Microconfetti Ovalbumin Vaccine in Vivo

The ability of MC to form a stable depot in vivo was verified by injecting medium sized indocyanine green (ICG)-loaded MC subcutaneously and measuring ICG fluorescence by an in vivo imaging system (IVIS). The MC depot remained at the injection site for a least 9 days (Figure 34). Next, the dose of resiquimod delivered either by MC or in soluble form was evaluated in an OVA vaccine model in vivo (Figure 35). Mice were immunized on Days 0 and 21, and serum was collected on Day 28 to measure anti-OVA antibody titers. Five batches of medium sized MC with increasing weight loadings of resiquimod were used in order to deliver increasing doses of resiquimod (0, 0.02, 0.1, 1, and 10 µg/mouse) while maintaining the same mass of MC delivered to each mouse (Table 3, Figure 36). Interestingly, all MC groups resulted in significantly higher total anti-OVA IgG titers compared to OVA only, and there was no effect from resiquimod dose delivered by MC (Figure 35A). This is similar to our findings in vitro where blank MC were efficient at MHC I cross-presentation and unaffected by resiquimod loading, suggesting that the MC themselves are immunologically active without adjuvant loading. All resiquimod-loaded MC demonstrated enhanced total IgG titers compared to soluble resiquimod combined with OVA (Figure 35A). Furthermore, all MC groups afforded total IgG titers comparable to alum combined with OVA, a widely used adjuvant in many FDA-approved vaccines (Figure 35A) (149, 195). Similar trends were observed with the IgG1 antibody subtype (Figure 35B).
TLR agonists have previously been combined in vaccine formulations to promote skewing of a Th1 phenotype, which contributes to the generation of a cellular immune response driven by effector CD8+ T cells (152, 154, 161). Resiquimod is a TLR 7/8 agonist and has previously been shown to promote Th1 skewing in pre-clinical vaccine formulations (196, 197). However, in this study no dose of soluble resiquimod tested was able to generate this response over PBS or OVA. Only upon delivery via MC was the highest dose of resiquimod (10 µg) found to be effective in producing IgG2c anti-OVA antibody subtype, which is indicative of a Th1 skewed immune response (Figure 35C). This indicates that MC formulation may increase the potential for resiquimod to promote a Th1-skewed response. Alum did not result in detectable IgG2c antibodies, which was expected as it has historically promoted a Th2 immune response (195). These results illustrate the flexibility of MC to generate a more balanced immune response depending on the adjuvant loaded.

Next all three sizes of MC with and without resiquimod loading were evaluated in the OVA vaccine model (Figure 37). All blank MC sizes were found to produce similar levels of total IgG (Figure 37A) and no detectable IgG2c (Figure 37B). These results suggest that the differences between size observed with blank MC internalization and MHC presentation in vitro were not influential enough to produce difference in the humoral response in vivo. Comparable to the results from the previous OVA vaccine study assessing resiquimod dose (Figure 35), medium blank MC, along with small blank MC, produced the same level of total anti-OVA IgG as alum, suggesting that no additional adjuvant is necessary for MC to mount a strong humoral response within the small – medium size range. Although the amount of IgG produced by large blank MC was not significantly lower than small and medium MC, it was significantly lower than alum. Furthermore, large blank MC resulted in titers more closely aligned with soluble OVA. Because the same mass
of MC was delivered across the MC groups, substantially fewer large MC particles were administered compared to small and medium MC, reducing the incidence of MC interaction with APCs. This, combined with the lower overall frequency of large MC internalization in vitro, could explain the limited impact of large MC on the humoral response. Similar to other depot forming adjuvants, blank MC were found to adsorb OVA and enhance MHC presentation (149). Furthermore, in a previous study, Kumar et al. investigated spherical and rod-shaped polystyrene particles in an OVA vaccine model and found that their un-adjuvanted rod-shaped particles also promoted a primarily Th2 response, as indicated by high anti-OVA IgG1 titers and the absence of the Th1 skewed subtype, IgG2a (for BALB/c mice) (183).

With resiquimod loaded MC, size was a more influential parameter in the humoral immune response. Small and medium resiquimod-loaded MC vaccines resulted in higher total IgG anti-OVA antibody titers compared to large MC (Figure 37A). Furthermore, small and medium blank MC produced comparable levels of total IgG titers as their resiquimod-loaded size counterparts. Interestingly, large MC was the only size to differ in titer level produced based on resiquimod-loading, yet here, resiquimod-loaded large MC resulted in lower total IgG titers compared to blank (Figure 37A). Small and medium resiquimod-loaded MC both stimulated IgG2c production. However, small exhibited the strongest Th1 skewed response among MC sizes and was the only group that significantly increased IgG2c titers over OVA alone (Figure 37B). Considering the enhanced MHC I cross-presentation of MC observed in vitro and the production of IgG2c antibodies in vivo, small and medium resiquimod-loaded MC could likely be used to induce a cellular immune response. In contrast, large resiquimod-loaded MC did not elicit any IgG2c antibody titers (Figure 37B). It is unclear if this is due to size or its slow release profile observed in vitro (Figure 19A).
MSRs are high aspect ratio particles that have previously been investigated as biomaterials-based vaccines (171-173). MSRs with adsorbed GM-CSF and the TLR 9 agonist, CpG, were found to enhance Th1 and Th2 skewed antibody production and immune protection in a prophylactic cancer model (171). Interestingly, longer MSRs (88 µm) that were similar in length to large MC, were found to recruit a greater number of DCs to the injection site, which correlated with higher antigen presentation and germinal center B cells in draining LNs, compared to 37 µm long MSRs, which fall within the range of medium size MC (171). The authors attributed this to increased pore size within the 3D depot created by the longer MSRs; APC internalization was not investigated due to their length. In this study, large MC loaded with resiquimod resulted in lower total IgG antibody production compared to medium and small MC. Other studies have also found that biomaterial scaffold pore size influences DC recruitment and infiltration (170, 198). One possible explanation for the superior humoral response by small and medium MC is that the higher incidence of small and medium MC internalization observed in vitro could be more influential compared to the impact of depot architecture. Overall, these results demonstrate that MC are a promising vaccine platform that can be tuned by size and adjuvant loading to elicit a desired immune response.

3.4 Conclusions

Herein, the injectable, depot-forming MC platform was investigated for the first time for the application of a vaccine. Both MC size and adjuvant (resiquimod) loading were explored as tunable parameters and were found to generate different immune responses. Three distinct populations of MC were fabricated with lengths ranging from 10 – 90 µm and widths ranging from 0.7 – 5.5 µm. All sizes of MC were found to be internalized by DCs and macrophages. OVA readily adsorbed to the particle surface and MC facilitated improved MHC I and II presentation.
by DCs. The adjuvant activity of resiquimod was enhanced when loaded into MC in an OVA vaccine model in vivo. All three MC sizes elicited a humoral immune response and stimulated production of anti-OVA IgG antibodies after subcutaneous immunization. When delivering 10 µg of resiquimod, small and medium resiquimod-loaded MC resulted in Th1 skewing as indicated by increased IgG2c titers, whereas large MC exhibited a primarily Th2 immune response. These results illustrate the potential for the MC vaccine platform to tailor a range of immune responses by changing size and loading of resiquimod.
Scanning electron micrographs of electrospun scaffolds made with different polymer concentrations (225, 300, and 400 mg/mL) and polymer solution flow rates (0.25, 1, 4 mL/hr) were investigated in order to identify three unique fiber diameters for generating different MC sizes. Columns: changing polymer concentration, (A, B, C) 225 mg/mL; (D, E, F) 300 mg/mL; (G, H, I) 400 mg/mL. Rows: changing polymer flow rate, (A, D, G) 0.25 mL/hr; (B, E, H) 1.0 mL/hr; (E, F, I) 4.0 mL/hr. Scale bar is 5 µm.
<table>
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<th>Microconfetti Size</th>
<th>Resiquimod Dose In Vivo (µg)</th>
<th>Microconfetti Dose In Vivo (mg)</th>
<th>Resiquimod Loading (% w/w)</th>
<th>Encapsulation Efficiency (%)</th>
<th>Endotoxin (EU/mg)</th>
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<tr>
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<td>93.7 ± 1.77</td>
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<td>0</td>
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Table 3 Microconfetti Batches for In Vitro and In Vivo Studies

Microconfetti size, in vivo resiquimod dose (µg), in vivo microconfetti dose (mg), resiquimod loading (% w/w), resiquimod encapsulation efficiency (% EE), and endotoxin values (EU/mg) for each batch of microconfetti made for in vitro and in vivo studies.
Figure 17 Evaluation of Microconfetti Dimensions by ImageJ

Representative scanning electron micrographs were taken at different magnifications listed in the top left corner for small (A), medium (B), and large (C) MC. Yellow lines are measured MC taken by ImageJ. Scale bars are 50, 100, and 500 µm for A, B, and C, respectively.
Figure 18 Fabrication of Microconfetti

(A) Schematic of MC fabrication. Ace-DEX scaffolds are electrospun and then homogenized to fragment the fibers into MC. Representative scanning electron micrograph of electrospun scaffold. Scale bar is 5 µm. (B) Scanning electron micrographs of small, medium, and large MC at different magnifications. Scale bar is 25 µm. Width (C) and length (D) measurements taken by ImageJ. Data represented as mean ± standard error of the mean. (E) Injectability of MC through a 26 G needle. Increments of 0.2 mL were pushed through the needle, degraded, and normalized to 0.2 mL of that volume pipetted to represent the intended MC dose. (F) Table containing electrospinning fabrication parameters (polymer concentration and flow rate) and resulting MC dimensions (length, width, and aspect ratio). Aspect ratio calculated by the average length divided by width. Data represented as mean ± standard deviation. MC: microconfetti. ****p < 0.0001.
Figure 19 In Vitro Microconfetti Resiquimod Release Profile and Bioactivity in BMDCs

(A) Percent (%) of resiquimod released over time in PBS at pH 7.4 from small, medium, and large MC. Cytotoxicity of blank MC (B) and resiquimod-loaded MC (C) after incubation with BMDCs for 24 hours as determined by LDH assay. Supernatants from BMDCs treated for 24 hours with blank (open symbols) and resiquimod-loaded (closed symbols) small MC (D), medium MC (E), and large MC (F) were evaluated for TNF-α secretion by ELISA. Independent experiments (n = 2 – 4) in triplicate were performed. Data represented as mean ± standard error of the mean. BMDCs: bone marrow derived dendritic cells, MC: microconfetti, MPs: electrosprayed microparticles, Resi: resiquimod.
<table>
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</thead>
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</tr>
<tr>
<td>Large</td>
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</tr>
</tbody>
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**Table 4 Resiquimod Release from Microconfetti**

Table displaying time in days to 50% of resiquimod released. Data represented as mean ± standard deviation.
Figure 20 Bioactivity of Electrosprayed Microparticles and Soluble Resiquimod

Cytotoxicity and TNF-α cytokine secretion from BMDCs treated with electrosprayed microparticles (MPs) and soluble resiquimod. Cytotoxicity of BMDCs were with MPs (A) and soluble resiquimod (B) for 24 hours in vitro determined by LDH assay. Supernatant was evaluated for TNF-α secretion by ELISA from BMDCs treated for 24 hours with blank (open symbols) and resiquimod-loaded (closed symbols) electrosprayed MPs (C) and soluble resiquimod (D). Independent experiments (n = 2 – 4) in triplicate were performed. Data represented as mean ± standard error of the mean.
**Figure 21 Cytokine Secretion from BMDCs Treated with Microconfetti**

IL-1β (A) and IL-6 (B) secretion from BMDCs treated for 24 hours with blank and resiquimod-loaded MC and resiquimod-loaded electrosprayed MPs. Independent experiments (n = 2) were performed in triplicate were performed. Data represented as mean ± standard error of the mean.
Figure 22 Confocal Microscopy of BMDCs Treated with Microconfetti

Confocal microscopy images of BMDCs treated with 12.5 µg of small, medium, and large BODIPY-MC after 24 hours. Panels from left to right: DIC, CellTracker (blue), LysoTracker (red), BODIPY-MC (green), merged (blue, red, and green). Scale bar is 20 µm. BMDC: bone marrow derived dendritic cells, MC: microconfetti, NT: no treatment.
Figure 23 Microconfetti Internalization by Antigen Presenting Cells

(A) Confocal microscopy of MC internalization by APCs. Representative 3D renderings of z-stacks created in the Imaris microscopy image analysis software displaying MC internalized within BMDCs (A) and RAW 264.7 cells (B). Cells were treated with BODIPY-MC (green) for 24 hours and then stained with CellTracker (blue) and LysoTracker (red). Scale bar is 10 µm. (C) Quantification of MC internalization after 24 hours verified by z-stack. Percent of cells with one or more internalized MC. Data represented as mean ± standard error of the mean. **p < 0.01, ****p < 0.0001.
Figure 24 Microconfetti Internalization by BMDCs, Orthogonal Views

Confocal microscopy images of BMDCs treated with 12.5 µg of small, medium, and large BODIPY-MC after 24 hours. Cells were treated with BODIPY-MC (green) for 24 hours and then stained with CellTracker (blue) and LysoTracker (red). Representative orthogonal projections from z-stacks.
Figure 25 Microconfetti Internalization by BMDCs, 3D Renderings

Confocal microscopy images of BMDCs treated with 12.5 µg of small, medium, and large BODIPY-MC after 24 hours. Cells were treated with BODIPY-MC (green) for 24 hours and then stained with CellTracker (blue) and LysoTracker (red). Representative 3D renderings of z-stacks created in the Imaris microscopy image analysis software displaying MC internalized within BMDCs. Far left panel displays maximum intensity projections of z-stacks and right two panels show 3D surface renderings of the z-stacks at two different rotational views. Scale bar is 10 µm. NT: no treatment.
**Figure 26 Quantification of Microconfetti Uptake by BMDCs**

BMDCs were treated with 12.5 µg of medium and large BODIPY-MC for 24 hours. (A) Average number of internalized MC per cell. White arrows pointing to small MC. Scale bar is 10 µm. (B) Average number of internalized MC among cells with one or more MC internalized (MC\(^+\)). Data represented as mean ± standard error of the mean. **\(p < 0.01\), ****\(p < 0.0001\).
Figure 27 Confocal Microscopy of RAW 264.7 Cells Treated with Microconfetti

Confocal microscopy images of RAW 264.7 cells treated with 12.5 µg of small, medium, and large BODIPY-MC after 24 hours. Panels from left to right: DIC, CellTracker (blue), LysoTracker (red), BODIPY-MC (green), merged (blue, red, and green). Scale bar is 20 µm. NT: no treatment.
Figure 28 Microconfetti Internalization by RAW 264.7 Cells, 3D Renderings

Confocal microscopy images of RAW 264.7 cells treated with 12.5 µg of small, medium, and large BODIPY-MC after 24 hours. Cells were treated with BODIPY-MC (green) for 24 hours and then stained with CellTracker (blue) and LysoTracker (red). Representative 3D renderings of z-stacks created in the Imaris microscopy image analysis software displaying MC internalized within BMDCs. Far left panel displays maximum intensity projections of z-stacks and right two panels show 3D surface renderings of the z-stacks at two different rotational views. Scale bar is 10 µm.

