IN-VIVO STUDY OF BRAIN TUMOR MIGRATION VIA
ELECTROSPUN NANOFIBER IMPLANTS

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Presented to
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IN-VIVO STUDY OF BRAIN TUMOR MIGRATION VIA ELECTROSPUN NANOFIBER IMPLANTS

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To the undergraduate researchers in the Bellamkonda Lab
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<tr>
<td>GBM</td>
<td>Glioblastoma Multiforme</td>
</tr>
<tr>
<td>PCL</td>
<td>Polycaprolactone</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>SeeDB</td>
<td>See Deep Brain optical clearing solution</td>
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<td>HFIP</td>
<td>Hexafluoro-2-propanol</td>
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<td>HFIP</td>
<td>Dimethyl formide</td>
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SUMMARY

Glioblastoma multiforme (GBM), one of the deadliest forms of human brain cancer, migrates to different parts of the brain via the white matter tracts. This behavior is the basis for biomaterial research currently done to mimic white matter tracts so that GBM migration can be investigated. While there have been many in-vitro studies done on GBM migration with electrospun nanofiber films, only one in-vivo study has been done on GBM migration. Encouraged by our findings on GBM cell migration on aligned fiber films published in Nature Materials, we proposed to make two new implant designs, the aligned conduit implant and the silicone tube implant and utilize these nanofiber films to investigate GBM cell migration from inside the brain to outside of the brain. It was found that the silicone tube implants had a design flaw that hindered GBM cell migration from the tumor. The aligned conduit implant facilitated GBM migration significantly with a p-value of $2.01 \times 10^{-4}$. Quantification of migration was done using a recently introduced SeeDB protocol, which greatly expedited analysis time. The results from this investigation show that it is possible to design a brain implant that is able to remove GBM tumor non-invasively and will add to the advancement to biomedical technology in this field.
CHAPTER 1: INTRODUCTION

Glioblastoma multiforme (GBM) accounts for nearly 50% of reported malignant gliomas, the most common type of primary brain tumor in adults. GBM also accounts for approximately 70% of the 22,000 new cases of malignant primary brain tumors that are diagnosed in adults in the United States each year. Despite advances in surgical techniques, neuroimaging, and adjuvant modalities such as chemotherapy and radiation, this class of cancer remains resistant to treatment. Median survival rate of patients diagnosed with GBM remains dismal (i.e., about 1 year) with tumor recurrence and progression inevitable in almost all cases. Clinical observations suggest that these GBM tumors migrate as single cells, particularly along white matter tracts, despite the fact that white matter is an inhibitory substrate for neurite outgrowth and astrocyte migration. The reasons for this phenomena is currently not yet fully understood. However, this phenomenon has been a basis for many biomaterial models to mimic the white matter tract to induce migration of GBM and other malignant brain tumors.

Since GBM tumors migrate along white matter tracts, there have been attempts to mimic white matter with electrospun nanofiber films. As the anisotropic elongated structures and nanotopography accurately reflect the mechanical and structural cues present in the brain extracellular matrix (ECM) of the white matter tracts, electrospun nanofibers of polycaprolactone (PCL) with average diameters of 400 nm to 600 nm have been used to study GBM migration. It was also shown that aligned PCL nanofibers provide structural advantage for promoting cell migration. The most promising model proposes to use core-shell electrospun nanofibers to mimic white matter tract topography to examine the migration of malignant brain tumors.
However, despite the various attempts for mimicking white matter tract to examine migration of malignant brain tumors, there has only been one in-vivo study to confirm the prospect of an electrospun nanofiber implant for the migration of GBM. \[14\] This study showed that in a rat model, human GBM U87 cancer cells efficiently migrated on aligned fibers and had effect on controlling the tumor volume.

