Cell and Nanomaterial-Based Approaches for Diagnosis and Chemotherapy of Metastatic Cancer Cells

By

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Submitted to the Department of Biological Engineering on May 21, 2010 in Partial Fulfillment of the Requirement for the Degree of Master of Engineering in Biological Engineering

Abstract

Metastasis is a multistep process during which tumor cells separate from a primary tumor, penetrate the bloodstream, evade host defenses, and colonize distant organs. This final and fatal step in tumor development is the cause of more than 90% of cancer related deaths. Therapies and diagnostics can be targeted to metastasis at three points in its progression: the primary tumor, the secondary tumor, and circulating tumor cells (CTCs). While much work has focused on primary tumors, less effort has concentrated on targeted isolation, detection and therapy of deeply penetrated metastases and CTCs. Here, I discuss cell and nanomaterial-based approaches for detecting and ablating these malignant populations. The number of CTCs in the blood directly correlates with disease progression; however, the lack of definitive markers has limited their isolation and characterization. I have demonstrated the potential use of platelets as a cell-based marker for isolation and detection of CTCs. Using phage display technology, it was possible to identify candidate peptides specific to mesenchymal-like tumor cells that may mimic the motile and aggressive CTC population. In order to detect and ablate metastases and CTCs, M13 bacteriophage was engineered into a platform for simultaneous tumor targeting, imaging, and therapy. Single-walled carbon nanotubes (SWNTs) and doxorubicin, a chemotherapeutic agent, were loaded on phage for fluorescent near-infrared imaging and cytotoxicity of metastatic lesions, respectively. The near-infrared optical properties of SWNTs in the "second window" make them promising candidates for imaging nascent and deeply seeded tumors. This approach provides an 'all-in-one' platform for targeted fluorescence imaging and efficient drug delivery and may allow for real-time monitoring of tumor response to drug regimens.

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Chapter 1: Isolation of Circulating Tumor Cells (CTCs) with Non-Epithelial Markers

1.1 Background

1.1.1 Circulating Tumor Cells and Metastasis

Metastasis is a multistep process in which tumor cells separate from a primary tumor, penetrate the bloodstream and lymph nodes, evade host defenses, exit circulation and colonize distant organs\(^1\). This final and fatal step in tumor development is responsible for more than 90% of cancer related deaths; as a result, detecting and characterizing migrating and invasive tumor cells, the precursors of metastasis, are key obstacles in the battle against cancer\(^2\). Circulating tumor cells (CTCs), cells that have detached from a primary tumor and circulate in the bloodstream, may be the seeds of metastasis – CTC levels in peripheral blood are a predictor of overall survival in patients with metastatic disease. Unfortunately, little is known about the molecular characteristics of these cells. Two hurdles stand in the way of CTC characterization. First, since these cells are extremely rare (1 in \(10^9\) cells in blood), it is difficult to enrich CTCs from a large background of blood cells. Second, a lack of non-epithelial markers for CTCs may prohibit the isolation of the cells most primed for metastasis.

The formation of metastasis is an inefficient process. Nearly \(10^6\) tumor cells per gram of tumor tissue can be introduced into the bloodstream each day\(^3\). Epithelial cells from primary tumor tissue are unequipped to survive in circulation, as they have a rigid phenotype and are dependant on anoikis. As such, within 5 minutes of entering circulation, over 85% of these cells disappear\(^4\). The circulating tumor cells (CTCs) that persist may be resistant to anoikis and primed to overcome the mechanical and immunological stresses of the blood such as the shearing effects of hemodynamic forces and the cytotoxic activity of natural killer cells\(^1\). Enumeration of CTCs in the peripheral blood of cancer patients has diagnostic potential: 5 or more CTCs per 7.5ml of blood in patients before first-line
chemotherapy is a better indicator of overall survival than conventional imaging procedures\textsuperscript{5}. Beyond enumeration, molecular characterization of CTCs may provide a non-invasive method to diagnose, treat, and monitor malignancy using blood samples. CTC based diagnostics have been limited by the ability to isolate and enrich rare CTC populations from the blood. Further, a lack of proven enrichment technologies limit genomic and proteomic analysis of CTCs, as leukocyte contamination distorts molecular analysis of rare cells. As such, the most pressing problem in CTC research is the purification and enrichment of malignant CTCs.

1.1.2 Circulating Tumor Cells and the Epithelial to Mesenchymal Transition

Previous attempts to purify CTCs have employed flow cytometry\textsuperscript{6}, fibre optic array scanning\textsuperscript{7}, immunomagnetic beads\textsuperscript{8}, microfluidic separation\textsuperscript{9}, and high throughput optical imaging\textsuperscript{10, 11}. Most of these technologies have relied on an epithelial marker, epithelial-cell-adhesion-molecule (EpCAM), to distinguish tumor cells from leukocytes and erythrocytes in the blood. EpCAM is frequently overexpressed in a number of epithelial carcinomas in the lung, breast, prostate, head and neck, and liver\textsuperscript{9}. However, while EpCAM has been useful in establishing proof-of-concept of the diagnostic potential of CTCs, it may not be a comprehensive CTC marker.

While epithelial cells in a solid tumor may robustly express EpCAM, disseminated tumor cells in the bloodstream are believed to undergo an epithelial to mesenchymal transition (EMT) that induces migratory and invasive properties, upregulates stem cell properties, inhibits apoptosis and senescence, contributes to immunosuppression, and downregulates epithelial characteristics\textsuperscript{12}. The mesenchymal state is associated with the capacity to metastasize and differentiate as well as evade chemotherapy\textsuperscript{11}. Previous work isolating CTCs yields cells with an epithelial phenotype reminiscent of the primary tumor\textsuperscript{13}. Gene and protein expression studies have also relied on such epithelial markers to isolate CTCs\textsuperscript{14, 15}. However, this approach may fail to screen for highly malignant disseminated cells with mesenchymal characteristics. Cells that have undergone an EMT and are poised to seed a secondary tumor may not express EpCAM and would be invisible to
current CTC detection technologies. Thus, a more comprehensive marker for post EMT CTCs must be used to isolate, enrich, and subsequently characterize cells.

1.2 Cell-based Methods for Circulating Tumor Cell Isolation

1.2.1 Background: Circulating Tumor Cell Interactions with Platelets

In addition to undergoing EMT to survive in the blood, evidence suggests that CTCs evade the immune response and extravasate from the vasculature by interacting with cells in the blood\(^1\) (Figure 2a). The most compelling evidence is the inhibition of metastasis by depletion of platelets and the restoration of metastatic potential after platelet repletion in a mouse model\(^16\), \(^17\). Platelet/fibrinogen clots surrounding the tumor cells may shield them from natural killer cell surveillance. In mice lacking functional natural killer cells, fibrinogen deficiency was not a determinant of metastatic potential\(^8\). Platelets also promote vascular extravasation of CTCs by releasing vascular endothelial growth factor (VEGF) and promoting vascular hyperpermeability\(^1\). These platelet interactions are up regulated in the tumor microenvironment. Thrombin, a potent platelet activator, is up regulated at hypoxic sites such as the tumor. Further, tumor cells activate platelets by secreting cysteine proteases and ADP\(^19\). This interplay between platelets and tumor cells is vital for successful metastasis and may provide insight useful in developing new therapeutic strategies to fight metastasis. Recent work has shown that platelets can induce an EMT in tumor cells via secretion of TGF-β (Personal Communication, Myriam Labelle – Hynes Lab, MIT). This work may show that EMT may not be restricted to the primary tumor, and that epithelial-like cells that enter the blood may undergo an EMT after interacting with platelets.

This comprehensive body of work points to a vital role for platelets in metastasis. By co-opting platelets, tumor cells are protected from cytotoxic forces and effectively escorted to a secondary site. While little is known about the molecular characteristics of CTCs, platelets are well studied. As such, they may represent a powerful marker in CTC isolation. Here I outline an approach in enumerating CTCs using platelets as a marker. Using flow cytometry, I isolated complexes with the surface expression of a platelet but with the size and
complexion of a tumor cell. These platelet tumor cell complexes may be the seeds of metastasis.

1.2.2 Formation and Characterization of Platelet/Tumor Cell Complexes \textit{in vitro}

1.2.2.1 Proof of Concept Studies

In order to investigate platelet/tumor cell complexing, I used flow cytometry to identify cells that were the size of tumor cells (10-20 microns) but had the surface expression of platelets. Two platelet markers were used to identify platelets: CD41 and P-selectin. CD41 (integrin $\alpha_{IIb}$) associates with CD61 (integrin $\beta_{IIb}$) to form the gpllb/IIIa (CD41/CD61) complex. This complex is expressed on platelets, megakaryocytes and early hematopoietic progenitors, and binds to fibrinogen, fibronectin, vitronectin, von Willebrand factor, and thrombospondin. It is necessary for platelet adhesion and aggregation\textsuperscript{20}. Selectins (CD62) are a family of cell-surface proteins that bind carbohydrates and mediate cellular interactions with leukocytes. L-selectin is expressed on the majority of B and T lymphocytes and interacts with carbohydrates on endothelial cells. P-selectin (CD62P) is expressed on activated platelets and endothelial cells and binds to sialyl-lewis-x (sLe\textsuperscript{x}) on neutrophils and monocytes. Interestingly, aberrant expression of carbohydrates such as sLe\textsuperscript{x} is associated with tumor formation and metastasis\textsuperscript{21}.

Platelet activation is the culmination of the clotting cascade and can be induced by a number of molecules and proteins including thrombin and ADP. Acting through cell surface receptors, ADP activates platelets resulting in shape change, aggregation, and release of granule contents. ADP also causes a number of intracellular events including inhibition of adenylyl cyclase, mobilization of calcium from intracellular stores, and rapid calcium influx in platelets\textsuperscript{22, 23}. ADP mediated platelet activation triggers a positive feedback loop, as activated platelets secrete ADP in order to form a dense clot.
<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Size (microns)</th>
<th>Abundance (Per ml blood)</th>
<th>Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Circulating tumor cell (CTC)</td>
<td>10-30</td>
<td>Unknown ~1-100</td>
<td>Unknown EpCAM</td>
</tr>
<tr>
<td>Erythrocyte</td>
<td>6-8</td>
<td>4.5-6 E6</td>
<td></td>
</tr>
<tr>
<td>Platelet</td>
<td>2-4</td>
<td>150-450 E3</td>
<td>CD41, CD62P</td>
</tr>
<tr>
<td>Granulocyte</td>
<td>12-15</td>
<td>3-7 E3</td>
<td>GR-1 (Granulocyte differentiation antigen 1)</td>
</tr>
<tr>
<td>Monocyte</td>
<td>16-20</td>
<td>.22-.55 E3</td>
<td>F4/80, CD11.b</td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>8-10</td>
<td>1.45-3.3 E3</td>
<td>CD45</td>
</tr>
</tbody>
</table>

Table 1: Composition of blood by cell type. CTCs make up a miniscule and poorly described portion of patient blood. A number of markers for specific blood cells were used to identify CTCs using flow cytometry.

