Binding, Delivery, and Targeting: Improving Diagnostic and Therapeutic Technologies

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BINDING, DELIVERY, AND TARGETING: IMPROVING DIAGNOSTIC AND THERAPEUTIC TECHNOLOGIES

By

Nelson R. Salgado

A THESIS

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Master of Science

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Master of Science

BINDING, DELIVERY, AND TARGETING: IMPROVING DIAGNOSTIC AND
THERAPEUTIC TECHNOLOGIES

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Binding, Delivery, And Targeting: Improving Diagnostic and Therapeutic Technologies

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Advancements in diagnostics and therapeutics arise from acquiring new knowledge of biochemical and molecular processes. These breakthroughs can mean a quicker and more precise diagnosis, more efficient therapeutic treatments and a better quality of life for patients suffering from a disease. To better serve patients, scientists must find new and improved diagnostic tools and treatments. This starts by recognizing a shortcoming of the current methodologies or an unmet need in the medical field. The aim of the research of this thesis is to implement bionanotechnology strategies to generate new technologies to aid in the diagnosis and treatment of certain health conditions. The studies herein focus on binding of molecules to ligands, formulating delivery vehicles for drug transport, and targeting therapeutics to sites of injury.

In the first part of the thesis, work toward developing new diagnostic/sensing systems for bacterial-related diseases was performed. In that regard, we focused on understanding ligand binding to further unravel the mechanism of bacterial communication by using Pseudomonas aeruginosa as
the model system. Specifically, the work centered on developing a strategy for the preparation of the quorum sensing protein LasR, the sensing protein that we planned to use in our studies for the development of biosensors as diagnostics tools for bacterial disease or diseases with a heavy bacterial component. Protocols were optimized to obtain the soluble LasR ligand-binding domain (LBD) in the unbound state, which is essential for performing binding studies of the protein with the quorum sensing molecules of the bacterial communication system of *Pseudomonas aeruginosa*.

Next, the focus centered on the development of drug delivery technologies for small drugs and for stem cells. The first project aimed at designing a drug delivery system for transdermal administration of a small well-known drug. Specifically, the need to administer the erectile dysfunction drug sildenafil citrate locally resulted in the formulation of a liposomal delivery platform that shows promising characteristics. In a separate project, we addressed the current need for improved inflammatory bowel disease treatments by developing a nanocarrier platform for the targeted delivery of stem cells to the gut. The developed approach has shown in mice models of inflammatory bowel disease its ability to selectively home stem cells to the site of injury and inflammation in the intestine of the mice.
Acknowledgements

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Chapter 1: LasR Binding Studies and Generation of a Biosensor

1.1 Background

LasR and the Pseudomonas aeruginosa Quorum Sensing System

_Pseudomonas aeruginosa_ (P. aeruginosa) is an opportunistic Gram-negative bacterium that is very problematic for immunocompromised individuals; in particular, cystic fibrosis patients [Oliver, 2000]. The health danger of _P. aeruginosa_ arises when it becomes virulent, making it more resistant to medication and the human body’s immune system. Virulence is controlled by the bacteria’s quorum sensing (QS) system, which allows it to sense the population density in its environment [Schembri, 2003]. Once a certain threshold has been reached, the QS system activates genes that promote more aggressive pathological strategies, like biofilm formation and the release of virulence factors [Rumbaugh, 2000].

There are two QS systems in _P. aeruginosa_, las and rhl, which are responsible for the activation of various genes when they sense their respective quorum sensing molecules (QSMs) [Smith, 2003]. For the las system, the QSM is N-[3-oxododecanoyl]-L-homoserine lactone (3-oxo-C12 HSL), which directly binds to the LasR protein [Van Delden, 1998]. This binding happens in a region of the LasR protein termed the ligand-binding domain (LBD), which incorporates the first 173 amino acids of the protein sequence (figure 1.1.1). The binding pocket is composed of a region that interacts with the lactone head of the 3-oxo-C12 HSL through hydrogen bonding and a hydrophobic region that interacts with
the acyl tail of the ligand further stabilizes its docking (figure 1.1.2) [Bottomley, 2007].

Previously, our laboratory studied the role of the neurotransmitter serotonin (5-Hydroxytryptamine, 5-HT) in the activation of quorum sensing pathways. Through the use whole-cell biosensors, in vitro bacterial assays, and in vivo mouse models, our group was able to show that 5-HT activates the quorum-sensing network of P. aeruginosa; specifically, we demonstrated that this activation occurs through the LasR pathway [Knecht, 2016]. In order to further elucidate the mechanism of action of the hosts' neurotransmitters on the bacterial communication network, further understanding and characterization of the direct binding of 5-HT to the LasR protein needs to be achieved. The aim of the project described herein was to elucidate the binding of 5-HT to LasR. Additionally, this project aimed at creating a biosensor to study other possible activators of this quorum-sensing pathway. It was envisioned that this type of sensor would allow for the screening of various molecules that might interact with LasR and activate this pathway, providing with a tool for the study of the interaction between bacteria, their hosts, and the environment.
Figure 1.1.1 Structures of Quorum Sensing Molecules and LasR-LBD. A) C4-HSL is the quorum-sensing molecule (QSM) of the rhl network in *P. aeruginosa*. B) 3-oxo-C12 HSL is the QSM of the las system. C) Structure of the ligand-binding domain (LBD) of the LasR protein. Structure was obtained using the on-line tool SWISS-MODEL (Biasini, Marco, et al. 2014).

Figure 1.1.2 Docking of 3-oxo-C12 HSL in LasR-LBD. Docking of the QSM 3-oxo-C12 HSL is stabilized by the proximity of the acyl tail to the β-sheet of the structure shown, which is composed of various hydrophobic residues. LasR-LBD structure obtained from SWISS-MODEL based on the work by the Bottemley group.
1.2 Methods

Ligation Independent Cloning

Primers were designed according to the Novagen Xa/LIC kit protocol; they contained sequences complementary to the LasR ligand-binding domain (LBD), overhangs complementary to the pET-30 Xa/LIC vector, and a stop codon (fig. 1.2.1). The pET-30 Xa/LIC vector allows for the generation of a fusion protein incorporating a histidine tag and S tag, for purification and identification, at the N-terminal and an additional His tag at the C-terminal of your insert. Protein expression is IPTG (Isopropyl β-D-1-thiogalactopyranoside) inducible due to the presence of the lac operator in the plasmid. The plasmid also contains a gene offering kanamycin resistance for positive selection (fig. 1.2.2).

The designed primers were used to amplify the LasR-LBD from a strain of E. coli used in the lab as a whole cell sensor, pSB1075, which contain a plasmid with the lasR gene. The amplification was carried out using KOD hot start mix with thermal cycler settings of 95°C for 2 minutes followed by 24 cycles of 95°C for 20 seconds, 82°C for 10 seconds, and 70°C for 20 seconds. The resulting PCR product was ran on a 1% agarose gel to separate and excise the LasR-LBD insert DNA band (512 bp). Protein was purified from the excised band by using a gel extraction kit (Qiagen).

The purified LasR-LBD insert was treated with T4 DNA polymerase to generate the vector-compatible overhangs according to the user protocol. Annealing of insert and vector was accomplished by mixing and incubating the two for 5 minutes at 22°C followed by addition of 6.25 mM EDTA and a
subsequent 5 minute incubation at 22°C. Finally, the annealed reaction was transformed into NovaBlue GigaSingles competent cells and screened by colony PCR using insert specific primers.

To generate plasmid, overnight cultures of the competent cells were grown and the plasmid was isolate by following Qiagen’s plasmid mini-prep protocol.

