Engineering M13 Bacteriophage Platforms for Cancer Therapy Applications

by

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S.B., Engineering Sciences
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Submitted to the Department of Mechanical Engineering in Partial Fulfillment of the Requirements for the Degree of

Master of Science

at the

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June 2015

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Abstract

Two novel schemes for engineering M13 bacteriophage for application in the diagnosis, imaging and treatment of human tumors are proposed. Firstly, by exploiting the uniquely malleable biology of the M13 filamentous phage, we have engineered filamentous phages of shorter lengths by constructing our own set of small viral ssDNA that are packaged by M13 capsid proteins. These ‘inho’ phages can be sized to ~50nm and above in length. The small phage retains the M13 major and minor coat proteins which have previously been manipulated to serve as tethers to carry various therapy and imaging agents and target specific cancer sites. Now with the ability to control the aspect ratio of these rigid, rod-like phages we can further improve on M13 based cancer detection by optimizing for phage blood circulation and tumor extravasation. Secondly, we have added to our cancer targeting M13 platform collection by cloning for chlorotoxin display on the tail p3 capsid protein of M13. Chlorotoxin can induce passage across blood-brain barrier, targets for cancer cells, and specifically internalizes to glioma cells. Expression of chlorotoxin on M13 will allow us to capitalize on its strong affinity for tumors of neuroectodermal origin and expand the M13 therapy and imaging platform to tumor masses in the brain.

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ACKNOWLEDGMENTS

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INTRODUCTION

i. Problem Statement

Surgical resectioning remains the primary mode of cancer therapy today. However, general surgical debulking is highly dependent on the surgeon’s ability to successfully separate cancer tissue from its surrounding healthy tissue. The surgeon’s precision, especially in highly sensitive organs such as the brain, can directly affect patient survival rates [1]. Consequently, recent research has closely focused on improvements in monitoring and guidance systems used before and during surgeries. Alternate non-invasive treatment routes including direct therapeutic delivery or molecular level attacks at the tumor site have also been considered. Hence, current clinical procedures could highly benefit from the introduction of cancer markers and targeting carriers that can clearly define the boundaries of tumor masses.

ii. Thesis Overview

The object of this Master’s thesis is to expand on the functionality of M13 bacteriophage as an engineerable biomaterial for medical imaging and therapy applications particularly in the case of tumor diagnosis. In two parts, we first describe the assembly of M13 of smaller sizes, termed ‘inho’ phages, and also propose the display of chlorotoxin peptide on the tail capsid of M13. These phage manipulations can be applied to better characterize tumor extravasation of filamentous phage based probes and also optimizes M13 as a tool for targeting brain tumors.

iii. Phage Biology

Filamentous bacteriophages such as M13 are a family of single-stranded DNA (ssDNA) containing viruses that infect only gram-negative bacteria. M13 falls under the Ff class of viruses which infect host cells via the F conjugative pilus on the surface of male E.Coli. Ff viruses are parasitic rather than lytic in nature with the infected cells continuing to divide and grow, though at a much reduced rate. Phage proteins make up 1-5% of total protein synthesis in the cells and this metabolic load can reduce growth by up to 50%. However, the phage
reproduction cycle is very rapid where the first secretion of phage progeny appears within 15-20 minutes of infection, and M13 produces up to 2000 viral progeny per cell over its cell doubling time [2].

**Figure 1. M13 phage genome:** Location of each viral gene is shown with the direction of transcription indicated by arrows. The origin of replication hairpin is found in the intergenic region (between gII and gIV). The packaging signal is located between the (−) strand replication origin and gIV.

Upon infection, the phage ssDNA strand (+) is translocated to the cytoplasm where host enzymes synthesize the complementary strand (−), forming the double stranded parental or replicative DNA (rfDNA). The rfDNA is replicated to about 100 copies per bacterial cell and acts as the template for synthesis of ssDNA genomes for encapsulation [2]. The circular super-coiled
ssDNA occupies the axis of the M13 phage for almost its entire length. The size and base-pair distribution of this enclosed genome dictates the length of the phage. The wildtype M13 bacteriophages have a long, flexible rod-like structure with a diameter of about 5 to 6 nm and a length of 880 nm.

Figure 2. Structure of the M13 bacteriophage

The M13 phage genome encodes a total of 11 proteins—five of which are the main phage structural proteins. The five structural proteins—p3, p6, p7, p8, p9—are the body of the M13 phage and are inserted into the inner host cell membrane prior to assembly of the phage. p1, p11, and p4 are the morphological proteins that locate on the inner and outer membrane of the bacteria and coordinate to form a 7nm exit pore structure through which the assembling viral particle is extruded from the cell. Replication proteins—p2 and p10—facilitate the phage DNA synthesis while DNA binding protein, p5, localizes the intracellular ssDNA [3].