Platelet surface expression was confirmed by flow cytometry (Figure 1). Washed platelets were prepared from mouse whole blood (detailed in Materials + Methods). While unstained platelets showed no surface expression (Figure 1a,c), platelets stained positive for CD41 (Figure 1b) and CD62P (Figure 1d). As expected, platelets activated with ADP showed an up-regulation of P-selectin (Figure 1e).

In order to establish proof of concept of platelets as a marker for CTCs, experiments were carried out on well-characterized mouse tumor cells lines. The 393M1 (liver to lung adenocarcinoma) and Tearly (early stage 393M1) cell lines were used for these experiments because they represent different stages in tumor formation. While 393M1 cells are known to be metastatic, Tearly cells are isolated from an early stage primary liver adenocarcinoma and may not form metastasis (Personal Communication, Monte Winslow-Jacks Lab, MIT). Ideally, these cell lines allow for correlation of malignancy with platelet binding. The hypothesis was that those cells better primed for metastasis (393M1) would bind platelets more efficiently than earlier stage tumor cells or non-malignant cells (Tearly).
Figure 1: Confirmation of platelet staining and activation. (A,C) Unstained platelets are compared to platelets stained with (B) CD41 or (D) P-selectin. Addition of ADP activates platelets and up-regulates P-selectin expression (E). EpCAM staining represents a negative control, as this epithelial marker is not expressed on platelets.

In order to recapitulate tumor cell platelet binding in vitro, 393M1 and Tearly cell lines were trypsinized and mixed with platelet rich plasma (PRP) for an hour at 37 °C (detailed in Materials and Methods). Platelet-tumor cell complexes were then stained with platelet markers (CD-41, P-selectin), tumor cell markers (Ep-CAM) and 4', 6-diamidino-2-phenylindole (DAPI), a fluorescent dye that has a strong affinity for DNA. An outline of markers used for different cell types is shown in Table 1. Platelet-tumor cell complexes were identified by flow cytometry (Figure 2). In the absence of PRP, tumor cells did not stain for platelet markers CD41 or CD62P. When PRP was mixed with tumor cells, binding occurred and cells stained positive for both CD41 and CD62P (Figure 2c,d). Interestingly, cells stained positive for platelet marker CD62P, which is exclusively expressed on activated platelets. This result may suggest that tumor cells locally activate platelets by secreting ADP or thrombin.
Figure 2: Complexing of platelets and tumor cells. (A) Fluorescence microscopy image of platelets bound to a tumor cell. (B) Unstained 393 tumor cells were mixed with platelet rich plasma and stained for (C) CD41 or (D) CD62P. The percentage of cells staining for platelet markers is given in the upper right corner of the plots. Cells were gated on forward scatter, DAPI negative, and GFP positive. The addition of ADP did not increase platelet-tumor cell complexing (E,F), which may show tumor cell mediated platelet activation.

To test this hypothesis, platelets were activated by ADP and incubated with tumor cells (Figure 2e,f). No increase in platelet-tumor cell binding occurred after the addition of ADP, which suggests that tumor cells are activating platelets and triggering P-selectin expression. Further, experiments carried out on the Tearly cell line yielded similar results: Tearly cells complexed with and appeared to locally
activate platelets. While this data is in vitro, it may indicate that platelet binding is a universal process rather than one restricted to highly metastatic cell lines. Future work should be carried out to evaluate the mechanism of tumor-mediated platelet activation. ELISA or western blot analysis could probe for the amount of thrombin or ADP in a particular cell type. Further, inhibitors of these molecules could be used to suppress platelet binding. This work is particularly relevant in the context of recent work carried out by Myriam Labelle of the Hynes lab at MIT. Labelle has shown that platelet binding triggers an epithelial to mesenchymal transition in tumor cells and primes them for metastasis; platelets control EMT by secreting TGF-β (Personal Communication – Myriam Labelle, Hynes Lab, MIT).

While these initial experiments demonstrate tumor cell-platelet complexing, the extent of platelet-tumor cell binding was inconsistent. A potential reason for this inconsistency was the anti-coagulant used in whole blood isolation from mice. Initial experiments used blood drawn into sodium citrate tubes. Sodium citrate acts by chelating calcium ions necessary for selectin binding of carbohydrate moieties24. Calcium is thus needed for both homotypic interactions between platelets and for platelet/tumor cell binding. To circumvent this issue, hirudin, an anticoagulant that acts by inhibiting thrombin rather than chelating calcium, was used25. Platelet binding experiments were repeated using hirudin (Figure 3). Interestingly, platelet binding to tumor cells decreased as compared to experiments done in sodium citrate (Figure 3c,d and 3b,c). This may be because thrombin is required for tumor cell mediated platelet activation. Residual hirudin would therefore block platelet activation and prohibit binding. Alternatively, platelet-tumor cell binding may be a selectin-independent process and binding could be integrin mediated and calcium independent.
Calcium chelation may alter the dynamics of platelet-tumor cell complex formation. Hirudin, an anticoagulant that inhibits thrombin, was used to preserve physiological calcium levels. (A) Unstained 393 cells were compared to cells mixed with platelet rich plasma and stained for (B) CD41 or (C) P-selectin. The use of hirudin did not have a dramatic effect on complex formation.

1.2.2.2 Effect of Shear Flow on Platelet/CTC Complex Formation

In order to better recapitulate in vivo conditions, platelet-tumor cell suspensions were subjected to a uniform shear field. Hydrodynamic shear induced collisions augmented platelet-tumor cell binding (Figure 4c,d). A shear rate of 100s$^{-1}$ dramatically increased platelet binding to tumor cells when judged using CD41 as a marker. However, this shear force had seemingly no effect when using CD62P as a marker. This may be because at low shear forces (<100 s$^{-1}$), platelet binding is P-selectin-independent/Arg-Gly-Asp (RGD)-dependent. At higher shear forces (>800 s$^{-1}$) this process may become P-selectin dependent$^{26}$. As such, CD62P may be up-regulated at higher shear forces because it is a stronger and more specific interaction than RGD-based binding. In addition to platelet protein expression, tumor cell protein expression appears to be affected by shear forces (Figure 4g,h), as EpCAM seems be upregulated under flow conditions. Thus,
platelet activation and the fluid environment of the vasculature may have an effect on platelet-tumor cell adhesive interactions, and more work should be done to examine protein expression changes in response to fluid-mechanical forces.

Figure 4: The effect of shear flow on platelet and tumor cell protein expression. After applying a shear force of 100 s⁻¹ to a suspension of platelets and tumor cells, tumor cell forward and side scatter remained the same (A,B). However, platelet expression of CD41 seemed to increase (C,D), while platelet expression of P-selectin remained constant (E,F). It also appears that platelet/tumor cell complexing increased as a result of shear flow. Finally, tumor cell expression of EpCAM increased as a result of shear flow (G,H).
In order to evaluate flow cytometry as a tool for CTC isolation, 393 cells were spiked into whole blood and recovered via flow cytometry. Since these cells stably express GFP, their isolation can be confirmed by fluorescence. Tumor cells were spiked into mouse whole blood and incubated for 1 hr at 37 °C. After erythrocyte lysis, granulocytes, monocytes and lymphocytes were stained using markers outlined in Table 1. This counter-staining allowed for facile separation of blood cells. Using this method, I was able to resolve ~10 GFP positive tumor cells per 100,000 WBCs, or 1 tumor cell per 10^9 total blood cells (~10 ml of total blood). These initial experiments confirm flow cytometry as a viable method for enumerating and separating CTCs from peripheral blood.

After proof of concept studies in mice revealed platelet-tumor cell complexing to be a repeatable in vitro process, we turned to more relevant human cell lines. Human colonic carcinoma cell lines, LS180 and HT29, were tested for platelet binding. While LS180 robustly expresses sLex, an antigen for P-selectin on platelets, HT29 does not express this carbohydrate group (Figure 5). As such, I expected to see platelet binding to LS180 and negligible binding to HT29. Unfortunately, this was not the case, as neither cell line appeared to bind human platelets. Rather than a failure to bind platelets, this result may be an unfortunate experimental artifact. Antibodies to human isoforms of CD41 and CD62P on platelets appear to have been compromised during shipping from AbCAM. As a result, data from these experiments must be reproduced with a fresh batch of antibodies.
Figure 5: Sialyl Lewis X staining of HT29 and LS180. (A,B) While HT29 express Sialyl Lewis X, an antigen for P-selectin on platelets, at basal levels, (C,D) LS180 robustly express this carbohydrate group. (A) and (C) show unstained HT29 and LS180, respectively. While (B) and (D) show cells incubated with an antibody to CD15s.

1.2.3 Discussion and Future Work

Tumor cells bound by platelets are protected from shear forces and natural killer cell activity and may have a competitive advantage over other CTCs in seeding a secondary tumor. With the dearth of definitive CTC markers, platelets are a promising candidate for further evaluation. Here, I have delineated some early results investigating the propensity and mechanism of platelet-tumor cell binding in vitro. From these cursory experiments, it seems that platelets may not bind some tumor cells better than others – there is no clear correlation between metastatic potential and platelet binding. Also, tumor cells may locally activate platelets by secreting ADP or thrombin. Further, the surface expression of both tumor cells and platelets is altered in response to shear forces, and future work investigating CTCs should take this change in protein expression into account.
Work on a human model of platelet-tumor cell binding has been hampered by unfortunate complications with reagents. However, this work should be continued with the goal of identifying differences in platelet-tumor cell binding under varying shear forces and time courses. In addition, target proteins such as P-selectin, CD41 and carbohydrate groups such as sLe\(^\alpha\) should be systematically inhibited in order to investigate the mechanism of complex formation. The final step of this work \textit{in vitro} is to use FACS to sort out platelet-tumor cell complexes from whole blood and re-inject these complexes into mice in order to assay for metastatic potential. Since platelets protect from natural killer cell activity, it is imperative that these mice are not immuno-compromised, as the ability of tumor cells to overcome the immune system appears to be a critical step in metastasis mediated by platelets.

Once these \textit{in vitro} experiments have elucidated the mechanism of platelet binding and its role in metastasis, patient samples should be assayed for platelet-tumor complexes. Peripheral blood samples from patients with metastatic disease should be subjected to staining and subsequent flow cytometry. If platelet-tumor cell complexes exist, they should be isolated and perpetuated in culture. These complexes should also be injected into mice in order to investigate metastatic potential. Ideally, there will be a correlation between the number of platelet-tumor complexes and the severity of a patient's disease state. If this is the case, these complexes may be the focus of future therapeutic and diagnostic development.