NT: no treatment.
Figure 29 Confocal Microscopy of DC2.4 Cells Treated with Microconfetti

Confocal microscopy images of DC2.4 cells treated with 12.5 µg of small, medium, and large BODIPY-MC after 24 hours. Panels from left to right: DIC, CellTracker (blue), LysoTracker (red), BODIPY-MC (green), merged (blue, red, and green). Scale bar is 20 µm. NT: no treatment.
Figure 30 Ovalbumin Adsorption to Microconfetti Surface and Presentation of Ovalbumin Peptide

(A-B) Adsorption of Texas Red-OVA to the surface of small, medium, and large BODIPY-MC was characterized by flow cytometry. (A) Percent (%) of MC that are OVA+. (B) OVA adsorption represented by OVA median fluorescence intensity (MFI) for MC incubated without and with OVA. Relative MHC I (C) and II (D) presentation of OVA peptide facilitated by blank-MC after 24 hours, normalized to soluble OVA alone and compared to microparticles (MPs). Data represented as mean ± standard deviation. **p < 0.01, ***p < 0.0005, ****p < 0.0001 for comparisons between MC sizes. +++p < 0.0005, ++++p < 0.0001 for comparisons between MC of the same size with and without OVA incubation.
Figure 31 Ovalbumin Adsorption to Microconfetti Surface by Flow Cytometry

Representative flow plots of BODIPY vs. TexasRed-OVA fluorescence for BODIPY-MC incubated with and without TexasRed-OVA, and blank-MC to account for MC autofluorescence were run for all three sizes of MC (small, medium, and large). Overlay of gated blank-MC (red), BODIPY-MC (blue), and BODIPY-MC + TexasRed-OVA (orange).
Figure 32 Correlation of Ovalbumin Adsorption to Microconfetti Surface by Size

Forward scatter (FSC) MFI to approximate MC size. (E) Correlation of OVA adsorption (OVA MFI) with MC size (FSC MFI). Pearson’s correlation coefficient (r) and corresponding p-value are displayed on the graph.
Figure 33 Presentation of Ovalbumin Peptide by Blank and Resiquimod Microconfetti

MHC I (A) and II (B) presentation comparing medium size resiquimod-loaded microconfetti to blank microconfetti.
Figure 34 Microconfetti Depot Formation In Vivo

Female BALB/c mice (6-8 weeks old) obtained from Jackson Labs (Bar Harbor, ME) were injected subcutaneously with medium sized MC loaded with indocyanine green dye (ICG, 0.37% w/w) in the flank with 0.2 mL using a 26-gauge needle at a concentration of 5 mg/mL. Fluorescence of ICG (excitation 710-760 nm and emission 810-875 nm) was monitored on days 1 and 9 using the Perkin Elmer IVIS Lumina In Vivo Imaging System (Waltham, MA). Fluorescence color scale is in radiance (ρ/s/cm²/sr) with limits set at 5.00 x 10⁷ to 5.00 x 10⁹.
Figure 35 Resiquimod Dose Vaccine Study with Medium Sized Microconfetti

Mice were immunized on Day 0 (prime) and Day 21 (boost). Blood draws were done on Days -7 and 28. Total IgG (A), IgG1 (B), and IgG2c (C) endpoint anti-OVA antibody titers from day 28. Titers were log transformed and represented as mean ± standard deviation. ***p < 0.0005, ****p < 0.0001 for comparisons to soluble resiquimod dose counterpart. +p < 0.05, ++p < 0.01, ++++p < 0.0001 for comparisons to OVA only.
Figure 36 Scanning Electron Micrographs of Microconfetti from Resiquimod Dose Ovalbumin Vaccine

Representative scanning electron micrographs of each batch of resiquimod-loaded MC corresponding to their resiquimod dose delivered (10, 1, 0.1, 0.02, 0 µg resiquimod). Scale bar is 25 µm.
Figure 37 Effect of Microconfetti Size on the Humoral Immune Response to Ovalbumin Vaccine

Mice were immunized on Day 0 (prime), Day 21 (boost), and Day 35 (boost). Total IgG (A), and IgG2c (B) endpoint anti-OVA antibody titers from Day 42 serum collection. Titers were log transformed and represented as mean ± standard deviation. *p < 0.05, **p < 0.01, ****p < 0.0001 for comparisons between MC sizes. +p < 0.05 for comparisons to OVA only.
CHAPTER 4: FUTURE DIRECTIONS AND CONCLUSIONS

4.1 Polymeric Biomaterial Scaffolds for Tumoricidal Stem Cell Therapy

4.1.1 Summary

In 2019, the Central Brain Tumor Registry of the United States reported an annual average of 11,833 cases of GBM (199). Unfortunately, GBM has a devastating prognosis with a five-year survival of only 5.6-6.8% (1, 199). As such, new therapeutic strategies are needed to combat tumor recurrence. Because the BBB poses major barrier to the passage of common chemotherapeutics administered systemically, local drug delivery in the surgical resection cavity remains a promising strategy for combating GBM recurrence. However, poor drug diffusion across the brain parenchyma is a current limitation with local drug delivery strategies like drug loaded polymeric implants and direct injection because GBM is a diffuse and invasive tumor. Tumoricidal SC therapy offers several advantages that other locally applied therapies offer, such as bypassing the blood-brain barrier and delivering tumoricidal agents in the resection cavity, near the site where the tumor commonly reoccurs. Because SCs can migrate towards tumor cells in the brain, tumoricidal SC therapy has the potential to overcome drug diffusion limitations. Moreover, a substantial benefit is afforded by using a polymeric biomaterial scaffold for delivery of tumoricidal SCs. As discussed here, scaffolds enhance SC implantation efficiency in the GBM surgical resection cavity and extend the persistence of SCs in the brain. While the benefit of scaffolds has been established by several groups, investigation into the optimal scaffold properties for the function of supporting GBM killing by tumoricidal SCs has only recently begun.
In Chapter 2 the effect of scaffold degradation rate on the persistence of NSCs implanted in the surgical resection cavity was investigated (40). Fast and slow degrading electrospun scaffolds were composed of gelatin and Ace-DEX. The degradation rate of Ace-DEX was tuned by changing the CAC of the polymer, via the reaction time. Scaffold mass loss half-lives of 0.8 days and > 56 days in vitro were investigated. Short-term viability and output of the tumoricidal agent, TRAIL, by mouse NSCs seeded onto electrospun scaffolds in vitro were comparable to NSCs grown on tissue culture treated polystyrene. This suggests that the scaffold did not affect tumoricidal agent output. Importantly, NSC seeding efficiency was within the same range across both scaffolds. This accounts for any potential influence of cell number on persistence time in vivo.

The disparity in degradation rate between the two scaffolds in vivo was confirmed by measuring fluorescence of ICG-loaded scaffolds over time (40). Similar to previous studies, both scaffolds significantly increased the implant efficiency and persistence of mouse NSCs in the brain resection cavity after implantation, compared to NSC direct injection. Notably, scaffold implanted NSCs were still detectable and had a significantly higher BLI signal at day 120 compared to direct injection. While there was a trend of increased NSC persistence with slower scaffold degradation, there was not a statistical difference in time to NSC clearance between fast and slow degrading scaffolds (40). These results highlight the long-term impact on NSC persistence in the brain by supporting their implantation into the surgical resection cavity with a polymeric biomaterial scaffold.

4.1.2 Future Directions

Currently, hydrogels are the most widely explored scaffold type for tumoricidal SC therapy. Characterization of bulk hydrogel properties such as morphology, degradation rate, and
stiffness and their impact on tumoricidal SC function have yet to be explored. These specific hydrogel properties have been shown to influence SC differentiation in other therapeutic applications (77, 79, 200, 201). For example, NSCs generally have shown increased neuronal differentiation and migration when seeded onto hydrogels composed of polymers with high elastic moduli, similar to that of the brain (200, 201). Others have found that bulk hydrogel stiffness can influence NSC migration and direct differentiation. Hydrogels with lower stiffness favored cell spreading/branching and neuronal lineages, and higher stiffnesses enhanced NSC migration and differentiation into astrocytic lineages (79). Since tumoricidal SC therapy using bulk hydrogels heavily relies upon the tumor-tropic migration associated with SCs, it would be beneficial to screen hydrogel parameters to maintain this phenotype. The effect of scaffold properties on other SC functions, such as therapeutic agent output, should be evaluated as well.

Moreover, hydrogel properties could be exploited to enhance migratory SC-mediated GBM killing. Hynes et al. identified a poly-L-lysine/polyethylene glycol hydrogel that resulted in reduced NSC differentiation and enhanced migration (201). In 2017, Addington et al. found that HA-laminin hydrogels increased NSC migratory response to SDF-1 by upregulating the CXCR4 receptor for the application of TBI therapy (202). This pathway is the same pathway involved in SC tumor-tropism; as such, scaffolds composed of HA-laminin could improve SC migration towards distant tumor. Lastly, Cheng et al. developed a hydrogel capable of steadily releasing SCs from the hydrogel over time as it degraded (95). These hydrogel formulations could be evaluated for their ability to maximize tumoricidal SC therapy for GBM.

The potential therapeutic benefit for alginate microcapsule delivered SCs is substantial, as studies have indicated that this strategy offers the ability to utilize a universal SC line over autologous SCs, significantly reducing production time for the therapy. This is supported by the
result that survival of human MSC xenografts in alginate microcapsules was reported for at least eight weeks in immunocompetent animal models (33, 34). Future studies focusing on increasing this time span by optimizing microcapsule properties that have been shown to impact the foreign body response would be beneficial to that end. These properties include cationic polymer coatings, alginate chemical modifications, and changing pore size (92, 203-206). With regard to the type of therapy, anti-angiogenic therapy delivered by alginate encapsulated cells has demonstrated efficacy (30, 33, 34), and application of this therapy in the GBM surgical resection cavity could be beneficial in combating tumor recurrence. Use of alginate microcapsules for SC therapy is an area where further studies can lead to potentially clinically relevant therapies.

Similar to considerations made for hydrogels, further exploration into the optimal electrospun scaffold properties for tumoricidal SC therapy is needed. While electrospun scaffold parameters such as fiber diameter, coating, morphology by Sheets et al. and degradation (Chapter 2) have begun to be investigated for their impact on SC persistence in the resection cavity, studies on the influence of these parameters on SC phenotype and therapeutic efficacy remain to be done (39, 40). However, a substantial amount of work has been done evaluating the influence of electrospun scaffold properties such as polymeric biomaterial choice, fiber width, and fiber alignment on SC differentiation in other fields (101, 104-106, 207). Yang et al. found that PLA electrospun scaffolds with nanofibers increased the rate of differentiation compared to microfibers (207). In contrast, Christopherson et al. found that NSC differentiation was directed towards neurons when cultured on electrospun scaffolds composed of polyethersulfone with 283 nm wide fibers, whereas oligodendrocytes was favored on 749 nm fibers (105). Czeisler et al. found that 10 µm wide electrospun fibers with laminin coating increased NSC migration compared to either 800 nm fibers and 10 µm fibers without laminin (104). These results suggest that the influence of
scaffold properties on SCs are likely dependent on the specific polymeric biomaterial used. Studies on the impact of polymeric biomaterial choice and electrospun scaffold parameters on SC phenotype as it relates to tumoricidal SC function is an important next step. Furthermore, considering the suggested link between enhanced NSC growth in vitro by the 3D gelatin hydrogel and extended persistence in the brain by Sheets et al. (39), investigation into increasing electrospun scaffold porosity to promote SC growth could be beneficial.

4.2 Microconfetti Biopolymer Platform for Vaccine Applications

4.2.1 Summary

Polymeric biomaterials are used to enhance the efficacy of protein and peptide vaccine formulations, by a variety of mechanisms. Nano- and microparticles can be employed to increase antigen presentation by more efficiently shuttling antigen into APCs upon internalization (174, 175). Furthermore, they can encapsulate and deliver adjuvant to stimulate innate cell signaling that initiate different immune responses. On the other hand, scaffold vaccines can create an immunostimulatory depot similar to adjuvants such as MF59 and alum, which extend APC stimulation thereby enhancing the adaptive immune response (149-151). In Chapter 3, we sought to combine these two biomaterials-based approaches in applying Ace-DEX MC as a new vaccine platform.

High-aspect ratio, ribbon-like MC were fabricated by electrospinning followed by high-shear homogenization. This method allowed for precise control over MC fabrication, and three distinct sizes of MC, small (0.67 x 10.2 µm), medium (1.28 x 20.7 µm), and large (5.67 x 90.2 µm), were produced. MC were loaded with the adjuvant, resiquimod. Steady release rates of resiquimod were observed from MC, indicating their ability to create an immunostimulatory depot in vivo. Resiquimod-loaded MC stimulated inflammatory cytokine production in bone marrow
derived dendritic cells without incurring additional cytotoxicity in vitro. Interestingly, even medium and large MC were able to be internalized by antigen presenting cells and facilitate antigen presentation when OVA was adsorbed onto their surface. MC formed a subcutaneous depot upon injection in vivo. Blank MC of all sizes with adsorbed OVA were found to stimulate a humoral response. Adjuvant activity of resiquimod was enhanced by loading it into MC and small and medium sized MC effectively induced a Th1-skewed immune response.

4.2.2 Future Directions

Initial characterization of MC in Chapter 3 demonstrates the potential of MC as a new vaccine platform. The Th1-skewing determined by antibody subtype and enhanced cross presentation in vitro suggests that small and medium sized MC loaded with resiquimod are capable of engaging the cellular immune response. Future work should be done to characterize the cellular immune response in mice immunized with resiquimod-loaded MC, including but not limited to the evaluation of immune cell trafficking to the MC depot and OVA peptide specific T cells in the spleen and draining lymph nodes. Additionally, different adjuvants could be explored to demonstrate the versatility of this platform.

Since MC is composed of Ace-DEX, there is a unique opportunity to explore the effect of MC degradation on the resulting immune response. Collier et al. determined that the molecular weight and relative cyclic acetal coverage of Ace-DEX influenced drug release from MC injected subcutaneously (116). Furthermore, changing the degradation profile of Ace-DEX MPs has been shown to modulate antigen processing in vitro (178) and the adaptive immune response in vivo (179, 208). The MC evaluated in Chapter 3 was composed of 60% relative cyclic acetal coverage Ace-DEX, which is approaching the highest theoretical coverage and falls within the slowest degradation range defined in a number of studies utilizing Ace-DEX (176, 179, 208, 209). For
example, more rapid MC degradation may further enhance MHC I cross presentation, similar to the trend observed with MPs by Broaders et al. (209). In addition to evaluating the effect of MC degradation profile on the resulting immune response, the release of antigen from MC could be modified through different surface conjugation strategies.

Because this is only the first step in evaluating MC as a vaccine platform, MC were assessed in the vaccine model with a model antigen, OVA, which is a protein found in egg whites. Next steps should be to evaluate MC vaccines in a relevant disease model in order to better determine if MC are a viable vaccine. Prior research investigating Ace-DEX MP degradation on the immune response suggests that optimal degradation kinetics may be different based on the antigen, animal model, and disease state (179, 208). Previous scaffold vaccines have most commonly been applied as therapeutic cancer vaccines (163-167, 173). Because scaffolds persist for an extended period of time upon administration, they have the capability to extend immune stimulation. This extended stimulation has been found to be beneficial in generating and maintaining an immune response capable of overcoming the immunosuppressive tumor microenvironment (163). Considering the encouraging immunostimulatory activity of MC in Chapter 3, MC may be well suited for a therapeutic cancer vaccine application as well.