Table 1. Phage protein properties *(Table adjusted from Barbas III et al.)*

<table>
<thead>
<tr>
<th>Phage Protein</th>
<th>Function</th>
<th># of Amino Acids</th>
<th>Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>p2</td>
<td>DNA replication</td>
<td>410</td>
<td>46,137</td>
</tr>
<tr>
<td>p10</td>
<td>DNA replication</td>
<td>111</td>
<td>12,672</td>
</tr>
<tr>
<td>p5</td>
<td>binding of ssDNA</td>
<td>87</td>
<td>9,682</td>
</tr>
<tr>
<td>p8</td>
<td>major coat capsid</td>
<td>50</td>
<td>5,235</td>
</tr>
<tr>
<td>p3</td>
<td>minor tail capsid</td>
<td>406</td>
<td>42,522</td>
</tr>
<tr>
<td>p6</td>
<td>minor tail capsid</td>
<td>112</td>
<td>12,342</td>
</tr>
<tr>
<td>p7</td>
<td>minor head capsid</td>
<td>33</td>
<td>3,599</td>
</tr>
<tr>
<td>p9</td>
<td>minor head capsid</td>
<td>32</td>
<td>3,650</td>
</tr>
<tr>
<td>p1</td>
<td>assembly</td>
<td>348</td>
<td>39,502</td>
</tr>
<tr>
<td>p4</td>
<td>assembly</td>
<td>405</td>
<td>43,502</td>
</tr>
<tr>
<td>p11</td>
<td>assembly</td>
<td>108</td>
<td>12,424</td>
</tr>
</tbody>
</table>
The distal end of the M13 phage is the part of the phage that is assembled first and has approximately 5 copies of the two coat proteins p7 and p9. The p9 protein interacts with the negatively charged hairpin loop known as packaging signal sequence (PS) of the phage ssDNA and plays a major role in the initiation of assembly [4,5,6,7,8,9]. The PS tags viral DNA for encapsulation. Along the length of the phage, approximately 2700 copies of the p8 protein are required to fully coat the M13 virion ssDNA during elongation. As a result, the concentration of p8 proteins in the inner cell membrane is extremely high. The PVIII is α-helical in structure and wraps around the virion axis in a right-handed helical sense. The proximal end of the phage consists of approximately five copies of p3 and p6 each. The p3 protein is the largest and most complex component of the M13 structure and is extruded last during the assembly process. The amino terminus of the p3 was the first location used for the display of proteins and peptides on M13. The p3 protein is required for both termination of phage secretion and is also necessary for host binding during infection. Non-infectious, multi-length polyphage is produced in the absence of PIII capping protein [2].

The M13 bacteriophage exhibits nonlytic infection, simultaneous single and double stranded viral DNA with little size constraints on DNA length, and very high titer capacity. All of these characteristics have made the M13 a very popular tool for biomolecular applications.
CHAPTER 1: Reducing the length of M13 bacteriophage for improved *in vivo* trafficking to tumor

1.1 Project Goal

*Construction of smaller phage to improve on the blood trafficking of our M13 probe systems while retaining its multi-functionality which allows us to simultaneously target, detect, and deliver various agents to cancer masses.*

1.2 Background

The Biomolecular Materials Lab (Belcher Group) at the MIT Koch Institute has previously demonstrated that targeted M13 bacteriophage conjugated with fluorescent materials can perform *in vivo* molecular imaging of tumors. M13 filamentous bacteriophage is composed of a circular single stranded DNA (ssDNA) encapsulated by the major coat protein p8 and minor cap proteins p3, p6, p7, p9 (Figure 4) [10]. These proteins can be engineered to display or attach various targeting ligands and nanoparticles or drug molecules—effectively creating a phage shuttle that carry imaging or therapy agents to specifically targeted cancer cells [11].

However, this method can be limited by inefficient extravasation of the probe into vasculature due to the length of phage (880nm). To improve tumor penetration of phage, we are engineering a system to control and shorten the length of phage while maintaining its multi-functional capsid. Prior studies indicate the geometry and size can play a significant role in the transport, bio-distribution, and internalization of nanoparticles [12, 13]. Upon injection, nanoparticles must demonstrate the ability to evade immune macrophage during blood circulation, high marginalization or ability to escape the blood flow and reach the blood vessel walls, ability to extravase to the tumor intersitium, and finally capability to either bind or internalize to cancer cells [14]. Though spherical particles have been the norm in nanomedicine research, recent works indicate that non-spherical nanoparticles (i.e. rods, chains, ellipsoids) are most effective in these areas [15]. Not only are chain or rod-like structures more likely to avoid internalization by macrophages, their shape subjects them to certain torque and tumbling motion that increases contact with the vessel walls. Furthermore, oblong shaped particles are more likely to form multivalent occurrences essential for targeting, and in the case of the
filamentous bacteriophage, avidity of binding can be highly enhanced by the display of materials on all 2700 copies of the body p8 protein. On the other hand, size considerations must be made to accommodate for the high interstitial flow pressure typical of tumor masses. Due to high leakiness and reduced lymphatic drainage in the area, extravasation to tumor tissues is highly enhanced with smaller particles in the ranges below 100nm [14]. Hence, the proper aspect ratio of particles is vital to the design of biomedical nanoparticles [16]. By understanding how aspect ratio affects these tumor distribution properties, we can determine ideal length of phage for the different in vivo cancer applications we have demonstrated such as diagnostic imaging or drug delivery/therapy. To this end, a system to shorten the M13 bacteriophage platform is explored in this chapter.

**Figure 4. Previous M13 bacteriophage designs:** i). Tumor targeting at p3 end, fluorescent imaging agent at p9, and doxorubicin/drug release sites at p8. ii). Tumor targeting at p3 end and single walled carbon nanotube (SWNT—a deep tissue, near infrared imaging agent) binding at p8.

