Determining the Feasibility of a Liposomal Prototype Formulation for GC-C Agonist Peptide Drug Delivery

Determining the Feasibility of a Liposomal Prototype Formulation for GC-C Agonist Peptide Drug Delivery

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Abstract

The purpose of this study was to determine the feasibility of using a liposomal formulation for the systemic delivery of a GC-C agonist peptide drug. Peptide drugs have increased in numbers lately but are limited by delivery challenges. Liposomes can overcome some of those challenges. Peptides themselves are sensitive to processing conditions but GC-C agonists are stabilized by multiple disulfide bonds. Processing conditions, lipid types and drug solutions for preparing liposomes were evaluated. The drug load efficiency and peptide degradation were measured by quantitation of the drug and impurities by HPLC. The formation of multimers was monitored by size exclusion chromatography. Two prototype batches were stored at two temperatures to determine the stability of the liposomal formulation. The peptide degradation, multimer formation, and leakage rate was evaluated. The results discussed here show the feasibility of liposomes containing linaclotide to be developed as a formulation that may be functionalized with PEG or a mucoadhesive polymer that has potential to be used in an inhaler, nasal pump, or other new delivery system that can utilize a liposomal solution to deliver the drug to systematic targets, although further optimization is needed before a scaled-up, commercial formulation is realized.

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Chapter I

Introduction

One of the reasons that I have chosen to concentrate my studies on the field of nanotechnology and bioengineering is that I see clear advantages to incorporating these technologies into the drug development process. A drug is a chemical designed to bind to an active target within the body, and also designed to not affect other important functions as to not cause side effects, or an adverse reaction. Although the first activity in designing a good drug is to identify a molecule that will act specifically at the intended target, once such a molecule has been discovered there are many other factors that can impede the process of drug development. Such factors include: poor absorption (e.g. proteins and peptides), low solubility at physiological pH, insufficient cellular uptake, rapid drug elimination, and high nonspecific side effects (Burton, 1996). Some of these difficulties are related to molecular weight, which can cause poor permeability; lipophilicity, which can cause poor solubility into the blood stream; and total number of hydrogen bond donors or acceptors, which can impair permeability across membrane bilayers. The use of biotechnology to deliver the drug molecule to the intended target in a "nanocarrier" such as synthetic polymers, microcapsules, lipoproteins, liposomes, micelles, hydrogels, liquid particles and many others can overcome some of these challenges. To further enhance their performance, these nanocarriers can be made to slowly biodegrade, to react to external stimuli (pH or temperature), or to help the

attractiveness of a target by conjugating them with ligands specific for the certain characteristics of the pathological area.

Therapeutic Potential of Peptide Drugs

The drugs currently being developed at pharmaceutical companies can be divided into two major categories - traditional 'small molecule' drugs with typical molecular weights of <500 Da that generally have good oral bioavailability, and much larger 'biologics' typically with molecular weights >5000 Da that are not orally bioavailable and need to be delivered via injection. Due to their small size, conventional small molecule drugs may suffer from reduced target selectivity that often cause off target effects in humans that cause toxic side-effects, whereas protein therapeutics tend to be specific for their targets due to many more sites for hydrophilic and lipophilic interactions, but this comes at a cost of low bioavailability, poor membrane permeability, and metabolic instability. The next logical step for novel drug development should be development of new drug leads that fit between these two molecular weight extremes, with the goal of combining advantages of small molecules (cost, conformational restriction, membrane permeability, metabolic stability, oral bioavailability) with those of proteins (natural components, target specificity, high potency).

Peptides are biologically occurring relatively short chains of amino acid monomers linked by peptide (amide) bonds. Peptides are distinguished from proteins on the basis of size, and as an arbitrary benchmark can be understood to contain approximately 50 or fewer amino acids. Peptides have a wide range of applications in medicine and biotechnology. Insulin was the first therapeutic protein to be introduced to

treat insulin-dependent diabetes in the 1920s, and there are sixty FDA approved peptide drugs in the market, about 140 peptide drugs are in clinical trials and over 500 are in pre-clinical development (Busby, 2013). Peptide therapeutics represent an emerging class of drugs with great pharmacologic potential because of their high specificity, affinity, and molecular target recognition but orally administered peptides typically have low absolute oral bioavailability, so achieving systemic plasma concentrations is often a challenge.

GC-C Agonism as Potential Target

GC-C is a receptor for the endogenous peptide hormones guanylin and uroguanylin and is predominantly expressed on the apical surface of epithelial cells of the small intestine and colon. The endogenous hormones act on GC-C expressed on the luminal surface of intestinal epithelial cells to regulate intestinal fluid secretion in response to a meal. These small natural peptides, (one is 13 amino acids and another is 14 amino acids long), have multiple cysteines that form disulfide bond bridges that stabilize the molecule by keeping it locked in a compact structure. This structure is shown to protect them from enzyme degradation in the GI but they are not systemically available because of poor permeability and a short half-life in circulation. At my company, Ironwood Pharmaceuticals, we have developed orally administered, minimally absorbed peptides which are similar in structure to guanylin family of cGMP-regulating peptides that includes the natural hormones guanylin and uroguanylin (Hannig, 2014). One of these drugs, Linaclotide, is an orally administered 14-amino acid peptide agonist of GC-C which acts locally within the lumen of the GI tract to exert its effects without a need for systemic exposure.

The amino acid sequence and tertiary molecular structure of Linaclotide differ from those of guanylin and uroguanylin, which contain four cysteines, as opposed to the six within Linaclotide. These extra cysteines allow linaclotide to form an additional intramolecular disulfide bond. This third disulfide bond enhances the relative GC-C agonist potency of linaclotide by locking it into its active conformation, strengthening its binding affinity for GC-C and slowing the degradation rate in the GI track (Busby, 2013). Although the mechanism underlying Linaclotide's ability to improve frequency of bowel movements is clearly related to a pro-secretory action (Bryant, 2010), a key question relates to how it relieves abdominal pain and discomfort. Is this simply an outcome of reduced constipation or is it an independent process? A recent study suggests in the case of IBS-C, where visceral pain is one of the physiological symptoms, that Linaclotide has pain relieving properties independent of constipation relief. It was shown that Linaclotide significantly decreased colonic hypersensitivity in separate models of non-inflammatory and inflammatory visceral pain. The remarkable aspect of this finding is that these models displayed different types of hypersensitivity, and Linaclotide reversed each one of them (Brierley, 2012). This could potentially provide an underlying mechanism for the analgesia effects previously observed during in vivo animal studies and the pain relief effects reported in human clinical trials (Bryant, 2010).

However, a significant amount of additional investigation is required to definitively determine the precise mechanisms and effectors involved in this process and if it translates to analgesia in humans. In mammals GC-C has recently been identified as a key cGMP signaling component controlling appetite, and in the process identifying uroguanylin as a new satiety hormone (Brierley, 2012). These studies show that GC-C

receptors could be an attractive target for novel therapeutics outside the GI track. If discovered, they could potentially lead to the development of a novel, guanylin peptide – based therapeutic interventions in pain relief and obesity, as well as other studies that show potential in asthma, chronic obstructive lung disease or other airway diseases (Rozenfeld, 2013).

Delivery Challenges of Peptide Drugs

Some promising new peptides with novel therapeutic potential have been identified, but their clinical utility has been limited by delivery problems. The peptides are readily metabolized by enzymes within the body, and have difficulty transporting across cell membranes (Torchilin, 2007). Some peptide and proteins are also unstable and have a short half-life of a few minutes in aqueous solution as well as having poor permeability, so they can only be administered by parenteral injection if systematic exposure is needed to deliver the drug to its intended target. This delivery system is thought to be invasive, carries risk of infection and often leads to low patient compliance. This shows that new non-invasive delivery strategies for these biomolecules are needed. The optimized formulations for these drugs depend on construction of a suitable delivery system that offers both protection and improved cellular penetration. Although much research is aimed at improving oral formulations so patients can experience the ease of taking peptide drugs by mouth, the intestinal lining is the only possible non-invasive port of entry. The small peptides developed at Ironwood that have been designed with enhanced stability are good candidates for a new formulation that can increase systematic exposure. The peptides are stable enough to have the potential to have a longer half-life

in the body once permeability is achieved. The small size is an asset if the hydrophilic barrier into lipid tissue could be overcome. An extended release formulation could increase these potentials as well.

