Synthesis and Application of a Magnetic Nanoparticle Based Nano-complex for Targeted Cell Death in Glioblastoma Multiforme Cells

Matthew Tovar
University of Mary Washington

Follow this and additional works at: https://scholar.umw.edu/student_research

Recommended Citation
https://scholar.umw.edu/student_research/282

This Honors Project is brought to you for free and open access by Eagle Scholar. It has been accepted for inclusion in Student Research Submissions by an authorized administrator of Eagle Scholar. For more information, please contact archives@umw.edu.
Title: Synthesis and Application of a Magnetic Nanoparticle Based Nano-complex for Targeted Cell Death in Glioblastoma Multiforme Cells

Name of Candidate: Matthew Alexander Tovar

Approved by Examination Committee:

Dr. Leanna C. Giancarlo
Associate Professor of Chemistry
Sponsor

Dr. Kelli M. Slunt
Professor of Chemistry

Dr. Janet A. Asper
Professor of Chemistry

Dr. Randall D. Reif
Assistant Professor of Chemistry

Date Approved: April 29, 2019
Synthesis and Application of a Magnetic Nanoparticle Based Nano-complex for Targeted Cell Death in Glioblastoma Multiforme Cells

By

Matthew Alexander Tovar

Thesis submitted to the faculty of the University of Mary Washington in partial fulfillment of the requirements for graduation with Honors in Biochemistry

2019
ABSTRACT

In the last ten years, there has been little advancement in the treatment of the aggressive brain cancer Glioblastoma Multiforme (GBM). This work describes the synthesis of a superparamagnetic iron oxide (SPION)-based nanotherapeutic complex for use in targeting and killing aggressive mesenchymal GBM cells. The average sizes (and therefore magnetic properties) of the synthesized SPIONs are precisely tailored via a novel time-controlled approach utilizing a previously described electrochemical reaction. Through this synthetic method, the optimal particle size (OPS) where maximal thermal energy is released upon stimulation with an external magnetic field was determined to be 21 nm. The nano-complex was further modified to selectively target GBM cells by adding a heterobifunctional poly(ethylene) glycol polymer cross-linked to TWEAK (a GBM targeting ligand). Further investigation with FITC Annexin V/Propidium Iodide fluorescent probe and transmission electron microscopy showed that cells treated with the synthesized nano-complex showed both biochemical and morphological markers positive for programmed cell death. Thus, these nano-complexes show promise as a potential treatment agent for an otherwise untreatable disease.
ACKNOWLEDGEMENTS

I would like to express my immense gratitude first to Dr. Giancarlo for her endless support and guidance throughout the last three years. Her valuable and constructive suggestions and willingness to lend her time and expertise have been paramount to the success of this work. In addition, I would like to thank the Departments of Chemistry and Biology for lending supplies, research space, and additional guidance when needed. Particular thanks are given to Mrs. Angela Andrews, Dr. Amir Aslani, and Dr. Christine Brantner for lending their expertise in the fields of transmission electron microscopy (TEM) operation, superparamagnetic characterization, and TEM sample preparation, respectively, and Dr. Randall Reif for donating aliquots of Jurkat T-Lymphocytes for this work. Finally, I would like to thank Mrs. Irene Piscopo Rodgers for her generous continued financial support through the Irene Piscopo Rodgers and James D. Rodgers Student Research Fellowship throughout the last two years.
LIST OF ABBREVIATIONS

BBB: blood brain barrier
CoV: coefficient of variation
E: anisotropic energy barrier
$E_B$: anisotropic energy constant (4.6 kJ m$^{-3}$ for maghemite)
DMAP: 4-dimethylaminopyridine
DMPAP: 2,2-dimethoxy-2-phenylacetophenone
DNA: deoxyribonucleic acid
DOX: doxorubicin
EDC: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
EGFR: epidermal growth factor receptor
EMR: electromagnetic radiation
EPR: enhanced permeability and retention effect
$f_0$: ferromagnetic resonance frequency (~1 GHz)
$\eta$: liquid dynamic viscosity
$\mathbf{H}$: external magnetic field
HIF1A: hypoxia-inducible factor 1α
$^1$HNMR: proton nuclear magnetic resonance
ICP: intracranial pressure
IR: infrared
$J$: angular momentum quantum number
$k_b$: Boltzmann’s constant (1.38x10$^{-23}$ J/K)
$K_B$: dissociation constant
GBM: Glioblastoma Multiforme
$\lambda$: wavelength (of light)
$l$: angular momentum
$\mu$: magnetic dipole moment
$\mathbf{M}$: magnetization
mAb: monoclonal antibody
MES: 2-(N-morpholino)ethanesulfonic acid
MGMT: O$^6$-methylguanine-DNA methyltransferase
NF-κB: κ-light chain enhancer of activated B cells pathway
PBS: phosphate buffered saline
PI: propidium iodide
PS: phosphatidylserine
θ: easy axis angle
rc: critical radius
rh: hydrodynamic radius
RT: room temperature
RPMI: Roswell Park Memorial Institute
s: spin angular momentum
SAv: Streptavidin
SCB: sodium cacodylate buffer
SPION: Superparamagnetic Iron Oxide Nanoparticle
Sulfo-NHS: N-hydroxysulfosuccinimide
τB: Brownian rotation frequency
τN: Neel rotation frequency
T: temperature
t=time
TEM: transmission electron microscope
TNF: tumor necrosis factor
TRAF: TNFR-associated factor
TWEAK: TNF-like weak inducer of apoptosis
V: (particle) volume
VEGF: vascular endothelial growth factor
VEGF-R: vascular endothelial growth factor receptor
VSM: vibrating sample magnetometry
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>iii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>2</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>3</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>4</td>
</tr>
<tr>
<td>Glioblastoma Multiforme: Pathology and Molecular Pathogenesis</td>
<td>4</td>
</tr>
<tr>
<td>Glioblastoma Multiforme: Current Treatments and Challenges</td>
<td>5</td>
</tr>
<tr>
<td>Superparamagnetic Iron Oxide Nanoparticles: Theory And Applications</td>
<td>8</td>
</tr>
<tr>
<td>The TWEAK cytokine/Fn14 receptor axis</td>
<td>12</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>18</td>
</tr>
<tr>
<td>Reagents</td>
<td>18</td>
</tr>
<tr>
<td>General Experimental</td>
<td>20</td>
</tr>
<tr>
<td>SPION Synthesis and Characterization</td>
<td>20</td>
</tr>
<tr>
<td>Synthesis of Siloxane-Terminated PEG Molecule</td>
<td>22</td>
</tr>
<tr>
<td>SPION Functionalization</td>
<td>22</td>
</tr>
<tr>
<td>Addition and Evaluation of SAv</td>
<td>22</td>
</tr>
<tr>
<td>Addition and Evaluation of TWEAK</td>
<td>23</td>
</tr>
<tr>
<td>Cell Culture Conditions</td>
<td>24</td>
</tr>
<tr>
<td>Fluoromicroscopic Detection of SPION-Induced Programmed Cell Death.....</td>
<td>24</td>
</tr>
<tr>
<td>Evaluation of the Ultramorphologic Changes of SPION-Induced Apoptosis...</td>
<td>25</td>
</tr>
<tr>
<td>RESULTS AND DISCUSSION</td>
<td>27</td>
</tr>
<tr>
<td>SPION Synthesis and Characterization</td>
<td>27</td>
</tr>
<tr>
<td>SPION Biofunctionalization</td>
<td>40</td>
</tr>
<tr>
<td>\textit{In vitro} testing: U87 GBM Studies</td>
<td>52</td>
</tr>
<tr>
<td>CONCLUSION</td>
<td>58</td>
</tr>
<tr>
<td>WORKS CITED</td>
<td>60</td>
</tr>
<tr>
<td>CURRICULUM VITAE</td>
<td>63</td>
</tr>
</tbody>
</table>
LIST OF TABLES

TABLE 1: A tabulation of the change in temperature of a SPION colloidal suspension as a function of time at constant pressure, volume, and temperature.
LIST OF FIGURES

FIGURE 1: The molecular structure of Temozolomide.............................. 5
FIGURE 2: A graph depicting a generalized Langevin function................... 9
FIGURE 3: TWEAK trimerization leads to activation of the NF-κB pathway... 13
FIGURE 4: Endosomal nanoparticle uptake is dependent on particle size...... 15
FIGURE 5: SPIONs coated with a polymer undergo endocytosis............... 17
FIGURE 6: SPION experimental setup.............................................. 21
FIGURE 7: A scheme of the BEEM capsule/GBM cell setup..................... 24
FIGURE 8: ATR-FTIR spectra of SPION samples................................... 30
FIGURE 9: TEM images and trendline of SPION synthesis ..................... 32
FIGURE 10: Percent frequency of coefficients of variation...................... 34
FIGURE 11: SPION hysteresis showing size-dependent magnetic properties ... 36
FIGURE 12: SPION coercivity values attained from the hysteresis loops...... 37
FIGURE 13: SPION Temperature Gradients......................................... 39
FIGURE 14: FTIR tracking of polymer synthesis.................................... 42
FIGURE 15: 1H NMR tracking the conversion of molecule 2 to molecule 3.... 43
FIGURE 16: The mechanism of EDC/Sulfo-NHS covalent crosslinking....... 46
FIGURE 17: Fluorescence spectroscopy evidence of SAv linkage............... 47
FIGURE 18: The mechanism of TWEAK biotinylation........................... 48
FIGURE 19: Fluorescence spectroscopy evidence of TWEAK linkage........ 49
FIGURE 20: A TEM image of the SPIONs following Bio-conjugation........ 50
FIGURE 21: The molecular structure of propidium iodide ...................... 52
FIGURE 22: Controls for the fluorescence apoptosis assay..................... 53
FIGURE 23: Fluorescence microscopy depicting SPION-induced cell death... 54
FIGURE 24: TEM images tracking SPION internalization....................... 56
FIGURE 25: SPION Cross-specificity assay against Jurkat T-lymphocytes... 57
**Introduction**