### 1.3 Innovation Process

The length of the circular ssDNA plasmid packaged by M13 correlates with the size of the virion. Deleting unnecessary regions of the M13 phage genome does not significantly reduce the length as most of the ssDNA is essential for assembly. Preceding work by Specthrie
et al. created a plasmid that generates a minimal packaging genome and produced a mixed population of full length phage and smaller phage that are 50nm in length (though only 1-3% by mass of total population) [17, 18, 19]. We are extending on Specthrie et al. in two ways. The first is to construct a set of plasmids (termed “inho”) that generate package-able genomes with sizes listed in Table 2, which are much smaller than the 6407 nucleotides observed in wildtype M13. The second is to generate populations of a shorter size by removing the helper phage using a plasmid that contains all phage proteins but is unable to package its DNA. Only in the presence of both of these plasmids is phage production observed (Figure 8), where inho plasmids are packaged by the proteins translated from the unpackaged large RM13-f1 plasmid.

1.3.1 Packaged Genome

The M13 single stranded genome coding all protein genes are replicated and extruded from the body of the bacteria and wrapped in coating protein due to the respective presence of the f1 replication origin and packaging signal on the plasmid [2]. Here, we have generated a set of plasmid inserts of varying lengths which contain the packaging signal and the f1 origin and termination of replication but none of the phage protein genes. In effect, construct with our inserts produces ssDNA of a given length that signal to be packaged.

The plasmid map below gives a general outline of the inho constructs that produce our packaged genomes. In addition to ampicillin and tetracycline resistance and a ColE1 plasmid origin of replication, the construct encodes an insert comprising of the f1 replication origin and terminal and a packaging signal.

<table>
<thead>
<tr>
<th>Inho Construct</th>
<th>Genome Size (nucleotides)</th>
</tr>
</thead>
<tbody>
<tr>
<td>inho285</td>
<td>285</td>
</tr>
<tr>
<td>inho311</td>
<td>311</td>
</tr>
<tr>
<td>inho344</td>
<td>344</td>
</tr>
<tr>
<td>inho378</td>
<td>378</td>
</tr>
<tr>
<td>inho475</td>
<td>475</td>
</tr>
<tr>
<td>inho1310</td>
<td>1310</td>
</tr>
<tr>
<td>inho1960</td>
<td>1960</td>
</tr>
</tbody>
</table>
The sequence for inho285 further illustrates the key regions of code that facilitate the production of small package-able ssDNA from our inserts. Note that the final strand replicated for packaging includes the full DNA packaging signal but not the full f1 origin or termination regions. The size of the ssDNA is manipulated by the addition of base pairs via standard Gibson cloning between either the f1 origin and the packaging signal or packaging signal and the f1 termination region.
**Figure 6. ssDNA sequencing region of inho285 construct**

<table>
<thead>
<tr>
<th>F-1 Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’  TGGGCACTGCCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGAGTCCACGTTC</td>
</tr>
<tr>
<td>3’  ACCCGATTGCGGACTATCTGGCCAAAAGCGGAACTGCAACCTCAGGTCGAAG</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>PACKAGING SIGNAL</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>5’  TGGGCACTGCCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGAGTCCACGTTC</td>
</tr>
<tr>
<td>3’  ACCCGATTGCGGACTATCTGGCCAAAAGCGGAACTGCAACCTCAGGTCGAAG</td>
</tr>
</tbody>
</table>

**PACKAGED GENOME**

| 5’  TGGGCACTGCCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGAGTCCACGTTC |
| 3’  ACCCGATTGCGGACTATCTGGCCAAAAGCGGAACTGCAACCTCAGGTCGAAG |

**IHNO285**

| 5’  TGGGCACTGCCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGAGTCCACGTTC |
| 3’  ACCCGATTGCGGACTATCTGGCCAAAAGCGGAACTGCAACCTCAGGTCGAAG |

**15**
1.3.2 Protein Genome

In the absence of M13 protein coding in our packaged genomes, we constructed a plasmid which expresses all essential phage assembly components but itself lacks the packaging signal and f1 replication origin. Transformation of E.coli with the protein genome will lead to kanamycin resistance and to the production of the M13 assembly and coating proteins but no extrusion of phage will take place without properly labeled ssDNA available to package.

Figure 7. Protein plasmid map

1.3.3 Phage-inho Assembly

Co-tranformation of an inho and the protein construct into a competent bacterial strain (in our case xl-1 or DH5α) gives us the production of M13 assembly proteins and inho ssDNA copies that are ready to be packaged. Overnight amplification of a co-tranformed colony in
Amp/Kan LB media provide sufficient number of phage for analysis. Using these two plasmids in concert, we can then proceed to purification of the extruded phage-inhos for characterization.

**Figure 8. Schematic of phage-inho production:**
Inho plasmids when co-transformed with another plasmid (RM13-f1) coding for phage proteins produce short phage that packages the inho genomes, which control the length of the phage.

![Schematic](image)

The inho-phage samples (post PEG precipitation and SDS and heat lysing) were also run on Nu-PAGE gels for anti-p3 western blotting, Coomassie and Ponceau staining to check for the presence of phage capsid proteins. Inho-phage samples reacted with AlexaFluor488 was analyzed for labeled p8 proteins. (Phage coat proteins can be labeled with AlexaFluor fluorescent probes through NHS-ester reaction with the primary amines on phage proteins by 1hr incubation with dye in PBS buffer and dialysis to remove excess dye). P3 at 406 amino acids and 42,522 MW runs at between 60 to 50 kDa on Nu-PAGE 4-12% gel, while P8 coat protein 50 amino acids and 5,235 MW appear between 10 and 3.5 kDa (See Table 1). Inho phage major coat protein p8 and capping protein p3 are both present in our PEG precipitated samples signifying that inho construct phage have been successfully extruded from the bacterial cells.
Figure 9. Stained gels for capsid protein p3 and wrapping protein p8:
PEG precipitated (2.5% or 10%) and SDS and heat lysed phage were analyzed on a NuPAGE Novex 4-12% Bis-Tris Gel with 1% MES running buffer.