It is also worth exploring the unexpected observation of medium and large MC internalization by APCs in more detail. It is unclear if the unique geometry of the MC facilitates uptake of such long particles or if it is some intrinsic quality of Ace-DEX, such as surface chemistry or mechanical property. However, as PCL and PLGA electrospun scaffolds were unable to withstand the high sheer homogenization process to create MC, some other polymer would need to be identified to explore such a hypothesis. Furthermore, it would be interesting to determine if there are differences within APCs upon internalization of ribbon-like MC compared to spherical particles. We
hypothesize that increased MHC I cross presentation by medium sized MC compared to spherical Ace-DEX particles observed in Chapter 3 was due to a combination of the high internalization frequency of medium MC and adsorption of OVA to the surface of medium MC particles. However, the geometry of the MC could also be playing a role as well, such as increased endosomal rupture, promoting delivery of more OVA to the cytosol or intrinsic changes in cell signaling or gene expression within the cell. Such intrinsic changes that could contribute to increase cross presentation include signaling that results increased expression of MHC I or co-stimulation molecules on the surface of the APC.
1.1 Introduction

Glioblastoma (GBM) is an especially aggressive, grade IV central nervous system tumor. GBM is the most common primary brain tumor and accounts for 45-50% of all gliomas (210), and even with surgical resection, radiation, and chemotherapy, the median survival for GBM patients remains only 12 to 15 months (122, 210, 211). The primary cause of mortality for GBM is local tumor recurrence, with 90 - 95% of tumors recurring within 2 cm of the original tumor (212-215). As such, patient outcomes could be greatly improved by delaying or preventing local recurrence. Unfortunately, chemotherapeutic options are limited due to the inability of most drugs to cross the blood-brain barrier. One strategy to improve drug delivery to GBM and bypass the blood-brain barrier is interstitial chemotherapy. This can be achieved by lining the resection cavity with a material that releases drug as it degrades (216). This strategy is applied clinically with Gliadel®, a polyester and polyanhydride polymer wafer that is used in some GBM patients to deliver carmustine (BCNU) directly into the resection cavity (11). Unfortunately, Gliadel® only extends median survival by 10-18 weeks over placebo (217, 218). This is likely due to the low tumoricidal activity of BCNU against GBM and its rapid clearance from the brain after release from the polymer wafer (61, 219, 220). Additionally, tumor cells easily acquire resistance to alkylating agents like BCNU through the DNA repair mediated by the enzyme O⁶-methylguanine-DNA methyltransferase (MGMT) (221, 222). Low tumoricidal activity, easily acquired drug resistance,
and rapid clearance of BCNU from the brain highlight the need for exploration into alternative anticancer drugs for interstitial therapy.

One promising compound to combat GBM is doxorubicin (DXR). This highly potent chemotherapeutic agent induces cell death via multiple mechanisms of action, including DNA intercalation and interaction with topoisomerase II (223, 224). However, DXR is unable to be administered systemically against GBM due to its inability to pass through the blood-brain barrier and reach therapeutic levels in the brain (225). Interstitial delivery of DXR against GBM has been explored previously with some success. Local delivery of DXR was able to extend median survival and delay tumor growth, but ultimately complete remission was not achieved (226, 227). This is likely due to the sub-optimal DXR release kinetics from the polymeric devices utilized for interstitial therapy. Lesniak et al. has shown that interstitial DXR delivered via a polyanhydride polymer wafer was able to extend median survival of Fisher rats with intracranial glioma from 21 to 45 days, despite the fact that DXR release from the polymer wafer plateaued at approximately 14% after the first 50 hours (226). Lin et al. also found that DXR released from polyester polymer device was able to slow tumor growth in a subcutaneous flank model (227). Similar to the polyanhydride wafers, the polyester device displayed an initial release of DXR that plateaued after the first 2 days. This pattern of stagnant DXR release after an initial period of rapid release over the first few days is also seen in multiple polymer drug delivery platforms including polyanhydrides (226), polyesters (227, 228). and polyethylene glycol polyester composites (229, 230). The therapeutic efficacy of interstitial GBM treatment could be greatly improved with sustained DXR release from an optimal polymer matrix.

Aggressive cancers, such as GBM, recur in a matter of weeks. As such, polymer drug delivery platforms capable of rapid and sustained drug release are vital to suppressing local tumor
recurrence. Most commonly used polymers for interstitial drug delivery, such as polyesters and polyanhydrides, have relatively slow degradation rates on the order of months to years (231, 232). Additionally, polyesters degrade by bulk degradation, which can lead to spontaneous dumping of drug and potential unforeseen toxicity (233). An example of this can be seen in Manome et al., where poly(D,L-lactide-co-glycolide) (PLGA) sheets exhibited a sudden burst release of DXR around 30 days in vitro (234). Finally, polyesters create an acidic microenvironment as they degrade, which can alter drug activity and cause tissue toxicity (235-237). Taken together, these limitations regarding effective drug release and adverse polymer by-products highlight the need for a tunable drug delivery platform to expand the population of drugs that can be utilized for sustained interstitial drug delivery.

An alternative polymer for drug delivery is acetalated dextran (Ace-DEX). Ace-DEX is a novel polymer with tunable degradation rates ranging from days to months. It is synthesized by chemically modifying dextran with 2-ethoxypropene to render the polymer hydrophobic (118, 120). This reaction generates acyclic and cyclic acetals on the pendent hydroxyl groups of dextran in a time-dependent manner (119, 178, 209). The ratio between cyclic and acyclic acetals can be changed based on the length of reaction time (119, 178, 209). Under aqueous conditions, acetal groups are hydrolyzed, revealing the parent hydroxyl groups of dextran and resulting in Ace-DEX degradation. Since cyclic acetals are significantly more stable to hydrolysis than acyclic acetals, the time-dependent nature of acetal coverage allows for tight control of Ace-DEX degradation rate. The degradation by-products of Ace-DEX (dextran, acetone, and ethanol) are pH neutral and occur at exceedingly low concentrations, far below levels that would result in toxicity (119, 178). Our group has previously fabricated drug loaded Ace-DEX nanofibrous scaffolds using electrospinning, and demonstrated tunable drug release and bioactivity (118). The tunability of
Ace-DEX degradation rates combined with the safe, pH-neutral degradation products makes it a promising polymer platform for interstitial drug delivery against GBM. To advance this potential therapy towards translation to the clinic, it is imperative that we evaluate GBM treatment in the most relevant animal model.

The therapeutic efficacy of interstitial therapy against GBM is often tested in a simple subcutaneous flank or orthotopic tumors that fail to incorporate an essential component of multimodal therapy: surgical resection. As surgery is the standard of care for human patients, a preclinical animal model of GBM resection and recurrence is extremely beneficial. This is particularly important because surgical resection induces changes in the peri-tumoral microenvironment and influences tumor biology, growth, and invasion (60). We previously developed a fluorescent-guided surgical mouse model of GBM resection and recurrence (38, 66). With this model, GBM xenografts are implanted orthotopically and allowed to establish for several days. Under fluorescent guidance, approximately 90% of the tumor is removed by surgical resection and interstitial therapy can then be applied at the time of surgery (Figure 38). Here we describe the fabrication and utilization of electrospun DXR loaded polymer scaffolds comprised of Ace-DEX or poly(L-lactide) (PLA), a polyester commonly used for interstitial drug delivery. We then investigated the effect of polymer on DXR release from scaffolds in vitro. DXR release rates were then evaluated in a clinically relevant murine model of local GBM recurrence following partial surgical resection.

1.2 Materials and Methods

1.2.1 Chemicals and Reagents

Dextran (molecular weight 450-650 kDa), anhydrous dimethyl sulfoxide (DMSO, >99.9%), hexafluoro-2-propanol (HFIP, >99%), 1-butanol, triethylamine (TEA, >99%),
pyridinium p-toluenesulfonate (>98%), poly-L-lactide (PLA, molecular weight 260 kDa), thiazolyl blue tetrazolium bromide (MTT), and BCNU (99%) were purchased from Sigma (St. Louis, MO). Doxorubicin hydrochloride (DXR, >99%) was acquired from LCLabs (Boston, MA). 2-ethoxypropene was purchased from Matrix Scientific (Columbia, SC). High glucose Dulbecco's Modified Eagle Medium (DMEM) cell culture medium and fetal bovine serum were obtained from Corning (Corning, NY). Penicillin/streptomycin was purchased from HyClone (Pittsburgh, PA). Rat monoclonal antibodies against CD45 (clone I3/2.3, ab25386), F4/80 (clone BM8, #14-4801-82), and ImmPRESS HRP Anti-Rat Ig (#MP-7444-15) were acquired from Abcam (Cambridge, MA), eBioscience Inc. (San Diego, CA), and Vector Labs (Burlingame, CA), respectively. Rabbit polyclonal antibody against glial fibrillary acidic protein (GFAP, #Z0334) and protein block (#X0909) were both purchased from DAKO/Agilent Technologies (Santa Clara, CA). Bond™ Dewax Solution (AR9222), Bond™ Wash Solution (AR9590), Bond™ Epitope Retrieval Solution 1 (pH 6.0, AR9961), Bond™ Enzyme 1 (RE7160-CE), and Bond™ Polymer Refine Detection (DS9900) were all acquired from Leica Biosystems (Buffalo Grove, IL).

1.2.2 Cell Transfection and Cytotoxicity Assays

Human glioma cell lines U87-MG, LN-18, and LN-229 were purchased from American Type Culture Collection (ATCC; Manassas, VA; #HTB-14, #CRL-2610, and #CRL-2611, respectively). Custom vector synthesis services from Invitrogen (Carlsbad, CA) were utilized to generate mCherry-firefly luciferase (mCh-FL). The construct was packaged as a lentiviral vector in 293T/17 cells using a helper virus-free packaging system as described previously.(238)

Cells were cultured in DMEM medium supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum and maintained in the exponential phase of growth at 37°C under 5% carbon dioxide. Cells were seeded onto 96-well plates at a density of 5 x 10^3 cells per well and
allowed to grow to 50% confluency. DXR, at concentrations of 0.005, 0.025, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, or 50 µM, was incubated with the cells for 48 hours. MTT powder was dissolved in media at a concentration of 0.6 mg/mL and added to treated cells for 3 hours, allowing for metabolically active cells to reduce the MTT salt to form purple formazan crystals. Crystals were dissolved in isopropanol and the absorption at 560 nm was utilized to quantify cell viability normalizing values to an untreated control, using 670 nm as a reference wavelength. Experiments were repeated in triplicate and a best fit trend-line was used to determine the dose required to reduce cell viability by 50%. This procedure was repeated for BCNU at concentrations of 0.01, 0.05, 0.1, 0.25, 0.5, 0.75, and 1 mM.

1.2.3 Acetalated Dextran (Ace-DEX) Synthesis

Ace-DEX was synthesized and characterized as described previously (120). Briefly, lyophilized dextran was dissolved in DMSO in the presence of an acid catalyst, pyridinium p-toluenesulfonate. Dextran was reacted with 2-ethoxypropene under anhydrous conditions for 2 hours, before the reaction was quenched with TEA. Ace-DEX was precipitated in basic water and lyophilized. Afterwards, the polymer was dissolved in ethanol and centrifuged to remove impurities. Ace-DEX was then re-precipitated in basic water, lyophilized, and stored at -20°C until it was used. The relative extent of cyclic acetal coverage, which dictates the degradation rate of the polymer, was calculated via nuclear magnetic resonance (NMR, Varian Inova 400) based on a previously developed method (120).

1.2.4 Scaffold Fabrication

Ace-DEX or PLA was dissolved in organic solvents at 200 mg/mL with 5% or 10% (wt/wt) DXR and loaded into a glass syringe with a blunt 21 gauge needle. A bias of 20 kV (−10 kV to the collection surface and +10 kV to the needle) was applied over a 13 cm working distance at a flow
rate of 1 mL/hr. A tri-solvent system consisting of HFIP, butanol, and TEA was evaluated. Maintaining TEA constant at 1% v/v, HFIP and butanol ratios were varied to create three conditions: 90% HFIP and 10% butanol, 80% HFIP and 20% butanol, and 60% HFIP and 40% butanol.

1.2.5 Scaffold Characterization

Scanning electron microscopy was performed to assess the morphology of the electrospun scaffolds. Scaffolds were mounted on aluminum stubs using carbon tape and imaged at 2 kV on Hitachi S-4700 Cold Cathode Field Emission Scanning Electron Microscope. DXR encapsulation efficiency was defined as the ratio of empirical DXR loaded to theoretical loading. To quantify this, scaffold samples were weighed, dissolved in DMSO, and evaluated by optical absorption at 480 nm. The amount of DXR loaded was then determined by calibration curve. To evaluate DXR release in vitro, approximately 1 mg of scaffold was placed into dialysis cups with a molecular weight cutoff of 7 kDa and added to a sink of phosphate buffered saline (PBS, pH 7.4) stirring at 37°C. At predetermined time points, scaffolds were removed and stored at -20°C. Samples were then lyophilized, dissolved in DMSO, and evaluated by optical absorption at 480 nm. The amount of DXR retained in each sample was determined by a calibration curve.

1.2.6 Animals

Nude BALB/c mice were housed in groups of five in a vivarium maintained on a 12 hour light/dark schedule at 30°C and 50% relative humidity. Food and water were available ad libitum. For all surgical procedures, mice were anesthetized by vapor isoflurane and immobilized on a three point stereotactic frame (Stoelting, Kiel, WA). For all surgeries, subcutaneous carpofen (5 mg/kg) was administered prior to surgery and twice daily for 3 days following surgery for pain management.
All experimental protocols were approved by the Animal Care and Use Committees at The University of North Carolina at Chapel Hill, and care of the mice was in accordance with the standards set forth by the National Institutes of Health Guide for the Care and Use of Laboratory Animals, USDA regulations, and the American Veterinary Medical Association.

1.2.7 Maximum Tolerated Dose

An incision was made in the skin to expose the skull of the mouse. A small circular portion of the skull over the right frontal lobe, approximately 3 mm in diameter, was surgically removed using a bone drill (Ideal Microdrill, Harvard Apparatus, Holliston, MA) and forceps. A cranial window was created by leaving this portion of the skull open. The skin was closed with Vetbond Tissue Adhesive (3M, Maplewood, MN). Several days after cranial windows were established, the dura over the cranial window was cut away, and a section of the right frontal lobe was removed by aspiration creating a pseudo resection cavity approximately 1 to 2 mm deep. DXR loaded Ace-DEX scaffolds were placed in the resection cavity and the skin was closed with tissue adhesive. Mice were evaluated daily for two weeks to assess for gross toxicity as evidenced by weight loss or gait abnormalities. After 14 days, mice were euthanized by cardiac perfusion. Brains were extracted and fixed in formalin in preparation for histology.

1.2.8 Therapeutic Efficacy Against Recurrent GBM

The therapeutic efficacy of drug loaded scaffolds against local GBM recurrence was evaluated in an image-guided surgical model of GBM resection and recurrence as described previously (Figure 38) (60, 66). After establishment of a cranial window as detailed above, mice were again anesthetized by vapor isoflurane and immobilized on a stereotactic frame. Firefly-luciferase and mCherry transfected U87-MG cells were implanted stereotactically (1 x 10^5 cells in 3 µL of phosphate buffered saline at a rate of 1 µL per minute) in the right frontal lobe 2 mm lateral
to the bregma and 0.5 mm below the dura. Tumor growth was monitored by bioluminescent imaging (BLI, Perkin Elmer IVIS Lumina In Vivo Imaging System). Once tumors were visualized as being well established (1 to 2 weeks after tumor implantation), mice were anesthetized and immobilized. The cranial window was exposed, and dura was removed to expose the tumor. Under fluorescent guidance, the tumor was removed by aspiration. Following tumor removal, scaffolds were placed in the surgical cavity and the skin was closed with tissue adhesive. For Ace-DEX/10DXR, this required a total scaffold mass of 1.8 mg (which was equivalent to six, 3 mm circular hole punches). For PLA/10DXR this required a total scaffold mass of 1.65 mg (which was equivalent to four, 3 mm circular hole punches). Equivalent mass for blank polymer scaffolds was implanted. Serial BLI imaging was used to noninvasively quantify tumor growth starting the day after surgical resection and scaffold implantation (Day 1). A total of 42 mice were implanted with U87-MG tumors. Mice were excluded from the study on Day 1 if there was no evidence of tumor by BLI (“complete resection”, n=3), or if the BLI signal was outside two standard deviations of the average (“insufficient resection”, n=3). Mice were also excluded for extra-cranial tumor location, either in the initial tumor placement (n=2), or when the tumors recurred (n=5). Two other mice were also excluded; one began having seizures 41 days after tumor resection despite having no evidence of tumor (statistical significance remained the same regardless of inclusion); another contracted Corynebacterium bovis, which has been shown to affect tumor xenografts and drug sensitivity in nude mice (239). Excluded mice are detailed in Figure 39. This resulted in 27 mice being included in the study with the following breakdown for groups: no treatment control (n=5), Ace-DEX/blank (n=5), PLA/blank (n=6), Ace-DEX/10DXR (n=7), PLA/10DXR (n=4). Prior to resection, Day -1, established tumors had an average BLI signal of $4.1 \times 10^9 \pm 2.7 \times 10^9 \ \rho/\text{sec/cm}^2/\text{sr}$. The average BLI signal after resection, Day 1, was $1.8 \times 10^8 \pm 3.4 \times 10^8 \ \rho/\text{sec/cm}^2/\text{sr}$. Percent of
tumor resected was determined by the following equation: \( \text{Percent Resected} = 100 \times \left(1 - \frac{\text{BLI_{Day 1}}}{\text{BLI_{Day -1}}} \right) \). The average percent of tumor resected for all groups was 95.4% ± 9%. A box plot illustrating pre-resection BLI values for each mouse and a table detailing average BLI prior to resection (Day -1), percent tumor resection, and BLI after resection (Day 1) for each treatment group can be found in Table 5. Mice were evaluated daily and euthanized if they lost more than 15% body weight. The study was conducted for 120 days. After euthanasia by cardiac perfusion, the brain was extracted, formalin fixed and paraffin embedded, and saved for histological evaluation.

