Investigation of the MS2 bacteriophage capsid as an MRI-capable, brain-targeted nanoparticle platform

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INVESTIGATION OF THE MS2 BACTERIOPHAGE CAPSID AS AN MRI-CAPABLE, BRAIN-TARGETED NANOPARTICLE PLATFORM

by

Stephanie M. Curley

A Dissertation
Submitted to the University at Albany,
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INVESTIGATION OF THE MS2 BACTERIOPHAGE CAPSID AS AN MRI-CAPABLE, BRAIN-TARGETED NANOPARTICLE PLATFORM

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ABSTRACT

Novel methods are needed to traverse the blood-brain barrier (BBB) and deliver drugs to specific targets in the brain. To this end, MS2 bacteriophage was explored as a multifunctional transport and targeting vector. The MS2 capsid exterior was modified with two different targeting moieties for delivery across the BBB and targeting specific regions of interest in the brain. Successful modification of MS2 capsids with a brain targeting peptide and NMDAR2D-targeting antibody was confirmed by immunoblotting and fluorescence detection. To measure transport efficiency of MS2 particles across an in vitro BBB model, a highly sensitive RT-qPCR protocol was developed and implemented. Finally, in order to demonstrate the potential of MS2 as a drug delivery vehicle, interior loading of capsids was investigated with the MRI contrast agent Gd-DOTA modified with psoralen. While the focus of this dissertation was to develop a nanoparticle platform toward a specific purpose, we have also developed a foundational toolkit for use with any system amenable to the functionalization chemistries and PCR protocols presented herein, ultimately offering longevity and further application of the techniques developed in these studies.
ACKNOWLEDGMENTS

Throughout my time at CNSE, there have been numerous people who have helped and supported me. I have said multiple times that I feel as though I was raised by the village, and this has never been more true than during the last year of my PhD. Without these people and their support, this degree might not have been possible. Support has come in many forms, from personal to professional, monetary to emotional, and has involved anything from text messages across the country to face-to-face meetings in the lab. Below is just a small subset of those who have been there for me throughout the last four years, but I would like to say that I appreciate everyone at CNSE, including professors, post-docs, students, and staff, for their constant support, as well as putting up with all of my griping and grumbling.

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<tr>
<td>AP2</td>
<td>Angiopep-2</td>
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<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
</tr>
<tr>
<td>BCEC</td>
<td>Brain capillary endothelial cell</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C\textsubscript{T}</td>
<td>Cycle threshold</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<tr>
<td>CMT</td>
<td>Carrier-mediated transport</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>DCN</td>
<td>Dorsal cochlear nucleus</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>DOTA</td>
<td>1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid (or Tetraxetan)</td>
</tr>
<tr>
<td>DTPA</td>
<td>Diethylenetriaminepentaacetic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>Ex/Em</td>
<td>Excitation/Emission</td>
</tr>
<tr>
<td>FAM</td>
<td>6- Carboxyfluorescein</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast protein liquid chromatography</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>Gd</td>
<td>Gadolinium</td>
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<tr>
<td>GPC</td>
<td>Gel permeation chromatography</td>
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HEPES  4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IC    Inferior colliculus
ICP-MS Induced coupled plasma mass spectrometry
IgG   Immunoglobulin G
InsR  Insulin receptor
LDLR  Low density lipoprotein receptor
LRP1  Low density lipoprotein receptor-related protein 1
MEMRI Manganese-enhanced MRI
MRI   Magnetic resonance imaging
MWCO  Molecular weight cut-off
NHS   N-hydroxysuccinimide
NMDA  N-methyl-D-aspartate
NMDAR2D NMDA receptor subunit 2D
pAF   P-aminophenylalanine
PAMAM Poly(amidoamine)
PAMPA Parallel artificial membrane permeability assay
PBS   Phosphate buffered saline
PCL   Poly(ε-caprolactone)
PCR   Polymerase chain reaction
PEG   Polyethylene glycol
PPI   Poly(propylene imine)
qPCR  Quantitative PCR
RBMEC Rat brain microvascular endothelial cell
<table>
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<tr>
<td>RMT</td>
<td>Receptor-mediated transcytosis</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse-transcriptase PCR</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<tr>
<td>SDS PAGE</td>
<td>SDS polyacrylamide gel electrophoresis</td>
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<tr>
<td>SMCC</td>
<td>Succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate</td>
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<tr>
<td>SMPEG</td>
<td>PEGylated SMCC</td>
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<tr>
<td>SPB</td>
<td>Succinimidyl-[4-(psoralen-8-yloxy)]-butyrate</td>
</tr>
<tr>
<td>SPDP</td>
<td>Succinimidyl 3-(2-pyridyl)dithio)propionate</td>
</tr>
<tr>
<td>ssRNA</td>
<td>Single-stranded RNA</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline Tween 20</td>
</tr>
<tr>
<td>TEER</td>
<td>Trans-endothelial electrical resistance</td>
</tr>
<tr>
<td>TfR</td>
<td>Transferrin receptor</td>
</tr>
<tr>
<td>TR</td>
<td>Traut’s Reagent</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
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<td>UV-Vis</td>
<td>UV-Visible</td>
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<tr>
<td>VLP</td>
<td>Virus-like particle</td>
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<tr>
<td>ZO-1</td>
<td>Zonula occludens-1</td>
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CHAPTER 1

USE OF NANOMATERIAL SYSTEMS FOR OVERCOMING THE BLOOD-BRAIN BARRIER

1.1 Introduction

Delivery of imaging agents and pharmaceutical payloads to the central nervous system (CNS) is essential for efficient diagnosis and treatment of brain diseases. However, therapeutic delivery is often restricted by the blood-brain barrier (BBB), which prevents transport of clinical compounds to their region of interest. Therefore, an innovative approach to facilitate transport of these molecules across the BBB and into the brain is a crucial area of research. A multifunctional nanoparticle system is needed that 1) can be loaded with functional molecules, 2) transport across the BBB, and 3) localize to specific regions in the brain. Development of a nanoparticle platform that meets all of these needs while also being biocompatible, biodegradable, and easily amenable to mass production, has been a largely unmet challenge in the biomedical industry. Many potential delivery platforms exist and are currently being studied for brain delivery, but few meet all of the requirements for use in the clinic. The following sections provide a brief introduction into brain targeting strategies and how they have been coupled with nano-delivery systems for the treatment and diagnosis of brain diseases, as well as proposing a platform that has as yet been unused for brain targeting and delivery: the viral nanoparticle.
1.2 Motivation

There are numerous conditions, diseases, and disorders that can negatively affect the central nervous system (CNS). These neurological pathologies can range from disruption of cell-cell interactions, to changes in the levels of brain chemicals, to cellular degeneration and physical damage.\(^1\) One problem prevalent in medicine is that many diseases of the CNS can most often only be diagnosed using histology post-mortem. Clinical diagnosis of people living with these conditions is usually accomplished through observation of symptoms and diagnosis by exclusion of other diseases. For example, Parkinson’s disease is diagnosed via the classical symptoms absent in other neurodegenerative diseases, such as tremors combined with muscle rigidity, slow movement (bradykinesia), and confusion.\(^2\) General symptoms can be open to interpretation, are often incredibly subjective, and usually depend on the experience of the clinician. In some cases, similar diseases can often be indistinguishable, which generates difficulties in disease treatment. In other cases, even when the condition is known, treatment options are not often available because drugs are unable to successfully transition from the laboratory to the clinic, and often fail during clinical trials because \textit{in vivo} conditions are not met during the testing process.

In fact, entering the CNS \textit{in vivo} poses a unique obstacle in that it is not accessible through traditional delivery routes (i.e., use of the circulatory system). Therapeutic delivery to the brain is often restricted by the BBB. The BBB is the structure formed by the endothelial cells lining the capillaries of the brain and the tight junctions between them. These tight junctions often prevent paracellular transport of molecules between cells from the blood to the CNS (Figure 1-1A). The natural function of the BBB is to protect the brain from neurotoxic and foreign molecules. However, because the BBB is so efficient and effective, it also prevents
potential drug treatments and imaging dyes used for diagnosis from entering the brain. The BBB has been known to block nearly 100% of large molecules and approximately 98% of small molecules from entering the brain for treatment purposes. Efflux transporters on the endothelium can also eject the few molecules capable of transporting into the brain, which further reduces the therapeutic effectiveness of these clinical agents. The BBB is therefore a huge obstacle for drugs and imaging dyes, preventing diagnosis and treatment in patients.
Figure 1-1. Strategies for bypassing the BBB. A) Paracellular transport between endothelial cells occurs throughout most of the body, and is lacking in the endothelium of the BBB. B) Injection into the brain parenchyma has been used to completely avoid the BBB when treating patients. C) BBB disruption is accomplished by injection of osmolytes or chemical agents, or via focused ultrasound. Use of this technique causes loosening of the tight junctions of the endothelium, allowing clinical agents access to the brain. D) Transcellular transport refers to cell mechanisms that transport molecules directly through a cell. E) The transcellular lipophilic pathway allows the passive diffusion of small, lipophilic molecules through the BBB and into the brain. F) Carrier-mediated transport (CMT) is a type of facilitated transport that utilizes specific proteins to move molecules from the environment into and through the cell. G) Cationic molecules can be endocytosed via adsorptive transcytosis nonspecifically and transported through the cell. H) Specific ligands bind receptors and are endocytosed and transported through the cell via receptor-mediated transcytosis.
Researchers have investigated ways to cross the BBB and deliver drugs and imaging dyes without negatively affecting the patient. Often, methods of direct administration to the brain are too dangerous or inconvenient for use in the clinic. This unmet need has therefore opened a new avenue for use of nanomaterials in CNS disease treatment. Nanomaterials have a distinct advantage over other molecules, in that they can often be targeted to their site of interest by conjugation to targeting moieties such as antibodies, peptides, or oligonucleotides. Additionally, nanocarriers can be used to encapsulate multiple clinical agents to prevent their degradation during circulation, and increase the concentration delivered at the target site to reach a therapeutic effect. Various nanoscale materials have been used for these purposes, ranging from synthetic polymeric materials to biologically derived nanomaterial systems.\textsuperscript{4} Biologically derived materials have been shown to be more promising for use in the clinic, as they are often biocompatible and biodegradable, making them unlikely to cause the negative cellular effects often seen with synthetic delivery systems, such as frustrated phagocytosis.\textsuperscript{5}

1.3 General Techniques for Overcoming the BBB

Before development and expanded use of targeted nanoparticle systems, clinicians and researchers had much more difficulty overcoming the BBB when designing drugs and treating patients. Early approaches to therapeutic delivery attempted to utilize the common clinical administration routes of intramuscular, intravenous, and subcutaneous injection. However, for all of these routes, the particles end up in the circulatory system, requiring them to cross the BBB. As systemic administration of most drugs and imaging dyes for CNS treatment and diagnosis was found to be ineffective, researchers studied other techniques for treatment and delivery. Previous strategies have focused on bypassing the BBB entirely through use of direct
parenchymal injections or BBB disruption, while more recent strategies have focused on use of a molecular 'Trojan horse' to hijack transport mechanisms already used by the cells of the BBB.

1.3.1 Localized injection

One possible route to bypass the BBB is to deliver therapeutic agents locally by direct injection into the brain parenchyma (Figure 1-1B). Intraparenchymal injection requires an invasive surgical procedure, and is commonly used in the laboratory setting as it validates the clinical agent’s ability to treat the target disease before investigating the effects of the therapeutic agent when administered systemically. Unfortunately, therapeutics administered via direct injection have been shown to be limited by a diffusion radius of only millimeters from the injection site, which is impractical for whole-brain diseases.⁶,⁷ Although intraparenchymal injection allows complete evasion of the filtering mechanisms of the BBB, it is not a viable approach for routine delivery due to the multiple invasive injections required for diagnostic and treatment purposes.

Clinical agents can also be injected intrathecally to bypass the BBB. Using this administration route, the therapeutic is injected into the spinal canal and has access to the brain via the cerebrospinal fluid (CSF). Intrathecal injection is often used for pain management or chemotherapy, but has application in delivery of clinical agents to the brain to manage other conditions, such as meningitis.⁸,⁹ This route has many advantages over intraparenchymal injection, as it is much less invasive and can be used to deliver multiple rounds of injections. However, there is an additional layer of cells between the CSF and the brain that must be traversed before therapeutic agents can access the brain, which may hinder the transport of clinical agents and make this delivery technique ineffective.¹⁰,¹¹ Various other administration
routes have been studied for brain delivery, and the reader is directed elsewhere for more information.11,12

1.3.2 BBB disruption

Systemic delivery is a more desirable delivery route for therapeutic delivery to the brain as it is well tolerated by patients and is much easier to administer. Additionally, using currently available clinical agents, such imaging agents for MRI or chemotherapeutics that have been shown effective in treating cancer in other areas of the body, is ideal. As mentioned earlier, the success of systemic delivery hinges on the ability of clinical agents to cross the BBB. As such, researchers have developed the technique of BBB disruption, which is accomplished using chemical agents such as bradykinin, osmolytes such as mannitol, or with focused ultrasound.11 Most disruption techniques function by promoting stress on the tight junctions between the endothelial cells of the BBB, effectively loosening the junctions and allowing paracellular transport of clinical agents into the brain (Figure 1-1C). Although this technique successfully allows clinical agents to pass from the blood to the brain easily,13–15 it also allows albumin and other neurotoxins through the BBB, which are capable of causing neuronal damage.16

1.3.3 Transcellular transport

Transcellular transport is a promising strategy for transport of clinical agents into the brain. The technique involves the transport of agents directly through the endothelial cells of the BBB (Figure 1-1D). Various types of cell transport have been targeted, including the transcellular lipophilic pathway, carrier-mediated transport, adsorptive transcytosis, and receptor-mediated transcytosis (RMT).11,17,18

Lipid-soluble molecules smaller than 400 Da are capable of BBB transport via the transcellular lipophilic pathway (Figure 1-1E),3 and many clinically available CNS drugs such as
opioids and anticonvulsants are believed to use this pathway to enter the brain. In fact, the average size of a CNS active drug is 357 Da. Researchers have attempted to convert water-soluble molecules incapable of crossing the BBB to lipid-soluble molecules that are capable of crossing it. However, there has been little success for this approach, as modifications for lipid solubility will often decrease the molecule’s affinity for its target receptor or increase the molecular weight of the molecule above 400 Da, both of which are counterproductive to BBB permeability. Additionally, the lipophilic pathway has other drawbacks for BBB targeting, including size limitations and non-specific uptake, as all cell membranes are capable of lipophilic transport.

Carrier-mediated transport (CMT) refers to the use of the highly specific transport proteins for glucose, amino acids, and nucleosides. These transport proteins can also be used to transport therapeutic molecules into the brain (Figure 1-1F). In fact, the large neutral amino acid transporter (LAT1) has been shown to transport drugs such as L-dopa, gabapentin, and baclofen into the brain. Although these transporters are useful for transporting molecules that mimic the structure of endogenous biomolecules, their uptake mechanism is too selective to be used regularly for nanomaterial systems. Transport proteins have been targeted by nanoparticle systems for BBB transport, but most of these efforts have been unsuccessful in utilizing CMT, with few examples of nanoparticle loads being directly shuttled via transporters.

Transcytosis differs from the above-mentioned transport systems in that it is vesicular-based. When the ligand binds to its target receptor on the apical (blood-facing) surface of the cell, the ligand-receptor complex is internalized, forming an endosome. This endosome is then transported through the cytosol and releases its cargo on the basolateral (brain-facing) side of the BBB. There are two different types of transcytosis: the nonspecific mechanism termed
adsorptive transcytosis, and the more specific receptor-mediated transcytosis (RMT). Adsorptive transcytosis is triggered by the electrostatic interaction of positively charged epitopes on the surface of the target particle and the negatively charged cell membrane\(^{27}\) (Figure 1-1G). This transport mechanism is intermediate between pinocytosis, the nonspecific uptake of molecules into the cell, and receptor mediated transcytosis. Although adsorptive transcytosis has been shown to successfully transport cationized serum albumin across the BBB,\(^{28}\) this transport mechanism has not been further studied for BBB transport, probably due to high likelihood of nonspecific uptake in other organs. Research involving targeted nanomaterial carriers has predominately focused on RMT, which requires specific receptor-ligand binding, resulting in endosome formation and transport through the cell (Figure 1-1H). RMT has been shown to accommodate particles up to hundreds of nanometers in size, and is therefore the most appropriate transport mechanism to target using nanomaterial systems.\(^{29,30}\) The most targeted receptors used for RMT brain transport studies have been the transferrin, insulin, and lipoprotein receptors, as they are highly expressed on the surface of the BBB epithelium.\(^{31}\) Targeting these receptors presents many challenges, such as navigating competition between endogenous ligands and targeted delivery platforms for binding. This can not only prevent delivery of therapeutic agents, but also prevent transport of necessary nutrients and proteins to cells, which could harm the patient.

1.3.3.1 Transferrin Receptor (TfR)

One well-known target for receptor-mediated transport across the BBB is the transferrin receptor (TfR), which is abundant on the apical surface of the endothelial cells of the BBB and undergoes RMT. However, as TfR is saturated by endogenous transferrin in the blood (mg/mL amounts), transferrin itself is rarely used as a targeting ligand.\(^{32}\) Alternatively, antibodies have
been developed and used to target TfR.\textsuperscript{33–35} OX26, the mouse monoclonal antibody against rat TfR, is the most studied targeting moiety, and has been shown to accumulate in the brain to a greater extent than in other organs.\textsuperscript{36,37} Conjugation with OX26 has been shown to help shuttle proteins and other small molecules across the BBB and into the brain, allowing them to reach a therapeutic effect.\textsuperscript{38–41} For studies in mice, the antibody 8D3 was developed to target TfR.\textsuperscript{42} This targeting moiety has proven to be successful in transporting nucleic acids across the BBB for therapeutic purposes.\textsuperscript{43} It has also been used to image beta-amyloid plaques in an Alzheimer’s disease mouse model.\textsuperscript{44}

Despite the many successes of TfR targeting in animals, there are also many drawbacks to targeting this receptor clinically. TfR is abundant on the surface of cells throughout the body, and as such can cause non-specific uptake, limiting the overall amount of therapeutic agent that can be specifically delivered to the brain. For example, studies have shown that after 24 hours, only 0.44\% of the OX26 conjugate injected systemically is present in the brain.\textsuperscript{45} Similarly, after 1 hour, only 1.5\% of the injected dose of 8D3 is present in the brain. Studies in Rhesus monkeys have also shown low brain uptake (0.3\% in 24 hours) when using the 8D3 antibody.\textsuperscript{46} Uptake of the clinical agent in peripheral organs could cause unwanted side effects and prevent a high therapeutic dose from accumulating in the brain, reducing the effectiveness of treatment. In addition to these drawbacks, the experimental antibodies 8D3 and OX26 are not suitable for use in humans, as mouse and rat antibodies used for therapeutic delivery could cause an immunological response. TfR-targeting antibodies must therefore be developed specifically for human patients. To overcome this, fusion proteins combining 8D3 or OX26 and human antibodies have been developed that produce no immune response in humans, but have similar targeting abilities.\textsuperscript{47}
1.3.3.2 Insulin Receptor (InsR)

The insulin receptor (InsR), like TfR, is highly expressed on the apical side of the BBB and has also been studied for receptor-mediated transport. Use of insulin itself as a targeting vector is problematic, as injection of additional insulin into the body could cause hypoglycemia. Additionally, the half-life of insulin in blood is 10 minutes, which would not be enough time to allow the accumulation of the clinical agent at the target site, or for a therapeutic effect to occur. Therefore, antibodies for this receptor have been developed for BBB targeting. The mouse antibody against human InsR, termed 83-14, has been shown to successfully deliver proteins to the brain, including glial cell line-derived neurotrophic factor (GDNF). A humanized insulin receptor monoclonal antibody (HIRMAb) has also been shown to transport into the brain when conjugated a TNFα decoy receptor for the abatement of brain inflammation. Although these antibodies have been designed to bind a different epitope of InsR than endogenous insulin, there are concerns about competition between these molecules for the same receptor, which could have adverse effects on glucose metabolism and bar further potential for use of these targeting moieties in the clinic.

