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The Development of a Synthetic Self-Adjuvanting Cancer Vaccine

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Declaration

I hereby declare that all the work contained in this thesis is the original work of my own research, with the exception of specific references to other sources. This thesis was written by me and was not submitted in whole or in part for any other degree or qualification. I certify that the work submitted is entirely mine except the *in vivo* experiments which have been done with help of Prof. Lindy Durrant's group.

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Abstract

Cancer testis antigens (CTA) have demonstrated high immunogenicity towards multiple cancer types, and due to their restricted expression profile, they warrant further attention as potential vaccine candidates. Covalently attaching an adjuvant to an antigen is an effective strategy to induce a strong, targeted immune response. This thesis describes a robust synthetic method to palmitoylate CTA peptides with a di-palmitoyl-*S*-glyceryl cysteine residue (Pam₂Cys). These adjuvants, known to effectively enhance the severity of an immune response, were covalently linked to the chosen CTA sequences to develop a self-adjuvanting, targeted cancer vaccine.

Liposomes can provide adjuvant activity either by enhancing antigen delivery or by activating innate immune responses. To examine this, a CTA antigen bearing an N-terminal Cys was conjugated covalently to the outer envelope of liposome particles via conjugation to a lipid carrying an appropriate electrophilic headgroup. The palmitoylated CTA sequences with native Pam₂Cys were also inserted into the lipid bilayer of the liposomes via the palmityl 'tails' and these strategies of adjuvant and antigen incorporation into the particle formulation compared. The immune responses generated by the self-adjuvanting CTA peptide vaccine constructs, self-adjuvanting CTA peptides formulated into liposomal vaccine formulations, and the liposomes carrying the conjugated peptide antigens onto outer envelope of the nanoparticles were examined in vivo using healthy mouse models.

Chapter 1 gives an introduction on the human immune system and how it acts against foreigner antigens, leading to vaccine development. This chapter also discusses the CTA peptides and several types of adjuvants including Pam₂Cys and liposomes. Previously reported syntheses of palmitoylated peptides are discussed. **Chapter 2** describes our efforts towards the preparation of the lipid building block (Pam₂Cys) and studies towards the conjugation of this component to the CTA peptides. **Chapter 3** discusses the synthesis of CTA peptides that carry the Pam₂Cys adjuvant. The adjuvant was covalently linked to the selected CTA

sequences successfully to develop a self-adjuvanting peptide-based vaccine using two of the synthetic methods discussed in Chapter 2. Two CTA sequences were selected for this project. A fragment of the CTA, NY-ESO-1₁₅₇₋₁₆₅, that has been shown to produce a strong anticancer immune response and the BAGE4₁₈₋₃₉ peptide, an under-explored melanoma antigen. Further engineering of the peptide antigens to include a solubility tag was also undertaken. The adjuvant was conjugated to these versions of the CTA to produce vaccine constructs with higher aqueous solubility. **Chapter 4** describes the successful formulation of liposomes decorated with the BAGE4₁₈₋₃₉ antigen via reaction with a maleimide linker. The chapter initially describes the synthesis of a maleimide-containing lipid formulation of this compound into liposomes. This is followed by the successful optimisation of Cys-BAGE4₁₈₋₃₉ conjugation, resulting in a BAGE4Cys-maleimide-liposome formulation. Also, a second formulation that incorporates the native Pam₂Cys linked to the BAGE4₁₈₋₃₉ was formulated for comparison. **Chapter 5** describes the biological evaluation of the vaccines described in the previous chapters: Pam₂Cys-SK₄-NY-ESO-1₁₅₇₋₁₆₅, BAGE4₁₈₋₃₉ antigen, BAGE4Cys-Mal-lipo formulation, Pam₂Cys-SK₄-BAGE4₁₈₋₃₉, and formulated Pam₂Cys-SK₄-BAGE4₁₈₋₃₉ liposome by readout of T-cell response using ELISpot assay. The results showed an immunogenic response was elicited by the Pam₂Cys-SK₄-NY-ESO-1₁₅₇₋₁₆₅ conjugate; however, it was not strong enough to be taken to cancer models. The data also shows that the BAGE4₁₈₋₃₉ sequence is immunogenic, however, the immune response of the BAGE4Cys-maleimide-liposome formulation is not effective. Unfortunately, both of the last two vaccine candidates; Pam₂Cys-SK₄-BAGE4₁₈₋₃₉ administered alone and formulated into liposomes, were toxic to the model which led to sacrifice of the animals within the first 24 hrs, thus, study was stopped.

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List of Abbreviation

A/Ala Alanine
Ac₂O Acetic anhydride
APC Antigen-presenting cells
ARC Accelerating Rate Calorimetry
Boc Tert-Butoxycarbonyl
C/Cys Cysteine
COMU (1-Cyano-2-ethoxy-2-oxoethylidenaminoxy)dimethylamino-morpholino-carbenium hexafluorophosphate
CTC 2-Chlorotrityl chloride
CTA Cancer testis antigens
D/Asp Aspartic acid
DCM Dichloromethane
DIC Diisopropylcarbodiimide
DMF N,N-dimethylformamide
DMSO Dimethyl sulfoxide
DTT 1,4-Dithiothreitol
DCC *N,N'*-Dicyclohexylcarbodiimide
DIPEA Diisopropylethylamine
DSC Differential Scanning Calorimetry
DLS Dynamic Light Scattering
DMTMM (4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride)
EDC 5-Ethynyl-2'-deoxycytidine
E/Glu Glutamic acid
Eq./ equiv. Equivalents
Et₂O Diethyl ether
ESI-MS Electrospray ionisation mass spectrometry
EDT Ethanedithiol
EtOAc Ethyl acetate
F/Phe Phenylalanine
Fmoc Fluorenylmethyloxycarbonyl
G/Gly Glycine
HATU Hexafluorophosphate Azabenzotriazole Tetramethyl Uronium

HBTU N,N,N',N'-Tetramethyl-O-(1H-benzotriazol-1-yl)uranium
 hexafluorophosphate
 HCTU O-(1H-6-Chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium_
 hexafluorophosphate
 HOAt 1-Hydroxy-7-azabenzotriazole
 HFIP Hexafluoroisopropanol
 HOBt 1-Hydroxybenzotriazole
 HPLC High-performance liquid chromatography
 HRMS High-resolution mass spectrometry
 K/Lys Lysine
 MeCN Acetonitrile
 MgSO₄ Magnesium Sulfate
 MeOH Methanol
 MHC Major Histocompatibility complex
 Mal Maleimide linker
 NaHCO₃ Sodium hydrogen carbonate
 NCL Native chemical ligation
 NMM *N*-Methylmorpholine
 NMR Nuclear Magnetic Resonance
 Oxyma Ethyl cyano(hydroxyimino)acetate
 PEG Polyethylene glycol
 PBS Phosphate Buffered Saline
 pI Isoelectric point
 Q/Gln Glutamine
 S/Ser Serine
 SPPS Solid-phase peptide synthesis
 TBTU 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethylaminium
 tetrafluoroborate
 tBu Tert-butyl
 TCEP Tris (2-carboxyethyl) phosphine
 TIS Triisopropylsilane
 Trt Trityl
 TFA Trifluoroacetic acid
 TAA Tumour-associated antigens
 VA-044 2,2'-Azobis[2-(2-imidazolin-2-yl)propane] Dihydrochloride

W/Trp Tryptophan

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Introduction

1.1 Cancer

According to the World Health Organisation, cancer is the second leading cause of the death worldwide, responsible for around 10 million deaths in 2020 alone.¹ Cancer involves the development of abnormal cells which reproduce uncontrollably and spread throughout the body rapidly invading and destroying normal body tissue and organs. There are several factors that contribute to the wide spread of cancer. These factors can be biological, such as age, gender, and inherited genetic defects, environmental factors such as exposure to UV radiation, radioactive materials, or carcinogenic chemicals, or lifestyle-related factors like diet, the use of tobacco and other drugs, alcohol and tanning which involves excessive exposure to ultraviolet light. There are several available treatments for cancer such as surgery, radiation therapy, chemotherapy, and stem cell transplant. Even though these types of treatment are associated with the reduction of cancer-related morbidity and mortality, new, effective therapeutic approaches are urgently required. This is because of the limited access developing countries have to the more expensive frontline treatments and the development of therapeutic resistance mechanisms that slow the improvements in the clinical results associated with current therapies.

1.2 Immune system

Immunity refers to the human body's capacity to defend itself against pathogens via recognition of antigens. Antigen is a substance that triggers an immune response, binding to a specific antibody or T-cell receptor.² Antigens can be toxins, chemicals, bacteria, viruses, proteins, peptides, polysaccharides, lipids, nucleic acids or other substances that come from outside the body. Tumour associated antigens (TAA) are antigenic material, proteins or peptides, produced from cancer cells. Normal cells either don't produce these antigens or do so in much smaller amounts.

The human immune system is divided into innate and adaptive systems which work constantly to provide efficient immune responses against foreign material. Critical components of the innate immune system include immune cells, such as macrophages, neutrophils, natural killer (NK), and dendritic cells (DCs) which guide the immune response to target any foreign material. The innate immune system carries general resistance through a range of continuously working defensive measures, anatomical and physiological barriers. These barriers include intact skin, mucous membranes, low stomach pH and bacteriolytic lysozyme in tears, saliva and other secretions which provide the first-line of protection against pathogenic agents,³ however; it does not provide a response which is unique to any particular pathogenic agent. The adaptive system on the other hand is specific and more complex. The main components of it include B cells and T cells. B cells are mainly responsible for the production of proteins, called antibodies that bind specifically to foreign material (e.g., bacteria and viruses). T cells are classified in two major groups: T cells that activate B cells and assist in antibody creation, and killer T cells that actively destroy cells. Adaptive immune cells kill viruses or bacteria using data provided by DCs and other inborn cells, and store information about these pathogens, generating long-lived cells that remain dormant but that can easily re-express effector functions when they encounter particular antigens. This feature provides the adaptive immune response with the capacity to demonstrate immune memory, allowing it to contribute to a more successful host response when particular pathogens or toxins are encountered a second time, often decades after the initial response, which is considered to be a key feature of adaptive immunity.⁴ The adaptive immune system can be divided into humoral (antibody-mediated) and cell-mediated components.

Humoral (antibody-mediated)

Humoral immunity refers to antibody production by B-lymphocytes or (B cells). It is termed '*humoral*' because it involves substances found in the humours or body fluids. The humoral immune system responds to pathogen antigens that are freely circulating, or outside the infected cells.

The antibodies produced will encounter these antigens and bind with them to destroy them either by neutralizing them and preventing reproduction, causing lysis (destruction of cells by a lysis), or initiating phagocytosis.

Cell-mediated components

Cellular immunity is mediated by T-lymphocytes (T cells). There are three types of T cells, based on their function: helper, suppression, and cytotoxicity. The helper T-cells stimulate the immune response of other cells like B cells, the T-suppressor cells regulate the immune response level and efficiency, and the cytotoxic T-lymphocyte (CTL) cells identify and kill infected cells and activate phagocytes to eliminate pathogens.

Antigens are recognised and are expressed on the cell surface by an antigen-presenting cell (APC), a cell that displays antigen bound by major histocompatibility complex (MHC) proteins (class I or class II). T cells then bind to the MHC-antigen complex leading to differentiation of the T cell into a cytotoxic T cell (when the binding occur between $CD8^+$ T cell surface molecule and MHC I), and helper T-cells, activating B cells, resulting in the production of antibodies and a humoral response (when binding occur between $CD4^+$ T cell surface molecules and MHC II)⁵ as shown in Figure 1.

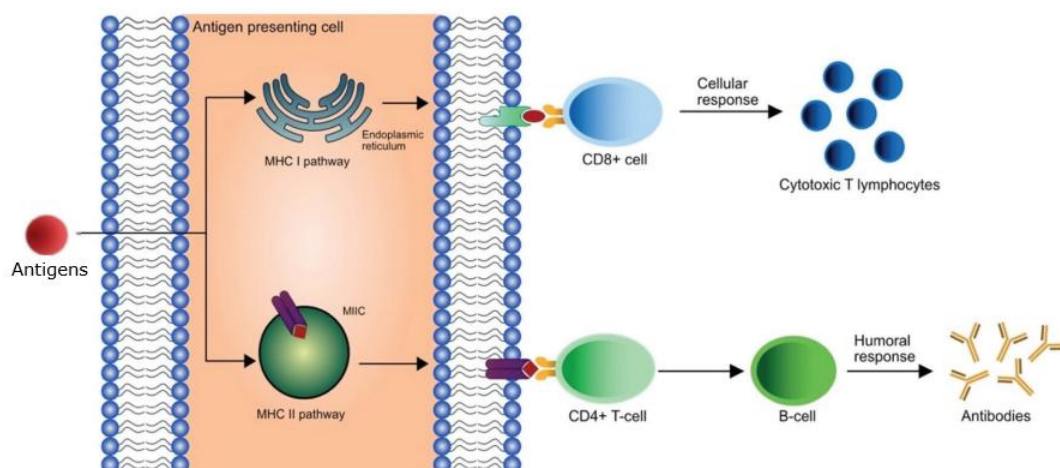


Figure 1: Schematic of antigen processing through the MHC I or MHC II pathways inside an APC. The result of the process is either cellular or humoral immune responses.⁶

Several studies have been performed to investigate the immunological mechanisms through which pathogens are identified and eradicated from the human body, and to understand the process of establishing immune memory to better respond to future exposures. This knowledge has impacted greatly on vaccine design, allowing the identification of specific immunogens that are able to stimulate immune responses, which then provide protection. Generally, the response to antigenic material can be divided into two phases: the innate and adaptive responses.⁷ When exposed to a foreign agent for the first time the innate immune system will work by DC or macrophages or both together (APC) which will uptake this antigen and process it and then present it via MHC I or II.⁸ T cells can recognise this complex and either memory or naive will be activated or reactivated. The key role of a vaccine is to activate T cells that are specific for the antigen that the vaccine is comprised of and once these cells are activated they go back to the site of antigen production (infection or tumour) and recognise the antigens presented at this site to destroy any cells or foreign material that produces the antigen. In this way, T cells can eliminate the infection or tumour cells.