1.3 Phage Based Approaches to Isolate CTCs

1.3.1 Isolation of Mesenchymal like CTCs Using Phage Display

The shift from an epithelial to mesenchymal phenotype is mediated by a number of transcription factors: Snail, Zeb, E47, KLF8, FoxC2 etc. In particular, TWIST has been shown to be essential for metastasis and EMT in breast carcinomas\textsuperscript{27}. The Weinberg group has shown that up regulation of TWIST leads to degradation of cell-cell adhesion, downregulation of epithelial markers (cytokeratin, E-cadherin), and upregulation of mesenchymal markers (vimentin, N-cadherin)\textsuperscript{28}. Yang and colleagues went on to create a stable mesenchymal-like cell
line by constitutively up-regulating TWIST in mammary carcinoma cells\textsuperscript{29}. Using these two cell lines (TWIST +, TWIST -) as a proxy for mesenchymal-like and epithelial-like tumor cells, we have used phage display of a library of random peptide 7-mers fused to minor coat protein pIII of M13 bacteriophage to select for peptides that bind to mesenchymal-like cells and not to epithelial-like cells (Figure 6).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{biopanning.png}
\caption{Schematic of biopanning approach. A library of \textasciitilde10\textsuperscript{10} phage clones was incubated with mesenchymal-like (TWIST +) tumor cells, bound and internalized phage was collected and incubated with epithelial-like (TWIST -) tumor cells. Unbound phage was collected and this sequence was repeated three times to enrich the phage population. After enrichment, phage clones were sequenced and mesenchymal binding peptide candidates (MBP) were identified.}
\end{figure}

M13 is a filamentous virus that infects bacteria, consisting of five proteins that encapsulate the viral DNA. It is approximately \textasciitilde1 \textmu m in length and 6-7 nm in diameter. M13 phage is an attractive vector for peptide display since its genotype relates directly to its phenotype. Consequently, peptides can be genetically engineered into the multiple coat proteins of the M13 phage for display. Previously, others have displayed peptides and proteins on the coat proteins with affinity towards epitopes\textsuperscript{29, 30}, antibodies\textsuperscript{31} and mammalian cells for targeting\textsuperscript{32, 33}. Our lab has engineered a phage vector for peptide display on two coat proteins, p3 and p8 and we have demonstrated multiple display of the M13 scaffold to grow and nucleate various inorganic materials\textsuperscript{34-42}. M13 filamentous bacteriophage is an excellent biological building block due to its multiple peptide display system, controllable length, and functionality as a nanoscale scaffold for nanoparticle organization.

Initial biopanning results (detailed in Materials and Methods) yielded 12 candidate mesenchymal binding peptide (MBP) sequences (Figure 7).
Figure 7: Biopanning results. 12 candidate sequences for the mesenchymal binding peptide are aligned above. The 7-mer library is book-ended by two cysteine residues, which form a disulfide bond. The consensus sequence is proline and serine rich, which is common for peptides that bind surface proteins.

While the library did not collapse to a handful of sequences, there are a number of conserved motifs between the sequences (Figure 7). The 7-mer sequences are given by positions 2-8 and are sterically constrained by two cysteine residues. The consensus sequence is proline and serine rich. Proline is a unique amino acid as its side chain is cyclized onto the backbone nitrogen. As a result, the confirmation of proline and the preceding residue are restricted. The PxxP motif is a common motif in peptides specific for proteins, as the prolines form a continuous hydrophobic strip around the surface of the helix and allows for ideal hydrogen binding sites. As such, these isolated sequences are promising candidates for further investigation.

1.3.2 Discussion and Future Work

Peptides isolated via phage display should be further investigated for binding to mesenchymal-like cells. The most straightforward experiment to be carried out is a competitive ELISA in which phage specific for mesenchymal-like cells are incubated with cells and competed off with free MBP. If this competition is successful, one can estimate the \( K_D \) of peptide binding. Once a peptide has been found using phage display, it can be used to isolate mesenchymal-like CTCs from peripheral blood.
In order to isolate CTCs from peripheral blood, the MBP will be biotinylated and attached to 50nm streptavidin coated Fe$_2$O$_3$ superparamagnetic bead, and magnetically labeled cells will be isolated using magnetic-activated cell sorting (MACS)$^{44}$. MACS employs a steelwool column that is magnetized by placing it in a .6 Tesla magnetic field. The magnetic column acts as a sensitive filter for magnetically labeled cells and allows for a 5-log-fold enrichment of viable cell populations$^{44}$. As proof of concept of the isolation approach, TWIST expressing mesenchymal-like cells will be spiked into whole blood to mimic a CTC population, and MBP functionalized magnetic beads will be added to the solution to label CTCs. After erythrocyte lysis, leukocytes and tumor cells will be applied to a magnetic column (Miltenyi Biotec) – magnetically labeled tumor cells will be retained by the column while leukocytes and plasma proteins will be discarded. Cells can be eluted from the column by removing the external magnetic field or by competing with free MBP. Several rounds of purification can be carried out in order to enrich tumor cells. If necessary, negative selection can be carried out by labeling Cd45$^+$ leukocytes with biotinylated antibodies and running the sample through a streptavidin functionalized affinity column. This iterative approach allows for control of the purity of isolated CTCs. By closely monitoring the number of cells spiked into blood and the number of cells successfully isolated, the efficiency and resolution of this approach can be quantified. Unfortunately, this approach comes with a number of limitations. Functionalization of beads to the MBP may alter the specificity of the peptide. As such, if MACS fails to isolate tumor cells, conjugating MBP to a smaller bead with a different shape may allow for better purification. Additionally, this approach is limited by the number of MBP ligands on the surface of the tumor cells. If these ligands are of low-abundance, cells will have few magnetic nanoparticle bound and a low magnetic moment, which may prohibit their isolation using MACS and may require the use of an expensive multi-tesla magnet. While the MBP may show affinity for mesenchymal-like cells in vitro, this affinity may not translate well into clinical samples. Since CTCs in the blood are likely more heterogeneous than a cell line, the MBP may only isolate a subset of CTCs. In addition, this subset may not represent a particularly malignant group of cells. A number of modifications can be
made to the phage display protocol to direct the selectivity of the MBP. Increasing the number of alternating rounds of negative and positive selection during biopanning can increase its specificity. By negatively selecting against leukocytes in addition to epithelial-like cells, a MBP can be identified that does not bind to blood cells. In order to better recapitulate in vivo conditions, selection can be carried out against detached cells in media rather than cells adherent to a plate, as gene expression may change dramatically in circulation\textsuperscript{45}.

In order to generate global gene expression profiles, RNA will be extracted from isolated mesenchymal-like cells and applied to the Affymetrix Genechip Human Genome U133 Plus 2.0 Array, which allows for analysis of over 47,000 transcripts\textsuperscript{46}. To benchmark expression, results will be compared to profiles from epithelial-like cells. In order to confirm that the isolation procedure has no effect on gene expression, expression signatures from mesenchymal-like cells isolated from blood will be compared to signatures from mesenchymal-like cells isolated directly from culture. If there is a dramatic difference in gene expression attributable to the isolation procedure, a different procedure may be implemented. For example, the MBP could be functionalized to microposts in a microfluidic device. While leukocytes will flow through the device, mesenchymal-like CTCs will be captured. These cells can then be eluted from the posts by competition with free MBP. This MEMS device is beneficial in that physiological flow conditions may preserve the viability of CTCs; however, it is limited by its low throughput, as only small volumes of blood can be run through each chip at a time. Conversely, a change in gene expression of mesenchymal-like cells after incubation in blood could be due to interactions with blood cells (personal communication, Myriam Labelle – Hynes Lab, MIT). To test this hypothesis, cells in culture could be incubated with whole blood. After washing the cells to remove unbound blood cells, gene expression analysis would be carried out.

After these proof of concept studies have confirmed that MACS is a feasible CTC enrichment strategy that does not alter gene expression, this approach can be used to characterize CTCs from patients with varying stages of metastatic disease. In collaboration with surgeons, blood and primary tumor samples will be collected
from patients that have undergone surgery to excise primary epithelial tumors. Since the cell lines from previous experiments are mammary carcinoma based, we will initially try to obtain clinical samples from patients with advanced breast cancer. Magnetic beads conjugated to the MBP will be used to purify CTCs from the blood samples. Since CTCs are so rare, multiple rounds of isolation will be carried out to ensure minimal leukocyte contamination. Since an iterative isolation procedure may be harmful to cells, we will have to empirically determine how many rounds of isolation are appropriate. Gene expression of isolated CTCs will be compared to expression from primary tumor cells from the same patient and to the TWIST inducible mesenchymal-like and epithelial-like cell line from the Weinberg group. When comparing patient CTC and patient tumor gene signatures, we will look for genes that are significantly up/down regulated when cells enter the bloodstream. We would expect to see up-regulation of mesenchymal markers and down regulation of epithelial markers as confirmation of an EMT. Further, we can correlate gene expression of CTCs with clinical characterization of the patient’s disease stage. Cancer can be staged from 0 (carcinoma in situ), to stage IV (evidence of metastasis)\(^47\). Using principal component analysis (PCA), we may be able to correlate CTC gene expression with that of primary tumor cells and tumor grade. PCA may be useful in identifying a handful of candidate genes that are responsible for survival in the blood and metastatic potential. Future work would be carried out by examining protein expression levels of these candidate genes, as there tends to be no correlation between gene and protein expression levels\(^48\). While the utility of gene expression data is limited, it may prove beneficial by yielding a small number of candidate genes for further characterization\(^49\). In addition to gene and protein expression analysis, a simpler correlation between CTC number and malignancy will be studied. Large numbers of circulating epithelial cells in peripheral blood translate into poor clinical outcomes. By isolating mesenchymal-like rather than epithelial-like tumor cells from the blood, we may be able to classify malignancy more accurately and precisely, as mesenchymal-like cells, rather than epithelial-like cells, may be the precursors of metastasis.
The paucity of CTCs isolated using MACS may prohibit gene expression analysis. Ten nanograms of total RNA from CTC and primary tumor samples is needed to prepare biotinylated hybridization targets with the Affymetrix small sample target labeling assay\textsuperscript{50}; this system is designed to reproducibly amplify 10-100ng of total RNA using T7 RNA polymerase. Assuming that each cell has approximately 30pg of total RNA and 2\% of total RNA is mRNA\textsuperscript{51}, about 330 tumor cells are needed in order to get reproducible gene expression results. If a typical blood draw is 5ml, CTC counts would have to be >60/ml in order to carry out such genetic analysis. As such, microarray analysis will only be possible if MACS can reproducibly enrich a large CTC population or if analysis is restricted to patients with high CTC counts.

An emerging alternative to microarray analysis that is well suited to rare and heterogeneous CTCs is single-cell quantitative PCR with reverse transcription (qRT-PCR) (Fluidigm Biomark system). This approach is advantageous in that it can distinguish between pooled and single cell expression, a short coming of the microarray approach\textsuperscript{52, 53}. Single cell resolution will be important to find subpopulations of malignant CTCs and to understand how a single CTC can give rise to a tumor\textsuperscript{54}. The drawback of this approach is that it will not yield a global gene expression profile. Instead, we can examine the expression of a group of query genes. These genes would include EMT hallmark genes as well as housekeeping genes such as Actb (beta-actin) and Hprt1 (hypoxanthine guanine phosphoribosyl transferase 1 in order to normalize and benchmark analysis.

1.4 Materials and Methods

**Blood Collection**

Blood was obtained from Research Blood Components (human blood) or Bioreclamation LLC (mouse blood). Blood was typically drawn into sodium citrate tubes. In order to avoid calcium chelation, blood was drawn into hirudin tubes (Diapharma). Mouse blood was shipped overnight on dry ice, while human blood was shipped day of on dry ice. Blood was always used within two days of receipt.
**Platelet Preparation**

Whole blood was centrifuged at 800g for 7 minutes in a tabletop centrifuge. Platelet rich plasma (PRP) was collected and the remaining blood cells were again centrifuged for 7 minutes at 800g. The platelet poor plasma was collected and mixed with the PRP to obtain the desired platelet concentration.