1.3.3.3 Low Density Lipoprotein Receptor-Related Protein 1 (LRP1)

Ligands of the lipoprotein receptor, specifically the drug molecule aprotinin, have been shown to have an eight-fold higher transcytosis efficiency over in vitro and in vivo compared to transferrin receptor-mediated transport across the BBB. As a result, more work has focused on targeting members of the low density lipoprotein receptor (LDLR) family, specifically the low density lipoprotein receptor-related protein 1 (LRP1) receptor. LRP1 is known to have over 30 distinct ligands. Many of these ligands, such as aprotinin and apoE, are capable of transport across the BBB, and have been previously used to facilitate transport of nanomaterials into the
However, due to the low transport efficiency of these molecules, researchers have designed targeted peptides based on the amino acid sequence homology between these ligands. From a BLAST search, three proteins were discovered to align with the Kunitz domain of aprotinin, and from this sequence alignment, 96 peptides were generated, termed the “angiopeps.” These peptides, approximately 3 kDa in size, were studied for their ability to transcytose across an in vitro BBB model derived from a bovine capillary endothelial cell (BCEC) monolayer. Angiopep-2 was found to be the most promising of the angiopeps, having a seven-fold higher transcytosis efficiency than the drug aprotinin and approximately seventy-fold higher transcytosis efficiency than transferrin across an in vitro BBB model. This peptide has been shown to target LRP1 and used for targeting many different nanomaterial-based delivery systems and drug conjugates across the BBB. Negative systemic effects associated with targeting the LRP1 receptor are considered minimal compared the biological effects possible for targeting TfR and InsR. Although angiopep-2 conjugates could be taken up nonspecifically throughout the body and cause side effects, studies have shown that there is little uptake in peripheral organs, with the most uptake occurring in the brain, liver, and spleen.

1.4. Biological nanoscale platforms for drug delivery to the brain

Use of nanoscale platforms in conjunction with the above-mentioned RMT-targeting strategies is crucial to the success of diagnoses and treatments in CNS diseases. Nanoscale platforms often provide targeted delivery of multiple clinical agents, allowing accumulation of the clinical molecules at the specific target site and therefore increasing the probability that a therapeutic effect will be attained. Targeting the transferrin, insulin, or LRP1 receptors accessible on the epithelium of the BBB with nanoparticle systems conjugated to receptor-
specific ligands is a highly studied area of research. Many targeted nanomaterial platforms have been used for BBB delivery purposes, ranging from synthetic systems such as PEG-PCL NPs\textsuperscript{58,59} and carbon nanotubes\textsuperscript{60} to biologically derived nanomaterials. When conjugated to angiopep-2, synthetic particles successfully transported through \textit{in vitro} BBB models, into rat brains \textit{in vivo}, and have also been targeted to glioma cells in the brain.\textsuperscript{57–60} There are some disadvantages, however, to using polymeric nanoparticles in biological systems, such as a lack of biodegradability and the potential for material toxicity, which can compromise the integrity of the BBB by inducing the opening of tight junctions in the brain.\textsuperscript{62} Fabrication of polymeric nanoparticles often requires complex protocols and toxic reagents during synthesis.\textsuperscript{63} Additionally, many polymeric nanoparticles have a largely polydisperse size distribution that can range by hundreds of nanometers. Controlling this polydispersity is often possible, but can be challenging, and is rarely feasible for commercial use.

A large size range affects nanoparticle localization within both individual cells and throughout the body, which can influence drug delivery efficiency. For example, nanoparticles larger than 200nm may be shuttled to spleen and the liver, rather than crossing the BBB and reaching their target.\textsuperscript{64} Nanoparticles less than 10nm in diameter are rapidly cleared from the body by the kidneys, which filter out and excrete nanoparticles with urine.\textsuperscript{65} Therefore, having a monodisperse size distribution of particles ranging between 20 nm and 200 nm in diameter is ideal, as this promotes a prolonged circulation time, discourages cellular degradation, and helps facilitate proper cellular localization.\textsuperscript{66} While synthetic nanoparticles lack many of these characteristics even after in-depth analysis and optimization, biologically derived nanomaterials have many of these properties without any modifications required, especially those that self assemble. Many different biologically based nanoplatforms have been used for brain delivery,
including liposomes, dendrimers, protein-drug conjugates, and biological nanoparticles (Figure 1-2). These materials have been selected for their biocompatibility, biodegradability, and ease of preparation and functionalization.

**Figure 1-2.** Biologically derived nanomaterial systems used for brain-specific drug delivery. Molecular graphics images were produced using the UCSF Chimera package from the Computer Graphics Laboratory, University of California, San Francisco.

1.4.1 Liposomes

Liposomes are spherical particles formed from a lipid bilayer. This bilayer is often derived from the amphiphilic phospholipids that make up the lipid bilayer of the cell membrane,
but can also be formed from synthetic amphiphilic lipids. Micelles vary from liposomes in that they are composed from a monolayer rather than a bilayer of amphiphilic lipids. Micelles have been previously used as drug delivery systems, and have been reviewed extensively by others. Synthetic amphiphilic liposomes are unrelated to the work performed here, and as such, will not be further discussed. Phospholipids consist of a polar ‘head’ end and a nonpolar ‘tail’ end. The polar end of the phospholipid is hydrophilic, while the nonpolar tail region of the phospholipid is hydrophobic. The shape a phospholipid aggregate forms is related to its head group size, chain volume, and chain length. These components are combined in the formula for molecular packing number ($N_s$):

$$N_s = \frac{v_0}{l_0 a_e}$$ (1)

Where $v_0$ refers to the surfactant tail volume, $l_0$ is the tail length, and $a_e$ is the effective area per head group of the phospholipid. When introduced to water, phospholipids with $N_s \approx 1$ will spontaneously aggregate into a spherical bilayer structure, which also conveniently encases any other molecules present in solution. This strategy can be used to encapsulate both hydrophobic and hydrophilic molecules. Due to their typical size of 500 Da or greater, liposomes are mainly taken up through adsorptive-mediated transcytosis or RMT. Cationic liposomes have been previously shown to cross the BBB. The main disadvantages to using liposomes are nonspecific uptake throughout the body and a short clearance time, which can be overcome by conjugation of targeting ligands and polymer chains to the liposome surface. The polymer chain polyethylene glycol (PEG) specifically has been used to minimize non-specific adsorption and uptake, extend circulation time, and reduce immunogenicity of many nanoparticle carriers, including liposomes.
The accessibility of reactive functional groups present on the exterior surface of certain liposomes makes these nanomaterials relatively easy to modify. Antibodies, peptides, and brain-permeating carbohydrates such as mannose have all been used to further improve the transport of liposomes across the BBB. Strategies for conjugating these targeting moieties involve use of the streptavidin-biotin interaction, covalently binding the targeting moiety to the surface, and coupling of targeting ligands to PEG chains already bound to the liposome surface. Many different antibody-conjugated PEGylated liposomes (immunoliposomes) have been developed to target the BBB. Conjugating both PEG and targeting moieties to the surface of liposomes has introduced some difficulties, including steric hindrance for antibody-target interaction and overall instability of the liposome structure. Researchers have been able to overcome this by conjugating the brain-targeting ligands to the distal end of the PEG bound to the liposome. Tethering occurs at the tips of 1-2% of the PEG strands. This process was successful in conjugating OX26 and 8D3 to the surface of PEGylated liposomes. Both targeted liposomes were shown capable of BBB uptake and transport when loaded with luciferase and β-galactosidase reporter genes and radioactively labeled daunorubicin.

1.4.2 Dendrimers

Dendrimers are polymeric compounds comprised of a central core, a branching unit, and a terminal end group. They can be based on synthetic nanomaterials, (e.g., poly(amidoamine) dendrimers (PAMAMs) and poly(propylene imine) dendrimers), or on biological molecules (e.g. carbohydrates and amino acids), or a combination of both. Glycodendrimers incorporate carbohydrates into their structure, whereas peptide dendrimers incorporate amino acids or peptides. Advantages of dendrimers over other nanomaterial systems include their ease of preparation, ease of surface modification, capability for controlled release of clinical compounds,
and their versatility.\textsuperscript{84} Exclusively synthetic cationic dendrimers (i.e., dendrimers containing terminal amine groups) are rarely used as delivery systems, due to their high toxicity via disruption of cell membranes.\textsuperscript{85,86} Conjugation of sugar moieties to terminal amines or further incorporation of carbohydrates into the dendrimer structure have been used as strategies to make these nanomaterials more biocompatible. Most dendrimers used for BBB targeting incorporate the synthetic component poly(propylene imine) (PPI) with carbohydrates. Poly(propylene imine) glycodendrimers with a maltotriose coating conjugated to fluorescein have been shown capable of crossing the BBB without the conjugation of targeting moieties.\textsuperscript{87} The positively charged glycodendrimer coating allows uptake by adsorptive-mediated transcytosis. This capability has sparked great interest in studying the effect of these compounds on brain diseases. For example, the effects of PPI glycodendrimers on amyloid beta plaques in Alzheimer’s mice have been extensively studied. One of these combination dendrimers with a maltose coating on the surface was capable of crossing the BBB and reducing the toxicity of amyloid beta plaques in the mouse brain.\textsuperscript{88} Another PPI glycodendrimer was complexed with copper (II) to study fibril formation,\textsuperscript{89} and yet another was used to eliminate \textit{PrP}\textsuperscript{Sc} prions that formed \textit{in vitro} from scrapie, a neurodegenerative disease.\textsuperscript{90} Interestingly, the glycodendrimers themselves are capable of providing the therapeutic effect, without the additional need of carrying a clinical agent. This was originally shown with PAMAM dendrimers, which are capable of destroying prions \textit{in vivo}.\textsuperscript{91} Dendrimers are also amenable to cargo loading and delivery, due to their polymeric, porous structure.\textsuperscript{92}

Other biologically derived dendrimers, such as peptide dendrimers and dendrimers formed by enzymatically-synthesized glycogen, have not been investigated for BBB delivery, but are capable of surface modification and have shown success in delivering compounds \textit{in vivo}.\textsuperscript{93}
The idea that they could also be used with BBB targeting moieties for CNS delivery is not unreasonable, especially if they have distinct advantages over other dendrimers in vivo. However, further studies are needed to confirm the efficacy and safety of these biological dendrimers.

1.4.3 Protein-drug conjugates

Another strategy for delivering clinical agents to the brain involves direct conjugation of the targeting moiety and therapeutic. Antibody-protein conjugates have been created to target TfR using the OX26 and 8D3 antibodies for BBB delivery, as mentioned above. One group used biotinylated bFGF and reacted it with OX26 bound to streptavidin (SA). The biotin-avidin interaction is regularly exploited in chemistry and biology due to its simplicity and high conjugation efficiency. These bio-bFGF-OX26-SA conjugates were used to provide a neuroprotective effect in rats with ischemic stroke. Researchers determined that OX26 was capable of transporting bFGF into the brain, which then reduced the infarct volume by nearly 80%. The biotin-avidin interaction has also been used to link brain-derived neurotrophic factor (BDNF) and vasoactive intestinal peptide (VIP) to OX26, and results show that this conjugate was also capable of BBB transport and modulated other therapeutic effects in vivo. Other conjugation strategies for formation of protein-drug conjugates include maleimide (thioether), thiol, and amide linkages (Table 1-1).
Table 1-1. Commonly used linkages for ligand conjugation.

<table>
<thead>
<tr>
<th>Linkage Type</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avidin-Biotin</td>
<td>(38), (39), (40), (44), (79), (81), (95), (96)</td>
</tr>
<tr>
<td>Maleimide (Thioether)</td>
<td>(43), (82), (83), (98)</td>
</tr>
<tr>
<td>Disulfide</td>
<td>(97), (98)</td>
</tr>
<tr>
<td>Amide</td>
<td>(80), (98), (100)</td>
</tr>
</tbody>
</table>

Although producing drug conjugates is considered inexpensive, simple, fast, and efficient, the delivery efficiency of drug-antibody conjugates is relatively low compared to other compounds. Studies have shown that antibody penetration alone into the brain is exceptionally low, on the order of 0.1-0.2% of circulating antibodies in vivo cross into the brain.\textsuperscript{99} Peptide-drug conjugates, especially those conjugated to angiopep-2, have shown much higher transport efficiency in vivo. ANG1005 is a paclitaxel-angiopep-2 conjugate generated by the company Angiochem to treat brain cancers.\textsuperscript{100} Due to the success of these compounds in animals, ANG1005 was developed further for clinical trials. Phase II trials were completed in September 2017, and Angiochem is currently preparing for Phase III clinical trials.\textsuperscript{101}
Figure 1-3. Conjugation and loading strategies needed for a clinically relevant theranostic nanoparticle system. A) Therapeutic loading, B) surface functionalization, and C) imaging agent loading of the nanoparticle platform can be combined to generate the ideal theranostic delivery system.

1.4.4 Nanoparticle systems

Nanoparticle platforms for CNS delivery are plentiful, with some utilizing the targeting moieties mentioned above, while others are capable of BBB transport on their own. Each platform has its own advantages and disadvantages during the steps of synthesis, functionalization, and cargo loading, which can make selecting the most efficient and effective
nanoparticle system difficult. However, all have the advantage that they are capable of surface modification for targeting and carrying multiple clinical molecules to release at the target site, which makes them much more valuable than single-conjugate systems (Figure 1-3). Releasing multiple therapeutic molecules at the target site is more likely to produce a therapeutic effect and less likely to cause side effects with a smaller administered dose. This feature is what makes nanoparticles so popular in biotechnology applications.

1.4.4.1 Synthetic nanoparticle systems

Nanoparticle platforms based on synthetic materials such as gold, polymers, and carbon nanotubes have especially been used for brain-targeted delivery. These materials are often chosen for their relative inertness and ease of manufacture. However, they may range in size by hundreds of nanometers, which can affect their cellular localization both in vivo and in vitro. It has been shown that polyamidoamine-poly(ethyleneglycol) (PAMAM-PEG) and poly(ethylene glycol)-co-poly(ε-caprolactone) (PEG-PCL) nanoparticles that range from 20-200nm may localize to the lysosomes of U87 MG cells and C6 glioma cells in vitro rather than being transported. Typical strategies for BBB targeting with synthetic nanoparticles have involved targeting LRP1 by conjugation with angiopep-2. However, 4nm glucose-coated gold nanoparticles have also been used for brain transport in vitro. Interestingly, these particles localized in the cytosol, rather than the lysosomes. Researchers also found that when the GLUT-1 transporter was inhibited, it did not affect transport of the glucose-coated particles across the BBB. They concluded that the coating on the 4nm particles affected their transport, rather than specific receptor targeting. Alternatively, targeting LRP1 with angiopep-2 was successful in increasing the number of PEG-PCL, PAMAM-PEG, and gold nanoparticles, and carbon nanotubes transported into the brain. Although these synthetic systems are all viable
options for targeted brain delivery, their overall safety and the high likelihood and effects of accumulation in untargeted areas makes their future use in the clinic questionable.

1.4.4.2 Chitosan nanoparticles

Chitosan is a naturally occurring cationic polymer made from chitin, a polysaccharide derived from the shells of crustaceans. Simply mixing chitosan with pentasodium tripolyphosphate forms chitosan nanoparticles (CNPs) that can range from tens to hundreds of nanometers in diameter.\textsuperscript{104} CNPs have many advantages, as they are easy to make, their size is relatively easy to control, they have high capacity for loading, can continuously release entrapped proteins to provide a sustained release effect, and show pH-dependent swelling behavior that could be exploited \textit{in vivo} for drug release.\textsuperscript{105,106} Due to their positive surface charge, CNPs are capable of transport across an \textit{in vitro} BBB model without requiring a targeting moiety.\textsuperscript{107} However, introducing OX26 to the surface of CNPs via the avidin-biotin interaction was also successful in transporting loaded particles into the brain for treating brain ischemia.\textsuperscript{108} Dual targeting of CNPs is also possible, with one moiety (anti-TfR) targeting the BBB, and the other targeting astrocytes for cargo delivery.\textsuperscript{109} These particles have many different functionalities that can be exploited for future BBB targeting and transport.

1.4.4.3 Viral nanoparticles

Virus-based nanoparticles have many advantages over nearly every other nanoparticle system, as they are capable of self-assembly, have a relatively monodisperse size distribution varying only by 1-10nm, are often within a size range of 20-50nm, and are fully amenable to mass production.\textsuperscript{110} Viruses are also easily modified both on the interior and exterior due to their protein cage structure, which is comprised of reactive groups such as amines, thiols, and carboxylic acids. Pores on the surface of some viruses allow them to be loaded with different
compounds for therapeutic delivery.\textsuperscript{111-114} Virus-like particles (VLPs) are a subset of viruses that have had their genetic material removed, which helps alleviate fears the public has about the safety of these particles. However, removal of the viral genome is often unnecessary when using viral nanoparticles, as most of these particles are derived from viruses that attack plants, insects, or bacteria, and are therefore non-infectious to humans. The benefit of using viruses is that they can be produced in their host at high levels not often seen with the synthesis of other drug delivery platforms. For example, plant viruses such as cowpea chlorotic mottle virus (CCMV) and cowpea mosaic virus (CPMV) can be produced in gram quantities in their host plants.\textsuperscript{115} Yields of milligram to gram amounts are also seen in bacteriophage production.\textsuperscript{116} Interestingly, industry has the capacity and means to produce bacteriophage in large quantities, as vaccine manufacture is already established and optimized, so mass production of bacteriophage nanoparticles is feasible in the future.