**Glioblastoma Multiforme: Pathology and Molecular Pathogenesis**

Brain cancers represent a clinically diverse, unique, and deadly set of tumors. Within the brain there exist two main classes of cells: neurons and glial cells. By far, most primary intrinsic tumors diagnosed are cancers of glial cells, otherwise referred to as gliomas. Within the glioma subcategory, Glioblastoma Multiforme (GBM) is the most prevalent, accounting for 56.1% of all diagnosed gliomas between the years 2012-2016.\(^1\)

Pathologically, these tumors are defined by having a diverse intratumoral morphology with areas high vascularity mirroring areas of tissue necrosis. At the cellular level, GBM displays high cellularity, nuclear atypia, polynucleism, and increased mitotic activity.\(^2\)\(^-\)\(^4\)

The patterns of the molecular pathogenesis in GBM mimic those traditionally seen in other types of aggressive cancer. Hallmark alterations includes overexpression and mutations of the following proteins (among many others): epidermal growth factor receptor (EGFR), O\(^6\)-methylguanine-DNA methyltransferase (MGMT), hypoxia-inducible-factor-1-alpha (HIF1A), and vascular endothelial growth factor (VEGF) and numerous Tumor Necrosis Factor (TNF) superfamily proteins.\(^2\)\(^-\)\(^3\)

Overall, the net effect of these alterations acts to increase the ability of the cancerous cells to survive and proliferate within healthy parenchymal tissue. Healthy neural tissue is affected in one of two ways due to this tumor. First, healthy tissue is directly destroyed through exposure of pro-inflammatory agents leaked from neoplastic cells such as cytokines and chemokines. This results in focal neural deficits typically seen in patients suffering from strokes. Second, increased tumor size leads to increased vasogenic edema which in turn leads to increased intracranial pressure (ICP). Increased
ICP has the capacity to cause devastating negative effects to the brain centers responsible for maintaining our ability to survive.\textsuperscript{5} Even with the most aggressive and concurrent treatments available in modern medicine, the median survival time of patients with GBM is 12-15 months. This survival time has not significantly changed over the last 10 years.\textsuperscript{2,6}

**Glioblastoma Multiforme: Current Treatments and Challenges**

The traditional first-line chemotherapeutic treatment for malignant primary intracranial gliomas has been Temozolomide. Temozolomide (Figure 1) is a small, nonpolar imidazotetrazine derivative of dacarbazine (a DNA alkylating agent) with a unique ability of being able to cross the blood brain barrier (BBB).

![Figure 1: The chemical structure of Temozolomide, the primary chemotherapeutic agent against GBM](image)

Temozolomide is initially administered as a prodrug. In the liver, this drug is metabolized to its active form, diazomethane, through a mechanism initially proposed by Shealy \textit{et al.}\textsuperscript{7} The diazomethane zwitterion then travels through the bloodstream to both the site of the tumor and other highly vascular sites within the body where it induces DNA methylation at either the \textit{O}-6 or \textit{N}-7 sites of the guanine base.\textsuperscript{8} In theory, DNA methylation should induce a cascade that prevents cellular replication and induces apoptosis; however, in practice, this is not the case. It has previously been determined that 55-65\% of GBM patients possess overexpression of the DNA repair enzyme MGMT.\textsuperscript{9} MGMT overexpression effectively reverses any DNA methylation induced by
Temozolomide. In addition, diazomethane (the active form of Temozolomide) targets any rapidly dividing cell line, to include epithelial cells producing hair follicles, endothelial cells lining the lumen of the stomach, and hemocytoblast cells. The latter cells are located in the bone marrow and are responsible for differentiating into erythrocytes and leukocytes (a process known as hematopoiesis).\textsuperscript{10,11} Although some side effects are relatively inconvenient at their worst (hair loss, persistent nausea), myelodysplasia (or disruption of hematopoiesis) in particular can be detrimental to the patient’s long-term prognosis. Several studies have shown that iatrogenic myelodysplasia by treatments with Temozolomide results in lymphopenia, anemia, and acute hepatopathy.\textsuperscript{11} This in turn can lead to sepsis, tissue hypoxia, renal failure, and liver failure. Overall, disease progression is observed in nearly all patients treated solely with this chemotherapeutic agent.\textsuperscript{3}

The second-line chemotherapeutic option for GBM patients is Bevacizumab. Bevacizumab is a human monoclonal antibody (mAb) whose antigen is VEGF. As the mAb circulates throughout the blood, it binds to VEGF.\textsuperscript{12} This prevents the ligand from reaching its receptor (VEGF-R) which is overexpressed on the surface of the cancer cell. Overall, this causes anti-proliferative and anti-angiogenic effects. It is interesting to note that Bevacizumab performs the majority of its antitumoral effects outside the environment of the tumor bed itself, as its molecular weight prevents it from crossing the BBB. Again, when this treatment was placed into practice, it proved statistically ineffective at preventing tumor recurrence and growth as the cells in the tumor would begin to overexpress proteins such as hypoxia inducible factors (HIFs) that would promote subsequent angiogenesis.\textsuperscript{13,14}
Immunotherapies are treatments that rely on the training of the body’s T-Cells to recognize, attack, and kill neoplastic cells. Though this has proved successful in other types of cancers, particularly in the blood cancer realm, GBM is unique in that it promotes global sequestration of the body’s T-cells to the point that GBM patients display lymphopenia mimicking that of AIDS patients. This process is further enhanced through any iatrogenic lymphopenia. Without white blood cells, immunotherapy is rendered ineffective. The final two options available in modern medicine to treat GBM, surgical resection and radiotherapy, fail mainly due to GBM’s ability to proliferate and migrate many millimeters away from the main tumor bed into seemingly healthy parenchymal tissue. As a result, neither surgeons nor radiation oncologists can guarantee 100% removal of the neoplastic cells. After surgical resection and/or radiotherapy, the remaining neoplastic cells proliferate into new tumors, contributing to tumor recurrence and further treatment resistance.

In summary, no one sole treatment is completely effective in improving the long-term prognosis of GBM. Though current treatments have a marginal effect on the eradication of the tumor core, little-to-no effect is seen at the invasive rim cells that migrate away from the main tumor mass. These untreated neoplastic cells then lead to tumor recurrence. This deadly cycle continues for 12-15 months until the patient succumbs to the physiological effects of neuronal necrosis and increased ICP. For the cancer to be completely eradicated, the invasive rim cells responsible must be specifically and irrevocably targeted and killed.
Superparamagnetic Iron Oxide Nanoparticles: Theory and Biomedical Applications

Superparamagnetic Iron Oxide Nanoparticles (SPIONs) have previously been explored as cytotoxic agents for the treatment of cancer.\textsuperscript{24-26} They do so by inducing thermal denaturation of biological macromolecules necessary for cellular survival and proliferation. This so called “cellular hyperthermia” occurs due to the nanoparticle exhibiting a unique magnetic property known as superparamagnetism. The property of superparamagnetism occurs at the nano-scale when an anisotropic magnetic dipole moment ($\mu$) exists.

To fully understand the magneto-thermodynamics of superparamagnetic material, it is imperative to understand the magnetic behavior of typical paramagnetic materials. The Brillouin function shows that paramagnetic magnetization ($\mathbf{M}$) is dependent both on the external magnetic field ($\mathbf{H}$) and the total angular momentum quantum number, $J$.\textsuperscript{18} $J$ itself is described as the summation of the spin angular momentum ($s$) and the orbital angular momentum ($l$) vectors. Because a large number of electrons exist in nanoparticles, the value of $J$ approaches infinity.\textsuperscript{19} Thus, the Brillouin function simplifies to the Langevin function ($L$), which relates the susceptibility ($\chi = \frac{M}{H}$) of a paramagnetic material to $\mathbf{H}$:\textsuperscript{20}

\begin{equation}
L(\xi) = \coth \xi - \xi^{-1},
\end{equation}

where $\xi = \frac{\mu H}{k_B T}$ is proportional to the applied $\mathbf{H}$, $k_B$ is Boltzmann’s constant, $T$ is temperature, and $\mu$ is the magnetic dipole moment.\textsuperscript{20} The $\chi(\xi)$ graph shows that at high $\mathbf{H}$ values, $\chi_{\text{SPION}}$ becomes saturated. This observation is one of the pinnacle features that defines paramagnetic materials.
The magnetization of a superparamagnetic material behaves identically to what is predicted by the Langevin paramagnetism. The *key difference* between these two materials, however, lies in the fact that the $\mu_{\text{net}}$ of a superparamagnetic material is the summation of all electrons within that material. Thus, the massive amounts of electrons present in the nanoparticle results in extremely large magnetic moments relative to the constituent subatomic particles. This is the mathematical basis for distinguishing superparamagnetic materials from normal paramagnetic materials.

Another key difference that distinguishes superparamagnetic nanomaterials from atomic particles is the presence of uniaxial anisotropy. The magneto-thermodynamics of SPION growth dictate that, up to a specific size, it is energetically favorable for the $\mu$ of the SPION’s constituent atoms to align in a uniaxial manner (i.e. all atomic $\mu$s run in the
same direction).\textsuperscript{21} Above this critical size, however, the magneto-potential energy the material attains reaches a limit. To reduce the large amount of internal energy formed at large particle sizes, the $\mu$s fragment into two or more magnetic domains. When only one magnetic domain is present, the SPION can be described as uniaxially anisotropic. To switch the direction of the $\mu$, an energy barrier, known as the anisotropic energy barrier, must be applied. The anisotropic energy barrier is dependent on both particle volume and identity. Its magnitude is given by

$$E = E_B V \sin^2 \theta,$$

where $V$ is particle volume, $E_B$ is the material’s anisotropy constant (4.6 kJ m$^{-3}$ for maghemite), and $\theta$ is the angle between the material’s easy axis (the native direction of $\mu$) and magnetization direction.\textsuperscript{22}