**Figure 1.2.1 LIC Primers.** Primers were designed using Serial cloner to find LasR-LBD complementary sequences and to predict melting temperatures.

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<td>Forward</td>
<td>5’ ggtattgaggggtcgcatggcccttggttacggttttt 3’</td>
<td>Vector compatible overhangs</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’ agaggagagtagtagcctcatttgtcgtaccgatgcga 3’</td>
<td>Start codon</td>
</tr>
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**Figure 1.2.2 pET-30 Xa/LIC Vector Map.** Plasmid from Novagen, showing important sites. LasR-LBD insert also shown. His = histidine.
Transformation into Expression Host

LasR-LBD plasmid was transformed into BL21(DE3)pLys S competent cells. This strain of *E. coli* is engineered to be lysogenic for the λ-DE3 prophage, which encodes for T7 RNA polymerase under the control of the *lac* UV5 promoter. Additionally, this strain of bacteria contains pLys S; encoding for T7 lysozyme which can inhibit basal levels of T7 RNA polymerase. This allows for tighter control over target protein expression.

A stock vial of BL21(DE3)pLys S from Promega was thawed over ice before adding 45 ng of plasmid DNA and incubating in ice for 5 minutes. Cells were heat shocked at 42°C for 30 seconds before being placed back on ice for 2 minutes. SOC media was added and cells were incubated at 37°C, 250 RPM for 1 hour. Bacterial solution was spread on LB agar plates containing 35 µg/mL kanamycin to select for colonies containing the plasmid. These colonies were used to make glycerol stocks.

Protein Expression

Cultures of BL21(DE3)pLys S containing the LasR-LBD plasmid were made using 5 mL of terrific broth and 50 µg/mL kanamycin. They were incubated overnight at 37°C, 250 RPM. Following day the cultures were refreshed with 500 mL of terrific broth and 50µg/mL kanamycin. Cultures were incubated at 37°C, 250 RPM until reaching an OD$_{600}$ of approximately 0.5 before inducing the expression of the LasR-LBD with 0.5 mM IPTG. Induction of protein was allowed to proceed at 30°C, 250 RPM for 4 hours. Cell pellet was collected by centrifuging at 10,000 x g for 10 minutes at 4°C. Pellet was resuspended in lysis
buffer (50 mM Tris HCl, 100 mM NaCl, 1 mM EDTA, pH 8) and 10 µL of Lysonase reagent was added to break down the cell walls and DNA, thereby, allowing for higher protein recovered. Solution was sonicated on ice for 15 minutes (1 second on, 1 second off) to lyse cells. Lysed cell solution was centrifuged at 16,000 x g to collect inclusion bodies (IB). IB pellet was resuspended in denaturing buffer (100 mM NaH$_2$PO$_4$, 10 mM Tris HCl, 8M Urea, pH 8) and incubated at room temperature for 30 minutes.

**Ni-NTA Protein Isolation and On-column refolding**

One of the features of the pET-30 vector is the addition of a 6 histidine tag at the N-terminal of the expressed protein. The imidazole side chain of the histidine residue readily forms bonds with the transition metal Nickel ions in the Ni-NTA agarose beads, allowing for the separation of tagged proteins [Bornhorst, 2000]. To this end, 2 mL of Ni-NTA resin was added to a 15 mL gravity column and washed with 10 mL of MilliQ water followed by 4 mL of denaturing buffer.

The denatured protein solution was added to the column and incubated on a rotator at room temperature for 1 hour. Flow through was collected for further studies. Bound protein was incubated with 5 mL of 6 M urea solution (100 mM NaH$_2$PO$_4$, 10 mM Tris HCl, pH 8) for 5 minutes before allowing it to flow through and collecting sample. Step down refolding was accomplished by repeating this with 4 M, 2 M, 1 M, 0.5 M urea solutions. The final solution used to elute the protein from the Ni-NTA column was PBS with 300 mM imidazole to out compete the histidine side chains.
Protein Solubility

The LasR-LBD is soluble in the presence of its natural ligand, N-3-oxododecanoyl-L-homoserine lactone (3-oxo-C12), and has provided a crystal structure to study binding interactions [Bottomley, 2007]. However, in the absence of its natural ligand, obtaining soluble LasR-LBD has proven to be difficult resulting in various optimization attempts.

Expression of the LasR-LBD was induced under the presence of 10 µM 3-oxo-C12, 1 mM 5-HT, or alone, in 50 mL LB broth. Bacterial cells were lysed using EMD Millipore’s BugBuster Protein Extraction reagent with lysonase. Millipore’s protocol was followed to separate inclusion bodies. Soluble protein fraction was added to a Ni-NTA column to isolate tagged LasR-LBD as described above. Column was washed twice with wash buffer before eluting with 250 mM imidazole. Aliquots were taken and mixed with equal volumes of Laemmli sample buffer containing 2-mercaptoethanol to reduce disulfide bonds. Samples were added to a Tris-glycine gel for SDS-PAGE and subsequent Coomassie protein stain (fig. 1.3.1)

SDS-PAGE and Coomassie Stain

To visualize the presence of LasR-LBD and to estimate purity of protein solution, protein gels were ran and stained. In short, 20 µL aliquots were taken from samples and mixed with 20 µL of Laemmli buffer (with 355 mM 2-mercaptoethanol) from Bio-Rad. The solutions were incubated at 90°C for 5 minutes to denature the proteins before being placed on ice for 10 minutes.
Protein solutions (30 µL) were added to 4 – 20% Tris-glycine gels from Bio-Rad and placed under a voltage of 175 V for 30 minutes. Gels were immersed in Coomassie staining solution (40% MeOH, 7% glacial acetic acid, 0.0125 % w/v coomassie dye) and heated in microwave for 1 minute before incubating on rocker at room temperature for 1 hour. To remove excess stain, the gel was transferred to destaining solution (40% MeOH, 7% glacial acetic acid) and incubated on a rocker at room temperature overnight. Gels were imaged on an illuminated surface.

**BCA Protein Concentration Analysis**

The bicinchoninic acid assay (BCA) is a way to quantify the concentration of protein in a solution. This is accomplished through the reduction of Cu\(^{2+}\) ions to Cu\(^{+}\) by the peptide bonds of the proteins. The bicinchoninic acid chelates the Cu\(^{+}\) ions forming a complex, which strongly absorbs light at the 562 nm wavelength. The reaction is incubated at 37°C to increase the rate, allowing for increased sensitivity and reducing the effect of unequal amino acid composition [Olsen, 2007].

Pierce’s BCA Protein Assay Kit was used to quantify solutions after protein expression. The kit protocol was followed for microplate assays with one exception; the standard vials were not used. Instead, bovine serum albumin (BSA) standards were made by dissolving 2 mg BSA in 1 mL of the same buffer used for the unknown samples and performing a serial dilution to obtain the more diluted standards. Standards and samples were run in triplicate in a black clear bottom 96 well plate.
Tryptophan Fluorescence Binding Study

Tryptophan (Trp) residues are responsible for a great deal of the intrinsic fluorescence of proteins. In some cases, for example, when there is a tryptophan residue in the binding pocket of a protein, the binding of ligands can cause a change in the fluorescence due to a change in the local environment of the residue [Pollard, 2010]. The LasR binding pocket has a tryptophan residue that interacts with the lactone carbonyl group of the 3-oxo-C12 HSL [Bottomley, 2007]. With this in mind, Trp fluorescence experiments were carried out.