a) Gel proteins are transferred for anti-p3 western blotting for wildtype (M13KE) and inho475 phage
b) Gel imaged with Typhoon imager for Alexa488 emission from fluorophore labeled p8
c) Gel directly stained with Coomassie show p3 capsid protein

The phage were also purified by PEG precipitation, digested with DNase for 1 hr—to remove all external DNA found in the suspension solution, and finally lysed by SDS and heat before analysis on a TBE-PAGE gel which was stained with SYBR-Gold. We observed different genome sizes for each of the inho constructs and we see the correct relative changes in genome size (Figure 10: The gel ladder for these genome runs do not necessarily reflect size as the electrophoresis is affected by the circular and single stranded nature of the phage DNA). Sequencing of the packaged ssDNAs further confirms that our inho phage batches all contain the designed inho genome.
Figure 10. Gel analysis of packaged genomes: TBE-PAGE gel of the inho constructs, stained with SYBR-Gold. Inho phages were purified by PEG precipitation, incubated in DNase, and finally lysed with SDS and heat for the gel run.

1.3.4 Phage-inho Visualization

The physical size of the inho-phage must be visually confirmed. Here, we have chosen atomic force microscopy as our main mode of imaging. Due to bacterial debris pelleted along with phage during PEG precipitation, additional methods of phage extraction from the bacterial growth medium were used in order to acquire clean images. Purification becomes especially important when dealing with small phage which can be hidden by contaminating particles and salts. Best results were obtained through Ni-NTA affinity column purification or dialysis in deionized water over 72 hrs at 12-14kD molecular weight cutoff. For Ni-NTA columns, inho-phage was produced by using a RM13-f1 plasmid clone coding for three histidines per p8 wrapping protein (DDAH). Expression of his6 tag on the tail p3 protein proved to be insufficient for column binding. His3 should be displayed on all 2700 p8 copies which allows for improved avidity binding to the nickel column. DDAH p8 phage was kindly supplied by John Casey, PhD:

\[5'GATGATGCGCACGTGCACTGGGAGGATCCCGCAAAAGCGGCCTTTGACTCCCTGCAAGCCTCAGCGACC\n\]
\[CGAACATATATCGTTATGCGGCGATTGTTGGTGATCATTGTCGGCGCAACTATCGGTATCAAGCTGTT]
TAAGAAATTCACCTCGAAAGCAAGC 3'). Protein plasmid p8 was replaced by DDAH p8 via standard Gibson cloning and inho phage was amplified using DDAH protein plasmid.

For AFM samples, a total of 1e11 phage are diluted to 100ul with millipore deionized water and deposited on mica for 1hr before drying with argon gas. Initial examination (Veeco Model MMAFMLN) found that each of our inho construct products show a distribution of sizes in spite of the uniform profile of the packaged genome lysed from the products. This points to the production of polyphage.

Figure 11. Inho475 phage cleaned by dialysis or affinity binding

a) AFM capture of inho475 polyphages (72 hrs dialysis)

b) AFM capture of inho475 phage (NiNTA elution)

c) AFM Capture of NiNTA beads & inho475 phage
1.3.5 Controlling Polyphage

We find that with our system, phage length is highly dependent on the number of inho genomes packaged within one virion, which is governed by the ratio of available body wrapping-p8 to capping-p3 proteins during production. The termination and capping process in the extrusion of ssDNA to form competed phage at the bacterial wall membrane is a less than efficient process [2]. Oftentimes, there may exist phages of double or greater lengths—polyphage—that form as a result of packaging more than one ssDNA. With the inho phage, we have very short ssDNA’s while the number of p8 body and p3 capping protein produced by the RM13-f1 plasmid remains regulated at levels meant to package wildtype phage. The frequency of capping protein encounter during the assembly process could be too low when we force the system to package short ssDNA. The speed of wrapping and the speed of capping are not optimized for small phage. Consequently, with increasingly smaller ssDNA, we observe greater polyphage events. The larger inho constructs (i.e. inho1960) prove to be more uniform in size.

The distribution and hence yield of the phage-inho can be improved by regulating the available coating and capping proteins. With high p8 to p3 count, we have less p3 available to cap and end assembly of phage resulting in more polyphages (phage containing multiple genomes). Alternatively, with low p8 to p3 ratio, we create more inho phage that package only a single genome. The level of p3 and p8 can be adjusted by tweaking the ribosomal binding site and promoter regions of the protein genes in order to either down or upregulate the expression of the protein through the translation or transcription of the RM13-f1 protein plasmid. We chose to test new protein plasmids with modified ribosomal binding (RBS) sites for the p3 and p8 genes. Different RBS have varying binding efficiencies which has a significant influence on the overall translational efficiency.