Due to the accessibility of functional groups on virus surfaces, it is conceivable that many different targeting ligands can easily be conjugated to their surfaces. Indeed, conjugation of targeting moieties to MS2 bacteriophage runs rampant, with numerous MS2-ligand complexes developed. MS2 is a non-enveloped, 28nm diameter icosahedral virus that has been used as a drug delivery vehicle for a multitude of diseases.\textsuperscript{110,117-122} This virus displays 540 amine groups on its surface readily available for reaction.\textsuperscript{123} Conjugation of targeting moieties to MS2 is simple, fast, and efficient, and has been accomplished numerous times with various aptamers for cell targeting.\textsuperscript{110, 117-120,122,124} Additionally, due to its RNA-containing hollow core, MS2 is capable of being loaded via nucleotide-mediated interactions.\textsuperscript{112}
1.5 Scope of the thesis

When endeavoring to diagnose and treat diseases of the CNS, dual functionalization of the nanoparticle exterior with two different targeting ligands is necessary for transport across the BBB and to a specific region. Additionally, the ability of the nanoparticle system to carry cargo is vital for delivery of therapeutic and imaging agents to the sites of disease. The success of a brain-targeting nanoparticle platform hinges on these three core necessities, which is summarized in Figure 1-4. As viral nanoparticles, specifically bacteriophages, have many of the capabilities necessary for brain delivery, we have chosen to leverage this nanoparticle platform to our advantage for CNS disease diagnosis. Here, we have developed a nanoparticle system based on the MS2 bacteriophage capsid for BBB targeting and delivery, and investigated the effects of surface modification on targeting and transport and the ability to load the virus via nucleotide-mediated interactions. The overall strategy to develop MS2 for specific brain targeting and delivery of MRI imaging agents involved chemical functionalization of the exterior of the viral capsid and pore-based loading of the capsid interior. We investigated this hypothesis with the following chapters:

- **Chapter 2** investigated the surface functionalization of MS2 with a brain-targeting peptide. The ability to control the number of targeting moieties conjugated and the effect of a longer spacer arm on conjugation efficiency to the capsid surface were explored. In **Chapter 5**, these particles were studied for their ability to transport over an *in vitro* BBB model.

- In **Chapter 3**, the MS2 particles developed in Chapter 2 were further modified on the exterior for specific region of interest targeting of NMDAR2D receptors.
• Chapter 4 investigated the possibility of loading the capsid interior with MRI functional agents via nucleotide-mediated interactions.

Figure 1-4. Strategy for the development of a brain targeting nanoparticle platform for MRI contrast agent delivery based on the MS2 bacteriophage capsid. MS2 should first be loaded through surface pores with contrast agent, which is modified to interact with RNA in the interior. Surface functionalization with antibody, then brain-targeting peptide will enable particles to transport across the BBB and to a specific region of interest.
1.5 References


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CHAPTER 2

DEVELOPMENT OF ANGIOPEP-2-CONJUGATED VIRUS CAPSIDS FOR

BBB TARGETING AND TRANSPORT

2.1 Introduction to BBB targeting via the LRP1 receptor

Various nanoparticle systems have been used for BBB targeting. However, one highly versatile platform that has yet to be used specifically for brain targeting and transport is the viral particle, specifically the MS2 bacteriophage capsid. MS2 is a well-studied, $3.5 \times 10^6$ Da icosahedral $T=3$ quasi-symmetric virus approximately 28 nm in diameter that has recently emerged as a potential drug delivery vehicle.\(^1\)\(^2\) This virus solely infects the bacteria $E.\ coli$ through binding of the virus’ maturase protein to F pili, making it non-harmful to humans.\(^3\) The MS2 bacteriophage is straightforward to produce and purify, remains structurally stable between pH 4.5-12, and can withstand temperatures up to 68°C.\(^4\) The MS2 capsid is comprised of 180 coat proteins (~13.5 kDa each) that can be chemically modified through the 540 available lysine residues on the capsid exterior surface. MS2 can be decorated with a maximum of 574 ($\pm$ 33) fluorescein molecules per virus, yielding approximately 3 lysine residues per coat protein available for reaction.\(^5\) Additionally, the MS2 capsid contains 32 pores with diameters ranging from 1.6-1.8 nm each, with an interior core containing one single-stranded, positive sense RNA (ssRNA).\(^6\) The size of these pores is large enough for small molecules to enter the capsid interior. The void volume present in the interior, when accounting for the ssRNA coiled inside, is approximately 7nm in diameter, making it possible to load the capsid with imaging agents or drugs via nucleotide-mediated interactions.\(^7\) The many natural advantages of the MS2
bacteriophage capsid make it a fitting model viral system for development of a drug delivery platform targeted to the brain.

The simple structure of the MS2 bacteriophage capsid and relatively small genome of 3,569 nucleotides have enabled many different strategies for surface modification of the virus. For example, cysteine residues have been genetically introduced on the capsid interior for modification by thiol-click reactions\(^8\) or by targeting tyrosine residues (tyrosine 85) with oxidative coupling reactions.\(^9\) The unnatural amino acid p-aminophenylalanine (pAF) can be genetically incorporated onto the capsid exterior surface, to be subsequently modified with an aldehyde to form an oxime linkage between the virus and another molecule.\(^1,8\) More commonly, however, strategies for covalent conjugation of groups to the capsid exterior rely heavily on direct modification of the exposed amine groups on the surface. It has been shown that there are three available primary amines on each capsid protein that are presented on the exterior of the fully formed capsid: the coat protein N-terminus, lysine 106, and lysine 113.\(^9\) Therefore, most conjugation strategies have targeted these functional groups, as they do not interfere with nucleotide-mediated loading, and because there are nearly 540 possible reactive groups available on the surface. Amine-based coupling strategies are plentiful, as amines are very good nucleophiles, and include reactions with active esters, aldehydes, anhydrides, acyl azides, or epoxides to form a covalent linkage. The most specific amine-targeted coupling strategy incorporates n-hydroxysuccinimide (NHS) ester-based linkers, such as SPDP (succinimidyl 3-(2-pyridyldithio)propionate) or SMCC (succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate). The amine-NHS ester reaction results in formation of a covalent amide bond that is not easily reduced. NHS esters are exceptionally reactive, and as such have half-lives of nearly 1 hour at room temperature in a pH 8 solution.
Figure 2-1. Heterobifunctional crosslinkers comprised of one NHS group and one maleimide group for use in MS2-AP2 conjugation. a) SMCC has a relatively small spacer length of 0.83nm. b) SMPEG has a much larger spacer length of 9.4nm and will be used to extend the Angiopep-2 from the capsid surface.

In this work, the native primary amines on the surface of the MS2 capsid were targeted for modification and conjugation of angiopep-2 (AP2) for BBB targeting. AP2 has been shown to have the greatest transcytosis efficiency over the BBB over any other targeting ligand, and is currently in clinical trials for use in humans. Additionally, its small size relative to antibodies (2876 g/mol, compared to ~150,000g/mol) makes it amenable to conjugation strategies that would link multiple ligands onto the MS2 surface. Before conjugation of AP2 to the MS2 capsid, the capsid was activated with the linker SMCC or PEGylated SMCC (SMPEG$_{24}$) (Figure 2-1). Both heterobifunctional linkers contain an NHS group on one end that reacts specifically with
amines to create a covalent bond. The other end consists of a maleimide group, which is specific for reaction with thiol groups, and takes advantage of thiol-click conjugation by thiolene reaction. Click chemistries allow for a very rapid (less than one hour) reaction. A free thiol has been added to the C-terminus of AP2 (shown in Figure 2-2, next page) for later conjugation to our linkers. Our conjugation strategy for MS2 is outlined in Figure 2-3. In the following studies, we investigate ability to control the number of targeting moieties conjugated to the virus and the effect of spacer length on the number of conjugated groups.

![N-TFFYGGSRGKRNNFKTEEYC-C](image)

**Figure 2-2.** Modified Angiopep-2 Amino Acid Sequence. A cysteine has been added to the C-terminus to provide a free thiol group for conjugation purposes (yellow), and the fluorophore FAM was added to the N-terminus for tracking purposes (green).
**Figure 2-3.** Reaction Scheme for MS2-AP2 Conjugation. The outer surface of the MS2 capsid contains 540 amine groups, which will specifically react with the NHS ester group of SMCC or SMPEG to form MS2-SMCC or MS2-SMPEG, respectively. After purification by GPC or FPLC, maleimide group of the MS2-SMCC conjugate is available to react with free thiols in solution. When we add our thiol-activated Angiopep-2, it can react with the maleimide, forming a covalent bond, irreversibly conjugating the peptide to the MS2 capsid.

**2.2 Experimental Methods**

2.2.1 Materials

Unless otherwise noted, all chemicals were purchased from Sigma Aldrich (MO).
Angiopep-2-FAM conjugated to cysteine (FAM-TFFYGGSRGKRNNFKTEEYC) (AP2) was synthesized by GenScript Biotech Corporation (NJ). SMPEG$_{24}$ was purchased from Fisher Scientific (MA). All experiments were conducted with reagent-grade chemicals.

2.2.2 MS2 growth and purification

MS2 wild-type bacteriophage was initially provided by David Peabody, PhD (University of New Mexico), then cultivated and purified as previously described. Briefly, a single colony culture of C3000 Escherichia Coli (ATCC 15997) was infected with $10^9$ plaque-forming units of MS2 phage and allowed to propagate. Cell debris were pelleted by centrifugation and MS2 was precipitated using polyethylene glycol (PEG$_{6000}$, 12% w/v). Precipitated phage was collected by centrifugation and resuspended in 50 mM Tris-HCl buffer (pH 7.0) supplemented with 10 mM MgSO$_4$. PEG was removed from suspension by chloroform extraction. MS2 was further purified from solution in a CsCl step gradient for 4 hours at 38,000 RCF followed by a CsCl equilibrium gradient for 24 hours at 180,000 RCF in a SW40-Ti rotor (Beckman-Coulter, CA). The MS2 band was removed and dialyzed for 16 hours against 2 L of 20mM NaPO$_4$, pH 7.4, with the buffer changed 3 times.

2.2.3 Preparation of MS2-SMCC and MS2-SMPEG

MS2 bacteriophage was reacted with the heterobifunctional crosslinker SMCC (succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate)) at a 1:3000 molar ratio or SMPEG$_{24}$ (PEGylated SMCC) at a 1:1500 ratio in 20 mM sodium phosphate buffer at pH 7.4 for one hour at room temperature. MS2 conjugates were purified using a PD-10 Desalting Column (5k molecular weight cutoff (MWCO), Sephadex G-25 M by GE Healthcare, UK) equilibrated with 20 mM sodium phosphate buffer at pH 7.4. The obtained samples were then concentrated using a Vivaspin 500 concentrator with a 30kDa (MS2-SMCC) or 100kDa (SMPEG) molecular...
weight cut off (Sartorius, Germany) and measured for MS2 protein content using a Nanodrop spectrophotometer (ND1000, ThermoFisher Scientific, MA). The concentration of MS2 was calculated using the absorption coefficient for MS2, \( e_{260} = 8.03 \text{ mL/mg} \).\textsuperscript{11} A maleimide assay (modified from Wängler et al\textsuperscript{12}) was used to determine the number of incorporated SMCC. Briefly, MS2-linker conjugates were reacted with 370-fold excess cysteamine hydrochloride to MS2 for one hour. After the reaction, the remaining cysteamine was back-titrated with 20-fold excess Ellman’s reagent (5,5'-dithiobis-(2-nitrobenzoic acid)), and absorbance at 412 nm was measured. The number of conjugated linkers (SMCC or SMPEG) per MS2 was calculated by using the following equation:

\[
\frac{\text{linker}}{\text{MS2}} = \frac{\Delta A}{\varepsilon_{\text{ER}} M} (2.1)
\]

where \( \Delta A \) represents the difference between the absorbance values at 412 nm before and after addition of Ellman’s reagent, \( \varepsilon_{\text{ER}} \) represents the extinction coefficient of Ellman’s reagent at 412 nm (14150 M\(^{-1}\) cm\(^{-1}\), as show by Collier\textsuperscript{13}), and M represents the molarity of MS2 in solution. After concentration, samples were also washed with 200uL sodium phosphate buffer four times in the concentrators to remove any remaining unconjugated linker.

\textit{2.2.4 Preparation of MS2-AP2 particles}

After purification of the MS2-SMCC conjugates, the samples were reacted with either a 1:50 or a 1:500 molar ratio of MS2 to AP2 for one hour in the dark at room temperature in 20 mM phosphate buffer, pH 7.4. All samples were purified using a PD-10 Desalting Column and concentrated using Vivaspin concentrators as described above.

\textit{2.2.5 Analysis of AP2 conjugation efficiency}

The amount of AP2 in the MS2-AP2 samples was determined using an AP2-FAM fluorescence standard curve at 495nm excitation (the excitation wavelength for FAM), and
540nm emission. The sample fluorescence was compared to the standard curve to calculate the AP2 concentration in the sample. The MS2 concentration was determined using a Nanodrop spectrophotometer as described above (absorbance at 260 nm). Using these concentration values, the molar ratio of AP2 to MS2 was calculated.

2.2.6 UV-Vis and Fluorescence Spectroscopy

UV-Vis and fluorescence measurements were performed using a Tecan Infinite M200 plate reader (Tecan Group Ltd., Switzerland). For elution experiments, peak absorbance spectra values (260 nm for MS2) and peak fluorescence spectra values (495 nm excitation, 540 nm emission for AP2) were determined for each fraction. UV-visible absorbance peaks and fluorescence peaks were used to confirm AP2 conjugation to MS2.

2.2.7 SDS-PAGE analysis of MS2-AP2

MS2 concentration was determined using a Nanodrop spectrophotometer and samples were loaded into a Mini-PROTEAN Precast Gel (Bio-Rad, CA) along with a Precision Plus Protein™ dual color standard ladder (Bio-Rad). The samples were run in 1x Tris-Glycine-SDS buffer for 15 minutes at 60 V for stacking and an additional 60 minutes at 125 V. Samples were first imaged using fluorescence at 488nm excitation, 520nm emission in a ChemiDoc MP Imaging System (Bio-Rad) before staining with Brilliant Blue Colloidal R Solution for 10 minutes at room temperature. The Coomassie-stained gel was destained with five changes of 10% acetic acid in deionized water and imaged.

2.2.8 DLS analysis of MS2-AP2

Mean particle diameter was determined in 20 mM sodium phosphate buffer pH 7.4 (0.5 mg MS2/mL) using dynamic light scattering (DLS, Malvern Zetasizer Nano, UK). Mean particle diameter measurements were averaged over three measurements using 15 scans at 10 second
duration each at $20^\circ$ C. The percent intensity algorithm in the Malvern Zetasizer collection software was used to calculate the particle diameter.

2.3 Results and Discussion

2.3.1 Conjugation of heterobifunctional linkers to MS2

Viral particles, including MS2, have been demonstrated as drug delivery platforms that are capable of self-assembly, form physically stable structures, and are easily modified with functional moieties.\textsuperscript{3,8,9,14-16} Wild-type MS2 has approximately 540 lysine groups readily available for reaction on its surface.\textsuperscript{5,6} These lysine groups have previously been used to conjugate various linkers and targeting moieties to the capsid surface.\textsuperscript{8,14,15} It has been shown that there are three primary amines on each capsid protein monomer available on the exterior of the fully formed capsid (N-terminus, lysine 106, and lysine 113).\textsuperscript{9} In this study, these groups were targeted for MS2 surface modification using the heterobifunctional linkers SMCC or SMPEG for click-conjugation chemistry (Figure 3). These linkers were chosen for their highly specific reactivity with primary amines and ability to react at neutral pH.\textsuperscript{17}

To confirm surface modification of the capsids with SMCC or SMPEG, a maleimide assay was used to detect the number of reactive maleimides available on the MS2 capsid surface after reaction. This assay was conducted by back-titrating thiol-reacted maleimides with Ellman’s reagent. Ellman’s reagent is used to detect the number of free thiols present in solution, which is subtracted from the excess number of thiols originally introduced to give the number of maleimides present. Results of maleimide back titration of MS2-SMCC conjugates showed that 111 ($\pm$ 10) SMCC reacted per MS2, indicating a coupling efficiency of about 20% of the total number of primary amines exposed on the capsid exterior (Table 1). Considering the large excess
of SMCC used for reaction (~6 SMCC per amine on the capsid surface), the number of SMCC conjugated could be limited by several reasons. One possibility is that an SMCC linked to the capsid surface prevented additional SMCC from reacting by steric hindrance. However, considering the length of the crosslinker (less than 1 nm), this seems unlikely. Another explanation could be that the capsid surface itself is restricting the access of the SMCC for reaction. Some amine groups are likely more exposed than others, and will therefore react first, while others are more buried on the surface and require more time to react. Additionally, a large amount of the NHS groups on the crosslinkers in solution may hydrolyze during the course of the reaction, reducing the total number available for crosslinking to MS2 surface amines.

MS2-SMPEG conjugates were determined to have 44 (± 16) SMPEG bound per MS2 (Table 1). Unlike the shorter (less than 1 nm) SMCC spacer length, SMPEG has a length of over 9 nm, which is nearly one third of the diameter of the MS2 capsid. This larger size (vs. SMCC) spacer could cause steric hindrance between reacting groups and limit the number of SMPEG bound compared to SMCC. Rather than extending from the surface linearly, due to its length, the SMPEG spacer will more likely form a globular structure on the surface of the MS2, taking up more surface area than expected and preventing other linkers from binding near where one has already bound. Indeed, the decrease in the number of linkers bound when determined by maleimide assay confirms the PEGylation introduces steric hindrance, as there was a reaction efficiency of less than 10% of the total number of primary amines available on the MS2 capsid, compared to the 20% by reaction with SMCC. One disadvantage of the maleimide assay, though, is that it only detects the number of available maleimides on the MS2 surface. This provides a maximum number of available sites for AP2 conjugation. However, if the maleimides have reacted with another compound in solution (i.e. other thiols or the MS2 capsid itself), these
reactions would not be detectable by use of the maleimide assay, lowering the effective reaction efficiency. This possibility is explored in a later section.

Interestingly, but not unexpectedly, SMCC and SMPEG reaction efficiency decreases with age (time after opening and exposing to the lab environment). Linkers that were more than six months old tended to have a lower conjugation efficiency overall compared to linkers that had more recently been opened (e.g., ~70 SMCC per MS2 compared to 111 SMCC per MS2). This is consistent with the high reactivity of NHS esters, which will hydrolyze when exposed to water or the water vapor present in air. Crosslinkers used for the experiments here were less than three months old, to ensure the highest possible number of linkers could be conjugated and minimize effects from NHS hydrolysis.

Table 2-1. Characterization of MS2-AP2 Conjugates.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Groups Conjugated</th>
<th>Measured Size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS2</td>
<td>-</td>
<td>29.8 ± 0.7</td>
</tr>
<tr>
<td>MS2-SMCC</td>
<td>111 ± 10</td>
<td>30.6 ± 0.5</td>
</tr>
<tr>
<td>MS2-SMCC-AP2 (1:50)</td>
<td>37 ± 4</td>
<td>32.6 ± 0.4</td>
</tr>
<tr>
<td>MS2-SMCC-AP2 (1:500)</td>
<td>77 ± 4</td>
<td>36.2 ± 1.2</td>
</tr>
<tr>
<td>MS2-SMPEG</td>
<td>44 ± 16</td>
<td>36.8 ± 1.1</td>
</tr>
<tr>
<td>MS2-SMPEG-AP2 (1:50)</td>
<td>20 ± 6</td>
<td>37.7 ± 1.2</td>
</tr>
<tr>
<td>MS2-SMPEG-AP2 (1:500)</td>
<td>41 ± 2</td>
<td>39.0 ± 1.3</td>
</tr>
</tbody>
</table>

2.3.2 Conjugation of AP2 to MS2-SMPEG or MS2-SMCC

To impart BBB targeting functionality, MS2-SMCC and MS2-SMPEG were reacted with thiolated AP2 using a thiol-click reaction. Size exclusion chromatography was used to purify MS2-AP2 nanoparticles from excess AP2, and to verify conjugation. This technique separates
molecules based on size through a column of porous beads. Molecules smaller than the column cutoff are caught in the beads and have a longer column retention time than large molecules, which pass around the beads and elute earlier.