Non-Invasive Routes of Drug Administration

For most of the industry's existence, pharmaceuticals have primarily consisted of simple, fast-acting chemical compounds that are dispensed orally (as solid pills and liquids) or as injectables. During the past three decades, however, formulations that control the rate and period of drug delivery (i.e., time-release medications) and target specific areas of the body for treatment have become increasingly common and complex. Since IV formulations are invasive, carry risk of infection and often lead to low patient compliance, other options for drug delivery are always needed. Some non-invasive drug delivery systems include topical ointments or creams, nasal sprays, ophthalmic drugs such as eye drops, and transdermal patches that use diffusion through the intact skin for systemic rather than topical distribution. Most peptide drugs have a high molecular weight and are poorly lipid soluble, so are not absorbed via skin or mucous membranes, or have very slow absorption. There are biotechnologies under investigation that can enhance absorption such as synthetic polymers, microcapsules, lipoproteins, liposomes, micelles, lipid particles and many others that are currently applied or under development. To further enhance their performance, all these drug carriers can be made slowly biodegradable, stimuli-reactive (pH- or temperature-sensitive), and targeted (by conjugating them with ligands specific towards certain characteristic components of the pathological area) (Torchilin, 2007).

One drug delivery system that is especially attractive is inhalation directly into the lungs. The lungs as a delivery route is attractive because the surface area of the lungs is large, and mucosal permeability is relatively easy because the walls of the alveoli are thin. Also, the activity of drug-metabolizing enzymes is lower in the lungs than in the stomach. The inhaled drug route also has the advantage of acting locally at the lung site and therefore allows lower doses to be utilized for therapeutics designed to treat pulmonary illness, such as lung tumors, asthma, COPD or pulmonary hypertension which could lower any chance of side effects or systematic cytotoxicity effects (Smola, 2008 and Tewes, 2010).

Although a few inhalable peptide formulations are available, such as Pulmozyme™ and Exubera™ there are many challenges to delivery and patient compliance, as well as challenges to manufacturing and testing to control and ensure quality and consistency of the micro-particles on scale-up and commercialization (Tewes, 2010). So while inhalation therapy is promising, it is not yet optimized. Some of the most influential factors affecting performance of an inhaled particle are particle-particle interactions (agglomeration) and size of the particle. In order to have a controlled, systematic effect, the particles need to reach the alveoli, and many studies have shown that particles with a median aerodynamic diameter of 1-5 μm deposit maximally into the distal lung. Smaller particles, although able to penetrate deeper into the optimal alveoli area, can cause irritation and are more prone to be exhaled (Smola, 2008). Agglomeration can cause the particles to clump together and reduce their ability to penetrate deep into the lung.

Nasal delivery is another needle-free, non-invasive and painless formulation; it does not require sterile preparation, can be self-administered and facilitates the rapid onset of both local and systemic drug action. The nose to brain pathway opens the door for targeting drugs to the central nervous system (Johnson, 2005). Similar to other mucosal routes of drug delivery, the physical barrier of the nasal epithelium is the main limitation with respect to drug absorption, especially for large molecules such as proteins and peptides. Another important obstacle is the rapid clearance which limits the time available for drug absorption. There are also fewer enzymes such as proteases that are present in the nasal mucosa and the mucus layer when compared to oral delivery. In vivo studies for the nasal administration of optimized formulations have showed that there was a 14-fold increase in the bioavailability of nerve growth factor, compared to administration of the peptide alone (Vaka, 2009). The low permeation of peptides and proteins through the nasal mucosa can generally be improved by using formulation technologies to loosen or widen the tight junctions (Johnson, 2005). In nasal delivery technologies, manipulation of the tight junctions is considered a practical approach since it is a reversible process and applicable to all drugs, in theory. Nasal formulations also have to be optimized for a controlled particle size and need lack of agglomeration to reach optimal nasal cavities for maximal absorption and minimal clearance.

Liposome Formulation

A liposomal formulation is an example of a nanocarrier that can be used with an inhalation or a nasal formulation. Liposomes could also potentially enhance the permeability of transdermal or topical formulation. These liposomes, which form a

phospholipid bilayer packet around the molecule, can protect peptides from aggregation, and give lower exposure to the rest of the body while it is acting selectively at the site. Biological stability of liposomes is dependent on the presence of agents such as proteins that interact with liposomes upon systemic application. There have been many strategies to enhance the biological stability of liposomes that improve the liposomal drug delivery in vivo and increase the circulation time in blood stream. In vivo stability of liposomes is also dependent on their charge. In serum, there are several proteins that are both positively and negatively charged. Liposomes with neutral charge are found to be more stable as they have much less electrostatic affinity towards proteins. One of the most popular and successful methods to obtain long-circulating biologically stable liposomes is to coat the surface of the liposome with poly(ethylene glycol), PEG. The coating of these vesicles by surface PEGylation results in "stealth" liposomes. The PEGylation modification enables extended blood circulation time of the drug and diminishes the mononuclear phagocyte system uptake which is an advantage over the conventional liposomes (Curic, 2013). Mucoadhesive polymers are another novel drug delivery additive that can help liposomes attach to tissue or to the surface coating of the tissue for targeting various absorptive mucosa such as ocular, nasal, pulmonary, buccal, vaginal etc. Mucoadhesive polymer liposomal nanoparticle formulations have been shown to give more effective and prolonged action in comparison to non-coated systems, in both oral and pulmonary administration of peptide drugs (Takeuchi, 2001).

The liposome formulation has been shown to stabilize other peptide drugs, such as VIP (vasoactive intestinal peptide) (Sejourne, 1997). Insulin (Ohnishi, 2015) and calcitonin (Murata, 2013) are two other peptides that have been formulated into

liposomes, although these articles also discuss the degradation of peptides during processing condition when preparing the liposomes. Formulation research and development has enabled Ironwood to design, manufacture, and commercialize a stable drug product for oral delivery, which is a capsule containing spray-dried beads. The bead composition, capsule design and formulation of the spray dry solution have been carefully optimized by the addition of stabilizing excipients to achieve the commercial room temperature stable formulation. This data supports the hypothesis that a non-invasive, systemic delivery system, once designed for permeability, can also be optimized by formulation development for stability and scalability.

My company needs to be able to screen early development peptide drug molecules for systemic activity, which could potentially lead to new understanding of the GC-C receptor pathway and lead to novel therapeutic ideas to develop in the future. I propose a formulation that incorporates the peptides in an aqueous buffer into an optimized liposome formulation that could then potentially be further formulated for use in an inhaler, a nasal spray, or as an oral drug. Although the use of liposomes for enhancing the systematic bioavailability of peptides is not novel, their use in stabilizing GC-C agonist peptide drugs has not been tested. I hypothesize that a liposomal formulation can be developed and optimized to stabilize the peptides against potential chemical degradation due to the decreased probability of interaction between molecules, as well as enhancing permeability by lipid-lipid interaction. The liposomal formulation also allows the use of buffers that are best for stabilizing disulfide bonds that are prone to reduction in solution. The peptides are generally more stable in an acidic buffer system, which is more tolerable in a liposomal formulation than a neat solution because the lipids

would buffer mucous tissue from irritation. The increased permeability of this liposomal formulation would allow recently developed GC-C agonist peptide drugs reach new targets systematically, which will enable researchers to explore the role of G-CC in many new disease types.

The formulations which enhance permeability into the blood stream should also stabilize the peptide drug so it can be scaled up into a commercial product. One of the most common forms of chemical instability for these GC-C agonists is the formation of multimers. Peptide disulfide-bond multimers were found to increase by the reduction of the disulfide bonds in neutral or slightly basic solutions and the crossing of these bonds with cysteines on neighboring molecules when reformed to create dimers and to a lesser extent, trimers and higher order multimers. Generation of these multimers can be affected by stress temperature, stress time, ionic strength of the solution, and the concentration of peptide in solution. Although the peptides are not stable against multimer formation in basic solution, too acidic an environment can induce hydrolysis, and so solution formulations must be carefully optimized for each molecule.

My hypothesis is that it is feasible to load the hydrophilic peptide drug linaclotide into a liposomal formulation which potentially could reduce degradation rate of the peptide and the formation rate of multimers. I also hypothesize the GC-C agonist drug Linaclotide is inherently more stable than other peptide drugs due to small size and locked conformation from 3 disulfide bridges such that it is feasible to use harsh processing conditions of liposome preparation using hydration, mechanical dispersion methods.

Chapter II

Materials and Methods

The following is a list of materials and descriptions of methods used for experiments.