1.3 Vaccine development

Early vaccines were prepared to contain live pathogens that were attenuated by reducing the virulence of the pathogen while maintaining viability or contain pathogens inactivated by exposure to high temperatures.⁹ However, although these methods continue to be used in the 21st century, they are associated with several drawbacks, as some of these vaccines have failed to achieve sufficient potency and efficacy.¹⁰ Additionally, the potential for reversion to virulence among the live-attenuated vaccine strains and the incomplete inactivation of bacteria or viruses contained in vaccines has occasionally resulted in post-vaccination disease occurrence. These disadvantages have reduced confidence in such approaches. Therefore, more innovative vaccine strategies have been introduced to address problems associated with vaccine design and implementation. Purified antigen vaccines are a new approach with generally improved safety profiles relative to live-

attenuated and whole-inactivated pathogen-based vaccines. However, they are often less immunogenic, due to the elimination of the organism's pathogenic characteristics¹¹ and likely associated with their inability to induce APC maturation.⁷ Induction of APCs, such as DCs, play a critical intermediate role between the innate and adaptive responses, and has a strong influence on the direction of the adaptive immune response, thus inducing the antitumor T-cell immunity.^{7,12} MHC1 is the main actor of tumour immunity; therefore, an ideal vaccine should induce an innate immune response that will be able to direct the adaptive immune response towards the efficient inactivation and removal of a particular pathogen, followed by immune memory growth.⁷ For a cancer vaccine in particular to be optimally efficacious, it should induce both humoral and cellular immune responses. Therefore, to overcome the low immunogenic abilities of highly purified antigens, an adjuvant, which are substances that are combined with the vaccines to enhance the immune response, is used. Adjuvants work by recruiting professional antigen-presenting cells APCs to the vaccination site, increasing the delivery of antigens to APCs, or by activating APCs to produce cytokines and by triggering T cell responses¹³, resulting in stronger immune response.¹⁴ Self-adjuvanting peptide-based vaccines contain an antigenic peptide covalently bound to an adjuvant. These constructs have been demonstrated to be promising immunotherapy tools.¹⁵

1.4 Cancer-Testis Antigens

Successful immunisation against infectious diseases indicates that discovering key antigens (and TAAs) associated with the immune-mediated rejection of tumours may facilitate the production of highly selective and efficient anti-tumour vaccines. Thus, selecting the appropriate antigen has a significant impact regarding the success of the vaccine. There are several factors that need to be considered to determine a suitable cancer-antigen candidate. These factors include tissue expression profile, avidity of the antigen-specific T-cell response, the presence or absence of pre-existing immune tolerance, and the

commonality of tumour antigens among patients and tumour types.¹⁶ In the immune response to the tumour, T cells play critical roles, however, there is a growing body of evidence which indicates the essential role that B-cell-mediated immunity and other immune system components may play in tumour rejection.^{17,18} Therefore, searching for antigens that trigger an immune responses in both B-cells and T-cells is the first step toward vaccine success. The various types of tumour antigens are summarised in Table 1.¹⁹

Table 1: Summary of the various types of tumour antigens.¹⁹

Antigen type	Antigen	Tumor type
Universal tumor antigens	Telomerase, hTERT, survivin, mdm-2, CYP-1B1	All
Cancer testis antigens	MAGE, BAGE, RAGE, GAGE, NY-ESO-1	Breast, prostate, testicular, bladder, melanoma, gastric, esophageal, hepatocellular, renal, lung, head and neck
Tissue-specific differentiation products	PSA	Prostate
	Tyrosinase, Mart-1/Melan-A, gp100, trp-1, trp-2	Melanoma
Mutated gene products	CDK-4-R24C	Renal
	β -catenin	Colon
	p53	Colon, breast, lung
	k-ras	Colon, pancreas, lung, esophageal
	bcr-abl	Leukemia
	MUM-1	Leukemia
Overexpressed self-antigens	HER-2/neu	Breast
	Proteinase-3	Leukemia

	Mucin-1	Multiple myeloma, pancreas, gastric, breast
	WT-1	Leukemia
	MART-1/melan-A	Melanoma
	Mesothelin	Pancreas, ovarian, peritoneal, mesothelioma
Idiotypes	Ig, TCR	Lymphoma

TAA's have the ability to induce adaptive immunity either by triggering the expansion of CD8⁺ CTLs, which can destroy tumour cells, or by activating B-lymphocytes, resulting in a humoral response in the form of tumour-specific antibodies, or initiation TAA's of both of these processes.²⁰ CTA are a specific group of TAA's with a high immunogenicity profile.²¹

The CTA are a family of antigens encoded by 276 genes. Across the family, they share features of immunogenicity and high tissue-restriction; their expression is restricted to male germ-line cells and expressed abnormally in various types of cancer.^{22,23} Unlike bacteria and viruses, which appear foreign to our immune system, cancer cells closely resemble our normal, healthy cells and as a result autoimmunity can often occur when using a cancer antigen as a vaccine, but this is not the case for the CTA. This is because the male germ-line cells do not actively express HLA-class I genes which means they cannot present antigens to T cells.²⁴

Therefore, when CTA are expressed in cancer they will be considered as neoantigens and will stimulate the immune responses that are strictly cancer specific. Moreover, it has been demonstrated that CTA including NY-ESO-1, MAGE-A, and SSX can induce humoral and cellular immune responses and associated with cytolytic activity of tumour immunity.²² These features make CTA a target for further research and investigation in the field of cancer immunotherapy and vaccine development.

MAGE-1 (melanoma-associated antigen 1) was the first successfully cloned human tumour antigen in 1991²⁵ as it had been reported that MAGE-1 was able to trigger a spontaneous CTL response in autologous melanoma patients.^{26,27} Subsequently it was found that MAGE is highly expressed in testis cells and it not limited to melanoma but also found in many different types of cancer.²²

Using the same genetic strategy of T-cell epitope cloning on melanoma patients additional members of the CTA family were identified: BAGE and GAGE, which both share most of the previously described features of CTA.²² The BAGE gene is expressed in 22% of melanomas, 30% of infiltrating bladder carcinomas, 10% of mammary carcinomas, 8% of head and neck squamous cell carcinomas, 6% of non-small cell lung carcinomas, and also in 15% of ovarian cancer. BAGE has been shown to promote a cellular immune response.²² Moreover, the amino acid length of this CTA sub-family is considered to be short (ranging from under 50 to 180 amino acids) which make them ideal candidates for vaccine development using a chemical synthesis strategy. There are several studies that provide evidence of immunological responses as well as some favourable clinical responses that indicate using CTA as a cancer vaccine may be a successful strategy for cancer therapy.^{20,21,28,29} NY-ESO-1 and MAGE-A are further examples of CTA that reported promising results in several studies.³⁰ It has been reported that melanoma and ovarian cancer patients demonstrated substantial activation of both humoral and cellular immune responses and increased survival in

response to NY-ESO-1 and MAGE-A vaccines, as well as adoptive T-cell transfer.^{31,32} However, as discussed, peptide antigen may provide an immune response but not at the level required to combat cancer. Using a suitable and effective adjuvant along with a CTA should elicit strong anti-tumour responses for actively targeted immunotherapy.²⁰

1.5 Adjuvants

Adjuvants can be defined as any compound that can induce stronger immune response against a co-administered or covalently attached antigen. They have been administered clinically for more than 90 years and are components of more than 30 approved vaccines.^{33,34} There are several examples of adjuvants including mineral salts, microbial products, emulsions, saponins, cytokines, polymers, microparticles, and liposomes.^{35,36,37,38} The enhancement of immune response with adjuvant delivery is associated with several practical advantages such as reducing the amount of antigen required in individual vaccine doses. The improved efficiency of the immune response can result in fewer vaccine doses required to achieve immunity. Dose reduction combined with antigen conservation may have significant consequences for increasing global vaccine availability. Adjuvants may also boost immune responses in populations that generally have weaker responses to vaccines, such as in infants, the elderly, and the immunocompromised.³⁹

The selection of adjuvant with suitable human administration properties is limited. Aluminium considered as one of the earliest discovered adjuvants, was first used in human vaccines in 1932 and was the only adjuvant used in licenced vaccines for nearly 70 years.³⁹ Another early adjuvant effort was a mineral oil-in-water emulsion (Freund's incomplete adjuvant); however, this adjuvant is no longer used in human vaccines because it is over-reactogenic. It has been demonstrated that antibody production is increased rapidly when aluminium adjuvants are employed providing a strong antibody-mediated immune response; therefore, this adjuvant works most efficiently when co-administered with vaccines targeting pathogens destroyed primarily by antibodies.⁴⁰ However,

aluminium is not appropriate for eliciting cell-mediated immunity and therefore fails to prevent intracellular pathogen infections.⁴¹ In addition, it is not appropriate for the administration of mucosal vaccines, as alum formulated vaccines do not develop mucosal antibodies following administration.⁴¹

Another type of adjuvant is lipopolysaccharide (LPS) which is a natural adjuvant synthesized by Gram-negative bacteria.⁴² It is a potent activator of the immune system through its recognition by Toll-like receptor 4 (TLR4) and the subsequent pro-inflammatory signalling cascade.⁴³ LPS is composed of three main structures, an O-antigen, oligosaccharide core, and lipid A, Figure 2, with each of these playing a different role in the function of LPS, lipid A is responsible for the potent adjuvant activity of LPS.⁴⁴ While LPS is a potent adjuvant, its ability to contribute to vaccine reactogenicity due to its strong biological activity, has prevented its clinical use in vaccines.⁴⁵ In the 1970's, Edgar Ribi systematically subjected LPS from *Salmonella minnesota* to chemical modification in order to separate its desirable immunostimulatory properties from its endotoxic effects.⁴⁶ This led to development of monophosphoryl lipid A (MPL), Figure 3, which is defined as a detoxified version of endotoxin lipopolysaccharide that boosts adaptive immunity via TLR4.⁴⁷

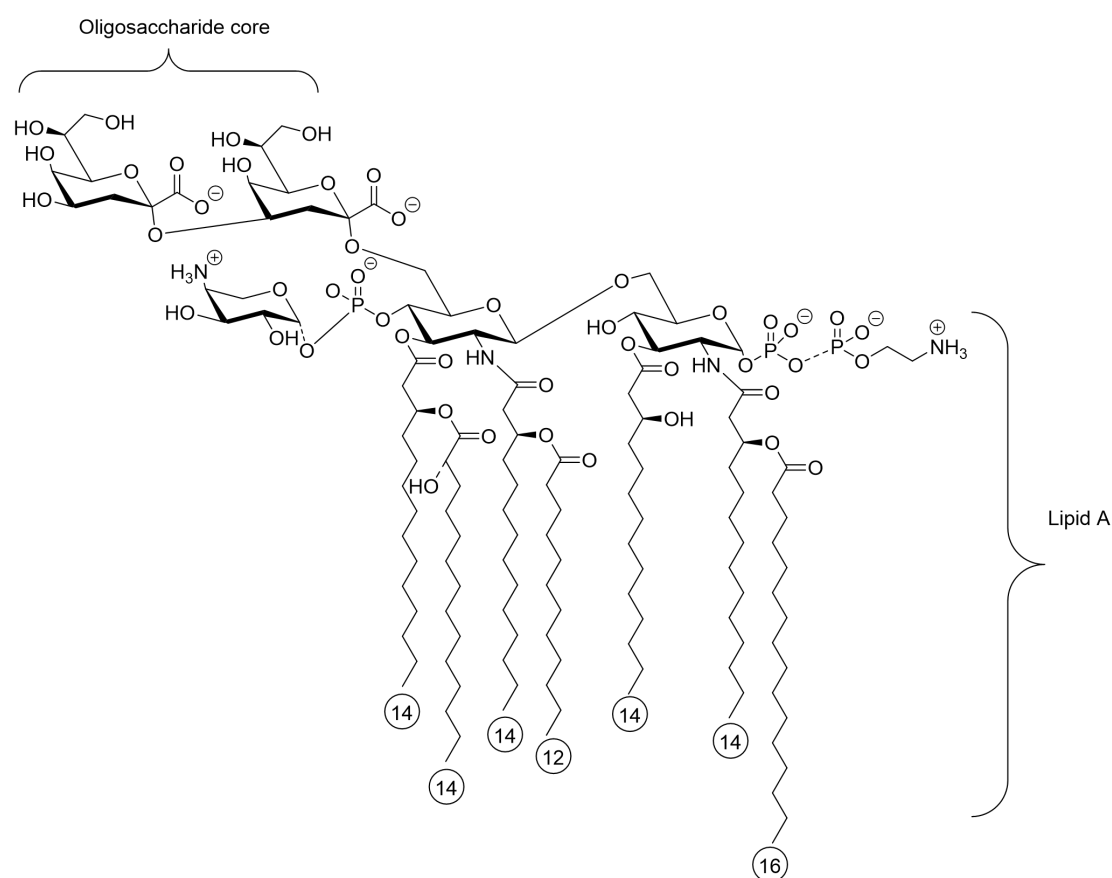


Figure 2: Structure of LPS as prepared from *S. Minnesota*, showing oligosaccharide core, and lipid A.^{47–49}

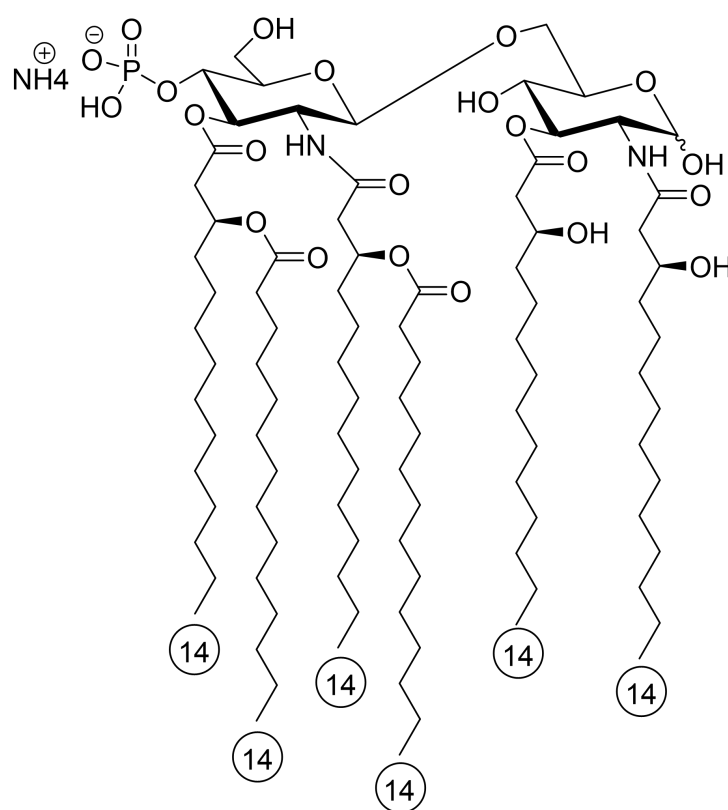
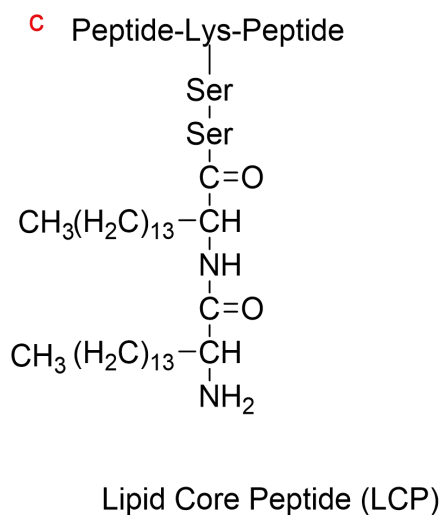
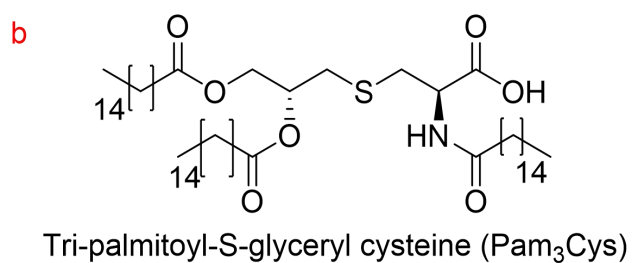
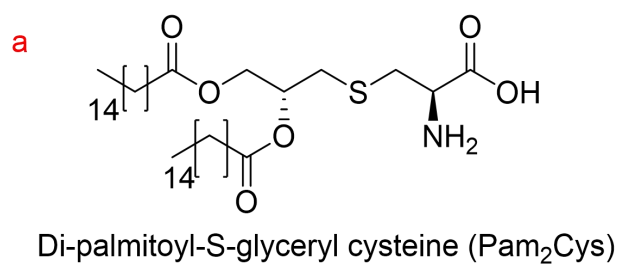


Figure 3: Structure of the major components of MPL adjuvant prepared from LPS *S. minnesota*.⁵⁰

MPL has been used effectively in human-administered vaccines,⁴⁹ however, the search for additional, non-toxic, and effective adjuvants for human use, with the potential to elicit immune responses in both the cellular and mucosal forms, remains a priority for future vaccine development efforts. The binding of bacterial or synthetic lipids as an adjuvant to synthetic peptide antigens has also been investigated. The most common lipid moieties used in the production of lipopeptide vaccines are bacterial lipoprotein analogues [e.g., tri-palmitoyl-*S*-glyceryl cysteine (Pam₃Cys) and di-palmitoyl-*S*-glyceryl cysteine (Pam₂Cys)], lipid-core peptide (LCP)¹¹ and multiple antigen lipophilic adjuvant carrier (MALAC) systems, Figure 4^{7,8,51}, which are synthetic lipopeptide vaccine development systems produced using synthetic lipid amino acids (lipophilic adjuvant carriers).^{7,8,51} Gram-negative lipoproteins have three fatty acid chains, while gram-positive bacterial lipoproteins have two. Although the acyl chains are heterogeneous in terms of length

and saturation level, palmitic acids (containing 16 carbons) are most frequently found.⁴ Several lipopeptide vaccines using lipid adjuvants have advanced to human clinical trials, showing a high level of protection, with little to no side effects reported.⁴