Each sample was monitored for either absorbance at 260nm by the MS2 RNA (MS2 and MS2-AP2), or fluorescence of the FAM-labeled AP2 at Ex/Em 495/540nm (AP2, MS2-AP2). A representative figure of purification of AP2 from MS2-AP2 particles is shown in Figure 2.4. Unmodified MS2 is eluted from the column within the first 2mL of volume and peaks at 1mL eluted (Figure 2-4A, grey), whereas unmodified AP2 is eluted over the full 5mL, and peaks after 3mL eluted (Figure 2-4B, grey). After a 1 hr incubation, the MS2-SMCC-AP2 sample showed co-elution of AP2 (Figure 2-4B, black) with MS2 (Figure 2-4A, black), indicating successful conjugation of AP2 to MS2. MS2-SMPEG-AP2 particles showed a similar elution profile (data not shown). The elution profile illustrates that MS2 elutes from the column earlier than AP2 overall, but there remains some AP2 (aggregates or possibly dimers) that elute in the same volume as MS2. A small percentage of the MS2-AP2 sample may therefore be unconjugated AP2, but considerably more of the co-eluted AP2 is likely conjugated to MS2 capsids. Before further investigation of the MS2-AP2 particles, excess unconjugated AP2 was removed via spin filtration to verify that unconjugated AP2 in the sample did not affect other results.

Fluorescence analysis indicated conjugation of 37 (± 4) AP2 per MS2 capsid for MS2-SMCC-AP2 (1:50), yielding a coupling efficiency of only 33% (number of AP2 bound per total number reacted) (Table 2-1). When reacted with 500 AP2 per MS2, however, the coupling efficiency jumped to nearly 70% (AP2 per SMCC linker bound). Additionally, when reacted with MS2-SMPEG at a 1:50 or 1:500 ratio, the number of AP2 bound dropped slightly, to 20 (± 6) and 41 (± 2) AP2 per MS2, respectively (Table 2-1). The variation in AP2 conjugated when
reacting with 1:50 versus 1:500 ratios proves that we can control the number of AP2 conjugated to MS2. Interestingly, despite the large excess of AP2 used, we were unable to conjugate the maximum possible number of AP2 when reacting with MS2-SMCC. One possible reason for less than 100% conjugation efficiency may be due to steric hindrance between AP2 molecules on the surface of the MS2 capsid. Also, the spacer length of SMCC only 0.83nm, which may also cause steric hindrance between the AP2 and capsid surface itself, preventing AP2 from binding. SMPEG, however, does not have this issue, as the longer spacer length extends the reactive maleimide end from the surface of the MS2, preventing steric hindrance between the AP2 and the capsid surface. This allows the reaction efficiency to be nearly 100% when reacting with a 1:500 ratio of MS2 to AP2.
Figure 2-4. Representative elution profiles from MS2, AP2, and MS2-SMCC-AP2 (1:50) with a 5 kDa MWCO size exclusion column. A) MS2 in solution was monitored by absorbance at 260nm. Note that the MS2-AP2 sample is eluted from the column in the same volume fractions as the MS2 only control, indicating the presence of MS2 in the sample. *Curves were normalized to maximum absorbance. B) Angiopep-2-FAM in solution was monitored by fluorescence at an excitation wavelength of 495 nm and emission at a wavelength of 540 nm. The MS2-AP2-FAM sample shows fluorescence in the same volume fractions as the MS2 control above, showing co-elution of MS2 and angiopep-2-FAM in the sample, indicating successful conjugation. **Curves were normalized to maximum fluorescence.
2.3.3 Characterization of MS2-AP2 particles

SDS-PAGE analysis was used to confirm covalent conjugation of AP2 to MS2 and analyze the conjugates formed (Figure 2-5). In the Coomassie-stained gel (Figure 2-5A), the MS2 control (lane 2) shows a band at 13.5 kDa (arrow 1, Fig 2-5A), representing the MS2 capsid protein monomer, and a band at 45 kDa (arrow 2, Fig 2-5A), representing the maturase protein. The MS2-SMCC-AP2 (1:500) sample (lane 3) also shows a band at 13.5 kDa representing the MS2 monomer, as well as an additional band that migrated more slowly than the first band, located at approximately 16.5 kDa (arrow 3, Fig 2-5A). This additional band represents MS2 capsid protein modified with one (16.5 kDa) AP2 molecule (~3 kDa) per MS2 capsid protein. There is a fourth band at approximately 25 kDa, representing crosslinking between MS2 capsid proteins (arrow 4, Fig 2-5A). The MS2-SMCC-AP2 (1:50) sample (lane 4) has both the control MS2 band and the crosslinked MS2 band, but is lacking the 16.5 kDa band representing AP2 conjugated to MS2 capsid protein. Conjugation of SMCC to the MS2 capsid did not significantly change the size of the MS2 capsid monomer (data not shown). The MS2-SMPEG-AP2 samples (lanes 5 and 6) both have a band at 16.5 kDa, but the molecular weight of SMPEG24 is nearly 1.4 kDa, so these bands could represent two SMPEG conjugated per MS2 rather than conjugation of AP2.
Figure 2-5. Representative SDS PAGE of MS2-AP2 conjugates. A) Coomassie-stained gel, B) fluorescence-imaged gel at 488/520nm, C) merged Coomassie-stained (blue) and fluorescence (green) images. Images are of the same gel. Lanes assignments are the same for all images. Lane 1 represents the protein ladder standard. Lane 2 shows the MS2 control sample. Lanes 3 and 4 are the MS2-SMCC-AP2 samples reacted 1:500 or 1:50 MS2:AP2, and lanes 5 and 6 are the MS2-SMPEG-AP2 samples reacted at 1:500 or 1:50 MS2:AP2. Arrow 1 refers to the 13.5 kDa MS2 band, arrow 2 refers to the 45 kDa maturase band, arrow 3 refers to the shifted 16.5 kDa band representing 1 AP2 conjugated per MS2, arrow 4 refers to the MS2 capsid protein dimers formed, and arrow 5 refers to the 19.5 kDa band representing 2 AP2 conjugated per MS2.

The SDS PAGE gel was also imaged fluorescently before staining to further identify the composition of the bands and determine the extent of AP2-FAM labeling. Interestingly, as can be seen in the merged imaged (Figure 2-5C), the fluorescent image shows additional bands not present in the Coomassie-stained gel. In lane 3 (MS2-SMCC-AP2 1:500), when overlaid with the Coomassie-stained gel, the fluorescence gel shows the presence of a fluorescently labeled band at 16.5 kDa (arrow 3, Fig 2-5C) and 19.5 kDa (arrow 5, Fig 2-5C), representing capsid protein modified with one or two AP2 molecules per MS2 capsid protein, respectively. These results
suggest that some capsid proteins are capable of conjugating more than one SMCC-AP2 complex, while others conjugate none. This may be affected by the ability of the lysine residues to react due to differences in surface exposure.\textsuperscript{18} Additionally, there are bands around 25 kDa representing MS2 capsid protein dimers, dimers conjugated to one AP2, and dimers conjugated to two AP2. The final fluorescent band around 45 kDa may represent formation of an MS2 capsid protein trimer conjugated to one AP2. The MS2-SMPEG-AP2 samples show two fluorescently labeled bands representing conjugation of one (~17 kDa) or two (~20 kDa) AP2 per MS2 capsid protein. Due to the low concentration of protein in these bands, they are not present in the Coomassie-stained gel.

All of the samples have bands around 25 kDa or larger that do not represent maturase protein. These bands result from crosslinking between two or more MS2 capsid proteins. While MS2 lacks exposed thiol groups on the surface of the capsid for reaction, the conjugated maleimide could have a side-reaction with a closely situated amine group if the local microenvironment is favorable. Although the maleimide-thiol click reaction tends to be specific, proximity of an amine to the maleimide group has been previously shown to allow maleimide-amine reactions to occur.\textsuperscript{19} This would cause the maleimide assay to underestimate the number of SMCC that reacted with the capsid initially, reducing the effective conjugation efficiency determined by the assay. As these maleimides are unavailable to react with free AP2 when the ligand is introduced, they do not affect the conjugation efficiency of AP2. Reaction of MS2 with SMPEG\textsubscript{24} rather than SMCC actually decreases this crosslinking, but also reduces the number of AP2 conjugated due to steric hindrance.

Dynamic light scattering (DLS) was also used to confirm surface modification of the particles. When SMCC was added to the MS2 surface, the hydrodynamic radius did not change
significantly (Table 2-1). However, with addition of SMPEG and/or AP2, the particles increased in size up to nearly 10 nm. Addition of AP2 itself, at both a 1:50 and 1:500 ratio, was capable of increasing the hydrodynamic diameter from 29.8 nm for the unmodified capsid to 32.6 nm and 36.8 nm, respectively. PEGylation of the SMCC increased the capsid size even more, to 37.7 nm for 1:50 and 39.0 nm for 1:500 ratios of MS2 to AP2. It is notable that, despite the 10 nm length of SMPEG, the hydrodynamic radius of the MS2 capsid only increased by about 7 nm. This indicates that the SMPEG spacer does not extend linearly from the MS2, instead spreading along the capsid surface, confirming our earlier hypothesis that SMPEG may be creating steric hindrance on the capsid surface to prevent more linkers from binding.

2.4 Conclusions

Targeting and transporting across the BBB is the first step in delivering clinical agents to the brain. Previously, the synthetic peptide angiopep-2 (AP2) has been conjugated to polymeric nanoparticle platforms and used as a targeting moiety for BBB transport.\textsuperscript{20-22} Although these systems have been successfully modified and characterized, none have studied the ability to control the number of AP2 conjugated, nor the effect of the number of ligands conjugated on transport. Additionally, none have investigated the effect of spacer length on conjugation efficiency.

Here, we have used the MS2 bacteriophage capsid as a nanoparticle platform for conjugation of AP2 for BBB targeting. We have been able to conjugate up to 77 AP2 per MS2-SMCC, with up to two AP2 conjugated per capsid protein, when reacting with a 1:500 ratio of MS2 to AP2. When reacting with a 1:50 ratio of MS2 to AP2, only 37 AP2 per MS2 are conjugated, indicating the number of AP2 conjugated can be controlled using molar ratios.
PEGylation of the crosslinker caused a lower AP2 reaction efficiency (20 and 41 AP2 per MS2 conjugated, when reacted at 1:50 or 1:500 ratios of MS2:AP2), but also reduced crosslinking between MS2 capsid proteins.

The maleimide assay only indicates a conjugation efficiency of 20% for MS2-SMCC. However, this assay is limited by its ability to only detect free (unreacted) maleimides in solution. If these maleimides have already reacted (i.e., by crosslinking to another capsid protein), they will not be detected, reducing the observed conjugation efficiency. The SDS PAGE results do show that the crosslinking is occurring, indicating a greater linker conjugation efficiency that is seen in the maleimide assay. The importance of the maleimide assay, though, is that it provides a maximum number of possible AP2 reaction sites, allowing us to calculate the effective reaction efficiency for the AP2 conjugation.

Controlling the number of groups conjugated to a nanoparticle platform is crucial for the optimization of a therapeutic delivery system. When testing nanoparticles in vitro, it is important to determine the number of conjugated targeting moieties required for targeted delivery to be effective, and costs can be minimized if an optimal number can be found. Investigation of the effects of spacer length on the number of conjugated groups and uptake and transport in vitro is also valuable, as steric hindrance between the platform and targeting moiety can affect ligand binding to target. Taken together, the results shown here provide a foundational toolbox for a set of different applications that extend beyond BBB research and targeting, and can provide guidance for not only MS2 functionalization with AP2, but also functionalization of other nanoparticle systems with different targeting moieties that react using the same principles we have presented here.
2.5 References


CHAPTER 3
INVESTIGATION OF VIRAL CAPSIDS FOR TARGETING BRAIN HYPERACTIVITY

3.1 Introduction to Tinnitus Targeting and Treatment

The conjugation of two targeting moieties to nanodelivery platforms is critical for transport of these systems over the BBB and specific region of interest targeting and treatment after systemic administration of these nanoparticles in the clinic. In order to investigate the application of MS2 as a dually targeted nanoparticle platform for targeting specific brain regions, we focused on targeting the NMDA receptor 2D subunit, the overexpression of which in the inferior colliculus is associated with the perception of tinnitus. The rationale for using this target receptor is highlighted below.

Tinnitus, commonly known as the sensation of “ringing in the ears,” is characterized as a persistent sound perceived by individuals in absence of external acoustic stimulation. Tinnitus has been known to affect over 50 million people in the United States, and can be attributed to a myriad of causes.\(^1\) Although usually deemed a symptom of an underlying issue, perception of tinnitus alone can often be debilitating, preventing those affected from functioning in everyday life. This debilitating form of tinnitus affects nearly 3 million people in the United States, a large subset of which is military veterans. In fact, tinnitus and hearing loss have consistently been the top two service-connected compensated disabilities for those who have returned from serving in the Middle East.\(^2\) Damage to the inner ear and brain are signature injuries from these conflicts, as they are regularly encountered from battle explosions and gunfire. Associations that have the
responsibility to provide health care for veterans, such as the Veteran’s Health Administration, are also impacted by the amount of tinnitus-related disabilities in the veteran community. As there are no treatment options for tinnitus, therapies at the Veteran’s Administration medical centers focus on education, counseling, and sound-based therapy. However, these management techniques vary in effectiveness from person to person, and they only focus on learning to live with the condition, rather than alleviating the underlying cause.

Figure 3-1. Schematic of the current scientific theory for the cause of noise-induced tinnitus. Hearing loss caused by acoustic trauma induces hyperactivity in the central nucleus of the inferior colliculus of the auditory cortex. This hyperactivity has been associated with an upregulation in the gene expression of N-methyl-D-aspartate (NMDA) receptors.
Acoustic trauma resulting in hearing loss is a well-known trigger for development of tinnitus (Figure 3-1). Acoustic trauma can result in physiological changes in the discharge of auditory neurons, and have secondary physiological effects on the auditory cortex. These physiological changes can also cause an imbalance in auditory neuron excitation or inhibition, which can destabilize circuits in the brain. This destabilization may induce synaptic readjustment of the neurons in the auditory cortex in order to compensate for this shift from the norm. In sum, neuronal hyperactivity, spontaneous firing of neurons, and/or increased neuronal synchrony are thought to be the key elements that result in the perception of tinnitus caused by acoustic trauma.

Recently, researchers have found that inducing tinnitus in rats via salicylate injection or noise exposure results in neuronal hyperactivity specifically localized to the inferior colliculus (IC) and the dorsal cochlear nucleus (DCN) of the midbrain. The IC is the primary auditory center of the midbrain, and receives input from brainstem nuclei in the auditory pathway and the auditory cortex. Outputs from the IC go to the medial geniculate nuclei of the thalamus, which then extends to several other auditory cortical areas. By using a modified form of magnetic resonance imaging, termed manganese-enhanced MRI or “MEMRI,” studies have enabled researchers to better visualize these areas affected by tinnitus, indicating a target area for treatment. These hyperactive regions of the brain in induced tinnitus animal models have been associated with the upregulation of certain proteins, including N-methyl-D-aspartate (NMDA) receptors. Studies have shown that NMDA receptor subunits, including the 2D subunit (NMDAR2D), have a more than tenfold increase in gene expression, making these receptors a prime candidate to investigate as a potential target for the delivery of clinical agents to these regions of hyperactivity.
Figure 3-2. Reaction of Traut’s Reagent (2-iminothiolane) with antibody. When reacted with primary amines, Traut’s Reagent undergoes a ring opening reaction that adds a free thiol group.

Here, we have chosen to investigate targeting these hyperactive regions of the brain to enhance imaging using antibodies specific for the NMDAR2D subunit as a proof-of-principle for dual modification of the exterior of the MS2 capsid. To facilitate nanoparticle modification, the MS2-SMCC conjugation strategy from Chapter 2 was applied to conjugate both the antibody and AP2. Conjugation of IgG to MS2, however, presents some difficulty, as IgG does not contain free thiol groups for maleimide reaction. To maintain our strategy, IgG was first reacted with Traut’s Reagent (2-iminothiolane), which undergoes a ring opening reaction when attacked by the primary amines present on the IgG surface (Figure 3-2). After this modification, MS2-SMCC and IgG-SH were reacted to form MS2-IgG (Figure 3-3). Additional reaction of the remaining SMCC groups with AP2 generates MS2-IgG-AP2.
**Figure 3-3.** Overall Strategy for MS2-IgG Conjugation. First MS2-SMCC and IgG-SH are reacted to form MS2-IgG. MS2-IgG is then either loaded with SYBR Gold nucleic acid dye (1) or conjugated with AP2 (2), then analyzed for conjugation efficiency.

### 3.2 Experimental Methods

#### 3.2.1 Materials

Rabbit anti-NMDA receptor 2D antibody was purchased from Alomone Labs. Angiopep-2-FAM conjugated to cysteine (FAM-TFFYGGSRGKRNFKTEY) (AP2) was synthesized by GenScript Biotech Corporation (NJ). Traut’s Reagent was obtained from Toronto Research Chemicals (Ontario, Canada). SYBR™ Gold nucleic acid gel stain was obtained from Life Technologies (CA). All other reagents were purchased from Sigma Aldrich unless specified. Experiments were conducted with reagent-grade chemicals.

#### 3.2.2 MS2-IgG Conjugation

SMCC-modified MS2 particles were generated as previously described. Before reaction
with MS2-SMCC, the NMDAR2D IgG was first activated with Traut’s reagent, 2-iminothiolane. IgG was reacted in a 1:5 ratio of IgG to Traut’s reagent in a PBS-EDTA buffer (150mM NaCl, 20mM NaPO4, 5mM EDTA, pH 7.4) for 1 hour at room temperature, forming thiolated IgG (IgG-SH). IgG-SH was then purified using a PD-10 Desalting Column (5k molecular weight cutoff (MWCO), Sephadex G-25 M by GE Healthcare, UK) equilibrated with PBS-EDTA buffer. The obtained samples were then concentrated using a Vivaspin 500 concentrator with a 30 kDa molecular weight cut off (Sartorius, Germany) and measured for IgG content using a Nanodrop spectrophotometer (ND1000, Thermo Fisher Scientific, MA). The concentration of IgG was calculated using the absorption coefficient for \( M_w = 150,000 \) IgG, \( \varepsilon_{280} = 210,000 \) M\(^{-1}\) cm\(^{-1}\).