1.2.9 Statistical Analysis

Statistical analysis of normalized mouse weights after scaffold implantation was performed by two-way analysis of variance with post-hoc Bonferroni test using GraphPad Prism (La Jolla, CA). Statistical analysis of overall and progression free survival rates was performed by proportional hazards regression with adjustment for the initial tumor size after surgical resection using IBM SPSS Statistics 24 (Chicago, IL).

1.2.10 Histology

Whole mouse brains were fixed in formalin, processed and embedded in paraffin, and step sectioned at 4 \( \mu \text{m} \) thickness using 300 \( \mu \text{m} \) gaps. Sections collected at each level were stained for histopathology using hematoxylin and eosin (H&E) and immunohistochemistry (IHC) to evaluate for signs of toxicity. H&E staining was performed using an Autostainer XL from Leica Biosystems. For IHC, rat monoclonal antibodies against CD45 and F4/80 and rabbit polyclonal antibody against glial fibrillary acidic protein (GFAP) were utilized. IHC was carried out in the fully automated Bond™ Immunostainer (Leica Biosystems Inc). Slides were dewaxed in Bond™ Dewax Solution and hydrated in Bond™ Wash Solution. Antigen retrieval for CD45 and GFAP
was performed for 20 min at 100ºC in Bond™ Epitope Retrieval Solution 1 (pH 6.0) and in Bond™ Enzyme 1 for 5 minutes followed by a 10 minute protein block. After pre-treatment, slides were incubated for 30 minutes with CD45 (1:100) and GFAP (1:2500), and for 1 hour with F4/80 (1:100). Detection of all antibodies was performed using Bond™ Polymer Refine Detection. For CD45 and F4/80, the secondary antibody was replaced with ImmPRESS HRP Anti-Rat Ig. Stained slides were dehydrated and sealed with a glass coverslip. Positive and negative controls (no primary antibody) were included for each antibody. H&E and IHC stained slides were digitally imaged using an Aperio ScanScope XT (Leica Biosystems) with a 20x objective.

1.3 Results

1.3.1 GBM Sensitivity to DXR

Sensitivity to DXR was tested in three GBM cell lines: U87-MG, LN-229, and LN-18, and then compared to BCNU (Figure 40). The concentration required to reduce cell viability by 50% (IC\textsubscript{50}) after 48 hour incubation is listed in Table 6. All three GBM cell lines were more sensitive to DXR than BCNU by at least 200 fold. These are comparable to literature values (240, 241).

1.3.2 Scaffold Fabrication and Characterization

Drug-eluting polymer scaffolds were fabricated by electrospinning. The organic solvent system and drug loading were varied to determine their respective role in DXR release. First, Ace-DEX scaffolds containing a fixed loading of 5% (wt/wt) DXR (Ace-DEX/5DXR) were electrospun varying the solvent system ratio of HFIP to butanol to 90:10, 80:20, and 60:40. The solvent system affected both fiber width and burst release of DXR. Increasing the volume fraction of HFIP generated wider fibers (Figure 41A-C), which correlated with a faster burst release of DXR (Figure 41E), and the scaffolds exhibiting a more vibrant hue of orange, the color of DXR.
The effect of DXR loading on release rate was also investigated. Figure 3a-c illustrates that increasing DXR loading from 5% to 10% (wt/wt) had no effect on fiber morphology. Increasing DXR loading did result in a higher burst release of DXR (Figure 42F); however, afterwards the rate of DXR release is less pronounced (data not shown). To directly compare Ace-DEX to a polyester, one of the most commonly used polymers for drug delivery, we electrospun a scaffold composed of PLA under identical electrospinning conditions with 10% DXR loaded (wt/wt) (PLA/10DXR). Ace-DEX and PLA scaffolds had similar morphology (Figure 42A-E) with high encapsulation efficiencies of $85 \pm 5\%$ and $94 \pm 12\%$, respectively. Evaluating DXR release in vitro over time, PLA/10DXR scaffolds had a higher burst release compared to Ace-DEX/10DXR, with $46 \pm 7.1\%$ and $28 \pm 1.6\%$ of DXR released in the first 24 hours respectively. However, PLA/10DXR scaffolds only released 3% of DXR over the next 34 days. By contrast, Ace-DEX/10DXR released 27% of DXR over the same extended time frame. Although the two scaffolds ultimately released similar amounts of DXR (approximately 50% over 35 days), the steady-state release from Ace-DEX scaffolds is faster than that from PLA, which releases the majority of the DXR within the first 24 hours (Figure 42G, Table).

1.3.3 Maximum Tolerated Dose

Prior to testing the efficacy of DXR loaded Ace-DEX scaffolds against GBM, in vivo toxicity was evaluated. Mice were weighed and evaluated daily for gross signs of toxicity. Scaffold mass, DXR dose, and mouse weight are detailed in Table 7. Mice implanted with Ace-DEX/10DXR scaffolds at 200 $\mu$g per mouse exhibited a 5% decrease in weight (Figure 43). This was notable given that other groups gained approximately 5% weight over this same time period. Mice implanted with the highest DXR dose, 200 $\mu$g, also displayed delayed surgical wound healing compared to other mice, including the second highest dose, 100 $\mu$g (data not shown). When
evaluated by histology, dose-dependent toxicity at doses as low as 100 µg were seen with Ace-DEX/10DXR scaffolds after 14 days (Figure 44). The effect of Ace-DEX(blank) scaffold compared to Ace-DEX/10DXR at the 200 µg dose was also evaluated by IHC staining. Concurrent coronal sections were stained for F4/80, GFAP, and CD45 to show presence of macrophages, activated microglia, and immune cell infiltrates, respectively, at the site of scaffold implantation (Figure 45). As demonstrated by IHC staining, higher levels of DXR led to increased glial cell activation and immune cell infiltrates, primarily macrophages, compared to unloaded Ace-DEX scaffolds.

1.3.4 Efficacy Against Recurrent GBM

Treatment with Ace-DEX/10DXR scaffolds statistically improved survival rates compared to Ace-DEX(blank) (p < 0.005) and ‘no treatment’ (p < 0.005) with four mice surviving to the end of the study (Day 120) (Figure 46A,B). Of these four surviving mice, only one had BLI evidence of tumor at the end of the study. Notably, the quantified BLI for this mouse decreased to just 20% of its original signal the day after resection (Day 1) over the length of the study (120 days) (Figure 46C). In contrast, only one mouse treated with PLA/10DXR survived to the end of the study, and over this time, the tumor BLI signal increased by more than eight fold (Figure 46B,C). Treatment with PLA/10DXR scaffolds statistically improved overall survival rates compared to ‘no treatment’ control group (p < 0.05), but was not significant compared to PLA(blank) (Figure 46A). Tumor recurrence occurred for all mice in the ‘no treatment’ control group. As expected, unloaded polymer scaffolds, Ace-DEX(blank) and PLA(blank), had no effect on GBM recurrence compared to ‘no treatment’ controls. U87-MG tumors regrew at exponential rates for all control mice. BLI measurements throughout the study for each mouse can be found in Figure 47.

Non-invasive imaging with BLI offered the opportunity to track tumor growth in real time. Figure 46D illustrates progression free survival, defining ‘disease progression’ as a five-fold
growth in tumor as measured by BLI. Treatment with Ace-DEX/10DXR scaffolds statistically improved progression-free survival rates compared to Ace-DEX/blank (p < 0.01) and ‘no treatment’ (p < 0.001) with four mice exhibiting no disease progression for the duration of the study. Treatment with PLA/10DXR scaffolds also statistically improved progression-free survival rates compared to ‘no treatment’ (p < 0.01), however all mice exhibited disease progression by day 38 (Table 8).

As shown in Figure 46E, tumor size after resection was quite variable. To attempt a more uniform comparison between treatment groups, mice with similar tumor sizes are highlighted in blue in Figure 46E and their individual tumor growth is graphed in Figure 46F. In this direct comparison, treatment with Ace-DEX/10DXR led to robust tumor suppression. By comparison, treatment with PLA/10DXR led to an initial decrease in tumor size and control of tumor regrowth for the first 2 weeks, but ultimately the tumor growth rate reached exponential rates similar to untreated control mice.

1.4 Discussion

1.4.1 GBM Sensitivity to DXR

Sensitivity to DXR was compared to BCNU, the drug currently used for interstitial GBM therapy in Gliadel® wafers, in three established human GBM cell lines: U87-MG, LN-229, and LN-18 (Table 6). GBM cells lines were found to be approximately 200 fold more sensitive to DXR than BCNU. This illustrates that DXR is highly potent against GBM and could be an enormously beneficial option for GBM patients. However, because systemic treatment with DXR is associated with severe peripheral toxicity, including irreversible cardiotoxicity, combined with the inability of DXR to cross the blood-brain barrier at therapeutic levels, DXR remains an untapped potential for GBM therapy (225, 242).
1.4.2 Scaffold Fabrication and Characterization

We next fabricated drug-eluting polymer scaffolds for interstitial DXR therapy. Scaffolds were fabricated by electrospinning, a technique that generates a thin, flexible, fibrous scaffold which is ideal for implantation in the brain (118). Scaffold flexibility allows the implant to contour to the shape of the resection cavity, maximizing contact surface area, while the thin nature of the scaffold reduces the risk of mass effects. This is in contrast to Gliadel®, which is a hard disk that cannot easily cover contoured surfaces. Importantly, electrospinning is an inexpensive and scalable technique that can be used to control drug delivery kinetics by varying parameters such as solvent system, drug loading, and polymer platform. These variables were separately investigated to determine their role in DXR release.

To ensure sustained DXR release over an extended period of time, a slowly degrading Ace-DEX polymer with 60% relative cyclic acetal coverage was utilized for scaffold generation. First, the organic solvent system and drug loading was varied to determine their respective effects on DXR release from Ace-DEX. Varying the organic solvent system affected both fiber width and burst release of DXR. Increased volume fractions of HFIP generated wider fibers (Figure 41A-C), a faster burst release of DXR (Figure 41E), and brighter orange scaffolds (Figure 41D). These effects are likely from two separate phenomena. The increase in fiber diameter is likely due to the higher volatility of HFIP, leading to more rapid solvent evaporation. The increase in burst release is likely dependent on drug and solvent system compatibility and indicates that DXR is concentrated at the surface of the fiber rather than well distributed throughout (228, 243). This is confirmed visually, as scaffolds with higher burst releases were a more vibrant hue of orange, the color of DXR. Increasing DXR loading from 5% to 10% weight loading led to a higher burst release, likely due to the hydrophilicity of DXR. Since high DXR burst release reduces the overall
drug reservoir for controlled and sustained release, the solvent system with a lower volume fraction of HFIP, 60% HFIP and 40% butanol, was used for the remainder of the studies.

Polyesters are one of the most commonly used polymers for drug delivery (227, 228, 234, 244-250), however their slow degradation rates can be a limiting factor for effective drug delivery. To investigate the role that polymer platform has on DXR release a scaffold composed of PLA was fabricated under identical electrospinning conditions to Ace-DEX. Controlling for fabrication parameters (polymer concentration, DXR loading, solvent system, and flow rate) allows for a direct comparison between Ace-DEX and PLA. Both scaffolds had nearly identical morphologies as visualized by electron microscopy (Figure 42C,E), however, the release rate of DXR varied quite drastically (Figure 42G). PLA/10DXR scaffolds were found to exhibit a high burst release followed by almost no release over the rest of the 5 week study. This is consistent with the literature, where Zeng et al. showed a rapid release of DXR from an electrospun PLA scaffold that stagnated over time (228). By contrast, Ace-DEX/10DXR released approximately 25% as a burst release and then another 27% of DXR in a controlled manner over the remaining 5 weeks. Although the two scaffolds ultimately released similar amounts of DXR (approximately 50% over 35 days), the steady-state release from Ace-DEX scaffolds is faster than that from PLA, which releases the majority of the DXR within the first 24 hours. This differential release rate will allow investigation into the role of DXR release kinetics as determined by polymer platform in controlling GBM recurrence.

1.4.3 Maximum Tolerated Dose

Prior to testing the efficacy of DXR loaded Ace-DEX scaffolds against GBM, in vivo toxicity was evaluated. To mimic tumor treatment, cranial windows and a resection cavity approximately 3mm in diameter and 1 to 2 mm deep were created in the parenchyma of nude mice.
DXR loaded Ace-DEX scaffolds were then implanted into the surgical cavity. To increase the total dose of DXR administered, the scaffold drug loading was maintained and overall mass of scaffold implanted was increased. To achieve the highest DXR dose tested (200 µg), 2.2 mg of total Ace-DEX/10DXR scaffold mass was required. Interestingly, all but the highest dose of DXR, 200 µg, was well tolerated as measured by mouse weight loss. The tolerated dose was higher than expected based on literature where Kooistra et al. found that the highest non-toxic tolerated dose of intrathecal DXR for Sprague Dawley rats was 20 µg (251). This discrepancy may be due to the fact that the dose is not entirely contained intra-cranially (as the cranial window is left open), differences between rats and mice, and the controlled release of drug from the scaffold, compared to bolus delivery. Although by histology, dose-dependent toxicity was seen at doses as low as 100 µg, to maximize therapeutic dose within tolerable toxicity (Figure 43), 150 µg DXR was tested for therapeutic efficacy.

1.4.4 Efficacy Against Recurrent GBM

Surgical resection is part of the standard-of-care for GBM patients. However, the majority of preclinical studies are conducted in mouse or rat models that lack surgical resection of the primary tumor (226, 244, 252-254). To mimic clinical conditions, tumor cells were implanted into the parenchyma of mice. Once tumors were well established, the established primary GBM mass was resected using fluorescent-guided microsurgery. The surgical resection greatly transforms the brain microenvironment with studies showing tumors re-grow more aggressively than the original primary tumor (60). These findings underscore the significance of utilizing this clinically relevant mouse model for pre-clinical GBM therapies.

As expected, tumors for control mice (untreated and blank scaffolds) regrew rapidly. However, treatment with DXR loaded scaffolds composed of both Ace-DEX and PLA led to
statistically improved survival rates compared ‘no treatment’ controls (Figure 46A, Table 8). Despite the slow steady state release of DXR from PLA/10DXR scaffolds, treatment with PLA/10DXR extended median survival from 29 to 63 days over PLA/blank. However, the lack of statistical significance differs from previous reports in the literature where Lesniak et al. (226) and Lin et al. (227) found that DXR loaded polyester or polyanhydride devices were able to significantly extend median survival and delay tumor growth over empty polymer devices. Since overall and progression free survival rates of PLA/10DXR were significant when compared to the ‘no treatment’ control group, the low sample size of our study may be a contributing factor in the lack of statistical significance when compared to PLA/blank. Additionally, the surgical murine model used in this study is unique in its clinical significance, with more aggressive tumor recurrence than primary GBM models. The complexity of this model may have highlighted limitations with PLA delivery of DXR. Moreover, the total therapeutic dose of DXR implanted for interstitial therapy in this work was lower than previous studies (226, 227). This suggests that the ratio of drug to tumor size may be an important factor in interstitial therapy.