Table 3 outlines the 8 variations tested. XL-1 bacteria transformed with only p3 modified RM13-f1 plasmids was grown to O.D. 0.75, resuspended in SDS and heated to test for p3 production. Western blot for p3 show increased p3 concentration for StartMUT, B0032, and B0034&StartMUT. While very little p3 protein was detected for B0032 samples, the strongest p3 band was exhibited by the B0034&StartMUT modified RM13-f1.
Table 3. P8 & P3 binding site modifications

<table>
<thead>
<tr>
<th>P3 Modification</th>
<th>P8 Modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>![Image of DNA sequences]</td>
<td>![Image of DNA sequences]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SITE</th>
<th>Sequence</th>
<th>SITE</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>StartMUT</td>
<td>GTG→ATG</td>
<td>RBS1</td>
<td>CTGATGGAGGCA</td>
</tr>
<tr>
<td>B0030</td>
<td>ATTAAGAGGAAA</td>
<td>RBS2</td>
<td>CTGATGGAGGCA</td>
</tr>
<tr>
<td>B0032</td>
<td>TCACACAGGAAAG</td>
<td>RBS3</td>
<td>TTGATGGAGGCA</td>
</tr>
<tr>
<td>B0034 &amp; StartMUT</td>
<td>AAGAGGGAGAAA</td>
<td>RBS4</td>
<td>CTTATGGAGGCC</td>
</tr>
</tbody>
</table>

Inho-phage produced using the eight protein plasmids were also analyzed for phage count. TBE-PAGE gels for packaged genome showed the greatest concentration for inho phage made with B0034&StartMUT for p3 modified RM13-f1 and RBS3 for p8 modified RM13-f1 (little or no phage genome is detected from RBS1, RBS2, and RBS4). Anti-p3 western blot of lysed inho phage also indicate that B0034&StartMUT and RBS3 produce the greatest number of capped phage. UV-Vis measurements of inho-phage samples provide us with an estimation of the concentration of phage particles we can achieve with our modified protein plasmids. Regular RM13-f1 give us readings of 1e12 pfu/mL.

High genome and p3 and high pfu/mL readings for B0034&StartMUT and RBS3 indicate that the two modifications successfully increased inho-phage yields. However, imaging of the various RM13-f1 reveal that RBS3 samples are heavily populated by polyphage while B0034&StartMUT help to mitigate polyphage events. While binding site modifications on p3 works to increase p3 production to boost inho-phage extrusion and capping, alterations to the p8 binding site were meant to reduce p8 productions. RBS1, RBS2, and RBS4 achieved this reduction in the system but ultimately resulted in extremely low phage production. RBS3 on the other hand appears to increase p8 production and hence the frequency of polyphage assembly.
Phage concentration (Figure 12) is given by absorbance peak at 269nm and correction at 320 nm by below relation [3]:

\[
Phage\ Concentration = \frac{(A_{269} - A_{320})(6 \times 10^{17})}{7200} \text{ pfu/mL}
\]

Figure 12. Phage concentrations produced with modified RM13-f1: (n=5, stdev error)

Another approach to copy regulation of the available proteins and the package-able ssDNA is through the plasmid origin of replication. Given the high copy number ColE1 origin on our inho construct and the low copy number p15a origin of our RM-f1 protein plasmid, we can choose to increase protein production through incorporating high copy pUC origin of replication in our RM-f1 plasmid. On the other hand, we may also reduce our small inho ssDNA numbers by replacing the high ColE1 origin with another of low copy numbers. This would help reduce the concentration of ssDNA accessible for multiple packaging events, but also lowers the total number of inho-phages that can be produced per bacterial cell.

The number of capping proteins could also be drastically increased by including extra copies of the p3 gene in our plasmid system. Figure 13 demonstrates inho construct with a repeat p3 gene outside of the ssDNA coding region. We could likewise reconstruct the RM13-f1 plasmid to contain extra protein genes. However, western blotting and phage count for inho-phage made with the extra p3 coding region did not reflect increased inho production as well as our B0034&StartMUT clone.
Figure 13. Protein and inho plasmids with extra g3 on inho construct

We must note as well that the amplification time during the growth of the infected bacteria and production of phage has an impact on the number of polyphage. Prolonged growth time contributes to the frequency of polyphage packaging which implies that strains on the host bacterial systems are reflected in the extrusion process of the phage. Amplification of phage is optimal below 15 hrs of bacterial growth where we reach high phage yields but do not begin to produce polyphage as a result of overcrowding in the growth media.

Overall, we find that inho1960, inho475, and inho285 exhibit base phage sizes of ~280nm, ~100nm, and ~50nm respectively as given in Figures 14-16, AFM's captured with phage generated with protein plasmid modification B0034&StartMUT. The images divulge the increasing difficulty of managing polyphage for the smaller genomes or the shorter inho-phages as formerly discussed.
Figure 14. AFM images of inho1960:
   a) Inho1960 sample cleaned with Ni-NTA column
   b) Single and double genome packaging
   c) Inho1960 phage produced with B0034 and StartMUT modifications
   d) Basic size of the inho1960 phage
Figure 15. AFM images of inho475:

a) Inho475 polyphage
b & d) Inho475 produced with B0034 and StartMUT
c) Inho475 basic size
Figure 16. AFM images of inho285:

a) Inho285 phage and polyphage produced with B0034 and StartMIT.
1.3.6 Inho-Phage Separation by Size

Considering the difficulty of manipulating the phage protein system in order to perfectly match ssDNA size to the correct ratio of wrapping proteins during assembly, techniques for separating polyphage from inho-phage samples were devised. Current lab methods for separating small proteins by size encompass a variety of technologies including chromatography, gel separation, gradient centrifugation, and precipitation [20, 21,22,23,24]. Adapting these systems to phage is made difficult by the geometry and size of the filamentous virions—the high aspect ratio and substantial molecular weight of even the inho phage.