Materials

Egg phosphatidylcholine (Egg PC, CAS#97281-44-2, Avanti#840051C), 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC, CAS#63-89-8, Avanti#850355C) were purchased from Avanti Polar Lipid, Inc. (Alabaster, AL, USA) as vials of 25mg/mL dissolved in chloroform and were used without further purification or dilution. Cholesterol (Chol) was purchased from Avanti Polar Lipid, Inc. (Alabaster, AL, USA) in powder form (CAS#57-88-5, Avanti#700100P), and was dissolved in chloroform to a concentration of 400 ug/mL. Linaclotide, lot#PPL-MD11001204A (PPL Torrance CA, USA) was used as described in experiments. Chloroform, anhydrous was purchased from Sigma-Aldrich, CAT#372978-100ML. For pH=2 solution preparations, 0.1 normal hydrocholric acid solution (0.1 N HCl) was used (Fisher, ACS grade). Components used for buffers at pH=6 and pH=7.4 are sodium phosphate dibasic heptahydrate, and potassium phosphate, monobasic (ACS grade reagents from Sigma-Aldrich). Water for buffer preparation and mobile phase was dispensed from Milli-Q system at Ironwood. Mobile phase additives are trifloroacetic acid (Sigma-Aldrich, CAT#T6508); acetonitrile (JT baker, HPLC grade) and L-Arginine (Alfa-Aesar, CAT#A15738).

Methods

Liposomes were prepared, optimized and characterized according to the following methods. Encapsulation effiecency, peptide degradation, and multimer formation were determined by various HPLC separation techniques and calculations which are described here in detail.

Preparation of Liposomes

Liposomes were prepared from lipids of different sources to compare the effect of lipid composition. Conventional rotary evaporation–sonication method was developed. Appropriate amount of lipids and cholesterol (Egg phosphatidylcholine:Cholesterol or 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine:Cholesterol, molar ratio 5:1) dissolved in chloroform were mixed in a round bottom flask. Lipid film was formed inside the 50 ml round bottom flask by evaporating the organic solvent using Rotavapor, (Büchi,#R-215) under reduced pressure with various drying temperatures to compare the effect of process conditions. The film was dried in a vacuum oven overnight to ensure complete removal of the solvent. After addition of drug dissolved in aqueous buffer (various compositions to adjust pH and varied drug concentrations), the lipid film was incubated in a water bath set to a temperature above the T_c of lipid composition used for approximately 30s and then vortexed for approximately 30s. This cycle was repeated five times. Buffer, drug load, reconstitution temperature were tested in various experiments as further described in detail in results and discussion sections. The resulting liposomal suspension was incubated at 5ºC for 10 min and then ultracentrifuged (Beckman Coulter Optima MAX-

XP) at 50,000 × g for 1 hr at 4ºC under vacuum to separate free drug from liposome fraction.

Encapsulation Efficiency and Drug Loading Determination

Encapsulation efficiency was measured by HPLC. Concentration of linaclotide in the starting buffer solution (total drug), the free drug in the supernatant of samples, as well as degradation of encapsulated linaclotide was measured by RP-HPLC analysis, performed using an Agilent 1200 HPLC system consisting of a quaternary pump quaternary, refrigerated autosampler, thermostated column compartment, and a UV detector. The column used for the separation was a Waters YMC-Pack Pro C18, 3.0 x 150 mm, 3 um, 120 Å, at temperature of 40 ◦C. A gradient method using 0.1% trifloroacetic acid in water and acetonitrile for mobile phase was developed and qualified at Ironwood Pharmaceuticals. The mobile phase flow was set at 0.6 ml/min, injection volume was 10uL and the relative retention time was found to be 33.0 minutes. Concentration of linaclotide and degradents in samples was then determined by UV absorbance at 220nm.

The entrapment efficiencies of the various liposomes were determined by ultracentrifugation at 50,000 × g for 1 hr. The free drug in the supernatant was detected by HPLC. Total drug content of the stock solutions was also determined. The entrapment efficiency was calculated as the ratio of drug content within the liposomes to the total drug content of the starting solution. The drug content within the liposomes was calculated as the total drug content of the suspension minus the free (unencapsulated) drug.

The encapsulation efficiency was calculated as follows:

Concentration of encapsulated drug = concentration of total drug in stock solution – concentration of drug in supernatant.

$$EE\ (\%) = \left(\frac{concetration\ of\ encapsulated\ drug}{concentration\ of\ total\ drug}\right) \times 100\%$$

Subsequently, the drug loading was calculated as described:

$$DL\ (mg/mg) = \frac{concentration\ of\ drug\ (\frac{mg}{mL})\ \times volume\ added\ (mL)}{concentration\ of\ lipids\ \left(\frac{mg}{mL}\right) x\ volume\ added\ (mL)}$$

Measurement of Peptide Stability

Degradation of encapsulated peptide drug was determined by dissolving liposome pellet in 50/50 water/acetonitrile and measured by HPLC using the method conditions described above and comparing area response of peptide drug vs. related impurities for % purity value. Degradation was determined by measuring peptide purity by RP-HPLC as follows:

% degradation = % purity drug in starting buffer − % purity encapsulated drug

Multimer Determination

One of the most common forms of chemical instability for these GC-C agonists is the formation of multimers. Peptide disulfide-bond multimers increase by the reduction of the disulfide bonds in neutral or slightly basic solutions and the crossing of these bonds with cysteines on neighboring molecules when reformed to create dimers and to a lesser extent, trimers. These larger molecular weight multimer impurities are measured by SEC-HPLC (size exclusion chromatography). This method uses a Waters Alliance HPLC model e2695 with a binary pump, refrigerated autosampler, thermostated column compartment, and a fluorescence detector. The column used for size exclusion was TOSOH Bioscience TSKgel SW2000, 300x4.6mm, 4um, 120A and isocratic mobile phase consisting of 15mM Arginine, 0.1% trifloroacetic acid, and 17% acetonitrile in water. This method was developed and qualified at Ironwood Pharmaceuticals. The mobile phase flow was set at 0.2 ml/min, injection volume was 10uL and the relative retention time of dimers was found to be 13.0 minutes. Concentrations of linaclotide and multimers in samples was then determined by florescence area (excitation 273nm – emission 303nm) and are presented as % multimers as follows:

$$\%multimers = \frac{area\ of\ dimers\ and\ trimers}{area\ of\ linaclotide\ and\ other\ small\ impurities} \times 100\%$$

Chapter III

Results

Experiments were performed to prepare, optimize and characterize encapsulation of GC-C agonist peptide, linaclotide, into liposomes. Encapsulation effiecency, peptide degradation, and multimer formation were determined. The resulting drug formulations were then assessed for drug leakage and peptide stability upon storage conditions.

Preparation and Characterization of Liposomes

Liposomes were prepared as described in the method section. In the first set of experiments after optimization work, a cholesterol solution was prepared by dissolving 4.0 mg in 10 mL of chloroform to make a solution of 40ug/mL and 4 mL was pipetted into a 50 mL pear shaped flask with a glass Class A pipet. Using a 1 mL glass syringe, 600 uL of Egg PC solution of 25 mg/ml in chloroform was added to the flask and swirled gently to produce Egg PC:Choleserol molar ratio of 5:1. The flask was then placed on rotovap set at 100psi and 40C to evaporate chloroform until a thin lipid film was formed and dried overnight under vacuum.

For hydration, a suitable buffer at a temperature above the phase transition temperature of the phospholipid is employed. Once lipids are hydrated in the presence of

hydrophilic drugs, a portion of the drug gets entrapped inside the liposome and another portion remains in the bulk, outside the aqueous core of the liposome. From literature, it seems there are many factors that affect loading of liposomes with hydrophilic drug including lipid to drug ratio, concentration of drug solution, composition and pH of aqueous buffer and rehydration temperatures and dispersion methods, which are discussed in more detail in discussion section. Here, a solution of 2 mg/mL of linaclotide, a GC-C agonist peptide drug, was prepared in a 20mM phosphate buffer, pH=6.0 and pre-warmed to 52ºC. The solution has drug to lipid ratio of 1.2 (mg/mg) and is swirled manually (with a water bath set to 52ºC and vortex mixer as described in methods section) until all the lipids have been incorporated into the solution. The resulting product is a milky suspension of lipids which is allowed to stand for at least 30 min. at 10ºC for the complete swelling to give liposomes entrapped with drug. The resulting mixture was centrifuged and analyzed by HPLC and SEC to obtain results for entrapment efficiency and degradation of the peptide as described earlier. The entrapment efficiency was 21.4%; however the peptide showed 6.2% degradation.

In the next experiment, the conditions were repeated with batch#1 containing egg PC:Chol molar ratio of 5:1 and batch#2 of DPPC:Chol with molar ratio 5:1. Although conditions were kept the same, the entrapment efficiency was 3.9% for batch#1 and 3.2% for batch #2. The peptide degradation was 3.7% for batch#1, and 1.0% for batch#2.

Since the synthetic phospholipid component DPPC showed less peptide degradation and the encapsulation efficiency was not significantly different, it was used in the next experiments. This time all conditions were kept as previously described with the exception of batch #1 using 0.1 N HCl as buffer and batch #2 using 20mM phosphate

buffer at pH=7.4. The %EE was 5.4% with degradation of peptide 0.6% for batch#1 and %EE of 4.4% and degradation 0.9% for batch#2.