As evident in Figure 46E, residual tumor size after resection was quite variable. Unfortunately, because tumors do not grow homogenously and surgical conditions are not equivalent, it is difficult to ensure identical residual tumor size after resection. Although this variation is difficult in a research setting, this model closely mimics the clinical setting where incomplete or insufficient tumor resection outcomes are often a reality. In this way, the range of residual tumors at the start of treatment allows some insight into the robustness of each therapy. Not surprisingly, residual tumor size after surgical resection played a significant role in therapeutic efficacy. For the PLA/10DXR group, mice with larger residual tumors after surgical resection succumbed to tumors faster than those with smaller residual tumors. This is likely due to the slow
steady state release of DXR from PLA scaffolds which allowed tumor growth to outpace DXR release. The success of mice treated with Ace-DEX/10DXR was also dependent on residual tumor size. Mice with smaller residual tumors were more responsive to therapy than those with larger tumors. However, in contrast with PLA/10DXR, the size threshold for tumor recurrence was much higher for Ace-DEX/10DXR.

Due to this residual tumor size discrepancy, a more uniform comparison between therapies can be made by comparing representative mice with similar sized tumors after resection (highlighted in blue in Figure 46E). In this case, PLA/10DXR resulted in an initial decrease in tumor burden, possibly due to the burst release of DXR from the scaffold; however, tumor regrowth ultimately outpaced the slow steady state DXR release from the PLA scaffold (Figure 46F). In contrast, the release of DXR from Ace-DEX/10DXR scaffolds was high enough to result in complete remission for a residual tumor of the same size. This direct comparison helps to highlight the benefits of the higher steady state DXR release from Ace-DEX scaffolds.

1.5 Conclusions

Despite current treatments, local GBM recurrence and associated mortality is almost 100%. This is complicated by the fact that the blood-brain barrier limits efficacy of systemic chemotherapy. To combat recurrence and overcome the blood-brain barrier, local drug delivery to the tumor resection site is highly advantageous. Judicious selection of a polymer platform is essential to ensure sustained drug delivery. The most commonly used polymers for drug delivery are limited by slow degradation rates. Previous reports of polymeric delivery of DXR exhibited similar trends: rapid release of DXR over the first few days followed by slow steady state release. Although this led to improved median survival or tumor growth delays, to our knowledge no complete remissions have been achieved.
Here we addressed the need for an improved polymer platform for sustained interstitial drug delivery to prevent GBM recurrence. DXR was incorporated into nanofibrous scaffolds composed of PLA or Ace-DEX to directly compare the effect of polymer on DXR release rate. Although both DXR loaded scaffolds ultimately release approximately the same amount of DXR, PLA/10DXR scaffolds released the full payload within the first 24 hours, whereas Ace-DEX/10DXR scaffolds exhibited controlled and sustained release of DXR over the same period. PLA/10DXR scaffolds extended progression free survival over ‘no treatment’ control; however, all mice in this treatment group exhibited tumor growth by 38 days after treatment. The higher sustained DXR release from Ace-DEX scaffolds led to more robust suppression of tumor recurrence, leading to complete remission in 43% of mice. Future studies will further explore the tunability of the Ace-DEX polymer platform, to determine the optimal degradation rate to maximally suppress GBM recurrence.
Figure 38 Schematic of Mouse Model of Glioblastoma Resection and Recurrence

Tumors xenografts were implanted and allowed to grow for several days as monitored by bioluminescent imaging (BLI). Established tumors were then surgically resected under fluorescent guidance leaving positive tumor margins. Drug-loaded polymer scaffolds were then implanted into the surgical cavity and the wound was sealed. Post-treatment tumor growth was then monitored with BLI.
<table>
<thead>
<tr>
<th>Mouse ID</th>
<th>Reason for Exclusion</th>
<th>Time of Exclusion</th>
<th>Treatment Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>M124</td>
<td>Complete resection</td>
<td>After Treatment (Day 1)</td>
<td>PLA/10DXR</td>
</tr>
<tr>
<td>M126</td>
<td>Complete resection</td>
<td>After Treatment (Day 1)</td>
<td>PLA/10DXR</td>
</tr>
<tr>
<td>M106</td>
<td>Complete resection</td>
<td>After Treatment (Day 1)</td>
<td>PLA/blank</td>
</tr>
<tr>
<td>M644</td>
<td>Insufficient resection</td>
<td>After Treatment (Day 1)</td>
<td>PLA/10DXR</td>
</tr>
<tr>
<td>M660</td>
<td>Insufficient resection</td>
<td>After Treatment (Day 1)</td>
<td>Ace-DEX/blank</td>
</tr>
<tr>
<td>M651</td>
<td>Insufficient resection</td>
<td>After Treatment (Day 1)</td>
<td>No Treatment</td>
</tr>
<tr>
<td>M122</td>
<td>Tumor implanted extra-cranial</td>
<td>Before Treatment</td>
<td>N/A</td>
</tr>
<tr>
<td>M659</td>
<td>Tumor implanted extra-cranial</td>
<td>Before Treatment</td>
<td>N/A</td>
</tr>
<tr>
<td>M120</td>
<td>Tumor recurred extra-cranial</td>
<td>After Treatment (Day 23)</td>
<td>Ace-DEX/blank</td>
</tr>
<tr>
<td>M105</td>
<td>Tumor recurred extra-cranial</td>
<td>After Treatment (Day 13)</td>
<td>Ace-DEX/blank</td>
</tr>
<tr>
<td>M645</td>
<td>Tumor recurred extra-cranial</td>
<td>After Treatment (Day 30)</td>
<td>PLA/10DXR</td>
</tr>
<tr>
<td>M130</td>
<td>Tumor recurred extra-cranial</td>
<td>After Treatment (Day 27)</td>
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<td>M131</td>
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<td>M109</td>
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<td>After Treatment (Day 41)</td>
<td>Ace-DEX/10DXR</td>
</tr>
<tr>
<td>M132</td>
<td>Corynebacterium bovis</td>
<td>After Treatment (Day 10)</td>
<td>No Treatment</td>
</tr>
</tbody>
</table>

**Table 5 Mice Excluded from Therapeutic Efficacy Study**

Table details mice that were excluded from the study, the reason and time of exclusion, as well as the treatment group.
Figure 39 Tumor Size Prior to Treatment

(Left) Box plot illustrating quantified BLI the day before tumor resection and treatment (Day -1). Each data point represents an individual mouse. BLI units are in radiance ($\mu$/sec/cm²/sr). (Table) Table details average BLI prior to resection (Day -1), percent tumor resection, and BLI after resection (Day 1) for each treatment group.
Figure 40 Sensitivity of Glioblastoma Cell Lines to DXR

Normalized viability of a) U87-MG, b) LN-18, and c) LN-229 cells measured by MTT assay after a 48 hour incubation with DXR (●) or BCNU (△). Data points are the mean ± standard deviation.
Table 6 Sensitivity of Glioblastoma Lines to Chemotherapies

Concentration required to reduce cell viability by 50% (IC$_{50}$) after 48 hour incubation with DXR or BCNU for glioblastoma cell lines U87-MG, LN-18, and LN-229, as measured by MTT assay.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>IC$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DXR</td>
</tr>
<tr>
<td>U87-MG</td>
<td>0.13</td>
</tr>
<tr>
<td>LN-18</td>
<td>0.80</td>
</tr>
<tr>
<td>LN-229</td>
<td>1.10</td>
</tr>
</tbody>
</table>
**Figure 41 Solvent System Effect on Ace-DEX Scaffolds**

Scanning electron micrographs of acetalated dextran 5% (wt/wt) doxorubicin (DXR) (Ace-DEX/5DXR) scaffolds electrospun in a solvent system of hexafluoroisopropanol (HFIP) and butanol with ratios of (A) 90:10, (B) 80:20, and (C) 60:40. (D) Picture of 5% wt/wt loaded DXR scaffolds electrospun with HFIP and butanol with ratios of 90:10, 80:20, and 60:40 (from left to right). (E) Burst release of DXR from Ace-DEX/5DXR scaffolds electrospun with different HFIP to butanol ratios of 90:10 (black square), 80:20 (gray triangle), and 60:40 (white circle). Data points for all graphs are the mean ± standard error of the mean. Scale bar is the same for all images and represents 5 µm.
Figure 42 Morphology and Drug Release from Scaffolds

Scanning electron micrographs of (A) Acetalated dextran blank (Ace-DEX/blank), (B) 5% (wt/wt) doxorubicin (DXR) (Ace-DEX/5DXR), (C) 10% (wt/wt) DXR (Ace-DEX/10DXR), (D) poly lactic acid blank (PLA/blank), and (E) 10% (wt/wt) DXR PLA (PLA/10DXR) scaffolds. Scale bar is the same for all images and represents 5 µm. (F) Burst release of DXR from Ace-DEX/5DXR (○) and Ace-DEX/10DXR (■) electrospun with 60:40 ratio of HFIP to butanol. (G) DXR release from Ace-DEX/10DXR (■) and PLA/10DXR scaffolds (♦). Table describes DXR release kinetics from Ace-DEX/10DXR and PLA/10DXR scaffolds. Burst release = DXR released over the first 24 hours. Data points for all graphs are the mean ± standard error of the mean.
Figure 43 Toxicity of DXR Loaded Scaffolds in Vivo

Weight of mice (normalized to day 0) implanted with unloaded acetalated dextran (Ace-DEX) scaffolds or 10% w/w DXR loaded Ace-DEX scaffolds (Ace-DEX/10DXR) at DXR doses of 0, 50, 100, or 200 µg per a mouse. Statistical analysis by two way ANOVA with post-hoc Bonferroni where *p<0.05 with respect to all other groups. Data points are the mean ± standard deviation.
Table 7 Toxicity of DXR Loaded Scaffolds In Vivo

Mice weight starting on the day of scaffold implantation (day 0, D₀) and end of the study (day 14, D₁₄). The DXR dose as both total mass (µg of DXR per mouse) as well as (mg of DXR per kg) are also detailed.
Figure 44 Histological Toxicity Increasing Doses of DXR in Ace-DEX Scaffolds

Hematoxylin and eosin staining of unloaded Ace-DEX scaffold compared to 10% wt/wt doxorubicin (DXR) loaded Ace-DEX scaffolds with total DXR doses of 12.5, 25, 50, 100, or 200 µg per a mouse. Scale bars are the same for images at each magnification. Black boxes indicate location of magnified region of interest presented in subsequent row. Bright red regions are indicative of pooled blood.
Figure 45 Histological Toxicity of Ace-DEX Scaffolds

Comparison of unloaded Ace-DEX and Ace-DEX/10DXR Scaffolds. Coronal sections of mice euthanized 14 days after scaffold implantation with (Left) Unloaded acetalated dextran (Ace-DEX) scaffold (total scaffold mass of 2.2 mg) and (Right) 10% w/w Ace-DEX/10DXR scaffold (total scaffold mass of 2.2 mg) with total amount of doxorubicin (DXR) equal to 200 µg. Images show sections stained with hematoxylin and eosin (H&E), glial fibrillary acidic protein (GFAP, indicating glial reaction), CD45, (immune cell infiltrates), and F4/80 (macrophages). Scale bars are the equivalent for all images at the same magnification. Black boxes indicate location of magnified region of interest presented in subsequent column.
Figure 46 DXR Scaffold Efficacy Against Glioblastoma

(A) Kaplan-Meier curves of overall survival comparing survival rates of No Treatment (○, n=5), acetalated dextran blank scaffold (Ace-DEX/blank, □, n=5), 10% w/w doxorubicin (DXR) Ace-DEX scaffold (Ace-DEX/10DXR, △, n=7), polylactic acid blank scaffold (PLA/blank, ▼, n=6), 10% w/w DXR PLA scaffold (PLA/10DXR, ◆, n=4). Statistical significance by proportional hazard regression model adjusted for tumor size after resection where *p<0.05 with respect to Ace-DEX/blank, †p<0.05 and ††p<0.005 with respect to No Treatment. (B) Quantified BLI the day after tumor resection (Day 1). Mice that survived to the end of the study have markers which are colored green. (C) BLI images of mice who survived to the end of the study showing the reduction of BLI signal for Ace-DEX/10DXR compared to the increased signal for PLA/10DXR. BLI scale is in radiance (ρ/sec/cm²/sr). (D) Kaplan-Meier curves of progression free survival rates comparing survival of No Treatment (○, n=5), Ace-DEX/blank (□, n=5), Ace-DEX/10DXR (△, n=7), PLA/blank (▼, n=6), PLA/10DXR (◆, n=4). Tumor progression is defined as a five-fold increase in BLI signal. Statistical significance by proportional hazard regression model adjusted for tumor size after resection where **p<0.01 with respect to Ace-DEX/blank, ††p<0.01 and †††p<0.001 with
respect to No Treatment. (E) Quantified BLI the day after tumor resection (Day 1) Markers indicating representative mice with comparable starting tumor sizes are highlighted blue. f) Quantified BLI of a representative mouse from each treatment group illustrating tumor growth over time (highlighted in blue in e). No Treatment (○), Ace-DEX/blank (□), Ace-DEX/10DXR (▲), PLA/blank (▼), PLA/10DXR (◆).
Figure 47 Tumor Growth After Tumor Resection

Quantified bioluminescent signal for each mouse following resection.
Table 8 Statistical Analysis

Proportional Hazard Ratio (Adjusted for Tumor Size), Estimated Hazard Ratios, and 95% Confidence Intervals for Overall and Progression Free Survival Rates Across Treatment Groups.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Overall Survival</th>
<th>Progression Free Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hazard Ratio</td>
<td>P-value</td>
</tr>
<tr>
<td></td>
<td>(95% CI)</td>
<td></td>
</tr>
<tr>
<td>No Treatment</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Ace-DEX/blank</td>
<td>0.81 (0.20-3.27)</td>
<td>0.766</td>
</tr>
<tr>
<td>Ace-DEX/10DXR</td>
<td>0.09 (0.02-0.46)</td>
<td>0.004</td>
</tr>
<tr>
<td>PLA/blank</td>
<td>0.46 (0.12-1.75)</td>
<td>0.254</td>
</tr>
<tr>
<td>PLA/10DXR</td>
<td>0.16 (0.03-0.85)</td>
<td>0.032</td>
</tr>
</tbody>
</table>
2.1 Introduction

Glioblastoma (GBM) is an invasive grade IV brain tumor that accounts for 45-50% of all gliomas (210). The drug delivery obstacles presented by the blood-brain barrier (BBB) unfortunately lead to near 100% mortality, despite treatment with a combination of surgery, radiation, and adjuvant oral chemotherapy (temozolomide, TMZ) (210, 211). With 90 - 95% of GBM recurring within 2 cm of the original tumor (212-215), inhibiting local recurrence could significantly extend patient survival. Local drug delivery is a promising strategy to overcome the BBB and achieve high drug concentrations locally at the desired site (216, 217). One method to achieve this is direct infusion of drug to the tumor via catheter, however, there is a high risk of complications such as infection, catheter blockage, and incorrect placement (25, 26, 255, 256). Alternately, polymers can be used to deliver drugs. One such example is Gliadel®, a polymer wafer that releases the drug carmustine. Gliadel® is the first and only FDA approved polymer implant for interstitial therapy against GBM (11); however, it only extends median survival of patients by 10-18 weeks (217, 218). This could be due to a number of factors including low tumoricidal activity of carmustine, poor drug transport properties of carmustine in brain tissue (220), and sub-optimal release rate of carmustine from the polymer implant (257). The first two potential factors can be addressed by changing the drug; whereas, optimizing release rate is dependent on the drug, polymer, and implant fabrication method.
Paclitaxel (PTX), a microtubule stabilizing agent, is highly potent against GBM and has numerous characteristics that make it an ideal candidate for interstitial therapy (258). Although PTX is unable to cross the BBB when given intravenously (259), which led to failed clinical trials (260, 261), when delivered locally, PTX has improved drug transport properties compared to other chemotherapies (12, 262, 263). Directly compared to carmustine, PTX displayed low elimination within the tumor resection cavity, ten times higher drug concentration in the resection cavity, as well as six times further penetration into the surrounding brain (220). These improved pharmacokinetic factors resulted in positive tumor margins receiving two orders of magnitude higher drug exposure (220). Additionally, while a hindrance in systemic delivery, the hydrophobicity of PTX is a beneficial feature for interstitial drug delivery because it allows for precise release as the polymer degrades.