To further test the tumor cell migration in another in-vivo model of GBM the brain of green fluorescent protein (GFP) expressing transgenic mice, we have designed our brain implants such that the aligned electrospun nanofiber films become a pathway of migration for the GBM migration. In this context, two new implant designs were evaluated in this study. Once the implants are surgically fixed into mice brains, the tumor cells in the brain should align with the nanofiber films of the implants as demonstrated earlier.\[14\] The strategy is similar to that is observed earlier in the rat model where tumor cells will migrate along the nanofiber films from the tumor and to an apoptotic sink at the end of the films. At the apoptotic sink, the tumors cells could be destroyed by various means including the use of anticancer chemotherapeutics.
CHAPTER 2: LITERATURE REVIEW

Tumors in general can be benign or malignant, and in the case of brain tumors, the tumors can occur in different parts of the brain, and they may or may not be primary tumors. Benign tumors are clinically better manageable, while malignant tumors are generally more serious and are often a threat to life. Usually benign tumors can be surgically removed, and they seldom grow back, as opposed to malignant tumors, which are likely to grow rapidly and/or invade nearby healthy brain tissue. There are two types of brain tumors, primary and metastatic. A primary tumor can be defined as a tumor that has started in the brain while the metastatic tumor is a tumor that has spread to the brain from another part of the body.\[1\]

Among the primary malignant tumors in adults, the most common is the Glioblastoma multiforme (GBM), which accounts for nearly 50% of reported malignant tumors. It was reported that the GBM accounts for approximately 70% of the 22,000 new cases of malignant primary brain tumors that are diagnosed in adults in the United States each year. Also, it was reported that the medial survival rate of patients diagnosed with GBM is at about one year, with tumor recurrence and progression inevitable in almost all cases.\[2\] Current literature has shown that there is a pressing need for a cure for this tumor as it remains resistant to current treatments despite advances in surgical techniques, neuroimaging, chemotherapy, and radiation.\[3\]

Clinical observations suggest that these GBM tumors migrate as single cells, particularly along white matter tracts, despite the fact that white matter is an inhibitory substrate for neurite outgrowth and astrocyte migration.\[4\] The reasons for this phenomenon is currently not yet fully understood.\[5\] However, this phenomenon has been
a basis for many biomaterial-based models which have been created to mimic the white matter tracts to induce migration of GBM and other malignant brain tumors.

Earlier work has shown that there have been many attempts to create the suitable biomaterial for evaluating tumor cell migration, and the most popular biomaterial for the past two decades has been the Matrigel.\textsuperscript{[6-8]} Matrigel is a natural biomaterial from a mouse tumor extract that is popular, but bears little resemblance to the composition of the brain, being substantially different from normal brain tissue.\textsuperscript{[9]} Hence, the search for the biomaterials that facilitate tumor cell migration was ongoing until the introduction of electrospun nanofiber films that mimic white matter.

Current literature shows that there have been many attempts to mimic white matter with electrospun nanofiber films, with many different parameters to create the optimum material for tumor migration.\textsuperscript{[10-12]} This sudden surge in research into nanofiber films indicates that researchers are looking into the GBM tumors migrating along white matter tracts; a phenomenon, which may lead to further studies and possibly treatment for GBM. As the anisotropic elongated structures and nanotopography accurately reflect the mechanical and structural cues present in the brain extracellular matrix (ECM) of the white matter tracts, many researchers have started to use electrospun nanofibers of polycaprolactone (PCL) with average diameters of less than 1 µm to study GBM migration.\textsuperscript{[11, 12]}

As opposed to any form of topography of PCL nanofibers or films with aligned topography was shown to provide structural advantage for promoting cell migration, where the alignment probably mimicked white matter tracts most accurately.\textsuperscript{[10]} Among the different nanofiber models currently being used, the most promising model proposes
to use core-shell electrospun nanofibers to mimic white matter tract topography to examine the migration of malignant brain tumors.\cite{12} Similar to what was used in the core-shell model, the aligned core-shell electrospun nanofibers were used in the current study.
CHAPTER 3: MATERIALS AND METHODS

*SeeDB Optical Clearing Protocol*

SeeDB is a water-based optical clearing agent, which clears fixed brain samples within three days, and it is especially ideal for GBM brain tumor quantification as it clears the membrane without quenching the fluorescent dyes in the brain.\[15\] Fructose, distilled water and α-thioglycerol were used in the protocol, details of which can be found in Appendix A.

*Electrospun Nanofiber Tumor Guidance Implants & Experimental Set-up*

Two different types of implants have been proposed, where the two implants were carefully designed for guiding tumor cell migration in mice brain. The aligned nanofibers in the implants were fabricated by electrospinning as indicated in the Appendix A.