**Erythrocyte Lysis**

1ml of whole blood is centrifuged for 7 minutes at 800g. Remove the PRP and resuspend remaining cells in 1.5ml ACK buffer (.15M NH₄Cl, 10mM KHCO₃, .1mM EDTA). Allow cells to incubate with buffer for 10 minutes. Centrifuge for 3 minutes at 3000RPM and discard supernatant. Some red blood cells will remain. Resuspend cells in ACK, incubate and centrifuge. Discard supernatant. A small white pellet should be visible (white blood cells) – resuspend in PBS to desired concentration.

**Cell Lines and Culture**

393 and Tearly cell lines were a gift from the Jacks Lab (MIT). HT29 and LS180 were a gift of the Hynes lab (MIT). All cells were grown in Dulbecco’s Minimum Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) and 1% penicillin/streptomycin (Invitrogen, CA) at 37 °C in 5% CO₂.

**Freezing Cells**

Cells were preserved by freezing at -80 °C. A confluent 225 cm² culture plate was trypsinized and cells were resuspended in 10% DMSO, 90% FBS. Cells were frozen in isoproponal containers to control cooling.

**Cell Counting**

Cells were quantified and their viability assessed using a Vi-Cell Series Cell Viability Analyzer (Beckman Coulter). This machine was used with permission from the Lauffenberger Lab (MIT). While this machine was useful in quantifying mammalian tumor cells, both human and murine platelets were too small to be reproducibly counted by this machine. Instead, these cells were counted using a hemacytometer.
Formation of Platelet Tumor Complexes

Platelet rich plasma (PRP) or washed platelets are prepared and incubated with tumor cells for 1 hour at 37 °C on a rocker. Typically $10^6$ tumor cells were mixed with $10^8$ platelets in PBS in a volume less than 1ml. After incubation, complexes were separated by centrifuging at 1200 RPM for 5 minutes at room temperature (a small red ring is visible around the tumor cells at this step). If using $10^6$ tumor cells, a pellet should be clearly visible. The supernatant was discarded and the complex was stained for platelet markers using antibodies on ice (see above). Some experiments included 10uM ADP during the incubation step (1 hour) for platelet activation. To form complexes with cells in whole blood, $10^6$ tumor cells were added to a flow tube and centrifuged at 1200 RPM for 5 minutes. .5 ml of whole blood was added directly to the cells and lightly vortexed. This solution was incubated for 1 hour at 37 °C and then centrifuged at 1500 RPM for 7 minutes. The PRP and the buffy coat were carefully collected such that there was no erythrocyte contamination. These cells were then stained and run on FACS. White blood cells were counterstained with biotinylated CD45, GR-1, CD11b and F4180 incubated with streptavidin-PE-Cy7 beads. In order to recapitulate shear forces, the vasculature was mimicked by a simple pump apparatus. A Pump33 (Harvard Apparatus) was connected to plastic tubing (diameter = .5mm, length 500 cm). A shear force of 100s$^{-1}$ was applied using a flow rate of 1.3 ul/s on the Harvard pump. PRP and tumor cells were mixed and immediately applied to the pump for about 10 minutes.

Antibody Staining

100,000 – 1,000,000 cells were incubated in 100 ul volumes of PBS. Primary antibodies were added at 1:100 dilutions and secondary antibodies were generally added at 1:500 concentrations (see lab notebook for precise concentrations for specific experiments). Cells were incubated with antibodies on ice and protected from light for 30 minutes to 1 hour. After incubation with either primary or secondary antibodies, cells were washed 1-3x with PBS by centrifugation at 1200
RPM. In addition to surface markers, cells were incubated with DAPI immediately before analysis in order to test for cell viability.

**Antibody List**

1. P-selectin (Psel.KO.2.7) (Santa Cruz Biotech, Inc sc-101336) (Mouse anti-human)
2. PE Goat Anti-Mouse Ig (BD Pharmingen 550589)
3. PE Goat Anti-Rat Ig (BD Pharmingen 550767)
4. Purified Rat IgG1, k Isotype Control (BD Pharmingen 553922)
5. Mouse Monoclonal (104-2) to CD45.2 PE/Cy7 (abcam 25463)
6. Ep-CAM (G8.8) (Santa Cruz Biotech, Inc sc-53532)
7. Rat Anti-mouse CD41 MWreg30 (BD Pharmingen 553847)
8. Mouse Mab CD41 (reacts with human samples) alpha-2b [sz.22] FITC (abcam 19687)
9. Mouse Mab CD62P (reacts with human samples) (AK-6) (ab 33279)

**Flow Cytometry**

Flow cytometry was carried out on both LSRII and FACScan cytometers. High throughput automated cytometry was carried out using the LSRII.

**Flow Cytometry Data Analysis**

Platelet tumor complexes were selected on the following criteria. Side and forward scatter were characteristic of the tumor cell line. If using the 393 cell line, cells were GFP positive. Cells stained positive for platelet markers CD41 or CD62P. Cells were DAPI negative, indicating viability. Cells were CD45, GR-1, CD11b and F4180 negative, indicating that they were not blood cells.

**Panning**

Panning was conducted with three rounds of positive selection and three rounds of negative selection. The rounds were conducted in the following order: positive, negative, negative, negative, positive, positive. Positive selection was carried out against 393 cells (Jacks Lab) while negative selection was carried out against whole blood. Phage was amplified and titered between rounds of positive selection.
Positive Selection
1. Grow epithelial-like cells such that they are over-confluent
2. Prepare phage cocktail/solution: Mix 2E11 pfu in 1 mL 1x PBS with 1% BSA and protease cocktail
3. Incubate with 3ml serum-free media for 2 h, 37 °C. This is to allow for 'clearing' of the receptors (and possibly the glycocalyx).
4. After serum-free incubation, aspirate media and add 2E11 pfu/mL phage to (target) cells.
5. Incubate phage 1 h at 37 °C with gentle rocking to allow time for phage to be internalized.
6. After incubation, remove supernatant (contains unbound phage) by aspiration
7. To remove nonspecific binding phage, the cells are washed five times with 3 ml PBS supplemented with 1% BSA and 0.05% Tween-20 at room temperature in a flow tube
8. After washing add 1 ml of glycine (pH= 2.2, added by HCl) and incubate 8 min.
9. Remove the supernatant, repeat the wash with glycine, and collect the supernatant, transferring it into a microcentrifuge tube.
10. Add 150 uL Tris (pH ~9.1) to the solution in the micro-centrifuge tube, the ACID eluted fraction. This collects weakly bound phage to the cells. Save this fraction.
11. Recover the internalized phage by lysing the cells with 1 ml of 0.1% triethanolamine (Sigma) in PBS (pH 7.6) for 5 min at RT
12. Neutralize the internalized phage pool with 100 µl of 0.5 M Tris-HCl (pH 7)
13. Vortex and incubate for 30 min on ice.
14. Spin down the cell lysate and collect the supernatant (~1 mL). This is the CELL fraction. This fraction should have phage that showed affinity towards target cell.
15. Save 10% CELL fraction for titering. Use the rest of fraction for phage amplification.

Negative Selection:
1. Clear mesenchymal cells
2. Take 1E11 pfu of amplified phage from previous round and make phage cocktail in 1 mL sterile PBS with protease inhibitor.
3. Incubate cells with phage cocktail for 1 h, 37 °C.
4. Take the supernatant. This fraction will contain unbound phage that did not bind the mesenchymal-like cells and will be used for panning against metastatic liver cells.
5. GO TO Panning protocol.
Chapter 2: M13 Bacteriophage as a Platform for Targeted, Simultaneous Near-Infrared Imaging and Efficient Chemotherapy of Prostate Cancer Cells

2.1 Background: Nanomaterial-Based Therapeutics

Most systematic therapeutic approaches to cancer lack specificity and are not precisely controlled after injection. The ability to control the functionality and valency of nanomaterials allows for manipulation of pharmacokinetics and dynamics and can improve safety, potency, and efficacy over traditional tumor therapies and imaging techniques. Further, the size and shape of many nanomaterials yield a large surface area to volume ratio and allow for the containment and functionalization of an array of targeting, therapeutic or imaging modalities.

A number of nanomaterials have shown promise in a clinical setting and many more candidate chemotherapy and imaging agents have been successful in murine trials. For example, iron oxide and gadolinium (III) nanoparticles can be functionalized to targeting moieties in order to increase the relaxivity of water molecules in a tumor and subsequently enhance magnetic resonance images, which may allow for more precise imaging of tumors. Gold nanorods have been effective agents of thermolysis by acting as antennas to absorb infrared radiation and cause tumor cell necrosis. Single-walled carbon nanotubes (SWNTs), rolled-up tubes of graphene sheets, have also been recently studied for therapy, imaging and as fluorescent biosensors for nitric oxide and reactive oxygen species. Their quasi-one-dimensional architecture yields a large surface area for functionalization and drug loading. Further, SWNTs are an attractive candidate for biomedical imaging because of their high absorption of near infrared (NIR) light, cross-section of Raman scattering, and NIR band-gap fluorescence ranging from 900 – 1600 nm. NIR fluorophores with emission in the “second window” (900 – 1400 nm) may have greater tissue penetration than those in the first spectral window (650-900 nm). These probes are advantageous because of the low characteristic autofluorescence of biological species in the second window, which results in a
signal to noise ratio that may be 100 fold higher than that in the first window and may allow for precise and sensitive detection of nascent tumors.

Previous research has established SWNTs as viable imaging and therapeutic vectors. Several groups have shown SWNT mediated therapeutic delivery or NIR fluorescence imaging of tumors\textsuperscript{72}. Despite the recent success in utilizing SWNTs for imaging and therapy, it remains challenging to engineer multifunctional, biocompatible, and singly-dispersed SWNT complexes that retain fluorescence. Further, no single platform has demonstrated simultaneous fluorescent second window imaging and drug delivery, as therapeutic loading may limit the stability of non-covalently functionalized SWNTs.

The next generation of nanomedicines will have encoded multifunctionality that allows for targeted and simultaneous therapy and diagnosis (so called "theranostics"). While a number of nanomedicines used in the clinic target tumors using the enhanced permeability and retention effect (EPR), targeting to specific tumor markers may improve the efficacy and safety profiles of nanomaterials by reducing nonspecific cell uptake. In addition, the ability to simultaneously image and ablate a tumor will allow for real-time monitoring of the efficacy of a drug regimen.

M13 is a promising platform for nano-scale therapeutic and diagnostic delivery because of its genetic tunability and physical characteristics. M13 has been previously used in biomedical applications for epitope discovery\textsuperscript{73, 74}, gene delivery\textsuperscript{75}, antibody delivery\textsuperscript{76}, and \textit{in vitro} and \textit{in vivo} ligand discovery\textsuperscript{77-79}. M13 bacteriophage has five genetically modifiable proteins (proteins p3, p6, p7, p8, and p9). Expression of material-specific peptides or targeting motifs on various coat proteins allows for genetic control and tunability of its function\textsuperscript{40}; the malleability of the phage genome allows for directed evolution of phage proteins for biomedical applications. In addition, the highly ordered structure of the 2700 copies of the major coat protein, p8, and the filamentous shape of M13 (~6.5 nm in diameter and ~880 nm in length) enables the phage to multivalently and cooperatively interact with one-dimensional nanomaterials such as SWNTs, and the abundance of
genetically modifiable proteins on the coat of the phage allow for encoding of multifunctionality.