Solutions were made, at various concentrations, of the LasR ligand 3-oxo-C12 HSL by serial dilution in PBS. These solutions were mixed with the LasR-LBD protein that was expressed in the absence of ligand and purified as described in the on-column refolding section, to yield final concentrations of 11.7 µM LasR-LBD and C12-HSL at 1nM, 100 nM, 1 µM, and 10 µM. The various solutions were incubated at room temperature for 10 minutes before measuring fluorescence. Samples were transferred to quartz cuvettes prior to excitation at 295 nm. Emission scans were taken from 305 – 500 nm (fig. 1.3.2)

1.3 Results

Characteristics of the newly prepared LasR-LBD

LasR-LBD was expressed in the presence of 5-HT or alone and compared to expression with its natural ligand, 3-oxo-C12 HSL. The soluble fractions were isolated and probed for the presence of the correct protein by SDS-PAGE and Coomassie stain. There was no band observed for the LasR-LBD expressed in
the presence of 5-HT or alone as opposed to expression in the presence of 3-oxo-C12, which gave a strong band at approximately 24 kDa (fig. 1.3.1).

**Figure 1.3.1 Solubility of LasR-LBD in the presence of 5-HT.** Protein expression was induced with 0.5 mM IPTG for 4 hours before lysing cells and collecting soluble fractions. **A)** Expression in the presence of 10 µM 3-oxo-C12, **B)** alone, or **C)** 1 mM 5-HT. 4 to 20% Tris-glycine gels stained with Coomassie dye.

**Binding Properties of the LasR-LBD**

Refolding of the isolated LasR (unbound) in the column resulted in improved solubility (fig. 1.3.2), yielding a solution with a protein concentration of approximately 100 µg/mL as measured by BCA. This protein was used in a tryptophan fluorescence assay to study the binding of the LasR ligand 3-oxo-C12 by mixing the protein and ligand and exciting at 295 nm. Emission scans were recorded from 305 to 500 nm on a PTI fluorometer.

The addition of the natural LasR ligand, 3-oxo-C12 HSL, resulted in a reduction of the tryptophan fluorescence peak intensity in a dose-dependent manner. These preliminary studies suggest that the reduction in fluorescence intensity upon ligand binding to the LBD is most likely due to the interaction with the tryptophan residue in the binding pocket. Further experiments need to be performed to confirm these observations. It should be noted that this method
cannot be employed to study binding interactions with serotonin because serotonin emits fluorescence in the same wavelength as tryptophan, which is a precursor in the synthesis of serotonin. However, it could be used to screen for other molecules that activate or deactivate the quorum sensing system and are not inherently fluorescent.

Figure 1.3.2 Soluble unbound LasR-LBD after refolding. Protein was expressed without ligand and denatured. Gradual refolding achieved while in the Ni-NTA column. LasR-LBD size with tags is approximately 24 kDa.
1.4 Conclusions and Future Studies

Our group has previously shown that 5-HT can activate quorum sensing in the LasR whole cell sensing system. In this work, we attempted to further characterize this interaction by isolating soluble LasR-LBD, first bound to 5-HT, and later to its natural ligand 3-oxo-C12 HSL. While LasR-LBD could not be isolated in the presence of 5-HT, we were able to accomplish our goal with 3-oxo-C12 HSL. Even at 100 times the concentration of LasR’s natural ligand, addition of 5-HT did not result in any observable protein band in the soluble fraction. This could be due to the lower binding affinity of 5-HT for LasR-LBD as opposed to its natural ligand, 3-oxo-C12 HSL [Knecht, 2016]. Another possibility
is that 5-HT binds to a different binding pocket on the LasR protein that is not part of the LBD. The LasR-LBD construct only incorporates the first 173 residues of the natural LasR protein. It could be possible that 5-HT interacts in an area that was not expressed. To further explore this possibility, constructs can be generated that express the full LasR protein or the DNA binding domain. Expression of the protein constructs could then be performed in the presence of 5-HT and, after that, the solubility experiments should be designed and implemented.

Another goal of this project was to obtain soluble LasR-LBD unbound. To this end, various techniques were employed to improve the solubility of the protein alone. Performing on-column refolding during the Ni-NTA column isolation resulted in small quantities of soluble protein. This was used for a single tryptophan fluorescence experiment to attempt a quick sensing method for detecting binding. Addition of the LasR ligand, 3-oxo-C12 HSL, resulted in a measurable decrease of the tryptophan fluorescence peak intensity. This suggests that molecules docking to the LBD can indeed influence the tryptophan residue located in the binding pocket, leading to the generation of a possible sensing system. While further experiments are needed to characterize and fully develop this biosensing system, we envision that this method can be utilized for screening of molecules that can act as activating or inhibiting ligands of the P. aeruginosa bacterial communication system. With the ever increasing resistance to antibiotics, new therapeutic agents are needed. To that end, this
biosensing system could be useful in identifying molecules with antibacterial therapeutic properties.

The low solubility of this protein when not bound to its natural ligand, adds to the complexity of its preparation, making it difficult for the design of biosensing platforms that employ it as a sensing element. Addressing this drawback will require that the LasR-LBD be re-constructed as a fusion protein. For example, a potential solution would be to genetically fuse a solubility tag, such as the maltose binding protein or MBP, that can act as a chaperone to allow proper folding [Costa, 2014]. Another option for the future direction of this project is to generate a fusion protein that places the LasR-LBD on the surface of a bacterial spore by generating a fusion protein that includes a leading protein for the display and a reporter protein for measuring of protein-ligand interactions. In this scenario the LasR-LBD will be sandwiched between a spore surface protein and a fluorescent protein like GFP. We previously have shown that binding of a molecule to the LasR-LBD causes a structural change that leads to a decrease or increase in measured fluorescence [Knecht, 2011]. This technique has been employed previously by our laboratory and can serve as a way to circumvent the solubility issues, as well as to generate a biosensor that is an improvement over the already available whole-cell sensors [O’Connor, 2015].
2.1 Background

Erectile Dysfunction

Erectile dysfunction (ED) is a chronic condition common in aging males where there is an inability to maintain a penile erection, which is necessary for satisfactory sexual performance [NIH, 1993]. Because of the many processes that are involved in producing and maintaining an erection, the cause of erectile dysfunction can be attributed to many different reasons, including a neurologic failure to initiate the erection, an arterial failure to fill, or a venous failure to store blood in the male organ (figure 2.1.1A) [Dean, 2005]. The average incidence rate of erectile dysfunction is 26 new cases for every 1000 men annually. Erectile dysfunction is dependent on age, socio-economic state, and evidence of diabetes or heart disease [Feldman, 2000].

Erections require the flow of blood into the corpora cavernosa [Boolell, 1996]. Blood flow is controlled by the release of nitric oxide in the penis, which acts in smooth muscle cells to generate cyclic guanosine monophosphate (cGMP). This leads to the outflow of calcium ions resulting in smooth muscle relaxation (figure 2.1.1B). With the muscle in a relaxed state, the arteries dilate allowing increased blood flow into the penis [Goldstein, 1998]. A negative regulator in this pathway is cGMP-specific phosphodiesterase type 5 (PDE5), which catalyzes hydrolysis of cGMP to GMP [Turko, 1999]. Because of this, the major forms of treatment for ED are inhibitors of PDE5.
Current Treatment of Erectile Dysfunction

Current treatments for ED include oral drugs and intracavernosal/intraurethral medications. For example, alprostadil is a synthetic PGE$_1$ analogue that can increase the concentration of cAMP, which also leads to increased blood flow to the penis. This drug is usually administered as a gel suppository or as an injection [Hatzichristou, 2001]. Because of the discomfort associated with the administration routes, this drug is labeled as a second-line therapy.