![Figure 3-1. Silicone Implant on the left and a Conduit Implant on the right](image)

The aligned nanofibers were attached to the implants shown in Figure 3-1. The experiment was set-up such that there are four different conditions – Craniotomy, Smooth Conduit Implant, Aligned Conduit Implant and Aligned Silicone Tube Implant. The smooth conduit and the aligned conduit had the same implant design. The craniotomy served as the negative control for all conditions and the smooth conduit served as the negative control for the aligned conduit implant. At least 3 animals were used for each condition.
CHAPTER 4: RESULTS

SeeDB Optical Clearing of Mice Brain

Figure 4-1: SeeDB cleared GBM tumor brain sample a, 1-mm-thick section imaged at a magnification of 10x b, another section imaged at 2x c, 3D image of the brain using z-stack imaging by confocal microscopy.

Initial testing for SeeDB optical clearing protocol on mice brain with GBM was done before data quantification. The optical clearing effects of SeeDB at various magnification is shown in Figure 4-1. The bright green fluorescent regions represent GFP expressing GBM. The contrast between the non-tumor and tumor regions was clearly evident.
It was observed that the mice brain had merged with part of the aligned conduit implant, as illustrated in Figure 4-2a. The aligned conduit implant was closely attached with the mice brain at the region of insertion and where the aligned electrospun nanofibers had come in contact with the tumor. For the silicone tube implant, bottlenecking of GBM tumors on the implant was observed. The sheer volume of tumor migration in the tube implant was observed to be much less than those of the aligned conduit implant.
Figure 4-3: Representative image of GBM migration, aligned conduit implant vs smooth conduit implant scale bars at 500 μm a, cross section of the entire GBM tumor with aligned conduit implant visible to the left of the image b, cross section of the entire GBM tumor with smooth conduit implant – implant removed prior to imaging and quantification c, close up of image 4-3a, migration of GBM tumor towards the implant d, close up of image 4-3b, migration of GBM tumor towards where the implant

Representative images of GBM migration in aligned conduit implant and in smooth conduit implant can be seen in Figure 4-3. As shown in Figure 4-3a and 4-3c, the aligned conduit implant is present – the aligned conduit implant had attached firmly with the brain tissue, where removing the implant without significantly damaging the tissues was nearly impossible.
Implant Results

Figure 4-4: Ratio of migrated GBM tumor to total tumor in smooth film conduit and aligned film conduit samples, $n = 12$ and $\alpha = 0.05$, with student’s t-test. Brain sections of a mouse implanted with the smooth film conduit and another with the aligned film conduit were evaluated. 12 sections of each mouse at similar sections of the brains were measured for total tumor size and total migrated tumor size. Error bars represent standard error of the mean. $P$-value of $2.01 \times 10^{-5}$

Random sections from similar region of the brain from a mouse implanted with a smooth film conduit and that from an aligned film conduit condition were imaged and quantified. Twelve sections were taken from each mouse and were measured for total tumor size and total migrated tumor size. It was found that the difference in the ratio of migrated tumor size to total tumor size was statistically significant, with the aligned film conduit inducing a larger volume of tumor migration.
CHAPTER 5: DISCUSSION

SeeDB Optical Clearing Technique for Tumor Volume Quantification

The SeeDB optical clearing of the brain with GBM made quantification of the volume of GBM much more efficiently. The optical clearing technique allowed brain sections up to 1mm to be quantified accurately and precisely. The entire brain volume of the mice was not used for this study. However, the SeeDB protocol should therefore, as described, be a useful technique to be adopted for all subsequent in-vivo study for GFP expressing brain tumor migration.

Silicone Tube Implant

The silicone tube implant was initially designed based on cerebral shunts that are already being used in hospitals. Clinical testing and FDA approvals are already done for these cerebral shunts, and since the silicone implants are slight design modifications from the cerebral shunts, the process of having the silicone implants ready for use in clinical settings should allow for expedited approval for clinical use in comparison to the conduit implant. However, with the experiment showing negative results for the current design, the silicone tube implant should be redesigned to produce effective cell migration from the tumor.