To attempt to increase encapsulation efficiency, two more factors were varied and results compared. Batch#1 used a higher rehydration temperature of 62ºC and batch #2 was prepared by using drug solution of 6 mg/mL in phosphate buffer at pH=6.0 which corresponds to a drug/lipid ratio of 3.54% with all other preparation conditions as originally described. The results for Batch #1 were a %EE of 3.4% and %degradation of 0.6% and for batch #2, the %EE is 21.1% and % degradation is 1.5%.

Next, the higher drug load condition was repeated to determine if increase in %EE is proportional to this parameter. Batch #1 was prepared by using drug solution of 6 mg/mL in phosphate buffer at pH=6.0 which corresponds to a drug/lipid ratio of 3.54% with all other preparation conditions as originally described. Batch #2 was prepared by using drug solution of 6 mg/mL in phosphate buffer at pH=7.4 also with drug/lipid ratio of 3.54% with all other preparation conditions as originally described. Results show %EE for both is approximately 6%. Degradation for drug encapsulated at pH-6 was 1.8% and for pH-7.4 it was only 0.3%. A summary of experiments and results is shown in Table 1. The HPLC chromatograms of representative samples illustrating peptide degradation are shown in Figure 1.

Multimer Formation from Encapsulation

The encapsulated drug from each experiment was also tested for the formation of multimers, which are dimers and to a lesser extent, trimers of the parent peptide. These larger molecular weight multimer impurities are measured by SEC-HPLC (size exclusion

chromatography). The results show at drug load of 1.2%, all samples gave 0.2-0.3% regardless of other process conditions or pH values. However, at the higher drug load of 3.54% and pH=6.0, the multimer content increases to approximately 1%. Results are summarized in Table 1.

Table 1. Results of Optimizing Conditions for Preparing Linaclotide Encapsulated Liposomes.

Liposome type	Reconst. Temp.	pH of buffer	drug/ lipid (g/g)	% EE	% degradation	% multimer
PC:Chol (5:1)	52 ºC	6	1.20	21	6.19	Not tested
PC:Chol (5:1)	52 ºC	6	1.20	4	3.65	0.26
DPPC:Chol (5:1)	52 ºC	6	1.20	3	1.02	0.26
DPPC:Chol (5:1)	52 ºC	2	1.20	5	0.61	0.20
DPPC:Chol (5:1)	52 ºC	7.4	1.20	4	0.85	0.26
DPPC:Chol (5:1)	62 ºC	6	1.20	3	0.55	0.26
DPPC:Chol (5:1)	52 ºC	6	3.54	21	1.45	0.90
DPPC:Chol (5:1)	52 ºC	6	3.54	6	1.82	1.10
DPPC:Chol (5:1)	52 ºC	7.4	3.54	6	0.30	0.11

Peptide degradation was observed in experiments where egg PC was used, while liposomes prepared using DPPC showed less degradation. Reconstitution temperature and pH of buffer had no measurable effect on encapsulation efficiency (%EE) or peptide degradation. Increased drug load increased the formation of multimers at pH-6, but decreased multimers at pH-7.4, while having a positive effect on %EE overall.

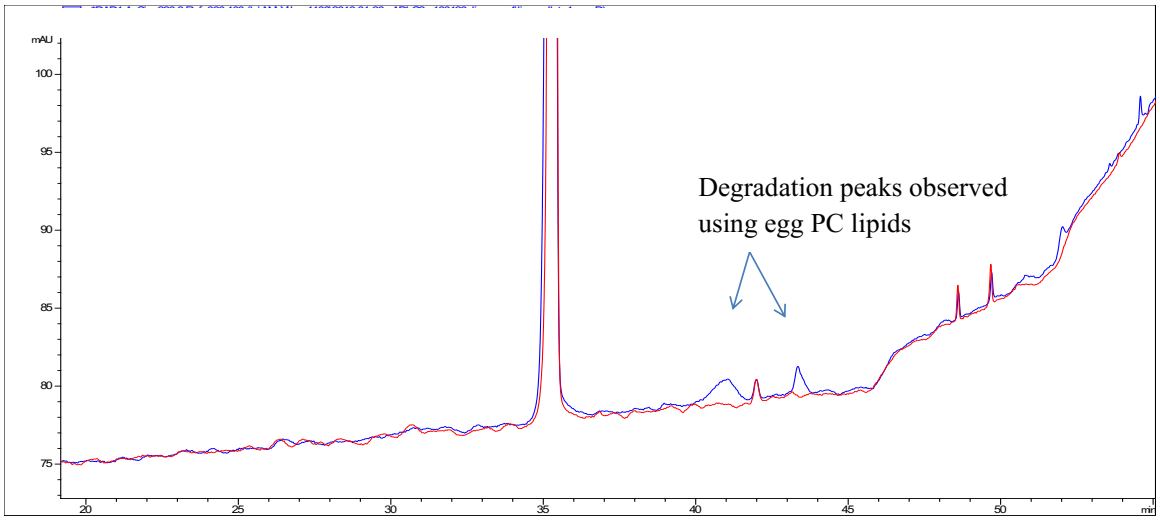

Figure 1. Overlays of UV Absorbance Showing Degradation of Peptide Drug from Encapsulation. Blue trace is linaclotide entrapped with eggPC:chol (5:1) and shows % degradation of 3.7%, while the red trace is linaclotide entrapped with DPPC:chol (5:1) and % degradation is much less at 1.0%. Results show specific impurity formation due to peptide degradation when liposomes are prepared using egg PC.

Experiments to Assess Stability in Storage Conditions

The next set of experiments was designed to evaluate the stability of the encapsulated peptides at various storage conditions. The last experiment described above produced two batches of linaclotide encapsulated in liposomes. Batch #1 was prepared by using drug solution in 20mM phosphate buffer at pH=6.0 and batch #2 was prepared by using drug solution in 20mM phosphate buffer at pH=7.4. Liposomes were prepared using lipid composition of DPPC:Chol (molar ratio 5:1), processing conditions of 52ºC for rehydration and a drug/lipid ratio of 3.54% was the same for both batches with all other preparation steps as previously described. The batches were then separated into 3 aliquots. One was analyzed immediately after preparation and separation by ultracentrifuge. One aliquot was placed at room temperature for 24 hours, and the third

aliquot was placed in a refrigerator at 5ºC for 5 days. The samples at the storage conditions were then ultracentrifuged, and the supernatant and liposome pellet were analyzed as previously described.

Drug leakage rate. Analyzing the concentration of drug in the supernatant over time at the storage conditions gives the leakage rate of the drug from the liposomes. For batch of liposomes at pH-7.4, the encapsulation rate of the drug starts at 5.7%, is reduced to 3.5% after 24 hours at room temperature and 1.5% after 5 days at 5C. Interestingly, although the liposomes at pH-6 show a similar leakage rate from 6.2% encapsulated to 4% after 24 hours at room temperature, the aliquot stored at 5C showed an increase in encapsulated drug to 13.3%. See Table 2 for results.

Table 2. Drug Leakage Rate at Storage Conditions

Drug Leakage Rate		
	pH-6	pH-7.4
%EE, T=0	6.20	5.70
% encapsulated after 24 hours at Room Temperature	4.01	3.52
Change, %leakage	-2.19	-2.18
% encapsulated after 5 days at 5C	13.27	1.50
Change, %leakage	+7.07	-4.20

Liposomes were able to encapsulate linaclotide. Approximately 2.2% of the encapsulated drug leaked out of both samples at various pH in the room temperature storage condition, showing the more flexible nature of the lipid barrier at this temperature. Interestingly, storage at refrigerated temperatures increased the % of drug encapsulated at pH 6, while the pH 7.4 sample shows drug leakage over time as expected. This difference can be explained by change in ionization state of the peptide.

Encapsulated peptide stability under storage conditions. The stock solutions of 6 mg/mL linaclotide prepared in each buffer pH were also aliquoted and stored at room temperature and 5ºC for controls. After 24 hours, the samples at room temperature were analyzed. For pH-6, the stock solution showed no degradation or multimer formation, while the encapsulated drug showed 0.66% degradation and the % multimers increased by 0.25%. For the pH-7.4 sample at room temperature, the stock solution gave 0.35% degradation and the encapsulated drug gave 0.6% degradation. Interestingly, although the stock solution at this pH showed multimer content increased by 0.24%, the multimer content in the liposome sample grew by less than 0.1%.