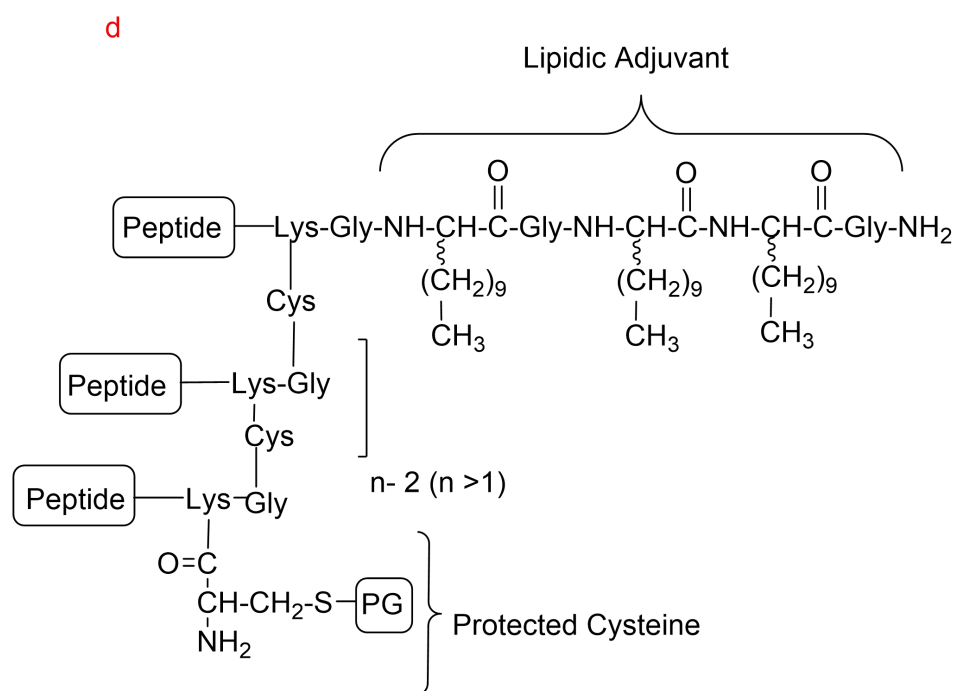


Figure 4: Structures of (a) Pam₂Cys, (b) Pam₃Cys⁵², (c) lipid-core peptide LCP⁴, and (d) multiple antigen lipophilic adjuvant carrier (MALAC)⁵³ systems.⁴

1.6 Lipopeptides

1.6.1 The mechanism of action for lipid adjuvants and their structure-activity relationship

Although adjuvants have been used in many vaccines, their mechanism of action still requires additional clarification. There are several suggested mechanisms that adjuvants could employ to stimulate an immune response. Such mechanisms include the constant release of antigen, recruitment of immune cells at the site of injection, stimulation of cytokines and chemokines.⁵⁴ Additional mechanisms involve an increase in antigen uptake and presentation to APC, activation, maturation of APC and promoting movement to the draining lymph nodes, and activation of inflammasomes.⁵⁵

Toll-like receptors (TLRs) are one of the pathogen-recognition receptor (PRRs) families that innate immune cells express to recognize infectious agents.⁵⁶ TLRs play essential roles in several adjuvant functions. Currently, 10 different TLRs have been identified in humans, with each TLR family having affinities for particular pathogen-associated molecular patterns (PAMPs).⁵⁷ TLR1, TLR2, TLR4, and TLR6 are the primary TLRs that recognise lipopeptide and glycolipopeptide vaccines. For bacterial lipopeptides, their synthetic derivatives (e.g., Pam₃Cys)⁵⁸⁻⁶⁰, and peptides modified with *N*-palmitoyl-lysine⁶¹, TLR2 is the primary signalling molecule. Generally, TLR2 combined with TLR6⁶² recognises di-palmitoylated lipopeptides (e.g., Pam₂Cys) and TLR2 combined with TLR1⁶³ recognises tri-palmitoylated lipopeptides (e.g., Pam₃Cys) with some exceptions.⁶⁴

The mechanisms that explain the adjuvant properties of bacterial lipopeptide are varied because TLR2 is expressed on many different cell types, including DCs, macrophages, and lymphocytes.⁶⁵ Preclinical studies demonstrate that using lipopeptides with antigens, either co-administrated or physically linked, can stimulate DC maturation, resulting in the upregulation of co-stimulatory signals and antigen-presenting molecules (e.g., MHC class II, CD80, CD83, interferon-gamma (IFN γ), interleukin (IL)-12).⁶⁶ Additionally, lipopeptide-associated antigens can induce macrophages to release cytokines⁶⁷, and enhance the maturation and activation of B-cells, which leads to improvements in antigen-specific IgG and IgM antibody production.⁶⁸ Moreover, using lipopeptides as adjuvants can enhance the generation of antigen-specific CTL responses.⁶⁹

Bacterial lipopeptides are the major structural elements found in the cell membrane, consisting of an acylated *S*-glycerylCys moiety, which can be conjugated to a peptide by the Cys residue. *Mycoplasma fermentas* macrophage-activating lipopeptide (MALP-2) and its synthetic analogues

Pam₂Cys and Pam₃Cys are the most widely used vaccine adjuvant bacterial lipopeptides⁷⁰ as they have been approved to be an effective technique for the induction of cellular and humoral immunity during vaccine development by several applications and some of these have proceeded to clinical development.⁷¹

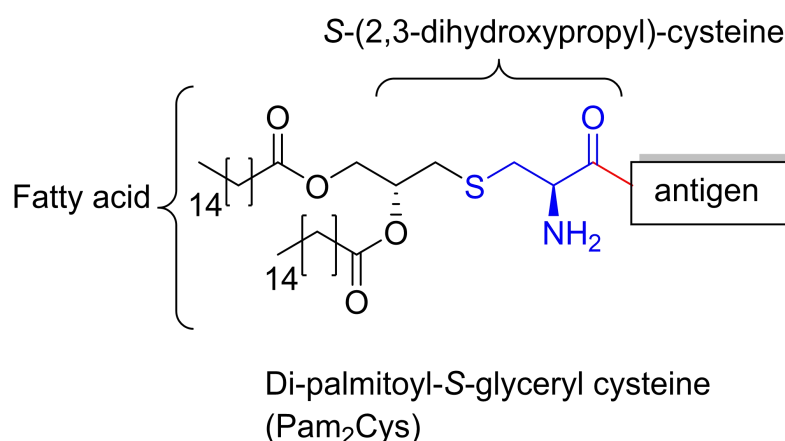


Figure 5: The site where the peptide can be conjugated to the Cys residue on an acylated moiety of S-glycerylCys.

The Pam₂Cys lipopeptide structure can be divided into three parts: two fatty acid components, the core structure S-(2,3-dihydroxypropyl)-Cys, to which the two fatty acids are attached via ester linkages, and the peptide component (Figure 5). Several structural details influence the activity of Pam₂Cys, as measured by nitric oxide release in macrophages.⁶³ TLR2 was observed to discriminate between the two Pam₂Cys stereoisomers; the natural *R* isomer (differing in the configuration around the asymmetric carbon atom of the 2,3-hydroxypropyl moiety) was 100 times more active than the *S* isomer.⁶³ The biological activity was reduced by 77% when the sulphur atom substitute with a methylene group⁷² and when the free N-terminus substitute with either an acetyl or palmitoyl group.⁷³ Also, the removal of either (or both) ester-bound fatty acids significantly lowered or eliminate specific biological activity.⁷³ The substitution of the two ester bonds, through which the two fatty acids are attached, by an amide bond also leads to the elimination of TLR2 agonist activity.⁷⁴ The influence of the

fatty acid length on Pam₂Cys activity was also investigated using HEK293 cells, through its ability to activate TLR2-specific cells and induce an antigen-specific humoral response.⁶⁶ It has been found that the lengths of alkyl chain of the two ester-bound fatty acid should be more than eight carbons.^{66,75} Pam₂Cys immunogenicity experiments in an *in vivo* BALB/c mouse model found that the LHRH- specific antibody response was dependent on the length of the alkane chains, with the order C18 = C16 > C12 > C8.⁶⁶ Therefore, C16 appears to be the best chain length to promote the TLR2 recognition of Pam₂Cys which has been confirmed as well by Agnihotri et al.⁷⁶

1.6.2 Lipopeptide synthesis

Several methodologies can be used to attach synthetic lipopeptides covalently to antigens. These approaches can be divided into synthetic and semisynthetic. Solid-phase synthesis is one of the most convenient synthetic methods. Chua, et. al⁷⁷ described, in detail, the techniques involved in the synthesis of a Pam₂Cys-based lipopeptide vaccine capable of stimulating the production of specific antibodies in vaccinated mice.⁷⁷ The synthesised lipopeptide vaccine consisted of a T-helper-cell epitope and a B-cell epitope, separated by a lysine residue (Lys), with the lipid moiety attached via two serine (Ser) residues (Figure 6).⁷⁷

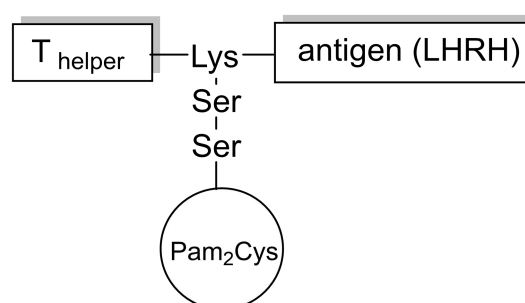


Figure 6: Structure of the lipopeptide vaccines.

The synthesis began with the B-cell epitope, LHRH, by using solid-phase peptide synthesis (SPPS)⁷⁷ followed by coupling of an Fmoc-Lys(Mtt)-OH to enable lipid attachment between the two epitopes. Then T-helper sequences were synthesised using SPPS and on completion the Mtt side chain protecting group of Lys was removed by 1% TFA, ensuring that the peptide remains on the resin. Two Ser residues were coupled to the exposed amine group of the intervening Lys sidechain and the Fmoc group removed from the second Ser residue to allow lipid attachment. Fmoc-S-(2,3-dihydroxypropyl) Cys was then coupled to the second Ser on the resin bounded peptide then palmitoylated by palmitic acid to yield the desire lipopeptide shown in Figure 6.

This strategy was also used by Waleed, H, et al,⁷⁸ with some modification. The Pam₂Cys-SK4-alkyne shown in Figure 7 was synthesised using the above Chua, et.al⁷⁷ SPPS method with the orthogonal sidechain protection at the C-terminal Lys which allowed on-resin deprotection and coupling to pentynoic acid before cleavage and purification. The Pam₂Cys-SK4-alkyne was then conjugated to the multiantigenic peptide azide shown using copper(I)-catalyzed alkyne-azide cycloaddition (i.e., Click Chemistry) (CuAAC) to give the multiantigenic lipopeptide 8Q_{min}/E6₄₃₋₅₇-Pam₂Cys, as shown in Figure 7.⁷⁸

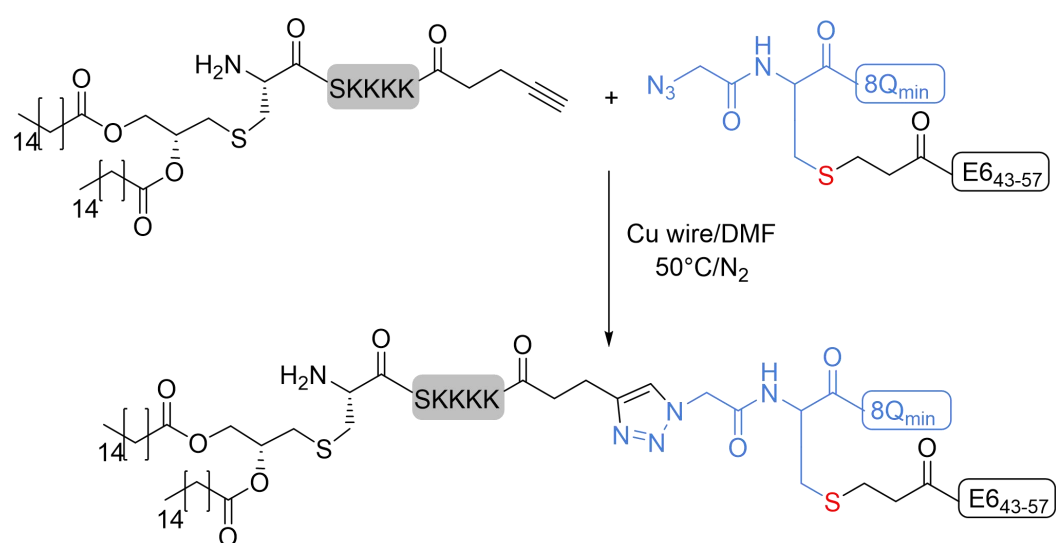


Figure 7: Synthesis of 8Q_{min}/E6₄₃₋₅₇-Pam₂Cys.⁷⁸

Synthetic lipopeptides can also be conjugated covalently to the desired antigen using a chemical ligation approach such as native chemical ligation (NCL). NCL is type of peptide ligation that results in a native amide bond linking two peptide sequences; it has been applied to the site-specific attachment of Pam₂Cys modules to desired antigens. The NCL reaction occurs between a peptide that contains a C-terminal thioester and another component that carries an N-terminal Cys residue; the general mechanism of native chemical ligation is shown in Figure 8.⁷⁹

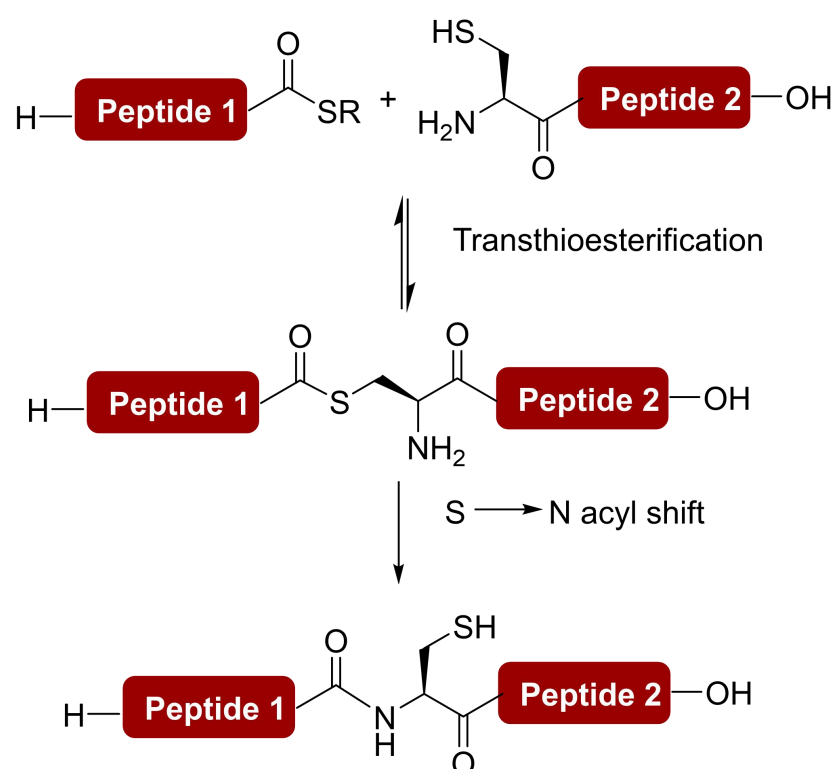


Figure 8: Scheme of native chemical ligation.