**PEG Precipitation**

Extraction of phage from the media is most commonly done through PEG8000 precipitation of phage [22]. Competing out bacteriophage from solution via the saturation of the medium through NaCl and PEG particles effectively crashes out wildtype phages. Specthrie *et al.* demonstrate that this crash out can be controlled by modulating for the percent PEG precipitation of phage samples. At low percent PEG ~2.5%, full size phages are more likely to precipitate while at high percent PEG ~15%, all phage sizes aggregate out of solution [17]. However, using gradient precipitation with our system proved to be an unreliable in separating out inho and polyphage. Nevertheless, high percentage PEG is imperative for the high yield for phage from our growth media. Most inho samples are precipitated at 10% to increase yields for analysis.

**FPLC**

In size exclusion chromatography, separation is based on differences in the size and shape of the molecules of interest. Column beads with limiting pore volumes exclude molecules above the cutoff molecular weight from entering and separate these particles from the bulk flow. Molecules that completely or partially enter the bead pores are fractioned according to their molecular weight such that the largest analytes elute first and the smallest last. Knowing the molecular weight of wildtype phage to be in the 1.5e7 Da range, Sepharose CL-2B beads with exclusion bracket 7e4 to 4e7 are ideal for
gel chromatography of our inho-phage batches. Using CL-2B beads (from Sigma) packed in Superdex200 column, we were able to perform fast protein liquid chromatography. UV profiles of our fractions highlight 3 distinct peaks at 15’, 35’, and 45’ (Figure 17). Western blot for capsid protein and TBE-PAGE gels for packaged genome signal the presence of phage in our first peak at 15’. Samples digested by DNase and/or dialyzed in buffer demonstrate reduced peaks at 35’ and 45’ revealing these later fractions to be mostly bacterial debris. FPLC fractions must be imaged to determine if the phage peak at 15’ is indeed small phage or if the fractions represent a mixture of polyphage and small phage. It is likely that large polyphages are drained in the void volume during elution and the peak represents single genome packaging phage.
Figure 17. FPLC profile of inho475 phage on Sepharose CL-2B column:
500 ul of concentrated inho samples run through CL2B300 Sigma in deionized water

a) UV reads protein peak at 15 min, 35 min, & 45 min for inho-phage sample PEG precipitated and digested with DNase

b) UV reads protein peak at 15 min, 35 min, & 45 min for inho-phage sample dialyzed for 72 hrs

c) UV reads protein peak at 15 min, 35 min, & 45 min for inho-phage sample run directly after PEG precipitation
**Gel Electrophoresis**

Gel electrophoresis separates molecules based on size and charge and is a technology well defined for most DNA/RNA and small protein applications. Though acrylamide gels have been used previously to separate M13 phage in this fashion, the method has been largely abandoned in relation to f1 virions [24]. Reminiscent of the DNA structure, the phage molecule is filamentous and negatively charged and should also be easily separated through a gel by applying an electric field. Here we have used low melting agarose gels to examine our inho-phage samples. 0.3 to 1% NuSieve GTC Agarose gel is cast in TAE buffer. Inho-phage suspended in PBS is incubated at room temperature for 1hr with AlexaFluor488 to label phage and proteins via NHS-ester amine reaction. The labeled samples are run in the agarose gel at 100V for 60minutes.

Agarose electrophoresis of inho-phage gives us 5 bands. Gel extraction and subsequent lysing and analysis reveal the presence of phage in the highest two bands. The agarose gel bands of the phage are rather disperse and reflect the difficulty of having particles of such high aspect-ratio move uniformly together through the gel pores. The bi-modal phage distribution also underlines mixed size population of our samples. It is possible that polyphage runs lower on the gel as its ‘floppy’ length can fold and squeeze through the gel pores rather like a globular mass. On the other hand, small phage

![Figure 18. Inho475 phage run on 1% NuSieve agarose gel (UV image)](image)
with its low aspect-ratio could behave more rigidly and be hindered in its passage through the gel.

1.3.7 Consolidating Co-Transformation Inho Plasmids

To further simplify the inho-phage assembly system, the protein and genome plasmids can be combined. The new construct including both the packaging insert and the M13 assembly insert gives us inho phages with a polyphage distribution just as demonstrated by the two plasmid system. While a single plasmid introduces co-dependence to the ssDNA and protein production process, the single plasmid system will aid in the efficiency of transformation and abridges the setup of our system.

Figure 19. Map of combined protein and inho plasmid
CHAPTER 2: Expressing chlorotoxin peptide on M13 tail proteins for passage across the blood-brain barrier

2.1 Project Goal

Construction of phage expressing chlorotoxin to allow for the passage of our M13 probe systems across the blood-brain barrier and targeting to human glioma cells.

2.2 Background

Chlorotoxin (CTX) is a 36 amino-acid (3995.8 Da) peptide derived from the venom of the *Leiurus quinquestriatus* scorpion. Its structure consists of an α-helix linked by three disulfide bridges to a small, three-stranded, antiparallel beta-sheet and a fourth disulfide bridge that links the N-terminal cysteine to the rest of the molecule (Figure 20). Recent studies show that the CTX peptide selectively binds to and invades malignant gliomas and tumors of neuroectodermal origin such as medulloblastomas, neuroblastomas, melanomas, PNETS, and small cell lung carcinoma [1]. While the molecular mechanism that cause chlorotoxin’s specificity for tumor cells is yet to be fully characterized, chlorotoxin has been evaluated in human clinical trials as a targeted radiotherapy and imaging agent [25]. These trials prove chlorotoxin to be safe for patient administration and define chlorotoxin as an important new treatment and imaging tool. Fluorophore conjugated chlorotoxin, termed ‘tumor paint’, has been effectively used to image various tumor types [26].