The samples stored at 5ºC were analyzed after 5 days. At pH-6, the stock solution gave 0.28% degradation, and the encapsulated drug was 0.43% more degraded than the initial sample. The multimers increased by 0.1% for the stock at pH-6, and 0.25% for encapsulated drug. At pH 7.4 and 5 days at 5ºC, the stock solution degraded by 0.13% and and liposome sample degraded by 0.52%. Again, although % multimer content increased for the stock solution by 0.26%, the % multimers in the liposomes only increased 0.15%. Multimer formation results are summarized in Table 3, and SEC overlays are shown in Figure 2. Peptide purity results are summarized in Table 4 and HPLC overlays showing degradation is shown in Figure 3. (See Appendix for Tables 3 and 4, and Figures 2 and 3).

Chapter IV

Discussion

Liposomes can encapsulate both hydrophilic and hydrophobic drugs in the aqueous core and/or the phospholipid membrane. Liposomes have been used to improve the therapeutic index of new or established drugs by modifying drug absorption, reducing metabolism, prolonging biological half-life or reducing toxicity. I proposed a formulation that incorporates a peptide drug in an aqueous buffer into an optimized liposome formulation that could then potentially be formulated for use in an inhaler, a nasel spray, or as an oral drug. Although the use of liposomes for enhancing the systematic bioavailability of peptides is not novel, their use in stabilizing GC-C agonist peptide drugs has not been tested. I hypothesized that it was feasible to develop and optimize a liposomal formulation to stabilize these peptides against potential chemical degradation due to the decreased probability of interaction between molecules. This formulation could then potentially be further optimized to enhance permeability by lipid-lipid interaction, and extend the half-life in circulation by extended release and stealth properties to hide the drug from being cleared. The liposomal formulation also allows the use of buffers that are best for stabilizing disulfide bonds that are prone to reduction in solution. The peptides are generally more stable in an acidic buffer system, which is more tolerable in a liposomal formulation than a neat solution because the lipids would buffer mucous tissue from irritation. The increased permeability of this liposomal

formulation would potentially allow recently developed GC-C agonist peptide drugs to reach new targets systematically, which could enable researchers to explore the role of G-CC in many new disease types.

Liposomes are small artificial vesicles of spherical shape that can be created from cholesterol and natural nontoxic phospholipids. Lipids forming liposomes may be natural or synthetic and liposomes are biocompatible and biodegradable which make them suitable for biomedical research and drug delivery formulations. Phospholipids are the main components of the eukaryotic cell membrane and lung surfactant and are considered as biocompatible since they are found to be biologically inert, with little to no associated toxicity. Phospholipids are amphiphilic molecules composed of a hydrophilic head-group including a phosphate group and a hydrophobic tail (Wauthoz, 2014). Because lipids are amphipathic (both hydrophobic and hydrophilic) in aqueous media, their thermodynamic phase properties and self-assembling characteristics shape their hydrophobic sections into spherical bilayers. Liposomes can trap both hydrophobic and hydrophilic compounds, avoid decomposition of the entrapped combinations, and release the entrapped at designated targets. They consist of one or more lipid bilayers surrounding aqueous units with particle sizes ranging from 30 nm to several micrometers (Akbarzadeh, 2013).

Liposome Preparation Design and Optimization

The manufactured liposome features are directly related to the preparation method. Although liposome formation may be spontaneous, some mechanical agitation is required. In order to have control over the size and structure of the liposomes that are formed, increase the efficiency of entrapment of the desired molecules, and prevent

subsequent leakage from the liposomes, different preparation methods have been devised. The amount of encapsulated drug is related with the size and the number of bilayers of the prepared liposome. Liposome size is a parameter in determining the circulation half-life of liposomes in the bloodstream, as well as a parameter for determining delivery type such as inhaled or nasal administration. There are many parameters that should be considered during the method selection such as: the physicochemical characteristics of the material to be entrapped and those of the liposomal ingredients, the nature of the medium in which the liposomes are dispersed, the effective concentration of the encapsulated material, optimum size, polydispersity and shelf-life of the liposomes for the intended application, batch-to-batch reproducibility and possibility of large-scale production of safe and efficient liposomal products.

According to the desired formulation and drug to be encapsulated, different liposome preparation methods can be employed. In these methods drug loading is passive and can be classified as mechanical agitation, solvent evaporation, solvent injection, and detergent solubilization. All the methods of preparing liposomes involve some basic steps; mixing the desired lipid components with a controlled molar ratio, drying down lipids from organic solvent, dispersion and hydration of lipids in aqueous media, mechanical agitation or sonication to control size and morphology and analysis of the final product. Here we are describing a solvent evaporation/rehydration method. For water soluble drugs such as peptides, liposomes are manufactured such that the hydrophilic materials are entrapped by using aqueous solution of these materials as hydrating fluid.

The starting point of this liposome preparation method is to prepare an organic solution of membrane lipids in order to ensure complete and homogenous mixing of all the components as they are required in the final membrane preparation. Compounds to be incorporated which are lipid soluble will be added to the organic solution, while compounds to be entrapped in the aqueous compartment of liposomes will be dissolved in the aqueous environment.

In this method, phospholipids are first dissolved in an organic solvent along with lipid soluble compounds to be incorporated in the liposome to ensure complete and homogenous mixing. The next step is the evaporation of the organic solvent by evaporating the solvent using a rotary evaporator connected to a vacuum pump to obtain a thin film of the lipid on the walls of a round bottom flask. The temperature, pressure, and time for drying can all affect the quality and stability of liposomes and need to be optimized. Evaporation of the solvent is followed by hydration of lipids with the aqueous medium. For hydration, a suitable buffer at a temperature above the phase transition temperature of the phospholipid is employed. The solution is swirled manually and mechanically with a bath sonicator or vortex mixer until all the lipids have been incorporated into the solution. The buffer type, reconstitution temperature and agitation time can affect the encapsulation efficiency and size distribution of the resulting liposomes. Once lipids are hydrated in the presence of hydrophilic drugs, a portion of the drug gets entrapped inside the liposome and another portion remains in the bulk, outside the aqueous core of the liposome. The aqueous volume enclosed within these lipid membranes is very small proportion of total volume used for preparation (5-10%). The

resulting product is a milky suspension of liposomes with entrapped peptide drug which are then analyzed for physical and chemical properties as well as stability over time.

Various types of liposomes can be prepared by different preparation methods, depending on the required application. In this study, a liposomal preparation technique for encapsulating peptide drug was developed and optimized. Peptides are known for instability and can be degraded during formulation processing conditions which is why there have been very few described in literature. Although I hypothesize that GC-C agonists like linaclotide are inherently stable enough for liposomal delivery, the conditions should be careful studied and optimized to both increase encapsulation and reduce any degradation.

Lipid Composition Selection

First, the liposome components to be used and compared are selected. Although traditionally natural egg yolk phosphatidylcholine has been used (Sejourne, 1997), other synthetic phospholipids may be a good alternative. Two different types of chemical degradation can affect the performance of the phospholipids bilayers; hydrolysis of the ester bonds linking the fatty acids to the glycerol backbone and oxidation of the unsaturated acyl chains, if present. The level of oxidation can be kept to a minimum by taking some precautions like starting with freshly purified lipids, deoxygenating solvents and aqueous solutions and storing under nitrogen. An alternative solution to the oxidation problem is to reduce the level of oxidizable lipids in the membrane by using saturated lipids instead of the unsaturated ones. Entirely synthetic and saturated phospholipids; DMPC, DPPC and DSPC, can be considered as a solution for the

oxidative degradation of liposomes. This is a good alternative to the natural egg components due to allergies to eggs in the general population, and synthetic lipids have the advantage of increasing batch to batch reproducibility and meeting FDA regulated GMP manufacturing compliance for a commercial product.

Dipalmitoyl-phosphatidylcholine (DPPC) is an inert and neutrally charged phospholipid, which has frequently been used for many studies because it is an important constituent of cell membranes; it is the major component in lung surfactants and often used as the main phospholipid to prepare liposomes for a wide range of applications. From previous work, exogenous liposomes encapsulating insulin delivered in rat lungs showed a significant enhancement of absorption of insulin into the systemic circulation with liposomes composed of palmitoyl group-based PC (i.e., DPPC or egg yolk PC), rather than non-palmitoyl group PC (i.e., DLPC, DMPC, DSPC, or 1,2-dioleoyl PC). The enhancement is dependent on the palmitoyl group content (i.e., 100% in DPPC and 32.5% in egg yolk PC) (Chono, 2009).

One of the main requirements for the use of liposomes as the drug carriers is the prolonged stability during its storage and also during its circulation time. In general, conventional DPPC liposomes show a moderate colloidal stability against aggregation (Toimil, 2012). The physical degradation, leakage and fusion of liposomes, can occur as a result of the lattice defects in the membrane introduced during the manufacture. Addition of cholesterol to the phospholipid mixture can be a solution to reduce or eliminate the transition. The presence of cholesterol prevents packing and aggregation by inducing orientation and more rigidity to the phospholipids. The presence of cholesterol

in the liposome also decreases the fluidity of the liposome bilayer, reducing leakage of the encapsulated drug over time at physiological temperatures.