This reaction leaves a Cys residue at the ligation site which can be converted to an alanine (Ala) residue by reduction via desulfurisation using tris (2-carboxyethyl) phosphine (TCEP) and 2,2'-azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride (VA-044) at room temperature (Figure 9).⁸⁰

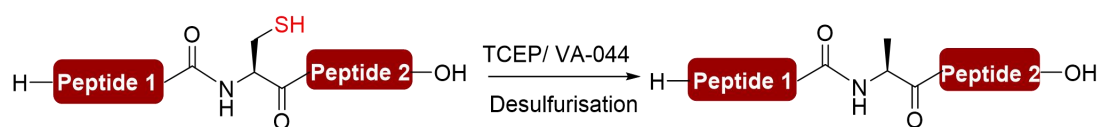


Figure 9: Scheme of desulfurisation of Cys to Ala.

NCL allows for convergent and chemoselective fusion of peptide fragments to generate larger constructs.⁸¹ It would also allow for the initial synthesis of a short and easily purifiable lipopeptide thioester fragment that could then be easily conjugated to the antigen scaffold. However, the main problem faced when performing conjugation of lipopeptides using NCL is the hydrophobicity of the lipopeptide fragment as it remains insoluble in aqueous solvents requiring the use of an organic solvent. NCL requires a buffer with a high concentration of chaotropic salt to denature the peptides (and facilitate the ligation), thus the %vol. of organic solvent that can be employed is limited.⁸² The self-adjuvating vaccine candidate shown in Figure 10 against *Mycobacterium tuberculosis*, synthesised by Ashhurst et al.,⁸³ is an example of the application of NCL to the conjugation of a Pam₂Cys-peptide to two antigenic peptides from the *Mycobacterium tuberculosis* proteins ESAT6 and TB10.4 using a one-pot NCL – desulfurization protocol.⁸³ In order to achieve the final vaccine construct, the two peptide components were synthesised via Fmoc-SPPS and the ligation between Phe-8 and Ala-9 of the ESAT6 epitope was undertaken.⁸⁴ In this ligation, the aqueous phosphate buffer (0.1 M, pH ~7) containing tris-(carboxyethyl)phosphine (TCEP, 50 mM) as a reductant and guanidine hydrochloride (Gdn.HCl, 6 M) as a denaturing agent were used. However, since the lipopeptide thioester, was insoluble in this buffer system, the non-ionic surfactant Tween-20 (0.5% v/v) was added as a solubilizing detergent.⁸³ So, lipopeptide thioester and peptide were dissolved in this buffer with addition of 2,2,2-trifluoroethanethiol (TFET, 2% v/v), used to convert the stable alkyl thioester to a reactive thioester.⁸⁴ With the crude, in situ desulfurization of the ligation product was performed to convert the Cys

to a native Ala residue at the ligation junction. Then purified by HPLC to get the final vaccine candidate in 34% yield.⁸³ The vaccine increased pulmonary immunogenicity when given mucosally to mice, producing potent Th17 responses in the lungs and multifunctional peripheral T cells.⁸³

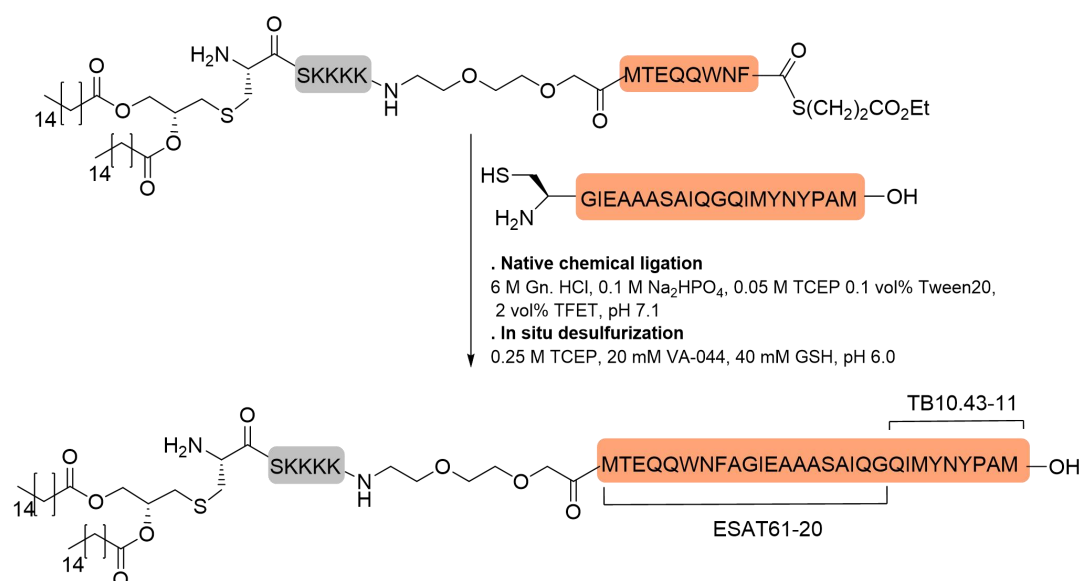


Figure 10: Self-adjuvant vaccine candidate structure, Pam₂Cys, as an adjuvant conjugated covalently to ESAT6 and TB10.4 immunodominant T-lymphocyte epitopes from Mtb.⁸³

1.7 Solid Phase Peptide Synthesis (SPPS)

As discussed in the previous section, SPPS has been used to synthesise lipopeptide components as well as the peptide antigen for peptide-based vaccines. SPPS was developed by Bruce Merrifield, leading to the Nobel Prize in Chemistry (1984) and it has many advantages compared with solution-phase peptide synthesis such as the relative ease of implementation, speed (since all the reactions are conducted in one single reaction vessel), and it can be partially or fully automated. Furthermore, the technique is efficient as there is no need to isolate and purify material after each step, increasing the overall yield.^{85,86} During SPPS, the initial amino acid is anchored to the insoluble solid support via

its C-terminus. The support (polystyrene resin) is pre-functionalised with an appropriate linker. A variety of linkers have been developed that allow the peptide to be cleaved under mildly acidic conditions; examples include 2-chlorotrityl chloride (CTC) and Wang linkers, affording a peptide bearing a C-terminal carboxylic acid, and Rink Amide, affording a peptide bearing a C-terminal primary amide, Figure 11.⁸⁷

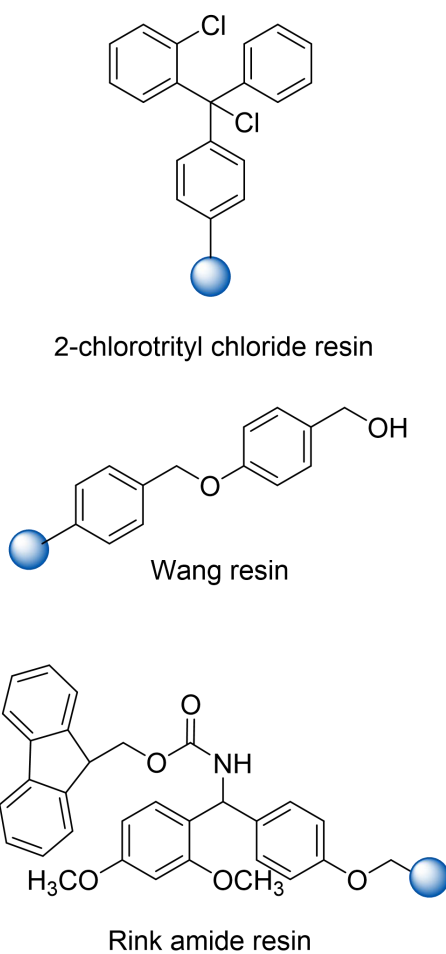


Figure 11: Structures of 2-chlorotrityl chloride, Wang, and Rink amide resin.

By using a solid support all unwanted solvent, reagents, and side-products of each reaction can be washed away from the resin using a porous filter, eliminating the need for purification. The initial resin loading has been identified as a critical factor in SPPS.⁸⁸ It is usually reported in mmol per gram of resin (mmol.g^{-1}). The loading of initial amino acid is critical to the peptide's final yield because it serves as the limiting reagent for all subsequent couplings.^{88–90} Overloading of resins is known to cause issues during synthesis.⁸⁸ Lower loading levels are more commonly used in SPPS because high loading levels have been linked to synthetic failures.⁸⁹ High loadings, in general, cause more crowding and, as a result, less reactive site accessibility on the resin. It can also increase chances of intermolecular interactions between the growing peptide chains, resulting in aggregation and synthesis failure.^{88,91} Therefore, the common loading levels of 0.3 to 0.5 mmol.g^{-1} and loading percentages of 50% to 70% , are considered as acceptable loading levels.^{89,92,93} Apart from the above advantages, SPPS also allows for chemoselective modification of the peptide chains using protecting groups that can be removed using conditions orthogonal to the linker and all other protecting groups.⁹⁴ SPPS employs a stepwise method of synthesis, in which each amino acid is added iteratively from the C-terminus, proceeding with the peptide sequence to the N-terminal end; the general scheme for this method is shown in Figure 12.

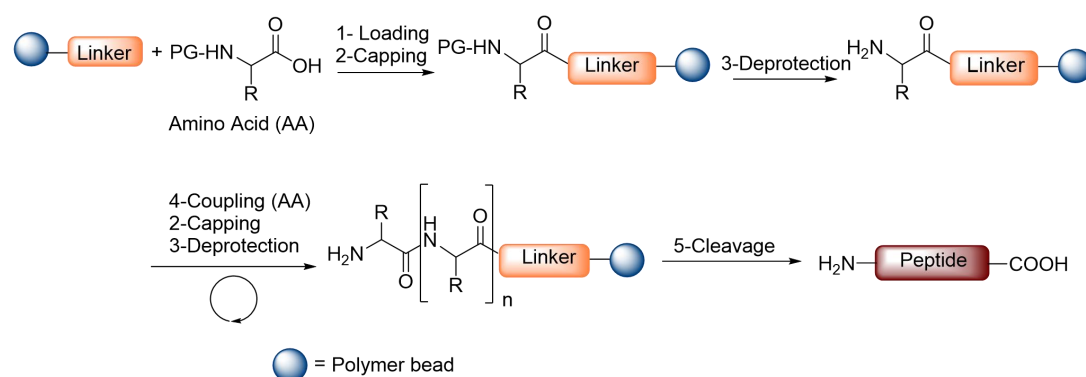


Figure 12: Steps involved in Solid Phase Peptide Synthesis (SPPS).

To avoid multiple addition and polymerization, the reactive side chains, both the incoming residue and those already incorporated in the chain, are protected by a temporary protecting group as well as the N-terminus of each amino acid. The fluorenylmethyloxycarbonyl (Fmoc) group is the most common alpha-amine protecting group in SPPS because it can be removed under mild basic conditions.⁸⁶ The base-labile Fmoc protecting group of the resin-bound amino acid can be deprotected by 20% piperidine in *N,N*-dimethylformamide (DMF). These mild removal conditions do not result in peptide cleavage and allowed the side-chains of amino acids to be protected using acid-labile protecting groups, such as trityl (Trt), tert-butyl (tBu) and tert-Butyloxycarbonyl (Boc), that can be removed using trifluoroacetic acid (TFA), Figure 13.

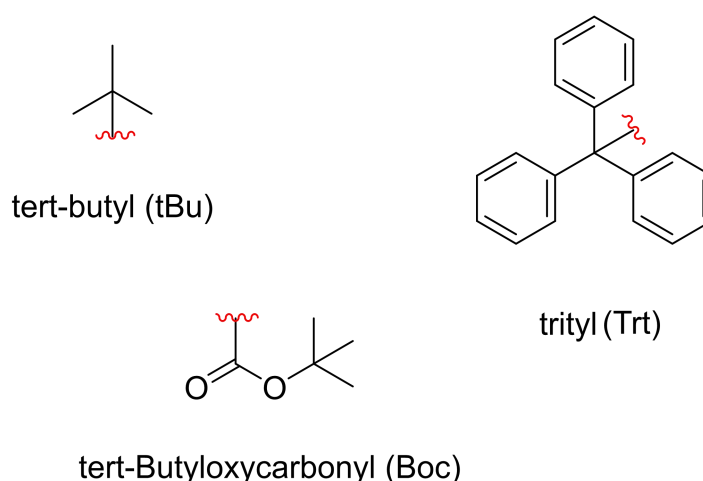


Figure 13: Structures of main acid-labile protecting groups, tBu, Boc, Trt.

The concept of orthogonal protecting groups can be taken further to include protecting groups that are not labile to acidic or basic conditions to allow selective deprotection and chemoselective modification of the peptide.⁹⁵ An example of such protection groups include acetamidomethyl (Acm), labile to oxidising agents while stable to acidic conditions and to alkaline aqueous solutions at 25 °C.⁹⁶ Another example of an orthogonal protecting group is methyltrityl (Mtt) which is highly acid labile that can be deprotected using 1% TFA (with scavengers).⁹⁷

The structure of protected Cys is shown in Figure 14 below with orthogonal protection on the N-terminus and sidechain, base labile Fmoc and acid-labile Mtt respectively.

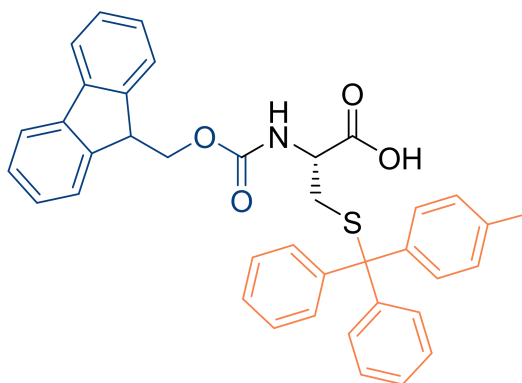


Figure 14: Example protected amino acid (Cys) showing the N-terminal protecting group (Fmoc, blue) and the side-chain protecting group (Mtt, orange).

The peptide chain is extended by forming an amide bond between the N-terminal amine of the resin bound amino acid and the C-terminus of the amino acid to be added to the sequence. Then the Fmoc protecting group is removed and the free N-terminal amine reacted with the next amino acid Fmoc-protected amino acid; this cycle is repeated to form the desired peptide. Coupling of an amino acid to the resin/peptide involves activating the C-terminus of the amino acid to be attached to the resin/sequence on the resin, using a coupling agent which reacts with the carboxylic acid to form an activated ester.^{98,99} Amide couplings can be completed in less than an hour.^{98,99} Two of the main classes of coupling reagents used are uronium salts and carbodiimides. There are many different phosphonium and uronium (aminium) reagents such as HBTU, TBTU, HATU, COMU, and HCTU. Examples of carbodiimides include, DIC, DCC, and EDC. However, drawbacks to these reagents include the potential for guanidinylation,^{100,101} racemisation, and anaphylaxis¹⁰¹ have encouraged the creation of more effective and safer reagents. It has been demonstrated that the combination of diisopropylcarbodiimide (DIC) and ethyl 2-cyano-2-(hydroxyimino)acetate (Oxyma) is safe, yields high coupling yields, and has low associated levels of racemization¹⁰², the

structures of each are shown in Figure 15. El-Faham and Albericio^{102,103} identified Oxyma as a safe and highly efficient additive to be used primarily in the carbodiimide approach for amide bond formation. Oxyma has demonstrated an exceptional ability to suppress racemization and coupling efficiency. Stability tests show that there is no risk of resin capping when using Oxyma as an additive under standard coupling conditions. Finally, calorimetry tests, (Differential Scanning Calorimetry (DSC) and Accelerating Rate Calorimetry (ARC)), show that Oxyma reduces the risk of explosion which is a risk when employing benzotriazole-based additives.¹⁰²¹⁰⁴ Therefore, the combination of DIC & Oxyma is usually employed for routine SPPS.

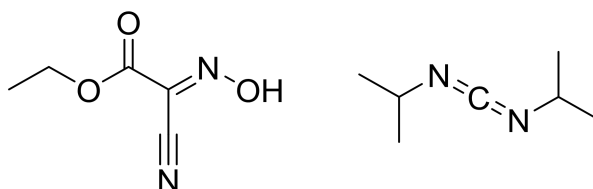


Figure 15: Chemical structures of Oxyma (left) and DIC (right).