When $H$ is applied to a SPION, magneto-potential energy is introduced to the system. The SPION reduces this incident energy in one of two ways. If the applied magneto-potential energy is above the anisotropic energy barrier, the particle undergoes Neel rotation, characterized by rotation of the magnetic moment itself. This rotation oscillates between the native direction of the SPION and the direction of the applied magnetic field with a frequency of

$$\tau_N = f_0 e^{\frac{-E_B}{k_BT}},$$

where $f_0$ is the ferromagnetic resonance frequency in the demagnetization field (along the order of GHz).\textsuperscript{23} This is the energetically favorable method of reducing the magneto-potential energy incident on the particle because the perturbation of the surrounding
solvent molecules is reduced by the physical material remaining immobile. Neel rotation, however, occurs only when the applied magneto-potential energy is greater than $E_B$. A different type of rotation, known as Brownian rotation, occurs when the energy of the applied magnetic field is less than $E_B$. With this rotation the SPION \textit{physically oscillates} between its native direction and the direction of the applied magnetic field with a frequency of

$$
\tau_B = \frac{4\pi \eta r_h^3}{k_B T},
$$

where $\eta$ is the viscosity of liquid medium the nanoparticle is rotating in and $r_h$ is the hydrodynamic radius of the particle.\textsuperscript{21} When the nanoparticle physically rotates against a liquid media, thermal energy is produced due to the friction of the nanoparticle surface and the surrounding liquid molecules. Thus, for SPION-induced hyperthermia to be effective, a sufficiently low amount of magneto-potential energy must interact with the SPION such that Brownian rotation occurs. If the energy of $H$ exceeds the anisotropic energy barrier, Neel rotation commences and the SPION gradually loses ability to physically rotate.\textsuperscript{21}

In summary, the effective release of thermal energy by the SPION relies on two key factors. First, the frequency of Brownian rotation (and therefore the magnitude of heat produced) is dependent on the hydrodynamic radius of the particles as seen in equation 4. Second, the theory of magnetic domains states that above a critical radius ($r_c$), the SPION loses its uniaxial anisotropic property as the single magnetic domain fragments into multiple domains. If this anisotropy is lost, the SPION has a $\mu_{\text{net}}$ at or near
zero and thus cannot rotate when exposed to $\mathbf{H}$. Thus, an optimal size exists that maximizes Brownian rotation but still retains its uniaxial anisotropy.

**SPION Ultralocalization: The TWEAK cytokine/Fn14 receptor axis**

Nanoparticles in general have shown to preferentially accumulate in the extracellular space surrounding tumors due to leaky and imperfect neovasculature (a property known as the enhanced permeability and retention, EPR, effect). As a result, preferential deposition in the extracellular matrix is achieved. This process is especially active at the liver, making SPIONs excellent candidates for detection of hepatic carcinomas. In this FDA-approved capacity, the particles induce inhomogeneities within the external magnetic field that are detectable via T$_1$, T$_2$, and T$_2^*$ Magnetic Resonance Imaging. They, however, are not yet FDA approved for the treatment of the neoplastic cells. As previously mentioned, when exposed to magnetic fields attaining a particular magneto-potential energy, thermal energy is produced via Brownian rotation. As with any hyperthermia treatment, one of the largest problems with this form of therapy is that intracellular “hot spots” exist where the produced thermal energy destroys structures surrounding it but structures far away from the thermal energy release site remain unaffected. Due to poor control of where SPIONs localize in prior studies, little gross cytotoxic effect has been seen with prior intracellular hyperthermia experiments.

Of the many enzymes and other proteins overexpressed in GBM, one in particular offers promise in promoting GBM-specific nanoparticle uptake. The Fn14 receptor is a 102 amino acid type I transmembrane protein that acts as the receptor for a cytokine known as the TNF-like weak inducer of apoptosis (TWEAK). When TWEAK binds to Fn14, a signaling cascade begins. First, Fn14 trimerization occurs. This is shortly followed by TNFR-associated factor (TRAF) binding to the cytoplasmic tail of Fn14.
The binding of TRAF to Fn14 activates numerous signaling cascades, most notably, the nuclear factor κ-light-chain-enhancer of activated B-cells (NF-κB) pathway (Figure 3). This pathway leads to the production of gene products that promote cellular survival, proliferation, and angiogenesis. Thus, it is intuitive that this pathway is one of the many intracellular signaling pathways that is upregulated in the pathogenesis of neoplastic cells.

Figure 3: A graphic showing the activation of Fn14 by TWEAK. Binding of the soluble TWEAK cytokine (sTWEAK) induced Fn14 trimerization, TRAF association, and activation of the NF-κB pathway. Image adapted from Winkles.

The overexpression of Fn14 is highly correlated with the invasiveness of the cancer subtype. Invasive rim cells tend to be the most aggressive and proliferative of GBM neoplastic cells, and, therefore, they have previously been found to exhibit constitutive NF-κB activation. Thus, it is logical that invasive rim cells have largely overexpressed Fn14 receptors compared to the normal parenchymal cells surrounding them. In fact, Fortin et al. showed via immunohistochemical detection that all invasive
rim cells analyzed in their study exhibited “moderate to strong” immunohistochemical detection of the Fn14 receptor protein. In contrast, a similar immunohistochemical assay for Fn14 on non-neoplastic glial cells showed that all cells showed weakly positive-to-negative detection. From these data, it is clear that the Fn14 receptor can be used as a portal to selectively enter into neoplastic cells because only neoplastic cells significantly overexpress the protein in the environment of the central nervous system.

As discussed, the BBB remains one of the most significant challenges to the delivery of therapeutic agents to distant neoplastic cells in the central nervous system. The interface of the BBB with the vascular system lies within the capillary bed. At this level, three concentric layers of cells (endothelial cells, pericytes, and astrocytes) envelop the capillary bed. The concentric layer most proximal to the lumen of the capillary vessel consists of a layer of negatively charged phospholipid heads. Maghemite nanoparticles are known to have a negative surface charge (i.e. zeta potential: ζ); therefore, electrostatic repulsions would prevent them from interacting and therefore undergoing transcytosis through the BBB. To circumvent this, scientists have previously coated the surface of nanomaterials with lipophilic polymers such as poly(ethylene) glycol. Doing so effectively reduces the surface charge of the nanocomplex and allows transcytosis through the BBB. Thus, polymeric nanomaterials have been found to be the useful material for endovascular targeting of invasive rim cells.

TWEAK-modified nanotherapeutics have previously been shown to have high affinity and specificity towards targeting only cells displaying the Fn14 receptor \(K_{D,(-)}^{Fn14}=1.62\ \text{nM}\) versus \(K_{D,(+)}^{Fn14}=0.024\ \text{nM}\) both in vivo and in vitro. From this, it is logical that nanoconjugates decorated with the TWEAK protein will target and interact
with the exterior cellular membranes of neoplastic GBM cells. Uptake of any nanotherapeutic has largely been linked to both the size and shape of the therapeutic. He et al. shows that 15 nm gold nanoparticles (AuNPs) that endocytosed into GBM cells, escaped from the endosomal system, and deposited within mitochondria and nucleus (Figure 4). More importantly, He et al. showed that this endosomal escape occurred only when particles were along the order of 20 nm and attained a spherical morphology (4.a). Particles whose sizes were greater than 50 nm (4.b) and attained non-spherical morphologies (4.c) remained trapped within the endomembrane system and thus did not undergo endosomal escape.

Figure 4: TEM micrographs (courtesy of He et al.) showing that the uptake of nanoparticles within the endomembrane system is largely dependent on both the size and geometric shape of the SPION, where particles that are approximately 15 nm could undergo endosomal escape (4.a) while those larger than 15 (4.b) and with non-spherical morphologies (4.c) did not undergo endosomal escape. He et al. found that eventually, 15 nm spherical AuNPs accumulated in the mitochondria, nucleus, and lysosomes of GBM cells.39
This observation by He et al. is reproduced in a study by Liu et al., where 90 nm SPIONs were observed to preferentially deposit both within the intermembrane space of mitochondria (figure 5) and within the nucleus. It is through this preferential deposition into organelles via Fn14-mediated nanoparticle internalization that we propose to ultralocalize the thermal energy produced from SPION Brownian rotation. This study sets the foundations for creating a novel treatment for GBM based on the thermal energy released by SPION rotation. In this study, we have successfully synthesized SPIONs via a straightforward and inexpensive electrochemical redox reaction. We demonstrate that size, and therefore magnetic properties, of a SPION can be precisely tailored by controlling the duration of iron oxidation. Following reaction optimization and bioconjugation of optimally sized nanoparticles, both fluorescence and transmission electron microscopy indicated that the GBM cells underwent programmed cell death within 3 hours of SPION nano-complex administration. Preliminary data also shows that SPIONs induce programmed cellular death only in cells that overexpress the Fn14 receptor. These results indicate that the optimized nano-complex structures described here possess great promise in acting as targeted, cytotoxic agents towards aggressive GBM cells.
Figure 5: A schematic of the internalization of SPIONs (courtesy of He et al.) that proposes SPIONs coated with a stearic-polyethyleneimine polymer undergo endosomal escape following internalization. These SPIONs are proposed to travel to both the nucleus and mitochondria. This is seen via TEM in figure 5B, where SPION internalization and subsequent mitochondrial deformation is shown by the orange arrows. The black arrows show healthy mitochondria.\textsuperscript{40}
Materials and Methods

Reagents

- Lithium chloride (Fisher)
- Solid iron (Fisher)
- Phosphate buffered saline (PBS, pH=7.4, 0.1 M) (Fisher)
- 2,2 dimethoxy-2-phenylacetophenone (DMPAP) (Fisher)
- 4-dimethylaminopyridine (DMAP) (Fisher)
- Succinic anhydride (Fisher)
- 3-Mercaptopropyltrimethoxyamine (Fisher)
- Trimethylamine (Fisher)
- 2-(N-morpholino)ethanesulfonic acid (MES) (Fisher)
- Lyophilized streptavidin (SAv) (Fisher)
- 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Fisher)
- N-hydroxysulfosuccinimide (Sulfo-NHS) (Fisher)
- Biotin-4-fluorescein (Fisher)
- EZ-link micro sulfo-NHS-biotinylation kit (Fisher)
- Annexin V/Propidium Iodide fluorescence apoptosis kit (Fisher)
- Glutaraldehyde (Fisher)
- Paraformaldehyde (Fisher)
- Sodium Cacodylate (Fisher)
- Osmium Tetraoxide (Fisher)
- Fetal Bovine Serum (Fisher)
- Gentamycin (Fisher)
• Poly(l)-lysine (Fisher)
• HEPES Buffer (Fisher)
• Cell Apoptosis Kit (Invitrogen)
• Allyl PEG10-OH (PolySciences)
• TWEAK cytokine (R&D systems)
• U87 Glioblastoma Multiforme (ATCC).
• Dulbecco’s Modified Eagle Media (DMEM) (Millipore Sigma)
• LX-112 Epoxy Substitute Resin (EMS)
• Uranyl Acetate (EMS)
• Size 3 BEEM Capsules (EMS)
• Jurkat T-Lymphocytes (Courtesy of Dr. Randall Reif)
• Penicillin (Courtesy of Dr. Randall Reif)
• RPMI 1740 (Courtesy of Dr. Randall Reif)
• Doxycycline (Courtesy of Dr. Lynn Lewis)
General Experimental