In the experiments described here, I screened different liposomal formulations, using various types of phospholipids such as egg PC and DPPC and added cholesterol during optimization of the liposomal particles. I also optimized the process conditions (such as concentrations, temperature, stirring time, etc.) in a controlled way to form the prototype liposomes. I then measured encapsulation efficiency, peptide degradation and the formation of multimers by various HPLC techniques for separation, detection, and calculations as described in Methods section.

The initial experimental conditions were designed from background research and optimization experiments. A composition of natural egg phosphatidylcholine: cholesterol (egg PC:chol) 5:1 molar ratio was prepared using a solvent evaporation of lipid mixture method which was dried at 40ºC under vacuum for 1 hour, and held at room temperature overnight to remove any residual chloroform from the lipid film. A buffer solution of 20mM phosphate at pH=6.0 was prepared, and used to dissolve 2.0 mg/mL of linaclotide. Hydration of the dry lipid film is accomplished simply by adding an aqueous medium to the container of dry lipid and agitating. The temperature of the hydrating medium should be above the gel liquid crystal transition temperature (T_c or T_m) of the lipid. The drug in buffer solution was then used to rehydrate the thin film of lipids by addition to the pear shaped flask which is then swirled in a water bath that is set to 52ºC. For our experiments this temperature was chosen due to calculation of DPPC (Tc: 41.6 ±0.3°C) and Chol (T_c: 76.0±0.5°C) (Wauthoz, 2014) which would give our test composition of 5:1 PC:chol a T_c of approximately 48ºC, and 52ºC should be above this transition temperature. After

addition of the hydrating medium, the lipid suspension should be maintained above the T_c during the hydration period. This is done by cycling between water bath and vortexer every 30 seconds to achieve mechanical agitation at the elevated temperature. The resulting mixture is allowed to cool as the liposomes form at 2-8ºC before processing for analysis.

The first experiment showed a better than expected encapsulation efficiency of 21.4%. The 6.2% degradation of the encapsulated drug before any prolonged storage was however a sign that the peptide might not be stable enough for a liposomal formulation. This degradation could be due to the stress of the processing parameters, the chemical incompatibility of the lipid materials, or the concentration and ionization state of the drug in solution.

The next experiment was designed to test the incompatibility of the materials while keeping the other experimental factors the same. Batch#1 contained egg PC:Chol molar ratio of 5:1 and batch#2 was DPPC:Chol with molar ratio 5:1. This time the %EE was 3-4% for both and more consistent to what was observed in previous scouting and method development batches. Interestingly, the synthetic DPPC component only shows 1% degradation while the egg PC gave approximately 4% degradation for the second experiment. This can be attributed to the many impurities in the egg PC, including unsaturated lipids susceptible to oxidation.

pH Optimization of Buffer for Drug Solution

Since the liposomes prepared with synthetic phospholipid component DPPC showed less peptide degradation and the encapsulation efficiency was not significantly

different, it was used in the next experiments. Next, the pH of the buffer solution was varied. Hydrolysis type of chemical degradation of the ester linkages in the phospholipid structure occurs most slowly at pH values close to neutral. In general, the rate of hydrolysis has a V-shaped dependence, with a minimum at pH 6.5 and an increased rate at both higher and lower pH. Linaclotide is more stable in solution at acidic pH, although the peptide can also undergo hydrolysis at low pH. Multimers can form at neutral to basic pH because the disulfide bonds can be reduced and reformed with neighboring molecules, especially in concentrated solutions. The charge of a peptide drug with an isoelectric point depends on the pH of the solution (Ohnishi, 2015). The small peptide drug has more than one pKa, and undergoes multiple ionizations states depending on the pH of the buffer.

Another consideration is that the aqueous solubility of the drug is limited, and in general increases with pH close to neutral. In fact, 2 mg/ml is near the limit of solubility at pH=2. The next experiments were performed in 0.1 N HCl which is approximately pH=2 and 20mM phosphate buffer at pH=7.4. For both batches, the %EE was 4-5%, the %degradation was 0.6-0.9%, and multimer formation was 0.20-0.26%, all of which were consistent with previous liposome preparations encapsulated with drug at pH=6.0. This shows the pH of buffer or ionization state of the peptide does not impact the encapsulation process.

The next two experiments were performed in an attempt to increase the encapsulation rate of the hydrophilic drug. Batch#1 used a higher rehydration temperature of 62ºC and batch #2 was prepared by using drug solution of 6 mg/mL in phosphate buffer at pH=6.0 which corresponds to a drug/lipid ratio of 3.54% with all

other preparation conditions as originally described. The elevated rehydration temperature is to ensure efficient spontaneous micelle and bilayer formation from the lipid thin film in an attempt to increase the encapsulation efficiency. The second batch has an increased concentration of drug in solution; 6 mg/mL is close to the limit of solubility in aqueous buffer. This should increase the amount of drug trapped in the liposomes, although the stability of the peptide can be affected by increased concentrations and there is increased risk of precipitation at refrigerated temperatures. The first batch at 62ºC and all other parameters the same as previous gave comparable results for both %EE, peptide degradation, and multimer formation, showing the elevated rehydration temperature has little or no effect. However, the higher linaclotide concentration in buffer and therefore higher drug/lipid ratio gave both increased encapsulation efficiency of 21% and slightly more degradation including multimer formation (1.45% peptide degradation and 0.9% multimer formation).

Next, it was decided to repeat the experiment to see if the higher %EE could be controlled by the higher drug/lipid ratio. The repeat experiment was conducted at two different pH levels since then stability at storage conditions also need to be tested to assess the feasibility of liposomes for linaclotide drug delivery, and pH is a known factor for peptide degradation. Both batches used a 6 mg/mL linaclotide solution in 20mM phosphate buffer and a drug/lipid ratio of 3.54%, all other liposome preparation condition held the same. Batch #1 was prepared at pH=6.0 and batch#2 at pH=7.4. The encapsulation efficiency was around 6% for both. This is about double the %EE from experiments using 2 mg/ml of drug and drug/lipid ratio of 1.2%, but not as high as the previous experiment, although all efforts were made to repeat condition exactly. The

analysis of the drug however did show repeatable results. For pH-6, the peptide degradation was 1.8% and 1.1% multimer content was observed. This is very similar to the previous experiment with the same conditions. The batch prepared at pH-7.4 but with the increased drug/lipid ratio showed much less degradation and multimer formation, at only 0.3% and 0.1% respectively. This condition seems to be the most promising one tested here for going forward to evaluate the feasibility of using liposomes for drug delivery of linaclotide to the blood stream. These samples were then aliquoted as described below for stability at storage condition assessment.

Stability at Storage Conditions Evaluation

The next set of experiments was designed to evaluate the stability of the encapsulated peptides at various storage conditions. The last experiment described above produced two batches of linaclotide encapsulated in liposomes. Batch #1 was prepared by using drug solution in 20mM phosphate buffer at pH=6.0 and batch #2 was prepared by using drug solution in 20mM phosphate buffer at pH=7.4. Liposomes were prepared using lipid composition of DPPC:Chol (molar ratio 5:1), processing conditions of 52ºC for rehydration and a drug/lipid ratio of 3.54% was the same for both batches with all other preparation steps as previously described. The batches were then separated into 3 aliquots. One was analyzed immediately after preparation and separation by ultracentrifuge. One aliquot was placed at room temperature for 24 hours, and the third aliquot was placed in a refrigerator at 5ºC for 5 days.

The results show that there is a pH induced effect on the peptide degradation and multimer formation of the drug encapsulated in the liposomes. The liposome formation

and passive loading at pH=6.0 degraded the peptide by 1.8%. This most likely occurred in the rehydration step when the peptide was in contact with the lipids in the gel phase at the elevated temperature of 52ºC. However, the liposome formation and passive loading at pH=7.4 only gave 0.30% degradation, which points to a more stable ionization state of the peptide at this pH in the presence of the lipids at the elevated temperature. The formation of multimers was also much less at the higher pH of 7.4.

On storage at room temperature and 5ºC, both samples gave a similar increase in degradation and multimer formation. Although the sample at pH-6 started with higher degradation and multimer content, the increase upon storage was the same for both. The finding I found to be the most interesting was that although it is known that multimer formation is faster at pH levels above neutral, and this trend was shown in the control, stock drug sample at pH=7.4, the entrapped peptide actually showed less multimer increase upon storage than the control, suggesting a protecting effect. Although the entrapped drug started with 0.26% multimers, it only grew by 0.08% on storage at room temperature, whereas the stock solution started at 0.15% multimers but then grew by 0.24% after the 24 hours at this temperature. The effect measured was small and may be within the variability of the method, so the experiments should be repeated and the study time extended to see if the protection effect is real. In any case, it seems that the liposomal prototype formulations for linaclotide was stable enough to ensure proper dosing in pharmacology experiments when stored at the conditions studied here.