There appears to be an assumption in the literature that a long and sustained release that still maintains a ‘therapeutic dose’ is optimal for interstitial therapy. Yet, the actual effect of release rate on GBM therapy has not been rigorously explored. This may be due to the fact that many commonly used polymers, such as poly(lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA), utilized for interstitial therapy are limited to slow degradation rates on the order of months. Furthermore, despite efforts to modify formulation to increase the rate of PTX delivery, the steady state release rate from these polymer implants remains ≤1% per day (Figure 48A, C) (228, 244, 245, 247-249, 262).

While polyester polymer platforms are ideal for slow and sustained release, there is a need to investigate a broad range of faster release rates. This could be addressed through the use of acetalated dextran (Ace-DEX), a biodegradable polymer with tunable degradation rates ranging from hours to weeks (120). Ace-DEX is a versatile polymer platform which has been extensively
utilized for nanoparticle and microparticle vaccine delivery formulations (177). In addition to tunable degradation, Ace-DEX polymer is acid sensitive, which could also prove beneficial for interstitial therapy against GBM as Ace-DEX implants could respond to the lower pH of the tumor microenvironment with an increase in drug release (264, 265).

In this work, we vary drug release rate by changing polymer composition while maintaining scaffold formulation to pinpoint the role of PTX delivery rate on efficacy of interstitial therapy. Furthermore, we investigated the impact of the physiological pH of the GBM tumor microenvironment on the release of PTX from polymeric implants. PTX-loaded scaffolds were tested in orthotopic mouse models simulating two clinical situations: (1) primary tumor resection and subsequent recurrence, and (2) a distant tumor metastasis in the brain. Additionally, we evaluated the acid-sensitivity of the Ace-DEX scaffold in response to the tumor microenvironment in vitro and in vivo.

2.2 Methods

2.2.1 Chemicals and Reagents

Dextran (molecular weight 450-650 kDa), anhydrous dimethyl sulfoxide (DMSO, >99.9%), pyridinium p-toluenesulfonate (>98%), triethylamine (TEA, >99%), hexafluoro-2-propanol (HFIP, >99%), 1-butanol, poly-L-lactide (PLA, molecular weight 260 kDa), and phosphate buffer pH 6.8 were purchased from Sigma (St. Louis, MO). LE agarose was obtained from VWR (Radnor, PA). Paclitaxel (PTX, >99%) was acquired from Medkoo Biosciences Inc (Morrisville, NC). 2-ethoxypropene was obtained from Matrix Scientific (Columbia, SC). High glucose Dulbecco's Modified Eagle Medium (DMEM) cell culture medium and fetal bovine serum were purchased from Corning (Corning, NY). Penicillin/streptomycin was acquired from Hyclone (Pittsburgh, PA).
Cell transfection

Human glioma cell line U87-MG was purchased from American Type Culture Collection (ATCC; Manassas, VA; #HTB-14). mCherry-firefly luciferase (mCh-FL) was acquired from Invitrogen (Carlsbad, CA). The vector was packaged as a lentivirus in 293T/17 cells as described previously. U87-MG cells were transfected for 24 hours and purified by puromycin selection. Cells were maintained in at 37°C in DMEM supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum.

2.2.2 Acetalated Dextran Synthesis

Ace-DEX was synthesized and characterized as described previously (119). In short, lyophilized dextran and acid catalyst, pyridinium p-toluenesulfonate (1.55% wt/wt), were dissolved in DMSO. Dextran was reacted with 2-ethoxypropene (20% v/v) under anhydrous conditions for 20 minutes, 2 hours, or 3.5 hours to yield fast, medium and slow degrading Ace-DEX, respectively. These polymers are termed: Ace-DEX/fast, Ace-DEX/med, and Ace-DEX/slow. The reaction was quenched with triethylamine (TEA). The resulting polymer was then precipitated in basic water (400 µL of TEA in 1 L of DI water, pH 9) and lyophilized. Next, the polymer was dissolved in ethanol and centrifuged to remove impurities. The resulting Ace-DEX was re-precipitated in basic water, lyophilized, and stored at -20°C. Relative cyclic acetal coverage was calculated via nuclear magnetic resonance (NMR, Varian Inova 400, Figure 49) as previously described. Briefly, Ace-DEX polymer is degraded in deuterium oxide and deuterium chloride resulting in the degradation products: dextran, ethanol, and acetone. Acetone and ethanol peaks were integrated and normalized to the glucose peak (integration = 1.00). Since the degradation of one cyclic acetal produces one acetone molecule and the degradation of one acyclic acetal produces one acetone.
and one ethanol molecule, the percentage of cyclic acetal relative to acyclic acetal coverages can be determined.

2.2.3 Paclitaxel-Loaded Scaffold Fabrication

Ace-DEX or PLA was dissolved at a concentration of 200 mg/mL in a tri-solvent system consisting of HFIP, butanol, and TEA at a ratio of 59%, 40%, 1% v/v respectively. PTX was added at 20% wt/wt. The polymer-PTX solution was loaded into a glass syringe with a blunt 21-gauge needle and slowly extruded using a syringe pump at a flow rate of 1 mL per hour. A voltage bias of 20 kV was applied over a 13 cm working distance and the resulting scaffold was collected and stored at -20°C until further use. A PTX-loaded Ace-DEX scaffold (Ace-PTX) was electrospun from each Ace-DEX polymer with varying degradation rates (Ace-DEX/fast, Ace-DEX/med, and Ace-DEX/slow), to generate three distinct scaffolds, termed Ace-PTX/fast, Ace-PTX/med, and Ace-PTX/slow, respectively. PTX-loaded PLA scaffolds are termed PLA-PTX/v.slow.

2.2.4 Scaffold Characterization

Scanning electron microscopy (SEM) was used to evaluate scaffold morphology. Palladium sputter coated scaffolds were imaged at 2 kV on a Hitachi S-4700 Cold Cathode Field Emission Scanning Electron Microscope. Fiber diameter was measured using ImageJ.

PTX encapsulation efficiency (EE) was defined as the ratio of measured PTX loaded to theoretical loading, using the following equation: $EE = \frac{\text{Measured PTX}}{\text{Theoretical PTX}} \times 100$. The theoretical PTX loading for each scaffold was 20%. To measure actual PTX content within each scaffold, six samples (approximately 1 to 2 mg each) were cut from each scaffold, weighed, dissolved in acetonitrile at a known concentration, and evaluated by high performance liquid chromatography (HPLC, Agilent Technologies, Santa Clara, CA). HPLC used an Aquasil C18 column (4.5 x 150 mm) with acetonitrile and water at a ratio of 90% and 10% v/v as an eluent at a rate of 1 mL per
minute. PTX was detected at 227 nm and a retention time of 1.9 minutes. The concentration of PTX was determined by calibration curve.

2.2.5 In Vitro Drug Release and Mass Loss Studies

PTX release from scaffolds in vitro was performed as described previously (102). Briefly, three pre-weighed scaffold samples for each time point were added to a sink of phosphate buffered saline (PBS, pH 7.4) stirring at 37°C. At specific time points, samples were removed, washed with basic water to remove salts from PBS solution. Scaffold samples were lyophilized and re-weighed to establish the percentage of scaffold mass lost. The scaffold sample was then dissolved in acetonitrile and evaluated by HPLC to determine the amount of PTX retained in each sample. This process was repeated using phosphate buffer at pH 6.8 to evaluate the effect of pH of scaffold degradation and PTX release.

PTX released from scaffolds in the presence of GBM cells was tested in vitro using a 3D agar assay (Figure 50). Thick agar gels were created by adding 0.5 mL of 0.5% wt/v agar dissolved in 50% complete DMEM media and 50% PBS to a 48 well plate and allowed to solidify at 4°C overnight. Thin agar gels were created similarly by adding 0.2 mL of agar to a 48 well plate. Thick agar gels were then placed in a 24 well plate in 0.5 mL of 50% complete DMEM media and 50% PBS. U87-MG cells were implanted into thick agar gels by injecting 1 µL containing 5 x 10⁴ cells in a 3 x 3 grid approximately 2 mm below the surface of the agar. Pre-weighed PTX-loaded scaffolds were placed on the thick agar gels and covered with thin agar gels. The media surrounding gels was replaced every 2 to 3 days and scaffolds were removed after 3, 7, or 14 days and lyophilized. This process was repeated using agar gels not containing U87-MG cells. HPLC was utilized to determine remaining PTX content. The area under the curve was approximated by trapezoidal rule.
2.2.6 Animals

Female athymic nude mice (Crl:NU(NCr)-Foxn1^nu) obtained from University of North Carolina Chapel Hill Animal Studies Core were used for in vivo experiments. All experimental protocols were approved by the Animal Care and Use Committees at The University of North Carolina at Chapel Hill, and care of the mice was in accordance with the standards set forth by the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the American Veterinary Medical Association. For all surgical procedures, mice were anesthetized by vapor isoflurane and stabilized on a three-point stereotactic platform. Meloxicam (5 mg/kg, subcutaneous) was administered prior to surgery and once daily for the following 3 days for pain management.

2.2.7 Pharmacokinetic Study

To evaluate pharmacokinetics of PTX released from scaffolds in the brain in mice, PTX loaded scaffolds were implanted into a resection cavity made in the right frontal lobe. To achieve this, the cranium was exposed and a small circular portion of the skull over the right frontal lobe was removed using a bone drill (Ideal Microdrill, Harvard Apparatus, Holliston, MA). A resection cavity (approximately 3 mm in diameter and 1-2 mm deep) was created by aspiration of superficial brain tissue. Scaffolds with a total dose of 200 µg PTX were implanted into the resection cavity and the skin was closed with Vetbond tissue adhesive (3M, Maplewood, MN). Mice were then euthanized at specified time points. At the time of euthanasia, brains and remaining scaffold were removed. The brain was cut in half along the longitudinal fissure separating the two hemispheres. Brain tissue was snap frozen, weighed, and homogenized in PBS using a VWR bead mill homogenizer. PTX was extracted from tissue using protein precipitation in methanol and 1% formic acid, dried under nitrogen, reconstituted in 50% acetonitrile and 0.1% formic acid, and
evaluated by LC-MS/MS to quantify the amount of PTX in each hemisphere. The lower limit of quantification was 12.7 ng of PTX per gram of tissue.

### 2.2.8 In Vivo Release With and Without Tumors

To determine if an intracranial tumor affected PTX release, mice bearing U87-MG tumors underwent partial tumor resection and had either Ace-PTX/med or PLA-PTX/v.slow scaffolds implanted in the resection cavity. Mice were euthanized at days 10, 14, or 21, at which point scaffolds were removed. Removed scaffolds were lyophilized, dissolved in acetonitrile, and evaluated by HPLC against a standard curve to determine remaining PTX. Additionally, from the glioblastoma resection model, at the time of euthanasia for tumor burden, scaffolds were removed and remaining PTX was measured by HPLC. Similarly, Ace-PTX/med and PLA-PTX/v.slow scaffolds were placed into a resection cavity made in the right frontal lobe of mice without tumors. At specified timepoints (Ace-PTX/med: 1, 7, 10, 14, 21, 28 days; PLA-PTX/v.slow: 1, 7, 10, 21, 28, 75, 85 days) mice were euthanized and scaffolds removed. PTX content remaining in scaffolds was determined by HPLC. Linear trendlines were fitted to the data and compared to scaffolds removed from tumor bearing mice.

### 2.2.9 Histology

The histologic effect of PTX-loaded scaffolds on the brain after seven days was evaluated in nude mice. Similar to in vivo release and biodistribution studies, scaffolds are implanted into a resection cavity made in the right frontal lobe. Scaffold masses corresponding with two doses of PTX are utilized, a low dose of 75 µg and a higher dose of 200 µg (corresponding to the doses utilized for the resection model and the distant metastasis model). These doses are approximately equivalent to 3.5 and 10 mg/kg. Mice with resection cavities but without any scaffold implanted and mice implanted with Ace-DEX/blank scaffolds were utilized as controls. After seven days,
mice were euthanized by cardiac perfusion. Brains were removed, formalin fixed, and paraffin embedded. Brain tissues were sectioned through the resection cavity in the coronal plane and stained with hematoxylin and eosin.

### 2.2.10 Glioblastoma Resection Model

A model of GBM resection and recurrence was used to evaluate PTX-loaded scaffolds as described previously (102). Briefly, a vertical incision was made to expose the cranium of the mouse and a small circular portion of the skull over the right frontal lobe was removed using a bone drill. Leaving the skull open to establish a cranial window, the skin was closed with Vetbond tissue adhesive. One week later, firefly-luciferase and mCherry transfected U87-MG cells were implanted ($1 \times 10^5$ cells in 2 µL of PBS at a rate of 1 µL per minute) within the cranial window approximately 0.5 mm below the dura. Tumor growth was monitored by bioluminescent imaging (BLI, Perkin Elmer IVIS Lumina In Vivo Imaging System). Eight days after tumor implantation, under fluorescent guidance, most of the tumor was removed by aspiration. Following tumor removal, scaffolds were placed in the surgical cavity and the skin was closed with tissue adhesive. For placement of the scaffolds in the cranium of mice, circular punches 3 mm in diameter were taken from the larger scaffold. The total scaffold mass required to achieve a PTX dose of 75 µg was determined by weight and required 0.38 mg, 0.38 mg, 0.41 mg, and 0.38 mg for Ace-PTX/fast, Ace-PTX/med, Ace-PTX/slow, and PLA-PTX/v.slow respectively.

A total of 75 mice were injected with tumors, randomized to treatment group, underwent tumor resection, and had scaffolds implanted. The total dose of PTX implanted per mouse via scaffolds was 75 µg. Mice were excluded from the study if the BLI signal on Day 1 was outside two standard deviations of the average (“insufficient resection”, n=9) or if the tumor regrew on the external portion of the cranium, rather than internally (n=4). One mouse was also excluded for
incorrect initial tumor placement. Excluded mice are detailed in Error! Reference source not found.. This resulted in 61 mice being included in the study with the following breakdown for groups: no treatment (n=5), Ace/blank (n=6), PLA/blank (n=5), Ace-PTX/fast (n=9), Ace-PTX/med (n=10), Ace-PTX/slow (n=8), PLA-PTX/v.slow (n=9). An additional group, termed ½ Ace-PTX/fast + ½ Ace-PTX/slow (n=9), was included which utilized half a dose of Ace-PTX/fast scaffold and half a dose of Ace-PTX/slow scaffold both placed in the resection cavity (maintaining the overall dose of PTX at 75 µg). Mice were evaluated daily and euthanized if they lost more than 15% body weight or exhibited signs of distress or gait abnormalities. The study was conducted for 75 days.

