

Towards the photo-triggered release of antimicrobial peptide GL13K tethered to phospholipid bilayer delivery vehicles

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ABSTRACT

Towards the photo-triggered release of antimicrobial peptide GL13K tethered to phospholipid bilayer delivery vehicles

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Deaths due to antimicrobial-resistant bacteria have surpassed those caused by HIV/AIDS and malaria. Antimicrobial peptides (AMPs) are a promising solution, although their delivery has challenges including poor proteolytic stability and the need for repeated high, localised dosages, traditionally restricting them to topical applications. We explore the use of biocompatible phospholipid bilayers as a platform from which to deliver the antimicrobial peptide GL13K with spatiotemporal control.

In this thesis, the initial focus pertains to the design of a photocleavable GL13K nanoplatform, anchoring peptide to the liposomal surface, as each constituent component had to be considered and many synthesized. GL13K was synthesized with both an *o*-nitrobenzyl photolabile group and an azide moiety selectively added to the central lysine residue. The nanoplatform is designed such that the bilayer will have an exposed alkyne group. This enables the peptide to be tethered to the phospholipid bilayer using a copper(I)-catalyzed azide-alkyne cycloaddition click reaction, post liposome fabrication. Once the platform was designed, synthesized, and the liposomal vehicles shown to be stable, successful tethering of the peptide was analysed and the GL13K able to adopt the bactericidal β -strand conformation, but not β -sheets when tethered to the liposomal surface. The nanoplatform was shown to have the capability of delivering bactericidal dosages of the AMP justifying future development towards an application in treating chronic wounds infected by persistent biofilm-forming bacteria.

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CONTRIBUTION OF AUTHORS

The experimental work, data analyses, and written thesis were prepared by Kailen Kroeger under the supervision of Dr. Christine DeWolf, who assisted with experimental design, data interpretation and writing.

Dr. Marc-André Bonin with the Peptide Synthesis and Imaging Probe Platform at Université de Sherbrooke assisted with the development of the synthetic pathway and carried out the synthesis detailed in Appendix 1 yielding the GL13K-linker conjugate.

Dr. Heng Jiang with the Centre for Biological Applications of Mass Spectrometry at Concordia University performed the mass spectrometry experiments on the GL13K-linker-lipid compound.

Dr. Chris Law with the Centre for Microscopy and Cellular Imaging at Concordia University performed the microscopy experiments on the GL13K-linker-lipid compound.

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List of abbreviations

AgNP	silver nanoparticle
AMP	antimicrobial peptide
AMR	antimicrobial resistance
ATR-FTIR	attenuated total reflectance Fourier-transform infrared spectroscopy
BCA	bicichoninic acid
BODIPY	boron difluoride containing dipyrromethene group
CD	circular dichroism
CF	carboxyfluorescein
CuAAC	copper-catalyzed azide-alkyne cycloaddition
DLS	dynamic light scattering
DMSO	dimethylsulfoxide
DOPA	1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphate
DOPC	1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine
DOPG	1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol)
DO-propargyl-PC	dioleoyl propargyl phosphatidylcholine
ESI-MS	electrospray ionization mass spectrometry
FRET	fluorescence resonance energy transfer
HEPES	2-[4-(2-Hydroxyethyl)piperazin-1-yl]ethane-1-sulfonic acid
Ln-UCNP	lanthanide doped upconverting nanoparticle

LUV	large unilamellar vesicle
MIC	minimum inhibitory concentration
MLV	multilamellar vesicle
MRE	mean residue ellipticity
NBD	nitrobenzoxadiazole
NLC	nanostructured lipid carriers
NP	nanoparticle
NVOC	6-nitroveratryloxycarbonyl
oNB	<i>o</i> -nitrobenzyl
PDI	polydispersity index
SLB	supported lipid bilayer
SLN	solid lipid nanoparticle
THPTA	tris-hydroxypropyltriazolylmethylamine
TIRFM	total internal reflectance fluorescence microscopy

Chapter 1. Introduction

1.1. Antimicrobial resistance, biofilms and the worst-case scenario

In recent years, deaths due to antimicrobial resistance (AMR) have surpassed HIV/AIDS and malaria.¹ In 2019, 4.9 million deaths were associated with bacterial AMR globally, of these 1.27 million were in low and middle income countries.^{2,3} The WHO predicts that if no action is taken now, by the year 2050 upwards of 10 million deaths will occur each year due to AMR.⁴

Currently there are several classes of antibiotics that target different vulnerabilities of bacteria, by inhibiting growth or reproduction, limiting protein synthesis, or targeting cell membrane constituents. The problem is that many bacteria have achieved resistance by developing stronger physical barriers, enzymes that degrade or remove the antibiotic, or by bypassing the afflicted biosynthetic pathway entirely.⁵

To further complicate the issue, many bacteria are imbued with the ability to develop biofilms, where they form complex extracellular matrices,⁶ can communicate through quorum sensing,⁷ and have an increased antibiotic resistance due to a stochastic cycling between active and somnolescent states.⁸ Unfortunately, infected wounds can develop into persistent infections if a biofilm takes hold, as outlined in Figure 1.1. This is a result of the biofilm's increased ability to evade antibiotic treatment, and higher innate antibiotic resistance, compounded by the increased prevalence of antimicrobial resistant bacteria.⁹ As a response, antipersister strategies are becoming a popular area of research, and specifically antimicrobial peptides and other peptidomimetics have been highlighted as viable treatment regimes against broad-spectrum multidrug resistant bacteria (Section 1.2.1).¹⁰

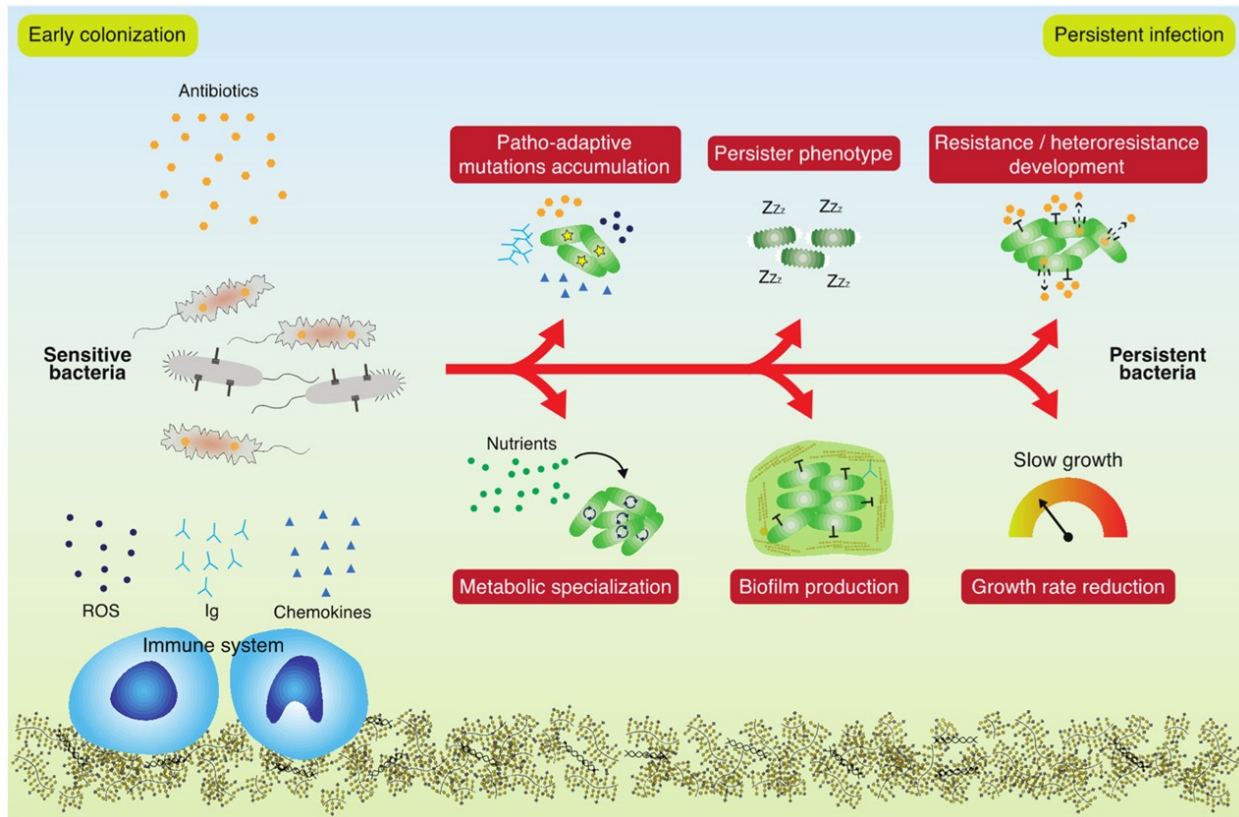


Figure 1.1 The process of persistent bacterial infection development.¹¹

1.2. Modern aspects of treatment addressing antimicrobial resistance

The age of antibiotics has been relatively short, with the discovery of penicillin by Alexander Fleming occurring less than 100 years ago.¹² Unfortunately, the birth of antibiotics coincided with the observation of antibiotic resistance, which was noted in Fleming's publication in 1929, a year after his initial discovery.¹² Regardless, at the time, penicillin satisfied a dire need for treatment options for infections, and its production was rapidly scaled up while significant research efforts were directed towards understanding antibiotics and their potential application.^{13,14} As more reports emerged of antibiotic resistance, the early response to combat this resistance was to develop more antibiotics with varying mechanisms and structures in order to stay ahead of resistance.^{14–16} Unfortunately again, this led to the development of multidrug resistant strains, proving the inadequacy of this response.¹⁷ The scale of the issue became increasingly apparent, leading to global efforts and surveillance to combat the growing problem.¹⁸ These efforts resulted in the development of strategies and antibiotic alternatives to circumvent an era of no effective treatments to bacterial infections.^{18,19} Much of today's research

in the field focuses on exploring responsible antibiotic use through more efficient treatments and investigating novel non-traditional/non-antibiotic therapies to address bacterial infections.^{20–22} Alternatively, the use of novel drug carriers is being investigated for the repurposing of traditional antibiotics, due to increased bioavailability and targetting capabilities while encapsulated in nanocarriers.²³

1.2.1. Antimicrobial Peptides

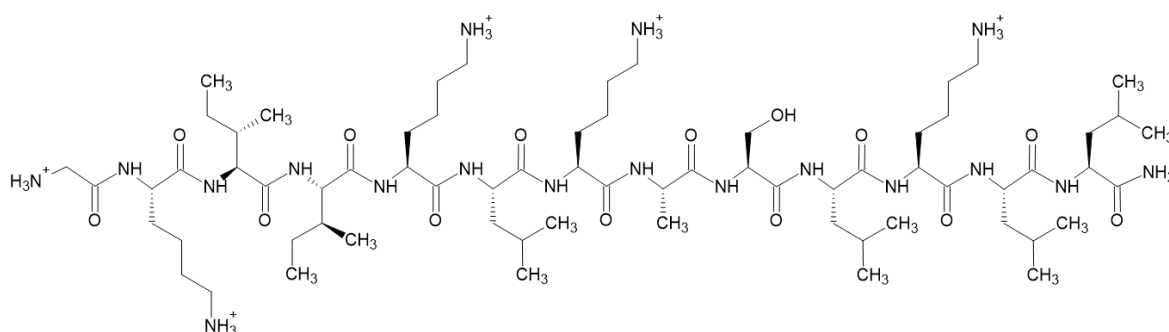
Antimicrobial peptides (AMPs) occurred first naturally, in the form of host defense peptides. These peptides serve immunological purposes in living systems and evolved to satisfy the need of protecting a host from threatening microbes. Traditionally, they were believed to act through membrane disrupting mechanisms but have recently been shown to also have activity against biosynthesis and metabolic pathways in pathogens.^{24,25} Further, these promising peptides have also shown significant activity in reducing or eliminating bacterial biofilms known to have increased resistance to traditional antibiotics.^{26,27} Their strength comes from their diversity, both in sequence, structure, and mechanism of action. Figure 1.2 demonstrates the vast array of structures formed by AMPs, with structures containing α -helices, β -sheets or a combination thereof.²⁸ With a few outlying exceptions, pervasive resistance against AMPs has not been seen. The significance of AMP activity is that it often comes from membrane disruption activity, and the difficulty to alter membrane composition, due to metabolic limitations and the vast number of systems integrated within the membrane, acts as a high barrier to overcome in order to develop resistance.²⁹ Interestingly, the onset of membrane disrupting activity often coincides with a conformation change of the peptide, initiated by the change in electrostatic environment in proximity to microbial membranes due to differences in membrane composition.^{30,31} This is significant as it also allows for specificity of the peptides, as disruption of all biological membranes would lead to cytotoxicity. AMPs are not without weakness, one major shortcoming being their susceptibility to protease degradation and thus rapid clearance from the body and the resulting frequent need to be delivered in repeat, high dosages.³² As a response, researchers have had to develop more complex and inventive deployment mechanisms.



Figure 1.2 Schematic demonstrating the structural diversity of many antimicrobial peptides, grouping peptides based on backbone torsion angles. Peptide structures are seen to span a broad range of α -helical, β -sheet and combinations of the two conformations, suggesting a large degree of difficulty for resistance to be developed.²⁸

1.3. *GL13K Antimicrobial Peptide*

As the choice therapeutic of the novel treatment designed in this thesis, an overview of the antimicrobial peptide GL13K is discussed here. The development of GL13K showcases the power of combining the native capabilities of host defense proteins with rational design. The origin of the peptide begins with the study of SPLUNC2 parotid secretory protein (PSP) for its antibacterial and anti-inflammatory properties. Peptides derived from PSP were shown to have antibacterial properties through an agglutinating mechanism.³³ Bactericidal activity was achieved through lysine substitutions aimed at increasing the net charge of the peptide. The result was a 13 residue peptide (GKIIKLKASLKLL-NH₂) coined GL13K, with a net charge of +5 and activity against both Gram positive and Gram negative bacteria.³⁴



GL13K sequence: **GKIIKLKASLKLL**-NH₂ (+5)

Figure 1.3 Structure and sequence of GL13K antimicrobial peptide.

1.3.1. Membrane specificity

The membrane specificity of AMP GL13K has previously been studied using liposome and supported lipid bilayer (SLB) model membranes, using 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DOPG) lipids to represent eukaryotic and bacterial membranes, respectively. The experiments showed that GL13K would bind DOPG bilayers with high affinity, leading to membrane rupturing effects. These same traits were not observed for DOPC bilayers. The significance of having selectivity for DOPG bilayers is that it suggests that the driving force for the peptide's activity is mediated by electrostatic interactions, since PG headgroups are anionic and PC headgroups zwitterionic. Further, it was shown that GL13K folds into a different conformation, preferentially forming β -sheets in proximity to the model bacterial membranes.³⁵ The electrostatic driving force has been shown to apply to more complex bacterial models as well as cancer cell model membranes, wherein the latter can also carry a net negative charge with the overexpression of phosphatidylserine.³⁶ Further, cholesterol, found in eukaryotic cell membranes but not those of prokaryotes has been shown to attenuate membrane disruption, offering another degree of protection against cytotoxicity.³⁷

1.3.2. Mechanism of action

Generally, the activity of antimicrobial peptides comes from two key characteristics: their amphipathic nature, and cationic charge.³⁸ The amphipathic nature of a peptide is a result of secondary structure folding, and both α -helical and β -sheet forming peptides occur. The β -sheet

forming peptides are more rare and generally require disulfide bridges or cyclization to stabilize the structure.^{28,39,40} The cationic characteristic is also significant, as it provides an electrostatic attraction to bacterial membranes that carry a negative charge. The structural diversity offered by peptides of different sizes, charges, side chains and secondary structures they can form, has led to many proposed mechanisms by which the peptides may act (Figure 1.4).⁴¹

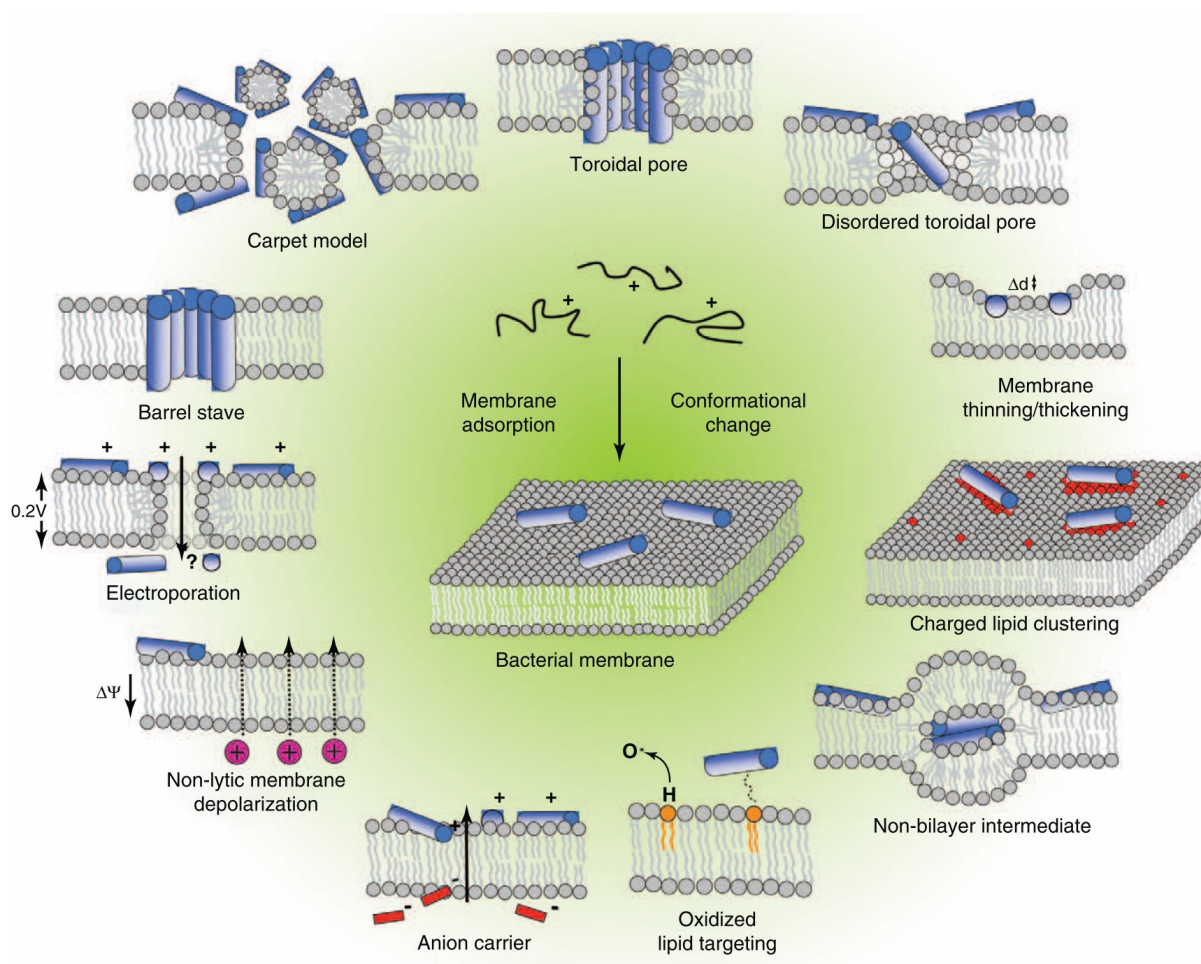


Figure 1.4 Schematic representation of some of the various mechanisms of action of AMPs.⁴¹

These mechanisms can be divided into three classes seen as the traditional AMP mechanisms of action: barrel-stave, carpet, and toroidal-pore models.⁴² The carpet model works by peptide aggregates forming at the bilayer surface, placing strain on the bilayer and pulling off material as micelles, disrupting the membrane. The other two models are pore forming mechanisms, where peptide inserts into the membrane and disrupts the continuous bilayer.⁴¹

GL13K is known to selectively form β -sheets in the presence of anionic bilayers.³⁵ Many AMPs that form β -sheets assemble into amyloid fibrils,⁴³ although the large cationic charge of GL13K may prevent this.⁴⁴ Instead, the peptide has been found to form crystalline anti-parallel β -sheets at the air-water interface in the presence of anionic monolayers.⁴⁵ This behavior suggests that the peptide is antimicrobial due to membrane activity via a carpet model.