Assessment and implications of leakage rate of drug from liposomes. Another effect that was studied in these experiments was the leakage rate of the liposomal formulation.

Permeability of liposome membranes depends highly on the membrane lipid composition, as well as on the encapsulated material. Large polar or ionic molecules will be retained much more efficiently than low molecular weight lipophilic compounds. The release rate of the drug is also controlled by the type of lipids used to form the liposomes. A high glass transition temperature (T_c or T_g) means the liposomes will be more rigid and the speed of drug release will be slow (Smola, 2008). The presence of cholesterol in the liposome also decreases the fluidity of the liposome bilayer, reducing leakage of the encapsulated drug over time at physiological and storage temperatures.

From the results of these experiments assessing the encapsulation rate over time, the drug seems to release slowly at room temperature for both pH samples. The %encapsulated started at approximately 6%, and about 2% was released over the 24 hour period giving a final encapsulation of 3.5% to 4% for both. However, at the low temperature, the pH-7.4 showed leakage over the 5 days as expected, but the pH-6 sample showed an increase in concentration of drug by 7% for a total of 13.3% encapsulation. This can be explained in two ways.

The pH of the buffer can affect the charge state of both the lipids and the peptides. The interaction of the peptide with the liposome as well as the resulting localization of the drug in the liposome formulation can possibly be changed by dispersing in the different pH of media (Ohnishi, 2015). Partially negative charged liposomes allow efficient trapping of cationic peptides (Smola, 2008) and it has been reported that like charged liposomes and drugs repel and negatively affect loading efficiencies (Guo, 2003). Another factor is that the peptide is at the limit of solubility at 6 mg/mL in buffer, and so the low temperature and change in ionization state can cause the peptide drug to

precipitate out and therefore become trapped inside the liposome. As the concentration inside slowly becomes higher than the 6mg/mL limit, the effect is multiplied as precipitation happens faster.

The effect of leakage and drug loading based on ionization state of both the liposomes and peptide at various temperatures needs to be investigated further.

Conclusion

Peptides have a wide range of applications in medicine and biotechnology. Some promising new peptides with novel therapeutic potential have been identified, but their clinical utility has been limited by delivery problems. At my company, Ironwood Pharmaceuticals, we have developed orally administered, minimally absorbed 14-amino acid peptide agonists of GC-C. These unique peptides are stable enough to be orally administered and can withstand the GI track. They are agonists of GC-C which act locally within the lumen of the GI tract produce therapeutic effects without a need for systemic exposure. However, recent studies show that GC-C receptors could be an attractive target for novel therapeutics outside the GI track and could potentially lead to the development of a novel, guanylin peptide – based therapeutic intervention in asthma, chronic obstructive lung disease and other airway diseases. The inhaled drug route also has the advantage of acting locally at the lung site, and mucosal permeability is relatively easy because the walls of the alveoli are thin. It is known that activation of GC-C regulates intestinal fluid homeostasis, preventing dehydration and intestinal obstruction. In addition to this key function recent advances indicate GC-C signalling has a multitude

of additional and unexpected functions, which are diverse and occur within and outside of the gastrointestinal tract (Brierley, 2012).

My company needs to be able to screen early development peptide drug molecules for systematic activity, which could potentially lead to new understanding of the GC-C receptor pathway and lead to novel therapeutic ideas to develop in the future, as well as for feasibility as a commercially viable shelf-stable product. The main formulation challenge for these therapeutic peptides is stability in solutions. The other challenges are low permeability across membranes and clearance by the immune system with systematic exposure.

In this study, I evaluated the feasibility of using a liposomal formulation for the systematic delivery of a GC-C agonist peptide drug. I showed the feasibility of encapsulation and performed initial optimization of preparation conditions for a liposomal formulation. As part of optimizing a process to load peptide drug candidates into the liposome, an analysis method to determine drug loading or encapsulation efficiency of encapsulated drug had to be developed. Optimization and evaluation of existing methods to analyze purity of peptide before and after formulation with lipid components had to be performed including size exclusion to determine multimer formation rate.

By characterizing drug leakage and stability during processing and storage, the potential of these prototypes to be used as drug delivery systems was evaluated. Results show Egg PC and DPPC both were able to entrap GC-C agonist peptide drug, although degradation of peptide was seen immediately using liposomes composed of the egg PC.

Various conditions for the process of liposome preparation and drug loading had very little effects on the encapsulation efficiency, although pH and temperature were found to play a role during the storage experiment. I analyzed the resulting particles from optimization experiments and results varied from 3% to 21% EE. From literature, passive loading for hydrophilic drugs after optimization is typically 10-35%. I found that a drug/lipid ratio of 3.5% was able to consistently give a %EE of around 6%.

Processing conditions had little effect on peptide stability when using the synthetic DPPC lipids. Although pH of buffer of drug solution used for reconstitution of liposomes did show some effects on peptide degradation and multimer formation, all systems tested show this prototype is suitable for further development as a stable solution that can be used as a drug delivery formulation. Since degradation was shown to be minimal and all drug solutions studied were above 90% pure, it is also feasible for these liposomal systems to be processed further, such as adding PEG coating, or sonication to form smaller liposomes.

The chemical and physical stability was tested over time at various storage conditions. It was found that both pH-6 and pH-7.4 samples were chemically stable, but only pH 6.0 at refrigerated condition was able to keep the drug encapsulated. In fact, storage at this pH and temperature increased the concentration of the drug in the liposomes and should be further investigated as ionization of both the liposomes and the peptide could be used as a tool for active loading to increase the dose of linaclotide in the formulation. The leakage of the drug at the physiological pH of 7.4 and higher temperature is acceptable because this was designed to be an extended release of peptide into the blood slowly over time, as the intended receptor, GC-C, is outside the cell.

The results discussed here show the feasibility of liposomes containing linaclotide to be developed as a formulation that may be functionalized with PEG or a mucoadhesive polymer that has potential to be used in an inhaler, nasal pump, or other new delivery system that can utilize a liposomal solution to deliver the drug to systematic targets, although further optimization is needed before a scaled-up, commercial formulation is realized.

Appendix

Table 3. Peptide Multimers

Peptide Multimers				
	pH-6		pH-7.4	
	Stock Drug	Entrapped Drug	Stock Drug	Entrapped Drug
Time 0	0.15	1.25	0.15	0.26
24 hrs. @ Room Temperature	0.16	1.50	0.39	0.34
5 Days, 5ºC	0.24	1.50	0.41	0.41

Change (% multimer formation)				
	pH-6		pH-7.4	
	Stock Drug	Entrapped Drug	Stock Drug	Entrapped Drug
%Multimer Formation from Entrapment		1.10		0.11
%Multimer Formation from Storage, 24 hrs @ R.T.	0.01	0.25	0.24	0.08
%Multimer Formation from Storage, 5 Days @ 5ºC	0.09	0.25	0.26	0.15

Linaclotide formed multimers during the entrapment process. At pH-6, the multimers increased by 1.1%, although at pH-7.4, only a 0.11% increase in multimers was observed. The multimers did not increase significantly for stock solution (control) at pH-6 upon storage, but entrapped drug showed increase in multimers by 0.25% for both room temperature and refrigerated conditions. At pH-7.4, multimers did form in stock solution (control) upon storage, 0.24% at room temperature and 0.26% refrigerated. The entrapped drug showed less multimer formation at pH-7.4 for both storage conditions suggesting a protecting effect.