First-line treatments refer to the less invasive oral options, which include the PDE5 inhibitors sildenafil, tadalafil, and vardenafil, in addition to apomorphine SL that stimulates the dopamine receptors that result in penile erection [Hatzichristou, 2001]. From these oral treatments, sildenafil citrate was the first effective treatment for ED in clinical studies [Goldstein, 2001]. Despite its success as an FDA approved first-line treatment, systemic administration of sildenafil citrate has various side effects, which can be serious depending on the situation. Some of the more common side effects include flushing, headache, nasal congestion, and visual changes [Moreira, 2000]. The more serious side effects include hypotension and heart attack, which can occur more frequently in individuals with pre-existing cardiovascular disease and who are on nitrates [Cheitlin, 1999]. This generates a need for a local administration of sildenafil to reduce the likelihood of off-target effects by avoiding systemic circulation and lowering the required dose. Additionally, because of the sensitive area of administration, a more gentle delivery route would be optimal.
Transdermal Delivery

Transdermal delivery has many advantages over other delivery routes. Administering a drug through the skin compared to oral methods avoids the first pass effect of the liver, which can metabolize the drug before it reaches its destination [Prausnitz, 2008]. This often requires the administration of high doses of a drug to take into account for the liver inactivation. In contrast, localized targeted delivery of a given drug can result in lower doses needed to achieve the desired efficacy. Compared to localized injections of drugs, transdermal delivery is pain free and does not generate medical waste or cause any risk of irritation or infection from the use of needles.

The major obstacle to transdermal delivery of many drugs is the hard-to-penetrate most superficial layer of the skin, the stratum corneum (SC) [Naik, 2000]. The SC is composed of corneocytes, terminally differentiated keratinocytes, anchored in a highly lipophilic extra cellular matrix (ECM) [Christophers, 1971]. This lipophilic environment along with the highly structured arrangement of the corneocytes is responsible for the SC’s strong barrier function; reminiscent of a brick and mortar assembly in a building’s construction [Elias, 1981].

There are many options that have been developed for the transdermal delivery of drugs. Dissolving the drug in a solution containing chemical agents that can temporarily disrupt the SC, like ethanol or sulphoxides, is one method used to delivery therapeutics. A side effect of these SC disrupting agents is skin
irritation. Another method utilized to ease the passage of drugs through the skin is iontophoresis, where a small current is generated in the skin to push ionized drugs through the skin [Naik, 2000]. This option is not easily feasible in the context of erectile dysfunction. It would require at the very least the placement of a patch containing the electrodes and the drug, which is difficult to achieve on a flaccid penis. Thus, a more suitable transdermal delivery method is required to effectively deliver drugs into the penis for therapeutic effect in erectile dysfunction.

**Liposomes as Drug Delivery Agents**

Liposomes are bilayered vesicles made of phospholipids; just like the membranes of cells (figure 2.1.3) [Dua, 2012]. These vesicles have been utilized in transdermal delivery because of their ability to encapsulate drugs and facilitate their passage through the skin. The traits of liposomes can be altered to meet most delivery needs. The size of liposomes can be manipulated through process such as sonication and extrusion to give vesicles ranging in size from 50 nm to 5000 nm [Torchilin, 2005]. They can be made to be more deformable to squeeze through the skin or more rigid by the addition of different molecules to the bilayer [Honeywell-Nguyen, 2002]. Targeting moieties can be added to the surface to home the vesicles to areas of interest [Park, 2001]. All the options available to modify liposomes to meet drug delivery needs made them the ideal choice for the transdermal delivery of a small drug like sildenafil citrate.
**α-Tocopheryl phosphate Properties and Liposome Drug Delivery**

Alpha–tocopheryl phosphate (αTP) is a natural derivative of vitamin E that can be found in various animal tissues [Gianello, 2005]. αTP is generated from the phosphorylation of the hydroxyl group on α tocopherol. This phosphate group makes the αTP amphiphilic, with both a hydrophobic tail and hydrophilic head group [Negis, 2005]. This characteristic allows αTP to integrate into the bilayer of the liposomes and helps alter the vesicles properties [Meybeck, 1995]. Additionally, as a vitamin E derivative, αTP may be able to soothe skin irritation and offer antioxidant properties [Meybeck, 1995], [Nishio, 2011]. The use of antioxidants has been shown to be beneficial in ED [Azadzoi, 2005], as such; the incorporation of αTP into the delivery vehicle may boost the therapeutic effect of the sildenafil citrate treatment.

The aim of this project is to formulate a transdermal delivery platform for the erectile dysfunction drug sildenafil citrate that incorporates the vitamin E derivative αTP into liposomes for the design and development of optimal therapeutic agents. To this end, the production of liposomes needs to be optimized and characterized for size, drug encapsulation efficiency, and enhanced permeation through the skin barrier.
Figure 2.1.1 Erection Pathway. A) Main processes in the initiation and maintenance of an erection. B) Biochemical pathway leading to increased blood flow into the penis. NO = nitric oxide, GC = guanylyl cyclase, GTP = guanosine triphosphate, cGMP = cyclic guanosine monophosphate, 5’ GMP = guanosine monophosphate, PDE5 = phosphodiesterase 5.
Figure 2.1.2 Drugs for Erectile Dysfunction. Drugs used to treat erectile dysfunction are all aimed at increasing blood flow into the penis. The most common drugs, including sildenafil, are inhibitors of the PDE5 enzyme, a negative regulator of blood flow.
Figure 2.1.3 Formation of liposomes. Phospholipids are composed of a phosphate head group linked to fatty acid tails by glycerol making them amphiphilic molecules. When they are in a concentration higher than their CMC (critical micelle concentration) they spontaneously form vesicles.

2.2 Methods

Liposome production

For this project liposomal vesicles were prepared using the bilayer components dipalmitoylphosphatidylcholine (DPPC) and α – tocopheryl phosphate (αTP). Studies were undertaken to optimize the molar ratio of DPPC:αTP. To this end, different molar ratios were tested, including 4:1, 2:1, and 1:1 with the purpose of comparing the size and the zeta potential of the vesicles they generated.

DPPC was dissolved in dichloromethane in a round bottom flask, making sure that the volume of the solution did not exceed half the total volume of the flask. The DPPC solution was placed in a rotary evaporator set to 35°C and 50
RPM to boil away the organic solvent and leave a lipid film. The lipid film was vacuum dried overnight to remove any remaining organic solvent. The next day, the αTP (different molar ratios) and sildenafil citrate (molar ratio 2:1, DPPC:drug) were dissolved in PBS by warming the solution to 42°C and vortexing. This solution was used to hydrate the lipid film for 2 hours at 45°C and 50 RPM. The solution was stored at 4°C.

**Vesicle size reduction**

Studies have shown that smaller sized liposomes can permeate through the skin more efficiently as compared to their larger counterparts [Verma, 2003]. To reduce the size of the vesicles formed from the initial encapsulation of sildenafil citrate, two methods were employed. First, the liposomal solution was sonicated in a water bath. The bath was set to 45°C and the flask containing the liposomal solution was lowered until the level of the solution. At this point, the sonication was turned on and left for 30 minutes at room temperature. The frequency of the vibrations broke down the larger vesicles, reforming as multiple smaller vesicles. This step resulted in smaller vesicles with mostly unilamellar membranes. Another method used to reduce vesicle size was extrusion. Liposomal solution was passed through a 0.2 µm filter using an extrusion device purchased from Avanti Polar Lipids. The extruder was maintained at 45°C during passage of solution. The liposomal solution was passed through the filter using syringes a total of 25 times. This led to a smaller distribution of sizes for the vesicles centered around 200 nm in diameter.
Size and zeta potential measurement

In order to obtain estimates for the size of the vesicles in our solutions we used Dynamic light scattering (DLS). This technique measures the change in intensity from the light scattered by particles in solution moving by Brownian motion. Taking this information and plugging it into the Stokes-Einstein equation can yield the hydrodynamic radius of the particles, or the radius including anything moving along with the particle [Bhattacharjee, 2016].