Ellman’s Assay was used to determine the number of free thiols incorporated on the surface of IgG. MS2-SMCC was then reacted with IgG-SH in a 1:60 ratio overnight at 4°C. After reaction, MS2-IgG was purified from excess IgG by fast protein liquid chromatography (FPLC) using a Superose 6 column (10/300 GL, GE Healthcare, UK) mounted on a ÄKTApurifier system (GE Healthsciences, PA). Purified samples were pooled and concentrated using a Vivaspin 6 concentrator (30k MWCO, Sartorius, Germany). MS2-IgG was subsequently loaded with SYBR Gold or modified with angiopep-2 (AP2) and analyzed.

### 3.2.3 MS2-IgG SYBR Gold Loading

After purification of MS2-IgG conjugates, samples were incubated with a 1:2500 molar ratio of MS2 to SYBR Gold (SG) for one hour at room temperature in PBS-EDTA buffer. MS2-IgG-SG particles were purified from excess SYBR Gold using a PD-10 Desalting Column and concentrated using Vivaspin 500 concentrators (30kDa MWCO) as described above. Unmodified MS2 was loaded with SYBR Gold and purified as above as a control.

### 3.2.4 MS2-IgG-AP2 Conjugation
MS2-IgG conjugates were reacted with AP2 at a 1:300 molar ratio of MS2 to AP2 for one hour at room temperature in PBS-EDTA buffer. After incubation, MS2-IgG-AP2 particles were purified using a PD-10 Desalting Column and concentrated using Vivaspin 500 concentrators (30 kDa MWCO) as described above.

3.2.5 UV-Vis Spectroscopy

UV-Vis measurements on IgG-SH fractions were performed using a Tecan Infinite M200 plate reader (Tecan Group Ltd., Switzerland). For elution experiments, peak absorbance spectra values (280 nm for IgG) and peak fluorescence values (495nm excitation, 540nm emission) were determined for each fraction. UV-Vis analysis of MS2-IgG fractions was accomplished using the ÄKTApurifier system. UV-visible absorbance peaks were used to confirm purification of MS2-IgG from excess antibody and both UV absorbance peaks and fluorescence peaks were used to confirm MS2-IgG-AP2 purification from excess AP2.

3.2.6 Analysis of MS2-IgG conjugation efficiency

MS2-IgG-SG and MS2-IgG-AP2 samples were analyzed by dot blot to confirm conjugation. Samples were blotted onto dry nitrocellulose paper in triplicate. After the samples dried, the membrane was blocked in 5% milk in Tris-buffered saline-0.1% Tween 20 (TBST). The membrane was incubated with secondary anti-rabbit antibody overnight at 4°C and rinsed, then west femto substrate (Thermo Fisher Scientific) was used for chemiluminescent blot visualization of the antibody in a ChemiDoc MP Imaging System (Bio-Rad, CA). Fluorescent imaging at 488nm/520nm was used to confirm the presence of SYBR Gold-tagged MS2 or fluorescently tagged AP2 in the sample.

3.2.7 Recognition of antigen by MS2-IgG

Western blot was used to determine if the MS2-IgG conjugate was capable of identifying
the NMDAR2D protein from auditory regions of the brains of tinnitus rats. Cell lysate from the inferior colliculus (IC), auditory cortex (AC), central nucleus (CN), and prefrontal cortex (whole brain control) were obtained from the Holt Lab at Wayne State University (Detroit, MI). Protein concentration was determined using a BCA assay (Pierce, Thermo Fisher Scientific) and samples were loaded into a mini-Protean gel (Bio-Rad) with Precision Plus Protein™ dual color standard ladder (Bio-Rad) and run in 1x Tris-Glycine-SDS buffer for 15 minutes at 60 V for stacking and an additional 60 minutes at 125 V. Samples were transferred to a polyvinylidene fluoride (PVDF) membrane using a TurboBlot transfer unit (Bio-Rad) and blocked for 1 hr in 5% powdered milk in TBST. The membrane was incubated with MS2-IgG, anti-NMDAR2D IgG control, or GAPDH (Glyceraldehyde 3-phosphate dehydrogenase, Santa Cruz Biotechnology, TX) control overnight. The blots were rinsed, and incubated with primary anti-MS2 antibody and secondary anti-rabbit antibody (MS2-IgG) or secondary anti-rabbit antibody (IgG control and GAPDH) for 1 hour at room temperature. West femto substrate (Thermo Fisher Scientific) was used for band visualization in a ChemiDoc MP Imaging System. Band intensities were quantified using ImageJ software standardized to the average intensity of the blot, and NMDAR2D band intensity was normalized to the corresponding GAPDH controls.

3.2.7 DLS analysis of MS2-IgG

Mean particle diameter was determined in PBS-EDTA buffer pH 7.4 (0.5 mg/mL MS2) using dynamic light scattering (DLS, Malvern Zetasizer Nano, UK). Mean particle diameter measurements were averaged over three measurements using 15 scans at 10 second duration each at 20° C. The percent intensity algorithm in the Malvern Zetasizer collection software was used to calculate the particle diameter.
3.3 Results and discussion

3.3.1 MS2-IgG Conjugation

There are two main challenges for delivering clinical agents to areas of the brain affected by disease: 1) targeting to a specific region of interest in the affected area, and 2) transport across the blood-brain barrier. These two demands require researchers to generate drug delivery platforms capable of dual targeting, by conjugating two different targeting moieties to the surface of the delivery system. Dual functionalization of these nanoparticle systems is essential for the diagnosis and treatment of brain diseases by small molecules. Here, we targeted overexpression of NMDAR2D associated with noise-induced tinnitus as a proof-of-principle to show that our particle is capable of dual exterior surface modification and specific region of interest targeting. Being able to reach regions of hyperactivity and receptor upregulation to deliver clinical agents such as contrast agents or drugs is essential for better understanding the origin of the perception of tinnitus and possibly finding a treatment option. As we already showed (in Chapter 2) that the MS2 bacteriophage capsid can be successfully modified with AP2, we leveraged this strategy to also conjugate the SMCC-modified MS2 capsid to anti-NMDAR2D IgG.
Figure 3-4. Formation of MS2 aggregates when reacted with antibodies that have been thiolated multiple times. Strict control over molar ratios is necessary to avoid crosslinking between MS2 capsids.

The reacted SMCC on the modified MS2 contains a maleimide group that will react with a free thiol. However, IgG does not contain free thiols (only disulfide linkages between its heavy and light chains), and must therefore be modified before it will react with MS2-SMCC. Traut’s reagent (2-iminothiolane) is a molecule that, when attacked by a primary amine on a protein, undergoes a ring opening reaction to conjugate a free thiol to the protein’s surface. IgG has approximately 90 lysine residues on its surface that will react with Traut’s reagent and thiolate the surface of the IgG.10 In this study, this reaction was performed at a very low ratio of IgG:TR (1:5) in order to minimize crosslinking between MS2 capsids after reaction of IgG-SH with MS2-SMCC. The large amount of SMCC on the MS2 surface (>100) makes it possible that, if each IgG has any more than one thiol conjugated per IgG, MS2 capsids could effectively link together by thiolated antibodies (Figure 3-4, previous page). Ellman’s assay showed that, with only a 1:5 ratio, we have been able to conjugate approximately 1 thiol per antibody on average.
Figure 3-5. Elution profile from MS2-SMCC and IgG-SH with a 5kDa MWCO size exclusion column. MS2 in solution was monitored by absorbance at 260nm, and IgG in solution was monitored by absorbance at 280nm. Note that MS2 and IgG are eluted from the column in the same volume fractions. *Curves were normalized to each sample’s maximum absorbance.

When purifying modified IgG from reaction byproducts, IgG-SH is eluted within the first 2mL of the Sephadex GPC column, similar to MS2-SMCC (Figure 3-5). Due to the low molecular weight cutoff of these columns (~5kDa), they cannot be used to purify MS2-IgG from unbound antibody. Therefore, fast protein liquid chromatography (FPLC) was used instead, with a Superose 6 column capable of separating molecules that are 5,000 to 5,000,000 Da in size. As shown in Figure 3-6, the Superose 6 column was capable of separating MS2-IgG from unbound IgG.
Figure 3-6. Representative FPLC Plot of MS2-IgG Conjugates. MS2 (0.2mg/mL) is eluted from the FPLC column within the first 15mL (black), whereas IgG (2mg/mL) is retained until nearly 15mL (purple). The MS2-IgG plot in green shows a comparable elution pattern with the purified molecules when eluting from the column, indicating purification of unbound IgG from the MS2-IgG sample. The third peak represents albumin present in the IgG sample.

3.3.2 Analysis of MS2-IgG-SG Conjugates

To confirm conjugation of MS2 and IgG, MS2 was loaded with the nucleic acid intercalating dye SYBR™ Gold after conjugation to IgG. The fluorescent dye was used as a tag for the presence of MS2 in the sample. A dot blot was used to determine if MS2 and IgG were conjugated. This technique is similar to a western blot, but instead of running the sample through a gel, the sample is blotted onto a porous membrane, and then probed for the presence of the target group. Incubation with HRP-bound secondary antibody and subsequent reaction with substrate and enhancer provides a chemiluminescent signal. The presence of IgG in the MS2-
IgG-SG sample is evident in Figure 3-7, where the blot shows the chemiluminescent signal emitted (left). On the right side of Figure 3-7, fluorescence emission at 488/520nm (Ex/Em) shows the expression of the SYBR™ Gold dye, indicating the presence of MS2. The dot blot therefore indicates conjugation of intact MS2 and IgG, as they are both present in the MS2-IgG-SG sample.

**Figure 3-7.** Dot blot of MS2-IgG-SG. Left: Chemiluminescent probe showing the presence of IgG in the sample. Right: Fluorescent blot showing SYBR™ Gold dye in the sample, indicating the presence of intact MS2.

IgG blots were also diluted to 16, 4, and 2 ug/mL IgG (corresponding to 1:1, 1:4, or 1:8 IgG/MS2) to calculate the amount of IgG present in the sample (Figure 3-8). After densitometry analysis of the IgG blots, we calculated that on average 0.35 IgG were bound to each MS2 particle, indicating that only about one out of three MS2 is successfully modified. This low reaction efficiency could have a few causes. First, a low molar ratio of IgG to Traut’s reagent
(1:5) is used for thiolation, conjugating only about one thiol per IgG. Due to the substantial size of the antibody (typically 14.5nm x 8.5 nm x 4.0 nm), the probability that one of the SMCC crosslinkers effectively reach a free thiol on the surface of an antibody may be relatively low.\textsuperscript{11} One thiolation point (extending only 0.81 nm from the IgG surface) may not provide enough surface modification of the IgG to effectively conjugate more antibodies per MS2. Another possibility for low conjugation efficiency could be the formation of disulfides between IgG-SH molecules during processing, preventing their future reaction with MS2-SMCC. However, EDTA is also present in the IgG-SH solution, which should function to minimize disulfide formation between free thiols.

**Figure 3-8.** Dot blot of IgG dilutions in triplicate. Each blot corresponds to a ratio of MS2 to IgG for different concentrations of IgG. 1:1 IgG to MS2 corresponds to 16 ug/mL IgG, 1:4 IgG to MS2 corresponds to 4 ug/mL IgG, and 1:8 IgG to MS2 corresponds to 2 ug/mL IgG.

Dynamic light scattering (DLS) showed a significant increase in the hydrodynamic diameter from 29.8 ± 0.7 nm to 34.9 ± 1.6 nm with the addition of IgG to the MS2. The MS2-
IgG diameter is also larger than that of the MS2-SMCC-AP2 particles made with a 1:50 ratio of MS2 to AP2 (32.6 ± 0.4 nm). Interestingly, the diameter of the MS2-SMCC-AP2 particles reacted with a 1:500 ratio is larger than the MS2-IgG particles, though not significantly (36.2 ± 1.2 nm). This suggests that the IgG alone extends less than 5nm from the surface of the MS2 particle. As mentioned earlier, IgG is about 14 nm in length by 8 nm in height, so it could be expected that the full antibody extending out from the MS2 surface would increase the size even more than it does. However, if the antibody is lying on the MS2 surface, rather than extending out, this length is appropriate, as the width of an antibody is only a few nanometers.\textsuperscript{12} While this allows the IgG to be easily recognized by secondary antibody, this structural arrangement may affect the antibody’s recognition of the antigen (NMDAR2D). This possibility is explored in a later section.

\textbf{Figure 3-9.} Elution profile for MS2-IgG-AP2 with a 5kDa MWCO size exclusion column. MS2 present in the sample is monitored by absorbance at 260nm (grey curve), whereas AP2 elution is monitored by fluorescence at 495/540nm (black curve). *MS2 absorbance values and AP2 fluorescence values were normalized to the peak absorbance or peak fluorescence, respectively.
3.3.3 Analysis of MS2-IgG-AP2 Conjugates

After confirmation of IgG conjugation to MS2, dual modification of the MS2 particle was investigated. MS2-IgG was reacted with AP2 in a 1:300 ratio of MS2 to AP2 and purified using column chromatography. Fractions were taken up to 2.75mL and monitored for absorbance at 260nm or fluorescence at 495/540nm. The elution profile is shown in Figure 3-9. As can be seen in the plot, after a one-hour incubation, the MS2 and AP2 in the MS2-IgG-AP2 sample co-elute from the GPC column, indicating successful dual modification of the MS2 particle.

A dot blot was used to further confirm dual functionalization of the MS2 surface. MS2-IgG-AP2 was blotted onto a nitrocellulose membrane with IgG as a chemiluminescent control and FITC-BSA as a fluorescent control. FITC-BSA was used in lieu of AP2 as the small size of the peptide (~2.8 kDa) caused it to leak from the membrane during the blocking and antibody incubation steps (data not shown). The resulting dot blot is shown in Figure 3-10, where the chemiluminescent probe is displayed on the left, and the fluorescent probe is displayed on the right. The blot shows both the presence of IgG and AP2 in the sample by chemiluminescence and fluorescence detection, further indicating successful dual modification of the MS2 particle.
Figure 3-10. Dot blot of MS2-IgG-AP2. Left: Chemiluminescent probe showing the presence of IgG in the sample. Right: Fluorescent blot showing the presence of AP2 (fluorescently tagged with FAM) in the sample.

Overall, the sequence of reactions and controlling the molar ratios between MS2 and targeting moieties are critical for dual surface modification. Reaction of one targeting moiety may reduce the number of reactive groups on the surface of the particle so much that the second targeting moiety is unable to be conjugated. Here, we chose to react the IgG-SH with MS2-SMCC before reaction of the particle with AP2. The large size and low surface modification on the antibody limits the conjugation efficiency of the IgG to less than 1%, whereas conjugation of AP2 is limited mostly by steric hindrance between the peptide and the MS2 surface. Therefore, reaction of IgG before AP2 is necessary to allow the IgG more reaction time before the smaller AP2 group is introduced to scavenge the remaining SMCC groups on the MS2 capsid. If we reversed this reaction order, the smaller AP2 would take up the most reactive SMCC on the MS2 surface first, making the subsequent maleimide-thiol reaction with IgG even less likely.
Additionally, the AP2 may buffer the IgG from the MS2 surface, further preventing the antibody from contacting any of the free maleimides remaining on the surface for reaction.

3.3.4 Recognition of NMDAR2D by MS2-IgG

After thiolation of the IgG and conjugation to MS2, confirmation that the Fv portions of the antibody were still functional and able to recognize antigen (NMDAR2D) was necessary. MS2-IgG particles were used to probe samples of brain lysate from different regions of the auditory cortex in tinnitus-positive rats. The results of the western blot are shown in Figure 3-11. Product literature indicates NMDAR2D has two distinct bands, one at 145 kDa and one at 75 kDa. When using MS2-IgG as a probe for NMDAR2D, there are bands at 145 kDa and 75 kDa for the inferior colliculus as a whole and the central nucleus of the IC, two regions of the auditory cortex that have been shown to be associated with high NMDAR2D expression in tinnitus rats. Additionally, the MS2-IgG probe was also able to recognize NMDAR2D in the whole brain sample at 145 kDa and 75 kDa. However, the 145 kDa band for NMDAR2D is not present in the whole auditory cortex sample, but the 75 kDa band is present, demonstrating that MS2-IgG can recognize some form of the NMDAR2D antigen in each area of the brain investigated.
Figure 3-11. Western blot for NMDAR2D. MS2-IgG was used as a probe to detect NMDAR2D in tinnitus rat brain samples (left). As a positive control, anti-NMDAR2D antibody was used to probe for the presence of NMDAR2D in tinnitus rat brain samples (right). IC: inferior colliculus, AC: auditory cortex, CN: central nucleus of the inferior colliculus, WB/WB1: whole brain control, WB2: whole brain control four times diluted.

3.4 Conclusions

For nanoparticles to deliver clinical agents to the brain, they must be capable of 1) BBB targeting and 2) specific region of interest targeting. Dual modification of nanoparticle surfaces with different targeting moieties is key for the success of brain delivery platforms. While many studies have shown successful modification of nanoparticle surfaces with brain targeting molecules such as angiopep-2,13,14,15 few groups have endeavored to conjugate two different targeting moieties to the exterior of a brain-targeted nanoparticle system. One group conjugated both AP2 and an EGFR antibody to the exterior of polymeric nanoparticles for specifically targeting glioblastoma.16 Interestingly, although MS2 particles have been ‘dually functionalized’ on the exterior and interior,17 no one has yet to conjugate two different targeting moieties to the exterior for dual functionalization purposes.
Here, we have modified the MS2 bacteriophage capsid with two targeting ligands, an antibody and a BBB targeting peptide, as a proof of concept for targeted delivery to the brain. Results show that MS2 and IgG were successfully conjugated after an overnight reaction, but with low conjugation efficiency (~0.6%). Others have previously shown conjugation up to one IgG per MS2 capsid when reacting at a 3:1 ratio (~33% conjugation efficiency). However, their conjugation strategy relied on use of aniline-containing MS2 capsids (paF) for highly efficient oxidative coupling.\textsuperscript{18} Our conjugation strategy utilizes the amines present on wild-type MS2 to conjugate heterobifunctional crosslinkers that will react with IgG-SH. Although less efficient, our strategy is straightforward and less intensive, and has been shown capable of conjugating IgG to the MS2 surface.