If coupling reactions fail to reach completion, this can lead to 'deletions', i.e., a certain proportion of the peptide material will be missing a single amino acid or more.¹⁰⁵ These incomplete sequences make purification challenging, so, to prevent the production of such sequences, all unreacted amine sites are capped. After the coupling step, the peptide resin is treated with an excess of acetic anhydride (Ac_2O) and base (pyridine), to acetylate any free amine left over from the coupling step. The acylated truncated sequences can be easily separated from the desired full length peptide during HPLC purification. Coupling of sterically bulky β -branched amino acids such as valine (Val), isoleucine (Ile), and threonine (Thr) often results in partial coupling and may require microwave assistance or additional coupling attempts.

Once complete, treatment of the resin with a strong acid, usually TFA (cleavage conditions dictated by the linker), cleaves the polypeptide from the resin, deprotecting the side chain protecting groups at the

same time. Finally, the target polypeptide is purified, using reverse-phase high-performance liquid chromatography (HPLC), lyophilised into a powder, and analysed by analytical HPLC and electrospray ionisation MS (ESI-MS). Once the lipopeptide vaccine constructs have been synthesised, they can be formulated into higher order structures, such as lipid nanoparticles (liposomes) for drug delivery in an aqueous solution.

1.8 Liposomes

1.8.1 Liposomes as delivery system

Liposomes are the most popular and extensively studied vehicles for targeted drug delivery.^{106–109} They are spherical vesicles formed by cholesterol and natural phospholipid amphiphiles consisting of a hydrophilic head (phosphate group) and hydrophobic tail (containing fatty acid hydrocarbon chains).¹¹⁰ This unique structure allows a wide variety of drugs to be encapsulated within these vesicles, Figure 16, hydrophobic molecules can be introduced into the bilayer membrane while the hydrophilic molecules can be inserted into the aqueous center.¹¹¹ It is also possible to conjugate hydrophilic molecules to the surface of the liposome.¹¹¹

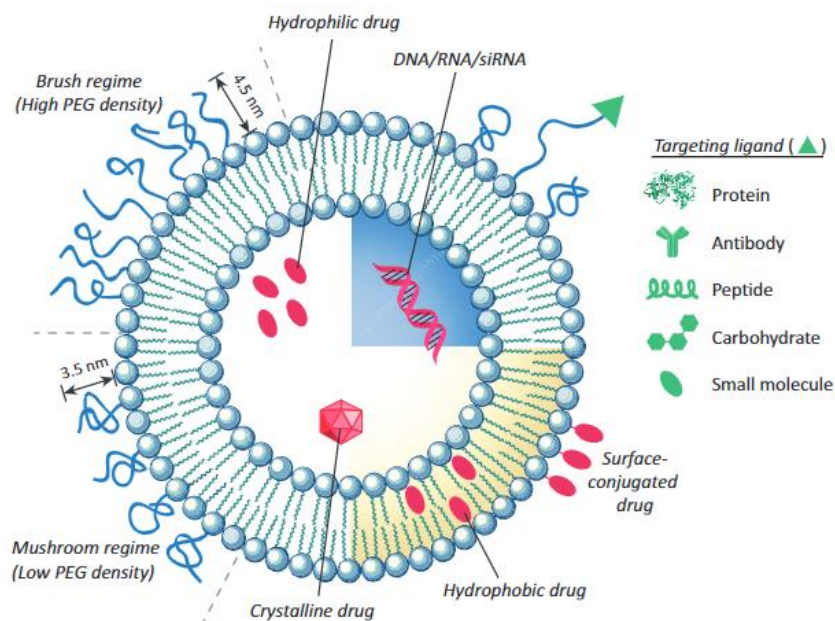


Figure 16: Variations with liposomal vaccine delivery system.¹¹²

1.8.2 Classification of liposomes; size, and charge

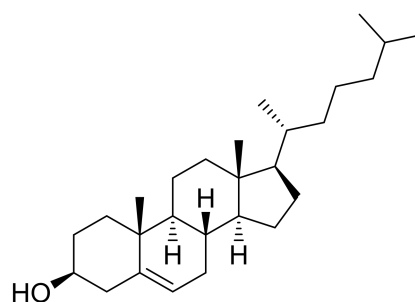
There are many different factors by which liposomes can be classified, including number of bilayers, size, and charge. Based on the layers and size, there are two main categories: multilamellar vesicles (MLV) and unilamellar vesicles. In unilamellar liposomes, the vesicle has a single phospholipid bilayer sphere surrounding the aqueous solution. Depending on the size of the particle, these liposomes can be further split into two categories: large unilamellar vesicles (LUV) which are usually greater than 100 nm and small unilamellar vesicles (SUV), 20-100 nm.¹¹³ Multilamellar liposomes have an onion structure made of several unilamellar layers.¹¹⁴ Size is an important factor to consider in order to formulate high quality liposomes. This is because it is one of the elements that affects the liposome-cell interaction. The circulation half-life of liposomes is also mainly controlled by the size of the particle. Liposomes smaller than 100 nm in diameter have a longer half-life in the blood.¹¹⁵ Larger liposomes were found to be eliminated from blood

circulation faster by reticuloendothelial system (RES) cells.^{115,116}

Liposomes can also be classified based on charge, i.e., either cationic or anionic. Charged liposomes are typically highly stable because charged particles repel each other, reducing their ability to aggregate. Cationic liposomes are used in wide variety of applications such as a delivery tool for negatively charged macromolecules, for example DNA and RNA, due to their negative charge and relatively large size that restricts passive diffusion into cells.¹¹⁷ Thus, cationic liposomes are also used in gene therapy to encapsulate nucleic acids (e.g., mRNA) via electrostatic attraction.¹¹⁸ An example of this is the anti-COVID-19 vaccine where an mRNA coding for the protein spike of the Coronavirus, encapsulated in liposomes that are intended to remain stable in the bloodstream until they are processed by phagocytic cells.^{119,120} Cationic liposomes interact with negatively charged glycoproteins (such as mucins) on the surface of corneal epithelial cells, increasing their affinity and contact duration with the target cells and, consequently, their ability to deliver drugs more effectively.¹²¹ However, positive charge may affect blood circulation and cause electrostatic interactions with anionic species in the blood, as well as promoting an increase in liposome aggregation, which reduces localization, limiting their spread and thus site of action.^{122,123} Furthermore, cationic liposomes tend to be taken up by cells near the injection site due to electrostatic association with cell-surface anionic moieties such as proteoglycans, limiting their distribution.¹²⁴ The addition of polyethylene glycol (PEG) to decorate the surface of these liposomes shields the particles from nonspecific interactions with RES cells (phagocytes) which provides protection from circulating proteins, improving drug efficiency by increasing systemic circulation time.^{125,126} Compared with neutral and cationic liposomes, anionic liposomes are less stable in the bloodstream with higher clearance rate.^{127, 128} However, they are commonly used for topical drug delivery due to their ability to improve the absorption properties through the skin barrier.¹²⁹ Due to poor packaging of DNA and lower transfection efficiency, anionic liposomes have not been researched as extensively for DNA delivery as

cationic liposomes.^{130,131} This could be explained by the ineffective interaction between DNA molecules and anionic lipids, which is caused by the repellent electrostatic interaction between these negatively charged species.^{132,133} However, there are several strategies have been studied to solve this issue. One strategy involves combining anionic liposomes with polycationic protamines to act as an electrostatic bridge between the liposome and the nucleic acid to improve the packaging of nucleic acids into anionic complexes.^{134,135} Another study by Patil et al.¹³⁶ showed that anionic liposomes may successfully entrap large amounts of DNA by complexation with divalent Ca^{2+} ions. These liposomes were composed of lipids of physiological origin and plasmid DNA, mediated by divalent Ca^{2+} .¹³⁶

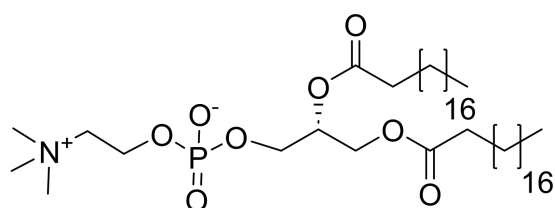
In order to achieve an optimized liposome formulation, the lipid composition is highly important. This is because the lipid composition has a critical impact on the liposomal properties including: particle size, stability, and electrical charge.^{137,138} The main components of liposome are cholesterol and non-toxic phospholipids. Cholesterol is usually used in liposome preparations in a ratio of around 30-50 mol% of the total lipids to improve the rigidity and stability of the liposomes.^{139,140} Sterols such as cholesterol are hydrophobic compounds consisting of four-ring structures as shown in Figure 17.



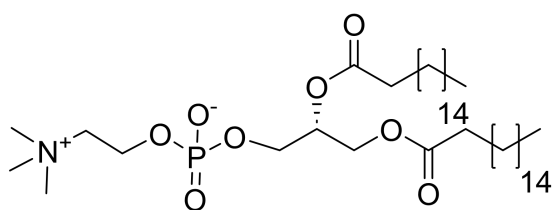
Cholesterol

Figure 17: Cholesterol structure.

The structure of phospholipid consists of two main groups: the hydrophilic head group and the lipid component. The head group could be negatively/positively charged, or neutral. The charge of the hydrophilic group provides stability through electrostatic repulsion which reduces aggregation. The hydrophobic lipid component varies in the acyl chain length, symmetry, and saturation.¹⁴¹ The phospholipids used for liposome preparation often carry saturated hydrocarbon chains e.g., stearic and/or palmitic fatty acids, such as DPPC and DSPC, Figure 18.



1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DSPC)



1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC)

Figure 18: Structures of DPPC and DSPC.

Acyl chains carrying a degree of unsaturation are also employed, as are phospholipids carrying a mixture of fatty acids, DOTAP is an example (Figure 19).¹⁴² The diverse range of phospholipids commercially available provides a broad variety of choice when formulating liposomes. The 'rigidity' (or 'fluidity') and the charge of the bilayer are determined by the components used in the bilayer.^{137,143} For instance, unsaturated phosphatidylcholine species from natural sources (such as egg or soybean phosphatidylcholine) provide highly permeable and low stable properties.¹⁴⁴ However, liposomes made of saturated phospholipids, like dipalmitoyl phosphatidylcholine, produced rigid, nearly impermeable bilayer structures.¹⁴⁴ For charged liposomes, as an example, DOTAP is

commonly used to prepare cationic liposomes while oleic acid or DOPG is used to formulate anionic liposomes,¹⁴⁵ structures for DOTAP & DOPG shown in Figure 19.

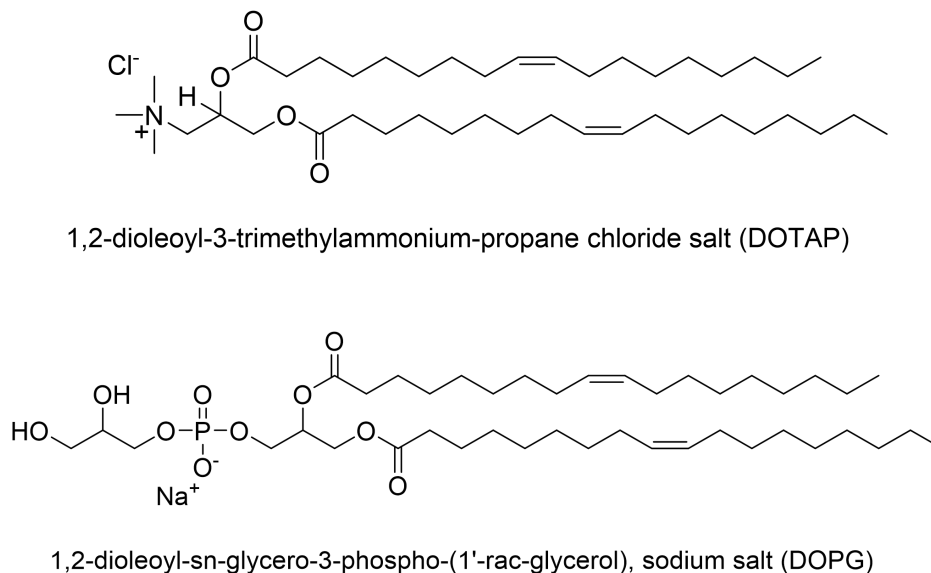


Figure 19: Structure of cationic lipid DOTAP and anionic lipid (DOPG).

1.8.3 Preparation of liposomes

There are different methods of preparation of liposomes, such as mechanical dispersion, solvent dispersion, and the detergent removal method¹⁴⁶ (removal of non-encapsulated material).¹⁴⁴ The general procedure for liposomal formulation involves four steps: drying down lipids from organic solvent to form a thin film, dispersing the lipid in aqueous media, purifying the resultant liposome, analysing the final product. Sonication is a mechanical dispersion method and it is the most well-used method to produce liposomes.¹⁴⁴ Either probe or bath sonicators can be used; probe sonicators yield smaller particles (100 – 200 nm) with low PDI values. Whereas bath sonicators are more suited to the formulation of larger particles (> 200 nm):

Probe sonication. The tip of a sonicator is immersed directly in the liposome dispersion. In this method, the energy is transmitted from the probe to the sample directly which makes the process more efficient and effective. However, since the energy is transferred directly to the sample, heat is generated. Therefore, the vessel must be immersed in a water/ice bath to reduce the temperature of the solution. The duration of this process should be limited to avoid the de-esterified of lipid and titanium contamination.¹⁴⁷ Usually this method is used for small scale batches.

Bath sonication. The liposome dispersion is placed into a bath sonicator. The temperature of the lipid dispersion can be controlled and larger batches can be prepared.¹⁴⁸

1.8.4 Characterization of Liposomes

Characterization of the liposomes plays an important role in the understanding of the functionality of the particles. It is important to analyse the main aspects of the formulated particles such as size, homogeneity, and surface charge to assure their suitability for in vitro and in vivo applications. Dynamic Light Scattering (DLS) is used to calculate liposome size distribution analysis, polydispersity index (PDI), and zeta potential (surface charge).¹⁴⁹ This technique measures the fluctuation of light scattered from particles undergoing Brownian motion due to collisions between suspended particles and solvent molecules.¹⁵⁰ DLS can be also used to analyse the intensity fluctuations which is important for determination of size distribution, indicating the homogeneity, represented by the poly dispersity index (PDI). PDI value ranges from 0.0 (perfectly uniform size sample) to 1.0 (highly polydisperse sample with multiple populations).¹⁵¹ A PDI of 0.3 and below is acceptable and indicates a homogenous population of liposome.^{152,153} Moreover, the surface charge (zeta potential) can be analysed by DLS. As mentioned previously, the stability of a particle

dispersion is associated with the surface charge of the liposome. When the zeta potential is greater than 35 mV or less than -35 mV, the particles are considered stable.¹⁵⁴

1.8.5 Functionalization of liposomes with targeting ligands.

Liposomes have emerged as an effective vaccine carrier, promoting humoral and cell-mediated immunity to a variety of bacterial, protozoan, and viral antigens as well as to tumour cell antigens as shown in animal immunisation research,¹⁵⁵ and have also been successfully used in clinical trials.^{156,157} Functionalizing the surface of liposomes with targeting ligands is one of the main methods used in this field. By attaching targeting ligands to the surface of liposomes, such as proteins, peptides, carbohydrates, or monoclonal antibodies, active targeting can be achieved.¹⁵⁸ As a result, targeted liposomes can only be taken up by cells that overexpress the receptor for the moiety, enhancing therapeutic results by increasing efficacy and lowering off-target toxicity.¹⁵⁸

To date, most of the liposomal vaccines tested have been made by antigen entrapment.¹⁵⁹ However, antigen-entrapped liposomes and surface-coupled antigens of liposomes are reported to induce different types of immune responses.¹⁶⁰ That is, antigen-entrapped liposomes have been shown to induce antigen-specific IgE antibody production,¹⁶¹ while antigen coupled to the surface of liposomes induced substantial IgG antibody production with a minimal amount of IgE antibody production as shown by ovalbumin-liposome,¹⁶² tetanus toxoid¹⁶³ or Shiga-like toxin,¹⁶⁴ which are antigens coupled to liposomes. Antigen-liposome conjugates are therefore considered to be suitable vaccine candidate strategies that cause minimal allergic reaction.¹⁶⁵