1.3.3. Practical applications

Recently, work investigating D-enantiomer peptides has been gaining interest due to their ability to evade protease activity, increasing the bioavailability of such therapeutics.⁴⁶ In the same vein, the D-enantiomer of GL13K is gaining interest (D-GL13K). This second-generation AMP has enhanced antimicrobial activity against both gram positive and gram negative bacteria, as compared to the L-enantiomer,⁴⁷ and lacks hemolytic activity or human cell toxicity.⁴⁸ Further, D-GL13K has been shown not to lead to bacterial resistance, even when used repeatedly at sub-inhibitory concentrations.⁴⁷ Both enantiomers are effective against biofilms, but do not achieve bacterial eradication without supplemental antibiotic.^{27,47} D-GL13K has also been shown to have potential for anticancer treatment, having shown activity against human lung and breast cancer cells.³⁶

GL13K has also been a promising candidate for coatings on dental implants and medical devices. The surfaces of these devices are common starting points for bacteria growth and biofilm formation, frequently leading to failure of the device.⁴⁹ Silane has been used to covalently link the peptide to titanium surfaces, which has been shown to prevent the proliferation of bacterial cells while not interfering with adhesion and growth of host tissue cells, such as human gingival fibroblasts.^{50–53} The peptide has been incorporated into a variety of other coatings as well, including enamel,⁵⁴ polyetheretherketone,⁵⁵ silver nanoparticle AMP composites,⁵⁶ and hydrogel,⁵⁷ which all have medical device/implant coating applications.

Nanocarriers have also been used as delivery vehicles for the peptide. These carriers offer protection from protease enzymes that can degrade the peptide, while also offering size tuning capabilities for targeting delivery to specific tissue.⁵⁸ These carriers generally consist of biocompatible, inert compounds. Two such examples are the employment of tetrahedral framework nucleic acids which favorably interact electrostatically with the peptide,⁵⁹ and the

electrostatic loading of GL13K into nanophytoglycogen particles of optimal size to deliver the peptide as an aerosol into the lung alveoli.⁶⁰

Most important is the broad-spectrum activity of GL13K. Listed below (Table 1.1) are several persistent infection and biofilm forming bacteria, some associated diseases, and the mechanism by which they are targeted by GL13K.

Table 1.1 The treatment capability of GL13K against bacterial species.

Bacteria	Disease associated	GL13K treatment capability
<i>Pseudomonas aeruginosa</i>	Cystic fibrosis, COPD, chronic wounds, primary ciliary dyskinesia ¹¹	Bactericidal activity of both L and D-GL13K and kills biofilm ^{27,61}
<i>Staphylococcus aureus</i>	Cystic fibrosis, implants (surgical, etc.), chronic wounds, primary ciliary dyskinesia ¹¹	GL13K coatings cause antibiofilm activity ⁶²
<i>Streptococcus gordonii</i>	Dental implant infections ⁶³	GL13K is bactericidal ³⁴ and prevents biofilm formation ⁵²
<i>Porphyromonas gingivalis</i>	Dental implant infections ⁶³	GL13K is not bactericidal ³⁴ but does prevent attachment ⁵³
<i>Escherichia coli</i>	Urinary tract infections ¹¹	Bactericidal ³⁴
<i>Acinetobacter baumannii</i>	Chronic wounds ⁶⁴	Bactericidal activity of both L and D-GL13K ⁶¹
<i>Klebsiella pneumoniae</i>	Chronic wounds ⁶⁴	Bactericidal activity of both L and D-GL13K ⁶¹

1.4. Nanoparticles in an antibiotic context

Nanoparticles (NPs) offer many opportunities for tackling a post-antibiotic era. They can be defined as a particle of any type of material but must have at least one dimension on the nanometer scale (<100 nm).⁶⁵ Due to their broad definition, NPs can take on a diversity of forms, including crystallites, clusters, and amorphous particles. Additionally, their composition spans a wide range of materials and configurations, such as nanotubes, dendrimers, micelles, liposomes,

polymers, and dots, among others. Interestingly, at this scale some materials exhibit unique optical⁶⁶ and magnetic⁶⁷ properties that are distinct from both individual molecules and bulk materials. One final trait that has significance to the medical field is that as particle size decreases, the surface to volume ratio increases. Consequently, small particles have a much higher surface area for the same amount of bulk material. This means that any inherent properties of the surface, or any surface functionalization, are enhanced at the nanometer scale.

NPs for the treatment of bacterial infections can be divided into two categories: those with inherent antimicrobial activity, and those used as a vehicle for the delivery of other antibiotics. A classic example of a material with inherent antimicrobial activity is silver nanoparticles (AgNPs). These particles offer broad-spectrum activity against bacteria, fungi and viruses with facile synthesis.⁶⁸ AgNPs are suspected to kill bacteria through one of two mechanisms. First, electrostatic interaction of the particles may lead to aggregation on the bacterial cell membrane.⁶⁹ Alternatively, the release of Ag^+ ions can interact with thiol moieties on membrane associated proteins, leading to protein denaturation and disruption of the bacterial envelope. Once inside, Ag^+ can shut down metabolic processes, as well as DNA and protein synthesis.⁷⁰ Unfortunately, AgNPs have also been found to be potentially cytotoxic, raising concerns for their use in human subjects.⁷¹ A more recent example is amino acid based carbon dots, which have shown promising antimicrobial activity. Their biocompatible starting material offers a reduced risk of cytotoxicity.⁷²

1.5. Nanoformulations – Liposomes, Lipid Nanoparticles and Supported Lipid Bilayers

The years from 2019 to 2023 has been an incredibly interesting time for lipid-based drug carriers. With the onset of the COVID-19 pandemic and novel mRNA vaccines to treat the virus, lipid nanoparticles had a huge effect on many peoples' lives. Liposomes are a naturally occurring form of lipid nanoparticles as they can form spontaneously when adding phospholipids to water. They are defined as any closed lipid bilayer and therefore can come in many sizes, forms, and configurations. Size ranges from small (<100 nm) to large (100 nm - 1000 nm) to giant (>1000 nm) and they can have one concentric bilayer (unilamellar) or many (multilamellar) or even many nonconcentric bilayers (multivesicular). One other characteristic of liposomes is their surface charge, which can be manipulated by altering the lipid composition. The constituent

phospholipids of the liposomal formulation can be anionic, cationic, or zwitterionic, offering tunability of the surface potential. Generally, the charge is altered to maintain colloidal stability as electrostatic repulsion needs to outcompete attractive van der Waals forces in order to prevent aggregation of the liposomes.^{73,74}

The utility of liposomes as drug delivery vehicles comes from the three discrete environments they comprise. There is an aqueous interior, a hydrophobic lipid bilayer, and an exterior liposomal surface. The aqueous core can be loaded with hydrophilic compounds, such as small molecule drugs, or nucleotide-based therapeutics such as RNA. One such therapeutic currently being used is DepoDur™ where liposomes are used for the extended release of morphine sulfate during an epidural.⁷⁵ The phospholipid bilayer is a convenient pocket to store and protect effective small molecule drugs that have poor pharmacokinetics due to low water solubility. The first FDA approved liposomal therapy was Doxil®, that delivers the highly lipophilic anticancer drug doxorubicin for the treatment of ovarian cancer.⁷⁶ The exterior surface of the liposome also offers many opportunities for tethering. The outer leaflet of the bilayer can be used either for carrying therapeutics, similar to the other locations discussed, but is also often used for functionalization. The two most common functionalities are the attachment of a targeting ligand, such as a specific antibody, or the tethering of a polymer such as polyethylene glycol (PEG) to protect the liposome and increase stability.⁷⁷

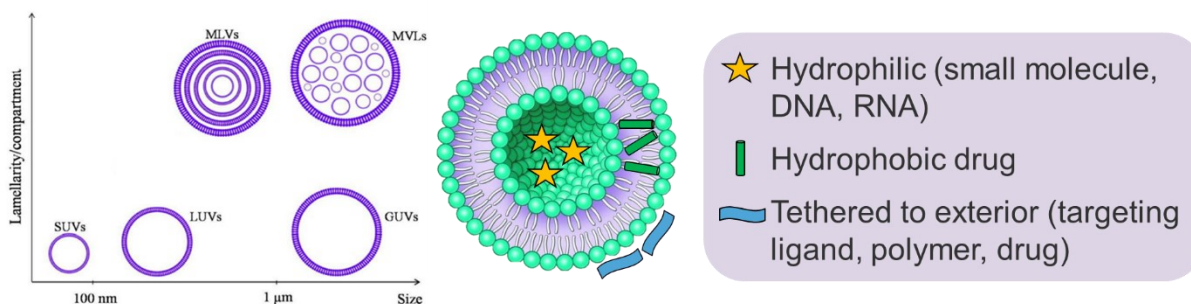


Figure 1.5 Left: Graph depicting size classes and bilayer configurations of liposomes.⁷⁸ Center and right: Overview of environments available for drug loading within a liposome.⁷⁹

Other classes of lipid nanoparticles include cationic lipid nanoparticles, solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC). Cationic lipid nanoparticles are made through the complexation of cationic lipids with highly anionic nucleic acid therapeutics. The main purpose

of these particles is to facilitate the delivery of the nucleic acid by masking its charge (which hinders diffusion into target cells), and protecting it from nuclease degradation.^{80,81} SLNs and NLCs were developed to overcome two limitations of liposomes: the requirement of organic solvent in their fabrication, and the difficulty with large scale fabrication, homogenization, and sterilization. Their structures differ from liposomes in that they have a surfactant exterior, with a solid lipid core, and in the case of NLCs, also liquid-crystalline domains.⁸²

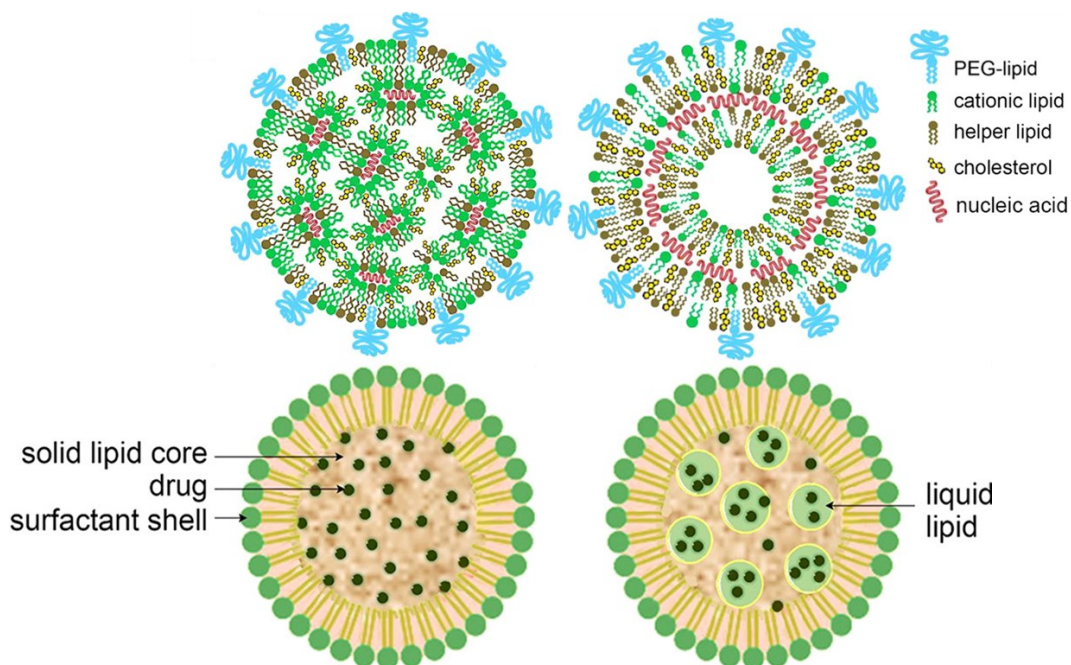


Figure 1.6 Schematic representation of cationic lipid nanoparticles (top), solid lipid nanoparticles (bottom left) and nanostructured lipid carriers (bottom right).⁸²

Lipids can also be used in conjunction with other nanoparticles. Liposomes themselves have been used as delivery vehicles for smaller particles, for example nanodiamonds,⁸³ or combined with dendrimers to form dendrimersomes⁸⁴ or polymer-lipid hybrid nanoparticles.⁸⁵ One of the most promising use cases of lipids however, is for encapsulation. There are several advantages of lipid encapsulation, as it can confer biocompatibility, can prevent aggregation and render particles water dispersible. Further, the lipid coating may be used as a platform for functionalization at the particle surface.

One example that will be highlighted is the formation of an SLB on the surface of lanthanide doped upconverting nanoparticles (Ln-UCNP). This application is of particular relevance to this

thesis as it is hoped that the lipid formulation developed, and peptide functionalization shown in this work will be applied as an SLB for similar particles. The Ln-UCNPs have the interesting characteristic of upconversion through a two-photon absorption process, which can allow the absorption of light in the near infrared region, and subsequent emission of ultraviolet light. Lipid encapsulation is particularly important for these particles for two reasons. First, the particles have an oleate cap and are therefore not dispersible in water,⁸⁶ and second, water quenches the upconversion process if allowed contact with the particle surface, by allowing excited electrons to relax to ground state through non-radiative processes. A water-impermeable lipid bilayer acts as a barrier, increasing upconversion efficiency.⁸⁷

The specific Ln-UCNPs being coated are LiYF₄ diamond shaped scaffolds co-doped with Tm³⁺ and Yb³⁺. Lanthanide dopant ratios can be adjusted for tuning of the emission intensities at certain wavelengths.⁸⁸ The diamond crystal conformation of the particles makes coating very difficult as it is highly faceted and has acute angles, yet conversely the equivalent surface energy of each of the faces promotes a homogeneous coating.⁸⁷ It was found that a lipid formulation of 64:7:29 mol% 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphate (DOPA) to DOPC to cholesterol was capable of successfully forming the bilayer, as demonstrated in Figure 1.7. DOPA is used as the main lipid in this formulation due to its anionic headgroup. The oleate capped Ln-UCNPs have an inherent positively charged surface. DOPA headgroups in the inner leaflet, therefore, will interact electrostatically with the surface of the particle, and through hydrophobic interactions with the oleate. Having an entirely negatively charged membrane would be unstable due to the repulsion forces between the headgroups, so zwitterionic DOPC is used to allow for some spacing between the headgroups. Cholesterol is added to form a denser bilayer, decreasing water permeability.⁸⁷ More recently, a photo-switching azobenzene-modified-lipid has been used to confer the ability to deliver hydrophobic drug molecules embedded in the lipid bilayer, by manipulating the lipid packing, triggerable by excitation with 980 nm light.⁸⁹

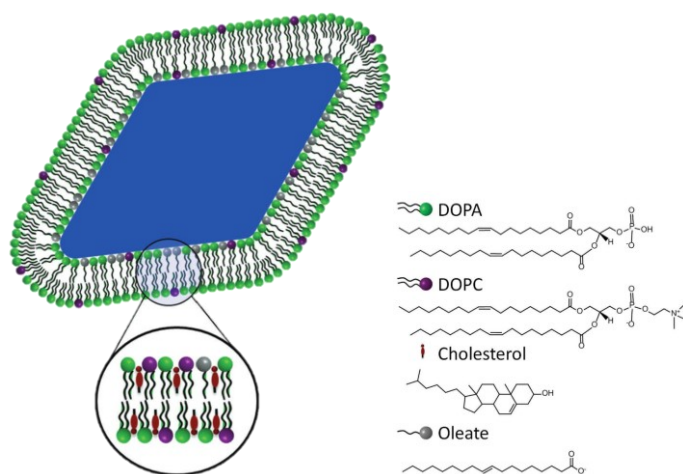


Figure 1.7 Schematic representation of a supported lipid bilayer encapsulated nanoparticle.⁸⁷

1.6. An opportunity for a novel treatment

An opportunity exists to combine elements of liposomal drug carriers, peptide therapeutics and photoactivated release. While this approach should have more widespread applications, the focus of this work was development of a nanoformulation towards the treatment of wounds with persistent infections. In the longer term, combination with upconverting nanoparticles as the source of energy for the photoactivation is envisioned, which then guides the component design. A review of relevant concepts is presented below.

1.6.1. Peptides and Light – Photocaged Therapies

Photolabile groups undergo a chemical response upon interaction with a photon, typically leading to a cleavage and release of some chemical species.⁹⁰ This characteristic offers spatial and temporal controls that have been leveraged by synthetic chemists for some time.⁹¹ More recently, biological⁹² and biomedical⁹³ applications have been developed, leading to a new genre of ‘photocaged’ therapies. These therapies encompass compounds, typically biomolecules such as proteins, that are protected from degradation or have their biological activity hindered until activation by light irradiation.⁹⁴ This concept can be applied to our proposed formulation, where the photolabile group can be used to tether the AMP to the delivery liposome until triggered release via light.

Careful consideration must be given as to which photolabile group would be suitable for tethering GL13K to the liposome surface. A few factors need to be considered: the compound

needs to be water soluble and stable and should not require light of a wavelength less than 200 nm to catalyze the cleavage mechanism since amide bonds absorb in this region as well. Further, the group and cleavage products must be biocompatible, and have reasonable kinetics for the application.⁹⁵ Some interesting examples of photocages include coumarin based groups and boron difluoride containing dipyrromethene groups (BODIPY). Coumarin based groups satisfy the above conditions, and also have fluorescent properties. This creates opportunities to monitor the reaction, or use the photocage as a tag.⁹⁶ Alternatively, the π conjugation of BODIPY allows for photolysis using visible light excitation.⁹⁷ However, due to concerns about hydrophobicity, and incorrect absorption wavelengths, these groups were not selected for our application.

A promising option, due to its absorbance between 300-400 nm with high quantum yield, and ability to leave the peptide without any vestigial fragments after the cleavage, is an *o*-nitrobenzyl (oNB) type group. Further, this type of photolabile linker is frequently applied in photocontrolled drug delivery and wound dressing applications.⁹⁸⁻¹⁰¹ A general reaction scheme for oNB bound to an amide,⁹⁶ along with the generally accepted photolysis mechanism,^{102,103} are presented in Figure 1.8. The cleavage proceeds through a phototautomerization before the formation of a hemiacetal, which fragments to release a carboxyl terminated product.¹⁰² When this carboxyl group is bound to an amine, CO₂ will be released spontaneously. Frequently, a version of oNB called 6-nitroveratryloxycarbonyl (NVOC) is used, with two methoxy groups as substituents on the aromatic ring, which is known to increase the absorbance at wavelengths >350 nm.⁹⁶ It has also been shown that these methoxy groups can be swapped for allyl ethers allowing the group to be used as a linker, without significantly impacting the absorbance spectrum of the compound.¹⁰⁴ Two such spectra are demonstrated in Figure 1.9, where the NVOC compound bound to a phospholipid incorporated in a liposome¹⁰⁵ is compared to the allyl ether version being utilized as a linker within a cyclic peptide.¹⁰⁴ Notably, broad absorbance is observed in both cases at wavelengths <400 nm, with maxima at approximately 350 nm, and as photolysis occurs, this absorbance decreases.