2.2.11 Distant Metastasis Glioblastoma Model

A vertical incision was made to expose the cranium of the mouse. A burr hole was made in the left frontal lobe approximately 3 mm lateral of bregma and U87-MG cells were implanted (5 x 10^3 cells in 1 µL of PBS at a rate of 1 µL per minute). During the same surgery, an approximately 3 mm circular piece of the skull over the right frontal lobe was removed using a bone drill. A resection cavity approximately 2 mm deep was created using aspiration to mimic primary tumor debulking. PTX-loaded scaffolds were placed in the surgical cavity and the skin was closed with tissue adhesive.

A total of 20 mice were utilized for this study. The total dose of PTX implanted per mouse via scaffolds was 200 µg. Two mice were excluded from the study as statistical outliers (excluded by interquartile range). This resulted in 18 mice being included in the study with the following breakdown for groups: Ace/blank (n=4), Ace-PTX/fast (n=5), Ace-PTX/slow (n=4), and ½ Ace-PTX/fast + ½ Ace-PTX/slow (n=5). Mice were evaluated daily and euthanized if they lost more than 15% body weight or exhibited signs of distress or gait abnormalities.
2.2.12 Statistical Analysis

Statistical analysis was performed using GraphPad Prism (La Jolla, CA). Paclitaxel release from scaffolds at pH 7.4 and 6.8 was compared by linear regression. Area under the curve of paclitaxel release on 3D agar gels was performed by Student t-test. In vivo paclitaxel release with and without tumors was compared by linear regression. Normalized bioluminescence was analyzed by two tailed Mann Whitney U test. Survival curves were evaluated by Log-Rank (Mantel-Cox) test.

2.3 Results and Discussion

2.3.1 Effect of Polymer Scaffold on Paclitaxel Release

GBM is a near fatal diagnosis, with minimal therapeutic options due its location behind the BBB. Interstitial drug delivery of PTX via polymer implant is a promising approach; however, the role of PTX release rate has yet to be thoroughly examined. Ace-DEX is a tunable polymer platform that will allow investigation into faster PTX release rates so that the role of PTX delivery rate on efficacy of interstitial therapy against GBM can be better understood.

Ace-DEX is a biodegradable polymer produced by chemically modifying dextran with 2-ethoxypropene which adds acyclic and cyclic acetals to the polymer backbone in a time-dependent manner (119, 120, 178). Due to the variable hydrolysis rate of cyclic and acyclic acetals, the time-dependent nature of acetal coverage allows for tight control of Ace-DEX degradation. Longer reaction times result in more cyclic acetals, which are significantly more stable to hydrolysis than acyclic acetals. Varying the reaction time from for 20 minutes, 2 hours, or 3.5 hours, resulted in Ace-DEX polymer with 46% (low), 52% (medium), and 61% (high) cyclic acetal coverage (CAC) as calculated by NMR (Figure 49). Due to the inverse relationship between CAC and degradation, these corresponded to fast, medium, and slow Ace-DEX polymer degradation.
rates, respectively. PLA was used to provide an established polymer with a very-slow degradation rate.

Poly-(14) copolymer (PCPP-SA 20:80), the polymer platform of Gliadel®, has also been explored for PTX delivery against GBM. Walter et al. formulated PTX within a PCPP-SA (20:80) polymer using compression molding to generate 11 mg wafers 2.5 mm in diameter, which extended median survival in a rat model of glioma (252). However, when this formulation was scaled for primate testing (increasing the wafer to 200 mg and 10 mm in diameter) the reduction of the surface area to volume ratio led to a significant reduction in PTX release rate (Figure 48B,D) (12, 252). To avoid this translational impediment, polymer implants can be formulated with a nanostructure that will maintain a high surface area to volume ratio and ensure consistent drug release kinetics when scaled.

Three distinct PTX-loaded Ace-DEX scaffolds (Ace-PTX) were fabricated by electrospinning using three separate Ace-DEX polymers with varying degradation rates (fast, medium, slow). The resulting PTX-loaded scaffolds are termed Ace-PTX/fast, Ace-PTX/med, and Ace-PTX/slow, respectively. PTX-loaded PLA scaffolds were similarly fabricated and termed PLA-PTX/v.slow. Electrospinning generates thin, flexible, nano- or micro-fibrous scaffolds. In addition to scalability, electrospun scaffolds are ideal for implantation in the soft tissue of the brain. The thin nature of electrospun scaffolds reduces the risk of mass effects, while its compliance allows the scaffold to easily conform to the resection cavity thereby maximizing contact surface area. All scaffolds were fabricated under identical parameters (polymer concentration, drug loading, solvent system, and flow rate), varying only the polymer platform, to result in comparable morphology (Figure 51A-D). Fiber diameter was evaluated (Figure 51G) and on average ranged from 0.56 to 1.32 µm. Interestingly, there was a decreasing trend in fiber
diameter with decreasing release rate. PTX-loading had similar high encapsulation efficiencies: Ace-PTX/fast (99%), Ace-PTX/med (98%), Ace-PTX/slow (92%), and PLA-PTX/v.slow (98%). The polymer utilized led to drastically different PTX release rates (Figure 51E) that correlated tightly with scaffold degradation (Figure 51F). For Ace-DEX scaffolds, changing polymer reaction time from 20 minutes to 3.5 hours led to in vitro PTX release ranging from 14.1, 2.9, and 1.3 percent per day. Limited PTX was released from PLA scaffolds in vitro which is in agreement with literature, wherein release studies were performed in the absence of proteinase K (228).

2.3.2 Effect of Polymer Scaffold on Pharmacokinetics and Histology

The pharmacokinetics of PTX released from scaffolds in the brain over time was evaluated in mice. To mimic clinical standards, scaffolds were implanted within a resection cavity in the right hemisphere of the brain (Figure 52A). This is important, as studies have shown this type of traumatic brain edema to greatly affect drug diffusion (61, 266). At specified timepoints from 1 to 28 days, mice were euthanized and the PTX exposure in the 2 hemispheres was measured by LC-MS/MS. Ace-PTX/fast scaffolds resulted in the highest exposure of PTX in both left and right hemispheres (Figure 52B-D). Within 1 day, Ace-PTX/fast resulted in higher PTX concentrations within the right, scaffold-bearing hemisphere than achieved with all other scaffolds over 28 days. Additionally, on day 7, Ace-PTX/fast had at least 50-fold higher PTX concentration compared to other scaffolds (Figure 52C). In the other extreme, PLA-PTX/v.slow led to the lowest PTX concentrations within the right hemisphere and had PTX concentration below level of quantification (12.7 ng/g) in the left hemisphere for all timepoints except day 7 (Figure 52B-C). At early timepoints (days 1 and 7), Ace-PTX/med and Ace-PTX/slow had comparable PTX concentrations within the right hemisphere. However, at days 14 and 28, Ace-PTX/med resulted
in higher PTX concentrations compared to Ace-PTX/slow. Within the left hemisphere, Ace-PTX/med led to higher concentrations of PTX on days 1, 14, and 28 compared to Ace-PTX/slow.

PTX distribution in the brain from polymer scaffolds has been explored by other groups (244, 247, 252). Notably, Ranganath et al. reported biodistribution over time in a murine brain from a scaffold (implanting a total dose of 200 µg PTX) with analogous release rates to Ace-PTX/slow (244). Although it is difficult to directly compare results since the brain tissue was sectioned differently, their results appear to show much higher levels of PTX being retained in the brain and the concentration increased over time. This may be because their experimental model did not include a resection cavity, and as such, the implanted scaffold was further away from the brain ventricles which would affect drug retention and diffusion. Similarly, Lee et al. evaluated PTX content in the murine brain 28 days after scaffold implantation (247). This scaffold had a similar in vitro release rate to PLA-PTX/v.slow and also implanted a total dose of 200 µg of PTX. Like Ranganath et al., comparisons are difficult due to the differences in tissue sectioning and lack of resection cavity; however, this group also illustrated higher concentrations of PTX retained in the brain. Lastly, Walter et al. assessed PTX concentration in a rat brain released from a polymer implant (252). The in vitro release rate of PTX from this implant was not linear and therefore less comparable to the current work.

The effect of PTX loaded scaffolds on brain histology was evaluated after seven days at total dose of 75 µg and 200 µg of PTX (Figure 53). There were no gross histological effects with increasing dose or with increased release rate at this timepoint. Additionally, mice were evaluated daily throughout this period and maintained a body condition score of 3 with no hunching, head tilt, gait abnormalities, or other signs of toxicity. While systemic toxicity was not directly investigated, the total overall PTX dosage given via scaffolds was 75 µg or 200 µg (3.75 mg/kg,
10 mg/kg). Notably, this dose was given over a sustained period of time as dependent on scaffold release rate. In the literature, the maximum tolerated dose for bolus Taxol® (6 mg/mL of paclitaxel formulated in a 1:1 ratio of ethanol and Cremaphor EL) is 20 mg/kg and most of the observed toxicity is attributed to the vehicle (267). When PTX is delivered within liposomes or polymer particles, the associated toxicities are greatly diminished (268, 269). As such, it is likely that with the local administration of PTX at a lower overall dose given over a sustained timeframe via scaffolds without Crephamor EL or ethanol, there will be limited systemic toxicity.

2.3.3 Effect of Paclitaxel Release Rate on Post-Surgical GBM Recurrence

To determine the therapeutic effect of PTX release rate against local tumor recurrence, scaffolds were evaluated in a clinically relevant surgical model of GBM (Figure 54A). First, a large fluorescent and bioluminescent tumor was allowed to establish for 8 days in the right frontal lobe of nude mice. The tumor was then partially removed under fluorescent guidance leaving behind positive tumor margins that rapidly regrows if left untreated. At the time of surgery, mice were randomized to treatment group and blank polymer scaffold controls or PTX-loaded scaffolds were placed in the resection cavity with the following groups: (1) No Treatment (resection cavity left empty), (2) Ace/blank, (3) PLA/blank, (4) Ace-PTX/fast, (5) Ace-PTX/med, (6) Ace-PTX/slow, (7) PLA-PTX/v.slow, and (8) ½ Ace-PTX/fast + ½ Ace-PTX/slow. The average percent of tumor resected for all groups was 98.2 ± 3.6%. Tumor BLI for each group before and after resection can be found in Figure 55A-B. Treatment group (8) ½ Ace-PTX/fast + ½ Ace-PTX/slow is made with half a dose of each individual scaffold, as such they will retain their original release kinetics. The release of 75 µg of PTX for this group has been modeled compared to the three other Ace-PTX scaffolds (Figure 55C).
Treatment with Ace-PTX/fast, Ace-PTX/slow, and the combination of ½ Ace-PTX/fast and ½ Ace-PTX/slow scaffolds together (maintaining a total dose of 75 µg PTX) led to statistically significant reduction in tumor growth by bioluminescence over controls (Figure 54C). Representative bioluminescent images for PTX scaffold treatment groups compared to No Treatment control are shown in Figure 54B. Additionally, the combination of ½ fast and ½ slow PTX release significantly inhibited tumor recurrence compared to all PTX-loaded scaffolds with singular release rates. Interestingly, Ace-PTX/med and PLA-PTX/v.slow did not significantly affect average tumor growth compared to controls (Figure 54C). This could be due to the variability between mice in each group, with a range of complete responders and non-responders leading to large standard deviations. Because of this, survival can give a more accurate view of the percentage of mice responding to each PTX release rate. When compared this way, PTX-loaded scaffolds of all release rates were able to statistically improve overall survival with respect to No Treatment control (p <0.001), but there was no statistical difference between singular release rates (Figure 54D). Ace-PTX/fast and Ace-PTX/slow were the singular release rates that led to the highest long-term overall survivals of 44% and 50%, respectively. We hypothesize that this may be due to two separate mechanisms; Ace-PTX/fast is able to rapidly control remaining post-surgical tumor margins, whereas Ace-PTX/slow offers long term GBM tumor control through sustained PTX release. Combining the strengths of both the fast and slow release rates, the combination group where half the PTX dose is delivered via Ace-PTX/fast scaffolds and the other half of the dose is delivered via Ace-PTX/slow led to the highest overall survival of 78%. This was statistically significant compared to control groups, No Treatment and Ace-DEX/blank (p < 0.0001), as well as treatment groups, Ace-PTX/med and PLA-PTX/v.slow (p < 0.02). Ace-PTX/med and PLA-PTX/v.slow had the lowest long-term survival from the treatment groups, with
only 20% and 22%, respectively. For PLA-PTX/v.slow we hypothesize that the very slow release rate may have led to a sub-therapeutic dose. However, the relatively low overall survival rate of Ace-PTX/med was surprising. It is possible that the release rate was both too slow to address positive tumor margins while being too quick to maintain longer term tumor suppression. While a very slow and sustained release of PTX from polyester implants has been shown in the literature to be beneficial (244, 245), utilizing Ace-DEX we were able to expand the range of faster release rates and demonstrate that these can result in profound differences in survival and tumor growth.

2.3.4 Effect of Paclitaxel Release Rate on Untreated Distant GBM Metastasis

To evaluate if PTX loaded scaffolds could affect a distant GBM tumor, the three best release rates, (1) Ace-PTX/fast, (2) Ace-PTX/slow, (3) ½ Ace-PTX/fast + ½ Ace-PTX/slow were evaluated in a newly developed contralateral hemisphere model of GBM (Figure 56A). A small tumor was implanted in the left frontal lobe at the same time as scaffolds were implanted into a resection cavity in the right frontal lobe approximately ~0.5 cm apart. This model is clinically relevant because 90-95% of GBM tumors recur with 2 cm of the original tumor location (212-215). Additionally, by including a surgical cavity in the right frontal lobe, we are still generating the irregular fluid flow created by a primary tumor resection that is consistent with clinical setting. In this model, only Ace-PTX/fast achieved statistically improved survival compared to a blank scaffold control (Figure 56D). We hypothesize that the fast release is necessary to create the gradient to drive drug diffusion deep enough into the brain to suppress tumor recurrence. The fast release creates a high PTX concentration in the resection cavity which according to Fick’s Law will lead to farther penetration of PTX. This is supported by our pharmacokinetic study (Figure 52) in which Ace-PTX/fast led to the highest PTX concentration in the left hemisphere. Additionally, Ranganath et al. found that due to the low elimination of PTX from the brain, PTX
penetration away from a polymer implant is primarily controlled by diffusion.\textsuperscript{25} These results differ from the resection model where the scaffolds were implanted in closer contact to the positive tumor margins after surgery. The complex nature of GBM will likely necessitate different approaches depending on the clinical situation. Future studies will be needed to investigate if the ideal release rate is drug or tumor specific to better advance interstitial therapy toward the clinic.

### 2.3.5 Paclitaxel Release in Response to Tumor Microenvironment

The hydrolysis of the acetal pendant groups on Ace-DEX follows first order kinetics relative to the hydronium ion. As such, the rate of hydrolysis is 10 fold faster with each unit of pH decrease (270). This renders Ace-DEX polymer acid-sensitive which has been previously exploited in vaccine formulations to release cargo under acidic conditions associated with endosomes and lysosomes (177, 271). We hypothesize that this may translate to the subtle extracellular pH changes that occur with common pathological conditions such as solid tumors (264, 265). Indeed, Gerweck et al. published the extracellular pH for patient matched GBM and normal tissue, finding that the average extracellular pH of GBM tumors was 6.8 (264).

To investigate whether PTX-loaded Ace-DEX scaffolds could respond to the tumor microenvironment with a change in PTX release rate, a release study was performed in phosphate buffer at pH 6.8 (Figure 57) and compared to release in PBS at pH 7.4. All Ace-PTX scaffolds degraded faster and released PTX more quickly at pH 6.8 than 7.4 (Table 10). By comparison, PLA-PTX/v.slow scaffold release and degradation were unaffected by this change in pH.