Native CTX as well as fluorophore conjugated tumor paint display the ability to cross the blood-brain barrier (BBB). The BBB acts as the interface between the blood and the brain and regulated the traffic of nutrients and metabolites to the brain. The BBB vastly limits the delivery of imaging and therapeutic agents to the brain. The permeability of the BBB to nanoparticles is affected by a number of factors including size, charge, and surface chemistry of the particles [27, 28]. To allow for selective passage across the BBB, nanoparticles are often complexed with ligands to facilitate receptor-mediated transport. As CTX has been shown to permeate intact BBB in both animals and humans with brain tumor, it is an ideal trafficking peptide for nanoprobes designed for brain tumor diagnosis.
CTX also has a distinct cellular localization and uptake pattern in human glioma versus normal cells. While CTX disperses in the cytoplasm in normal human dermal fibroblasts, it localizes near the Golgi in human glioma, lung carcinoma, and vascular endothelial cells [29].

On the other hand, M13 bacteriophage has been postulated to be able to cross the BBB as well. While long in length, the structure of M13 is extremely narrow in diameter and easily falls under the hydrodynamic size limit of particle that freely pass the BBB [1, 25]. Engineering the M13 platform to enable easy passage across the BBB would be particularly advantageous in concert with deep tissue imaging technologies. The multifunctional M13 shuttle could play a significant role in the imaging detection, drug treatment, and surgical removal of brain tumors which require extremely precise handling for patient safety.

**Figure 20. Chlorotoxin structure:**

α-helix linked by three disulfide bridges to an antiparallel beta-sheet and a fourth disulfide links the N-terminal cysteine to the rest of the molecule in blue.

*(Adapted from http://chemistry.umeche.maine.edu, 2013)*
2.3 Innovation Process

Phage display is not only useful as a laboratory technique for the study of protein and DNA interactions, but has also enabled us to engineer phage as a useful transporter of imaging and therapeutic agents. Here we develop a phage clone that encodes for chlorotoxin on the p3 capping protein of the M13 phage. The display of CTX on phage p3 tail protein will allow us to extend our bacteriophage based imaging and therapy platform to tumors in the brain.

2.3.1 Chlorotoxin-P3 Clone

The 36 amino acid sequence of the chlorotoxin peptide is incorporated into the gene for p3 as illustrated below. Cloning work here follows NEB Phage Display guidelines at the p3 site. Both Gibson assembly and enzyme digestion–ligation cloning methods were used in order to better guarantee positive insertion of the chlorotoxin sequence.

Figure 21. Map of M13KE modified with chlorotoxin
The CTX 108 nucleotides were extended with base pairs on both sides from the enzyme digestion region of p3 to 147 bps and purchased on standard pUC57 plasmid from GenScript. The cloning vector M13KE was purchased from NEB. The viral vector and the chlorotoxin vector was digested with Eag I and Acc65 I enzymes. The viral vector was dephosphorylated, and both digestions were agarose-gel purified. Purified vector and insert was ligated using T4 DNA ligase at 16°C overnight and electro-transformed to competent XL-1 blue cells. Transformed cells were incubated for 1 hr and plated on TET/XGAL plates and incubated at 37°C overnight. Blue plaques were amplified and DNA sequenced to confirm the insertion of oligonucleotides to express CTX on p3. (In Gibson, 20-40 nucleotide primers were used to PCR sticky ended CTX-insert and open up the RM13-f1 vector at the p3 gene. Vector pieces and insert were incubated with Gibson Assembly MasterMix (NEB) per NEB protocol).

Imaging of M13-CTX bacteriophage produces regular, infectious 880nm phage and no polyphage are produced using the chlorotoxin phage plasmid suggesting that the p3 capping protein remains intact and
functional. Western blot for p3 protein also show that CTX-p3 run slightly higher due to the added weight of the chlorotoxin peptide.

2.3.2 Chlorotoxin P3 & Gold Nanoparticles

The chlorotoxin peptide is highly positive in charge, an attribute which is part of the reason for its ability to cross the BBB and target to tumor areas where the extracellular matrix is particularly negative in charge [1]. When suspended with negatively charged citrate stabilized Au nanoparticles, CTX-phage electrostatically interacts with the nanoparticles at its CTX-p3 end.

Figure 24. Chlorotoxin-phage with negatively charged gold interaction at p3:

a) M13-CTX with

b) M13KE phage with gold binding peptide at p3

c) M13KE phage with negatively charged gold
Previous peptide sequence for gold affinity on p3 and wildtype in gold solutions are shown for comparison in Figure 24.

2.3.3 Work towards Chlorotoxin-Phage Affinity & Internalization to Glioma

Due to the somewhat complex structure of the chlorotoxin peptide, particularly its many disulfide bridges, it is important to verify that the chlorotoxin displayed on the p3 of M13 properly folded during assembly and remains functionally sound. To this end, various cell assays were designed to better characterize the p3-CTX.

Wiranowska et al. describes the cellular uptake and localization of chlorotoxin to the perinuclear Golgi region in glioma cells and hypothesizes that we may be observing clathrin-mediated endocytosis [29]. To assess the viability of our CTX-phage, we tested for similar endocytosis activity with CTX-phage in collaboration with the Floyd Lab at the MIT Koch Institute.