1.6.2. Click chemistry approaches to tether an antimicrobial peptide

A click chemistry approach can be used to attach the peptide to the exterior of the liposomes containing an alkyne-terminated (propargyl) lipid. Click chemistry is a subcategory of synthetic reactions that has many advantages over other traditional synthesis strategies. The criteria for a reaction to be considered ‘click chemistry’ include an aqueous reaction with little oxygen sensitivity and a high yield of stereospecific product with little to no side products, which when present are easy to separate.¹⁰⁶ Since its inception, reactions in this class have been used for drug discovery due to their ability to form complex chemical structures with diverse functional groups and heterocycles in relatively short sequences of reactions.^{106,107}

The pathway proposed in this thesis exemplifies a subclass of click chemistry: the bioconjugate reaction, wherein one or more biomolecules are either tethered or combined covalently. This application of click chemistry takes advantage of the mild reaction conditions so as to not damage sensitive biomolecules. Further, the high specificity of the reactions provides a high degree of selectivity, allowing for site-specific modification of proteins or nucleotides. Reactions can therefore be applied to label target compounds for imaging,¹⁰⁸ to tether them to delivery vehicles such as nanoparticles,¹⁰⁹ or to create bioconjugates for purposes such as biosensing.¹¹⁰

More recently, this genre of reaction has shown use in bioorthogonal chemistry, in which reactions are executed *in vivo*. This is feasible due to the gentle reaction conditions and their compliance with the environment within biological systems. The gain associated with using click chemistry for these processes is a large increase in specificity. A target biomolecule can be inoculated with a bioorthogonal chemical reporter, *i.e.* some functional group with the desired reactivity but not susceptible to biological reactions, and later probed for this chemical reporter. This allows for *in vivo* site-specific modification of many biomolecules, including proteins, lipids, glycans and nucleic acids. In turn, these biomolecules can therefore be easily tagged or given added functionality.¹¹¹

The Diels-Alder reaction, discovered in 1928 by Otto Diels and Kurt Alder, is often regarded as an early predecessor of click chemistry. Although it predates the formal concept of click reactions, the Diels-Alder reaction was the first side-product free reaction with a remarkable degree of stereoselectivity.¹¹² The Staudinger ligation was the next reaction introduced with a

high value add for bioconjugation. It has the ability to couple an azide group with a phosphine compound, resulting in an amide bond.¹¹³ The main drawback of this reaction is its relatively slow reaction rate, making modern reactions a more common choice. Arguably the largest leap in progress for click chemistry was the invent of the copper-catalyzed azide-alkyne cycloaddition (CuAAC), which was used to formally define these reactions in 2001 by Sharpless.¹⁰⁶ This reaction involves the reaction of an azide moiety with an alkyne using a Cu^{I} catalyst following the mechanism described in Figure 1.10 and will be evaluated in this thesis for the tethering of the GL13K peptide to a liposome nanocarrier. More recent examples of click reactions generally aim to broaden the applicable functional groups, (e.g. thiol-ene reaction¹¹⁴ and oxime ligation¹¹⁵) or to eliminate copper as a catalyst as it can be toxic in applications involving cells (e.g. strain-promoted azide-alkyne cycloaddition¹¹⁶ and tetrazene ligation¹¹⁷).

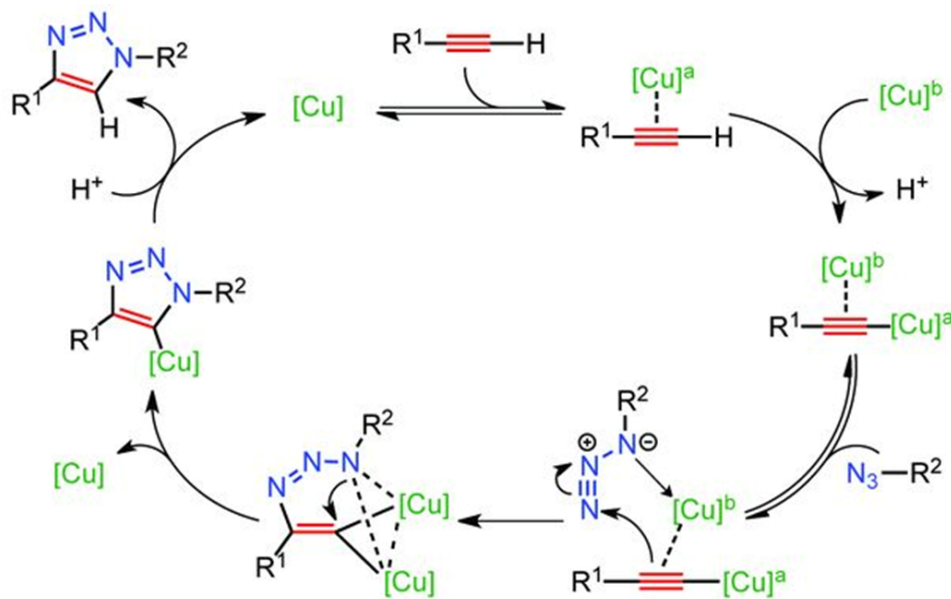


Figure 1.10 Cu^{I} catalysed azide-alkyne cycloaddition click reaction mechanism involving coordination of two Cu^{I} atoms which allows the alkyne to act as a nucleophile. An electron from the triple bond will attack the nitrogen at the 3rd position of the azide. This will push the electrons towards the first nitrogen, causing it to interact with the copper in the intermediate. The copper ions will leave, resulting in this 1,2,3-triazole product with high selectivity.¹¹⁸

1.6.3. Specific Objectives

Based on this literature review, the selected approach was to tether the peptide GL13K covalently to the exterior of liposomes using a lipid anchor, and an intermediate photolabile linker that would cleave upon irradiation with UV light. The specific thesis objectives are to:

1. design a novel photolabile peptide linker such that cleavage should occur without any vestigial fragments;
2. demonstrate attachment via click reaction of the peptide to the external leaflet of a phospholipid bilayer;
3. evaluate release of the peptide upon exposure to UV light.

Chapter two presents an overview of the experimental methods used in this thesis and Chapter three presents the results and discussion thereof. The thesis conclusions and an outlook for future research in this area are provided in Chapter 4. The appendices provide details of the chemical synthesis that was carried out for us by the Peptide Synthesis and Imaging Probe at Université de Sherbrooke (Appendix 1) and the calibration curves used for all assays (Appendix 2).

Chapter 2. Experimental methods

2.1. Materials

Phospholipids (1,2-dioleoyl-sn-glycero-3-phosphate (DOPA), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (N-propynyl) (DO-Propargyl-PC), 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DOPG), 1-Oleoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn-Glycero-3-Phosphocholine (18:1 12:0 NBD PC)) were purchased from MilliporeSigma (St. Louis, USA), at purity $\geq 99\%$ and used without further purification. Cholesterol ($>99\%$ purity), HPLC-grade chloroform, tris-hydroxypropyltriazolylmethylamine (THPTA, 95% purity), copper(II) sulfate pentahydrate ($\geq 98\%$ purity), (+)-sodium L-ascorbate ($\geq 98\%$ purity), phosphate buffered saline tablets, iron(III) chloride hexahydrate (97% purity), ammonium thiocyanate ($\geq 97.5\%$ purity), NaCl ($>99\%$ purity), Atto 565 NHS ester (HPLC purity $\geq 90\%$), 5(6)-carboxyfluorescein mixed isomers ($\geq 95\%$ purity), acetonitrile ($\geq 99.9\%$ purity), formic acid and anhydrous dimethyl sulfoxide (DMSO) were purchased from MilliporeSigma (St. Louis, USA). 2-[4-(2-Hydroxyethyl)piperazin-1-yl]ethane-1-sulfonic acid (HEPES) was purchased from BioShop Canada Inc. at 99.5% purity. Sodium bicarbonate and sodium hydroxide pellets were purchased from ACP Chemicals Inc. (Saint-Léonard, Canada). PierceTM bicinchoninic acid (BCA) Protein Assay Kit and Snakeskin Dialysis Tubing (10K molecular weight cut-off) were purchased from Thermo Fisher Scientific (Waltham, MA USA). GL13K peptide (95.10% purity) was purchased from CanadaPeptide (Montreal, Canada). Ultrapure water ($18.2 \text{ M}\Omega \text{ cm}^{-1}$) used was obtained from a MilliQ purification system.

2.2. Liposome fabrication

Stock phospholipid and cholesterol solutions made in chloroform are added to a round bottom flask in appropriate molar ratios. Then, the solvent is evaporated in a rotatory evaporator for 45-60 minutes to form a lipid film on the walls of the flask. Buffer, carboxyfluorescein fluorescent dye solution, or ultrapure water is then added in an appropriate volume to achieve the desired lipid concentration. The film is allowed to hydrate for 1-2 hours, before being vortexed at high speed for 5 minutes, forming multilamellar vesicles (MLVs). Then, the MLVs are reduced in size using an adapted method previously described by Mayer *et al.*¹¹⁹ The MLVs are extruded

through a polycarbonate filter with 0.2 μm pore size 41 times using a mini-extruder from Avanti Polar Lipids Inc. (Alabaster, AL, USA) to form large unilamellar vesicles (LUVs) with an expected diameter of 150-200 nm. Liposomal solutions are stored in the fridge and are used for a maximum duration of two weeks.

2.3. Copper(I) azide alkyne cycloaddition click reaction

Concentrations and procedure are based on the work of Hong *et al.* who optimized the CuAAC reaction for biomolecules in aqueous solutions against oxygen sensitivity of the reagents and to reduce oxidative stress caused by excess Cu^{I} .¹²⁰ Sodium ascorbate is used as an *in situ* reducing agent, allowing Cu to be kept in the less harmful oxidized state until the initiation of the reaction, where it catalyses the $\text{Cu}^{\text{II}} \rightarrow \text{Cu}^{\text{I}}$ reduction. Also, in order to sufficiently maintain the Cu^{I} state for the reaction, a chelating ligand is required as a catalyst. For bioconjugation reactions, the ligand may also act as a sacrificial reductant, protecting the biomolecules from unwanted oxidation. One such ligand is tris-hydroxypropyltriazolylmethylamine (THPTA).¹²⁰ Reagents were added at the concentrations and in the order described in Table 1. GL13K-linker conjugate concentrations were tailored such that they were in a slight excess of the available alkyne on the liposomes. The reaction proceeded under constant stirring with protection from light for 1.5 hours. Excess reagents were removed via dialysis.

Table 2.1 CuAAC Reagent Concentrations

<i>Reagent</i>	<i>Reaction Concentration</i>
CuSO_4	100 μM
THPTA	500 μM
alkyne liposomes lipid concentration	X (concentration selected based on application)
GL13K-linker conjugate	X + ~10% excess
Sodium ascorbate	2500 μM

2.4. Purification by dialysis

Dialysis was used to separate peptide-bound-liposomes from excess reagents used in the click reaction, or from peptide released post UV exposure. The 10K molecular weight cut-off pore size was selected as it is sufficiently large enough to allow diffusion of the peptide and click reagents through the pore, but too small for the liposome to pass. This technique is used both for purification and for peptide quantification. Dialysis tubing was prepared by wetting with ultrapure water. Sample was added into the interior of the tubing using a micropipette and clipped with dialysis clips. This tubing was suspended in ultrapure water using either a floater or bracket. For purification, the aim is to remove any excess reagents, so it was necessary to use ample dialysate. In all cases where dialysis was used for purification, a minimum of 300X the sample volume was used for dialysate, often achieved by replacing dialysate solution, over a minimum of 18 hours and stirring at constant speed, as described in the Snakeskin Dialysis Tubing user manual.¹²¹ When used for peptide quantification, the aim is to simply separate released peptide from the liposomes. Dialysate volume was therefore selected based on how much was needed to submerge the tubing. Samples in this experiment were dialysed for a minimum of six hours, enough time for equilibrium to be achieved. Due to the photolabile nature of the samples, they were dialysed under protection from light.

2.5. Stewart Assay

Quantification of phospholipids is achieved through the Stewart assay, which works based on the ability for ammonium ferrothiocyanate to complex phospholipids and form a coloured product, detectable in using a UV-Vis spectrophotometer.¹²² Other methods exist to quantify phospholipids, however the Stewart assay is unaffected by inorganic phosphate, allowing for phospholipid detection in a wider range of buffer solutions and without the need of strong acid for headgroup digestion.¹²³ Ammonium ferrothiocyanate has different degrees of complexation depending on the headgroup so calibration curves must be made for each lipid and lipid mixture, shown in Appendix 2.

Ammonium ferrothiocyanate solution was made by dissolving 2.703 g of ferric chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) and 3.040 g ammonium thiocyanate in 100 mL ultrapure water. Calibration curves were made using stock lipid solutions dissolved in chloroform and diluted in a

range from 0.02 mM to 0.2 mM with a total volume of 2 mL in chloroform and prepared in duplicate. Liposome samples were prepared as described in section 2.2 and an appropriate volume of lipid stocks between 0.1 and 0.5 mL were added to 2 mL chloroform. To all blanks, standards and samples, 2 mL of ammonium ferrothiocyanate solution were added. Vials were capped, and vortexed twice for 45 seconds each. The vials were centrifuged at 300 x g for 5 minutes. Note that if samples were not analysed immediately, they were protected from light. The aqueous phase was removed with a Pasteur pipette and the chloroform phase transferred to a cuvette for analysis by Agilent (Santa Clara, CA USA) 8453 UV-vis spectrophotometer, taking absorbance readings at 488 nm.

2.6. Dynamic light scattering (DLS) and ζ -Potential

For the confirmation of the hydrodynamic radius and stability of the formulation, as well as ζ -potential of liposomes, a Zetasizer Nano-S (Malvern Instruments Ltd, Worcestershire, UK) was used. Experiments were carried out in a disposable folded capillary cell (Malvern) at 10 measurements of 20 runs each. A volume of 0.7 mL and concentration range of 0.5-1.0 mM were used.

2.7. ATR-FTIR spectroscopy

The successful attachment of the peptide was evaluated using attenuated total reflectance Fourier-transform infrared spectroscopy (ATR-FTIR). Since the GL13K-clicked peptide has many IR active species, an approach of systematically adding one component at a time is taken for signal assignment. A Thermo Scientific (Waltham, MA USA) Nicolet iS5 FT-IR spectrometer equipped with an iD5 ATR accessory was used. A total of 64 scans were taken. Data were processed using Thermo Scientific Nicolet Omnic 9 software. To avoid interference from O-H stretch vibrations, lyophilized samples were used. Samples were lyophilized in a LyovaporTM L-200 Pro (Buchi, Switzerland), for a minimum of 24 hrs and under protection from light, until an endpoint was determined visually.

2.8. Raman spectroscopy

A Cora 5600 Raman spectrometer from Anton Paar (Graz, Austria) was used at a laser wavelength of 532 nm and 50 mW power level. For each sample, 10 averaged spectra with a 20

can count at 5000 ms per scan were used. Samples were prepared at ~0.5 mM in ultrapure water and taken from the same synthesis batch. Readings were taken in a quartz cuvette with a 1 cm path length.

2.9. Circular dichroism

Spectra were taken using a Jasco J-815 spectropolarimeter (Jasco Corporation, Essex, UK) equipped with an air-cooled 150 W xenon arc source. Measurements were taken as an average of 5 accumulated scans at 20 °C, in a quartz cuvette with a 0.2 cm path length. A background of ultrapure water was subtracted from each spectrum, and spectra were normalized to mean residue ellipticity (MRE) following Equation 2.1.¹²⁴

$$\text{Mean residue ellipticity } [\theta] = \frac{mdeg}{l * c * R} \quad \text{Equation 2.1. Mean residue ellipticity}$$

Where *l* is the pathlength of the cuvette in mm, *c* is the concentration in M and *R* is the number of amino acid residues.

2.10. Mass spectrometry

Electrospray ionization mass spectrometry (ESI-MS) was used for confirmation of successful click reaction. A Thermo LTQ Orbitrap Velos mass spectrometer (Waltham, MA USA) was used in both positive and negative modes to identify the formation of the peptide-lipid conjugate as well as analyse residual starting materials. Samples were prepared as described in sections 2.2 and 2.3, and diluted to 10 µM. They were injected alongside 30% acetonitrile and 0.05% formic acid and at a volume of 2 µL. Data were analysed using the software Freestyle™ 1.8 SP2 and *m/z* were compared to calculated theoretical mass of the compounds of interest, determined using the University of Warwick mass calculator.¹²⁵

2.11. Peptide quantification assay

Peptide quantification was achieved using the Pierce™ bicinchoninic acid (BCA) Protein Assay Kit purchased from Thermo Scientific™ (Waltham, MA USA). The method was adapted from the Enhanced protocol provided in the product documentation.¹²⁶ Briefly, BCA working reagent (WR) was prepared by combining 50 BCA reagent A with 1 part BCA reagent B. In individual Eppendorf tubes, 0.1 mL standard or sample was added to 2 mL WR, mixed, then incubated at

60°C for 30 minutes, before cooling to room temperature. Absorbances were read at 562 nm in an Agilent 8453 UV-vis spectrophotometer and corrected for a blank of ultrapure water. A standard curve was prepared for the GL13K peptide for a concentration range of 5 µg/mL – 250 µg/mL with 5 points prepared in triplicate (demonstrated in Appendix 2).

Peptide quantification was used in three instances. 1) to measure peptide attached to the liposomes via the click reaction, 2) to measure indirectly the peptide released after cleavage of the photolabile linker through UV exposure release through the difference of peptide on the liposomes before and after release, and 3) for direct measurement of peptide.

2.12. Liposome leakage carboxyfluorescein assay

A fluorescence assay is used to determine the stability of the bilayer by quantifying leakage from the interior space of the liposome. Carboxyfluorescein (CF) is the fluorescent dye that allows for this measurement since it has the ability to self quench through fluorescence resonant energy transfer (FRET) when in a high concentration. Liposomes can therefore be fabricated to contain high localized concentrations of the dye, then, through leakage or rupture, the dye is released. The resulting dilution by the surrounding medium produces a fluorescence signal.¹²⁷

The procedure was adapted from that used by Huang *et al.*¹²⁸ Carboxyfluorescein loaded liposomes are fabricated following the procedure outlined in section 2.2, using a HEPES buffered carboxyfluorescein solution for the hydration and extrusion steps (10 mM HEPES, 50 mM 5(6)-carboxyfluorescein mixed isomers (≥95%, Sigma Aldrich), 10 mM NaCl, buffered to pH=7.4). After extrusion, a solvent exchange is performed to remove CF from the surrounding space. This is done using size exclusion liquid chromatography via a Sephadex G-50 packed column and a column running buffer of 10 mM HEPES, ~107 mM NaCl at pH=7.4 whose osmolarity was brought within ±5 mOSM/Kg_{H2O} using an µOsmette micro osmometer (Precision Systems Inc.) and adjusting NaCl concentration. Fractions containing CF-loaded liposomes were determined by colour and verified by DLS (section 2.5), pooled and a final liposome concentration determined using the Stewart assay (section 2.4).

Leakage assays were measured *in situ* in a Cary Eclipse Fluorescence Spectrophotometer by Agilent Technologies (Santa Clara, CA USA). Liposomes were diluted to 2.5 µM to be within the sensitivity range of the instrument. Instrument parameters were set such that λ_{ex} =480 nm,

$\lambda_{em}=520$ nm, with excitation and emission slits both set to 5 nm. The liposome release experiments were run in three phases. First, the fluorescence intensity of just the liposomes is taken for 2 minutes to demonstrate a stable baseline. Next, the cuvette is removed, and the peptide added at the appropriate concentration, inverting the cuvette several times before placing the sample back in the fluorimeter and observing the fluorescence intensity for 1 hour. Finally, the cuvette is taken out again, and 10 μ L of a 2% (v/v) solution of Triton X-100 is added before monitoring the fluorescence intensity for 5 minutes. This induces complete rupture of all liposomes and allows for the maximum fluorescence intensity to be taken. Fluorescence intensities were then converted percentage of CF released using the equation described below.

$$\%CF \text{ Release} = \frac{F_t - F_0}{F_{max} - F_0} \times 100 \quad \text{Equation 2.2 \%CF Release}$$

Where F_t is fluorescence intensity at time t , F_0 is the initial intensity and F_{max} is the intensity after total rupture.

2.13. TIRF-microscopy

Total internal reflectance fluorescence microscopy (TIRFM) was used as a visual affirmation of the attachment of GL13K AMP to the liposome surface via the colocalization of a fluorescently labeled bilayer and a fluorescently tagged GL13K. TIRFM works by shining a laser of a particular wavelength onto a glass slide at an angle greater than the critical angle for total internal reflection. The only light shone through the slide and onto the sample is an evanescent beam that is intense enough to excite fluorophores but only penetrates a shallow depth, therefore imaging only the first 100-200 nm of the sample.¹²⁹ This technique provides high resolution with minimal background interference, and due to its shallow field of view is ideal for imaging cell surfaces or other lipid membranes.