DLS results indicate that conjugation of IgG increases the size of MS2 by nearly 5 nm. Other groups that have conjugated antibodies to MS2 have shown that the hydrodynamic diameter of the MS2 particles does increase by approximately 4nm, confirming our results.\textsuperscript{18} We successfully dually modified the exterior MS2 surface with both IgG and AP2, after reaction order and molar reaction ratios were carefully considered. This work is entirely novel, as there have been no studies that have demonstrated dual exterior modification of the MS2 surface. Lastly, MS2-IgG particles were shown capable of probing for the NMDA receptor 2D subunit from samples of brain lysate. We can therefore conclude that the antigen recognition ability of the anti-NMDAR2D IgG conjugated to MS2 is not lost by chemical modification, or by the steric hindrance caused by the possible structural arrangement of the IgG laying plane with the MS2 surface. Taken together, this work presents a novel strategy for dual functionalization of the MS2 exterior surface and demonstrates the functionality of NMDAR2D targeting groups, indicating the successful development of a nanoparticle platform for targeting brain hyperactivity.
3.5 References


CHAPTER 4

NUCLEOTIDE-MEDIATED LOADING OF MRI CONTRAST AGENTS INTO VIRAL CAPSIDS

4.1 Introduction

4.1.1 Brain Disease Diagnosis by Magnetic Resonance Imaging (MRI)

Methods for the detection of brain disease in live patients are severely lacking in the medical community. Many diagnoses rely on the ability of the physician to determine the disease that ails the patient and the best treatment options based on the medical expert’s interpretation of physically expressed symptom. Imaging techniques are a standard method of determining general brain structure in live patients. For example, magnetic resonance imaging (MRI) is regularly used to confirm the diagnosis of a stroke, tumor, or late-stage neurodegenerative diseases. Normally, hydrogen protons rotate freely throughout the body (Fig. 4-1A). MRI uses a magnetic field to align these protons in the patient (Figure 4-1B). Some of these protons align with the field, while others align opposite to the field. The majority of these alignments negate each other, but there are some that remain unpaired (Fig. 4-1B, green). A radio frequency pulse is emitted by the MRI machine, which causes a change in the orientation of the unpaired protons (Fig. 4-1C). After the pulse is turned off, these protons realign with the magnetic field, emitting electromagnetic energy (Fig. 4-1D). The machine measures this energy and converts the signal into an MRI image, which is interpreted by medical professionals.
Figure 4-1. Operating principle of magnetic resonance imaging (MRI). A) Hydrogen protons normally spin in random directions. B) Upon introduction of a magnetic field, the protons align either along the field or opposite of the field. Most of these protons form pairs and negate each other (blue), but some remain that are unpaired (green). C) When a radio frequency pulse is emitted, the unpaired protons briefly change orientation. D) When the pulse is removed, these protons rotate back into their original position, and release energy, which is detected by the MRI machine.

Although useful for diagnosis of many different diseases, MRI is limited by its ability to differentiate between normal and abnormal tissue. Additionally, the size of the abnormality must be large enough to visualize, giving MRI a relatively low detection limit. This technique is severely lacking for diagnosis of early stage neurodegenerative diseases or differentiating...
between degenerative diseases, diagnosing concussions, and discerning regions of general neuronal hyperactivity that may be negatively affecting the patient, such as the hyperactivity present in tinnitus. Establishment of a new method to effectively image these anomalies would greatly help in their diagnosis and eventual treatment in the future.

**Table 4-1.** Names and general structure of gadolinium-based contrast agents approved for use in the United States or European Union.

<table>
<thead>
<tr>
<th>Generic Name</th>
<th>Commercial Name</th>
<th>Structure</th>
<th>Chemical or Code Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gadoterate meglumine</td>
<td>Dotarem</td>
<td>Macroyclic</td>
<td>Gd-DOTA</td>
</tr>
<tr>
<td>Gadopentetate dimeglumine</td>
<td>Magnevist</td>
<td>Linear</td>
<td>Gd-DTPA</td>
</tr>
<tr>
<td>Gadobutrol</td>
<td>Gadovist</td>
<td>Macro cyclic</td>
<td>Gd-DO3A-butrol</td>
</tr>
<tr>
<td>Gadodiamide</td>
<td>Omniscan</td>
<td>Linear</td>
<td>Gd-DTPA-BMA</td>
</tr>
<tr>
<td>Gadobenate disodium</td>
<td>Multihance</td>
<td>Linear</td>
<td>Gd-BOTPA</td>
</tr>
<tr>
<td>Gadoteridol</td>
<td>Prohance</td>
<td>Macro cyclic</td>
<td>Gd-HP-DO3A</td>
</tr>
</tbody>
</table>

Fortunately, the development of contrast agents has upgraded the versatility of imaging techniques, allowing better visualization of affected brain regions and higher resolution for differentiating between normal and abnormal tissues. A list of currently used imaging agents is shown in Table 4-1. Contrast agents improve the visibility of internal tissue and the resolution of MRI by accelerating the relaxation time of hydrogen protons. Hydrogen protons in different tissues vary in their relaxation time, which provides MRI contrast. Introduction of a contrast agent such as gadolinium, which has 7 unpaired electrons, causes hydrogen molecules around the heavy metal to relax more quickly, enhancing the image in different areas of the same organ. The majority of contrast agents approved for use in the USA and European Union are gadolinium-based. Iron-oxide dextran-coated nanoparticle-based contrast medias have also been manufactured, specifically to enhance contrast in the liver, but these dyes are rarely used and few remain on the market. Gadolinium-based contrast agents are chelated with linear or
macrocyclic agents before introduction to the body (Table 4-1). At neutral pH, gadolinium forms an insoluble hydroxide complex with water. Gadolinium hydroxide will precipitate out of solution and deposit in tissue, potentially leading to harmful effects in the patient. Chelation is necessary to avoid this process. Additionally, macrocyclic chelators tend to be more stable than linear chelators, and are safer for patient use.²

Gadolinium-based contrast agents have been used extensively for brain imaging of strokes and tumors, as these pathological conditions compromise the BBB, allowing imaging agents access to brain tissue for image enhancement. When the BBB is intact, however, contrast agents are unable to enter the brain due to their hydrophilicity. Therefore, a novel strategy is needed to transport these molecules into the brain for image enhancement and potential diagnosis of currently undetectable brain pathologies.

4.1.2 Nucleotide-Mediated Loading of MS2 Viral Capsids

Use of nanoparticle carrier systems is crucial to the success of diagnosis and treatment in CNS diseases. Nanoparticle platforms are capable of providing targeted delivery of multiple clinical agents, allowing accumulation of the clinical molecules at the specific target site and increasing the probability that a therapeutic effect will be attained. Many different nanoscale platforms have been used for delivery to the brain.⁴⁻⁶ More recently, viral capsids have emerged as a highly versatile and modifiable nanocarrier platform capable of loading small molecules into an inner core via surface pores.⁷⁻¹⁰ The MS2 bacteriophage capsid has been especially well studied for this purpose. MS2 contains 32 pores with diameters ranging from 1.6-1.8 nm each, with an interior core containing one single-stranded, positive sense RNA (ssRNA).¹¹ These two features allow internalization of small molecules—including positively charged fluorescent dyes and porphyrins—and loading by nucleotide-mediated interactions.⁹ We have shown MS2
capable of loading with the nucleic acid dye SYBR™ Gold. After a thirty-minute incubation, the dye co-eluted with MS2 when purified by column chromatography, indicating that the SYBR™ Gold is loaded and interacting with the RNA inside of the MS2 capsid (Figure 4-2, previous page).

Figure 4-2. Elution profile of MS2 loaded with SYBR™ Gold nucleic acid dye. MS2 and dye co-elute from the column, indicating that the dye is interacting with the MS2 RNA on the interior of the viral capsid.

MRI capability has been previously conferred to MS2 by exterior covalent conjugation of Gd-DTPA. Here, we investigate the ability to load the MS2 interior with gadolinium-based contrast agents for MRI functionality, leaving exterior functional groups available for subsequent conjugation of targeting moieties. We have chosen to use the contrast agent Gd-DOTA
(gadoteric acid, trade name “Dotarem”) for loading of the MS2 capsid. To facilitate nanoparticle loading, however, modification of the gadolinium-chelator complex was necessary. The Gd-DOTA complex is negatively charged, which prevents it from interacting with the negatively charged RNA backbone inside the capsid. To overcome this, aminated Gd-DOTA was first reacted with the crosslinker SPB (succinimidyl-[4-(psoralen-8-yloxy)]-butyrate). SPB consists of an active NHS (N-hydroxysuccinimide) ester linked to a psoralen molecule. The NHS ester is specific for reaction with amine groups, which has been introduced to one arm of the DOTA (Figure 4-3, next page). Psoralen is a nucleic acid intercalating agent, which will be used to effectively link the MS2 RNA to the Gd-DOTA complex for MRI capability (Figure 4-4).
Figure 4-3. Formation of Gd-DOTA-SPB. DOTA has been aminated for reaction with SPB, which has an NHS ester capable of reaction with amines. The psoralen molecule on the opposite end of the SPB is used for intercalation into MS2 RNA.
Figure 4-4. Strategy for Gd-DOTA loading of MS2. SPB conjugated to Gd-DOTA can intercalate into the ssRNA inside the MS2 capsid. After a short exposure to UV light, SPB will form a covalent bond to the RNA backbone.
4.2 Experimental Methods

4.2.1 Materials

Unless otherwise noted, all reagents were purchased from Sigma Aldrich. P-NH$_2$-Bn-DOTA (DOTA-NH$_2$) was obtained from Macrocycles (Plano, TX). Succinimidyl-[4-(psoralen-8-yloxy)]-butyrate (SPB) and xylenol orange tetrasodium salt were obtained from Thermo Fisher Scientific. HEPES sodium salt was obtained from EMD Millipore (Germany). All experiments were conducted with reagent-grade chemicals.

4.2.2 Preparation of Gd-DOTA complexes

DOTA-NH$_2$ was mixed with GdCl$_3$ at a 1.1:1 ratio in deionized water and stirred at room temperature for two days. Removal of excess free Gd$^{3+}$ ions from solution was investigated by drop wise addition of NaOH or HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) to Gd-DOTA complexes and ICP-MS analysis. All complexes used for loading were purified by addition of a final concentration of 100mM HEPES to bring the pH to 8.2, then centrifugation of precipitated Gd(OH)$_3$ and collection of the aqueous upper phase. Trace Gd(OH)$_3$ was removed by filtration through a 0.2um PES (polyethersulfone) filter before reaction with SPB.

4.2.3 Analysis of Gd-DOTA chelation efficiency

Gd-DOTA chelates were analyzed for chelation efficiency after 48 and 72 hours using the xylenol orange test.$^{13}$ Briefly, xylenol orange (4mg in 250mL of 10mM acetate buffer) was mixed with 10uL of GdCl$_3$ (ranging from 100uM to 5uM gadolinium) or Gd-DOTA in a 10:1 ratio. After incubation on a rocker for 5 minutes, sample absorbance was read at 433nm and 573nm in a Tecan Infinite M200 plate reader (Tecan Group Ltd., Switzerland). A standard curve was made using the ratio of absorbances at 573nm and 433nm for GdCl$_3$, and the absorbance
ratios of the Gd-DOTA sample was compared to the standard curve to determine the amount of free Gd$^{3+}$ in solution.

4.2.4 Preparation and loading of Gd-DOTA-SPB conjugates

After purification, Gd-DOTA samples were reacted with SPB in molar ratios of 1:1, 1:10, and 1:100 DOTA:SPB at pH 8.2 over 24 or 48 hours in the dark. When reaction was complete, MS2 was incubated with Gd-DOTA-SPB in molar ratios of 1:10, 1:100, and 1:500 for 24 hours at 4°C. Both loaded and unloaded MS2 samples were exposed to UVA light (365nm, 4 watts) for 15 minutes at room temperature, then purified using a PD-10 Desalting Column (5k MWCO, Sephadex G-25 M by GE Healthcare, UK) equilibrated with 20mM NaPO$_4$ buffer (pH 7.4). Samples were pooled and concentrated using a Vivaspin 500 concentrator with a 30 kDa molecular weight cut off (Sartorius, Germany) and measured for MS2 content using a Nanodrop spectrophotometer (ND1000, Thermo Fisher Scientific, MA). The concentration of MS2 was calculated using the absorption coefficient for MS2, $\varepsilon_{260} = 8.03\text{mL/mg.}^{14}$ MS2-Gd-DOTA-SPB samples were analyzed using induced coupled plasma mass spectrometry (ICP-MS) for gadolinium content.

4.2.5 UV-Vis Spectroscopy

UV-Vis measurements on gadolinium-loaded MS2 fractions and for the xylenol orange test were performed using a Tecan Infinite M200 plate reader. For elution experiments, peak absorbance spectra values (260 nm for MS2) were determined for each fraction. UV-visible absorbance peaks were used to confirm MS2 particle stability after exposure to UVA light (365nm) for covalent conjugation of SPB to RNA. For the xylenol orange test, absorbance spectra values at 433 nm and 573 nm were determined for each sample and compared to a standard curve to calculate the molar amount of free Gd$^{3+}$ in solution.
4.3 Results and Discussion

4.3.1 Preparation, purification, and analysis of Gd-DOTA complexes

MS2 has previously been shown capable of loading with positively charged agents.\textsuperscript{9} Gd-DOTA, however, is negatively charged, which will prevent the nucleic acid interactions in the MS2 interior that are normally targeted for loading. Therefore, modification of the Gd-DOTA complex with SPB was necessary before loading into the MS2 capsid could be attempted.

Before reaction of DOTA-NH\textsubscript{2} and SPB, the chelation efficiency between Gd and DOTA-NH\textsubscript{2} was explored. A ratio of 1.1:1 Gd:DOTA was used for reaction, as this molar ratio has been used previously in literature for Gd-DOTA chelation.\textsuperscript{15} Indeed, the xylenol orange test showed that approximately 90.3\% of the gadolinium in solution was chelated after a 48 hour incubation, and approximately 90.8\% was chelated after a 72 hour incubation. This is to be expected, as we used a 1.1:1 ratio of Gd to DOTA in solution, thus the free Gd\textsuperscript{3+} remaining theoretically should be around 10\%. We chose to continue using a 48 hour incubation, as this was sufficient for the chelation reaction to be considered complete.

Reaction of DOTA-NH\textsubscript{2} with SPB requires a pH of 7-8 for efficient reaction. Additionally, purification of free Gd\textsuperscript{3+} from solution is crucial for \textit{in vivo} application. Fortunately, free Gd\textsuperscript{3+} will precipitate out of solution as Gd(OH)\textsubscript{3} at pH values greater than 6, allowing us to possibly accomplish both requirements simultaneously. Therefore, following studies were focused on exploration of controlled Gd\textsuperscript{3+} precipitation from solution through an increase in pH. A titration curve of 10mM GdCl\textsubscript{3} titrated with 100mM NaOH was used to study the potential to precipitate out all Gd\textsuperscript{3+} in solution with only a change in pH (Figure 4-4). The pH of a 10mM solution of GdCl\textsubscript{3} in water is 5.5. First, the pH of the solution was decreased to 3.9 with 100mM HCl, then titrated with 100mM NaOH to determine the point of precipitation. In the
titration curve, the equivalence point of GdCl$_3$ is at about pH 7, indicating the point at which Gd(OH)$_3$ begins to significantly precipitate out of solution. As the pH is increased, the amount of gadolinium detectable by the ICP-MS drops to zero (detection limit: parts per trillion). The curve indicates that with an increase to only pH 8, nearly all detectable free Gd$^{3+}$ is removed from solution.

**Figure 4-5.** Titration curve for free gadolinium titrated with 100mM NaOH. At pH 7, precipitation of Gd(OH)$_3$ from solution begins. After pH 8, nearly all free gadolinium has precipitated and been removed from solution. Detection of free gadolinium in solution was determined by ICP-MS.
As can be seen in Figure 4-4, after the precipitation begins with addition of NaOH, the buffering capacity of gadolinium drops significantly, reducing control over the pH change. Additionally, NaOH cannot act as a buffer, so use of a buffering agent capable of increasing the pH to 7-8 was necessary. HEPES has buffering capacity in the pH range of 6.8 to 8.2, and increases the pH to only 8.2 when added to a solution of GdCl₃, precipitating free Gd³⁺ from solution. A 100mM final concentration of HEPES was found ideal for precipitation, as this increased the pH to the appropriate value while precipitating out a minimal amount of Gd³⁺ from a Gd-DOTA solution (Figure 4-5). It is worth noting that, despite the high chelation efficiency (~90% of gadolinium chelated) before introduction of HEPES, increasing the pH to 8.2 and filtration of precipitated gadolinium removes approximately 40% of the original amount of gadolinium introduced to the solution. While 10% of this gadolinium is not chelated as per the molar ratios used, 30% of the gadolinium is effectively lost, either through incomplete chelation with the DOTA and subsequent precipitation or through capture in the filter membrane.
Figure 4-6. Effect of HEPES addition to Gd-DOTA chelates. Gd concentration was normalized to the starting amount of Gd in solution, 100uM. 10mM HEPES is used for precipitation of free gadolinium from solution for experiments. Note that some chelated gadolinium also precipitates, even with addition of a very small (10mM) amount of HEPES. Gadolinium was detected using ICP-MS.
**Figure 4-7.** The effect of Gd-DOTA-SPB reaction incubation time on MS2 loading. After a 24 hour and 48 hour incubation, MS2 loading with Gd-DOTA-SPB is not significantly changed. MS2 in samples was at a concentration of 1mg/mL. Gadolinium was detected using ICP-MS.

4.3.2 Analysis of MS2-loaded Gd-DOTA-SPB conjugates

All molar ratios used for reactions were compared to the concentration of DOTA present in the sample, as the DOTA concentration is the only consistent concentration throughout all experiments. After confirming that a controlled pH increase with HEPES can purify Gd-DOTA from free Gd$^{3+}$ and maintain the chelate complex, SPB-DOTA-NH$_2$ reactions were carried out. The NHS ester of SPB will be used for reaction with DOTA-NH$_2$, but there is the possibility that there could be some cross-reaction with the amine groups on the MS2 surface. Although this will not affect the amount of gadolinium loaded, it will prevent the conjugation of targeting moieties to the MS2 surface later on. To minimize the number of cross-reactions, we chose to react SPB and DOTA-NH$_2$ for 24 and 48 hours. NHS esters will hydrolyze in water, and as such have a short reaction half-life of about one hour at room temperature in pH 7.2 buffer. After 24 hours,
the number of reactive NHS in solution would be reduced by 6.0x10\(^8\) times; after 48 hours, the number would be reduced 3.6x10\(^{15}\) times. Allowing the reaction to proceed for 48 hours is more ideal, as it reduces the number of potential reactions SPB could have with MS2. As shown in Figure 4-6, the MS2 loading was not significantly affected by the time of Gd-DOTA-SPB reaction. Therefore, to decrease the possibility of SPB-MS2 cross-reaction, the 48 hour incubation time was used for all other DOTA-SPB reactions.

The effect of the ratio of DOTA:SPB on MS2 loading efficiency was also examined. Molar ratios of 1:1, 1:10, and 1:100 DOTA:SPB were used for reaction. As NHS esters are highly efficient, we expected there to be little difference in loading efficiency between the ratios. Indeed, as shown in Figure 4-7, the MS2 loading efficiency (MS2 incubated with a 10 times excess of DOTA) is not significantly affected by the DOTA:SPB reaction ratio. The 1:10 molar ratio for DOTA:SPB did have samples containing the highest gadolinium content, with values ranging up to 10.2 ppb gadolinium. As hydrolyzed SPB and reaction byproducts should not affect MS2 loading, these agents were removed from the sample during MS2 purification by column chromatography later on.
Figure 4-8. The effect of varying DOTA:SPB reaction ratios on MS2 loading. There was no significant difference between the samples, but the 1:10 ratio had the samples with the greatest amount of gadolinium loaded. MS2 in samples was at a concentration of 1mg/mL and reacted with Gd-DOTA-SPB in a ratio of 1:10 MS2:DOTA. Gadolinium was detected using ICP-MS.