The implant was observed to be inefficient in guiding the migration of GBM tumor from the brain to the apoptotic sink at the other end of the implant. With bottlenecking of the GBM tumor occurring at the opening close to edge of the implant where the tumor cells were supposed to have migrated through and up the tube, the GBM tumor cells were observed to have not been able to go through the small opening. This phenomenon of bottlenecking may be due to the fact that the openings were positioned
lateral to the direction of migration. It may also be due to the decrease in surface area as the GBM tumor cells had to go through the small opening of the tubes.

**Aligned Conduit Implant**

From the experiment, it was determined that the ratio of tumor volume migrated to the total tumor for the aligned conduit implant was significantly higher statistically when compared to the smooth aligned implant. The results indicated that the aligned conduit implant does indeed guide the tumor through the conduit. It must however be noted that the volume of the migrated tumor is small compared to the total volume of the brain for both the aligned and smooth implant with migration ratios at 6.35% and 3.96%, respectively. An improvement in the study could be using a mouse model with a smaller volume of GBM to examine the effectiveness of the aligned implants at a more containable tumor volume.

**Future Investigation with stem cells**

There have been several documented cases where there were tumor recurrences after brain tumors were removed surgically, and this is another problem to be considered with this study of non-invasive treatment of brain tumor cells. The reasons behind tumor recurrences could be due to tumor not removed completely during surgery, but the principal reason is due to the tumor stem cells that are in the brain. Whether tumor stem cells also have the capacity to migrate via aligned films is still unknown and a further study regarding this is needed urgently. If the problem of not being able to remove tumor stem cells is not solved, the prospects of using the aforementioned implant designs are bleak. Hence an in-vitro study of tumor stem cells should be done, and if the ingredients to the nanofiber films need to be changed, it should be done so accordingly.
CHAPTER 6: CONCLUSION

Novel designs of brain implants to induce GBM tumor migration \textit{in-vivo} have been evaluated in this experiment. The ratio of tumor migrated to the total tumor in the brain was evaluated for the conduit implant, and it was found that the aligned conduit implant was able to induce GBM tumor migration. The silicone tube implant had design flaws which caused bottlenecking at the beginning of the implant – further design iterations of the implant are needed. The results found in this investigation show that it is certainly possible to design a brain implant that is able to remove GBM tumor non-invasively, which indicate a promising future for brain tumor treatment.
APPENDIX A: MATERIALS AND METHODS

PCL Nanofiber Protocol

**10% PCL:**
- 4.5ml Hexafluoro-2-propanol (HFIP)
- 0.5ml Dimethyl formide (DMF)
- 500mg Polycaprolactone (PCL Beads)

**12% PCL:**
- 4.5 ml HFIP
- 0.5ml DMF
- 600mg PCL Beads

1. Wear nitrite gloves and work under the hood.
2. Label glass vial and put in stirrer.
3. Weigh the PCL beads.
4. Put DMF into vial using a glass pipette.
5. Add HFIP to DMF (will cause smoke) and start stirrer.
6. Add the beads.
7. Leave to mix overnight (takes >10 hrs for polymer to dissolve).

*The thicker the concentration of the polymer the thicker the fibers. (12% PCL)*

Spinning the aligned nanofiber film

1. Cut out aluminum sheet to appropriate size using the template.
2. Spray and wipe the aluminum with EtOH and tape tightly around the metal rotary collector.
3. With a 3ml syringe, take the needle from DCN solution and pump DCN to clean the needle.
4. Remove the needle and draw up 1ml of 12% PCL solution using the syringe.
5. Put needle back on and secure the syringe on the apparatus by putting the bar across the syringe and tightening the screw.
6. Place the clamp 2/3 from the tip of the needle.
7. Set the infusion rate on apparatus by clicking set → infusion rate → 1ml/hr → enter.
8. The distance from the tip of the needle to collection surface should be 8-10 cm.
9. Check to see if PCL solution is coming out of the needle and wipe with tissue.
10. Lower the safety cover, turn on the power, then turn on the voltage at 8-10 kilovolts.
11. Spin for 20 minutes and check with a flashlight to see that there is only one thread being spun.
12. If more than one thread, turn off the volt, then turn off power, stop the apparatus and wipe the needle. Turn on everything again.
13. After 20 minutes carefully remove the aluminum from the collector by cutting the tape with a scalpel.
Protocol for Implant Fabrication