Particle shape can greatly impact the cellular and tissue interactions of nanomaterials. Particles with a high aspect ratio (length to width ratio) have been shown to evade phagocytosis and can only be engulfed when cells interact with the particle ends. Investigators have shown that filamentous particles have circulation times about 10-fold longer than spherical particles. Further, surface charge or zeta potential (electric potential at the hydrodynamic slipping plane of a particle) is an important factor influencing the behavior of nanomaterials. Particles with a negative or neutral surface charge avoid non-specific cellular uptake better than particles with a positive charge. As such, the filamentous shape of M13 may help inhibit phagocytosis and also enhance circulation half-life in vivo, while the slightly negative zeta potential of the phage may reduce its nonspecific cellular uptake.

In the following section, I outline an M13 bacteriophage-based platform for manipulating single-walled carbon nanotubes (SWNTs) in biological solutions for multimodal near-infrared (NIR) fluorescent cell-specific imaging and therapy. SWNTs are stably dispersed by major coat proteins of the M13 bacteriophage through molecular recognition and retain band-gap fluorescence in the 900 – 1350 nm wavelength range, which may allow for deep tissue imaging. Minor coat protein p3, located at the proximal tip of M13, was genetically engineered to specifically target SPARC matricellular protein, a marker overexpressed on metastatic breast and prostate tumors. The targeted virus-SWNT complex serves as a NIR imaging agent and allowed for sensitive and selective NIR imaging of cells with varying expression levels of SPARC. Controlling M13-SWNT stoichiometry allowed further functionalization of the viral major coat with a chemotherapeutic agent for targeted drug delivery in vitro. Doxorubicin (DOX) delivered by the M13-SWNT complex inhibited cell growth 400x more efficiently than free DOX, and was restricted to SPARC expressing cells. This approach provides an 'all-in-one' platform for targeted NIR fluorescence imaging and efficient drug delivery.
Figure 8: A schematic of the M13 virus-based platform. (Figure components not to scale) (A) M13-SBP-SWNT-DOX complex. The filamentous shape of M13 bacteriophage and high aspect ratio allow for favorable physical interactions in vivo. p3 protein is engineered for targeting SPARC (SSPTGIN) and p8 protein is modified for SWNT binding and drug conjugation (DVYESALP). The secondary structure of a single p8 protein is shown inset and an inserted 8-mer peptide sequence is displayed on the amino-terminus of the p8 protein. The tyrosine residue in red is hypothesized to interact with hydrophobic SWNTs while the free carboxylate group on aspartic acid is used for drug conjugation. Doxorubicin is depicted with a blue sphere. (B) A helical structure of 2,700 copies of p8 protein along the M13 virus with five-fold rotational and two-fold screw symmetry, allowing for multivalent and cooperative binding of SWNTs. Rendering was based on a structural model of M13 virus, 2COW.pdb. (software: Swiss-PDB) (Courtesy of Hyunjung Yi)

2.2 Theranostic Properties of M13 Bacteriophage and Single-Walled Carbon Nanotubes (SWNTs)

2.2.1 Creation of M13-SWNT Complex (Hyunjung Yi)

To bind and disperse SWNTs non-covalently along the length of virus wrapped by p8 major coat proteins, 8mer peptide sequences displayed on p8 that show binding affinity towards SWNTs were identified through biopanning (see Materials and Methods). After several rounds of iterative panning against SWNT film, consensus sequences were identified. The three most frequently appearing clones, DPSRLANE, DKSIEPLP, and DVYESALP (designated p8cs#1, p8cs#2, p8cs#3...
respectively), were investigated for binding and dispersion of SWNTs. Well-dispersed HiPco (high-pressure CO decomposition) SWNTs in water with 2wt% sodium-cholate were dialyzed extensively against surfactant-free buffer in the presence of each virus clone\textsuperscript{83}. SWNT solution without virus was also dialyzed as a negative control. After dialysis, only p8cs#3 formed a homogeneous solution with SWNTs whereas other clones and negative control formed black aggregates (Figure 9). This solubility suggests that p8cs#3 had a binding affinity strong enough to disperse SWNTs in aqueous solution. Interestingly, only p8cs#3 had an aromatic residue, tyrosine (Y), which is thought to preferentially interact with the graphene sidewall of carbon nanotubes (Figure 8a)\textsuperscript{84,85}.

![Binding test results](image)

Figure 9: Binding test results. (A) Each clone was incubated with sodium cholate-dispersed HipCo SWNT in water and dialyzed against tris-buffered saline (TBS, Tris-HCl 100 mM, NaCl 150 mM, pH. 7.5) for three days with frequent buffer exchanges. The upper column has the highest virus concentration and each virus clone has duplicate samples. Only clone p8cs#3 shows homogeneous solutions while others
form black aggregates. (B) SWNT dialyzed with and without virus clone p8cs#3. After 24 hr, black aggregates are clearly observed for SWNTs dialyzed without virus. (Courtesy of Hyunjung Yi)

To further probe the binding mechanism of p8cs#3 to SWNTs, the hydrophobicity of the peptide sequence was calculated and plotted (Figure 10a)\textsuperscript{86}. The peptide insert of p8cs#3 has a hydrophobic moiety sandwiched within a hydrophilic region and this moiety is believed to interact with SWNTs. Meanwhile, p8cs#1 lacked a hydrophobic region and p8cs#2 showed lower hydrophobicity than p8cs#3. In general, π-π stacking and hydrophobic interactions are considered the primary interaction mechanisms between biological molecules and SWNTs\textsuperscript{84, 85}. Therefore, it is reasonable to conclude that the combination of π-π stacking and the hydrophobic properties of the p8cs#3 sequence are the driving force of the M13-SWNT binding. While the hydrophobic moiety is small, the overall interaction between SWNT and the virus is amplified by multivalent binding, as each M13 virus has 2,700 copies of p8 major coat protein that form well ordered structures with five-fold-symmetry and two-fold screw symmetry about the major axis (Figure 8b). Based on the crystallographic data of wild-type M13 virus (PDB ID: 2C0W), there are about 31 p8 peptides in series per 100 nm along the virus. This multivalent and cooperative binding scheme makes detachment of SWNT off the virus unlikely and energetically unfavorable.
Figure 10: SWNT binding and M13-SWNT complex. (A) Hydrophobicity plot of p8 peptide sequences of various virus clones. Hydrophobicity is calculated based on the Hopp-Woods scale. Window size is 5. (B) Proposed scheme of M13(p8cs#3)-SWNT complex. SWNT is bound along the groove of virus major coat proteins and the interaction is amplified by multivalent and cooperative binding. An arrow indicates an amino-terminus of a p8 protein. Rendering was based on 2CDW.pdb (software: mercury). (C) HRTEM images of SWNT bound along the M13 bacteriophage (outlined in red). Part of the phage major coat was burned off to clearly show SWNT bound along the virus. Arrows indicate a single SWNT. (Courtesy of Hyunjung Yi)

A proposed binding scheme for the M13 virus-SWNT is illustrated in Figure 10b. The assembled structure of p8 proteins has ten grooves of ~ 2.5 nm in width along the phage at the amino terminus of p8 proteins. Since the SWNT-binding peptides are expressed on the amino terminus of p8, SWNTs can interact with the SWNT-binding peptides along the grooves. In addition, because SWNT-binding peptides are inserted on the amino-terminus of p8 protein of wild-type M13, the amino-terminal peptides of p8cs#3 are longer and more flexible than wild-type M13. These longer and flexible amino terminal peptides can moderately reorganize to maximize the π-π stacking and hydrophobic interactions between the SWNT-
binding peptides and SWNTs while minimizing the surface exposure of hydrophobic SWNT surface. High-resolution transmission electron microscopy (HRTEM) was used to visualize the bound SWNT along major coat of the virus (Figure 10c). Part of the virus major coat proteins has been intentionally burned off during imaging, clearly identifying bound SWNT.

Although p8cs#3 phage has a strong binding affinity toward SWNT, the ratio of SWNT to phage affects the stability of the M13-SWNT complex solution. The complex was stable when the overall ratio of SWNT to virus was less than 4. However, when the ratio was increased to 30, the complex solution started to aggregate and eventually precipitated. A number of explanation can explain this phenomenon: first, as the ratio of SWNT to phage increases, SWNTs can form small bundles before they are bound by phage to form a complex. Second, when too many SWNTs are bound per phage, the hydrophobicity of the phage-SWNT complex increases and the whole complex can aggregate. Therefore, it is worth emphasizing that not only the binding affinity but also the overall ratio of SWNT to phage is critical to get a very stable phage-SWNT complex. In this context, the overall ratio of SWNT to p8cs#3 M13 phage was set to 1:1 throughout this study. Since the hydrophobic moiety of the p8 protein is small and approximately only 155 of 2,700 copies of p8 are bound by SWNT in the 1:1-M13-SWNT complex, the vast majority of the surface of the complex remains hydrophilic. In addition, the native negative surface charge of M13 contributes to the colloidal stability of M13-SWNT through electrostatic repulsion. The zeta potential of the complex is ~45 mV at pH 7.4 (Figure 11b); colloidal particles with absolute zeta potential above 15 mV are considered stable (Figure 11a) 87.
Figure 11: Colloidal stability of M13 phage (p8cs#3)-SWNT complex and its optical properties. (A) Phage disperse SWNTs in solution, while SWNTs crash out of solution in the absence of phage. (B) Zeta potential of the phage-SWNT complex. (C) UV-vis-NIR absorption spectra for SWNTs dispersed by 2wt% sodium cholate (starting SWNTs) in water and SWNTs dispersed by phage in PBS. (D) PL mapping data for starting SWNTs dispersed by 2wt% sodium cholate (top) in water and phage in PBS (bottom). The dotted lines are guides for red-shifting of peaks. The characteristic peaks of SWNT are preserved both in the absorption spectra (C), and PL spectra (D), even after formation of M13-SWNT, suggesting SWNTs are well dispersed and the complex is stable. (Courtesy of Hyunjung Yi)

These combined effects allow M13 to stabilize SWNTs in PBS, culture media, and serum (Figure 12) and prevent SWNT bundling, obviating the need for chemical functionalization.
2.2.2 Near-infrared Imaging of Tumor Cells using M13-SWNT Complex (Hyunjung Yi and Debadyuti Ghosh)

In order to investigate the optical properties of M13-SWNT, UV-vis-NIR absorption and PL spectra of the complex were measured and compared to starting sodium cholate-dispersed SWNTs (Figure 11c,d). The characteristic optical peaks of SWNTs, coming from van-Hove singularities of electronic band structure of SWNTs\(^{72}\) are preserved both in the absorption spectra and PL spectra after formation of M13-SWNT, suggesting that SWNTs are well dispersed. Peak broadening and red-shifting are observed in absorbance and fluorescence as previously reported in other systems where surfactants are replaced by polymers\(^{66}\) or other molecules\(^{88}\). Peak broadening and red-shifting are attributed to a perturbation of electronic structures of nanotubes induced by increased interaction of polar water with nanotubes, consistent to the proposed binding scheme where some of the SWNT surface is exposed to water. The peak shifts for the complex are similar to those from SWNTs dispersed by porous and grooved macromolecules such as glucose oxidase\(^{83}\). Therefore, our absorption and PL spectra suggest that closely packed sodium cholate molecules on SWNTs were successfully displaced by the phage (Figure 11c,d). Moreover, since the starting SWNT composition consists of metallic and semiconducting tubes, the clear PL peaks confirm that SWNTs are
well dispersed by phage with minimal bundling (Figure 11d). SWNTs dialyzed without phage showed no PL from semiconducting tubes due to quenching of fluorescence by metallic tubes, present in the starting SWNT composition, bundled with semiconducting tubes\(^{89}\).