**Figure 25. 6hour 1e11 CTX-phage incubation with 1e5 glioma cells:**
DAPI in blue for nucleus, GFP in green for Golgin-97 (used to mark for the golgi apparatus), AlexaFluor 647 in red for phage.
CTX-phage and control phage were labeled with AlexaFluor 647 and incubated with glioma cells at high and low titer for 15min, 30min, 2hrs, 4hrs, 6hrs, & 24hrs. Unfortunately, fluorescent imaging for localization of phage proved to be difficult due to noise from non-specific binding to the cell membrane. Figure 25 is representative of most images acquired for both CTX and wildtype phage.

In light of the general ‘stickiness’ of phage, we have reconfigured our experimental setup for more stringent washing steps, lower titers, and different imaging techniques. While internalization of phage via the same route as native chlorotoxin may not be easily discernable, the inhibition of cell surface gelatinase activity by chlorotoxin as observed by Deshane et al. could be a setup that better reflects the binding of phage to the surface receptors (i.e. matrix metalloproteinase-2) targeted by chlorotoxin [30]. We hope to see definitive results from both types of assays as we move forward with the chlorotoxin-phage.
CONCLUSION

i. Summary & Impact

Research discussed in the thesis has demonstrated a system for modifying M13 bacteriophage lengths and the potential for the usage of CTX-phage in the study and treatment of brain tumors. We have explored the various methods of manipulating the inho-phages to influencing the aspect-ratio of the phage and also of controlling the extrusion of polyphages. We look at purification and separation of inho-phage populations by size via Ni-NTA columns, PEG precipitation, dialysis, FPLC, and gel electrophoresis. Alterations to the capsid p3 and coat protein p8 production were also considered for size control. Inho-phages of base sizes of ~50nm, ~100nm, and ~280nm were illustrated. On another note, we synthesized chlorotoxin-phage which promises to be of particular effectiveness in extending the multi-functional M13 platform to brain tumors for diagnostic and therapy purposes.

M13 bacteriophage is a versatile material for nanomedical engineering as its multi-functional structure is genetically encoded and directly tunable. Past work with M13 at the Belcher Lab has established the efficacy of phage display in designing M13 for various biomedical and material applications. The inho-phage introduced here preserves its utility as an engineerable platform and will also allow us to explore the benefits of the new smaller geometries in trafficking to tumor sites [31]. Ultimately, our inphage platform carrying our imaging/therapy particles could target deeply embedded tumors and allow for early diagnosis and treatment of hard-to-reach tumors. The expression of the CTX peptide will also be of significance as we begin to explore deep tissue imaging of cancer. The brain is an organ which is especially difficult to reach past the BBB and image in depth. Recent development of the second-window near infrared fluorescent imaging for real time ovarian surgery monitoring at the Belcher Lab has paved the way for imaging biotissues at depth of up to 10cm. Combining the NIR imager with CTX-phage could be the critical next step to brain tumor treatment research.

Outside of cancer applications, we have additionally deliberated on the utility of the inho-phage in the production of aerogels and various scaffolds and biomarkers. The
modifiability of M13 bacteriophage size makes it a flexible new fibrous material that could be handled in a manner reminiscent of DNA origami and opens up the possibility of M13 phage based microstructures [32,33,34].

ii. Future Work

The initial assembly and cloning work described here must be carried forward to mice tumor models and further characterized for therapy applications. Using our inho system, we aim to move to cancer mouse models focusing on the trafficking of small phage. With our array of phage lengths, we will explore the effects of the phage aspect-ratio on tumor penetration and the vascular circulation and biodistribution of the phage. We additionally expect to functionalize the inho phages with our newly developed rare earth elements doped NaYF₄ nanoparticles to further build on the deep tissue imaging system established by the Belcher Group. We are excited to begin planning particle loading and circulation experiments for our smaller phages and to be considering various difficult-to-reach cancer models.

Moreover, we have begun working with the Floyd Lab to evaluate the function of the chlorotoxin-phage. We will continue our in vitro experiments against human glioma cell lines and expect to screen for internalization of CTX-phage and inhibition of cell surface gelatinase activity. On the other hand, the Floyd Lab has determined a setup for in vivo imaging of the crossing of nanoparticles across the blood brain barrier of mice. We are optimistic to soon be able to visualize our phage and evaluate its passage across the BBB.
i. Dynamic light scattering data for inho phage samples

DLS scattering curves for smaller phages inho285 and inho475 exhibit greater evidence of bimodal distribution inho phage and polyphage with hydrodynamic diameter peaks below 100nm and around ~300nm. Inho1960 is more uniform in size distribution with peak just above 100nm. For reference, general wildtype phage at 880nm reads between 100nm and 300nm, and peaks near 10000nm indicate large bacterial debris.

Figure 26. Inho285 phage DLS curves
Figure 27. Inho475 phage DLS curves

Size Distribution by Intensity

Size Distribution by Volume

Record 7: inho+Ph4 1  Record 8: inho+Ph4 2  Record 9: inho+Ph4 3
Figure 28. Inho1960 phage DLS curves
ii. Inho phage modified with Au binding peptide

The inho phage model remains functional for various phage display designs previously invented by the Belcher Group (as earlier illustrated by the expression of DDAH for nickel cleaning). We demonstrate below inho1960 clone with gold binding peptide which in 20nm gold solution aggregate to give the gold-phage network observed via AFM.

Figure 29. Inho1960 phage assembled with modified 9-f1 protein plasmid with Au-Binding Peptide
REFERENCES