Two fluorescent probes were selected for this procedure. To label the liposomal bilayer, a nitrobenzoxadiazole (NBD) based phosphatidylcholine lipid was added into the liposomal formulation at 1 molar percent, making the final formulation 63:7:29:1 DOPA:Propargyl PC:cholesterol:NBD-PC. The wavelengths of excitation and emission are 474 nm and 533 nm respectively. To label GL13K, ATTO 565-NHS ester was selected as it could be used to probe the peptide through amine coupling to any of the available lysines. The samples were prepared

following the liposome procedure in section 2.2, using labelling buffer at pH 8.3 in the hydration phase. Labelling buffer was prepared following the procedure described in the ATTO dye catalogue.¹³⁰ Namely 20 parts 0.01 M PBS were added to one part of a 0.2 M sodium bicarbonate solution pH adjusted to 9.0 with 2 M NaOH. The CuAAC click reaction was carried out following the procedure described in section 2.3 and after purification by dialysis, underwent the amine coupling to the probe. The ATTO 565-NHS ester was dissolved in DMSO to make a 20 mg/mL solution. Three molar equivalents in 8 μ L of this solution was added to the peptide clicked liposomes and incubated for 12 hours. Excess dye was removed via dialysis purification.

TIRFM samples were prepared by adding a drop of sample to a microscope slide cover, and images were captured using a Nikon (Minato City, Japan) Ti microscope equipped with a 100x Plan Apo lens (NA 1.49) and Photometrics Prime BSI camera (0.07 μ m/px). Illumination was provided by 488 and 561 nm lasers angled using the TIRF arm of the microscope to provide a highly inclined laminated optical sheet for efficient illumination of the sample, and appropriate filter cubes (488/10 ex, 525/50 em and 561/10 ex, 575lp em).¹³¹

Chapter 3. Results and Discussion

3.1. Designing an approach to tether an antimicrobial peptide to a phospholipid bilayer

An aim of this thesis is to design and construct a nanoformulation that will tether the antimicrobial peptide GL13K to a liposomal delivery platform, wherein the peptide therapeutic can be released *in situ* using UV light. Using a bilayer platform confers many advantages, as they are known to be biocompatible, can be tuned for form and function, and in the future can easily be adapted for nanoparticle encapsulation and nanoconstruct formation. Stimuli responsive liposomes are not a new concept and have been reported in the past for drug and AMP delivery. However, the general delivery mechanism is to use membrane disruption to rupture the bilayer and deliver a therapeutic from the interior aqueous space.^{132,133} To make a versatile delivery system that could be used for either liposomes or coated nanoparticles, this work will focus on tethering the peptide to the external lipid leaflet. This also allows delivery of the peptide without threatening the integrity of the bilayer, since release no longer means passing through the membrane. Also, in order to have a stimuli responsive characteristic, the peptide must be tethered in a manner that can be cleaved by a stimulus of choice, in this case photocleavage using UV light in the ~350 nm region.

Figure 3.1 below represents the final target compound developed as a result of this work. It comprises several components: 1) is the GL13K peptide (blue) attached to 2) an NVOC type photolabile linker (yellow) (discussed in Chapter 1.6.1) linked to 3) a triazole ring (the product of the CuAAC reaction mentioned below), that tethers the linker to 4) a dioleoyl phospholipid that can be embedded into a bilayer.

Since GL13K is an AMP with membrane disrupting activity, it is preferable that it be attached after bilayer and liposome formation. The peptide can be added post liposome fabrication taking advantage of CuAAC click reaction, as shown in Figure 3.2. This is made possible since an appropriate anchoring lipid, 18:1 DO-propargyl-PC (compound **1**), was available commercially. To this, GL13K can be tethered via a customized photolabile linker (compound **2**). However, GL13K with the photolabile linker had to be synthesized, and is discussed in the following section.

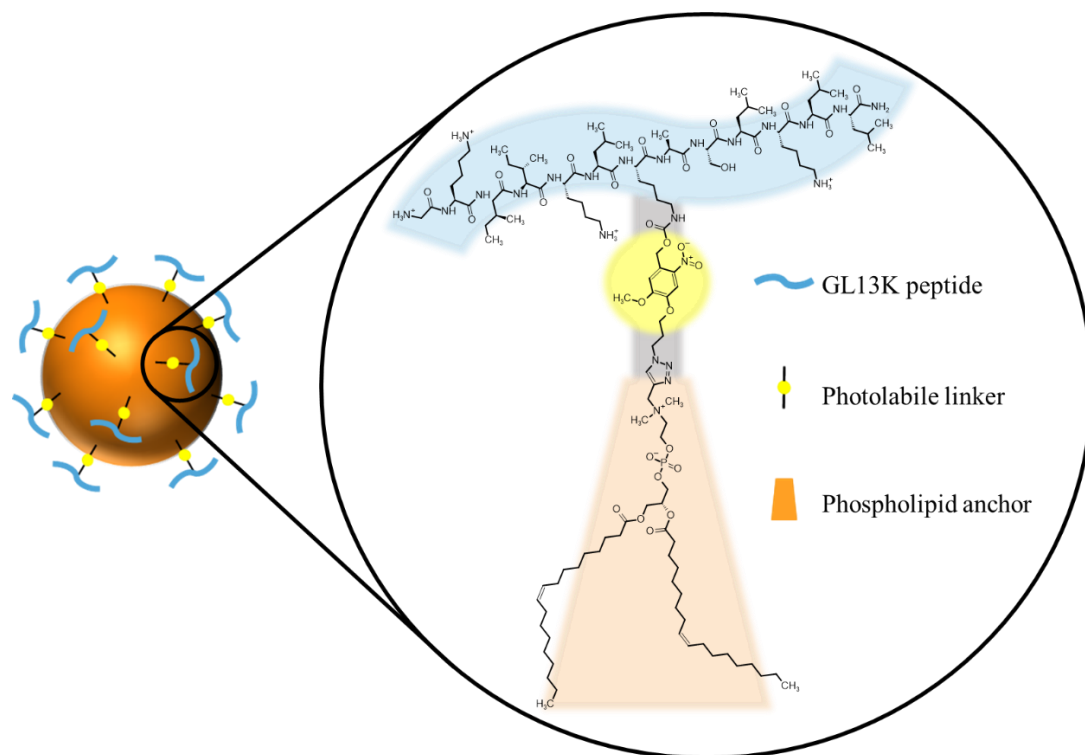


Figure 3.1 Schematic demonstrating the tethering of GL13K antimicrobial peptide to the exterior of a liposome via a photolabile linker and phospholipid anchor.

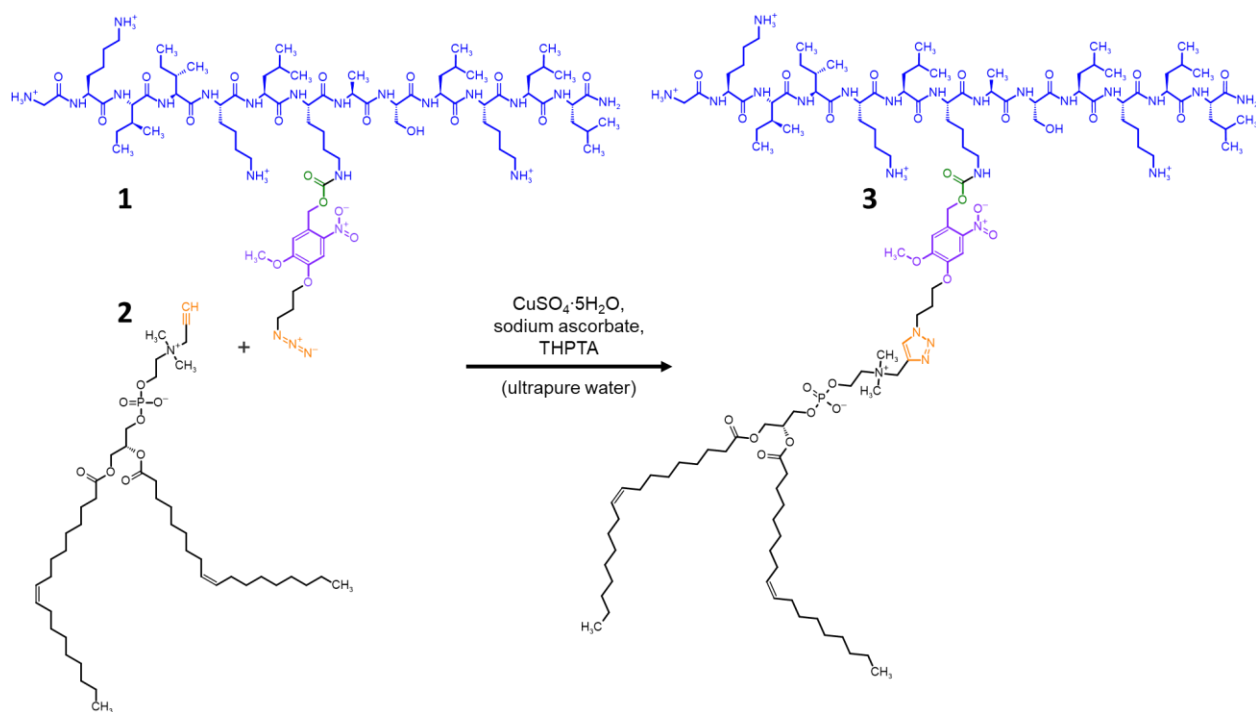


Figure 3.2 CuAAC click reaction tethering the GL13K-linker conjugate to DO-propargyl-PC.

3.1.1. Synthesis of the peptide-linker conjugate

Development of the synthetic pathway for the NVOC bound GL13K was undertaken in collaboration with the Peptide Synthesis and Imaging Probe at Université de Sherbrooke. The resultant synthesis pathway is modified from Hoffmann and Kazmaier,¹⁰⁴ in order to bind the linker to a lysine residue and add the azide group needed for the CuAAC reaction.

Beginning with vanillin, alkyl halide was added to the alcohol via an SN1 reaction, followed by nitration of the benzene to add the nitro group with regioselectivity. A reduction was employed to convert the aldehyde to an alcohol, followed by substitution of the bromine for the azide via another SN1 reaction, and finally the formation of the chloroformate group using phosgene. Once the chloroformate is formed, the *o*-nitrobenzyl containing linker can be added to the peptide by a reaction to a primary amine on a lysine residue. The linker was added selectively to the lysine at position seven through selective deprotection of that residue. An early goal of the treatment design was to limit the peptide's activity until after release. The reason for this is to limit membrane disruption until the *in situ* delivery of GL13K to increase the stability and shelf life of the formulation. The approach taken was to selectively tether the peptide via a central lysine residue (K7), with the aim of inducing sufficient steric hinderance and limiting its degrees of freedom. For a full synthesis procedure, see the outlined synthesis pathway as provided by the Peptide Synthesis and Imaging Probe at Université de Sherbrooke in Appendix 1.

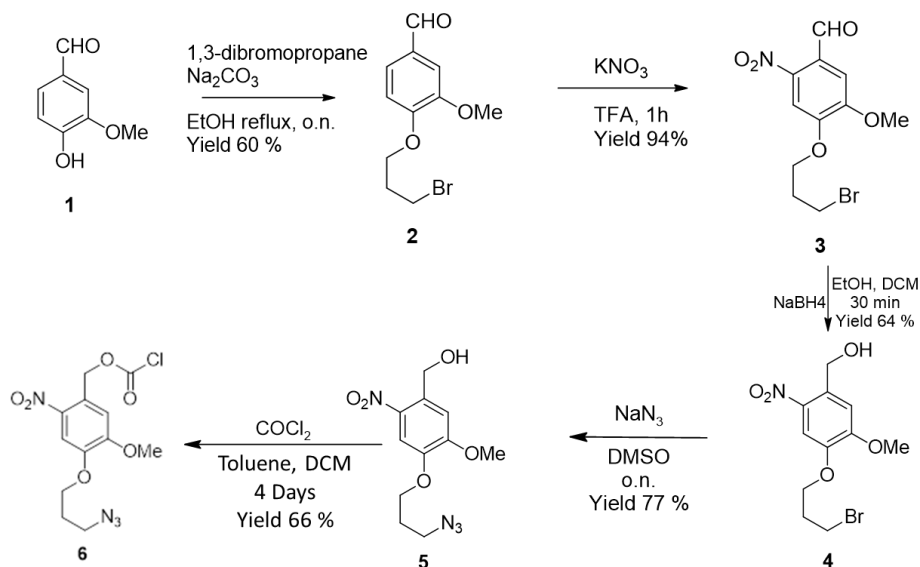


Figure 3.3 Synthesis by Peptide Synthesis and Imaging Probe Platform at Université de Sherbrooke.

3.2. Liposome size and uniformity

The liposome formulation was selected following a previously reported formulation, 64:7:29 molar ratio DOPA:DOPC:cholesterol, as discussed in Section 1.5.⁸⁷ This composition was selected for its potential of achieving the long-term goal of using a supported lipid bilayer on an upconverting lanthanide-doped nanoparticle, wherein the emission from the latter can be used to photocleave the peptide. Consideration was given as to how the DO-propargyl-PC should be incorporated. In order to neither impact the charge of the liposome (provided anionic DOPA) which was designed for use in conjunction with the UCNPs nor the membrane permeability (reduced via the inclusion of cholesterol), both of which serve important functional roles (as discussed previously), the DOPC in the original formulation is replaced entirely with DO-propargyl-PC, as is shown in Figure 3.4. DO-propargyl-PC is used for its modified headgroup containing an alkyne moiety. After liposome fabrication, this alkyne moiety will be presented on the liposome surface and offer the ability to bind to the azide containing GL13K-linker conjugate through the CuAAC click reaction.

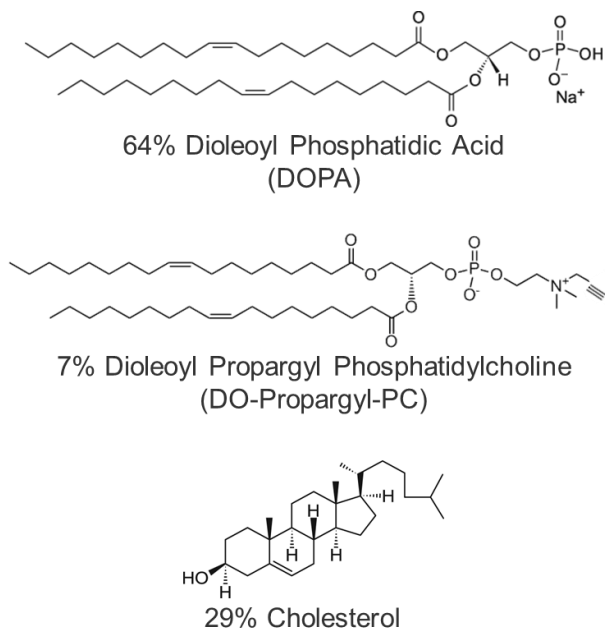


Figure 3.4 Structures and molar percentages of constituents in the liposome formulation.

It was important to evaluate the size and size changes for the liposomes throughout the fabrication process, for which DLS was used. This allowed us to evaluate the formulation

stability, including ensuring that the alkyne modified lipid did not significantly affect the concentric unilamellar bilayer. The size was also verified after the click reaction to ensure that the reaction conditions were not too harsh. DLS results are shown in Figure 3.5 and demonstrate satisfactory size uniformity. Comparing the Z-average size of the liposomes in the original formulation (64:07:29 DOPA:DOPC:Ch) to the new formulation substituting the alkyne modified-PC lipid for DOPC, the results show 171 nm and 170 nm, respectively. After the click reaction conjugating GL13K to the exterior of the liposome, the Z-average size increases to 180 nm, suggesting the addition of the peptide increases the hydrodynamic radius of the liposomes which is consistent with peptide binding on the exterior surface.

The polydispersity index (PDI), denoting the uniformity of the size distribution, increases from 0.166 before the reaction to 0.262 after the reaction, meaning the post-reaction liposomes are less uniform in size. This increase in PDI could have a number of causes, including aggregating peptide or liposomes, or large contaminants from click reagents or the purification process. Figure 3.5 shows that a secondary species in the micron size is introduced through the tethering of the peptide, however the majority of the liposomes remain at a similar size thus confirming that the membranes are not disrupted throughout the various stages of fabrication.

Table 3.1 DLS Z-average size and PDI for liposomes at various stages of GL13K tethering

Liposome	Z-Average Size (d.nm)	PDI
64:7:29 DOPA:DOPC:Ch	171	0.178
64:7:29 DOPA:DO-propargyl-PC:cholesterol	170	0.166
GL13K Clicked Liposomes	180	0.262

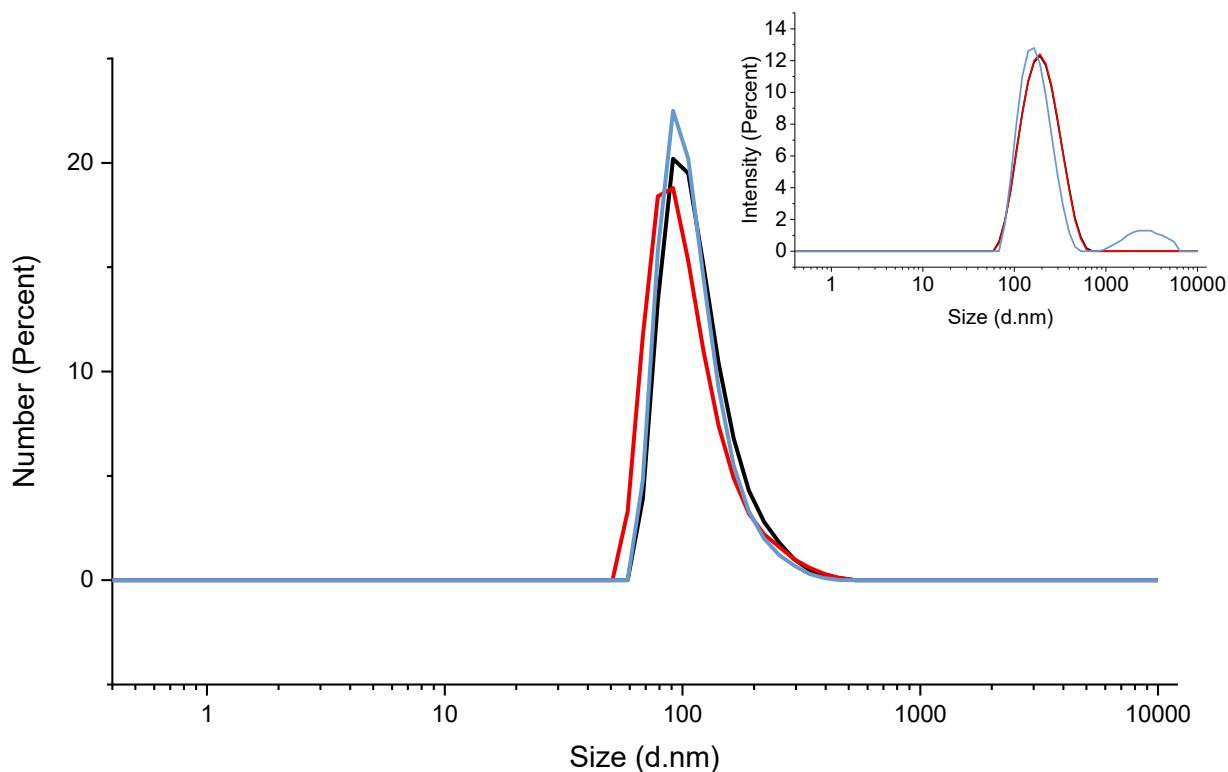


Figure 3.5 DLS results showing the uniformity of liposomes with hydrodynamic radii between 170 nm and 180 nm with control 64:07:29 mol% DOPA:DOPC:Ch liposomes (black), 64:07:29 mol% DOPA:DO-propargyl-PC:Ch liposomes (red) and GL13K tethered liposomes (blue). The inset shows the data plotted with respect to signal intensity, which shows the introduction of a larger species during peptide tethering.