After confirming p3 functionality of the phage in the presence of SWNTs on p8, we examined the ability of M13-SWNT to target metastatic prostate cancer cells by incorporating a targeting motif into p3 (Figure 8).

Expression profiling has identified secreted protein, acidic and rich in cysteine (SPARC) as a biomarker upregulated in various human cancers\(^{90, 91}\). SPARC, an anti-adhesive and promigratory matricellular glycoprotein, is upregulated in patients with aggressive melanoma, breast, brain, prostate, colon, and lung cancers\(^{92}\). In addition, SPARC may facilitate cancer cell invasion and dissemination and is correlated with poor prognosis, suggesting that it may be an excellent biomarker for identifying and staging metastatic lesions\(^{92}\). We investigated SPARC-specific targeting in metastatic prostate cancer cells using M13-SWNT as a NIR fluorescent probe. A binding peptide identified against SPARC (SPPTGIN, designated as SBP)\(^{93}\) was genetically engineered into the p3 minor coat protein of M13 (p8cs#3) (Figure 8). The targeted complex, M13-SBP-SWNT, was incubated with human metastatic prostate cancer cell lines LNCaP, C4-2B (SPARC positive; C4-2B is LNCaP derived line which metastasizes to the bone) and DU145 (SPARC negative) at physiological conditions and imaged in the NIR second window (950-1,400 nm) using a two-dimensional InGaAs camera (see Materials and Methods). C4-2B and LNCaP showed noticeable fluorescence (Figure 13a, bottom right, and middle), whereas DU145 control did not exhibit any fluorescence (Figure 13a, bottom left). Punctate fluorescence was observed in the cytosol, suggesting possible vesicular uptake (Figure 13a). To quantify targeting, cells were incubated with M13-SBP-SWNT and harvested for spectra measurements. The fluorescence intensity was integrated from 1,120 nm to 1,350 nm and normalized (Figure 13b, c). C4-2B and LNCaP exhibited eleven and five-fold greater intensities than the DU145 control, respectively (Figure 13c). Even after considerable incubation, there is negligible non-specific binding to control DU145 (Figure 13a-c). The imaging and
spectra suggest M13-SBP-SWNT can actively and discriminally target cells in vitro at relevant physiological conditions. This selective and sensitive fluorescence probe illustrates the potential of our phage-based approach for targeting and imaging in vivo metastases in the NIR regime.

Figure 13: Second window NIR imaging and analysis. (A) Brightfield (top) and NIR fluorescence images (bottom) of M13-SBP-SWNT targeting three prostate cancer cell lines with various expression levels of SPARC, C4-2B (++ SPARC), LNCaP (+ SPARC) and DU145 (- SPARC). Fluorescence from targeted cancer cells is clearly shown for both LNCaP and C4-2B while there is no obvious fluorescence signal from DU145. 20 μm-scale bar applies for all 6 figures. (B) PL spectra from targeting various cancer cells, normalized to the number of cells. (C) Integrated PL intensity from different cell lines. PL spectra were integrated from 1,120 nm to 1,350 nm and normalized by the number of cells counted after the PL.
measurement. C4-2B and LNCaP have about eleven times and five times stronger intensity than DU145, respectively. P values for LNCaP and C4-2B compared with DU145 were calculated using student t-test. * p<0.0001, ** p=0.014. p<0.05 is statistically significant. (Courtesy of Hyunjung Yi)

2.2.3 Targeted and Efficient Cytotoxicity using M13-SWNT Platform

To investigate the chemotherapeutic potential of the M13-SWNT platform, M13-SBP-SWNT complex was further conjugated with chemotherapeutic agents. Doxorubicin (DOX) is an anthracycline antibiotic and a common chemotherapeutic agent that acts by inhibiting topoisomerase II activity to produce stable DNA double strand breaks\(^9\)\(^4\). While it has been extensively used in the clinic to treat ovarian, breast, lung, uterine and cervical cancers, DOX injury to non-target tissues via induction of oxidative stress often results in limited DOX dosing regimens\(^9\)\(^5\). As such, efficient and targeted delivery of DOX to neoplastic lesions would lower non-specific toxicity and increase the clinical utility of DOX. DOXIL, a liposome encapsulated form of DOX, is used in the clinic to treat ovarian tumors\(^9\)\(^6\). More recently, Liu et al used pi-pi stacking to load DOX on SWNTs and showed improved efficacy over DOXIL in SCID mice\(^9\)\(^7\).

To actively deliver DOX to targeted cancer cells with fluorescent SWNTs, DOX was conjugated onto phage by forming a peptide bond between the free amine group on the drug and carboxylic acids (aspartic acid (D) and glutamic acid (E)) on the p8 major coat proteins of M13 (p8cs\#3)-SBP using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and Sulfo-NHS (Figure 8) after phage-SWNT complexation. To confirm DOX loading, the complex was precipitated by PEG-NaCl. The red color of DOX was used to visually confirm loading on M13-SBP-SWNT: after centrifugation, the SWNT-DOX pellet had a slightly red coloration while the SWNT pellet was black (Figure 14).
DOX loading was quantified by measuring optical absorption of the complex at 490 nm (the extinction coefficient of DOX at 490 nm is 23 cm$^2$/mg) (Figure 15a)$^{98}$. The quantity of loaded drugs on the M13-SBP-SWNT complex decreased as the overall ratio of SWNT to phage increased, eliminating the possibility of non-covalent loading of drug on the SWNT surface. An average of 390-450 DOX molecules were loaded per phage and DOX loading did not interfere with SPARC mediated targeting or SWNT fluorescence (Figure 15b). The final complex (M13-SBP-SWNT-DOX) is stable in PBS, and its cytotoxic potential was not diminished even after several weeks after complexation. This stable multifunctional phage construct allows for simultaneous targeted NIR imaging and cytotoxic drug delivery to tumor cells.
Figure 15: Doxorubicin conjugation to M13-SWNT complex. (A) UV absorption at 490 nm was used to quantify doxorubicin loading. (B) PL spectrum from M13-SBP-SWNT and M13-SBP-SWNT-DOX. Drug conjugation does not quench SWNT fluorescence, permitting simultaneous NIR fluorescence imaging and drug delivery.

In order to investigate the cytotoxic potential of DOX-SWNT-SPARC-phage, C42B (++ SPARC) and DU145 (- SPARC) cell lines were incubated with DOX-SWNT-SPARC-phage for 9 hours at 37 °C. Phage was removed, cells were washed with PBS and were subsequently grown for 14 hours in media. To evaluate cell viability, an MTT assay was performed and absorbance was read at 570nm. M13-SBP-SWNT-DOX drastically inhibited growth of SPARC-expressing C4-2B cells while it had a less significant effect on SPARC negative DU145 cells (Figure 16c), demonstrating targeted chemotherapy. In contrast, free DOX inhibited cell growth of both cell lines (Figure 16). Moreover, M13-SWNT-SBP-DOX was more than 245 times more efficient at inhibiting C42B cell growth than free DOX (Figure 16a). For C4-2B, the half maximal inhibitory concentration, IC₅₀, of free DOX was 29.5 µg/ml while that of M13-SBP-SWNT-DOX was 0.12 µg/ml. For DU145, the IC₅₀ for free DOX was 198 µg/ml while that of M13-SBP-SWNT-DOX was 28.9 µg/ml (Figure 16b). M13-SBP-SWNT did not significantly retard cell growth under the same condition used for the MTT assay for M13-SBP-SWNT-DOX, highlighting DOX as the mechanism of cell death rather than phage or SWNT.
Figure 16: Dose-response curves of M13-SBP-SWNT-DOX for C4-2B and DU145. (C) M13-SBP-SWNT-DOX inhibits growth of SPARC-expressing C4-2B cells about 35 times more efficiently than for SPARC negative DU145 cells. (A) Dose-response curves of M13-SBP-SWNT-DOX and free DOX for C4-2B. M13-SBP-SWNT-DOX is more than 245 times efficient at inhibiting C42B cell growth than free DOX, (B) while this difference is less pronounced in DU145 cells. IC\(_{50}\) values are shown in blue (A,B) and black (C).

<table>
<thead>
<tr>
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<th>C4-2B</th>
<th>DU145</th>
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<tr>
<td>M13-SBP-SWNT-DOX</td>
<td>Free DOX</td>
<td>M13-SBP-SWNT-DOX</td>
</tr>
<tr>
<td>0.120 µg/ml (0.52 µM)</td>
<td>29.5 µg/ml (220 µM)</td>
<td>28.9 µg/ml (219 µM)</td>
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Table 2: IC\(_{50}\) values for M13-SBP-SWNT-DOX and free DOX for C4-2B and DU145.

Time course of the cytotoxicity experiments is an obvious factor in determining efficacy and specificity of drug action. In order to quantify the impact
of incubation time on cytotoxicity, M13-SBP-SWNT-DOX complex was incubated with C42B cells for a fixed time (9 hours). Cells were washed with PBS and media was replenished. Cells were able to proliferate in media for 14, 24, 48 or 72 hours before the MTT assay was performed (Figure 17). The Szoka group at UCSF has shown that varying this second incubation time has a dramatic effect on the efficacy of doxorubicin treatment – as incubation time increases IC₅₀ decreases⁹⁹. Rather than an inverse relationship between IC₅₀ and incubation time, our experiments yielded a constant IC₅₀ regardless of incubation time, which could signal that the full cytotoxic potential of DOX is realized in the first 9 hours (Figure 17). Free DOX (Figure 17a) and M13-SBP-SWNT-DOX (Figure 17b) are shown for various time points. While the IC₅₀ remains constant, the steepness of the curves increases with along with incubation time. This means that lower doses of drug are less effective and higher doses of drug are more effective with increasing time. Rather than being a meaningful result, this outcome could be an experimental artifact. After the initial incubation with free DOX or M13-SBP-SWNT-DOX, a number of cells begin to undergo apoptosis. Those cells that survive this initial incubation proliferate quickly and repopulate the cells in the plate. At lower doses, more cells are left to repopulate the cell population, while at higher doses, more cells are undergoing apoptosis, and there are fewer cells to proliferate rapidly and mask the cytotoxic effects of the drug. The outcome of this experiment is poorly understood, and it may be more beneficial to vary the initial incubation with phage or free DOX in order to understand the kinetics of drug action.
Figure 17: Timecourse of doxorubicin mediated cytotoxicity. Cells were incubated with (A) free DOX or (B) M13-SBP-SWNT-DOX for 9 hours. After cells were washed and fresh media was replenished, cells were allowed to proliferate for 14-72 hours before the MTT assay was performed. Unexpectedly, this variable timecourse did not alter the IC50 of doxorubicin, indicating that the drug takes full effect in the first 9 hours.