There are a variety of reported methods to modify the surface of liposomes;¹⁶⁶ typically, functionalizing the liposome surface covalently has been achieved in two ways. The first method is to attach the targeted ligand of interest to a lipid first and then combine the functionalized lipid with other lipid elements to formulate liposomes.^{167,168} However, this approach is not practical for ligands that lose their active conformation in organic solvents or for ligands of a large size that complicate the synthesis of the functionalized lipid and its characterisation. This strategy thus works better for small ligands that are easy to manipulate and characterize. This method allows for full characterization of the targeted lipid which is an advantage. Examples of liposome conjugates made using this approach includes doxorubicin-loaded liposomes containing an anisamide ligand, displayed on the surface for targeting sigma receptors (membrane-bound proteins, which are overexpressed in several types of cancer), resulting in good affinity.¹⁶⁷

The second approach is to functionalize premade liposome by conjugating the desired targeting ligand, such as small molecules,¹⁶⁷ imaging agents,¹⁶⁹ peptides, large proteins, or antibodies, directly onto the liposome's surface.^{170–176} For this strategy, head group-modified lipids with a polyethylene glycol spacer functionalized at the end with amine, carboxylic acid, thiol, or maleimide groups provide excellent potential. Typically, only 5–10% of the total lipids in the liposomes are made up of these functionalizable lipids. On the exterior of the liposomes, some of the functional groups are exposed, allowing suitable chemistry (amide bond formation,¹⁷⁰ hydrazone bond formation,¹⁷⁷ thiol-maleimide addition,^{178,179} or disulfide bridge formation¹⁸⁰) to be carried out to modify the liposome surface, Figure 20. The great benefit of this method is that it requires only minimal amount of the desired ligand, which is helpful for attaching very large molecules like proteins.¹⁸¹ However, there are some disadvantages such as destabilization of the liposomes or conformational changes of the attached targeting molecules that may affect their targeting efficiency.^{182–184}

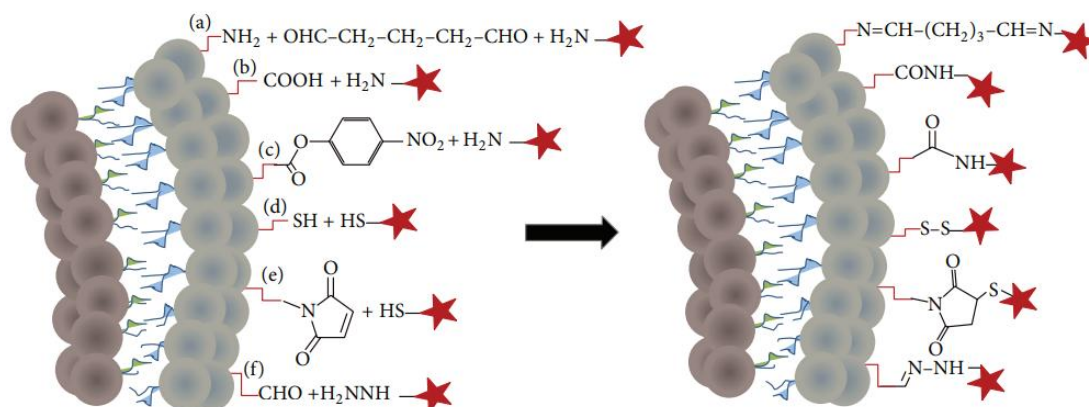


Figure 20: Classical methods for coupling ligands. Schematic representation of a cross section of a liposome bilayer containing functional groups to illustrate (a) crosslinking of primary amines by glutaraldehyde, (b) carbonyl-amine bond formation, (c) amide bond formation, (d) disulfide bond formation, (e) thioester bond formation, and (f) hydrazone bond formation. Coupling ligands are represented by red stars.¹⁸⁵

The functional groups present on the targeting molecule and the liposome control the approach chosen to covalently connect the desired targeting moiety to the liposome.¹⁸⁵ Appropriate bioconjugation techniques are discussed below, with a focus on Cys-selective methods.

Thiol-containing ligands

There are several elements that make Cys residues ideal chemical handles. The highly nucleophilic nature of the thiol group is one of the unique characteristics of Cys. The fact that this residue is one of the least abundant amino acids in the proteome (2%), makes modification at Cys advantageous. Therefore, site-selective modification is more likely because fewer Cys residues are likely to be present in a target sequence. Additionally, there is a high possibility that a protein antigen of interest will not include any Cys residues. This enables site-specific mutagenesis to be employed to include a non-native Cys residue. This makes Cys an attractive target for selective modification. There are many different

peptide and protein modification methods that can be applied using a Cys residue as a chemical handle including, but not limited to, alkyl halides via substitution reactions (as iodoacetamide) and Michael acceptors (e.g., maleimides).

Alkyl halides

This chemistry could be applied using different electrophilic probes such as α -halocarbonyls. The use of α -halocarbonyls for protein modification can be dated as early as 1935¹⁸⁶ where iodoacetamide was used to modify and study the Cys residues of keratin, as shown in Figure 21.

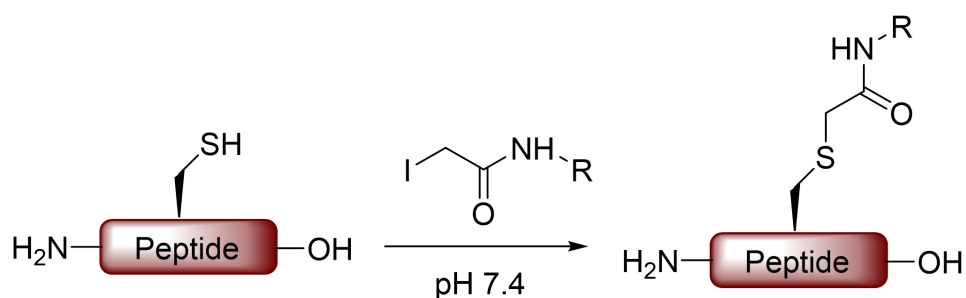


Figure 21: General scheme for the modification of Cys residues with iodoacetamide.

The thiolate anion of Cys reacts with iodoacetamide in a nucleophilic displacement ($\text{S}_{\text{N}}2$) forming an irreversible thioether bond between the probe and the protein.¹⁸⁷

Thiol-maleimide chemistry

Maleimides are an example of Michael acceptors - i.e., alkenes positioned next to electron withdrawing groups. The reaction proceeds via nucleophilic attack of the thiol into the alkene, followed by quenching of the resulting enolate (Figure 22).

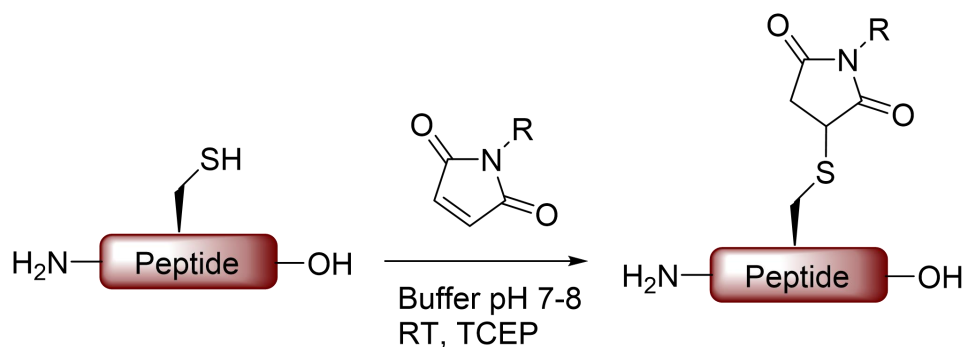


Figure 22: Generic modification using maleimides in buffered (phosphate, tris, etc.) solution at room temperature. A reducing agent, such as TCEP, is often included to ensure Cys remains in the reduced form.

Thiol-maleimide addition is a convenient and efficient method to functionalise the liposomal surface. The maleimide linker is a stable and convenient-to-handle moiety that quickly and covalently conjugates the thiol groups of Cys residues in proteins or peptides.^{188,189} Maleimides are highly chemoselective and, at neutral pH or lower, react with a thiol group 10^3 times more quickly than an amine group. However, amine addition can occur, particularly when the pH is raised or when organic co-solvents are added.¹⁹⁰ A reducing agent, such as TCEP or 1,4-Dithiothreitol (DTT), is included to ensure the Cys residue remains in the reduced form, preventing disulfide bonds formation.¹⁹¹ The conjugation of thiol-containing molecules to liposomes containing DSPE-maleimide derivatives was investigated in detail by Schelte et al.¹⁹² Maleimide-functionalized DSPE was prepared and used to formulate liposomes and thiol containing peptide at N-terminus conjugated to the outer surface of the particles.^{192,185} The commercially available DSPE-PEG-maleimide phospholipid is one of the phospholipids most commonly used to functionalize liposomes with thiol-containing ligands (antibodies, peptides or other ligands).¹⁸⁵ In one report, monomeric and tetrameric H2009.1 peptides for targeting $\alpha_v\beta_6$ integrins expressed on non-small cell lung cancer cells were attached to the surface of preformed liposomes containing DSPE-PEG2000-maleimide.¹⁷⁸ The efficiency of peptide coupling to the surface of the liposomes was 90% based on the amount of maleimide exposed on the liposome surface.¹⁷⁸

Aims and objectives.

As CTAs have demonstrated high immunogenicity towards multiple cancer types, and due to their restricted expression profile, they warrant further attention. Covalently attaching an adjuvant to an antigen is an effective strategy to induce a strong, targeted immune response. In this project, we will synthesise palmitoylated CTA peptides that have native and non-native di-palmitoyl-*S*-glyceryl cysteine residue (Pam₂Cys) linkages. These adjuvants will be linked covalently to the chosen CTA sequences to develop a self-adjuvanting, targeted vaccine, with the aim to develop vaccines that induce an effective but safe immune response against cancer, Figure 23. These synthetic systems mimic bacterial cell-wall lipopeptides, binding to TLR2 on the surfaces of dendritic cells, thus, activating one of the TLR pathways that are essential for the production of a cellular immune response. Because the antigen is covalently bound to the adjuvant, any resulting action should be directed toward this sequence, limiting the side effects that are often associated with separately employed adjuvants.

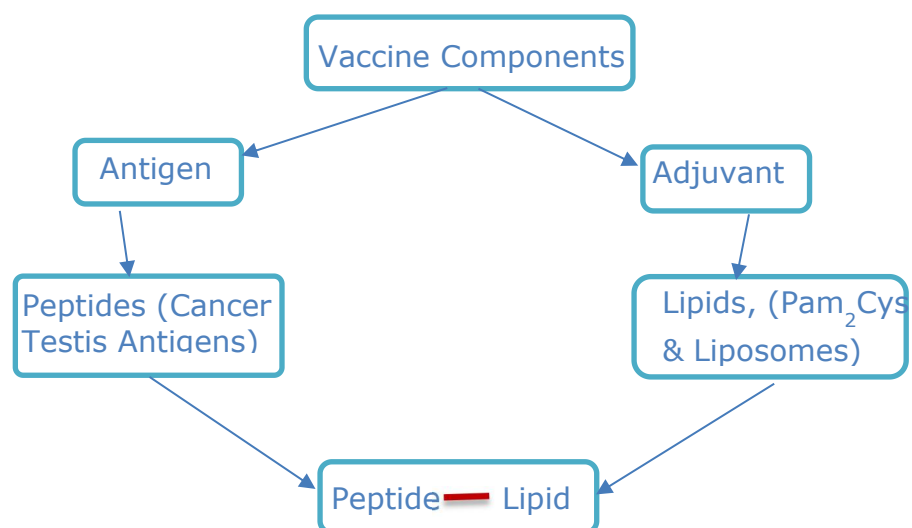


Figure 23: Diagram showing the main focus of this PhD project.

For the CTA, a small library of these proteins will be produced, starting with short sequences associated with B-melanoma antigen (BAGE), and the NY-ESO-1 protein families, for comparison. A robust method for installing palmitoylated Cys into peptides needs to be developed and optimised. Initial investigations will start with model peptide HAKTKEA, chosen from PAGE4¹⁹³, a subfamily of CT antigen, before moving to the BAGE4 CTA, and an immunogenic fragments of the NY-ESO-1 protein (NY-ESO-1₁₅₇₋₁₆₅: SLLMWITQC). First, The BAGE4 peptide will be synthesised using SPPS with Cys at the N-terminus, and then attached to the non-native maleimide-containing lipid formulated within a liposome. The maleimide linker can be synthesised as maleimide-containing lipid (**1**) (mal-lipid) and then used to formulate the liposome. This lipid (**1**) will be synthesised carrying a maleimide group attached to a glyceryl head group with two hexadecanol hydrocarbon chains attached via hydrolytically stable ether bonds. The synthesised maleimide lipid will be more cost effective and stable than the commercially available maleimide because it will be made using a di-ether linkage rather than a di-ester, which is a non-hydrolysable linkage. Beside the antigen attachment, using such lipid may provide an adjuvant activity. For the *S*-glyceryl palmityl adjuvant compounds, the (*R*)-stereochemistry is very significant (where the Cys thiol is directly attached) because it is the native form and, thus, is more biologically active.⁴ The stereochemistry of the proposed maleimide lipid mimics this preferred geometry (although the notation of the stereo centre is (*S*) due to the nitrogen replacing the sulfur of Cys) (Figure 24).

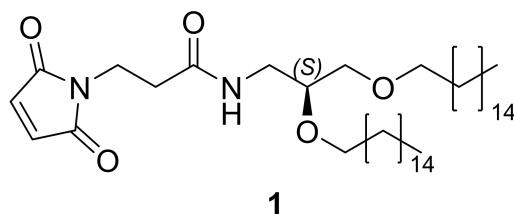


Figure 24: Structure of mal-lipid (**1**).

Using lipid structure (**1**) will allow the antigens to be conjugated conveniently to the maleimide linker that are exposed on the surface of the liposomes, Figure 25. This system can be applied to any antigen as any peptide/protein bearing an N-terminal Cys can be conjugated which is an advantage. This allows for the quick and high-yielding conjugation of the peptide to the outer envelope of the liposome and facilitates facile formulation of the liposomes; however, this method will not provide a native linker (Pam₂Cys), thus no significant adjuvant activities are expected, however this formulation will be used as an effective control to study antigen presentation.

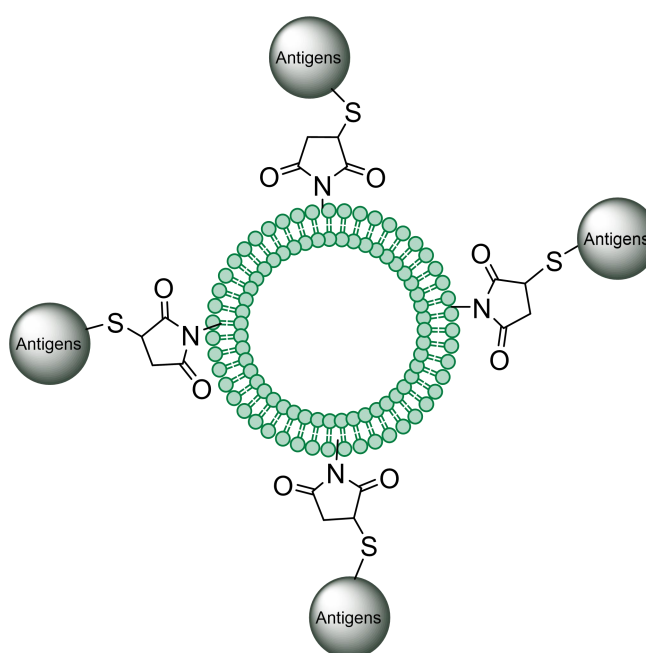


Figure 25: Final vaccine formulation of an antigen, conjugated to liposome via maleimide linker mimicking the native form.