Samples were diluted 10 fold by adding 100 µL of the solution to 900 µL of ddH$_2$O in a cuvette. The cuvette was placed in a Malvern Zetasizer and the instrument was set to take size measurements. Samples were run in triplicate.

Zeta potential describes the electrical potential at the slipping plane of a particle moving in a solution [Bhattacharjee, 2016]. It is used as a measure of the stability of a solution with dispersed particles. The further away from 0 mV, the less likely the colloidal solution is to have problems with aggregation [Ahmed, 2015]. To obtain the zeta potential of the liposomal solutions samples were diluted in ddH$_2$O 10 fold by adding 100 µL of the solution to the water in a cuvette. DLS on the Zetasizer was used in this situation as well, but this time a dip cell was inserted into the cuvette to produce an electric field. The settings on the instrument were changed to zeta potential before taking the readings. Samples were run in triplicate.
Encapsulation Efficiency

One of the most important characteristics to consider when making a liposomal delivery vehicle is encapsulation efficiency. Higher encapsulation efficiency means more of your drug is taking advantage of the enhanced qualities of the delivery vehicle. To test the encapsulation efficiency, the liposomes were made in ddH₂O and transferred to 100 MWCO filtered tubes (Pall). They were centrifuged at 10,000g for 20 minutes. The flow through was collected, labeled as free sildenafil, and used in a UV spectrophotometric analysis following a protocol established by Sparsha et al, 2012. Standard concentrations of sildenafil were made in ddH₂O and 100 µL of each was added to a clear UV 96 well plate in triplicate. 100 µL of free sildenafil was also added to the plate in triplicate. The absorbance at 291 nm was measured. The concentration of sildenafil in the samples was estimated from the standard concentration curve. This data was plugged into an equation, along with the total sildenafil concentration, to obtain the percent encapsulation efficiency (fig. 2.2.1).

\[
%EE = \left( \frac{S_t - S_f}{S_t} \right) \times 100
\]

**Figure 2.2.1 Percent Encapsulation Efficiency Equation.** $S_t$ is the total concentration of sildenafil citrate used in the liposomal preparation; $S_f$ is the concentration of free sildenafil (not inside the liposomes) obtained from the UV spectrophotometric analysis. Knowing these two values lets us plug them into the equation above to obtain the encapsulation efficiency.
2.3 Results

Liposomes with a molar ratio of 2:1 (DPPC:αTP) are smaller and have a more favorable zeta potential.

Liposomes were generated using different molar ratios of dipalmitoylphosphatidylcholine (DPPC) and α-tocopheryl phosphate (αTP) encapsulation equal amounts of sildenafil citrate. After hydrating the lipid film to generate the liposomes, the solution was sonicated for 30 minutes to reduce the size of the vesicles. There was no extrusion done to these liposomes. The DLS results show that the 2:1 (DPPC:αTP) ratio gives the smallest average vesicle diameter at approximately 98 nm (fig. 2.3.1A). This characteristic is favorable for transdermal delivery [Verma, 2003]. Additionally, the zeta potential of the 2:1 formulation was also the most favorable for a stable colloid solution at -48.4 (fig. 2.3.1B).

Figure 2.3.1 Liposome characterization. A) DLS results showing average diameter (nm) of vesicles for each molar ratio of DPPC to αTP (n=3). B) Zeta potential results for each molar ratio of DPPC to αTP (n=3).
Extrusion of liposomal solution results in more narrow size distribution of vesicles

To test the effect of extrusion on vesicle size, 2:1 molar ration liposomal solutions were passed through a 200 nm filter (Avanti Polar Lipids) a total of 25 times while at 45°C after the normal 30 minutes of sonication. These solutions were compared to liposomes that were just sonicated. DLS analysis of the solutions revealed that extruding leads to a more uniform size distribution (fig. 2.3.2). The peak size does not change significantly, but the range of the sizes greatly diminishes after repeated passage through a filter.

Figure 2.3.2 Effect of Extrusion on liposome size. Comparison of liposome size distribution for solutions (2:1) that were A) only sonicated and B) sonicated and extruded.
2:1 Liposomal formulation has an encapsulation efficiency of 92.2%

The 2:1 (DPPC:αTP) liposomal formulation was centrifuged in a filtered tube to separate the un-encapsulated sildenafil. This free sildenafil was plated next to standard concentrations of sildenafil in a 96 well plate and the absorbance was measured at 291 nm. From the generated standard curve (fig.2.3.3) the concentration of the free sildenafil was calculated and this value was plugged into the equation for encapsulation efficiency (fig. 2.2.1). The resulting %EE was 92.24 %.

**Figure 2.3.3 Sildenafil standard curve.** Absorbance at 291 nm of known standard solutions of sildenafil (blue dots) and unknown, free sildenafil (red dots). Line of best fit is calculated based on the standards.
2.4 Conclusions and Future Studies

The aim of this project is to formulate an effective transdermal delivery option to treat erectile dysfunction. To this end, we have employed a liposomal delivery vehicle to transport sildenafil citrate, the active ingredient in most current ED treatments, through the skin of the penis. This delivery vehicle is also incorporating α–tocopheryl phosphate, a vitamin E derivative, to enhance the therapeutic effect.

After testing different molar ratios of phospholipid to αTP, the 2:1 ratio proved to result in smaller vesicles. Additionally, when compared to previous formulations of liposomes, the incorporation of αTP seemed to generate smaller vesicles on average (data not shown). This adds another benefit to the use of this vitamin E derivative; as smaller sized vesicles have been shown to enhance permeation through the skin [Verma, 2003]. The zeta potential of the different liposomal solutions were not significantly different but they are all ideal for a stable colloidal solution [Hunter, 2013].

Extrusion of the liposomal solution proved to result in a more narrow size distribution as measured by Dynamic light scattering (DLS). For increased reproducibility in the manufacturing of the liposomes the size distribution should be minimized further. This can be accomplished by increasing the number of passes through the filter. Additionally, the filter pore size can be stepped down gradually during the passages to help reduce the range of vesicle sizes. The encapsulation efficiency of the 2:1 (DPPC:αTP) liposomes was measured to be 92.2%.
The next set of experiments to move this project forward should focus on the ability of this liposomal solution to permeate sildenafil citrates through the skin. To accomplish this aim a Franz, or vertical, diffusion chamber can be used. In short, rat skin can be excised and stretched over the opening of the diffusion chamber and the liposomal solution can be placed over the skin. PBS buffer will be added to the bottom chamber. The diffusion across the rat skin can be measured over time by taking samples periodically from the bottom chamber and probing for the concentration of sildenafil citrate. Comparing the permeation of the encapsulated drug to a solution of the drug alone would be a quick way to test the potential of this transdermal delivery vehicle as an erectile dysfunction therapy.
Chapter 3: Targeted Stem Cell Delivery in IBD

3.1 Background

Inflammatory Bowel Disease (IBD)

The worldwide incidence of individuals suffering from Inflammatory Bowel Disease (IBD), which encompass Crohn’s Disease (CD) and Ulcerative Colitis (UC), has been on the rise [Molodecky, 2012]. These diseases are characterized by severe inflammation and injury of the digestive tract resulting in pain, diarrhea, and loss of appetite [Xavier, 2007], among many other severe primary and secondary symptoms. Apart from reduced quality of life, IBD patients have to manage the rising direct health care costs, which average $125,404 per year [Colombel, 2010]. This cost is due to the high expense of current IBD treatments, the most effective being biologics that are very costly and aim to manage inflammation during flare-ups.