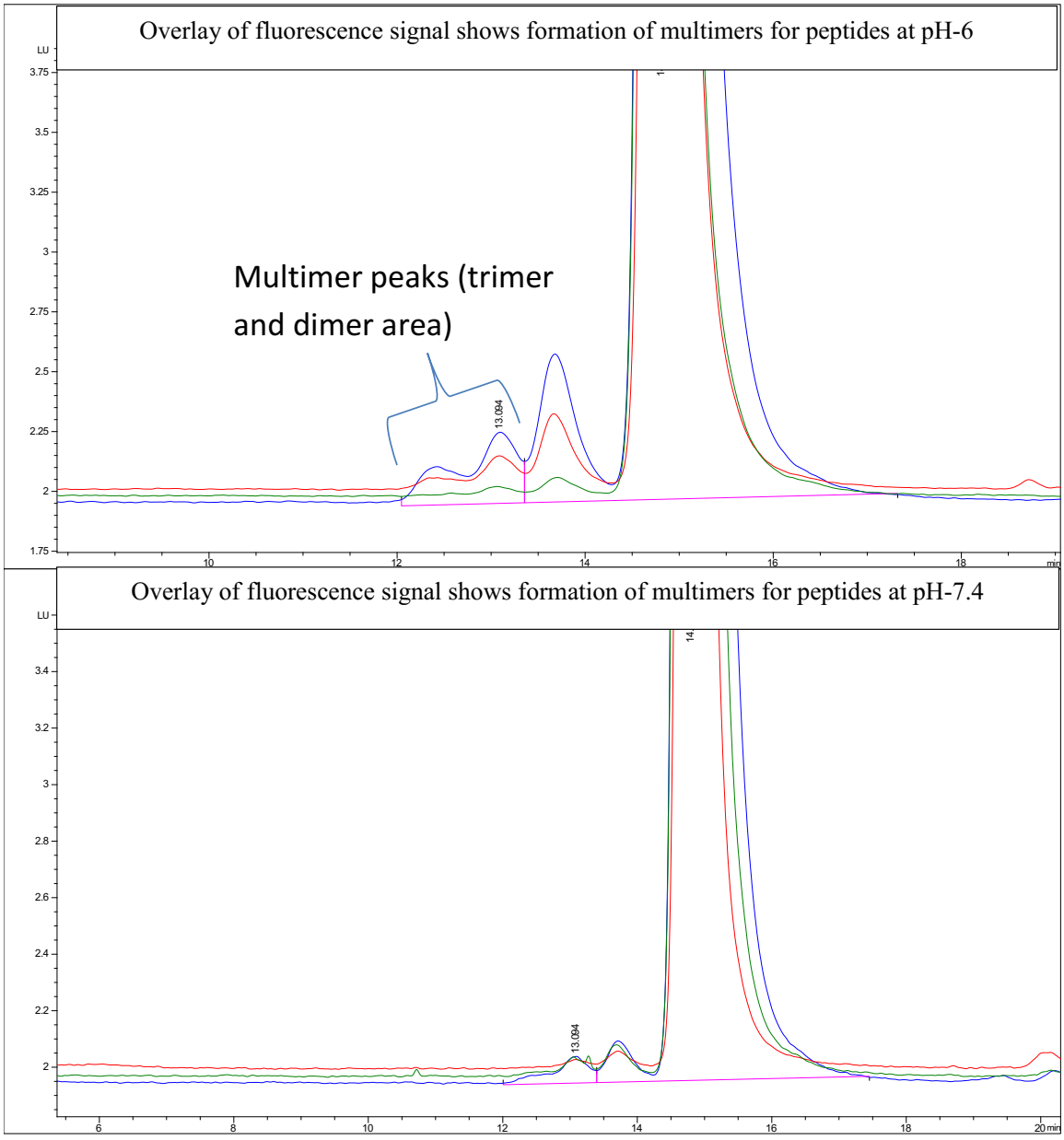

Figure 2. Multimer Growth After Processing and Storage. Green trace is stock drug solution, from supernatant, after processing; 0.15% multimers for both buffer pH solutions. Red trace is drug solution after encapsulation in liposomes, 0.26% multimers at pH-7.4 and 1.25% multimer at pH-6. Blue trace is encapsulated drug solution after 24 hours at room temperature; 0.34% multimers at pH-7.4 and 1.5% multimers at pH-6. (Multimer data for 5 day samples at refrigerated condition not shown, trends are similar to room temperature samples).

Table 4. Peptide Purity for Stability at Storage Conditions

Peptide Purity				
	pH-6		pH-7.4	
	Stock Drug	Entrapped Drug	Stock Drug	Entrapped Drug
time 0	99.2	97.4	99.3	99.0
24 hrs @ rt	99.3	96.7	98.9	98.4
5 days, 5C	98.9	97.0	99.2	98.5

Change (%degradation)				
	pH-6		pH-7.4	
	Stock Drug	Entrapped Drug	Stock Drug	Entrapped Drug
% degradation from entrapment		1.8		0.30
% degradation from storage, 24 hrs @ rt	0	0.66	0.35	0.60
% degradation from storage, 5 days @ 5C	0.28	0.43	0.13	0.52

The entrapment process degraded the peptide by 1.8% at pH=6, but only 0.3% at pH=7.4. After storage for 24 hours at room temperature and 5 days at refrigerated condition (5ºC), both samples showed slight increase in degradation as compared to stock solutions (controls) without significant differences between the samples.

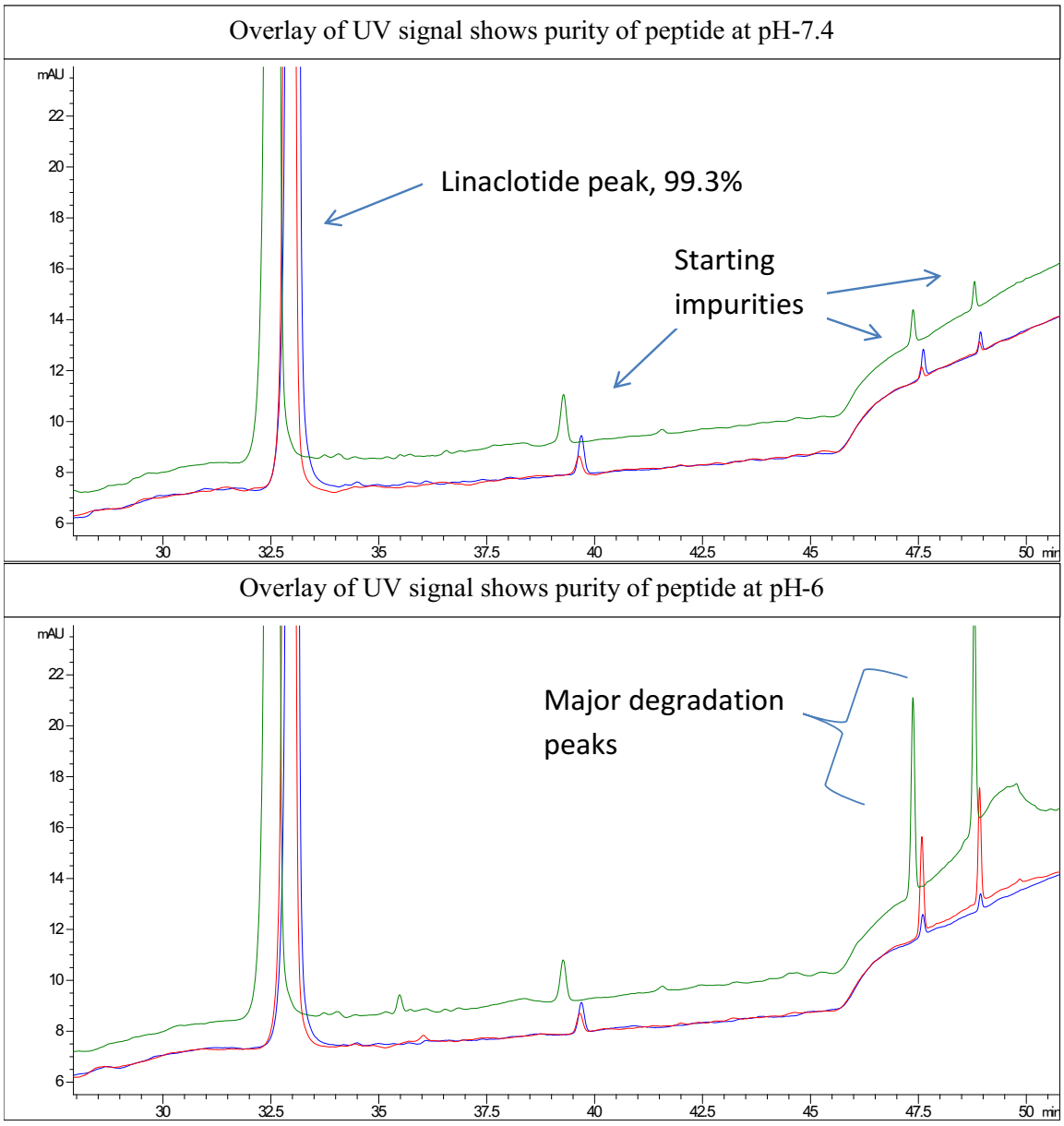

Figure 3. Peptide Degradation on Storage. Blue trace is stock drug solution, 99.2 – 99.3% pure linaclotide in buffer solutions. Red trace is drug solution after encapsulation in liposomes, 0.3% degradation at pH-7.4 and 1.80% degradation at pH-6. Green trace is encapsulated drug solution after 24 hrs. at room temperature; showing total degradation of 0.9% at pH-7.4 and 2.5% at pH-6. (Peptide purity data for 5 day samples at refrigerated condition not shown, trends are similar to room temperature samples).

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