To prepare the self-adjuvanting vaccines, we will explore three different methods to palmitoylate the BAGE4 and NY-ESO-1 peptide antigens with native linkages. First, we will synthesise and utilise an iodo-lipid probe (Pam₂I, **2**) which we will attempt to attach to the desired CTA sequences carrying a Cys residue at *N*-terminus as shown in Figure 26A. The second strategy involves the coupling of Fmoc-*R*-(2,3-dihydroxypropyl) cysteine (Fmoc-diol-Cys, **5**) to the peptide (antigen) on-resin followed by palmitoylation by palmitic acid (PA), Figure 26B, which provide us with a native linker but could present low coupling efficiency. Therefore, we will

also explore a third strategy by synthesising and attaching the entire Fmoc-Pam₂Cys (**3**) to the peptide while on resin, Figure 26C. Although the coupling efficiency of this method may also be low, we can compare it with the previous methods.

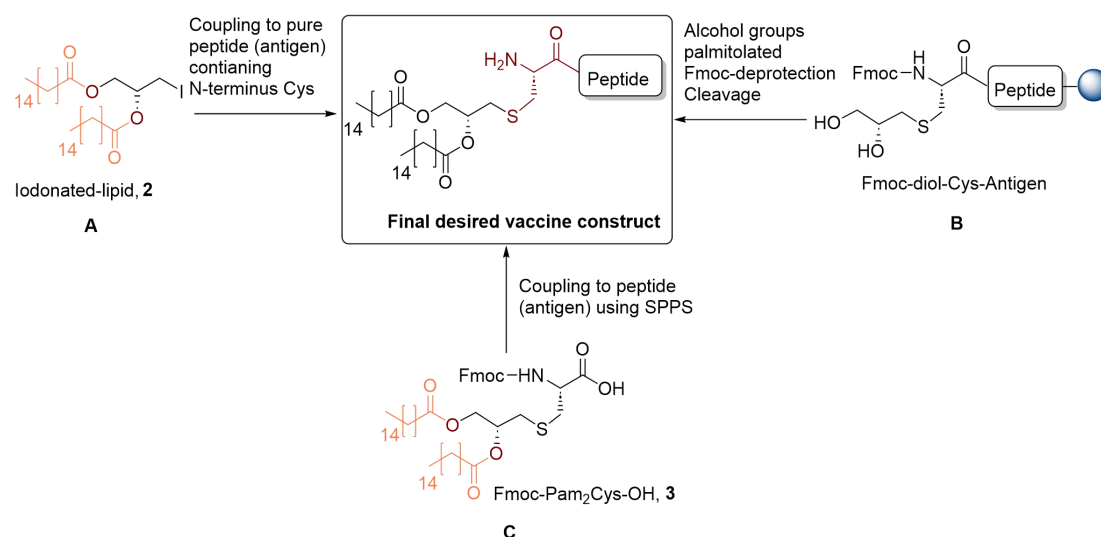


Figure 26: The three main approaches for palmitoylated CT antigens. A) First strategy where the synthesised Pam₂I (**2**) is coupled to the N-terminus Cys of purified antigen. B) Second strategy where the Fmoc-diol-Cys (**5**) is coupled to the peptide on resin first then palmitoylated by PA. C) Third strategy where the entire Fmoc-Pam₂Cys (**3**) residue is coupled to the peptide on resin using SPPS.

As discussed previously liposomes can provide adjuvant activity either by enhancing antigen delivery or by activating innate immune responses. To examine this, the most immunogenic of the palmitoylated CTA sequences will be attached, via their palmitoyl 'tails', into the lipid bilayer of liposomal nanoparticles. We will then compare the immune responses generated by the self-adjuvanting CTA vaccine constructs (Figure 26), self-adjuvanting formulated liposome vaccine construct (Figure 25), and liposome-antigen conjugate nanoparticles (Figure 27) in healthy mouse models. If the responses prove to be sufficient, we will move on to cancer models.

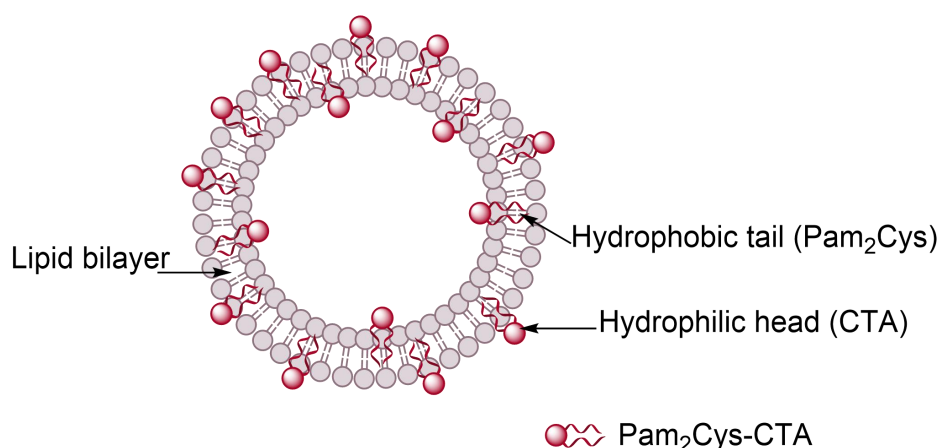


Figure 27: Self-adjuvanting formulated liposome vaccine construct.

The synthetic route **A** was novel at the time of investigation, but it was later described by Brimble.¹⁹⁴ Routes **B** and **C** have been previously described by Jackson & Brimble respectively.^{77,195} At the time of investigation Pam₂Cys-CT antigens had not been explored as vaccines candidates. However, during our investigations, Brimble described the synthesis of Pam₂Cys-NY-ESO peptide and tested the human and mouse TLR2 agonistic activities of lipopeptide homologues of Pam₂Cys using HEK293 cells.¹⁹⁴ However, the investigation of immune response by measuring the T-cell responses of Pam₂Cys-NY-ESO peptide in healthy mice is novel. The exploration of the Pam₂Cys-BAGE4 vaccine candidate is also novel.

Chapter 2: Synthesis and characterization of Palmitoyl building blocks.

2.1 Introduction

The main focus of this project is the synthesis and formulation of palmitoylated CTA peptides, and their evaluation as effective vaccines for a range of cancer types. This chapter describes our efforts towards the preparation of the lipid building block, Fmoc-Pam₂Cys (**3**) and studies towards the conjugation of this component to the CTA peptides. We initially proposed three potential routes towards Pam₂Cys-peptide conjugates. The first route starting with synthesis of iodo-lipid probe, Pam₂I (**2**), which will be then used to chemo selectively alkylate peptide carrying a Cys residue at the N-terminus as shown in Figure 28A. Thus, this strategy enables direct installation of the adjuvant into any peptide or protein antigen. The other advantages of this possible route include its operational simplicity, and a short synthetic route to the probe (**2**). There is also the possibility of conducting this conjugation in an aqueous solution with a co-solvent which is an advantage, considering that the Pam₂I (**2**) is applicable to the functionalisation of recombinantly expressed CTA protein. Furthermore, the use of 1:1 molar equivalents of pure starting materials (the iodo-lipid and the peptides), assuming efficient conjugation, may enable isolation of the product conjugate using size exclusion or dialysis (i.e., no requirement to separate product from excess peptide or lipid) resulting in high yield.¹⁹⁶ The main limitation of this route concerns the conjugation of **2** to a protein system with multiple native Cys residues, where chemo selectivity is not possible. However, the CTAs considered for this project do not contain any native Cys residues, therefore this limitation is not a concern. The second proposed route involves coupling Fmoc-*R*-(2,3-dihydroxypropyl) cysteine, Fmoc-diol-Cys (**5**), to the peptide (antigen) on-resin, followed by palmitoylation by palmitic acid, Figure 28B, which will result in a native linker but may have low coupling efficiency. As a result, a third strategy is possible, by attaching the entire Fmoc-Pam₂Cys (**3**) to the peptide prior to cleavage, as shown in Figure 28C. Although this method's

coupling efficiency may also be low; each method will be compared to identify the optimal strategy. Both of these last two potential routes to installing Pam₂Cys (B & C) employ solid phase chemistry. Therefore, optimisation of the coupling of the Pam₂Cys moiety should be possible. However, there are couples of disadvantages to be considered when coupling such a hydrophobic amino acid. These include the long synthetic route toward the amino acid building block, thus time consuming, which is not sustainable due to amount of organic solvent and reagent involved.¹⁹⁷ Furthermore, this strategy is considerably more expensive, and the purification step for the final desired product will be more complicated due to the presence of truncated peptide by-products and other side reaction products¹⁹⁸.

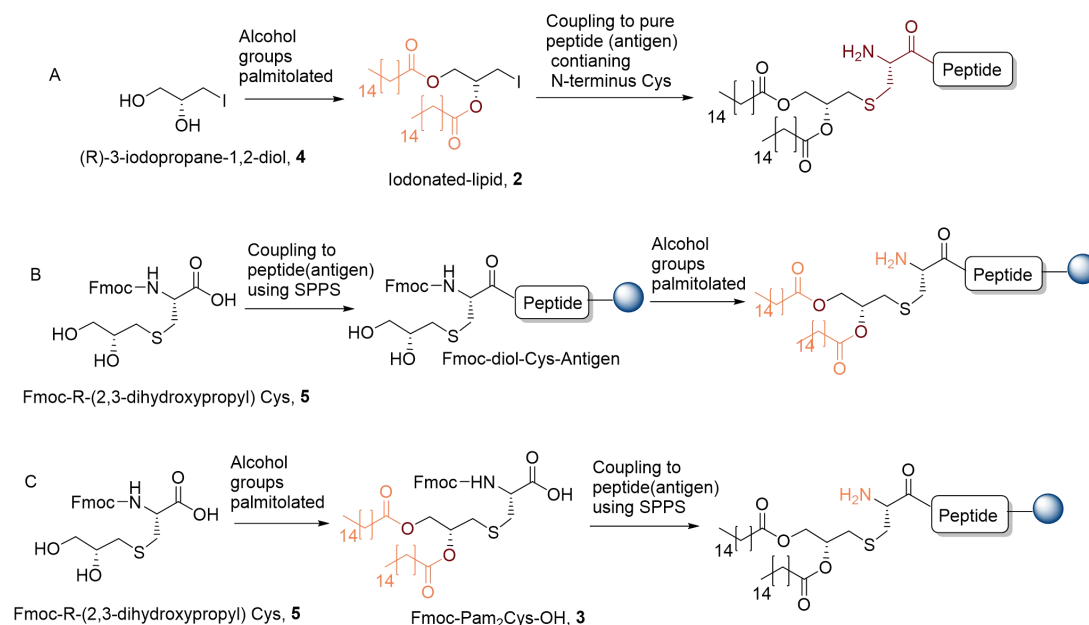


Figure 28: The three potential routes to Pam₂Cys-peptides.

The entire synthesis pathway of iodo-lipid probe (**2**) route will be discussed below in detail, this will be followed by a discussion regarding the optimisation of the conjugation of the lipid probe to chosen model peptide fragments first in order to be applied later using antigenic CT peptides. For the solid phase routes, the synthesis of initial building blocks (Fmoc-diol-Cys **5** & Fmoc-Pam₂Cys-OH **3**) will be discussed here

while the details of lipopeptides synthesis will be discussed in the next chapter.

2.2 Synthesis of Pam₂I (2)

As discussed previously one of the proposed routes to lipidated peptides is through Pam₂I (**2**), its structure can be divided into two parts: two fatty acid components and the core structure (*R*)-(2,3-dihydroxypropyl)-iodine, to which the two fatty acids are attached via ester linkages, structure shown in Figure 29. This probe should then be conjugated to the desired antigen via an N-terminal Cys to produce the lipidated peptide.

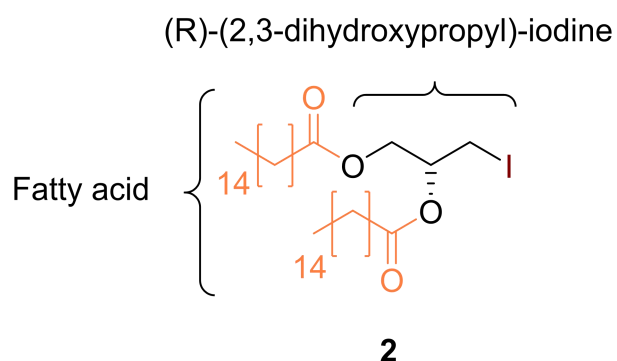
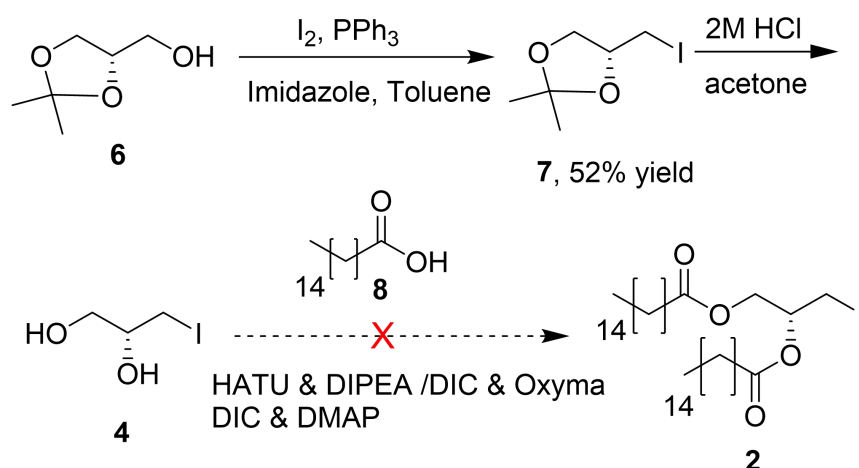


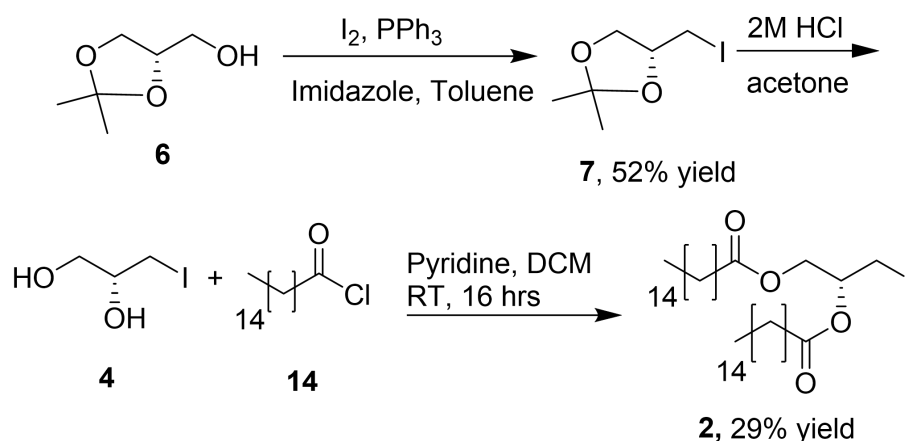
Figure 29: Structure of iodinated lipid (Pam₂I, **2**).

The synthesis of Pam₂I (**2**) was attempted via three different routes shown in Scheme 1, Scheme 2 and 5. The first method attempted was performed over three steps starting with iodination of alcohol protected as an acetal (*S*)-(2,2-dimethyl-1,3-dioxolan-4-yl) methanol (**6**), followed by deprotection of the ketal group to give the (*R*)-3-iodopropane-1,2-diol (**4**) with two free alcohol groups and palmitoylation using palmitic acid, Scheme 1.



Scheme 1: Pam₂I (**2**) synthetic scheme, route **1**.