MS2 was incubated with Gd-DOTA-SPB in ratios of 1:10, 1:100, and 1:500 MS2:DOTA, then exposed to UVA light (365nm) for 15 minutes to induce the photomechanism for psoralen reaction (see Figure 4-3). The UV light exposure was a cause for concern, as it has been shown that extended exposure to UV light can damage the MS2 capsid. Unloaded MS2 was exposed to UV light for 15 minutes, then fractionated by column chromatography to determine the effect of the exposure on the MS2 capsid. When compared to MS2 capsids that were not exposed to the UV light treatment, there is a small shift in the MS2 peak to the right, as the UV-exposed MS2 is eluted from the column in later fractions. This could indicate some capsid damage in the UV-exposed MS2 capsids, which would decrease our gadolinium loading yield.
Figure 4-9. Elution profiles for non-loaded MS2 capsids treated or untreated with a 15 minute exposure to UV light. The shift in the UV-exposed peak indicates that there could be some capsid damage after the 15 minute treatment. *Sample absorbance is normalized to the maximum absorbance fraction of each sample.

Initial gadolinium loading experiments were focused on determining the effects of modifying the SPB:DOTA reaction ratio and reaction time, and as such, a low concentration (1:10 DOTA:MS2) was used for MS2 loading. When the ratio of DOTA:MS2 was increased to 1:100 and 1:500, analysis of Gd-DOTA-SPB-loaded MS2 samples by ICP-MS determined that the 1:500 and 1:100 ratios of MS2:DOTA loaded much more gadolinium than the 1:10 ratio (Figure 4-9). The largest amount of gadolinium loading occurred with the 1:10 DOTA:SPB,
1:500 MS2:DOTA sample, corresponding to 1uM ± 0.3uM Gd, or about 3 gadolinium loaded per MS2. Here, it is assumed that any gadolinium detected in the sample by the ICP-MS is loaded and interacting with the RNA in the interior of the capsid. However, this assumption may be inaccurate, and the Gd-DOTA-SPB detected by ICP-MS could be interacting with the exterior of the capsid through Van Der Waals forces or electrostatic interactions. We have attempted to mitigate this by sample concentration and washing, which is rigorous and should remove the non-covalently-bonded gadolinium. Future studies should be focused on removal of the MS2 RNA and attempting to load this MS2 with Gd-DOTA-SPB, to unquestionably demonstrate that the RNA interaction alone is responsible for the loading.

![Graph](image.png)

**Figure 4-10.** The effect of MS2:DOTA incubation ratio on MS2 loading efficiency. MS2 was loaded with Gd-DOTA-SPB in ratios of 1:100 and 1:500 MS2:DOTA. The 500 times excess of Gd-DOTA-SPB loads significantly more gadolinium than the 100 times excess. MS2 in samples was at a concentration of 1mg/mL. Gadolinium was detected using ICP-MS.
4.4 Conclusions

As stated earlier, MS2 has been previously given MRI capability through conjugation of gadolinium chelates (Gd-DTPA) to its exterior.\textsuperscript{12} Here, we aimed to develop a multifunctional nanoparticle system capable of both targeting a specific region of interest and delivering imaging agents to this area. To meet this objective, the reactive groups on the exterior must be maintained for later reaction with targeting moieties, while the interior must be loaded with imaging agent. We have shown that after incubation with MS2 and subsequent purification of unloaded groups, gadolinium is detectable in the MS2-Gd-DOTA-SPB sample up to a concentration of 1μM, corresponding to approximately 3 gadolinium per MS2. In comparison to other groups, who have been able to conjugate over 500 Gd-DTPA to the MS2 exterior, the amount of gadolinium present in our sample is exceptionally low. However, it is important to recognize that our gadolinium loading strategy preserves the functionality of the MS2 exterior, allowing conjugation of targeting moieties in future.

The inefficient loading of Gd-DOTA-SPB into the MS2 could be due to two possibilities. First, the Gd-DOTA chelate is negatively charged, which, even with conjugation to the nucleic acid intercalating agent psoralen, may prevent the complex from interacting with negatively charged RNA. These repulsive forces may prohibit efficient loading of the interior of the capsid. This could be confirmed by using non-ionic chelating agents, such as Gd-DTPA-BMA (Omniscan), Gd-DO3A-butrol (Gadovist), or Gd-HP-DO3A (ProHance). These contrast agents are charge-neutral, and will therefore not be repulsed from interacting with the RNA on the interior, perhaps leading to a higher loading efficiency. Another possibility could be that the pore size of 1.4-1.8nm in the MS2 capsid is too small for the efficient migration of the large Gd-DOTA-SPB complex into the capsid interior, depending on the orientation of the complex. The
Gd-DOTA-SPB complex could require a much longer incubation time than the 16 hours provided in the above experiments.

Here, we focused on making the Gd-DOTA-NH$_2$ chelate capable for reaction with SPB and changing the reaction conditions for the DOTA:SPB reaction and its effects on MS2 loading. We have been able to minimize gadolinium precipitation when increasing the pH for SPB reaction, made a stable Gd-DOTA-SPB complex, and determined that the incubation time and molar ratio of the DOTA:SPB reaction did not significantly affect loading. We also showed that increasing the molar ratio of DOTA:MS2 for loading did affect the efficiency of loading. Future experiments could focus on increasing this molar ratio to determine if more gadolinium could be loaded, as well as changing the chelator complex to make the gadolinium chelates neutral.

In the clinical setting, the amount of gadolinium administered as a contrast agent to patients is on the order of 1mM. This value, as well as the untargeted MS2-Gd-DTPA particles fabricated by Anderson et al, is also significantly greater than the 1uM amounts of Gd-DOTA that we have been able to load into a 1mg/mL MS2 sample. However, our aim with these MS2 particles is for targeted delivery of gadolinium to the brain. Rather than employing the current clinical strategy of systemically injecting high concentrations of gadolinium necessary for the dye to reach its target tissue, targeted delivery will greatly reduce the amount of gadolinium necessary for injection by decreasing uptake and accumulation in non-targeted tissues and preventing side effects. Targeted delivery is safer for patients, less wasteful, and therefore less costly than inundating the body with high concentrations of contrast media, and may also increase contrast agent efficacy for imaging the brain, helping to better diagnose diseases in living patients.
4.5 References


CHAPTER 5

FEASIBILITY OF USING POLYMERASE CHAIN REACTION FOR MONITORING MS2 PARTICLE TRANSCYTOSIS

5.1 Introduction

5.1.1 In Vitro Blood-Brain Barrier Model Systems

The blood-brain barrier is frequently referred to as the ‘bottleneck’ for the development of new clinical agents to treat the brain. During development of new clinical molecules, many compounds and particles that are found to act on the brain during development (~100% of large molecule therapeutics and 98% of small molecule therapeutics) fail during in vivo studies, simply because they are unable to cross the BBB to act on the brain. For this reason, many in vitro models have been developed to pre-screen compounds for their permeability across the BBB. Not only do these systems allow screening of potential drug molecules, but they also provide an estimation of brain uptake and transport efficiency.¹

Various strategies have been developed to study the permeability of small molecules into the brain. Cell-free models, such as PAMPA (parallel artificial membrane permeability assay) have been introduced as high-throughput methods that do not require cell culture. PAMPA specifically uses a well-based system containing two compartments, a donor (apical, or blood side) and an acceptor (basolateral, or brain side) separated by a permeable membrane. Although PAMPA has been shown to be an accurate, reproducible, and low-cost model for BBB permeability, it does not take into account cellular transport processes, which can be targeted for improved transport efficiency.² ³ Additionally, due to its lack of cellular processes, PAMPA can
also overestimate the number of molecules that penetrate into the brain, as it does not model efflux transporters. Other BBB models incorporate cell culture into multi-well plates, which are constructed similar to the PAMPA system, but contain cells on the apical side of the membrane (Figure 5-1). Monocultures are frequently used as they only require the seeding and care of one cell type, while co-cultures and triple co-cultures are used to more accurately model the BBB in vitro.4

Figure 5-1. Multi-well systems used for in vitro BBB models. Pictured left is the monoculture setup of a multi-well system, with endothelial cells seeded on a porous membrane separating the apical and basolateral compartments. The Transwell chamber developed by Corning is shown on the right.

Many different cell types have been used to model the BBB, including primary cells and immortalized cell lines.1 Primary cells are known to form tighter junctions between them than immortalized lines, which better mimic the structure of the BBB and making them more successful model systems.4,5 Formation of tight junctions and a uniform cell monolayer in culture is critical for studying BBB permeability and transport across an in vitro BBB model. Tight
junctions prevent paracellular transport, which is key for screening compounds and studying transcytosis efficiency in vitro. Paracellular transport across a monolayer must be completely absent in order to properly and accurately test the permeability of a drug or delivery vehicle across the in vitro multi-well system. Tracer compounds can be used to monitor paracellular transport in vitro. These molecules are usually small (less than 100kDa), fluorescently labeled, and must not be a ligand for any endothelial transporters or receptors, or a substrate for endothelial enzymes. Labeled dextrans, bovine serum albumin (BSA), and sodium fluorescein are examples of these paracellular markers. In addition, the trans-endothelial electrical resistance (TEER) of a cell layer can be measured to monitor the formation of tight junctions in the monolayer. The TEER setup using an EndOhm is shown in Figure 5-2. Briefly, an electrode is placed in either compartment, the resistance is read (R_{cells}) and corrected for blank filter resistance (cell-free filter, R_{membrane}), and multiplied by the membrane area (A). The equation for this calculation is shown below:

\[(R_{cells} - R_{membrane}) \times A \quad (5.1)\]

TEER values are estimated to be over 1000 Ωcm² in mammalian systems, but values of 150-200 Ωcm² have been considered acceptable for assessing drug transport in vitro.
Figure 5-2. EndOhm TEER testing setup. Cells are seeded onto the Transwell membrane, the Transwell is then placed into the EndOhm chamber, and the two electrodes in the chamber are used to determine the resistance over the cell layer.

5.1.2 Polymerase Chain Reaction for the Detection of MS2 after Transcytosis

One major difficulty with the Transwell system we have chosen to use as the basis of our in vitro BBB model is the detection of transported molecules. Commonly, researchers often modify nanoparticle systems with fluorophores for detection in cell uptake and transcytosis studies.\textsuperscript{9-11} While this strategy is highly successful with larger nanoparticle systems, sub-50nm particles, such as MS2, can rarely be conjugated with enough fluorescent tags to make individual particles observable with fluorescence microscopy or detection via fluorometer. We explored fluorescent labeling and fluorescence detection of MS2 uptake and transport across the in vitro BBB. Even with maximum fluorophore-conjugated targeting moiety labeling, MS2 transport could not be detected using this approach.

In order to detect very low concentrations of MS2 particles transported across our in vitro BBB model, we explored a novel approach that utilizes reverse transcriptase polymerase chain
reaction (RT-PCR) amplification of the RNA that is an inherent component of every MS2 phage particle. Amplification and detection of MS2 RNA by quantitative RT-PCR (RT-qPCR) can accurately and reproducibly amplify MS2 RNA down to the nanomolar range after RNA extraction and purification. In order to avoid additional processing steps and maintain high yield in our study, we investigated freeze/thaw cycling as a means of releasing MS2 RNA from the capsid. Samples subjected to a single freeze/thaw cycle were then amplified by PCR without further RNA extraction or purification. C_\text{T} values obtained from qPCR were monitored and correlated to MS2 concentrations in solution to generate a calibration curve.

5.2 Experimental Methods

5.2.1 Materials

Rat endothelial cell medium (ECM-r), calf serum, endothelial cell growth supplement (rat), bovine plasma fibronectin, trypsin/EDTA solution (0.25%), trypsin neutralization solution (TNS), fetal bovine serum, Dulbecco’s PBS, and penicillin/streptomycin solution were purchased from ScienCell Research Laboratories (CA). Rabbit anti-Zonula occludens-1 (ZO-1) antibody, Alexa Fluor 388 goat anti-rabbit IgG (H+L), Alexa Fluor 568 phalloidin, Prolong Diamond Antifade Mountant, and 96 well glass-bottom plates were obtained from Thermo Fisher Scientific (CA). Primer sequences used for PCR probed for the MS2 capsid protein RNA sequence (the forward primer 5’-CGTTTCACAGGCTTACAAAGTACCT-3’ and the reverse primer 5’-CCAACAGTCTGGTGTGCGC-3’) were purchased from Integrated DNA Technologies (IA). All other reagents were purchased from Sigma Aldrich unless otherwise specified. Nuclease-free water and reagents were used for PCR.
5.2.2 Cell Culture

Rat brain microvascular endothelial cells (RBMECs, ScienCell) were seeded onto fibronectin-coated (2ug/cm²) surfaces and maintained in ECM-r supplemented with 5% calf serum, 1% endothelial cell growth supplement, penicillin (100 U/L), and streptomycin (100 ug/L), and cultured at 37°C under 5% CO₂. For passaging, cells were incubated with 5% trypsin-EDTA solution (in DPBS) for 10 minutes at 37°C, then rinsed with TNS and mixed with fetal bovine serum (20%) before centrifugation (1000 rpm, 10 minutes). Cells were seeded onto fibronectin-coated surfaces for further studies.

5.2.3 Immunocytochemistry

RBMECs were first investigated to verify formation of tight junctions. Cells were seeded at a density of 15,000 cells/cm² onto fibronectin-coated (2ug/cm²) glass coverslips and grown for 2, 4, 6, or 8 days and immunostained for DNA and ZO-1 expression. Samples were fixed in 4% formaldehyde in PBS for 10 min at room temperature. After fixation, samples were washed three times in PBS for 5 minutes, permeabilized for 15 minutes in 0.1.% Triton-X-100, and blocked for 1 hour in 3% BSA in PBS. Cells were incubated with primary antibody to ZO-1 (1:400) overnight, then rinsed three times in PBS for 5 minutes and incubated with secondary antibody (1:500) and DAPI (1:1000) in PBS for two hours at room temperature. Samples were washed three times in PBS for 5 minutes, then mounted onto glass microscope slides using Prolong Diamond Antifade Mountant and dried before imaging. Cells were imaged using a Nikon Eclipse 80i microscope at a magnification of 20x.

5.2.4 MS2-SMCC-AP2 Uptake

MS2-SMCC-AP2 particles were made as previously described in a 1:500 molar ratio (MS2:AP2). RBMECs were seeded at a density of 45,000 cells/cm² into 96 well glass-bottom
plates coated in fibronectin (2μg/cm²). After 8 days of growth, cells were incubated with 0.1 mg/mL of MS2-SMCC-AP2 (1:500) particles for 24 hours at 37°C. Uptake was monitored using an EVOS microscope (AMG, Washington) and a Leica SP5 confocal microscope (Leica Microscope systems, Germany) and images were acquired at a magnification of 20x using a dry objective and 1024 x 1024 image resolution in the confocal microscope. 62 stacks were acquired, then merged into a 318.2 um XZ plane image.

5.2.5 Detection of MS2 Particles Using Reverse-Transcription PCR

In order to determine if it was possible to release and detect MS2 RNA using a freeze-thaw strategy, MS2 samples at different concentrations were frozen at -20°C overnight, then thawed to room temperature. cDNA was generated from the MS2 samples using the SuperScript III First-Strand Synthesis System kit (Thermo Fisher Scientific, MA). PCR was performed using the GoTaq® Probe 2-Step RT-qPCR System (Promega, WI). Primers for the MS2 capsid protein RNA sequence were used at 400nM concentrations. For negative (no template) control reactions, nuclease-free water was substituted for MS2 samples. Cycle conditions were as follows: 95°C for 2 minutes; 35 cycles of 95°C for 30 seconds, 56°C for 30 seconds, and 72°C for 15 seconds; 72°C for 5 minutes. PCR product obtained was loaded into a DNA agarose gel (1.5% in TAE buffer, 0.4% ethidium bromide) along with a 1 Kb Plus DNA Ladder (Thermo Fisher Scientific) and run in TAE buffer for two hours at 100V. Ethidium bromide-stained bands were visualized in a ChemiDoc MP Imaging System (Bio-Rad, CA).

As a positive control, MS2 RNA was extracted from wild-type MS2 stored at 4°C using Trizol. 50μL of 5 mg/mL MS2 was mixed with 500μL Trizol. 100μL of chloroform was added to suspension, shaken vigorously for 15 seconds, then incubated at room temperature for 3 minutes. The sample was centrifuged at 10,000 RCF for 18 minutes at 4°C. The upper aqueous phase was
carefully removed and transferred to a new, RNase-free tube. An equal volume of 100% RNase-free ethanol was added to the solution, which was further purified using an RNeasy kit (QIAGEN, Germany).

5.2.6 MS2-SMCC-AP2 Transcytosis

MS2-SMCC-AP2 particles were made as previously described in 1:50 and 1:500 molar ratios (MS2:AP2). RBMECs were seeded at a density of 45,000 cells/cm² into Transwell® polycarbonate inserts (6.5mm, 0.4um pore size) coated with fibronectin (2ug/cm²). Cells were tested for TEER using an EndOhm-6 chamber with an EVOM2 epithelial voltohmmeter (World Precision Instruments, FL), and only wells with TEER values greater than 100 Ohm cm² were used for transport tests. Cells were incubated with 0.1 mg/mL MS2-SMCC-AP2 (1:50) particles or unmodified MS2 in the upper compartment of the Transwell for 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 6 hours, or 8 hours to preliminarily determine transport efficiency. To compare transport between more AP2-modified and less AP2-modified MS2 over a short-term time course, cells were incubated with 0.1 mg/mL MS2-SMCC-AP2 (1:50), MS2-SMCC-AP2 (1:500), or unmodified MS2 for 30 minutes, 1 hour, or 2 hours. After each time point, 600uL of media was removed from the bottom well of the Transwell setup and frozen at -20°C overnight. After samples thawed, they were analyzed for MS2 RNA content using real time reverse transcriptase qPCR.

5.2.7 Detection of MS2 Particles using Quantitative Reverse-Transcription RT-PCR

Real-time RT-qPCR was performed using the one-step Power SYBR® Green RNA-to-CT kit (Thermo Fisher Scientific). MS2 forward and reverse primers were used at 400nM. Cycle conditions were as follows: 30 minutes at 48°C; 10 minutes at 95°C; 40 cycles of 15 seconds at 95°C, and one minute at 60°C. A standard curve relating cycle threshold (Cₜ) values and MS2
concentration was produced using known concentrations of MS2 as determined by a Nanodrop spectrophotometer and the absorption coefficient of MS2, $\varepsilon_{260} = 8.03\ \text{mL/mg}$.\textsuperscript{14} MS2 content in transported samples was determined as a function of $C_T$ value related to the standard curve. All PCR samples were amplified in triplicate.

### 5.3 Results and Discussion

#### 5.3.1 RBMEC Tight Junction Development

Formation of tight junctions and a uniform cell monolayer in culture is critical for studying BBB permeability and transport across an *in vitro* BBB model. Paracellular transport across a monolayer must be completely absent in order to properly and accurately test the permeability of a drug or delivery vehicle across the *in vitro* multi-well system. Therefore, before testing particle transport across primary RBMECs, formation of tight junctions by the cells was monitored over time and verified by staining for the tight junction marker ZO-1 (Zonula occludens-1). Cells grown for 2, 4, 6, or 8 days, and then stained to determine the level of tight junction formation. As can be seen in Figure 5-4, tight junctions begin forming by Day 2 (5-4A). However, the junctions are not fully formed until at least Day 6 (5-4C), where the tight junction staining appears uniform over the cell layer. Additionally, TEER values were determined to be less than 100 Ohm cm$^2$ until Day 8 of cell growth. Thus, cell transport studies were not carried out until Day 8 in order to ensure that cells formed complete tight junctions.
Figure 5-3. Observation of RBMEC tight junction formation. Cells were grown on fibronectin-coated glass coverslips and stained with DAPI (blue) and immunostained for ZO-1 (green) after A) 2 days, B) 4 days, C) 6 days, or D) 8 days of growth. White arrows refer to the tight junctions developed at each time point. All images were taken with the same exposure time for 488nm excitation (300 ms). Scale bar is 100um.