The SPIONs were synthesized with a CH 600C series potentionstat. The SPIONs were analyzed using a Morgani FP 5005 transmission electron microscope (TEM), Perkin Elmer Spectrum RX-1 infrared spectrometer with Pike MIRacle ATR attachment, and LakeShore 7410 Vibrating Sample Magnetometer (VSM). The synthesized polymer was analyzed via IR spectrometry and $^1$HNMR Spectroscopy with an Oxford 300 MHz NMR. Progression of TWEAK crosslinking was monitored using a Horiba Jobin Yvon fluorimeter. Throughout cell death assays, GBM cells and SPIONs were exposed to a constant low energy (21 mT) magnetic field created by an air-core solenoid. Cell death was monitored utilizing a Nikon Eclipse Ts2R-FL epifluorescence microscope and the previously mentioned TEM.

SPION Synthesis and Characterization

The oxidative method of producing SPIONs was initially reported by Starowicz et al.\textsuperscript{41} It was employed here and optimized with various modifications. Most notably, the duration of synthesis was varied from 15 minutes to 60 minutes in place of varying [H$_2$O]. To create an adequate aqueous environment for nanoparticle synthesis, a beaker containing 200 mL of 0.100 M aqueous LiCl was sparged with Ar(g) for a period of 10 minutes prior to introduction of the electrodes. The gas was continually bubbled through the solution throughout the duration of the synthesis. Ag/AgCl acted as the reference electrode, Pt as the counter electrode, and Fe(s) as the working electrode. The complete experimental setup is seen in figure 6.
Figure 6: The experimental setup for SPION synthesis, where Ar(g) was bubbled into a solution of 0.100 M aqueous LiCl at RT. Fe(s) acted as the working electrode, Pt(s) as the counter-electrode, and Ag/AgCl as the reference electrode.

The reaction was run at a potential of 0.400 V and the duration of iron oxidation was manipulated to produce SPIONs of varying diameters. SPIONs were collected, centrifuged at room temperature (RT, 4000 rpm) and re-suspended in PBS. All samples were analyzed via TEM, Attenuated Total Reflectance Fourier-Transform Infrared Spectroscopy (ATR-FTIR), and VSM. Thermal energy output secondary to H-application was measured with $H_{\text{static}}=21$ mT in an air-core solenoid connected to a DC generator. The magnetic field was created by flowing 0.46 A of current through a 10cm long air-core solenoid attaining 360 turns of copper wire. The change in temperature of the aqueous SPION mixture with respect to time, $(\partial T/\partial t)_{P,V,n}$, was monitored by placing a digital thermometer into an aqueous suspension of SPIONs contained in a 3 mL glass
container and applying the static magnetic field over a period of 10 minutes. Values were normalized with respect to the mass of SPIONs in the suspension. \((\partial T/\partial t)_{p,V,n}\) of the pure solvent was subtracted from the attained values.

**Synthesis of Siloxane-Terminated PEG Molecule**

The polymer synthesis procedure was derived from Bloemen et al. and used with only minor modifications.\(^{42}\) Briefly, 1.990 g (4.0 mmol) of neat allyl-PEG10-OH (figure 14, molecule 1) was combined by stirring with 440 mg succinic anhydride and 9.7 mg DMAP for 16 hours at 50°C in a round bottom flask. The resultant product (figure 14, molecule 2) was precipitated with cold diethyl ether and vacuum filtered to remove any solid impurities and isolate the filtrate (molecule 2). Then, 1 mmol of molecule 2 was combined by stirring with 186 µL of (3-mercaptopropyl)-trimethoxysilane and 12.8 mg of DMPAP for 1 hour under 365 nm light at room temperature (RT), forming molecule 3 (see figure 14). The disappearance of the C=C π bond during this reaction was monitored via thin film IR spectroscopy on NaCl plates.

**SPION Functionalization**

To conjugate the synthesized polymer to the SPION, 3 mmol of molecule 3 was added to a beaker consisting of 100 mg SPIONs, 50 mL toluene, 2.5 mL triethylamine, and 50 mL of water. The mixture was ultrasonicated for 5 hours at RT. The PEGylated nanoparticles were precipitated by adding 50 mL of heptane and copious washing with acetone. The PEGylated particles were then dried in a vacuum oven overnight, massed, and resuspended in Nanopure water to achieve a final concentration of 50 mg/mL.

**Addition and Evaluation of Streptavidin (SAv)**

The conjugation of a GBM-targeting molecule (a cytokine known as TWEAK) took place in two steps. The first involved the conjugation of streptavidin (SAv) to the
SPION-COOH complex. To do this, 800 µL of the PEGylated SPIONs (50 mg/mL) was re-suspended in MES buffer (0.05 M, pH=5) and combined with 32 µL of 150 mM EDC (in MES Buffer) and 16 µL of 300 mM Sulfo-NHS (in MES buffer) for one hour at RT. The SPIONs were isolated by centrifugation, re-suspended in 800 µL PBS, and combined with 80 µL of 10 µg/µL SAv overnight. The final product was re-suspended in 800 µL PBS and stored at -20°C.

Fluorescence spectroscopy was performed to confirm the presence of SAv on the surface of the SPION using a biotin-4-fluorescein probe by combining 0.25 µL of 160 µM biotin-4-fluorescein to 3 mL SPION-SA v nanoparticles (50 mg/mL). Fluorescence quenching was assessed with a Horiba Jobin Yvon fluorimeter with λex/λem at 485 nm/525 nm, respectively.

**Addition and Evaluation of TWEAK**

The TWEAK ligand was biotinylated according to the protocol from the biotinylation kit. Briefly, 110 µL of biotin (50 mM in Nanopure water) was added to 1 mL of a TWEAK cytokine solution (1 mg/mL in Nanopure water) to achieve a 20-fold biotin:TWEAK ratio. The mixture reacted for 60 minutes at RT. A spin desalting column was prepared and the newly biotinylated protein sample was spun through the column via centrifugation at 1000g for 2 minutes at RT. The biotinylated TWEAK was added to the SPION-SA v complex by combining 0.25 µL of biotinylated TWEAK and 100 µL of the SPION-SA v suspension for 1 hour at RT. The presence of TWEAK on the SPION-SA v complex was determined via fluorescence spectroscopy using the same fluorescent probe and λex/λem described above.
**Cell Culture Conditions**

Human U87 Glioblastoma Multiforme cells known to express the Fn14 receptor were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and a 1:2000 ratio of Gentamycin:DMEM. The GBM cells were grown on 75 cm² plates at 37°C and 5% CO₂. Sterile media changes were performed every 3 days until the cells were ~80% confluent, then sub-cultured at a 1:5 ratio to maintain a stock cell line. Jurkat T-Lymphocytes, graciously donated by Dr. Randall Reif, were maintained in RPMI 1740 media supplemented with 10% FBS and 1% penicillin antibiotic.

**Fluoromicroscopic Detection of SPION-Induced Apoptosis**

The GBM cells were sub-cultured onto poly-L-lysine doped sterile glass slides. A sterile size 3 BEEM capsule was added with non-cytotoxic glue (3:1 w/w mixture of paraffin: Vaseline) to the glass cover slip, as seen in figure 7.

![Figure 7: A diagram of the coverslip/BEEM capsule setup. Briefly, BEEM capsules were inverted and added with non-cytotoxic glue to a poly-L-lysine-doped cover slip, acting as a physical barrier that isolates a select few number of cells. These cells are what were analyzed.](image)
20 µL of SPION nano-complexes (5 mg/mL) was added to different aliquots of cells within the BEEM capsule for periods of 20 minutes, up to 4 hours. In all cases, the SPIONs and cells were placed in a 21 mT magnetic field. The magnetic field was created as previously described using the air-core solenoid. The cells were suspended within the air core solenoid and exposed to the magnetic field for their respective durations of time. Incubation of the GBM cells with doxorubicin/sterile PBS in the absence of any magnetic field acted as the positive/negative controls, respectively. Glass slides were washed, first with cold PBS, then Annexin V binding buffer (50 mM HEPES, 700 mM NaCl, 12.5 mM CaCl₂, pH=7.4). Following this, 4 µL of a 50 µg/mL solution of propidium iodide and 20 µL of an Annexin V suspension was added to the cells for 15 minutes. Excess fluorophore was removed by additional washing in Annexin V buffer, and the cells were assessed for apoptosis and cell death via epifluorescence microscopy. SPION-TWEAK complexes were also tested against Jurkat T-lymphocytes by incubating 20 µL of SPION-TWEAK complexes (5 mg/mL) with the cells for 3 hours in the presence of the magnetic field. Fluorescence staining and microscopy took place as previously described.