Current treatments for IBD

Current treatments for IBD include systemic corticosteroids for mild symptoms, asacol (mesalazine) and immunosupressors such as azathioprine (imuran) or methotrexate, alone or in conjunction with biologics such as anti-TNFα antibodies [Colombel, 2010]. The latter although initially were reserved for cases where the previous therapies proved ineffective, but are now being used by the vast majority of patients with moderate to severe Crohn’s disease and Ulcerative Colitis. These therapies, which have multiple serious side effects, ignore the local damage present in the gut of IBD patients and aim to only
systemically reducing inflammation and somewhat improving the ulceration and injury to the tissue as well as closing fistulas. To improve the quality of life of patients, new therapy options need to be developed, ones that better regenerate the intestinal tissue without causing the widespread secondary effects that the biologics described above are responsible for. Herein, we propose to address this need by employing a nanocarrier platform for the targeted delivery of mesenchymal stem cells (MSCs) as our therapeutic agent to the gut.

Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are adult stem cells located in the bone marrow, adipose tissue, blood and liver [Campagnoli, 2001]. They have been used in medicine because of their ability to differentiate into various cell types and their secretion of soluble factors that promote tissue regeneration and modulate inflammation [Chamberlain, 2007]. These properties make MSCs of particular interest in the treatment of IBD but previous studies have had mixed results with regard to healing in IBD mouse models [Chen, 2013; Nam, 2015]. This is most likely because of differences in the models and administration routes. There is also evidence of potential complications from systemic MSC administration including off-target accumulation of cells, which can lead to tumorogenesis [Kurtz, 2008]. This has generated the need for targeted delivery platforms to home MSCs to the inflamed intestine. The group of Ko et al. has shown that targeting MSCs to the intestine using antibodies results in reduced inflammation in a dextran sodium sulfate (DSS)-induced mouse model of colitis
[Ko, 2010]. To circumvent the problems documented by these previous studies, we plan to manufacture a nanocarrier-based platform to home MSCs to the inflamed intestine in IBD with the goal of reducing inflammation and promoting tissue repair while minimizing the side effects observed in the reported studies.

Nanocarriers for the Targeted Delivery of MSCs

Nanocarriers are, typically, nanoparticles that can carry a therapeutic agent to different locations in the body. Many different nanoparticles exist that can be employed as nanocarriers, including polyamidoamine (PAMAM) dendrimers. PAMAM dendrimers are branched polymeric nanoparticles with various properties that make them ideal for biomedical use, including but not limited to, precise manipulation of their size and conformation, high structural homogeneity, functionalized surface groups and high biodegradability (figure 3.1.1) [Lee, 2005]. PAMAM dendrimers are synthesized one layer (or generation) at a time resulting in the identifiers G1, G2, G3, etc. Our research group has expertise in working with these dendrimer nanocarriers in a variety of targeted delivery applications, including the delivery of MSCs for wound healing [Daftarian, 2013]. Given our expertise with the PAMAM dendrimers nanocarriers, where we have demonstrated their high monodispersity, and retention on the cell membranes (figure 3.1.2), we believe that our choice of delivery platform will result in a method that can home the cells to the desired location in the gut via a molecular recognition molecule attached to the nanocarrier (figure 3.1.3), improve the efficacy of the delivery, reduce the number of cells needed for therapeutic action, which will then reduce the overall cost of treatment and
minimize the immunogenic response. Overall, the proposed approach aiming at regeneration of intestinal tissue by targeting MSCs should provide with an innovative efficacious therapeutic alternative to current systemic delivery of biologic agents.

**Figure 3.1.1 G5 PAMAM Dendrimer Structure.** Schematic depicting branched polymeric structure and spherical appearance of a generation 5 PAMAM dendrimer (shown as layers). The polyamidoamine dendrimer’s unique characteristic is the diamine core at its center. G5 PAMAM dendrimers are approximately 5 nm in diameter and have 128 primary amines on their surface, which are positively charged at physiological pH.
Figure 3.1.2 Dendrimer Nanocarrier Characterization. A) DLS measurement of particle size showing narrow peaks. B) TEM of a Mesenchymal stem cell 3 hours after addition of dendrimer nanocarrier. Previous data from Liu, Zhao-Jun, et al (2016).
Figure 3.1.3 Therapeutic Approach Using Targeted Nanoparticles for delivery of MSCs. A) Positively charged PAMAM G5 nanocarrier modified with a targeting peptide coat MSCs by interacting with the negatively charged plasma membrane. B) Targeted MSCs travel through circulation until reaching a site of inflammation where the targeting moiety can bind to its partner on the endothelial cell surface and anchor the cells.

3.2 Methods

Molecular Cloning and Transformation

The DNA sequence for the I domain of LFA-1 was subcloned into a pET11d plasmid using restriction site digest and ligation. LFA-1 (lymphocyte function-associated antigen 1) is a protein expressed on the surface of immune cells which binds to intercellular adhesion molecule-1 (ICAM-1) on the surface of
endothelial cells, specifically through the 180 amino acid region termed the I domain [Dustin, 1988].

10 ng of plasmid DNA was added to a thawed vial of *E. coli* BL21-DE3 competent cells and mixed by tapping gently on the tube. The solution was incubated on ice for 30 minutes followed by incubation at 42°C for exactly 30 seconds to heat shock the cells and allow the DNA to pass the plasma membrane. The solution was then quickly placed back on ice. SOC media was warmed before adding 250 µL to the DNA/competent cell solution and incubating at 37°C for 1 hour. The bacteria were spread on LB agar plates with ampicillin as a selection marker. After an overnight incubation at 37°C, colonies were picked to grow transformed bacteria carrying the plasmid for glycerol stocks.

**Protein expression and isolation**

Overnight cultures were made by picking from the BL21 bacterial glycerol stocks into 5 mL of LB broth containing 100 µg/mL ampicillin and incubating at 37°C and 250 RPM. The next morning the bacterial solutions were refreshed with 500 mL LB broth (100 µg/mL amp) and incubated at 37°C, 250 RPM until an OD$_{600}$ of 0.5. The expression of the I domain was accomplished by adding 1 mM IPTG and 10 mM MgCl$_2$ and incubating for 3 hours at room temperature, 250 RPM.

After the 3 hours, the bacterial solution was centrifuged at 10,000 x g and the pellet was resuspended in Homogenization buffer (50 mM Tris, 5 mM
Benzamide HCl, 2 mM EDTA, 2 mM DTT, 10 mM MgCl₂). Bacteria were lysed by sonication (1 second on and 1 second off for 20 minutes). Lysed cells were centrifuged at 16,000 x g for 10 minutes at room temperature. The pellet was resuspended in denaturation buffer (50 mM Tris, 6 M Guanidine HCl, 2 mM DTT, pH 8). The solution was incubated with rotation at room temperature for 2 hours before adding 80 mL of ice-cold renaturation buffer (50 mM Tris, 10 mM MgCl₂, 1 mM DTT, 5% glycerol v/v). Renaturation was allowed to proceed overnight at 4°C before centrifuging at 5,000 x g for 20 minutes. Protein pellet was resuspended in Phosphate buffer (10 mM Na₂HPO₄, 2 mM KH₂PO₄, 1 mM MgCl₂, 5% glycerol v/v, pH 7.4) and stored at -20°C.