5.3.2 Cellular Uptake of MS2-AP2 Particles

After confirmation of RBMEC tight junction formation, cell uptake of MS2-AP2 particles was monitored for 24 hours in a 96 well plate. Cell seeding density was increased to ensure formation of a confluent monolayer across the full surface of the glass-bottom well, and cells were incubated with 0.1 mg/mL of MS2-SMCC-AP2 (1:500) in media for various time
points. Using the EVOS fluorescent microscope, we were unable to detect uptake of MS2-AP2 particles, even after a 24-hour incubation (data not shown). Confocal z-stack images in the XZ direction also showed no uptake after 24 hours. Rather, particles remained suspended in solution above the cells and did not seem to be internalized (Figure 5-5). This could indicate that the MS2-AP2 particles were not taken up by the RBMECs seeded onto glass bottom plates, and/or that conjugation of AP2 did not influence uptake. More likely, however, is that any MS2 particles internalized by the RBMECs are likely too small to be visualized by fluorescence microscopy. Additionally, if the MS2 particles are efficiently transported through the cell, they should not accumulate at a high enough concentration inside of the cells to be visualized. The LRP1 receptor has been know to recycle back to the apical surface within 30 minutes.\textsuperscript{15} As such, this short time period would not be enough to capture the RBMECs transporting endosomes of small concentrations of MS2-AP2 particles. Lastly, cells may grow, express, and polarize differently on glass substrates as opposed to porous membranes, which are used in the transport experiments later on. Therefore, although we were not able to visualize fluorescently tagged MS2-AP2 particle uptake \textit{in vitro}, this does not prove that the particles will not be transported in a multi-well system.
Figure 5-4. Merged XZ slices of RBMECs incubated with MS2-AP2 particles. A) MS2-AP2 particles (green) remain above the cell layer (grey, striated). B) Cell monolayer and C) MS2-AP2 particles depicted separately.

5.3.3 Detection of MS2 Particles by PCR

As MS2-AP2 particles could not be visualized by fluorescence microscopy, the number of particles taken up and transported across an in vitro BBB model was expected to be relatively low and undetectable by use of a microplate reader. The sample concentration is diluted when transported from the apical to basolateral compartments and is time-dependent. Therefore, an alternative approach to fluorescence detection was explored based on amplification and detection of MS2 RNA using qRT-PCR. Additionally, to avoid the time-consuming processes of RNA extraction and purification, freeze/thaw was investigated as a method to release MS2 RNA from the capsid.

First, to ensure the freeze/thaw method would liberate RNA for successful RT-PCR, unmodified MS2 particles were frozen at a high protein concentration (5 mg/mL MS2, which corresponds to over 1000 ng/uL of RNA) overnight at -20°C. As a positive control, RNA was extracted and purified from unmodified MS2 in a separate experiment using Trizol. Synthesis of cDNA from MS2 RNA was achieved by reverse transcription, then PCR was performed on the cDNA product. Successful PCR amplification of the expected 100bp amplicon was confirmed by gel electrophoresis. Results are shown in Figure 5-6A. Both freeze/thaw and purified MS2
samples were amplified successfully using PCR (lanes 5 and 6 represent extracted MS2 RNA, and lane 7 represents MS2 RNA released by the freeze/thaw technique). Additionally, NRT controls (lanes 2, 3, and 4) had minimal amplification, making this method a viable option for detection of MS2 RNA.

Figure 5-5. Freeze/thaw of MS2 capsids is a viable method for release of RNA and PCR subsequent amplification. A) DNA gel of PCR product with lanes 2-4 representing PCR controls without reverse-transcriptase (lane 2 represents the NRT control for freeze/thaw MS2, lanes 3 and 4 represent the NRT controls for RNA extracted and purified from MS2). Lanes 5 and 6 represent purified control MS2 RNA, and Lane 7 represents freeze-thaw MS2 RNA. Lane 1 is the base pair DNA ladder. B) A standard curve was made from known concentrations of MS2 amplified and detected by qPCR. MS2 concentrations could be detected as low as 5 pM. All NRT values were greater than 34 or undetermined after 40 cycles.

Additionally, MS2 particles were diluted to known concentrations, then freeze-thawed, and amplified using RT-qPCR to make a standard curve relating $C_T$ value to MS2 concentration.
(Figure 5-6B). MS2 RNA was detectable by two step RT-qPCR down to 1 pM of MS2 capsid present in the sample.

5.3.4 MS2-SMCC-AP2 Transport Studies

After confirming that MS2 particles in solution could be detected by RT-qPCR, MS2-AP2 transcytosis across an in vitro BBB model was explored. First, MS2-SMCC-AP2 (reacted in a 1:50 molar ratio of MS2:AP2) particles were incubated with cells in the apical compartment over a long time course to preliminarily determine transport efficiency. As can be seen in Figure 5-7, MS2-AP2 particles are transported at a higher rate than MS2 alone after 4 hours of incubation, as detected by RT-qPCR. The number of MS2-AP2 particles transported continues to increase to the last time point at 8 hours. There is some baseline MS2 transport across the cell monolayer, indicating that the tight junctions may be somewhat leaky, allowing paracellular transport.
Figure 5-6. Transport of MS2-SMCC-AP2 (1:50, reacted molar ratio of MS2:AP2) over an in vitro BBB model. Preliminary transport results indicate MS2-AP2 transport occurs at a higher rate than MS2 after 4 hours of incubation. Transcytosed MS2 particle RNA was released by freeze/thaw and amplified and detected by one-step RT-qPCR. C₅₀ values for NRT controls and media controls were greater than 32 or undetermined after 40 cycles. Mean ± standard deviation of PCR samples run in triplicate, n=1 independent experiments.

While transport of MS2-AP2 (1:50) particles does occur at early time points, the difference between MS2 and MS2-AP2 (1:50) transport is not significant here. This was somewhat unexpected, as the LRP1 receptor is known to recycle back to the apical side of the cell after only 30 minutes, so the MS2-AP2 particles should have transported more efficiently at early time points. Therefore, a second transport study was carried out over a shorter time period
varying the ligand density on the MS2 particles to determine if a greater number of AP2 conjugated would increase the early MS2 transport efficiency. RBMECs were incubated with MS2-SMCC-AP2 that had been reacted in a 1:50 or a 1:500 molar ratio of MS2:AP2 (MS2-SMCC-AP2 (1:50) or (1:500), respectively) over a time course of 2 hours. As seen in Figure 5-8, MS2-AP2 (1:500) does transport more efficiently at earlier time points than unmodified MS2 or MS2-AP2 (1:50). The difference in transport efficiency could be attributed to the greater number of ligands per MS2 on the 1:500 particles compared to the 1:50 particles. This indicates that the number of AP2 conjugated to MS2 does have an effect on the uptake transport kinetics of RBMECs in vitro. Lastly, some MS2 transport occurs at each time point, but the number of MS2-AP2 (1:500) transported is significantly greater than the MS2 transported after 2 hours, and after only 1 hour, there is a trend in significance. This further indicates that conjugation of AP2 does increase the transport efficiency of the MS2 particles across an in vitro BBB system, and that the number of ligands conjugated per MS2 has some influence over the transport time.
Transport results indicate MS2-AP2 (1:500) transport occurs at a higher rate than MS2 or MS2-AP2 (1:50) at early time points. Transcytosed MS2 particle RNA was released by freeze/thaw and amplified and detected by one-step RT-qPCR. C_T values for NRT controls and media controls were greater than 32 or undetermined after 40 cycles. Mean ± standard deviation of PCR samples run in triplicate, n=3 independent experiments. 2-way ANOVA was used to analyze significance. ***p<0.001

5.4 Conclusions

Use of an in vitro BBB model for assessing the permeability and transport of targeted drug delivery vehicles is critical for pre-screening systems and estimating their baseline performance in vivo. After development of our brain-targeted MS2-AP2 particles in Chapter 2, it
was necessary to investigate their ability to transport across an *in vitro* BBB system to determine the effects of AP2 conjugation on transport efficiency. However, there were many difficulties in detecting the MS2-AP2 particles due to low concentrations accumulated in cells, the high efficiency of MS2 transport through the cell, and the limit of detection of fluorescence microscopy and microplate readers. To overcome these obstacles, we developed an RT-qPCR protocol for amplification of whole phage RNA, and used the process of freeze-thaw to rupture capsids and reliably release MS2 RNA. This method for the detection of transported particles is a novel technique that our nanoparticle system is uniquely suited for due to its underlying biology.

When testing MS2-AP2 particle transport, it was determined that conjugation of AP2 to the MS2 surface does increase transport over an *in vitro* BBB model compared to MS2 alone over an 8 hour time period. This was to be expected, as AP2 has previously been conjugated to other nanoparticle systems that have also shown increased transport efficiency when conjugated to this targeting moiety.\(^9\)\(^-\)\(^11\) Unlike other groups, however, we have also preliminarily studied the effect of the number of AP2 conjugated per particle on transport. Results show that, at early transport times, there is a significant difference in transport efficiency and kinetics between MS2 particles reacted at a 1:50 molar ratio of MS2 to AP2 compared to a 1:500 molar ratio of MS2 to AP2. MS2-SMCC-AP2 (1:500) were capable of much earlier transport than the MS2-SMCC-AP2 (1:50) particles, indicating that increasing the number of AP2 conjugated is capable of making particle transport more rapid and efficient. While these results are only preliminary, they suggest that the MS2-AP2 particles we have developed are capable of transport across an *in vitro* BBB model, and will therefore adequately perform when introduced *in vivo*. 

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5.5 References


**CHAPTER 6**

**SUMMARY AND FUTURE DIRECTIONS**

6.1 Summary

Delivery of imaging agents and pharmaceutical payloads to the CNS is essential for efficient diagnosis and treatment of brain diseases. However, therapeutic delivery is often restricted by the BBB, which prevents transport of clinical compounds to their region of interest. Therefore, an innovative approach to facilitate transport of these molecules across the BBB and into the brain is a crucial area of research. A multifunctional nanoparticle system is needed that 1) can be loaded with functional molecules, 2) transport across the BBB, and 3) localize to specific regions in the brain. Development of a nanoparticle platform that meets all of these needs while also being biocompatible, biodegradable, and easily amenable to mass production, has been a largely unmet challenge in the biomedical industry. To this end, we have developed a nanoparticle delivery system based on the MS2 bacteriophage capsid that has been successfully modified for transport and delivery of MRI contrast agents across the BBB and into the brain.

6.1.1 Surface Modification of MS2 with Angiopep-2

Targeting and transporting across the BBB is the first step in delivering clinical agents to the brain. Previously, the synthetic peptide AP2 has been conjugated to polymeric nanoparticle platforms and used as a targeting moiety for BBB transport. Although these systems have been successfully modified and characterized, none have studied the ability to control the number of AP2 conjugated, nor the effect of the number...
of ligands conjugated on transport. Additionally, none have investigated the effect of spacer length on conjugation efficiency.

We have modified the MS2 bacteriophage capsid with the linkers SMCC or SMPEG for conjugation of thiolated AP2 for BBB targeting. We have been able to conjugate up to 77 AP2 per MS2-SMCC, with up to two AP2 conjugated per capsid protein, when reacting with a 1:500 ratio of MS2 to AP2. When reacting with a 1:50 ratio of MS2 to AP2, only 37 AP2 per MS2 are conjugated, indicating the number of AP2 conjugated can be controlled using molar ratios. SMCC introduced some crosslinking between MS2 capsid proteins, reducing the observed reaction efficiency as determined by maleimide assay. PEGylation of the crosslinker caused a lower AP2 reaction efficiency (20 and 41 AP2 per MS2 conjugated, when reacted at 1:50 or 1:500 ratios of MS2:AP2), but also reduced crosslinking between MS2 capsid proteins.

6.1.2 Dual Surface Modification of MS2 Particles

Dual modification of nanoparticle surfaces with different targeting moieties is key for the success of brain delivery platforms. While many studies have shown successful modification of nanoparticle surfaces with brain targeting molecules, few groups have endeavored to conjugate two different targeting moieties to the exterior of a brain-targeted nanoparticle system. Although MS2 particles have been ‘dually functionalized’ on the exterior and interior, dual functionalization of the exterior with two different targeting moieties has not previously been attempted.

MS2 bacteriophage capsids were modified with two targeting ligands, an antibody and a BBB targeting peptide, as a proof of concept for BBB transport and specific region of interest targeting in the brain. Results showed that MS2 and IgG were successfully
conjugated after an overnight reaction, but with low conjugation efficiency (~0.6%). DLS results indicated that conjugation of IgG increases the size of MS2 by nearly 5 nm. We successfully dually modified the exterior MS2 surface with both IgG and AP2, after reaction order and molar reaction ratios were carefully considered. Lastly, MS2-IgG particles were shown capable of probing for the NMDA receptor 2D subunit from samples of brain lysate. We can therefore conclude that the antigen recognition ability of the anti-NMDAR2D IgG conjugated to MS2 is not lost by chemical modification, or by the steric hindrance caused by the possible structural arrangement of the IgG laying plane with the MS2 surface.

6.1.3 Nucleotide-Mediated Gadolinium Loading of MS2 Bacteriophage

In this thesis, we aimed to develop a multifunctional nanoparticle system capable of both targeting a specific region of interest and delivering imaging agents to this area. To meet this objective, the reactive groups on the exterior must be maintained for reaction with targeting moieties, while the interior must be loaded with imaging agent. We have shown that after incubation with MS2 and subsequent purification of unloaded groups, gadolinium is detectable in the MS2-Gd-DOTA-SPB sample up to a concentration of 1uM, corresponding to approximately 3 gadolinium per MS2. In comparison to other groups, who have been able to conjugate over 500 Gd-DTPA to the MS2 exterior, the amount of gadolinium present in our sample is exceptionally low. However, it is important to recognize that our gadolinium loading strategy preserves the functionality of the MS2 exterior, allowing conjugation of targeting moieties in future.

The majority of the work presented here was focused on making the Gd-DOTA-NH₂ chelate compatible for reaction with SPB, optimizing the reaction conditions of the
DOTA:SPB reaction, and determining the effects on MS2 loading. We have been able to minimize gadolinium precipitation when increasing the pH for SPB reaction, made a stable Gd-DOTA-SPB complex, and determined that the incubation time and molar ratio of the DOTA:SPB reaction did not significantly affect loading. Results also showed that increasing the molar ratio of DOTA:MS2 for loading also increased the loading efficiency.

6.1.4 Transport of MS2-AP2 Particles Across an In Vitro BBB Model

Use of an in vitro BBB model for assessing the permeability and transport of targeted drug delivery vehicles is critical for pre-screening systems and estimating their baseline performance in vivo. After development of our brain-targeted MS2-AP2 particles, it was necessary to investigate their ability to transport across an in vitro BBB system to determine the effects of AP2 conjugation on transport efficiency. However, there were many difficulties in detecting the MS2-AP2 particles due to low concentrations accumulated in cells, the high efficiency of MS2 transport through the cell, and the limit of detection of fluorescence microscopy and microplate readers. To overcome these obstacles, we developed an RT-qPCR protocol for amplification of whole phage RNA, and used the process of freeze-thaw to rupture capsids and reliably release MS2 RNA. This method for the detection of transported particles is a novel technique that our nanoparticle system is uniquely suited for due to its underlying biology.

When testing MS2-AP2 particle transport, it was determined that conjugation of AP2 to the MS2 surface does increase transport over an in vitro BBB model compared to MS2 alone over an 8 hour time period. We have also preliminarily studied the effect of the number of AP2 conjugated per particle on transport. Results show that, at early
transport times, there is a significant difference in transport between MS2 particles reacted at a 1:50 molar ratio of MS2 to AP2 compared to a 1:500 molar ratio of MS2 to AP2. MS2-SMCC-AP2 (1:500) were capable of much earlier transport than the MS2-SMCC-AP2 (1:50) particles, indicating that increasing the number of AP2 conjugated is capable of making particle transport more rapid and efficient. While these results are only preliminary, they suggest that the MS2-AP2 particles we have developed are capable of transport across an in vitro BBB model, and should adequately perform when introduced in vivo.

6.1.5 Conclusion

This thesis presents several studies of viral capsid modification, both on the exterior and interior, to impart BBB-targeting functionality to MS2 capsids. Although the focus of this work was to target hyperactive regions of the brain using a viral capsid, the methods used to surface functionalize the capsid could easily be applied to other nanoparticle systems and targets. The loading of SPB-modified molecules into MS2 capsids via nucleotide-mediated interactions could also be further applied to the loading of other molecules. Additionally, studies involving the process of freeze/thaw to rupture capsids for RNA liberation and development of an RT-qPCR protocol for amplification of whole phage RNA are further applicable to other viral systems for experimental detection beyond BBB transport, including detection of phage in samples from the living environment. Therefore, while the focus of this dissertation was to develop a nanoparticle platform toward a specific purpose, we have also developed a foundational toolkit for use with any system amenable to the functionalization chemistries and PCR protocols
presented herein, ultimately offering longevity and further application of the techniques developed in these studies.

6.2 Recommended Future Directions

The work outlined in this thesis demonstrates the utility of MS2 as a functional delivery vehicle for BBB transport and delivery of MRI contrast agents. To transition to in vivo studies, additional work is needed to improve reaction efficiency and yield, as well as more in vitro work to confirm functionality and transport. Future work is outlined in Table 6-1 below.

Table 6-1. Potential future experiments.

<table>
<thead>
<tr>
<th>Goal</th>
<th>Experiment</th>
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<tbody>
<tr>
<td>Confirm in vitro targeting of anti-NMDAR2D antibody using MS2-IgG-AP2.</td>
<td>Seed target cells (HEK293’s genetically modified to express NMDAR2D on the surface) in co-culture with RBMECs seeded onto a Transwell membrane. Study MS2-IgG-AP2 transport through the RBMECs to the target cells.</td>
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<tr>
<td>Determine the effect of Gd-chelator complex charge on MS2 loading.</td>
<td>Attempt MS2 loading with neutrally charged contrast agent, such as Gd-DTPA-BMA or Gd-DO3A-butrol.</td>
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<tr>
<td>Confirm Gd-DOTA-SPB loading is truly nucleotide-mediated.</td>
<td>Remove RNA from MS2 capsids and attempt Gd-DOTA-SPB loading.</td>
</tr>
<tr>
<td>Study the effect of linker size on transport across an in vitro BBB model.</td>
<td>Incubate RBMECS seeded on Transwells with MS2-SMPEG-AP2 or MS2-SMCC-AP2. Monitor transport of MS2 particles over time.</td>
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