**Evaluation the Ultramorphological Changes of SPION-Induced Apoptosis via TEM**

GBM cells were subcultured into small petri dishes. In a manner identical to the fluoromicroscopic assays, 20 µL of SPION nano-complexes (5 mg/mL) was added to different aliquots of cells for periods of 20 minutes, up to 4 hours in the low energy magnetic field (created as previously described). In all cases, the SPIONs and cells were placed in a constant 21 mT magnetic field using the air core solenoid as previously described. SPION internalization was halted by incubating the cells with a fixative consisting of 2.5 % v/v glutaraldehyde and 1% v/v paraformaldehyde in 0.12M sodium
cacodylate buffer (SCB) at pH=7.4 for 40 minutes at RT then overnight in the refrigerator. The next day, the cells were washed with SCB and stained with 1% OsO4 (in H2O) for 60 minutes. The OsO4 solution was decanted and the cells were again washed with SCB and incubated overnight with 1% uranyl acetate (in H2O) in the refrigerator. Following overnight staining with uranyl acetate, the cells were washed with cold water and dehydrated by rinsing with ethanol solutions of ascending concentrations (from 30% to 100%). The cells were then embedded in LX112 epoxy substitute resin. Modified BEEM capsules were added over the cells and filled with additional resin. The entire sample was placed in a 60°C oven and allowed to harden for 48 hours. Following this, BEEM capsules were harvested (with pliers) and sent to the George Washington University (GWU) Nanofabrication and Imaging center for thin-film slicing onto TEM grids with an ultramicrotome. Samples were sent back to the University of Mary Washington and analyzed under TEM.
Results and Discussion

**SPIION Synthesis and Characterization**

In this study a precise and direct correlation between duration of iron oxidation and the SPIION diameter was made. To our knowledge, this is the first time this correlation has been empirically determined, providing a relatively facile and new method of controlling SPION size. Measurements from vibrating sample magnetometry and thermogenic analysis empirically showed that there is an optimal nanoparticle size that releases maximal amounts of thermal energy when exposed to an external magnetic field. These optimized SPIONs were bio-conjugated with a cancer targeting molecule and show promise in inducing programmed cell death in a time-dependent manner.

Electrolytic synthesis of iron oxide nanoparticles initially produced a light-yellow solution that darkened and eventually turned a murky green color with particulates floating throughout the solution. The proposed iron oxidation reactions occur sequentially as follows:

(I): \[ \text{Fe(s)} + 2\text{OH}^- (aq) \rightarrow \text{Fe(OH)}_2(s) + 2e^- \]

(II): \[ 3\text{Fe(OH)}_2(s) \rightarrow \text{Fe}_3\text{O}_4(s) + 2\text{H}_2\text{O(l)} + \text{H}_2(g) \]

(III): \[ 2\text{Fe}_3\text{O}_4(s) + 2\text{OH}^- (aq) \rightarrow 3\gamma\text{-Fe}_2\text{O}_3(s) + \text{H}_2\text{O(l)} + 2e^- \]

The reaction begins with the oxidation of Fe(s) by an aqueous hydroxide species present in solution, forming iron (II) hydroxide (a green-blue species). The formation of the Fe(OH)$_2$ accounts for the noted color changes in solution. Its formation is also supported by evidence previously gathered from Starowicz *et al.*$^{41}$ In an anaerobic and aqueous
environment, Fe(OH)$_2$ a redox reaction via the Schikorr reaction (step II) to magnetite. The continued oxidation of magnetite strips electrons from the crystal structure and converts Fe$^{2+}$ to Fe$^{3+}$. The resultant electron holes induce defects in the crystal’s inverse spinel structure. This, in combination with all Fe atoms being in the 3$^+$ oxidation state, results in the formation of maghemite (reaction III). The lack of Fe$^{2+}$ and presence of electron holes leads to changes in the geometry of the nanoparticle’s crystal structure, best described by Wu et al.$^{43}$ Magnetite attains a face centered cubic spinel structure where Fe$^{2+}$ ions occupy half of the octahedral sites and the Fe$^{3+}$ ions are split among the other half of the octahedral sites and all tetrahedral sites.$^{43}$ In maghemite, however, Fe$^{3+}$ ions fill all tetrahedral sites and most octahedral sites, with the octahedral sites being filled with the electron holes.

The slight variance in structure between magnetite and maghemite has fairly significant biological consequences regarding stability and cytotoxicity. In any living system, Fe$^{2+}$ can be oxidized to Fe$^{3+}$ by H$_2$O$_2$ innately present in the cell (as a byproduct of mitochondrial oxidative phosphorylation), given as

$$(IV): \text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{HO}\text{●} + \text{OH}^-$$

This reaction, known as the Fenton reaction, produces harmful hydroxyl radicals that can result in unwanted and nonspecific cytotoxicity, potentially harming healthy tissue. Unwanted Fenton reactions can be circumvented by minimizing the presence Fe$^{2+}$ species in the nanoparticle structure. The utilization of this electrochemical method of synthesis best ensured this by continued application of an oxidative potential for 20 minutes.
Magnetite and maghemite appear identical in both VSM and TEM studies. To differentiate amongst the slight variances in crystal structure between the two materials, ATR-IR spectroscopy was utilized. Briefly, IR spectroscopy operates by introducing electromagnetic radiation (EMR) to a sample. This EMR induces different patterns of bond vibrations that can be utilized to, in this case, differentiate between maghemite and magnetite. It was originally reported by Tartaj et al. that bond vibration patterns in the 1000 cm\(^{-1}\) to 200 cm\(^{-1}\) range is the best for discriminating between maghemite and magnetite as these IR energies have been shown to be sensitive to the order-disorder characteristics of magnetic nanoparticles.\(^{44}\) The magnetite structure (which is more ordered than maghemite) produces two large signals at 570 cm\(^{-1}\) and 360 cm\(^{-1}\), while maghemite produces numerous vibrational peaks within the same region of interest. This increase in the number of absorption signals is associated with increase in the disorder of the material.\(^{45}\) ATR-FTIR spectroscopy gathered on the nanoparticles synthesized in this work is shown in figure 8.
Figure 8: IR spectroscopy of the nanoparticles synthesized in this work. The peak at 680 cm\(^{-1}\) along with the numerous other vibration signals indicate that a disordered maghemite crystalline structure is present in the nanoparticles.

The presence of numerous vibration signals within this IR range, along with a broad vibrational peak at 680 cm\(^{-1}\) (arising from Fe-O vibrations), all indicate that the synthesized nanoparticles retain a disordered crystalline structure. By definition, maghemite is considered to have a more disordered structure due to the presence of the electron holes induced within the octahedral sites of the crystal. Thus, by evidence of IR spectroscopy, it is asserted that the nanoparticles produced in this work retain a crystalline structure similar to maghemite.

As previously stated, maghemite displays superparamagnetic properties at the nanoscale. These properties largely depend on both the geometric size and number of
magnetic domains within the material. Thus, it was quickly realized that precisely control of SPIONs size was vital to this work. This was achieved by controlling the duration of synthesis of the electrochemical reaction. Diameters of SPIONs synthesized for differing durations of time were measured via TEM and plotted as a function of their respective duration of synthesis. Figure 9 shows the physical morphology of the SPIONs from TEM and the correlation between SPION size and duration of electrochemical synthesis.

From figure 9, it is clear that the SPIONs attain an electron-dense, spherical morphology. This is consistent with what was reported by Starowicz et al.\textsuperscript{41} The resultant SPION measurements were plotted as a function of the duration of electrochemical oxidation. When this was done, a linear trendline is achieved with a correlation coefficient of 0.97. Compared against the original literature, this correlation coefficient is greatly improved from what was originally reported by Starowicz et al. (see Starowicz figure 9).\textsuperscript{41} The reproducibility of this synthetic method was tested over twenty times by six different undergraduate researchers across multiple years, all with no significant deviance from the reported trendline. Thus, we can assert with high confidence that this method shows improved linearity and reproducibility compared to the original method initially reported by Starowicz et al.
Figure 9: (top) TEM images of a sample of SPIONs synthesized in this work. From TEM, these SPIONs appear as spherical, electron dense structures. (bottom) SPION size measurements were plotted as a function of the duration of synthesis (in minutes). From this relationship a correlation coefficient of 0.97 is achieved. This indicates that, with great precision, the size of the SPION can be controlled simply by varying the duration of the electrochemical oxidation reaction.
Just as important as average diameter of a SPION is the variance of individual SPION diameters from their respective means. As SPION size is largely correlated to magnetic properties, thermal energy output, and cellular internalization, having small variances from the mean SPIONs size is essential to both producing reliable thermal energy output and efficient cellular internalization. Here, SPION size variance is studied by employing the coefficient of variation (CoV), which normalized the standard deviation of a samples against the mean sample size, as seen in equation 5:

$$\text{CoV} = \frac{\sqrt{\frac{\sum(x-\bar{x})^2}{n}}}{\frac{\Sigma x}{n}} \cdot \frac{\sigma}{\bar{x}},$$

where $x$ is the individual value, $\bar{x}$ is the mean of a data set, $n$ is the number of samples in that data set, and $\sigma$ is the calculated sample standard deviation. The percent frequency of each set of CoV values was then calculated and is displayed in figure 10.
Figure 10: The percent frequency of a range of coefficients of variation (CoV=σ/μ) gathered from all SPION diameters measured. From this data set, it is shown that most SPIONs attained a CoV of less than 0.04 units. This indicates a small spread of data points amongst their respective means.

Figure 10 shows that most of the SPIONs attained a CoV value less than or equal to 0.04 units with no SPIONs analyzed having CoV values greater than 0.4 units. This indicates that the SPIONs analyzed achieved a very small spread from their respective means. Overall, this provides evidence that by varying the duration of electrochemical oxidation, it was not only possible to achieve precise average SPION sizes, but that the spread of data among each size category remained small. Thus, the synthetic method has not only proved to be reliable but also precise and accurately reproducible.
Following analysis of SPION size, the magnetic properties of the synthesized nanomaterials were analyzed utilizing vibrating sample magnetometry (VSM). In brief, VSM operates by mechanically vibrating a sample placed in a static magnetic field with a piezoelectric actuator. Based on Faraday’s law, movement of a magnetic material in a static magnetic field produces an electrical field. This electrical field is detected by the instrument by another electromagnetic coil, amplified, and shown on a computer. By varying the external magnetic field (H), the magnetization (M) also changes and a hysteresis loop (i.e. M(H) curve) is generated. Within the hysteresis loop, two values are of importance to this study. Saturation magnetization (M_{sat}) is defined as when the magnetization of the magnetic material can no longer be increased despite increases in the external magnetic field. A lower M_{sat} value indicates that a smaller magnitude of H is needed to achieve maximal magnetization. Theoretically, superparamagnetic materials would have low M_{sat} values as uni-magnetic materials would require low amounts of magneto-potential energy to achieve saturation. Coercivity (H_c) is defined as the points at which the M(H) curve crosses the x-axis. Its value describes the amount of magnetization leftover within the material after the external magnetic field has been completely removed. Ideal superparamagnetic materials have H_c values of 0; however, experimentally, superparamagnetism has previously been defined as H_c<0.01T. Figure 11 shows the M(H) curves of the SPIONs synthesized in this work.
Figure 11: M(H) curves of the SPIONs synthesized in this work taken via vibrating sample magnetometry. From this graph it is seen that 53 nm SPIONs attain the largest saturation magnetization while 21 nm SPIONs attain the smallest.