**Dendrimer acetylation**

Cationic PAMAM dendrimers have been shown to permeate across cell membranes to a higher extent when compared to neutral or anionic varieties [Kitchens, 2006]. This results in increased cytotoxicity from these dendrimers at physiological pH [Atwood, 2003]. Previous studies using endothelial cells have shown that functionalizing the surface amine groups of the dendrimers can lower their cytotoxic effect [Jevprasesphant, 2003]. To this end, acetylation of approximately 20% of the surface primary amines was carried out.

Acetic anhydride was used to acetylate 20 % of the amine groups of the dendrimers. The reaction was carried out in anhydrous methanol in a round bottom flask. Triethylamine was added in access to quench the acetic acid formed as a byproduct. The reaction was allowed to continue overnight at room temperature under an argon atmosphere. The methanol was removed by rotary
evaporation and the remaining film was dissolved in distilled water. The solution was first dialyzed against PBS for 8 hours followed by 3 rounds of dialysis in water using a 10,000 MWCO Slide-A-Lyzer cassette. Resulting solution was lyophilized and stored at 4°C.

Dendrimer-peptide complex formation

Generation 5 PAMAM dendrimers have a positive surface charge at physiological pH due to the many primary amines [Jevprasesphant, 2003]. This allows for the electrostatic interaction between these dendrimers and negatively charged peptides. The I-domain of LFA-1 was slowly mixed with the AcG5 PAMAM dendrimer in a molar ratio of 1:5 (protein: dendrimer) in sterile water. The solution was vortexed quickly before incubating at room temperature for 15 minutes. AcG5_LFA1 was stored at -20°C until ready to coat cells.

MSC Isolation and culturing

Mesenchymal stem cells are difficult to culture. Moreover, depending how they were isolated, passaged, and which media they were grown in, these cells can lose some of their therapeutic qualities. As such, a standard protocol must be followed in order to maximize the chances of obtaining therapeutically active MSCs. The protocol of Soleimani and Nadri [2009] is a simple method for obtaining functional MSCs.

In short, mice were sacrificed by asphyxiation with CO₂ and secondary euthanasia by cervical dislocation according to the recommendation of the American Veterinary Medical Association as outlined in animal protocol number
The ends of the bones were cut to allow access to the marrow. A 27-gauge needle was slightly inserted into the end of the bones and 1 mL of complete media (DMEM with 1% penicillin/streptomycin and 15% FBS) was flushed through the marrow cavity to collect content into a 10 mL tube kept on ice. The isolated marrow solution is filtered through a 70 mm strainer to remove clumps and bone particulates before performing a viable cell count using Trypan blue and a hemocytometer. Cells were plated at $2.5 \times 10^7$ cells/mL and incubated for 3 hours at 37°C and 5% CO$_2$ before changing media to remove non-adherent cells. Media was changed every 8 hours for 3 days followed by every 3 days for the next 2 weeks. After the 2 weeks the cells were washed with PBS and a quick 2-minute trypsinization at 25°C was performed to lift the MSCs from the plate. These lifted cells were cultured in a 25 mL flask, changing the media every 3 days. MSCs were transfected with either green fluorescent protein (GFP) for in vitro cell culture experiments, or luciferase for in vivo animal procedures.

**Cell culture and Binding studies**

In-vitro studies were performed to test the binding of the targeting peptide to the surface of endothelial cells. Human umbilical vein endothelial cells (HUVEC) were cultured in 6-well plates until reaching 100% confluency. MSCs were coated with the AcG5_LFA1 complex before being added to the wells (some wells received MSCs alone). After a brief incubation, the wells were washed with PBS three times. The MSCs constitutively express green fluorescent protein (GFP), allowing for the measurement of the fluorescence in the individual wells. The attachment of MSCs was compared between targeted
and non-targeted. The cells coated with the AcG5_LFA1 showed higher binding to the HUVEC (data not shown).

**DSS induced colitis mouse model**

Dextran sodium sulphate (DSS) is one of the most commonly used methods for inducing colitis in rodents (Wirtz, 2007). DSS has been found to be cytotoxic to the intestinal epithelial cells causing increased permeability of luminal antigens leading to a severe immune response (Araki, 2010).

The animals used for this study were male C57BL/6J mice from Jackson Laboratory. The mice were housed in the animal facility of the Biomedical Research Building at the University of Miami Miller School of Medicine, where they were kept on a 12-hour light/dark cycle and had free access to food and water.

DSS was dissolved in sterile drinking water to give a 3% weight per volume solution. This water was exchanged for the normal drinking water in the cages of the study mice. Animals were kept on the DSS treatment for 7 days while tracking their disease progression using the disease activity index (DAI). The criteria for scoring the DAI include: percent weight loss, stool consistency, and blood in the stool. Each of the criteria is scored in increased severity from 0 to 4. The scores are averaged and recorded daily during the DSS treatment. This was accomplished by weighing the mice daily, followed by placement in a container to collect fresh fecal samples. These samples were visually analyzed for consistency and a Hemoccult test kit was used to visualize blood in the stool.
Any mouse with a DAI of 3.6 or higher was sacrificed to avoid unnecessary suffering, in accordance with the animal protocol (number 15223). After the 7 days, the mice were switched back to normal drinking water.

**MSC treatment and In vivo imaging**

A day after the DSS treatment was completed; mice were placed under a warming lamp for 30 minutes to dilate blood vessels thereby improving visualization. Meanwhile, MSCs were coated with either AcG5_LFA1 targeted nanocarrier or AcG5_BSA non-specific control. Mice were placed in restraining device under light source to further visualize tail vein and 100 µL solutions containing 500,000 MSCs coated with AcG5-LFA1, ACG5-BSA, or nothing were administered by tail vein injection. The following day the mice received a 200 µL IP injection of a 15 mg/mL solution of d-luciferin 15 minutes before imaging. Imagining was performed on an IVIS machine while the mice were sedated with isoflurane. This allowed for the localization of the MSCs, which constitutively express luciferase. The imaging procedure was repeated 5 days after the injection to observe the long-term localization of the cells (fig. 3.2.1).
3.3 Results

MSCs coated with the AcG5-LFA1 nanocarrier have greater localization to the gut area of DSS mice after 24 hours.

The *in vivo* imaging results 24 hours after administration of the different MSC formulations shows an increased signal in the gut area for the MSCs coated with the targeted nanocarrier (AcG5-LFA1) compared to the non-specific control (AcG5-BSA) and the cells alone. This suggests that after the targeted cells are put into circulation they reach the gut and attach to the area of inflammation. There also seems to be no visible signal in any other part of the mouse, pointing to little off-target accumulation of the MSCs (fig. 3.3.1).
Additionally, after 120 hours the luminescence signal for the targeted MSCs has increased compared to the 24-hour time point, which is not seen in the other treatments (fig. 3.3.2). As an effect of this, the difference between the MSC-AcG5-LFA1 group and the others becomes more significant after 5 days (fig. 3.3.3). This suggests one of several possibilities. First, the MSCs that were targeted to the gut may be able to proliferate after reaching the inflamed intestinal environment. This would result in more cells constitutively expressing luciferase. Another possible explanation is that the targeted MSCs in the bloodstream circulate for several cycles and each time more of them anchor themselves in the gut, thereby, increasing the luminescent signal.
Figure 3.3.2 MSC localization after 120 hours. In vivo imaging performed 5 days after tail vein injection of different MSC treatments. One mouse per group is shown as a representation (n = 5 per group).
Figure 3.3.3 Quantification of fluorescence intensity. In-vivo images were analyzed using the IVIS software by PerkinElmer. Flux was measured in a predefined region of interest. \( n = 5 \) animals per group. PBS signal was below detection limit.