Conduit Implant

Materials
- 10 ml Dichloromethane (DCM)
- 1g Polyethylene glycol (PEG)
- 1g Polycaprolactone (PCL)
- Metal rod (diameter = 2.5 – 3.0 mm)
- PCL nanofiber film

Procedures
1. Mix 1g of PEG, 1g of PCL beads in 10ml DCM solution. Use heat if necessary.
2. Dip the metal rod vertically into the solution such that the solution caps the rod, and pull the rod out slowly.
3. Spin the rod for 30 seconds, allowing the solution to dry on the rod.
4. Repeat steps 2 and 3 four more times (5 dips total).
5. Leave the rod with the solution drying vertical overnight.
6. Carefully remove the solidified capped conduit from the rod.
7. Place the conduit in a tube with DI water and leave it overnight on an overhead spinner.
8. Remove the conduit and let it dry.
9. Cut the conduits such that the height is 3mm with two triangular teeth (2mm added to the 3mm height) on each side of the conduit.
10. Paste the aligned PCL films onto the conduit.

Silicone-tube Implant

Materials
- Silicone Tube (diameter 2 mm)
- Pink Laboratory Wax Glue
- PCL Nanofiber film

Procedures
1. Cut the silicone tube to lengths of about 10 cm each and fill one end with pink wax such that it fills up to 2mm from one end of the tube.
2. Cut two holes right below where the pink wax ends on the tube such that the holes are facing each other.
3. Put the nanofiber film into the tube via the open end and have it go around the two holes, covering and wrapping around the pink end.
# SeeDB Optical Clearing Protocol

Solutions needed to be prepared:

<table>
<thead>
<tr>
<th>Composition</th>
<th>Fructose</th>
<th>Solvent</th>
<th>α-thioglycerol</th>
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<tbody>
<tr>
<td>20% w/v</td>
<td>4g</td>
<td>Add distilled water to 20 mL</td>
<td>100 µL</td>
</tr>
<tr>
<td>40% w/v</td>
<td>8g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60% w/v</td>
<td>12g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>80% w/v</td>
<td>16g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100% w/v</td>
<td>20g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SeeDB</td>
<td>20.25</td>
<td>Add 5 mL distilled water</td>
<td></td>
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## SeeDB (standard)

1. Fix the sample in 4% PFA at 4oC with gentle shaking overnight.
2. Wash the sample in PBS three times (10 min each).
3. (optional step for fragile samples) Embed the sample in 1% agarose gel in PBS with desired orientation and then trim away extra portion to minimize the sample size. The surface of the sample should be close to the surface of agarose gel, because the working distance of commercially available objective lens is limited. Agarose embedding should not be used for large tissues, because agarose embedding reduces the penetration of SeeDB into the samples.
4. Incubate the sample in ~20 mL of 20% (w/v) fructose solution in 50 mL conical tube, and then place the conical tube on a tube rotator (~4 rpm) or a seesaw shaker (~17 rpm) for 4-8 hours, respectively. The incubation time is from 4 to 8 hours. A small piece of sample (e.g., slices) requires less time for optical clearing. Incubation should be performed at 25-37 C.
5. Incubate the sample in 40% (w/v) fructose for 4-8 hours as above.
6. Incubate the sample in 60% (w/v) fructose for 4-8 hours.
7. Incubate the sample in 80% (w/v) fructose for 12 hours. (Samples may no longer sink in 80% or higher concentrations of fructose.)
8. Incubate the sample in 100% (w/v) fructose for 12 hours.
9. Incubate the sample in ~20 mL SeeDB for 24 hours. The incubation time can be extended up to 48 hours. The transparency can be evaluated by eyes at this stage. If the sample is successfully cleared, the adult brain sample should look like amber under a light source.
10. If the clarity is still not enough for imaging, we recommend:

   A. (In this optical clearing session) Incubate the sample in ~20 mL SeeDB37 solution at 37 C (in an air incubator) with gentle rotation for 24 hours.

   B. (In the next optical clearing session) Trim away unnecessary portion of your sample to increase the penetration of fructose solution.

   C. (In the next optical clearing session) If the sample and fluorescent markers are heat resistant, try SeeDB37ht protocol.
REFERENCES