All SPIONs analyzed via VSM display sigmoidal curves. This indicates that all materials analyzed do not display diamagnetic or paramagnetic behavior, as both the former and latter attain linear trendlines in hysteresis loops. They are all either ferromagnetic/ferrimagnetic, or superparamagnetic. Figure 11 best displays the $M_{\text{sat}}$ behavior of the SPIONs with 53 nm particles attaining the largest $M_{\text{sat}}$ value (53 emu/g) and 21 nm particles attaining the smallest (8.9 emu/g). This observation provides the first evidence that 21 nm SPIONs have optimal superparamagnetic behavior as they require the least amount of incident magnetopotential energy to achieve saturated magnetization.
Figure 12 magnifies the origin of the graph in figure 11 to analyze the coercivity of the SPIONs.

Figure 12: A magnified view of the hysteresis loops presented in figure 9. From this view it is clear that 21 nm SPIONs display the greatest amount of superparamagnetic behavior. Additionally, by the working definition of superparamagnetism, 45 nm and 53 nm SPIONs are above the quantitative threshold (100 Oe=0.01T) of what is considered to be superparamagnetic.

Figure 12 shows two main important observations. As previously stated, the closer the \( M(H) \) curve approaches 0 on the x-axis, the more superparamagnetic the material is. By this definition, 21 nm SPIONs retain the most superparamagnetic behavior. This aligns with the observations gathered from figure 11 regarding the \( M_{\text{sat}} \) values as the magnetic dipoles of all atoms in superparamagnetic materials point in a single direction and therefore require low amounts of magnetopotential energy to align in the direction of \( H \). Secondly, both 45 nm and 53 nm particles surpass the quantitative threshold for what is
considered to be superparamagnetic. The sizes of these materials have become so large that fractured domains appear in the crystal, and thus, larger amounts of magnetopotential energy are required to achieve saturation. This is seen in figure 11 as both 45 nm and 53 nm particles require larger amounts of energy to achieve $M_{\text{sat}}$. To this point, a correlation has been made between SPION size and magnetic properties that supports what was initially theorized regarding ideal size-dependent superparamagnetism and the effects of magnetic domains diminishing those optimal properties. From here, a second correlation needs to be performed, linking optimal superparamagnetism to thermal energy output.

Thermal energy production was measured by suspending SPIONs in DI H$_2$O and placing the mixture in a low-energy magnetic field ($B=21$ mT). The change in temperature ($\Delta T$) of the solution was measured over the course of 10 minutes for all sizes of SPIONs. $\Delta T$ of pure solvent was background subtracted from all values and the resultant temperature gradients are shown in figure 13:
Figure 13: The change in temperature of a SPION solution over the course of 10 minutes in the presence of a low-energy magnetic field. From these data it can be seen that 21 nm SPIONs were able to induce the greatest ΔT over the course of 10 minutes.

Figure 13 shows that all SPIONs were able to induce changes in temperature of the aqueous solution. The derivative of the temperature gradients was taken with respect to time at constant pressure, volume, and moles of SPION. Presumably this temperature change was induced by the incomplete oscillatory rotations of the SPIONs in the mixture.

<table>
<thead>
<tr>
<th>SPION Size (nm)</th>
<th>( \frac{dT}{dt} )_{P,V,n} (°C s^{-1} g^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>0.053</td>
</tr>
<tr>
<td>21</td>
<td>0.058</td>
</tr>
<tr>
<td>45</td>
<td>0.051</td>
</tr>
<tr>
<td>53</td>
<td>0.052</td>
</tr>
</tbody>
</table>

Table 1: A tabulation of the change in temperature as a function of time at constant pressure, volume, and temperature. From these data, it is seen that 21 nm SPIONs produce the greatest change in temperature per unit time.
The results of this analysis are tabulated in table 1. Figure 13 shows that 21 nm SPIONs were able to induce the greatest \( \frac{dT}{dt}_{P,V,n} \) over the experimental course, followed by 19 nm, 45 nm, and 53 nm SPIONs (quantitatively supported by the tabulated data in table 1). Interestingly, this closely follows the degree to which each of the respective SPIONs display superparamagnetic behavior, with 21 nm SPIONs displaying the greatest and 53 nm SPIONs the least. In addition, the superparamagnetic SPIONs (19 nm, 21 nm) both induced larger \( \frac{dT}{dt}_{P,V,n} \) values than the non-superparamagnetic particles (45 nm, 53 nm).

Taken together, figure 13 and table 1 quantitatively show that the property of superparamagnetism is directly correlated to thermal energy output. Because superparamagnetism itself is correlated with particle size, thermal energy output is linked to SPION size and by manipulating the latter, the former can be controlled. It is through harnessing this thermal energy that thermal denaturation of vital intracellular structures that leads to Glioblastoma Multiforme cell death will be achieved.

**SPION Biofunctionalization**

It is well known that bare SPIONs lead to aggregation and activation of an immune response in *in vivo* and *in vitro* systems.\(^\text{42}\) To circumvent this, a poly(ethylene) glycol (PEG)-based polymer was added to the surface of the SPION. In addition to “cloaking” the SPION from the immune system, this polymer also contained a carboxylic acid functional group that was used in future steps to add the GBM-specific TWEAK cytokine. The reaction progressed as detailed in figure 14 and was monitored via IR spectroscopy. The IR spectra of the allyl-PEG10-COOH (molecule 2) contained a fingerprint region independently unique when compared to IR spectra of the starting
reagents (succinic anhydride and molecule 1) (data not shown). A carbonyl signal representing the formation of a C=O π-bond is present at 1730 cm⁻¹. This is highlighted by a blue box in figure 14. Following addition of the siloxane group via a click reaction under UV light, IR spectroscopy confirmed the loss of the C=C π bond at 1650 cm⁻¹ while retaining the C=O π bond previously mentioned. All spectra obtained agree with those originally reported by Bloemen et al.⁴² Although IR spectroscopy is facile and easily accessible, it was admittedly difficult to visualize the transformation between molecules 2 and 3, particularly the disappearance of the C=C π bond with solely IR spectroscopy. To ensure molecule 3 was synthesized, ¹HNMR spectroscopy was performed and the obtained spectra are seen in figure 15.
Figure 14: Infrared spectra of the allyl-PEG10-OH polymer (molecule 1), the carboxy-terminated PEG molecule (molecule 2) and siloxane-terminated PEG molecule (molecule 3). Between molecules 1 and 2, the emergence of a signal at 1730 cm\(^{-1}\) indicates the presence of a C=O group (highlighted by the blue box). Between structures 2 and 3, the disappearance of the signal at 1650 cm\(^{-1}\) indicates the loss of a C=C bond (highlighted by the red arrow).
Figure 15: $^1$HNMR spectra of the molecules 2 and 3. Of particular importance are the loss of peaks at $\delta=6.0$ ppm and $\delta=5.25$ ppm (highlighted by the red box). These signals are representative of hydrogens abutting the C=C $\pi$ bond and loss of these signals provides significant evidence that the C=C $\pi$ bond was lost. Thioester-specific peaks also emerge at $\delta=2.25$ and $\delta=2.5$ ppm, respectively in the $^1$HNMR of molecule 3.
The $^1$HNMR spectra between molecules 2 and 3 display both common and unique chemical shifts. For example, the large peaks at $\delta=2.25$ ppm and $\delta=3.75$ ppm account for the hydrogens in the repeating polymer subunit of the molecule. These peaks are retained between molecules 2 and 3. Of particular significance is the disappearance of signals at $\delta=6.0$ ppm and $\delta=5.25$ ppm. Chemical shifts in this range are traditionally indicative of vinylic hydrogens; therefore, loss of signals within this range provides strong evidence that the C=C $\pi$ bond was lost and replaced with the S-C bond upon the addition of the 3-mercaptopropyltrimethoxyamine group in molecule 3. Thioester-specific peaks at $\delta=2.25$ and $\delta=2.5$ also support the claim that the click-chemistry reaction occurred as described. This particular pattern of signal gains and losses is supported by the literature values originally gathered by Bloemen et al.\textsuperscript{42}

Sonication of the polymer, SPION colloid mixture, and a small amount of water induced the formation and collapse of microscopic vapor bubbles. Upon collapse of the vapor bubble, a large amount of energy is released to that local micro-environment. The theory is that this energy lowers the activation energy barrier that allows for covalent bonding of the polymer to the nanoparticle through the formation and interaction of sonolysis radicals. This mechanism was not further explored or empirically proven within the scope of this research. Following attachment of molecule 3 to the surface of the SPION via ultrasonication, the carboxylic acid terminus of the polymer was modified by EDC/Sulfo-NHS chemistry to create a suitable platform to first attach a SAv protein and then attach the cancer targeting molecule. Carbodiimide (EDC) molecules are favorable for crosslinking carboxylic acids because no portion of their structure becomes part of the
final bond between the two conjugated molecules. The carbon involved in the carbodiimide functional group is electrophilic and is a prime candidate for nucleophilic attack by the carboxylic acid alcohol. This forms an unstable \( o \)-acylisourea intermediate. To stabilize the intermediate for further reaction, \( N \)-hydroxysuccinimide is added via an additional nucleophilic reaction, with the alcohol on the latter species attaching the electrophilic carbonyl carbon on the \( o \)-acylisourea intermediate. A final nucleophilic attack by a primary amine on a protein of interest (either SAv or TWEAK) produces the final stable conjugate with two target molecules linked by an amide linkage. This crosslinking mechanism is depicted in figure 16.