3.3. The covalent tethering of GL13K to liposomes

In light of the peptide being cationic with a +5 charge, and the membrane of the liposome being anionic with its main constituent as DOPA, it is possible that in solution, the peptide is held in close proximity to the liposomes due to electrostatic interactions, rather than being covalently attached. Such non-covalent interactions are a valid way to entrap and deliver the peptide and have been demonstrated to be successful in the past.⁶⁰ However, the aim of this approach is for triggered delivery of the peptide with spatiotemporal control, so a covalent attachment is necessary with tethering via the photolabile linker.

Infrared spectroscopy was used to monitor key functional groups throughout the fabrication of the nanoplatform to verify that GL13K was, in fact, covalently tethered to the liposome. Figure 3.6 shows the spectra at each stage of fabrication, with the green and blue spectra representing

controls of the liposomes and the liposomes with native GL13K, respectively. The red and black spectra represent the nanoplateform before and after the click reaction. The green spectrum exhibits the expected spectrum for a phospholipid liposome containing cholesterol, with the main signals coming from sp^2 and sp^3 C-H stretching and C=O/C-O stretching from the ester linkages in the phospholipid. Some weaker signals from the hydrocarbon C-H bending are also present between 1300 and 1500 cm^{-1} . When GL13K is added, we see the appearance of the amide I and II bands in the blue spectrum, as well as a N-H stretch signal at 3270 cm^{-1} from the presence of the peptide. The red and black spectra contain all the aforementioned signals with one important addition, the red spectrum also shows a signal at 2095 cm^{-1} assigned to an azide stretching band. The presence of this band before the click reaction, and its absence after the click reaction in the black spectrum, along with the presence of the amide bands in both samples, confirms the successful conversion of the azide through the CuAAC reaction into a triazole ring, which is not IR active.

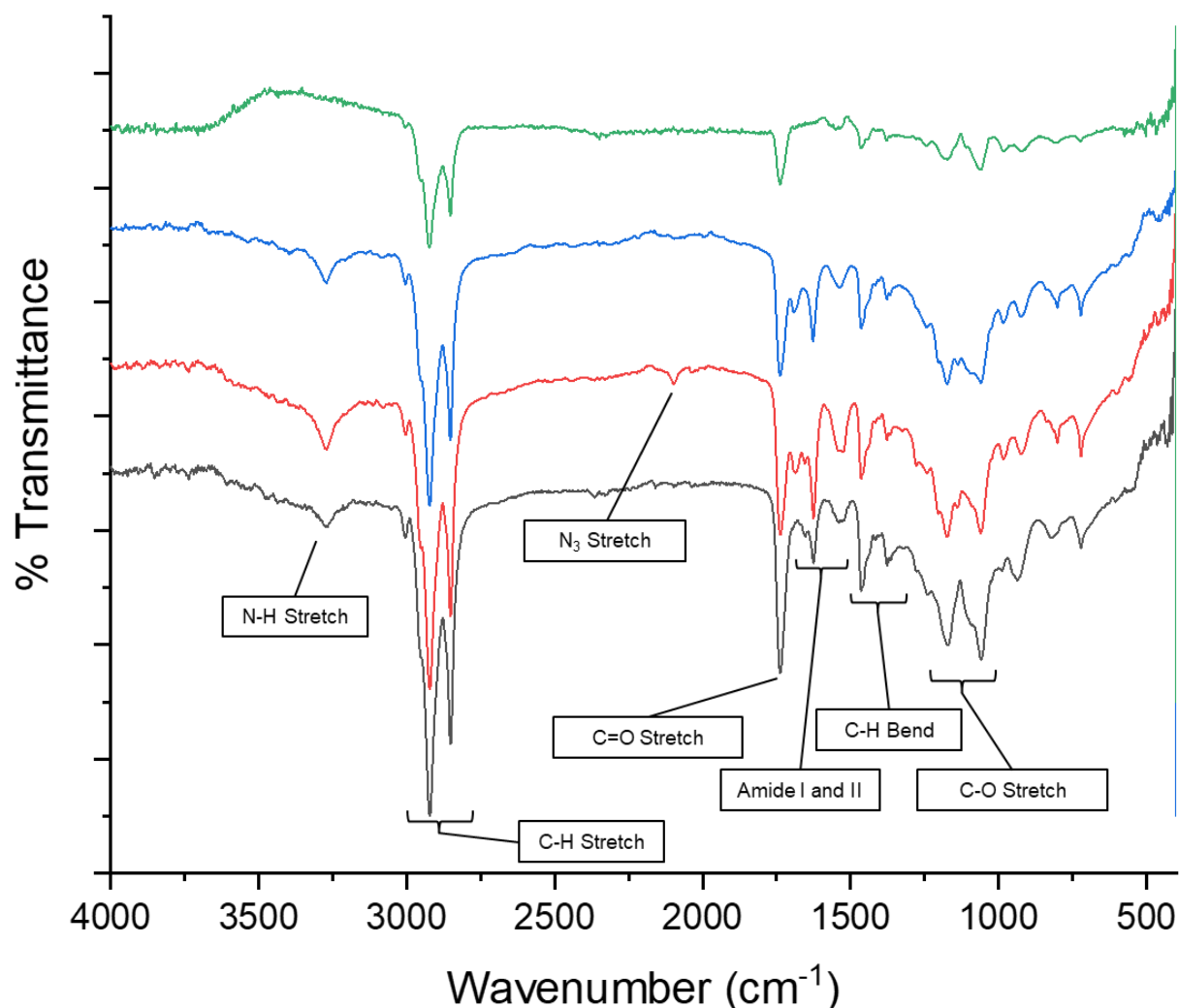


Figure 3.6 ATR-IR spectra of the nanoformulation surveying progressive stages of fabrication, including control liposomes (green), control liposomes with GL13K added at 7 mol% (blue), liposomes with DOPC replaced by 7% alkyne modified PC lipid and GL13K-linker compound added at 7 mol% (red) and GL13K tethered liposomes (black).

An attempt was made to monitor the attachment of the peptide using Raman spectroscopy. Raman spectroscopy has been used in the past to follow the *in situ* progress of similar reactions,¹³⁴ and has good potential since the 1,2,3-triazole product of the click reaction is Raman active.¹³⁵ Unfortunately, when spectra were taken at various stages of the nanoplateform formulation and peptide delivery, the spectra showed similar signals and low signal-to-noise, making it hard to differentiate real changes due to the presence of the functional groups of interest. We suspect that since there is a high ratio of phospholipid to attached peptide, that the

signal from the lipids overwhelms that of the 1,2,3-triazole, masking their appearance. Figure 3.7 shows strong bands at 3419 cm^{-1} and 3214 cm^{-1} coming from the amine in PC headgroup lipids, 1636 cm^{-1} from C=C, and weaker bands at 1409 cm^{-1} , 1294 cm^{-1} , 493 cm^{-1} from alkyl groups and potentially Si-O from the quartz cuvette.

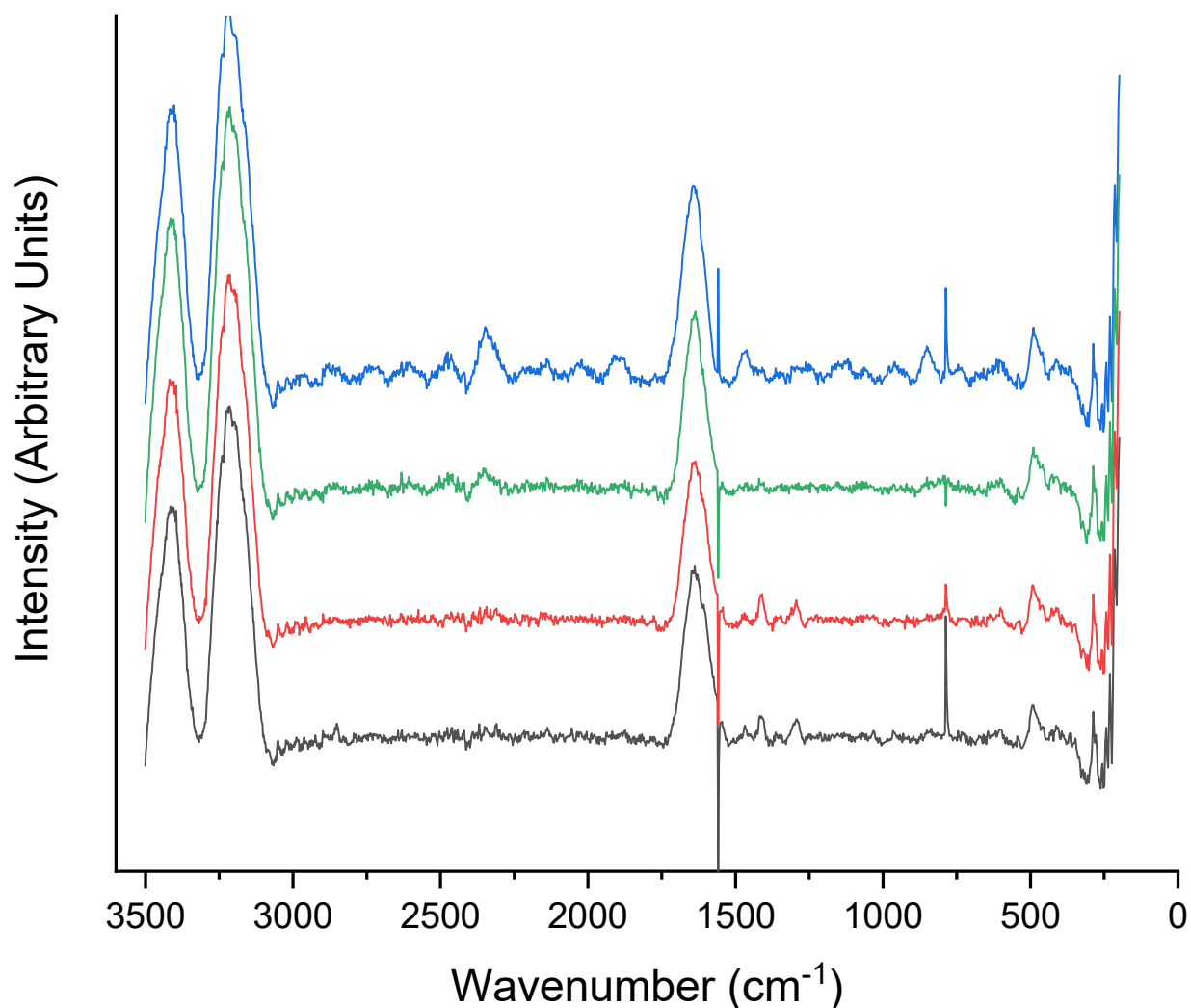


Figure 3.7 Raman spectra taken at each stage of the nanoplatform formulation and peptide release. The spectra represent the liposomes with the alkyne modified propargyl lipid (black), the GL13K clicked liposomes (red) and the formulation post UV exposure both before the peptide is removed by dialysis (green) and after (blue).

To complement the infrared and Raman spectra, TIRFM was employed to visually demonstrate the colocalization of the GL13K peptide and the liposomes. This was achieved by incorporating

an NBD-tagged phospholipid (18:1 12:0 NBD PC) in the liposome formulation and subsequently selectively labeling GL13K after tethering using an Atto 565 NHS ester dye. The use of distinct excitation wavelengths for the two labeling dyes, combined with the high spatial resolution offered by TIRFM, enabled independent imaging of the peptide and liposome using different light sources where individual GL13K-clicked liposomes could be resolved (Figure 3.8). The overlay image demonstrates the colocalization wherein the tethered peptide forms a halo (purple) around the liposome (yellow). Although the size of the halo likely corresponds to the higher relative fluorescence efficiency, the colocalization of the peptide label and lipid label suggest successful tethering.

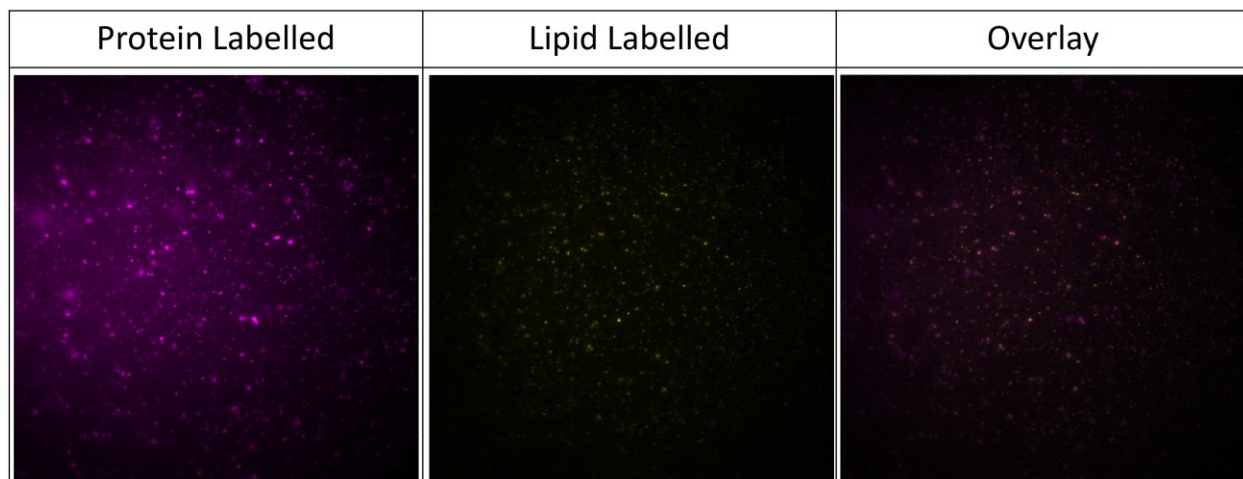


Figure 3.8 TIRFM image demonstrating the colocalization of fluorescently labelled GL13K (purple, λ_{ex} =561 nm, λ_{em} =590 nm) and liposomes (yellow, λ_{ex} =488 nm, λ_{em} =531 nm).

As a final confirmation that the GL13K is indeed covalently tethered to the alkyne modified lipid, a mass spectrum was taken of the liposomal formulation after the click reaction. The spectrum (Figure 3.9) exhibits the peaks for the double- and triple-charged ions for the lipid-conjugated peptide, with theoretical m/z of 1271.3425 and 847.8975 respectively, with both mass errors being within 0.4 ppm.

Thus, colocalization of the peptide and liposome has been demonstrated by TIRFM, with the presence of appropriate functional groups demonstrated by ATR-FTIR, and at the correct mass as demonstrated by MS, proving the successful formation of the target compound through click chemistry synthesis at the surface of a liposome. However, electrostatic interaction may still play

a role, and possibly prevent release of the peptide after cleavage of the photocleavable linker (to be discussed later).

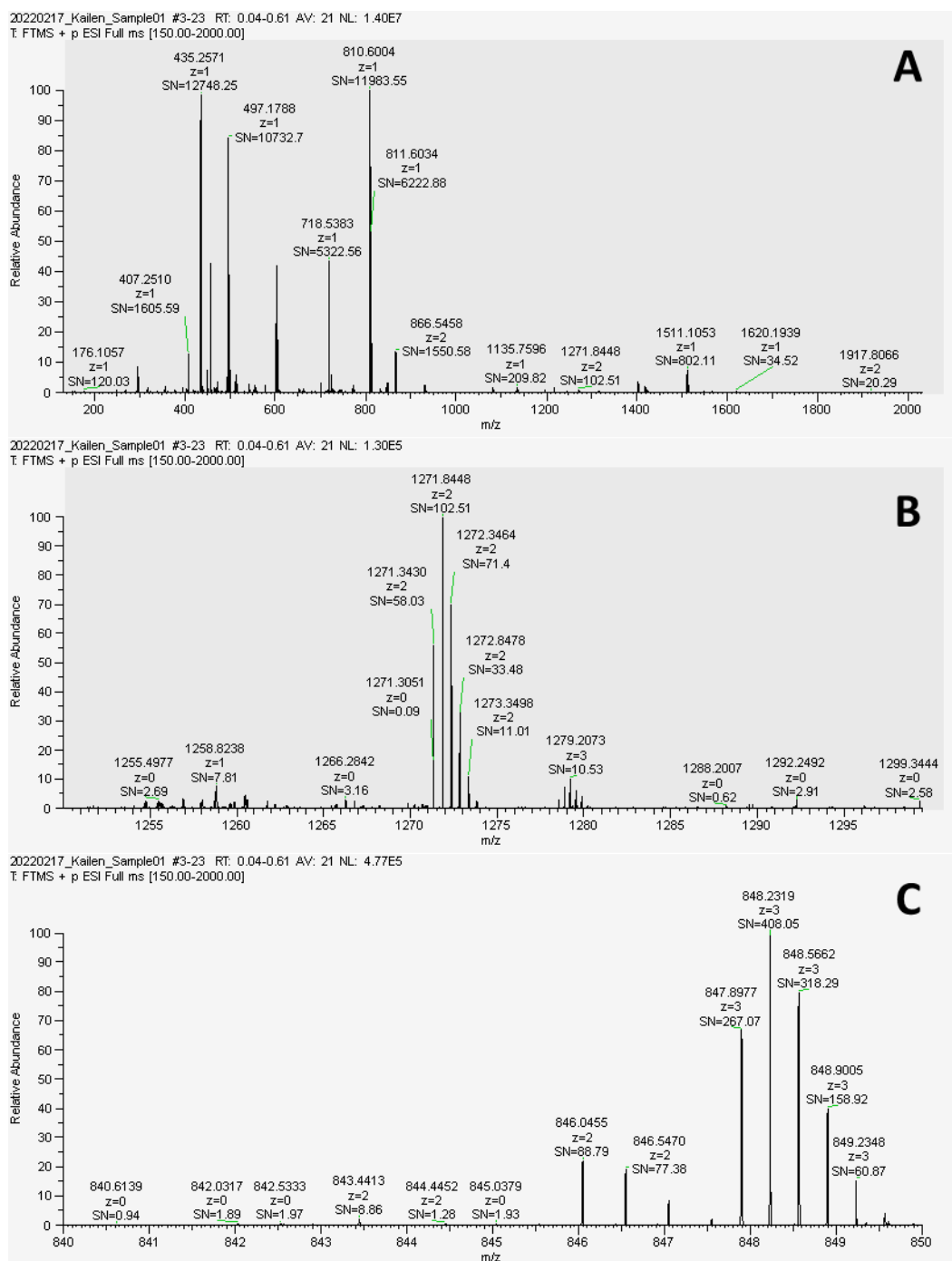


Figure 3.9 Mass spectrum of GL13K tethered liposomes with the full spectrum (A) and the expanded double charged ion peak (B) and triple charged ion peak (C).

3.4. GL13K Conformation and membrane activity

The activity of GL13K antimicrobial peptide has been well studied and is known to be closely linked to its conformation. The peptide is membrane active, achieving membrane disruption through a carpet model.³⁵ In brief, GL13K has a random coil conformation while free in solution, but proximity to an anionic membrane triggers a conformational change. The peptide takes on a β -strand structure, then aggregates into antiparallel β -sheets at the membrane surface.⁴⁵ These β -sheets are cumbersome and inflict strain on the membrane, leading to rupture and cell death. Studying the secondary structure of the peptide is therefore essential to understand its activity when bound to the liposomal delivery vehicle.