2.2.3.1 Intercellular Proteases as a Mechanism for DOX Release

In order for doxorubicin to take effect, it must be released from the phage. Researchers have demonstrated that doxorubicin linked to an amino acid via a peptide bond can maintain its cytotoxic potential100. After observing DOX mediated cytotoxicity using M13, we hypothesized that the drug was cleaved from the phage via intercellular proteases. For example, the amino-terminus of the genetically engineered p8 protein may be cleaved by chymotrypsin, a serine protease abundant in mammalian cells. Chymotrypsin is known to selectively cleave the carboxyl-terminus of aromatic residues and may cleave the tyrosine (Y) residue in the p8cs#3 sequence (Figure 8). In order to test this hypothesis, cytotoxicity was studied in the presence of serine and cysteine protease inhibitors (Figure 18). C42B cells were incubated with free DOX (Figure 18a) or M13-SBP-SWNT-DOX (Figure 18b). As expected, the protease inhibitor had little effect on the IC50 of cells incubated with free DOX (Figure 18a), as there is no need for DOX release. In contrast, cytotoxicity of cells incubated with M13-SBP-SWNT-DOX and protease inhibitor was greatly attenuated as compared to phage in the absence of protease inhibitor (Figure 18b). This result supports the hypothesis that drug is released from the phage.
intercellularly by proteases and that the function of the drug is maintained even if it is attached to a short peptide.

![Graph](image)

**Figure 18:** Doxorubicin is released from phage by intercellular proteases. C42B cells were incubated with (A) free DOX or (B) M13-SBP-SWNT-DOX in the presence or absence of protease inhibitor cocktail (Roche) and cell viability was assayed using an MTT assay. While M13-SBP-SWNT-DOX mediated cytotoxicity was reduced by protease inhibitor, that of free DOX remained constant, indicating that the protease inhibitor is preventing drug release from phage.

In order to more precisely confirm the mechanism of release, M13-SBP-SWNT-DOX was digested in vitro with chymotrypsin, and the resultant digested peptide fragments were analyzed using MALDI-TOF mass spectrometry (Figure 19). Chymotrypsin is a digestive enzyme that preferentially cleaves peptide bonds on the carboxyl side of tyrosine, tryptophan and phenylalanine (Figure 19a). These amino acids contain an aromatic ring that fits into the hydrophobic pocket of the enzyme. Chymotrypsin is prevalent in mammalian cells and chymotrypsin mediated DOX cleavage is a plausible mechanism for drug release from phage. Phage was digested with chymotrypsin and the resultant peptides were resolved using a reverse phase C18 column. M/Z spectra were analyzed by integrating the signal for each peak over each m/z integer value. As such, the output from m/z 500 to m/z 3000 was condensed to 2500 points before analysis. When conducting data analysis, only peptides in the periplasmic domain of p8 were considered for chymotrypsin digestion, as they are available for cleavage on the exterior of the phage (Figure 19a). A number of peptides expected from chymotrypsin digested
were identified (Figure 19b), and these results highlight this intercellular protease as a potential mediator of drug release.

![Periplasmic Domain](image)

Figure 19: MALDI-TOF to confirm chymotrypsin mediated DOX cleavage. (A) The full protein sequence of M13 bacteriophage major coat protein p8 is shown. The protein is translated with a signal sequence, which is cleaved after translocation. The rest of the sequence is comprised of a periplasmic domain and a transmembrane domain. Here, I assumed that the chymotrypsin only digested the periplasmic region. The SWNT binding peptide is shown in blue, and the aspartic acid for DOX conjugation is shown in red. Chymotrypsin cleavage sites are delineated by vertical lines. (B) MALDI-TOF m/z spectra are shown for whole phage. Several peptides characteristic of chymotryptic digestion were detected.

### 2.3 Trifunctional Phage

In order to take full advantage of the utility of M13 bacteriophage, we engineered a phage with triple functionality: targeting, imaging, and cytotoxicity (Figure 20). This phage was designed via genetic engineering and was not coupled to SWNT. Protein p3 has been modified to include the SPARC binding peptide (SSPTGIN), enabling cell targeting. The genomic copy of the p9 protein has been
modified to include a pelB leader sequence and a biotin acceptor peptide (BAP). Streptavidin coated fluorophores can be easily added to p9. Further, protein p8 has been engineered to include a cathepsin-B cleavable linker sequence for drug release (DFK). Drug is loaded on the asparagine via EDC chemistry and can be released in the cell by protease cathepsin-B\textsuperscript{100}.

![Image of phage with SPARC, DFK, and Streptavidin FITC labels]

Figure 20: Schematic of trifunctional 983 phage. Phage was genetically engineered for triple functionality. A SPARC binding peptide was cloned into p3 for tumor cell targeting (SSPTGIN). P8 was modified to include a cathepsin-B cleavable sequence for drug release (DFK). Finally, p9 was modified with a biotin acceptor peptide (GLNDIFEAQKIEWHE) for conjugation of imaging agents.

Trifunctional phage (983 phage) was biotinylated and streptavidin coated FITC particles were loaded on p9. Phage was incubated with SPARC + C42B cells or SPARC - DU145. Cells were analyzed for FITC signal using flow cytometry (Data Not Shown). The signal is extremely low from these experiments; however, this is to be expected, as there are only 5 copies of p9 per phage. Despite the low signal, C42B have a level of FITC staining 4x higher than that of DU145, as expected. There are simply not enough fluorophores bound to the phage. This experiment should be repeated with fluorophores with a brighter signal. DOX was loaded on trifunctional phage using EDC chemistry. Phage was pegylated and centrifuged in order to demonstrate DOX binding (Figure 21). The phage pellets have a distinct red color, indicating robust DOX binding.
DOX release by cathepsin-B was quantified by digesting phage with the protease. After digestion, phage was precipitated by PEG-NaCl and the supernatant was assayed for DOX using UV-VIS. DOX concentration was 50% higher in those samples digested by cathepsin-B. While this result does not yield a definitive confirmation of DOX release, it does provide a candidate mechanism of release for further study.

2.4 Discussion and Future Work

The M13-SBP-SWNT-DOX platform allows for simultaneous active targeting, NIR second window imaging, and chemotherapy *in vitro*. The M13 scaffold stably disperses SWNTs without losing their optical properties. The engineered phage encodes for cell-targeting functionality and is a carrier for drug conjugation and delivery, obviating the need to conjugate functional moieties directly to SWNTs and potentially compromise their stability and fluorescence. The phage-based platform circumvents these potential issues and allows for spatial control of targeting ligands and drugs with SWNTs. This versatile technology is powerful due to the interchangeability of its components, permitting study of a wide array of cancers. Potentially, any identified targeting ligands can be readily exchanged onto p3 for molecular recognition of various cancers. Additional chemotherapeutic agents
could be added or substituted by chemical conjugation or molecular recognition for specific targeted therapy of many diseases. Targeted therapy reduces the therapeutic dose of the drug and may prevent off-target cytotoxic effects (cardiomyocyte death for DOX).

Further work must be done to characterize the mechanism of drug release from phage. While preliminary experiments have shown that this process may be mediated by intercellular proteases, this hypothesis should be confirmed more precisely. Phage can be digested \textit{in vitro} and a cocktail of proteases, and drug release can be quantified by mass spectrometry. In addition, specific proteases can be knocked down in cells using siRNA and cytotoxicity can be assayed using MTT.

In order to enhance the cytotoxic effects of this platform, the favorable optical properties of SWNTs can be used for thermal ablation of tumors. SWNT have recently been used both \textit{in vivo} and \textit{in vitro} to destroy tumors using photothermal effect\textsuperscript{63, 65, 102-106}. Thermal ablation is particularly useful in treating multidrug resistant tumor cells that are resistant to common DNA alkylating chemotherapeutic agents. These chemotherapeutic agents target highly proliferative cells that are constantly replicating DNA and are vulnerable to bioenergetic catastrophe\textsuperscript{107, 108}. However, slower growing cells are less susceptible to these therapies and may repopulate tumors after incomplete chemotherapy. Thermal ablation functions by exceeding physical cell tolerances to heat (greater than 55 °C) and elicits a coagulative necrosis which involves protein denaturation and membrane lysis, in all cells regardless of phenotype\textsuperscript{102}.

While our phage complex has shown success \textit{in vitro}, it must be further characterized \textit{in vivo}. Experiments conducted on mice with C42B, LnCAP, and DU145 tumors will hopefully show simultaneous imaging and ablation. Ideally, tumor shrinkage can be tracked using NIR fluorescence derived from M13-SBP-SWNT complex. Mice can be imaged immediately before and at several timepoints after treatment in order to quantify efficacy. Perhaps the most interesting dimension to this project is the ability of SWNTs to image in the "second window" with greater tissue penetration depth and minimal background from autofluorescence. In order to test this capability, tumors will have to be grown,
rather than implanted in a deep tissue such as the prostate or pancreas. In these experiments, it may be prudent to use a LOX-CRE system (Jacks Lab, MIT) to spontaneously induce a tumor in the prostate and image it using M13-SBP-SWNT-DOX.

A number of safety concerns surround the phage-SWNT complex. The safety of M13 in the clinic was recently shown in a phase I clinical trial to identify patient-specific ligands. The bacteriophage was non-toxic, had no adverse allergic response, and elicited 'submaximal' humoral immune response\textsuperscript{109}. Further experiments should be done to understand the immunogenicity of M13 in greater detail in a mouse model. Repeated doses of phage over a long timecourse should be conducted in order to understand the clinical potential of M13 for drug delivery and imaging. A recent study showed that mice administered orally or intraperitoneally with a high dose of short carbon nanotubes did not show any growth or behavior abnormalities\textsuperscript{110}. The long characteristic half-life of filamentous particles \textit{in vivo} coupled\textsuperscript{81} with the initial safety profile of M13 in humans\textsuperscript{109} and biocompatible SWNTs\textsuperscript{72} cast this platform as a promising candidate for facile imaging and detection of metastases and simultaneous monitoring of the efficacy of drug therapy regimens; this initial work suggests the potential use of M13 in a clinical setting.