The presence of SAv was confirmed with a fluorescence quenching assay. Biotin-4-fluorescein is a fluorophore that emits light at 520 nm. When bound to SAv, however, the fluorophore’s ability to emit light is quenched. This property can be utilized to assess for the presence of SAv. Following SPION-SAv isolation, biotin-4-fluorescein was added and was assessed for quenching. This was repeated using PBS, SPION-PEG, and SPION (see figure 17). When the fluorophore binds to pure SAv, the fluorescent emission largely decreases, as expected. When the fluorophore was mixed with both uncoated SPIONs and SPION-PEG complexes, mild quenching occurred, though not to the extent as when the fluorophore is bound to SAv. This could be attributed to either a decrease in the amount of light reaching the fluorophore (likely through absorption of the light by the SPIONs) or nonspecific binding of the fluorophore to the SPION or polymer. When the fluorophore was mixed with the SPION-SAv complex, however, a decrease in emission was observed consistent with the fluorophore+SAv test. These results indicate the presence of SAv on the surface of the SPION.
Figure 16: The simplified mechanism by which EDC/Sulfo-NHS operates. Briefly, the hydroxyl on a COOH functional group performs a nucleophilic attack on a carbodiimide group, forming an unstable $\alpha$-acylisurea group. This reacts with sulfo-NH which bonds to the carbonyl carbon through a nucleophilic attack. The primary amine of a protein then attacks the same electrophilic carbonyl, and the sulfo-NHS group leaves as a nitroso compound. The final product is two target molecules linked through an amide bond.
Figure 17: Fluorescence spectroscopy assay of the SPION-SA v complexes with pure fluorophore (negative control), fluorophore with SA v (positive control), uncoated SPION, SPION-PEG, and SPION-SA v. The fluorophore exhibits large amounts of quenching when incubated with SPION-SA v in a manner similar to when incubated with pure SA v.

The purpose of the SA v was to utilize its strong affinity towards biotin to create a strong linker system that can connect the SPION to a biotinylated cancer targeting molecule. In this study, the targeting molecule used was the TNF-related weak inducer of apoptosis, or TWEAK, cytokine. Cytokines, like all proteins, possess numerous primary amines available for modification. These amine groups were manipulated with a Sulfo-NHS-Biotin linker available through the EZ-Link Sulfo-NHS kit, the mechanism of which is shown in figure 18.
Figure 18: The biotinylation mechanism of the TWEAK cytokine. Briefly, a primary amine attacks the carbonyl abutting the sulfo-NHS group. This causes Sulfo-NHS to act as a leaving group with the nitroso group eventually resolving to a tertiary amine with an alcohol group. The long alkyl chain linking the TWEAK to the biotin ensures there is adequate space between the biotin and the protein such that no steric hindrance would occur in the SAv-Biotin binding reactions.

In this mechanism, a nonspecific primary amine undergoes nucleophilic attack of the carbonyl carbon directly abutting the sulfo-NHS group. This induces Sulfo-NHS to leave as a leaving group with its nitroso group resolving to a tertiary amine following hydration with water. What is left on the TWEAK is a long alkyl chain connecting the protein to the
biotin molecule. The alkyl chain minimizes any steric hindrance the TWEAK protein would induce in the SAv-Biotin linking reaction.

The binding of biotin to SAv is one of the strongest noncovalent interactions in nature, thus, by introducing the biotinylated TWEAK molecule to the SPION-SAv group at RT under gentle shaking, the SAv-biotin noncovalent interaction was formed. Following isolation and resuspension of the SPION-TWEAK complexes, fluorescence spectroscopy was again utilized to confirm the presence of TWEAK on the SPION core with the same fluorophore. It was hypothesized that because all binding sites on the SPION-SAv complex were now occupied with biotinylated TWEAK, no significant fluorescent quenching would occur to the extent of what is seen in figure 17 as the fluorescence biotin would have few-to-none open binding sites to associate with. Figure 19 shows that negligible quenching occurred when the fluorophore was incubated with SPION-TWEAK complexes, confirming the original prediction.
Figure 19: Fluorescence spectroscopy of SPION-TWEAK complexes with a biotinylated fluorophore shows negligible quenching, indicating that the SAv sites on the SPION-SAv core are occupied with biotinylated TWEAK molecules.

The magnetic behavior, diameter, and thermogenic properties of the fully bioconjugated SPIONs were again characterized. TEM analysis of the SPIONs showed that the bioconjugated SPION complex grew, on average, by 33 nm +/- 6 nm to a final average size of 52 nm +/- 6 nm, as seen in figure 20.

Figure 20: A TEM image of the final bioconjugated SPION product, showing a size increased by approximately 30 nm to a final diameter of approximately 50 nm. The electron density around the periphery of the particle is notably less, likely corresponding to the presence of the polymer and protein network.

The magnitude of the observed growth is expected because the sizes of both the polymer and SAv protein are approximately equal to 29 nm. The observed size is within the error limits of the ideal increase in diameter (estimated by summing the length of the polymer and protein, respectively). Thermogenic analysis revealed a small decrease (-0.006°C sec^{-1}g^{-1}) in the thermal energy released by the SPION. This was likely due to the polymer and protein increasing drag (thus decreasing rotation frequency) in the aqueous medium.
The initial part of this work involved the development and optimization of a novel nanotherapeutic complex. This endeavor began by optimizing a previously described electrochemical method for synthesizing SPIONs. Through thermogenic and VSM analysis, an optimal size of 21 nm was elucidated. The surface of this optimally sized particle was decorated though the addition of both a polymer and a GBM-targeting molecule (known as TWEAK). The presence of TWEAK on the SPION was confirmed via fluorescence spectroscopy. Following development of the SPION-TWEAK nano-complex, its ability to selectively target and induce programmed cell death in U87 GBM cells was carried out in vitro with exciting and promising cytotoxic and specificity results.
**In vitro testing: U87 GBM Studies**

To test the *in vitro* effects of the SPION-TWEAK nano-complex, GBM cells were grown onto coverslips modified for optical microscopy by adding the inverted BEEM capsules. The cells were then treated with either doxorubicin (DOX; the positive control), sterile PBS (the negative control), or SPION-TWEAK complexes. During the mid-to-late stages of programmed cell death, cells signal macrophages for phagocytosis by upregulating the concentration of phosphatidylserine (PS) at the external cell membrane.\(^{48}\) Annexin V is a human anticoagulant Ca\(^{2+}\)-dependent phospholipid binding protein with a high affinity for PS. Thus, fluorescein-conjugated Annexin V (FITC-Annexin V) gives a reliable method for detecting cells marked for programmed cell death. Propidium iodide (PI) is a heterocyclic aromatic fluorophore that is typically impermeable to live cells. The molecular structure of this fluorophore is seen in figure 21.

![Molecular structure of propidium iodide](image.png)

**Figure 21:** The molecular structure of propidium iodide: a fluorophore that was used to detect cell death.

In dead cells with a denatured external cell membrane, the fluorophore is able to diffuse into the nucleus and intercalate with DNA. Figure 22 shows the positive and negative controls of Annexin V/propidium iodide staining, respectively. Positive controls were treated with cytotoxic concentrations of DOX and negative controls were treated with equimolar amounts of PBS.
Figure 22: U87 GBM cells were treated with either cytotoxic amounts of the apoptotic agent doxorubicin (a/b) or equimolar amounts of PBS (c/d), each for 6 hours, with the former being classified as the positive control for programmed cell death and the latter the negative control.

As figure 22 shows, significant green fluorescence emission is noted in cells treated with DOX, particularly around the periphery of the cell. This indicates that there is an externalization of PS at the external cell membrane. In addition, the red fluorescence emission in figure 22 (a) is indicative of PI intercalating with the DNA in the nucleus of the cell. Morphologically, the cell of interest appears shrunken and blebbed (figure 22
(b)), all consistent with the mid-to-late stages of programmed cell death. Neither of these fluorescence signals are seen in the negative control treatment of cells with PBS (as expected) (figures 22 (c)/22 (d)).

Figure 22 shows that the fluorescence dyes of choice perform well in discriminating cells that show biochemical signs of programmed cell death against those that do not. The same fluorophores were then used to assess for cellular death in GBM cells following exposure to SPION-TWEAK nano-complexes over a period of 3 hours in a constant low energy (21 mT) magnetic field. The results, shown in figure 23, show a clear and distinct trend towards cell death within the experimental timeframe.

Figure 23. Respective white light and fluorescence images of cells exposed to TWEAK-conjugated SPIONs for 20 minutes (a/b), 100 minutes (c/d), 2 hours (e/f), and 3 hours (g/h). From these images cells exposed to SPIONs undergo cell death through an apoptotic mechanism within 180 minutes following exposure to SPIONs. The size bar is 10 µm in all cases.

At 20 minutes of SPION exposure, no significant, morphologically relevant fluorescence emission is visualized (figure 23 (a)/23 (b)). Individual cells begin fluorescing after approximately 90-100 minutes of SPION exposure, and their morphology appears
blebbled, all consistent with the cells being in the late stages of apoptosis (figure 23 (c)/23 (d)). At 2 hours, red and green emission is seen simultaneously within the same cells, indicating that cell death has occurred (figure 23 (f)). At 3 hours, much of the fluorescence emission observed is red, indicating that most cells in this image are likely dead. Figure 23 provides evidence that SPION-TWEAK nano-complexes are able to initiate programmed cell death approximately 60-90 minutes following initial deposition. This timeline is similar to what has been previously reported by both He et al. and Yue et al. in their studies of the time-dependent SPION-induced programmed cell death of GBM cells. 39,40

To further explore the capabilities of SPION-TWEAK complexes to induce programmed cell death in the presence of a magnetic field, TEM was performed to both track particle internalization and analyze subsequent cellular morphology. To halt SPION internalization and any subsequent cellular interactions, the GBM cells were immersed in a fixative solution following relevant durations of time (20 minutes, 100 minutes, 2 hours, and 3 hours). The TEM studies, shown in figure 24, show that SPIONs internalize into the cell within 20 minutes of exposure (figure 24 (b)). Their presence in vesicles is apparent at least over the next 80 minutes (figure 24 (c)). Interestingly, at 100 minutes of cellular exposure, the average SPION diameter has notably decreased from 50 nm to approximately 15 nm. The nature of this decrease in size has not been fully elucidated and is the subject of future work. Cells exposed to SPIONs for 2 hours begin to show SPION accumulation in both the mitochondria (figure 24 (d)) and the nucleus (not shown), and at 3 hours of exposure the cells show heterogenous breakdown of the
chromatin, nuclear envelope, and large vacuole formation, all signs consistent with the late stages of programmed cell death.\textsuperscript{48,49}

Figure 24: Images taken via TEM of GBM cells. Healthy GBM cells display a morphologically intact nucleus with condensed chromatin and intact organelles (a; size bar 500 nm). After 20 minutes of SPION exposure, the nanoparticles are seen internalizing into the cell and are trapped in vesicles (b; size bar 20 nm). At 100 minutes (c; size bar 100 nm), SPIONs are still trapped in well-formed vesicles, though distinctly smaller than their known size at this point (\( \bar{x} = 15 \) nm). Cells exposed to SPIONs for 2 hours begin to show nanoparticle accumulation in organelles such as the mitochondria (d) and nucleus (not shown). By the third hour, nuclear envelope and chromatin breakdown along with large vacuole formation are visualized; all consistent with programmed cell death (e/f; size bar 100 nm and 500 nm, respectively).