The backbone conformation of the peptide was evaluated using CD and compared to previous work that demonstrated the conformational change of the peptide in proximity to phospholipid bilayers with varying lipid compositions and thus electrostatic environments.³⁵ The CD spectra shown in Figure 3.10 demonstrate that in the presence of the 64:7:29 DOPA:DO-propargyl-PC:cholesterol liposomes, the peptide backbone adopts the same β -conformation as was previously observed in the presence of DOPG liposomes. A similar structural transformation was observed for liposomes comprising between 25 and 75 mol% anionic lipids but is not observed in the presence of net neutral liposomes and thus has been attributed to an electrostatic driving force.^{36,37}

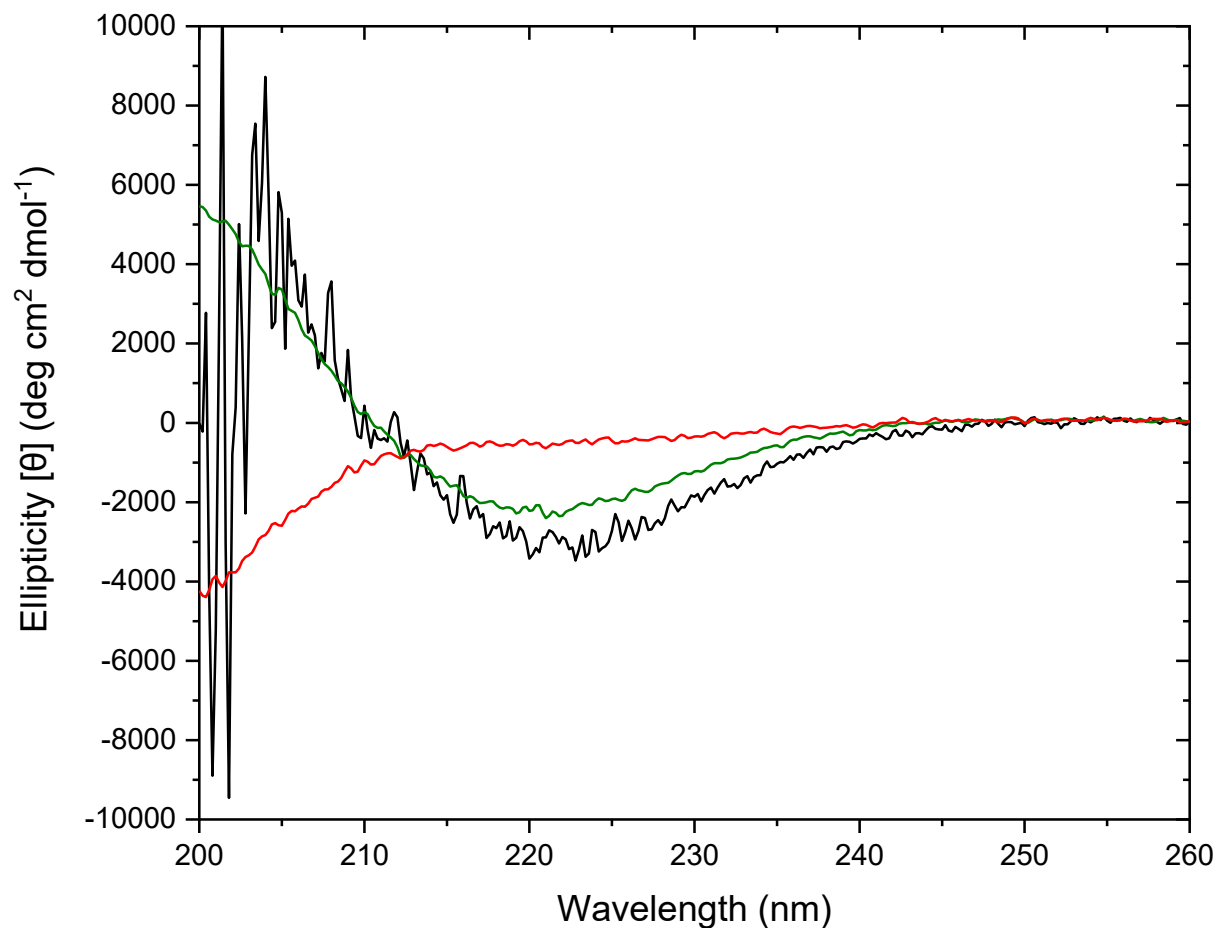


Figure 3.10 Far UV-CD spectra of GL13K in presence of DOPC (red)³⁵, DOPG (green)³⁵ and 64:07:29 DOPA:Prop PC:Ch (black) liposomes. Note that the previously reported DOPC and DOPG containing spectra are included here for comparison.

While CD gives valuable information on backbone structure, one of its shortcomings is that it provides no information of hydrogen bonding, meaning overall secondary structure can not be determined, i.e., it can not be discerned whether the peptide is forming a β -sheet or a β -strand while tethered. The possibility remains that the peptide is sufficiently restricted, preventing assembly into β -sheets. To gain hydrogen bonding information, one can return to the ATR-IR results presented in the previous section. One region that is particularly useful for protein IR is the amide I band, which has been extensively studied for its ability to discriminate secondary structure. The amide I signal will shift depending on the hydrogen bonding pattern of different groups along the backbone. The amide I band derived predominantly from C=O stretching, is not affected by side chains, and is sensitive to the backbone orientation and interactions in secondary

structures. Both random coil and alpha helices show an amide I band positioned at 1654 cm^{-1} , β -turns a signal around 1672 cm^{-1} and β -sheets lead to a splitting of the band into signals around 1633 cm^{-1} and 1684 cm^{-1} . With examination of the amide I region more closely, presented in Figure 3.11, a stark difference in the amide I bands can be observed.

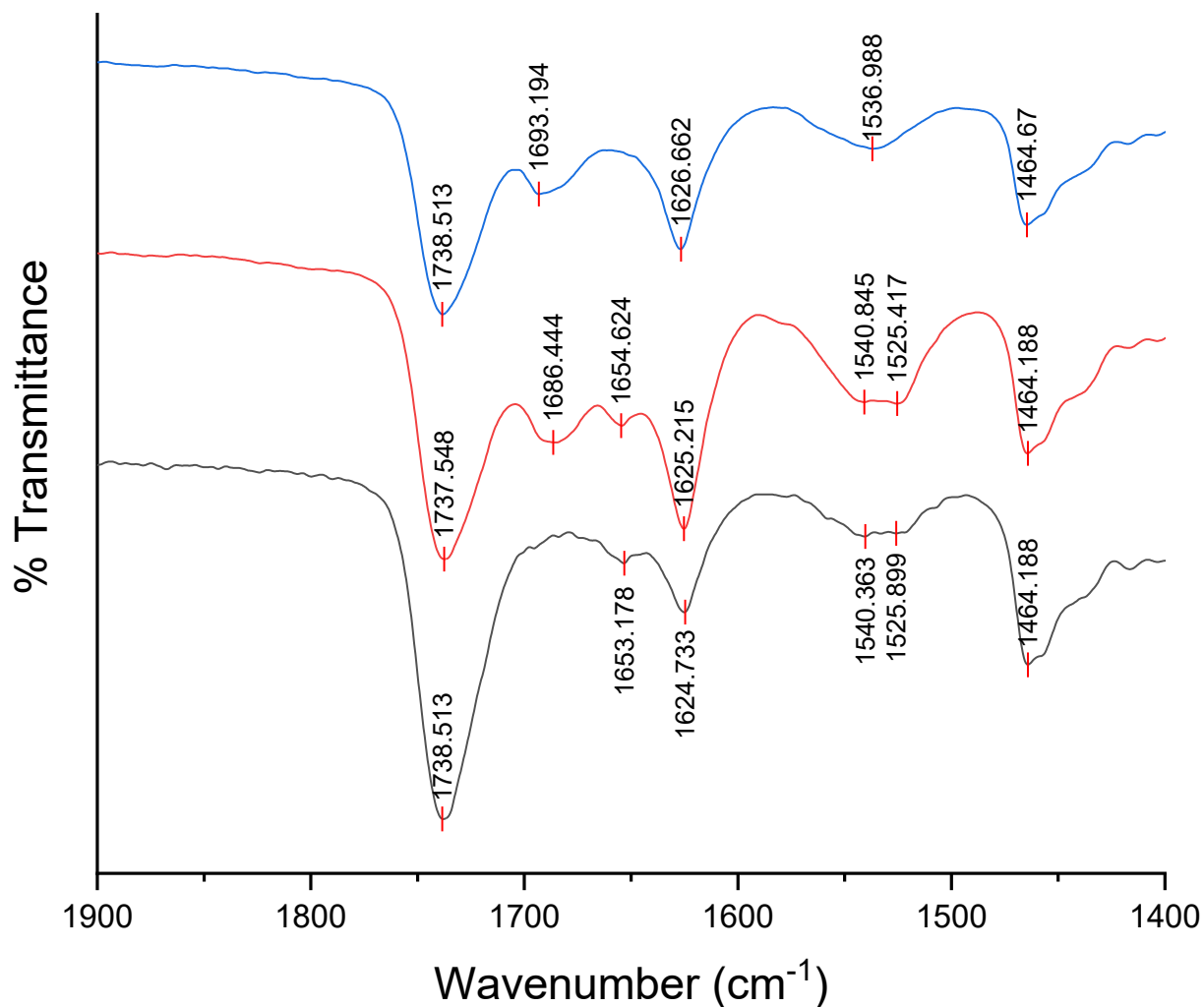


Figure 3.11 Expanded view of ATR-IR spectra presented in Figure 3.6 focused on the amide I and II band regions. The presented spectra are control liposomes with GL13K added at 7 mol% (blue), liposomes with DOPC replaced by 7% alkyne modified PC lipid and GL13K-linker compound added at 7 mol% (red) and GL13K tethered liposomes (black)

GL13K in its native state in the presence of the anionic liposomes (blue), exhibits a signal at 1693 cm^{-1} suggesting a β -sheet conformation. A band at 1633 cm^{-1} is expected to be present, but also eclipsed by other N-H bending vibration signals, shifting the peak to lower wavenumbers.¹³⁶

When GL13K is clicked to the liposomes (black), we see a shift to 1654 cm^{-1} , the region associated with random coil and alpha helices. This demonstrates lack of β -sheet formation, likely due to the restriction in mobility imposed by being tethered to the bilayer. Interestingly, when the GL13K-linker conjugate, added to the liposomes but not clicked (red), we see a coexistence of these two signals. This suggests that the peptide is present in both forms and gives some insight into the behaviour of GL13K. During the tethering process, the peptide folded state transitions from a free form in solution to a conformationally restrained state when bound to the linker, and finally to a state of both conformational and spatial restriction when tethered to the liposome. With each level of restriction, the peptide's capacity to form β -strands and β -sheets is impeded. Considering the necessity of β -sheet formation for GL13K activity, this represents a favorable advancement for a nanoplatfrom that emphasizes triggered activity.

Given that GL13K does undergo a conformation change, it is important to assess whether this transformation affects membrane stability, as structural transformation is an important component of the membrane disrupting activity of AMPs.³⁰ The extent of liposome rupture was determined using a CF leakage assay. CF is a fluorescent dye that has the ability to self-quench through fluorescence resonance energy transfer (FRET).¹²⁷ Liposomes are loaded with a high concentration of dye, which will have little to no fluorescence. CF in the surrounding medium is removed through column chromatography, so that when liposomes rupture and release CF, it will be diluted in the inter-liposomal space. This dilution leads to a measurable increase in fluorescence signal allowing for the degradation of the liposomes to be tracked. One of the design strategies was to add the linker to the peptide at the central lysine to limit its membrane activity until it is released. CF experiments were carried out in the presence of either native GL13K or the modified GL13K (referred to as peptide-linker conjugate). The native GL13K represents the peptide post cleavage from the liposome. The latter is necessary to ensure that the peptide with the linker modification would not disrupt the membrane prior to the click reaction.

The assay concentration must be tailored to account for the size of the liposome, as the CF contained within the internal compartment increases exponentially with size. In this assay, dilute concentrations of liposome, $2.5\text{ }\mu\text{M}$, were used in order to bring the fluorescence signal to within the range of the instrument. The peptide was added at a seven molar percent ratio to the lipid concentration, to simulate the amount bound to liposomes in the nanoplatfrom. For this reason,

the peptide is present at concentrations that are lower than its minimum inhibitory concentration (MIC). Although the peptide is present at concentration lower than its MIC, the amount of peptide per liposome is representative of the peptide to lipid ratios in the final nanoplatform. In Figure 3.12 below, the extent of membrane disruption as a function of peptide:lipid ratio is displayed and is similar to those previously reported.³⁵ While little disruption is observed at 7 mol% GL13K, the percent release is nonzero and remains observable.

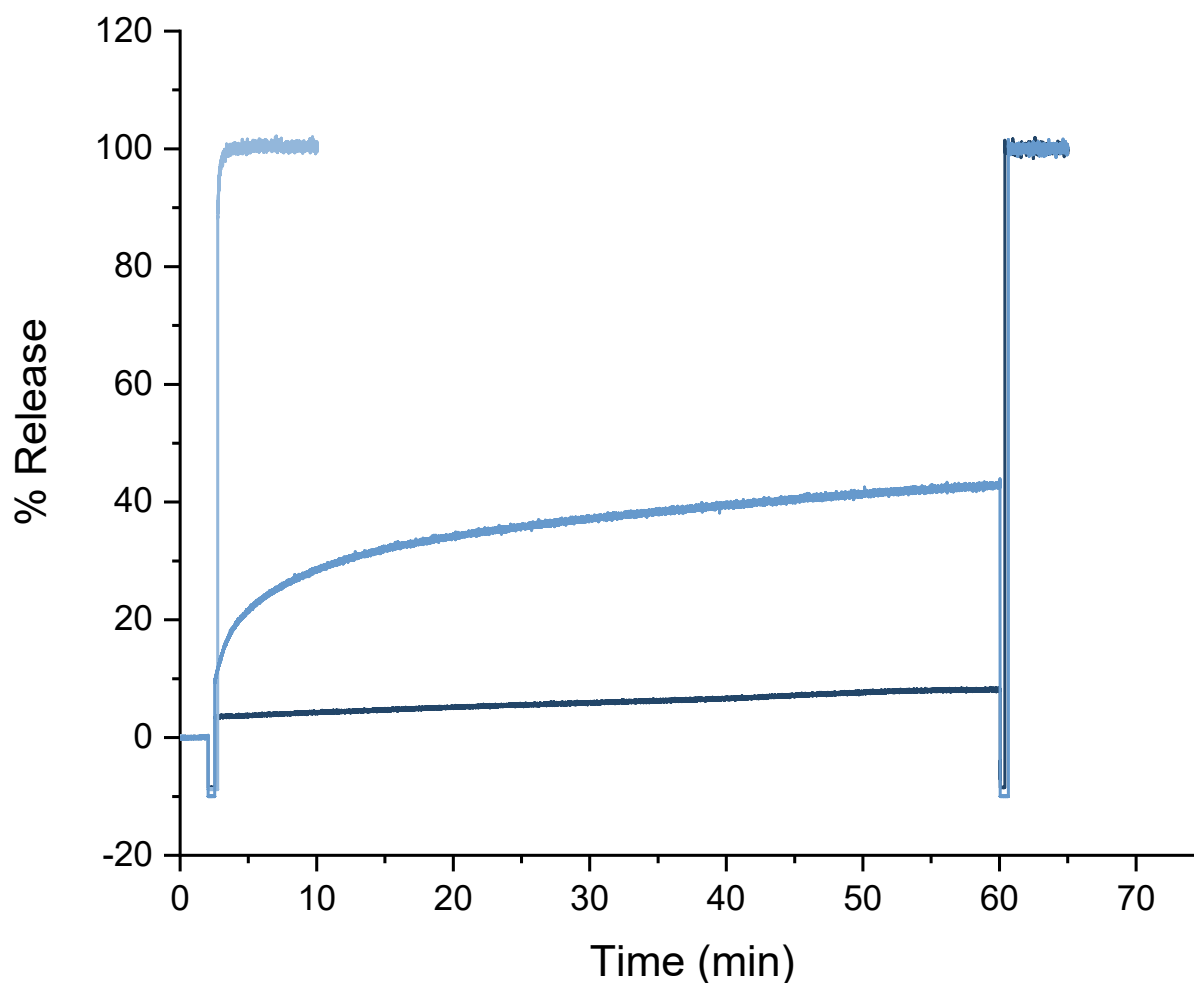


Figure 3.12 Liposomal membrane disruption CF assay on DOPG liposomes to demonstrate concentration dependence at increasing ratios of GL13K, specifically 7 mol% (dark), 14 mol% (medium), 70 mol% (light).

Initially, liposomes comprising 64:7:29 (mol%) DOPA:DO-propargyl-PC:Ch, the original lipid formulation were examined (Figure 3.13 A). With both unbound GL13K and with the peptide-linker conjugate, there was almost no CF release, meaning that neither peptide exhibited any

activity. It has been previously reported that the space filling effects of cholesterol hinders the activity of GL13K by altering membrane permeability.¹³⁷ To determine if cholesterol is enhancing the stability of our formulation, the experiment was repeated with liposomes containing comparable molar ratios of PC headgroup phospholipid to PA (90:10 mol %) but without cholesterol (Figure 3.13 B). Again, no activity was observed. To determine if this lack of activity was caused by the low peptide concentration (below MIC), the experiment was repeated with the same peptide concentration in the presence of pure DOPG liposomes, whose interactions with GL13K has previously been well studied.³⁵ In this instance, both free and linker-bound peptide showed activity (Figure 3.13 C). Interestingly, the conjugation of linker showed an increase in activity of the peptide, leading to an approximately 30% CF release, compared to approximately 7% without the linker. These findings suggest that even though complete rupture is not achieved, an anionic bilayer still induces active membrane disruption, even at low concentrations below the MIC of GL13K. The lack of activity of the 90:10 DOPA:DOPC liposomes suggests that the decreased surface potential, or perhaps differences in GL13K's ability to interact with the headgroups prevents its lysis of these liposomes, and we suspect that the addition of cholesterol further increases the stability of the formulation. These results justify a future study into the membrane disruption mechanics of GL13K at higher concentrations in order to prove the utility of this platform in infected wound treatment applications.

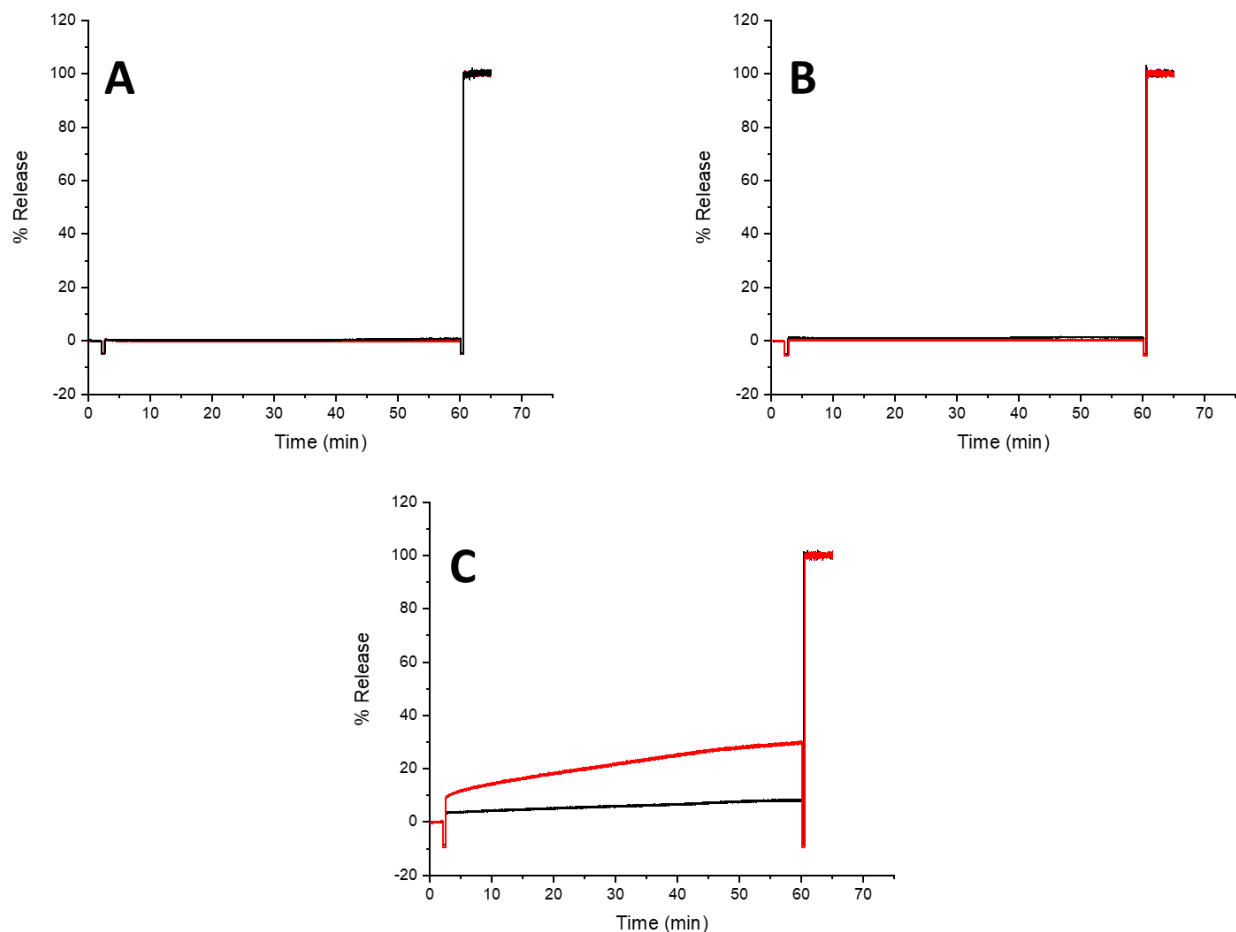


Figure 3.13 Membrane disruption CF assay comparing the effect of GL13K (red) and GL13K-linker conjugate (black) on 2.5 μM liposome systems: (A) 64:07:29 mol % DOPA:DO-propargyl-PC:Ch, (B) 90:10 DOPA:DOPC and (C) DOPG.