PTX-loaded scaffolds were then evaluated for release in vitro in a 3D agar assay comparing agar gels with and without GBM cells. This tested whether a relatively low number of adjacent GBM cells would affect PTX release from scaffolds over a short period of time. Although the pH of the medium was not directly measured, there was a color change in the phenol-red media
indicating a shift toward acidity in agar gels containing GBM cells. An increase in PTX release was seen for all Ace-DEX scaffolds incubated on agar gels containing GBM cells, however it was only significant for Ace-PTX/fast and Ace-PTX/med scaffolds (Figure 59). Based on the trend, it is possible that if this was extended to longer timepoints, which was limited due to cell viability, Ace-PTX/slow scaffolds might also be significant. As expected, there was no change in PTX release from PLA-PTX/v.slow scaffolds based on presence of tumor. This is expected as polyesters are not acid-sensitive at this pH range and PLA-PTX/v.slow did not display any increased release at pH 6.8.

To determine the effect of GBM tumors on PTX release from scaffolds in vivo, Ace-PTX/med and PLA-PTX/v.slow were implanted into a resection cavity created in the right frontal lobe of mice with and without tumors. At specified timepoints, scaffolds were removed from the brain and evaluated for remaining PTX. These results reflect in vitro PTX release studies, with Ace-PTX/med releasing statistically significantly more drug in the presence of GBM (Figure 58B) whereas PTX release from PLA-PTX/v.slow scaffolds did not change (Figure 59C). Interestingly, for Ace-PTX/med, the size of the tumor also appeared to affect the release rate (Figure 59), where scaffolds removed from mice with small tumors released less PTX than those with large tumors. Rohani et al. recently found that the acidic regions of a tumor are not restricted to hypoxic areas as previously thought, but the highly proliferative and invasive regions of the tumor surface are also acidic (272). We hypothesize that a larger tumor would have a higher acidic surface area of contact for the scaffold and lead to more drug release.

Acid-sensitive polymer systems have previously been utilized for anticancer therapies. However, to this point they have been mainly applied in nanoparticle formulations (273-278). Acid-responsive polymer fibers for drug delivery have also been explored, just to a smaller degree,
and largely utilized in acidic conditions outside the physiologic range of extracellular spaces (264). Two publications; Cui et al. (279) and Qi et al. (280) incorporated acetal and ortho-ester groups respectively within the backbone of a biodegradable polymer that they subsequently electrospun to form nanofibrous scaffolds. However, when investigating acid-sensitivity, only acidic pHS 5.5 and 4.0 were evaluated, which exceed the physiologic pH range of the tumor microenvironment. Jiang et al. developed polydopamine-coated electrospun fibers able to release positively charged molecules faster at a pH of 5.0 and 2.0 compared to neutral pH (281). Chunder et al. utilized electrospun ultrathin fibers of two weak polyelectrolytes, poly(acrylic acid) and poly(allylamine hydrochloride), to release methylene blue (a model for positively charged drugs) in a pH sensitive manner comparing pH 6 – pH 2 (282).

Zhao et al. demonstrated that incorporating sodium bicarbonate into PLA fibers allows for acid-sensitive release of ibuprofen (283) which was able to reduce inflammation in a murine wound model (284, 285). This same group also electrospun PLA scaffolds containing CaCO₃ capped mesoporous silica nanoparticles to release doxorubicin preferentially under acidic conditions (pH 5 and pH 3) (286). These scaffolds led to increased areas of necrosis visualized by histology in a subcutaneous tumor model. Similarly, Sang et al. incorporated sodium bicarbonate into electrospun gelatin fibers for increased release of ciprofloxacin at pH 5 compared to pH 7.4 (287).

Demirci et al. demonstrated pH sensitive release of ciprofloxacin from electrospun polymer fibers due to decreasing electrostatic interactions at lower pHS (7.4 and 5.5) (288). Lastly, while Tiwari et al. found pH sensitive release of doxorubicin from electrospun polycaprolactone fibers at physiologically relevant pHS (pH 7.4, 6.8, 5.5), they credit the increased release to increase in solubility of doxorubicin at lower pHS (289). To our knowledge, our work presented here
illustrates the first use of a drug-loaded polymer scaffold responding to the physiological acidic microenvironment of GBM.

2.4 Conclusions

The location of GBM behind the BBB severely limits systemic therapeutic options. As such, new and more effective chemotherapies cannot be easily utilized. Interstitial drug delivery is an innovative approach to combat GBM recurrence that occurs after surgical resection. However, limitations with current polymeric drug delivery platforms have led to a dearth of knowledge into the ideal drug release rate to optimize this therapy. Here we utilized PLA and Ace-DEX polymer platforms to investigate the role of PTX release rate on GBM interstitial therapy in a tumor recurrence model and distant metastasis model. PTX-loaded scaffolds with four unique release rates (fast, medium, slow, and very slow) were fabricated from Ace-DEX or PLA. In a surgical model of GBM resection, all PTX-loaded scaffolds improved survival with fast, medium, slow, and very slow release rates leading to overall survival of 44, 20, 50, and 22 percent, respectively. However, when half the dose of PTX was delivered from Ace-PTX/fast and half from Ace-PTX/slow scaffolds, overall survival was improved to 78%. In a distant metastasis model, only fast release led to improved survival. These outcomes illustrate that changing the rate at which the same dose of drug is delivered can lead to widely different outcomes. Ace-DEX polymer platform was also shown to be acid sensitive under physiologic conditions associated with GBM tumors. This translated to increased PTX release in vivo when a tumor was present. The facile synthesis, range of tunable degradation, and acid-sensitivity of the Ace-DEX polymer uniquely positions it as a promising drug delivery platform to expand interstitial therapeutic options against GBM.
Table 9 Exclusion of Mice from GBM Resection Model

Table details mice that were excluded from the study, the reason and time of exclusion, as well as the treatment group.

<table>
<thead>
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<th>Reason for Exclusion</th>
<th>Mouse ID</th>
<th>Time of Exclusion</th>
<th>Treatment Group</th>
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<td></td>
<td>M447</td>
<td>Day 1</td>
<td>No Treatment</td>
</tr>
<tr>
<td></td>
<td>M229</td>
<td>Day 1</td>
<td>Ace/blank</td>
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<td>Day 1</td>
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<td>Ace-PTX/slow</td>
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<tr>
<td></td>
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<td>Day 1</td>
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</table>
Figure 48 Published Paclitaxel Release from Polymer Implants

Images of published in vitro release curves were analyzed using Automeris WebPlot Digitizer. Data was exported to excel and plotted together on the same x and y axis for ease of viewing. A) PTX release from polyester implants from Li et al., Xie et al., Ranganath et al., Lee et al., Ong et al., Ranganath et al., Zeng et al. B) PTX release from polifeprosan 20 implants, the same polymer utilized for Gliadel. Blue lines from Walter et al. is PTX release from smaller tablets formulated for rats. Red line from Fung et al. is PTX release from a larger tablet formulated for primates. C) and D) are the same in vitro PTX release from previous publications with PTX-loaded scaffolds from this work overlaid for comparison. Ace-DEX scaffolds (Ace-PTX) fabricated using Ace-DEX polymer with varying degradation rates (fast, medium, slow) termed Ace-PTX/fast, Ace-PTX/med, and Ace-PTX/slow, respectively. PTX-loaded PLA scaffolds termed PLA-PTX/v.slow.
Figure 49 Representative 400 MHz 1H NMR Spectrum of Ace-DEX Degradation Products

Degradation products (dextran, ethanol, and acetone) in DCl/D$_2$O, with labels for the three important peaks: A = acetone −CH$_3$ peaks (6H, 1.78 ppm), and B = ethanol −CH$_3$ peak (3H, 0.73 ppm). Integrations were standardized for each glucose unit by C = two glucose ring hydrogens (2H, 3.5 ppm, integration ≡ 1.00). Normalized integration values of peaks A and B for each polymer (Ace-DEX/fast, Ace-DEX/med, and Ace-DEX/slow) is listed in the inset table.
Figure 50 Schematic of 3D Agar Assay

A PTX-loaded scaffold is placed on a thick slice of agar sitting in a pool of media and topped with a thinner slice of agar.
Figure 51 Effect of Polymer on Paclitaxel Release

Scanning electron micrographs of (A) Ace-PTX/fast, B) Ace-PTX/med, C) Ace-PTX/slow, and D) PLA-PTX/v.slow scaffolds. Scale bar is 5µm. E) In vitro release of PTX and F) mass loss from Ace-PTX/fast (green triangle), Ace-PTX/med (red square), Ace-PTX/slow (blue diamond), and PLA-PTX/v.slow (purple circle) scaffolds in vitro. Data points are mean ± standard deviation. G) Fiber thickness of each scaffold measured from scanning electron micrographs.
Figure 52 Pharmacokinetics of PTX Released from Scaffold in Mouse Brain

(A) Schematic of experimental process. Scaffolds (Ace-PTX/fast, Ace-PTX/med, Ace-PTX/slow, or PLA-PTX/v.slow) with a total dose of 200 µg PTX were implanted in a resection cavity in the right frontal lobe of mice. At specified timepoints, mice were euthanized, the brain was removed by dissection and partitioned for further analysis. Concentration versus time of PTX in the B) left and C) right scaffold-bearing hemisphere as analyzed by LC-MS/MS. Data points are mean ± standard error of the mean. D) Area under the curve (AUC) for PTX within the left and right hemispheres from PTX-loaded scaffolds over the first 7 days.
Figure 53 Histological Analysis of PTX Scaffolds

Hematoxylin and eosin staining of coronal sections of murine brain seven days after resection. Resection cavity was left empty (no treatment) or loaded with blank (Ace/Blank) or PTX scaffolds. Comparing total PTX doses of 75 and 200 µg per a mouse which corresponds with scaffold mass of 0.4 and 1.05 mg, respectively. Scale bars at 1x magnification are 1 mm, scale bars at 4x magnification are 0.5 mm. Black boxes indicate location of magnified region of interest presented in subsequent column.
Figure 54 Effect of PTX Release Rate on GBM Recurrence

(A) Schematic of murine model of GBM resection and scaffold implantation. Glioblastoma cells were implanted in the right frontal lobe. Tumor xenografts grow rapidly as monitored by bioluminescent imaging (BLI). Tumors were then surgically removed under fluorescent guidance leaving positive tumor margins. PTX-loaded polymer scaffolds were then implanted into the resection cavity. Post-treatment tumor growth was monitored with BLI. B) Representative bioluminescent images of mice after tumor resection. BLI scale is in radiance (p/sec/cm²/sr). C) Normalized bioluminescence for treatment groups over time. Data points are mean ± standard error of the mean. Statistical significance by Mann-Whitney U test where * p < 0.05 and ** p < 0.01 with respect to both No Treatment and Ace/blank between days 1 - 20, and ‡ p < 0.05 with respect to all the following groups: Ace-PTX/fast, Ace-PTX/med, Ace-PTX/slow and PLA-PTX/v.slow between days 9 - 41. D) Kaplan Meier survival curve. Statistical significance by Log-Rank test where † p <0.001 with respect to No Treatment and # p < 0.02 with respect to both Ace-PTX/med and PLA-PTX/v.slow.
Figure 55 Tumor Measurements Pre and Post Tumor Resection

(A) Prior to resection, Day 0, established tumors had an average bioluminescent signal of $2.4 \pm 1.6 \times 10^9$ photons per second. B) Approximately 24 hours after resection and scaffold implantation, the average bioluminescent signal was $2.9 \pm 6.0 \times 10^7$ photons per second. There is no statistical significance between groups either before resection or 24 hours after resection by ANOVA. C) Model of 75 µg of PTX release over time from Ace-PTX/fast (green), Ace-PTX/med (red), Ace-PTX/slow (blue), and $\frac{1}{2}$ Ace-PTX/fast + $\frac{1}{2}$ Ace-PTX/slow (orange).
Figure 56 Effect of PTX Release Rate on Distant GBM Metastasis

(A) Schematic of contralateral hemisphere model of GBM. Glioblastoma cells were implanted in the left frontal lobe. PTX-loaded polymer scaffolds were implanted into a resection cavity in the right frontal lobe. Post-treatment tumor growth was monitored with BLI. B) Representative bioluminescent images of mice after tumor resection. BLI scale is in radiance (p/sec/cm²/sr). C) Normalized bioluminescence for treatment groups over time. Data points are mean ± standard error of the mean. D) Kaplan Meier survival curve. Statistical significance by Log-Rank test where * p <0.05 with respect to No Treatment.
Figure 57 Effect pH on Paclitaxel Release

(A) Scaffold mass loss and B) Release of PTX over time at pH 6.8 for Ace-PTX/fast (green triangle), Ace-PTX/med (red square), Ace-PTX/slow (blue diamond), and PLA-PTX/v.slow (purple circle). Data points are mean ± standard deviation.
Table 10 Effect of pH on Scaffold Degradation and Paclitaxel Release

Table details degradation rate and PTX release rate for scaffolds determined by fitting trendline to data from Fig 1 and Supp Fig 5. Statistical significance was determined by linear regression comparing slopes, elevations, and intercepts. ** p < 0.01, ns = not significant.

<table>
<thead>
<tr>
<th>Scaffold</th>
<th>pH 7.4</th>
<th>pH 6.8</th>
<th>Significance</th>
<th>pH 7.4</th>
<th>pH 6.8</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ace-PTX/fast</td>
<td>14.1 ± 0.75</td>
<td>24.7 ± 1.66</td>
<td>**</td>
<td>14.1 ± 0.76</td>
<td>24.2 ± 2.04</td>
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<tr>
<td>Ace-PTX/med</td>
<td>2.2 ± 0.12</td>
<td>2.4 ± 0.14</td>
<td>ns</td>
<td>2.9 ± 0.16</td>
<td>3.1 ± 0.25</td>
<td>**</td>
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<tr>
<td>Ace-PTX/slow</td>
<td>0.7 ± 0.12</td>
<td>1.4 ± 0.06</td>
<td>**</td>
<td>1.3 ± 0.08</td>
<td>1.9 ± 0.21</td>
<td>**</td>
</tr>
<tr>
<td>PLA-PTX</td>
<td>0.2 ± 0.06</td>
<td>0.1 ± 0.08</td>
<td>ns</td>
<td>0.1 ± 0.02</td>
<td>0.02 ± 0.05</td>
<td>ns</td>
</tr>
</tbody>
</table>
**Figure 58 Effect of GBM Presence on Paclitaxel (PTX) Release in Vivo**

(A) Area under the curve (AUC) of PTX released from PLA or Ace-DEX scaffolds of varying degradation rates after 14 days of incubation on agar gels without (blue) and with implanted GBM cells (red). Data is represented as mean ± standard deviation with a sample size of 3. * indicates p < 0.05 by Student t-test. B) Percentage of PTX released from Ace-PTX/med scaffolds after intracranial implantation in mice without (blue) and with (red) tumors. Data points are mean ± standard error of the mean with a sample size of 3-7 per data point. †† indicates p < 0.005 by linear regression analysis. C) Percentage of PTX released from PLA-PTX/v.slow scaffolds after intracranial implantation in mice without (blue) and with (red) tumors. Data points are mean ± standard error of the mean with a sample size of 2-6 per data point, except for data points at days 38, 39, 52, 54, 55 which are scaffolds removed at the time of euthanasia for tumor burden from resection and recurrence study.
Figure 59 Effect of Tumor on Paclitaxel Release in Vivo

(A) Percentage of PTX released from Ace-PTX/med scaffolds after intra-cranial implantation in mice. Data points were stratified as in the presence of a small tumor (red triangle, bioluminescent signal < 200 fold over background), a large tumor (red triangle, bioluminescent signal > 200 fold over background) or without a tumor (blue diamond) at the time of scaffold removal. Best fit trendlines are fitted to the data. Each data point represents a scaffold removed from an individual mouse. B) Bioluminescent image of mouse with ‘small’ and ‘large’ tumors. BLI scale is in radiance (\(\text{p/sec/cm}^2/\text{s}\)).
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