### 3.4 Conclusion and Future Studies

The goal of this project is to target MSCs to the area of damage and inflammation in the gut as a therapeutic for IBD. To this end, we have employed a targeted nanocarrier platform incorporating a generation 5 (G5) PAMAM dendrimer complexed to the I domain of the cell surface protein LFA1 to coat the MSCs and help home the MSCs to the injured gut area. In a prior study, our team demonstrated promotion of angiogenesis and tissue repair in cutaneous wounds, employing this type of targeted delivery nanocarrier platform [Liu, 2016].

The systemic treatment with the targeted MSCs resulted in enhanced homing of the cells to the gut area of the DSS-induced colitis mice when
compared to the cells alone and a non-specific control. This suggests that the targeted delivery vehicle is selective and effective at allowing the circulating cells to attach to the inflamed area. Five days after administering the treatments, the difference in signal between the targeted cells and the controls is even more pronounced. This could be due to repeated rounds of circulation taking more cells to the area of inflammation over the extended period of time, thereby increasing the signal difference. Another possibility could be that the MSCs, once situated at the site of inflammation, proliferate. This would also lead to the increased signal observed at the longer time point.

To elucidate the reason for the increase in signal, proliferation experiments could be done where the MSCs are followed after they home to the inflamed tissue. Future studies for this project should be aimed at 1) further validating the targeting of the MSCs, 2) measuring the therapeutic effect of the targeted treatment, and 3) observing the effect of MSCs on epithelial cell proliferation and crypt cells dynamics.

To validate the targeting of the stem cells, the organ specific localization can be compared between treatments. After injecting the treatments the mice can be sacrificed and their organs harvested for an in vitro luciferase assay. The luminescent signal can be quantified and compared in different organs such as the intestines, liver, lungs, kidneys, and heart. This organ luminescent profile can be compared between treatment groups to validate that there is, indeed, more MSCs making it to the intestines when coated with the targeted delivery vehicle.
Measuring the therapeutic effect of the targeted treatment can be achieved by keeping track of the disease activity index (DAI) of the animals after injection of the MSCs. Seeing a decrease of the DAI score would indicate an amelioration of the symptoms. Additionally, histology can be used to compare the inflammation and damage in the intestines by looking at factors such as crypt disruption, immune cell infiltration, muscle thickening, goblet cell depletion, and crypt abscess [Geboes, 2000]. Finally, the permeability of the intestinal barrier can be measured by observing the passage of dye from the lumen to the inner layers using a FITC-dextran solution and a Fluorescein solution for capillary leakage into the lumen.

MSCs have been found to secrete various beneficial factors including PGE2 and TGFβ [Nighot, 2015; Park, 2002]. Both of these factors have been found to play roles in epithelial stem cell proliferation; a critical component of crypt regeneration after DSS injury [Davies, 2014; Santaolalla, 2013; Vamadevan, 2010]. Injecting the experimental mice with EdU (5-ethynyl-2’-deoxyuridine) moments before sacrificing and harvesting their intestines can allow us to study the proliferation of the cells in the tissue. EdU is an analogue of thymidine that is incorporated into the DNA during active synthesis. A fluorescent tag is then attached by click chemistry allowing for the quantification of DNA synthesis in the cell [Buck, 2008]. The effect of MSCs on the proliferation of epithelial cells can also be studied in vitro by harvesting colonoids from the intestine of DSS-induced colitis mice and co-culturing them with MSCs. Colonoids are obtained by harvesting the cells in the crypts of the intestine which
contain both epithelial cells and resident stem cells. The size, budding, survival, and proliferation of the colonoid cells can be measured and compared over time to elucidate the effect of the MSCs.

The preliminary in vivo imaging results suggest that the targeting of MSCs using a dendrimer nanocarrier and peptide results in enhanced homing to the gut area of DSS-induced colitis mice. This encouraging feasibility studies warrant further deep exploration and validation of this nanocarrier platform for the delivery of stem cells. Showing organ specific localization and a therapeutic benefit will make this a viable treatment option for individuals suffering from inflammatory bowel disease, helping to improve their quality of life.
Chapter 4: Closing Remarks

Biochemical and molecular methodologies are constantly allowing us to push the boundaries of human knowledge. Nowhere is this more crucial than in the medical field. New knowledge or insight into natural phenomena leads to new technologies that can enhance patient care. Generating new formulations for enhanced drug delivery as evaluated in this thesis for sildenafil and targeting methods as shown for the MSC therapy for IBD can give rise to new treatment options with lower incidence of side effects and more potent therapeutic outcomes. This can have an immensely positive effect on patient quality of life. Elucidating ligand-protein interactions can give insight into the mechanisms of disease pathogenesis as shown in the 5-HT and LasR cross talk studies and help in the developing of new screening tools for molecules with antibiotic effects. To make these types of medical advancements a solid foundation in the biochemical and molecular processes is crucial, as well as, a creative mind to devise new technologies based on these processes.

The studies on LasR and its binding to its natural ligand, and the neurotransmitter serotonin (5-HT), have resulted in the realization that 5-HT binding to LasR is not identical as that of the natural ligand. The preparation and isolation of soluble LasR-LBD in the presence of 5-HT was not possible. In contrast, LasR-LBD with the natural ligand (3-oxo C12 HSL) was readily obtained. This could be due to differences in the location of the binding domain for 5-H vs. that of the 3-oxo C12 HSL, which is established to be in the LBD of the LasR. Thus, it is possible that 5-H binds to a location in the protein that was
not present within the truncated LasR-LBD that we employed. In addition the stability of the LasR-LBD protein after binding could have been compromised. To investigate the binding of LasR-LBD we developed a fluorescence-based biosensor method for detection of quorum sensing molecules or other molecules that bind to this protein. The ability to employ this biosensor for screening of molecules that can potentially activate or inhibit this quorum-sensing network is important in the identification of new therapeutic antibacterial agents. The studies in this thesis are just the starting point to the exploration of the interactions between the bacterial quorum sensing protein LasR and a plethora of bacterial, host and environmental molecules that can interfere with the bacterial communication network.

Generation of a local therapeutic option for the treatment of erectile dysfunction resulted in a liposomal formulation, which incorporated the benefits of the vitamin E derivative α-tocopheryl phosphate to delivery sildenafil citrate through the skin. The delivery vehicle’s characteristics demonstrated its potential as a transdermal delivery option for sildenafil citrate. Future skin permeation studies to validate this method as a true therapeutic option are under way in our laboratories.

Finally, development of a targeted nanocarrier utilizing the biologically relevant PAMAM G5 dendrimer and taking advantage of the binding between the cell surface proteins LFA1 and ICAM1 during inflammation resulted in a targeted delivery system for mesenchymal stem cells. The in vivo imaging studies showed a localization of the targeted cells in the gut area when employing a DSS-induced
colitis mouse model. Studies aimed at investigating the therapeutic effect of the targeted stem cell treatment to both lower inflammation and heal the damaged intestinal tissue will demonstrate the potential this method has as an enhanced alternative to the current treatments.

The projects in this thesis are all aimed at generating new and enhanced diagnostic and therapeutic tools by elucidating new knowledge, while taking advantage of natural processes. They establish the groundwork for further study leading to actualized methods to help improve the quality of life of individuals with challenging diseases and health conditions.


Bhattacharjee, Sourav. "DLS and zeta potential—What they are and what they are not?." *Journal of Controlled Release* (2016).