Following these results, the SPION-TWEAK complexes were tested against other types of cell lines to ensure that the SPIONs were specific to only GBM cells. This was accomplished by incubating the SPION-TWEAK complexes with Jurkat T-lymphocytes
over the course of three hours. DOX and PBS again acted as the positive and negative controls, respectively. Fluorescence microscopy was then performed as previously described, and the resultant images are seen in figure 25.

Figure 25: Jurkat T-lymphocytes were incubated with either Doxorubicin (a/b), PBS (c/d), or SPION-TWEAK complexes (e/f). Minimal phosphatidylserine upregulation was noted in both the negative control and in the SPION-TWEAK samples while the positive control exhibited both increased phosphatidylserine expression and propidium iodide intercalation within the nucleus.

From figure 25 it is clear that the SPION-TWEAK complexes did not produce gross cell death as they did in the U87 GBM cells. In fact, only 1 cell out of the 20 exposed to SPION-TWEAK complexes showed biochemical evidence of upregulated PS at the external cell membrane, and no cells showed evidence of PI nuclear intercalation (figure 25 (f)). The dramatic difference in the capabilities of SPION-TWEAK to produce cellular death in GBM versus Jurkat T-Lymphocytes likely stems from the fact that the SPIONs
negligibly internalize into the lymphocyte cell line as these cells do not express the Fn14 receptor that TWEAK is a ligand for.\textsuperscript{50} In fact, within the environment of the central nervous system no cells aside from late stage neoplastic glial cells (such as GBM) substantially express the Fn14 receptor at the external membrane.\textsuperscript{6,38} Since non-neoplastic cells glial and neuronal cells are related to Jurkat T-lymphocytes in that both types of cells lack the Fn14 receptor, it is hypothesized that a similar result of no detectable programmed cell death would occur in the healthy parenchymal cells; however, proving this experimentally is the subject of future work.

**Conclusion:**

Glioblastoma Multiforme is regarded to many within the medical community as nature’s deadliest brain cancer. This work describes the novel synthesis of a SPION-TWEAK nano-complex for targeted induction of programmed cell death of glioblastoma cells. By employing a novel time-dependent approach on a previously described electrochemical synthesis, an optimal particle size was afforded that released maximal amounts of heat following excitation with a low energy magnetic field. The final SPION-TWEAK nano-complex was tested against both U87 Glioblastoma Multiforme cells and Jurkat T-Lymphocytes, and, utilizing a combination of fluorescence and transmission electron microscopy, sufficient biochemical and morphological evidence was gathered to assert that the SPION-TWEAK nano-complexes were able to induce programmed cell death exclusively in cells that express Fn14 at the external cell membrane.

From this promising \textit{in vitro} data we hope to gather more data regarding how the SPION-TWEAK complex induces death. Particularly, exploring the loss of mitochondrial membrane depolarization, which would indicate a loss of the ability to synthesize ATP is
of interest. Additionally, we hope to expand this therapy to an *ex vivo* model where nanoparticle internalization is analyzed in the environment of the tumor microenvironment. The destruction of cellular organelles would make programmed cell death of the invasive rim cells inevitable and irrevocable. In the GBM patient, this would increase disease survival and improved long-term prognoses.
Works Cited


Matthew A. Tovar

(830) 302-1225
mtovar@mail.umw.edu

Education

George Washington University School of Medicine Washington, D.C.

- METEOR Research Fellow
- Accepted June 2017 via Early Selection Agreement
- Doctor of Medicine, Anticipated May 2023

University of Mary Washington Fredericksburg, VA

- B.S. Biochemistry, Anticipated May 2019
  - ACS Approved Chemistry Curriculum
- University Honor’s Scholar
- Departmental Honors in Biochemistry

Grants, Awards, and Accomplishments

IAC Outstanding Biochemistry Award 2019
Chi Beta Phi Senior Key Award 2019
SURC Outstanding Oral Presentation 2019
Naval HPSP 4 Year Scholarship Recipient 2018
Chi Beta Phi National Meeting 1st Place in Undergraduate Research 2018
ACS National Meeting Outstanding Undergraduate Research Poster 2018
Earl G. Insley Chi Beta Phi Scholarship 2018
Irene Piscopo Rodgers Research Fellowship II 2018
First UMW student accepted to GWU School of Medicine Early Acceptance Program 2017

Irene Piscopo Rodgers Research Fellowship I 2017-2018
University Undergraduate Research Grant 2016-2017
P. Sukalo and J. Boarman Award 2016-2017
University Deans list (x5) 2015-2017
University President’s List Fall 2016
Eagle Scout 2014

Research Experience

Principal Undergraduate Researcher
The University of Mary Washington Fredericksburg, VA 2018- Present
Adviser: Dr. Robert Wells, Ph.D.

Analyzing the Neurobiological Connections Between Musical Chord Structures and Emotion
- Novel connections were made between the physical phenomena of acoustics, the biological phenomena of auditory sensation, and common musical chord structures seen in westernized music.

- Work culminated in the assembly of a senior-level research dissertation.

**Principal Undergraduate Researcher**
The University of Mary Washington Present
Adviser: Dr. Leanna Giancarlo, Ph.D.

**Synthesis of a Superparamagnetic Iron Oxide Based Nano-complex for Targeted Cell Death of Glioblastoma Cells**

- A novel synthetic method was utilized and optimized to create magnetic nanoparticles with optimal thermogenic properties. These optimized nanoparticles were bio-conjugated through the addition of a PEG-based polymer and a cancer targeting peptide.

- Transmission electron microscopy confirmed that the nanoparticles were selectively endocytosed into Glioblastoma cells. Epifluorescence microscopy was utilized to confirm that the nano-complexes induced apoptosis after approximately 3 hours of cellular exposure.

- Acquired merit-based funding through the Irene Piscopo Rodgers Research fellowship.

- Assembled and managed a research team consisting of 5 unpaid undergraduate students.

- An informal cross-institutional partnership was formed between George Washington University’s Magnetics Research Lab and my research group was formed. In this partnership, a post-doctoral fellow was recruited via a material-for-service trade where I purchased his instrument parts and he ran magnetic hysteresis tests on my samples.

- Presented results at 10+ conferences; one manuscript submitted for publication; one departmental honors dissertation prepared and defended.

**Assistant Undergraduate Researcher**
The University of Mary Washington Fredericksburg, VA 2015-16
Adviser: Dr. Lynn Lewis, Ph.D.

**Characterizing Di’ildio: A unique syphoviridae bacteriophage**
A novel, unique bacteriophage was isolated from the bacterium *Bacillus thuringiensis* subspecies Berliner DSM 350 that originally came from a soil sample collected in Fredericksburg, Virginia. Transmission electron microscopy, polymerase chain reactions, gel electrophoresis, primer synthesis, and bioinformatic software was used to characterize the bacteriophage.

- Operated as a member of a three-person team, to include one other undergraduate student and a professor.

**Professional Associations**
- Phi Beta Kappa Academic Honor Society
- American Chemical Society (University Faculty Liaison Officer 2017-2018)
- Chi Beta Phi STEM Honor Society (Society President 2017-2018; Society Senior Advisor 2019)
- Phi Eta Sigma Academic Honor Society

**Publications**


**Selected Conference Presentations**


Clinical Experience

Emergency Room Medical Scribe 2016-Present

Mary Washington Hospital, Stafford Hospital
Fredericksburg, VA

- Assisted physicians and PAs with documentation by conducting patient interviews, recording procedures, and reviewing charts.

- Provided additional miscellaneous clerical support for medical providers.

Emergency Room Technician 2015-2016

Stafford Hospital
Stafford, VA

- Provided patient care in a high-volume patient environment with a wide variety of medical and traumatic emergencies.

- Worked as a member of the code team in resuscitating adult, pediatric, and neonatal patients.

- Administered emergent diagnostic tests to patients upon orders of physician including administration and interpretation of 12-Lead EKG’s, urine and blood analysis tests, and rapid radiological studies.

- Performed medical procedures including sterile catheterization and setting fractures bones in temporary fiberglass casts.

- Assisted nurses in triaging patient acuity.

- Assisted in various emergent medical procedures such as laceration repair, reducing complex fractures, emergency thoracenteses, cricothyrotomies, rapid sequence intubation, and emergent anesthesia administration.

Emergency Medical Technician 2014-Present

Stafford County Fire and Rescue
Stafford, VA

- Operated as a member of a 2-3-person fire and rescue team. Independently provided basic life support and assisted paramedics in providing advanced life support medical care to patients suffering from acute medical emergencies.

References

Dr. Leanna Giancarlo, PhD. Associate Professor of Chemistry. Department of Chemistry, University of Mary Washington, Fredericksburg, VA 22401. 540-654-1407. lgiancar@umw.edu

Dr. Kelli Slunt, PhD. Professor of Chemistry. Department of Chemistry, University of Mary Washington, Fredericksburg, VA 22401, 540.654.1406. kslunt@umw.edu
Dr. Agostino Visioni, M.D. Attending Neurosurgeron. Mary Washington Medical Group Neurosurgery. divisioni@hotmail.com