2.5 Materials and Methods

**P8 phage-display Library Screening: a bio-panning**

For the bio-panning experiment, Hipco SWNTs were prepared in a form of thin films on glass substrates to maximize the direct contact of the virus to SWNT and a constructed p8 phage-display library was used. Ten µl of the library solution with 10\textsuperscript{10} viruses of 2 \times 10\textsuperscript{6} different p8 sequences were diluted with 250 µl of Tris-buffered saline (TBS, 100 mM Tris-HCl, 150 mM NaCl, pH 7.5) with different concentration of Tween 20 (TBS-T, Tween 20 concentration of 0.1-0.5 v/v%), applied to SWNT-films and incubated for an hour with gentle rocking. The SWNT-film was rinsed with one milliliter of TBS-T ten times to wash off unbound phage. Bound phage were eluted by 100 µl of 0.2 M Glycine-HCl, pH 2.2 and/or mid-log E.coli culture to harvest strongly bound virus not eluted by acid solution. The eluted phages were amplified and the same procedures were repeated.
for further rounds with increasing detergent concentration. After each round of panning, the numbers of eluted and amplified phage counted as PFU were measured using agar plates containing X-gal/isoproplβ-D-1-thiogalactopyranoside (IPTG)/tetracycline to set the input number of phage for each round the same. Also plaques from each round were amplified and DNA sequenced. DNA sequencing was done at MIT Biopolymers lab.

**Binding affinity test**

In the binding test, each virus clone with different concentrations was mixed with sodium-cholate dispersed SWNTs in water and dialyzed against TBS (100 mM Tris-HCl, 150 mM NaCl, pH 7.5, Tween 0.3 v/v%) using 96-well microdialyzer (Spectrumlabs.com) for 3 days with frequent buffer exchanges. Sodium-cholate dispersed SWNTs without any virus clones were also dialyzed as a negative control. After the dialysis, each solution was harvested and transferred to a new 96-well plate for visual comparison.

**Zeta potential measurement**

The concentration of virus solution used was 10^{12}/ml in water with 10 mM NaCl. The stock solution of virus (~ 10^{14}/ml) was initially dissolved in 10 mM Tris 15 mM NaCl before diluting in 10 mM NaCl in ddH_{2}O. The solution amount used to generate curve was 30 ml. The ionic concentration of the solution was set to 10 mM NaCl for all samples. The pH was then adjusted using 0.1 M NaOH until the pH was around 10. Zeta potential measurements were then made at an accumulation time of 15 with 5 measurements per sample at 20 V using DelsaNano (Beckman Coulter). Electrophoretic mobility was calculated using the Smoluchowski approximation (used for particles larger than 0.2 μm in 1 mM or great salt solution). pH was then adjusted with 0.1 M HCl, and zeta potential measurements were made at pH increments of 1 until pH 6-6.5. Then measurements were made at pH increments of 0.5.

**Genetic engineering for M13-SBP**

SPARC binding peptide (designated as SBP), SPPTGINGGG^{93}, was used for specific binding to SPARC. Oligonucleotides, 5' [Phos]-GTA CCT TTC TAT TCT CAC TCT TCA CCA CCG ACT GGA ATT AAC GGA GGC GGG TC -3' and 5' [Phos]-GGC CGA CCC GCC TCC GTT AAT TCC AGT CGG TGG TGA AGA GTG AGA ATA GAA AG-3', purchased from IDT (idtdna.com) were annealed to form a DNA duplex.
The cloning vector was extracted from virus p8cs#3 using standard miniprep kit (QIAGEN). The extracted vector was digested with Eag I and Acc65 I enzymes and dephosphorylated and agarose-gel purified. Purified vector and DNA duplex were ligated using T4 DNA ligase at 16°C overnight and electro-transformed to electro-competent XL-1 blue cells. Transformed cells were incubated for 1 hr and plated and incubated at 37 °C overnight. Blue plaques were amplified and DNA sequenced to confirm the insertion of oligonucleotides to express SBP on p3.

**Preparation of starting SWNTs**

As-produced and non-acid treated HiPco single-walled carbon nanotubes (SWNT), purchased from Unidyme, was diluted in a 2wt% sodium cholate aqueous solution. The diluted solution was homogenized for 1 hour, coup-horn sonicated for 10 min at 90% amplitude and then ultra-centrifugated at 30,000 rpm for 4 h to get individually dispersed SWNT. SWNT concentration was calculated using the extinction coefficient of HiPco SWNT at 632 nm, $e_{632}\text{nm}=0.036 \text{ L/mg} \cdot \text{cm}$. A final concentration of the SWNT solution, ~20-40 μg/ml was used in this study. This protocol produces a singly dispersed SWNTs with dimension about 1 nm in diameter and ~500 nm in length.

**Complexation of M13 with SWNTs**

1 ml of p8cs#3 virus solution with a concentration of $1.4 \times 10^{14}/\text{ml}$ was mixed with 4 ml of SWNT with concentration of 40 μg/ml. The solution was dialyzed for three days using dialysis membrane, MWCO of 12,000-14,000 (SpectraLabs.com). The dialyzing solution started with water with 10 mM NaCl and 0.64 mM NaOH. After the first three buffer exchanges, the ionic concentration of the buffer was step-wisely increased from 10 mM to 150 mM and PBS was used after water with 150 mM NaCl.

**PL measurement**

PL from SWNT was measured with a home-built near-infrared (NIR) PL microscope. An inverted microscope was coupled to a Princeton Instruments OMA V 1D InGaAs array detector through a PI Acton SP2500 spectrometer. As excitation sources, a 785 nm laser and a Xe lamp coupled to a monochromator were used for PL spectra and three-dimensional profiles, respectively. Detection ranges were 900 nm -1,350 nm. For PL imaging of targeted cells, nanotubes in the cells were excited by 658 nm laser and imaged...
and monitored using inverted microscope w. h a Princeton Instruments OMA V 2D InGaAs array detector and an AxioCam MRn charge-coupled device (CCD) camera.

**HRTEM**
For high-resolution transmission electron microscopy, JEOL 2010F TEM was used. For TEM analysis, virus-SWNT solutions were dropped on Cu Quanti-foil holy grid (TedPella), washed with ddH2O several times and dried.

**Cell lines and culture**
DU145 and C4-2B human prostate cancer cell lines were provided courtesy of Dr. Kimberly Kelly (University of Virginia). LNCaP human prostate cancer line was given as a gift by Dr. Juliana Chan (Massachusetts Institute of Technology). DU145 was grown in Dulbecco’s Minimum Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) and 1% penicillin/streptomycin (Invitrogen, CA) at 37 °C in 5% CO2. C4-2B were grown in T-medium (Invitrogen) with 10% FBS and penicillin/streptomycin. LNCaP were grown in RPMI medium (without phenol red), supplemented with 10% FBS, 1% penicillin/streptomycin, 1% sodium pyruvate, and 1% HEPES buffer.

**PL Imaging in vitro**
500,000 DU145, LNCaP, and C4-2B cells were plated on poly-lysine coated 35 mm glass bottom plates (MatTek Corporation). Cells were incubated with 2 ml of 0.29 µg/mL M13-SWNT in Dulbecco’s phosphate buffer saline (DPBS) with calcium and magnesium (Hyclone) for 12 h at 37 °C. Cells then were washed three times and resuspended with DPBS. For imaging, signal were collected for 5 -20 s.

**Targeting in vitro using PL spectrometry**
1,000,000 DU145, LNCaP and C42B cells were plated on 6-well plates (BD Biosciences) and incubated with 2 ml of 1.8 µg/mL M13-SWNT solution. Samples were washed three times with DPBS and harvested with trypsin (Hyclone). Cells were spun down and resuspended in 100 µL DPBS. Samples were placed in a 96-well glass bottom plate (MatTek) and PL spectra were measured in the wavelength of 1,000 – 1,350 nm. Samples were excited for five minutes. Spectra were subtracted against background. Cells were counted using a ViCell analyzer (Beckman Coulter, CA) and used to normalize PL intensity measurements. Samples were measured in triplicate.
DOX conjugation onto M13-SWNT-SBP

Doxorubicin (DOX) hydrochloride was purchased from Sigma-Aldrich and EDC and Sulfo-NHS were purchased from Thermo Scientific. 0.4mg of EDC and 1.1mg of sulfo-NHS were incubated with 10^13/ml M13-SWNT-SBP for 15 minutes in 3x EDC activation buffer (.3M MES, 1.5M NaCl, pH 6.0). The EDC reaction was quenched with 20mM 2-mercaptoethanol (Thermo Scientific). DOX was added at 50~100 µg/ml for the conjugation reaction and the reaction was allowed to proceed for 2 hours at room temperature in a fume hood. The conjugation was quenched with 10mM hydroxylamine to hydrolyze unreacted NHS and regenerate original carboxyl groups. After conjugation, the reactions was dialyzed against PBS for 2 days using dialysis membrane, MWCO of 12,000 - 14,000, (SpetraLabs.com) with frequent buffer exchange.

Cytotoxicity Assay

To evaluate cytotoxicity, M13-SWNT-SBP-DOX and M13-SWNT-SBP were incubated with C4-2B and DU145 cells for 9 hours at 37 °C in 50% DPBS, 50% PBS. Complexes were removed and cells were washed with PBS and were subsequently grown for 14 hours in complete DMEM or TMEM. An MTT assay was used to quantify cell growth. Cells were incubated with 12 mM MTT reagent for 4 hours. Cells were then washed and incubated with SDS-HCL for another 4 hours. The reaction was mixed, and the absorbance was read at 570 nm. Samples were measured in triplicate.

MALDI-TOF Mass Spectrometry

M13-SBP-SWNT-DOX was digested with chymotrypsin in order to understand the drug release mechanism. Chymotrypsin was reconstituted at 1mg/ml in 1mM HCl solution. Phage was incubated in digestion buffer (5x 500mM ammonium bicarbonate, 10mM CaCl, 500mM Tris HCl) in a 1ml total volume and pH was adjusted to 8.0. Cysteines were reduced using 10mM DTT, incubated at 60 °C for 45 minutes. After cooling to room temperature, 500mM iodoacetamide was added as an alkylating agent. This reaction is allowed to proceed for 30 minutes and was quenched with DTT. After alkylating and reducing the phage it was digested with chymotrypsin (1mg/ml) for 24 hours at 37 °C. Samples were stored at -80 °C until mass spectrometric analysis. Immediately before analysis, samples were resolved using a C18 column (Thermo Scientific).
**Mass Spectrometry Data Analysis**

Raw data from MALDI-TOF was bucketed into 1m/z increments by integrating spectra intensities. As such, the data set was condensed to 2500, each at a m/z integer value between 500 and 3000. This simplified data set was analyzed in Matlab.

**Cathepsin B digestion**

3 units of cathepsin B were activated at room temperature for 15 minutes in activation buffer (15mM EDTA 30mM DTT) in 30 ul total volume. Digestion buffer (25mM sodium acetate, 1mM EDTA, ph5.0) was pre-warmed to 37 °C. Phage and cathepsin B were added to the buffer and incubated at 37 °C for 24 hours. Phage was precipitated by incubation with 25% PEG-NACL on ice overnight and centrifugation at 10,000 RPM for 10 minutes.
Acknowledgements

I would like to acknowledge those who have helped with the completion of this project. I would like to thank Hyunjung Yi and Rana Ghosh for their contributions and guidance during the theranostic phage project. Hyunjung pioneered the coupling of SWNT to M13 and worked with Rana on the in vitro imaging experiments. I worked closely with Hyunjung on the cytotoxicity experiments. I would especially like to thank Rana for his mentorship over the years and for entertaining discussions on both science and sports. Finally, I would like to thank Angela Belcher for the opportunity to pursue this research during my M.Eng.
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