Finally, a liposome leakage assay was performed to evaluate the stability of the liposomes throughout UV exposure to verify that liposomes remain intact during irradiation and that GL13K released *in situ* does not degrade them. In this study, liposomes with GL13K covalently tethered via the photolabile linker were irradiated with 365 nm light for 30 minutes. Figure 3.14 demonstrates that the fluorescence does not increase significantly after the UV exposure, confirming that the release of peptide via UV exposure is a viable delivery mechanism. The lack of fluorescence and steady baseline at the beginning of the experiment also confirms that the formulation is stable while the peptide is tethered.

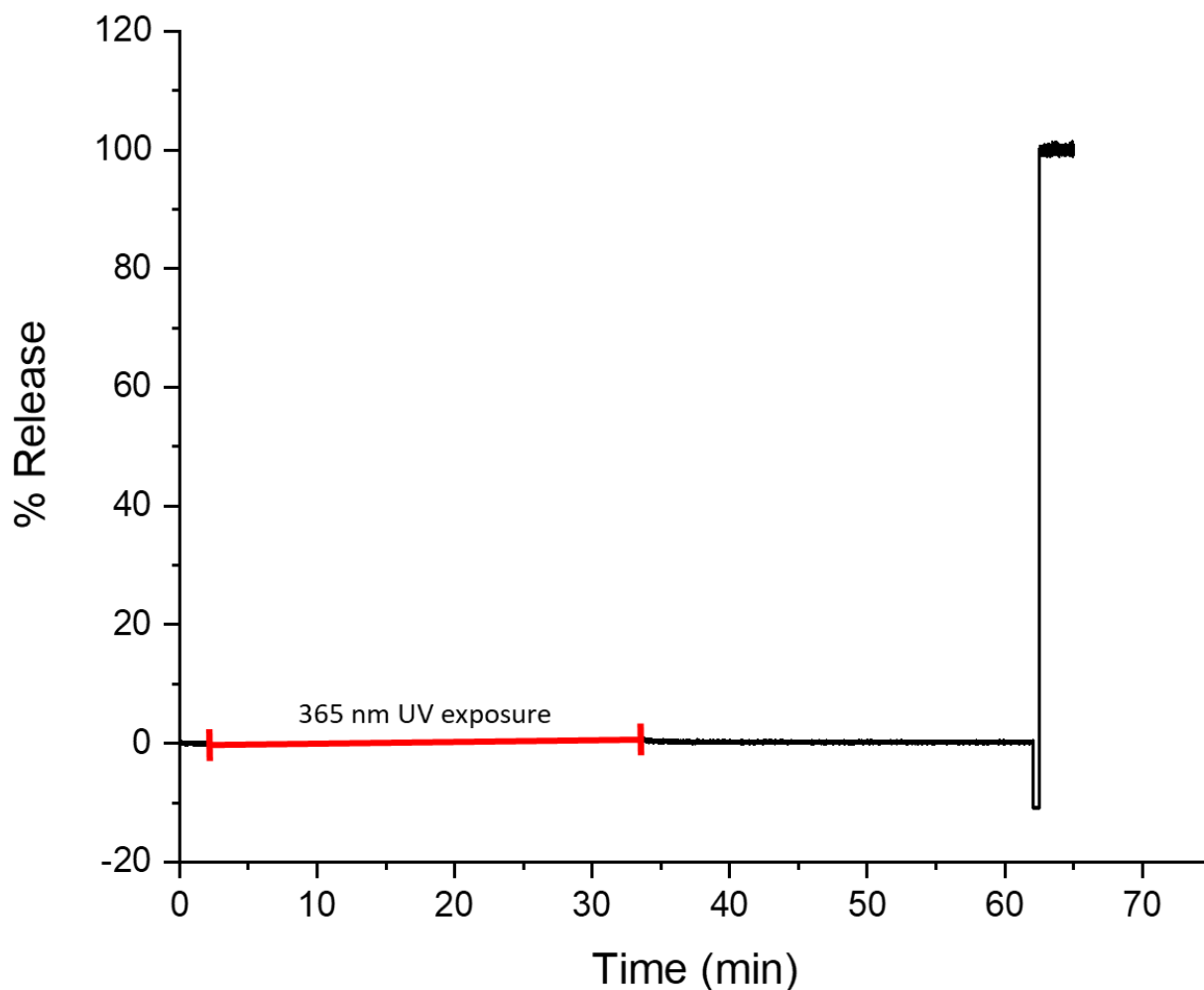


Figure 3.14 Liposomal membrane disruption assay on GL13K clicked liposomes including a 30-minute 365 nm UV light exposure to demonstrate membrane stability during *in situ* release.

3.5. Peptide quantification for attachment and release

An effort was made as well to quantify the amount of peptide attached to the liposomes, to determine the efficiency of the click reaction, and also to quantify the peptide released after UV exposure, in order to determine the delivery capacity. The BCA assay described in Chapter 2.11 was used to determine the concentration of GL13K.

In a preliminary experiment, GL13K-tethered-liposomes were irradiated with 365 nm light for 30 minutes and then dialysed to separate peptide from liposomes. However, the dilution incurred during dialysis yields a peptide solution that is too dilute for quantification by the BCA assay. To address this, a lyophilization step was added to the procedure in order to concentrate the

peptide. In this instance, the dialysate solution, containing any peptide that was cleaved and separated from the liposomes, was lyophilized and reconstituted in ultrapure water at the same volume of the initial sample (1 mL). The results of the peptide quantification are tabulated below (Table 3.2). Both the peptide removed via dialysis as well as peptide remaining on the liposomes were quantified, from liposomes with an initial lipid concentration of 0.5 mM. Additionally, liposomes were irradiated for an additional 4 hours (i.e., 4.5 hours total), to determine the impact of irradiation time on peptide release. In a standard prepared containing 200 $\mu\text{g/mL}$ GL13K, only 164.46 $\mu\text{g/mL}$ was detected after lyophilization, demonstrating that some loss, approximately 18%, can be expected when reconstituting the peptide.

For the experiments involving photoactivated release of GL13K from the liposomes (1 mL of 0.5 mM lipid), an average concentration of 7.26 ± 0.98 $\mu\text{g/mL}$ GL13K was released. The MIC of GL13K against *E. coli* and *P. aeruginosa* is 5 $\mu\text{g/mL}$ and 8 $\mu\text{g/mL}$ respectively.³⁴ This means that the liposomes are capable of delivering an effective amount of peptide from 1 mL of 0.5 mM liposomes. The release of only a portion of the peptide opens avenues for repeat dosages as will be discussed in Chapter 4. If we assume that all of the DO-propargyl-PC lipid is successfully tethered to GL13K (i.e., 7 mol% of the initial liposomal lipid concentration), then the theoretical maximum concentration of the peptide in the initial liposomal solution used herein is 49.8 $\mu\text{g/mL}$. It needs to be recognized that this is an overestimation, since 50% of the DO-propargyl-PC is expected to be found on the inner leaflet of the liposomal bilayer, making it not available for attachment. Thus, one can estimate that the maximum amount of peptide to be released is approximately 25 μg per mL of liposome formulation.

To assess the actual total peptide available for release, an attempt was made to perform the same peptide quantification assay using both the initial liposomal solution and the solution remaining after irradiation and dialysis. The peptide on the initial liposomal solution was found to be nearly double the theoretical maximum and the peptide remaining after release was also significantly above this maximum. Considering just the trends, the data are consistent with what was expected, a decrease in concentration due to peptide being released, and a longer UV irradiation leads to increased peptide release. However, the determined peptide concentrations for the initial liposomal formulation and the liposomal formulation after irradiation and dialysis cannot be a real representation of the peptide in solution, and another factor must be affecting the assay.

Table 3.2 GL13K release experiment peptide assay results.

	Sample	Calculated GL13K concentration ($\mu\text{g/mL}$)	Instrumental standard deviation ($\mu\text{g/mL}$)
Lyophilized dialysate samples	Method blank	-0.076	± 0.0109
	200 $\mu\text{g/mL}$ GL13K standard	164	± 0.0179
	GL13K released after 30 min UV exposure 1	8.19	± 0.0067
	GL13K released after 30 min UV exposure 2	6.24	± 0.0104
	GL13K released after 30 min UV exposure 3	7.36	± 0.0040
	GL13K Clicked Liposomes	93.2	± 0.0070
Samples from inside dialysis bag	Liposome 30-minute UV exposure	69.9	± 0.0134
	Liposome 4.5-hour UV exposure	61.2	± 0.0181

The same set of experiments were repeated, this time monitoring both the lipid and peptide concentrations, in order to account for any change in concentration during dialysis. Also, a control experiment was included with GL13K-tethered-liposomes that undergoes the same dialysis and lyophilization, omitting only the UV irradiation. In this case, the peptide concentration was found to actually increase in both samples, when it should have decreased. The lipid assay showed a drop in liposome concentration from 0.50 mM to 0.33 mM through dialysis, which also suggests there should have been a decrease in peptide concentration. Regardless, there is not a large difference between UV exposed liposomes and those without UV exposure.

Table 3.3 Direct comparison of UV irradiated GL13K-tethered-liposomes peptide assay.

Sample name	GL13K concentration (µg/mL)	Standard deviation (µg/mL)
GL13K concentration in initial tethered liposomes	32.231	±0.0222
GL13K on liposomes with no UV exposure	91.729	±0.0042
GL13K on liposomes with UV exposure	92.608	±0.0066

Two possible explanations for the anomalous high peptide readings from the internal dialysis compartment emerge. First, it must be noted that the BCA assay does not work by direct quantification of the protein, but rather the presence of amide bonds catalyses a reaction that creates a coloured product. For this reason, the assay does not reach an end point, and one must consider the possibility of interference from other components that may also catalyze the reaction and contribute to the signal. Cu^{II} is used in the assay as well as in the CuAAC click reaction and any remaining Cu^{II} would interfere with the assay.¹³⁸ Although it was expected that free Cu^{II} should be removed during the extensive dialysis purification, there is the potential for a strong electrostatic interaction of the positively charged cations with surface of the anionic liposome. Thus it is fully plausible that retention of Cu^{II} by the anionic liposomes would interfere with the assay and artificially inflate the resulting peptide detection for liposome-containing samples. The second explanation could be inherent with the design of the system. The electrostatic attraction of the liposomes could be too high to allow for GL13K to diffuse away, hence the small difference between liposomes that are irradiated with UV light and those not irradiated. However, while this can account for a high peptide concentration detected within the dialysis bag, it cannot be the cause of a signal significantly higher than the theoretical maximum. These results underscore the need for an alternate technique for accurate quantification of the peptide while attached to the liposome, and post release via UV irradiation, as will be discussed in detail in Chapter 4.

Chapter 4. Conclusions and future directions

The first objective of this thesis was the actual design of an AMP delivery vehicle, that could tether GL13K to a phospholipid bilayer in a manner such that release was triggerable using light in the UV range. Based on a literature review, a peptide-linker-lipid conjugate compound was elucidated. This compound took advantage of a customized NVOC-type photocleavable group that would release the peptide in its native form with no vestigial fragments. Due to the addition of an azide moiety to the NVOC linker, and the commercial availability of a headgroup modified alkyne phospholipid, the CuAAC click reaction could be used to tether the peptide at the liposomal surface. This allows for peptide attachment to occur after liposomal fabrication. The alternative of having the peptide prebound to the phospholipid would require the introduction of the membrane disrupting peptide during liposome fabrication, likely resulting in unsuccessful or unstable bilayer formation. The added advantage of adding GL13K to preformed liposomes is that all peptide is to be bound to the exterior surface, making for a more efficient nanoplatform. Additionally, of the proposed direction of incorporating an upconverting nanoparticle, the conjugation of the peptide to the lipid prior could hinder the formation of a conformal supported lipid bilayer. The synthetic pathway for photocleavable linker bound to the peptide was developed as a modified version of previously reported pathways,¹⁰⁴ and developed in collaboration with Peptide Synthesis and Imaging Probe at Université de Sherbrooke, who then successfully carried out the synthesis.

The second objective was to demonstrate the successful attachment of GL13K to the liposome surface. Liposomal stability was first confirmed throughout attachment. Colocalization of the peptide with the liposomes was demonstrated via TIRFM. Showing colocalization was however not sufficient proof of covalent attachment, due to the inherent electrostatic attraction between GL13K and the anionic liposomes. The covalent attachment was therefore confirmed by demonstrating the disappearance of an azide stretch band while maintaining the amide I and II bands throughout attachment via ATR-FTIR. This attachment was then reaffirmed using mass spectrometry to show the presence of the target compound.

The second objective was successfully attained, but there is an opportunity to improve the methodology. As mentioned in Chapter 3.3, similar CuAAC reactions have been monitored *in situ* taking advantage of the formation of the Raman active 1,2,3-triazole group.^{134,135} While

attempts made to similarly monitor the attachment of GL13K yielded spectra that were overwhelmed by signal from the liposomes, the signal at specific wavenumbers can be increased using a resonance Raman spectroscopy technique. This method involves the significant increase of target signal intensities through the selection of an incident wavelength of resonant amplitude to that of the target electron transition.¹³⁹ Due to the complex nature of the sample matrix while the peptide is anchored to the bilayer, signal amplification is likely necessary, and would allow for the monitoring of the reaction which in turn would allow reaction conditions to be optimized for efficient tethering.

Characterisation of the nanoplatform was also carried out. The first area investigated was the conformation of GL13K while tethered to the liposome vehicle. CD determined that the peptide is present in β -strand conformation while tethered to the anionic bilayer, similar to its behavior in model bacterial and cancer membranes.^{36,140} However, investigation of the amide I band in the IR spectrum revealed that the β -strands do not assemble into β -sheets necessary for the peptide's membrane disrupting activity. This activity was further studied with a liposome rupture assay that determined at the current peptide to lipid ratio (7 mol % GL13K), and 2.5 μ M liposomes, the membranes remain unperturbed throughout attachment and UV irradiation. One limitation of this study is that GL13K concentration is below its MIC, and future studies should be carried out at higher concentrations to ensure the nanoplatform can be used at the doses required for bactericidal activity.

Progress was made towards achieving the third objective of evaluating the efficiency of peptide release. Preliminary release results are promising, as after separation via dialysis and reconcentration using lyophilization, peptide cleavage from the liposomes as a result of UV exposure is found at a reasonable and effective concentration. The challenge lays in accurately quantifying the peptide directly bound to the liposomes, where the results always yielded concentrations above the theoretical maximum. This may be due to the high sensitivity and low specificity of the assay, the latter making it susceptible to interference from residual copper. It is proposed that the copper cations used in the synthesis may have an inherent electrostatic attraction to the anionic bilayer of the liposomes that would potentially hinder purification, and then catalyse the colorimetric assay generating artificially high readings. Regardless, using the current method, the experiments in this thesis demonstrate that quantification of the released

peptide can be carried out by directly measuring the released peptide, through dialysis and reconcentration via lyophilization.

A more rigorous quantification method could therefore be considered for future applications. One option would be to carry out elemental analysis, using an instrument such as an inductively coupled plasma mass spectrometer, to determine if copper is present and attribute the heightened signal to this contamination. An alternative method would be to use mass spectrometry for quantification, which is feasible as the GL13K-linker-lipid conjugate has already been identified with this method. Through calibration, the mass spectrum could quickly identify and quantify the amount of this species present. An added advantage of this method is that it could be used to simultaneously quantify the other phospholipids in the nanopatform and output a peptide to lipid ratio that could be used for evaluating attachment and release efficiencies.

Another area that can be improved upon is the efficient release and diffusion of the GL13K AMP after UV irradiation. Although this work shows that β -sheet formation does not occur on the surface of our liposomes, we suspect that the strong electrostatic interaction is preventing diffusion away from the particles. The lipid formulation of these liposomes was selected based on the future application of forming SLBs on the surface of Ln-UCNPs to tether the peptide, and it has been shown that this lipid ratio forms stable bilayers as the high percentage of anionic phospholipid binds electrostatically to the cationic surface of the nanoparticles.⁸⁷ Next steps should be directed toward developing an SLB formulation with a lower anionic lipid proportion, retaining a stable nanoparticle coating. One approach may derive from recent work demonstrating the removal of the oleate capping ligand and its replacement with a citrate monolayer, to which the SLB may be adsorbed.¹⁴¹ The significance of this method is that it renders the NP surface anionic, negating the need for anionic phospholipids, and opening the door to cationic formulations whose charge will not adhere GL13K upon release.

Development of a new liposomal formulation to facilitate the release of GL13K also opens the door to incorporating Ln-UCNPs into the nanopatform. These particles allow for the use of near IR rather than UV as the catalysing light source. While UV is a feasible stimulus for a topical wound dressing, it has the drawback of low tissue penetration and is damaging to genetic material.^{142,143} Alternatively, near IR does not damage DNA and has a greater depth of

penetration due to falling within a wavelength range where few biological materials absorb.¹⁴⁴ Incorporating near IR excitable Ln-UCNPs into the nanoplatform would improve the treatment for long-term use and allow for subdermal applications.

Another area of interest would be to study alternative residues in the GL13K sequence for attachment of the photolabile linker. This is feasible as it would require only the selective deprotection of different lysine residues for reaction with the custom photolabile linker at the chloroformate moiety. There are two reasons for this interest, the first being to restrict the membrane-lytic activity until post release. Currently the linker is bound to the central lysine 7 residue, but due to the β -strand structure observed by CD and ATR-FTIR, and DOPG membrane disruption by carboxyfluorescein assay, the peptide remains active. One caveat being that cholesterol incorporation and the spatial restriction provided by tethering seem to be preventing the formation of β -sheets on the surface of our delivery liposomes. The other reason for altering the peptide tethering site would be to protect the peptide from protease degradation in order to increase the shelf-life of future wound dressings as well as the bioavailability upon release. One promising way to do so would be to tether the peptide at both termini, creating a cyclic peptide that could be photo-released at either end, similar to previously reported configurations,¹⁰⁴ but while anchored to the bilayer. Both site specific protection, particularly at the termini, and conformationally restricting the backbone by cyclizing the peptide are viable means of protection against proteolysis.¹⁴⁵

The destination of the project is incorporation of the nanoplatform into a wound dressing with the intended application being to treat persistent infections with the ability to repeatedly re-dose the GL13K AMP without removing the bandage and risking reinfection of the wound. An ideal material for the dressing will need to be selected that will allow penetration of the stimulus light source and facile diffusion of the peptide towards the target site. There have been some reports of practical light responsive materials for similar applications. For example, sol-gel transitions can be induced in hydrogels,¹⁴⁶ or the release of antibiotic crosslinked to fibrous material.¹⁰⁰ However due to the phospholipid-based delivery vehicle presented in this work, a better suited material may be as an emulsion coating the surface of a bandage, to facilitate the diffusion of the peptide to the infection. Further, this work shows that complete release of the peptide is not achieved after a single 30-minute UV irradiation, opening the door to repeat dosing, however

further work is required to tune the formulation to allow for repeat dosing at functional peptide concentrations.

In conclusion, we have designed and constructed a liposomal nanoplatform capable of covalently tethering an antimicrobial peptide. We have demonstrated that the peptide can be released upon exposure to UV irradiation in sufficient quantities to cause membrane disruption in model membranes.

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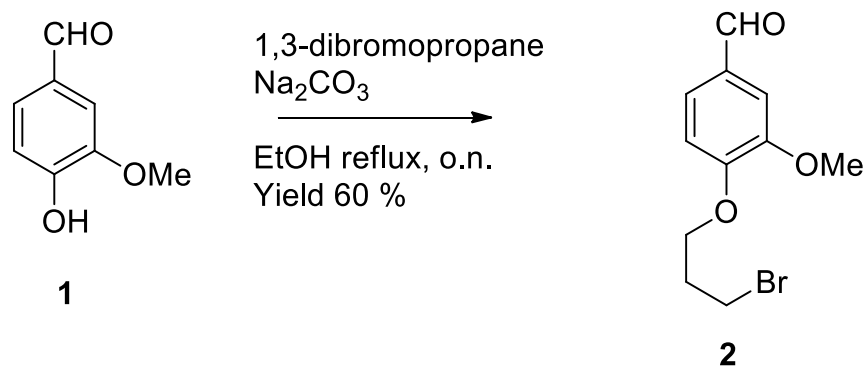
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Appendix 1

Each step of the synthesis pathway for the photolabile linker bound peptide is detailed below; the descriptions reproduced here were provided by the Peptide Synthesis and Imaging Probe at Université de Sherbrooke who carried out the synthesis.



To a suspension of **1** (3.00 g, 19.8 mmol, 1 eq) in ethanol (100 mL) was added sodium carbonate (4.18g, 39.5 mmol, 2 eq) and 1,3-bromopropane (4.00 mL, 39.5 mmol, 2 eq). The solution was under reflux overnight and the TLC analysis (eluent, 100% DCM) was performed to confirm the disappearance of the starting material. DCM (60 mL) was added and the reaction mixture was diluted with water (300 mL). The organic phase was collected and the aqueous mixture was extracted twice with DCM (60 mL). The combined organic layers were washed successively with water (60 mL) and brine (60 mL), and then dried with anhydrous magnesium sulfate. After filtration and evaporation of volatile solvents under reduce pressure, the crude product was purified by flash chromatography on silica gel (eluent, DCM 100%) to afford a pure compound **2** (3.22 g, yield of 60%) as a white powder.

^1H NMR (400 MHz, CDCl_3): δ = 9.87 (s, 1H), 7.45 (m, 2H), 7.02 (d, J = 8.0 Hz, 1H), 4.26 (t, J = 6.0 Hz, 2H), 3.93 (s, 3H), 3.65 (t, J = 6.5 Hz, 2H), 2.43 (quint, J = 6.0 Hz, 2H).

^{13}C NMR (100 MHz, CDCl_3): δ = 29.72, 31.97, 55.97, 66.49, 109.31, 111.70, 126.67, 130.27, 149.87, 153.65, 190.87.

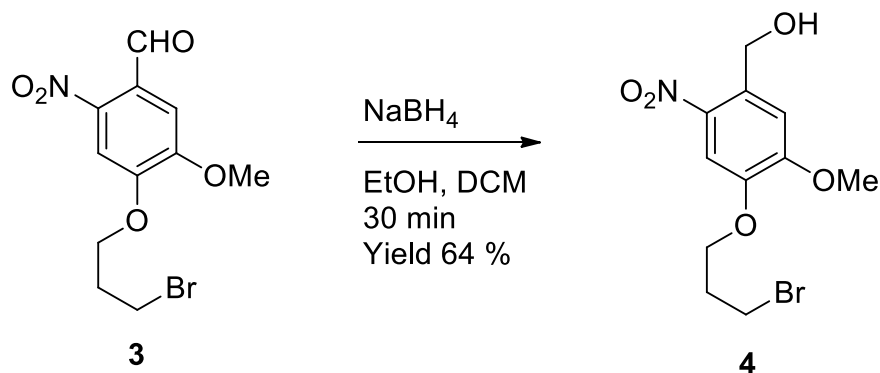
HRMS (ESI) : m/z $[\text{M}+\text{Na}]^+$ calculated for $\text{C}_{11}\text{BrH}_{14}\text{O}_3$: 294.9940 ; found : 294.9943

To a solution of **2** (3.12 g, 11.4 mmol, 1 eq) in trifluoroacetic acid (20 mL) was added potassium nitrate (1.15 g, 11.4 mmol, 1 eq). The solution was stirred on the magnetic stirrer for 1 hour and TLC analysis (eluent, 100% DCM) was performed to confirm the disappearance of the starting material. The trifluoroacetic acid was evaporated and the solid was put in the vacuum pump for 30 minutes. The organic solid was diluted with DCM (60 mL). The organic phase was washed with water (60 mL) and brine (60 mL), and then dried with anhydrous magnesium sulfate. After filtration and evaporation of volatile solvents under reduced pressure, the crude product was purified by flash chromatography on silica gel (eluent, DCM 100%) to afford a pure compound **3** (3.40 g, yield of 94%) as a white powder.

^1H NMR (400 MHz, CDCl_3): δ = 10.47 (s, 1H), 7.66 (s, 1H), 7.43 (s, 1H), 4.32 (t, J = 6.0 Hz, 2H), 4.01 (s, 3H), 3.65 (t, J = 6.5 Hz, 2H), 2.45 (quint, J = 6.0 Hz, 2H).

^{13}C NMR (100 MHz), CDCl_3): δ = 29.30, 31.64, 56.54, 67.07, 108.15, 109.82, 125.54, 143.55, 151.42, 153.37, 187.61.

HRMS (ESI) : m/z $[\text{M}+\text{Na}]^+$ calculated for $\text{C}_{11}\text{Br}_1\text{H}_{13}\text{N}_1\text{O}_5$: 339.9791 ; found : 339.9793



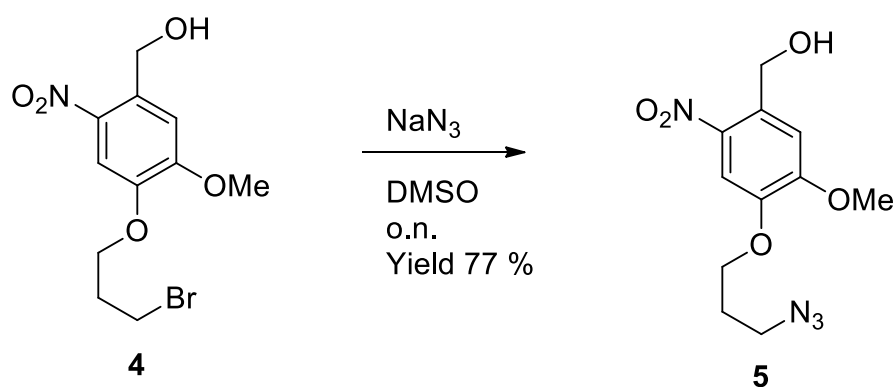
To a suspension of **3** (3.34 g, 10.5 mmol, 1 eq) in a mixture of ethanol (95 mL) and DCM (32 mL) was added sodium borohydride (1.60 g, 42.1 mmol, 4 eq). The solution was stirred on the magnetic stirrer for 30 minutes and TLC analysis (eluent, 100% DCM) was performed to confirm the disappearance of the starting material. The reaction mixture was diluted with HCl 1M (350 mL). The organic phase was collected and the aqueous mixture was extracted three times with DCM (50 mL). The combined organic layers were washed successively twice with sodium bicarbonate (50 mL) and brine (60 mL), and then dried with anhydrous magnesium

sulfate. After filtration and evaporation of volatile solvents under reduced pressure, the crude product was purified by flash chromatography on silica gel (eluent, 3:17 MTBE:DCM) to afford a pure compound **4** (2.16 g, yield of 64%) as a yellow powder.

^1H NMR (400 MHz, CDCl_3): δ = 7.75 (s, 1H), 7.18 (s, 1H), 4.97 (s, 2H), 4.24 (t, J = 6.0 Hz, 2H), 4.00 (s, 3H), 3.64 (t, J = 6.5 Hz, 2H), 2.60 (s, 1H), 2.41 (quintuplet, J = 6.0 Hz, 2H)

^{13}C NMR (100 MHz), CDCl_3): δ = 29.59, 31.97, 56.40, 62.84, 66.92, 109.79, 111.28, 132.53, 139.65, 147.09, 154.31.

HRMS (ESI) : m/z $[\text{M}+\text{Na}]^+$ calculated for $\text{C}_{11}\text{Br}_1\text{H}_{15}\text{N}_1\text{O}_5$: 341.9948 ; found : 341.9952

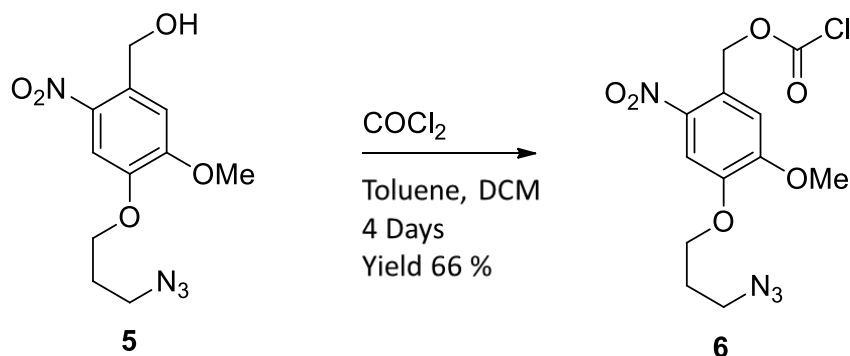


To a solution of **4** (2.16 g, 6.8 mmol, 1 eq) in DMSO (34 mL) was added sodium azide (0.44 g, 6.8 mmol, 1 eq). The solution was on the magnetic stirrer overnight and HPLC-UV-MS analysis (gradient, 30:95 ACN) was performed to confirm the disappearance of the starting material. The reaction mixture was diluted with ethyl acetate (30 mL) and water (100 mL). The organic phase was collected and the aqueous mixture was extracted three times with ethyl acetate (30 mL). The combined organic layers were washed successively twice with water (30 mL) and once with brine (30 mL), and then dried with anhydrous magnesium sulfate. After filtration and evaporation of volatile solvents under reduced pressure, the crude product was analysed with HPLC-UV-MS (gradient, 30:95 ACN) to confirm that the crude product was pure >99%, and then to afford a pure compound **5** (1.46 g, yield of 77%) as a yellow powder.

^1H NMR (400 MHz, CDCl_3): δ = 7.73 (s, 1H), 7.18 (s, 1H), 4.97 (s, 2H), 4.18 (t, J = 6.0 Hz, 2H), 4.00 (s, 3H), 3.58 (t, J = 6.5 Hz, 2H), 2.60 (s, 1H), 2.14 (quintuplet, J = 6.5 Hz, 2H)

^{13}C NMR (100 MHz), CDCl_3): δ = 28.43, 47.93, 56.35, 62.67, 66.09, 109.63, 111.06, 132.61, 139.46, 146.95, 154.27.

HRMS (ESI) : m/z $[\text{M}+\text{Na}]^+$ calculated for $\text{C}_{11}\text{H}_{15}\text{N}_4\text{O}_5$: 305.0856 ; found : 305.0863



To a solution of **5** (0.31 g, 1.1 mmol, 1 eq) in DCM (6 mL) was added a phosgene solution with a concentration of 20% in toluene (12 mL, 22.8 mmol, 20 eq). The solution was stirred on the magnetic stirrer for 4 days and the TLC analysis (eluent, 100% DCM) was performed to confirm the disappearance above 50% of the starting material. The phosgene solution was evaporated and the solid was put in the vacuum pump for 30 minutes. The crude product was purified by flash chromatography on silica gel (eluent, DCM 100%) to afford a pure compound **6** (0.25 g, yield of 66%) as a yellow powder.

^1H NMR (400 MHz, CDCl_3): δ = 7.78 (s, 1H), 7.02 (s, 1H), 5.74 (s, 2H), 4.19 (t, J = 6.0 Hz, 2H), 4.00 (s, 3H), 3.58 (t, J = 6.5 Hz, 2H), 2.15 (quint, J = 6.5 Hz, 2H).

^{13}C NMR (100 MHz), CDCl_3): δ = 28.39, 47.91, 56.43, 66.15, 69.80, 109.73, 110.62, 124.42, 139.66, 148.00, 150.32, 154.08.

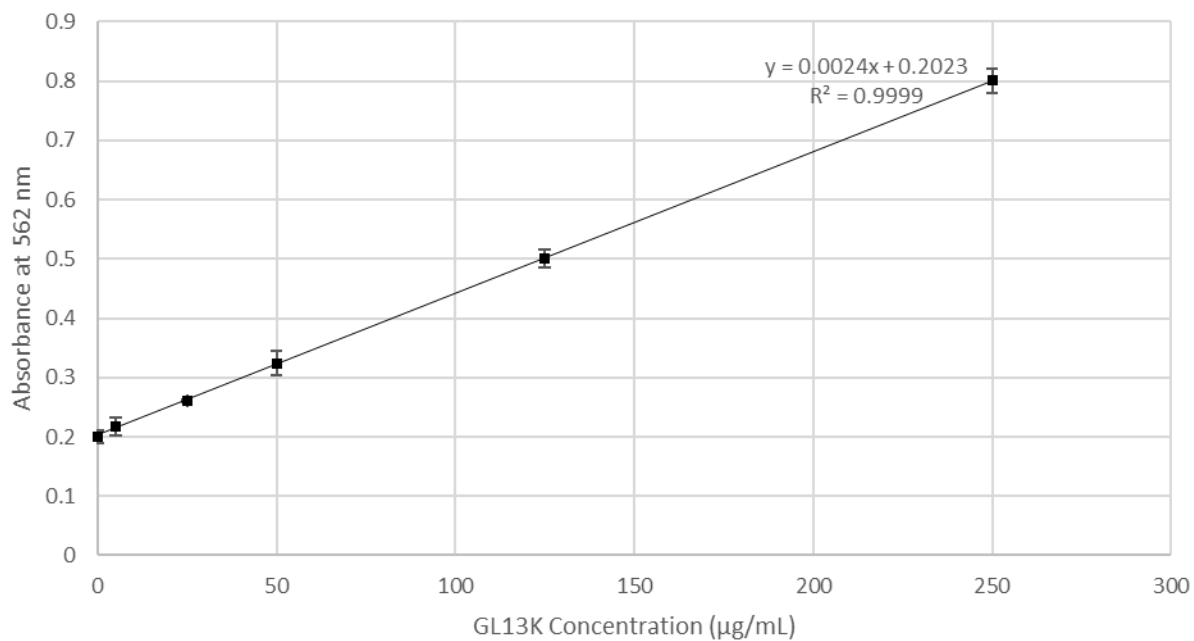
HRMS (ESI) : m/z $[\text{M}+\text{Na}]^+$ calculated for $\text{C}_{12}\text{H}_{13}\text{ClN}_4\text{O}_6$: 367.0416 ; found : 367.0416

The peptide GL13K was synthesized using a Symphony-X synthesizer from Protein Technologies, Inc. at a 100 μmol scale. The peptide linker conjugate was formed through selective deprotection of a Dde protecting group, and coupling of the chloroformate on the linker to the lateral chain of lysine at position 7. The peptide was cleaved from resin in TFA/TIPS/ H_2O

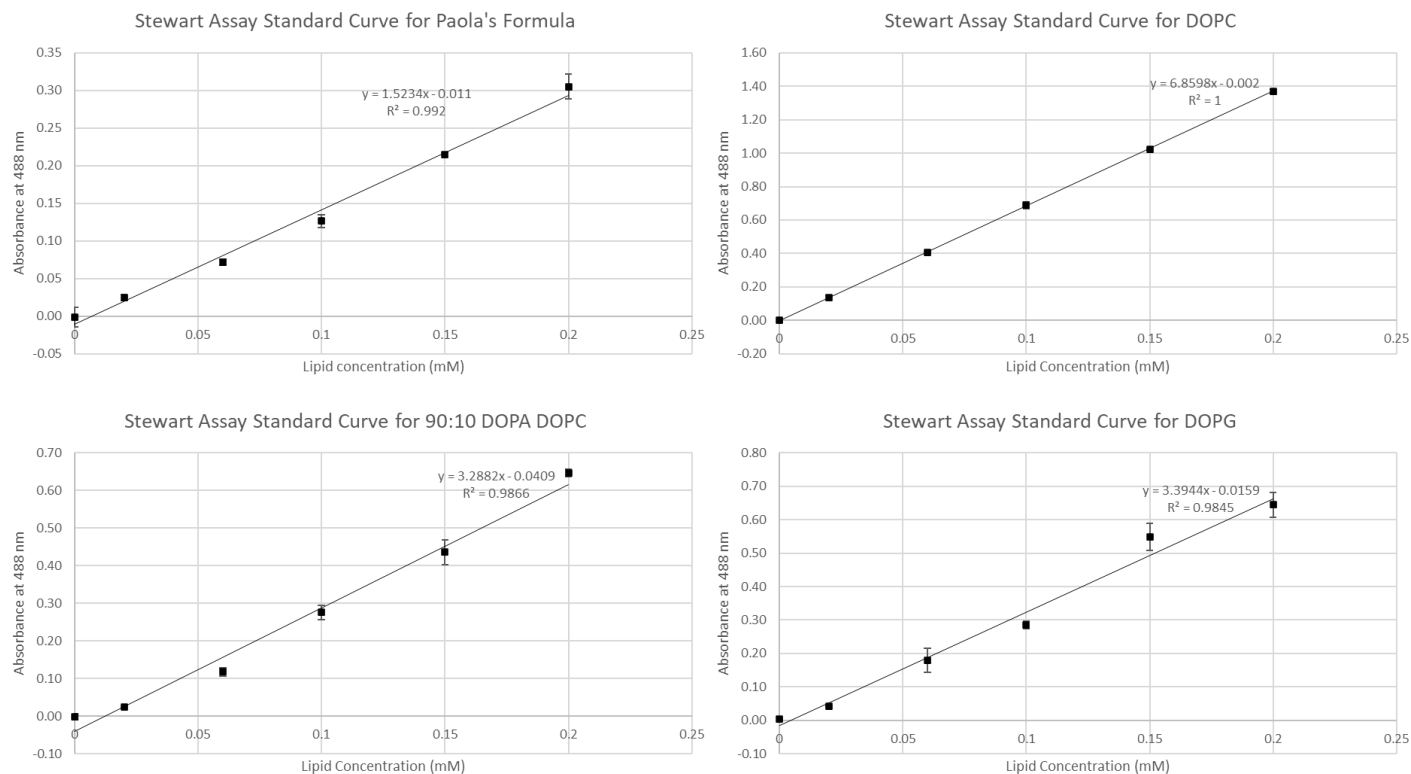
and precipitated in methyl tert-butyl ether before purification by reverse phase HPLC. Finally, the product was analysed by HRMS to confirm identity and purity >99%.

HRMS (ESI) : m/z calculated for $C_{80}H_{142}N_{22}O_{20}$: 1731.1 $[M+H]^{+1}$; found : 1733.3 $[M+H]^{+}$, 867.1 $[M+2H]^{2+}$, 578.3 $[M+3H]^{3+}$

Appendix 2



This graph demonstrates the calibration curve for the repeat peptide quantification experiment and direct comparison between UV exposed and no UV exposure experiment discussed in Section 3.5.



Calibration curves for Stewart lipid quantification assay used to quantify liposomes in CF experiments (Section 3.4) and peptide quantification experiments (Section 3.5).