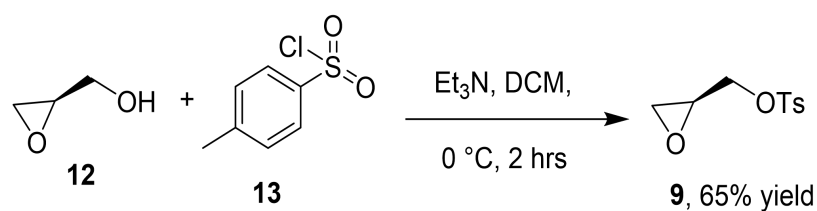
In the first step the iodo group was installed via activation of an alcohol protected as an acetal (**6**) with triphenylphosphine in the presence of imidazole and iodine, Scheme 1.^{199,200} After several attempts the optimised conditions of 2.6 equiv. of PPh₃ and I₂ and 1.5 of imidazole, under N₂ for 5 hrs in the absence of light resulted in the highest yield of 52%. In the second step, the ketal-protecting group in compound (**7**) was then removed under acidic conditions using 2M HCl, to produce the free alcohol groups that can be acetylated. However, in the last step several methods were attempted to enable the preparation of compound (**2**) using 3-iodopropane-1,2-diol, PA and several different coupling reagents, including HATU & DIPEA, Oxyma & DIC, DIC & DMAP, and DIC only. The reactions were monitored, using TLC and MS, at different time points starting from 1 hr to overnight. However, none of these reagents yielded the desired product. Therefore, further exploration of the palmitoylation of compound **4**, shown in Scheme 1, was carried out to develop a second route of Pam₂I synthesis. Esterification using palmitoyl chloride was performed, Scheme 2, which succeeded, producing the desired product (**2**) (135 mg, 29% yield).



Scheme 2: Pam₂I synthetic scheme, route 2

The above synthetic route was first trialled on inexpensive racemic compound (2,2-dimethyl-1,3-dioxolan-4-yl-methanol) before repeating with the correct required enantiomer *R*.²⁰¹ Lu et al.,¹⁹⁴ analysed and evaluated the effect of the stereochemistry on TLR2 activity. They found that the (*R*)-diastereomer gives a more robust immune response, enhanced macrophage, B-cell activation²⁰¹ leading to improved activation of dendritic cell²⁰² compared to the (*S*)-diastereomer.

An alternative method, where the esterification perform prior to iodination, reported by Ali & Robert²⁰³ was attempted to obtain the desired product (**2**) Scheme 5. This method involved the synthesis of a glycidyl tosylate²⁰⁴ (**9**) by conversion of the alcohol group in the glycidol (**12**) with a tosyl (OTs) group via the addition of toluene-*p*-sulfonyl chloride (**13**) in preparation for the next step as shown in Scheme 3. The synthesis was successful affording compound **9** in good yield. Simultaneously, the palmitic anhydride (**10**) was prepared using PA in present of DCC and under N₂, Scheme 4.

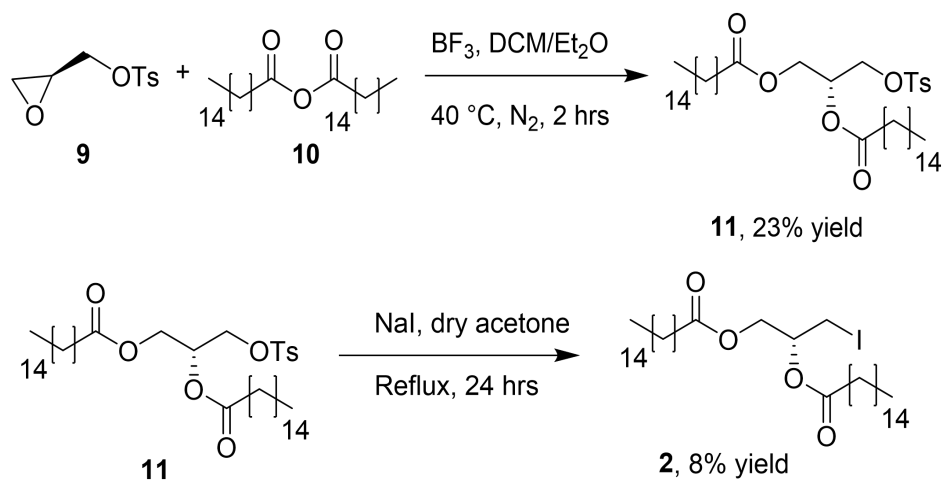


Scheme 3: Synthetic scheme of R-glycidol tosylate (**9**).



Scheme 4: Synthetic scheme of palmitic anhydride (**10**).

In the second step both compounds (**9**) & (**10**) were reacted in the presence of BF_3 , under N_2 and reflux to produce (*R*)-3-tosylatedpropane-1,2-diyl dipalmitate (**11**). The purification of this step was complicated due to the challenging separation of the PA from the desired product; addition of ammonia to the flash column solvent system enabled isolation of compound (**11**) in 23% yield, Scheme 5. ^1H NMR shows the presence of PA, however, since this should not affect the following step, the crude product was taken forward. Compound (**11**) was treated with NaI to produce the final compound (**2**), Scheme 5, purification by recrystallization afforded a very low yield (8%) with some impurities.

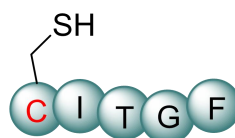


Scheme 5: Pam2I synthetic scheme, route **2**.

Since the overall yield of this synthesis was low (2%), also, the addition steps to create the starting materials were time consuming and expensive, the second synthetic route shown in Scheme 2 was approved for synthesising compound (**2**).

2.3 Conjugation of (*R*)-3-iodopropane-1,2-diol with CITGF model peptide

In order to optimize the conjugation conditions of inserting the iodo-probe, Pam₂I (**2**) into the desired antigen, we need first to attempt this in a simple model peptide system, thus the following model peptide **15** was synthesised using Fmoc-SPPS.



15

This peptide sequence, H-CITGF-OH (**15**), was chosen because it is a short convenient model peptide, so it can be easily synthesised. Also, it has an aromatic amino acid that can be detected using ultraviolet (UV,

for HPLC). Additionally, this model contains an N-terminal Cys as this represents our chosen attachment site among the canonical amino acids.²⁰⁵ The sulfhydryl group in the Cys side chain has enhanced nucleophilicity compared to the other canonical amino acids, thus this thiol is highly reactive, particularly in the physiological pH range at which the lysine (Lys) side chain amine is protonated. Accordingly, Cys reacts faster and more selectively than Lys, making coupling a highly desirable method via the Cys side chain.²⁰⁶ The peptide was synthesised successfully using Fmoc-SPPS with 88% yield, no purification was necessary.

The conjugation reaction was attempted under different conditions, using TCEP as a reducing agent to ensure the Cys thiol remains reduced. Considering that both the carboxylic moiety of the C-terminus and the N-terminal amine group could be involved in the reaction under basic conditions, the pH of PBS buffer was maintained around neutral.¹⁹⁶ Initially, the conjugation conditions were kept as mild as possible to enable application of this chemistry to proteins. Following are the conditions attempted.

Conjugation protocol **1** (@ **RT**)

Stock solutions of peptide (**15**) (0.01 M, 1 equiv.) and TCEP (0.02 M, 2 equiv.) were prepared in 0.1 M phosphate buffer (PBS) at pH of 7.4, and a stock of compound **2** (0.02 M, 2 equiv.) was prepared in 1:1 (0.1 M PBS): MeCN. Then, 100 µl of each compound was mixed (the solution was not completely soluble) and incubated at room temperature, overnight. The reaction was monitored by analytical RP-HPLC (10%-100% solvent B, see general methods in chapter 7). The starting material remained, without evidence of consumption, indicated that the conjugation reaction did not occur. To ensure full solubility of the reagents, compound **2** was dissolved in HFIP and the temperature increased to 45 °C as detailed below.

Conjugation protocol **2** (@**45 °C**)

A stock solution of peptide (**15**) (0.01 M, 1 equiv.) and TCEP (0.02 M, 2 equiv.) was prepared in 0.1 M PBS buffer, and a stock of Pam₂I (**2**) (0.02 M, 4 equiv.) was prepared in HFIP. 100 µl of each compound was mixed (note: the lipid was not completely soluble in the final solvent system of 33% HFIP in PBS buffer), and incubated at 45°C, over a 72-hour period. The reaction was monitored by analytical RP-HPLC. However, the desired product was not observed; this could be attributed to poor solubility of compound (**2**). An elevated temperature of 60°C, and a larger excess of compound (**2**) were applied but no progress was noticed, as only the peak for the starting material was shown by RP-HPLC and confirmed by MS.

Conjugation protocol **3** (@ **60 °C** & DMF as a solvent).

Larger proportions of organic solvent were initially avoided as these conditions would denature protein material. However, since we are focussing on the preparation of palmitoylated peptides, we can afford to move away from conditions that are appropriate for proteins. Therefore, DMF used as the solvent of the reaction with Pam₂I (**2**) (0.02 M, 2 equiv.) and using DIPEA (0.02 M, 2 equiv.) as the base in presence of TCEP (0.02 M, 2 equiv.) 100 µL of each mixed and the reaction mixture was agitated at 60 °C for overnight. The starting peptide H-CITGF-OH (**15**), (0.01 M, 1 equiv.) was consumed over this period (observed by HPLC). Figure 30, shows that the peak of starting material disappeared (confirmed also by MS) and a new peak eluted at a later retention time around 11 mins as well as a small peak eluted around 6 mins could be related to some impurities. The large shift in retention suggested the attachment of lipid; however, this could not be confirmed by MS (calculated mass: 1117.7 Da; observed mass 1159.9 Da), although the mass intensity was very weak. Analysis by MALDI confirmed this result; a +42 increase in mass can often be attributed to acetylation or CO₂ from incomplete Boc group removal (often on tryptophan), however, we do not believe either of

these events occurred for our peptide (the Fmoc-amino acids used for this model peptide are not protected with a Boc group). Regardless the aggressive reaction conditions used, it has been proven that the starting material has been consumed as discussed; however, the desired product has not been obtained.

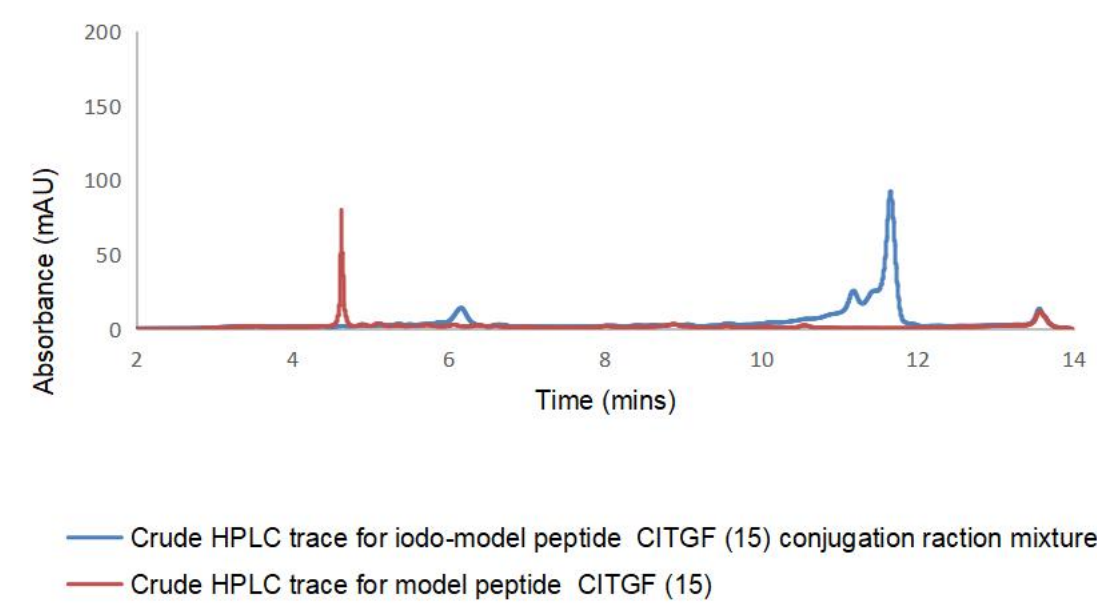


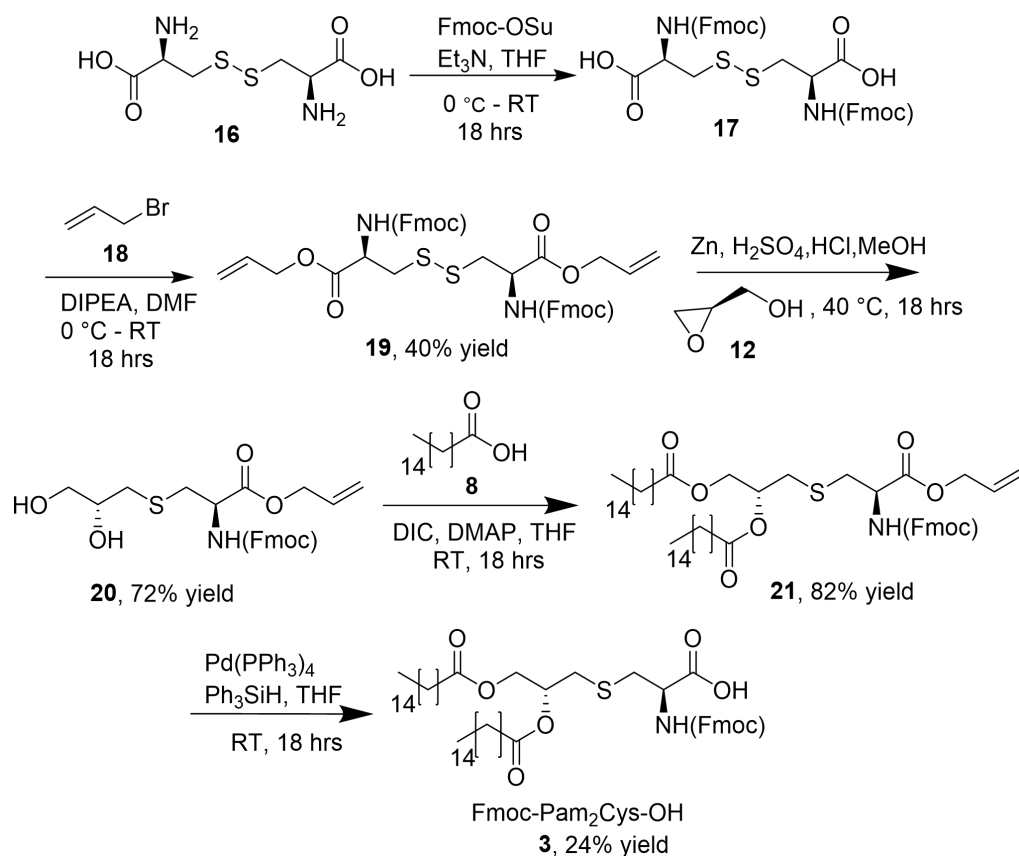
Figure 30: Analytical HPLC traces of crude model peptide CITGF (**15**), and the trace of reaction mixture. Analytical gradient 2-100% B over 10 mins, 280 nm.

The main advantage of employing the Pam₂I (**2**) compound is its ability to attach to any peptide/protein. However, due to the harsh conditions necessary to push the reaction to completion, and since the desired product could not be obtained and identity of the by-product not elucidation, we switched our focus toward the synthesis of Fmoc-Pam₂Cys-OH (**3**), which can be used to incorporate the adjuvant into peptides on the solid-phase.

2.4 Synthesis of Fmoc-Pam₂Cys-OH (Method 1)

Fmoc-Pam₂Cys-OH (**3**) is the building block of the third proposed route towards the Pam₂Cys peptide targets shown in Figure 28. Its structure is discussed in detail in Chapter 1, in brief, it is similar to that of Pam₂I (**2**) in containing two fatty acid components and the core structure of (*R*)-

(2,3-dihydroxypropyl), with a Cys residue replacing the iodine, attached via the thiol. The desired CTA can be then coupled to the C-terminal carboxylic acid of the Cys residue using SPPS. The synthesis of this building block achieved by two different synthetic routes as detailed below (Scheme 6).



Scheme 6: Synthetic scheme **1** for the synthesis of Fmoc-Pam₂Cys-OH (**3**).
207,208

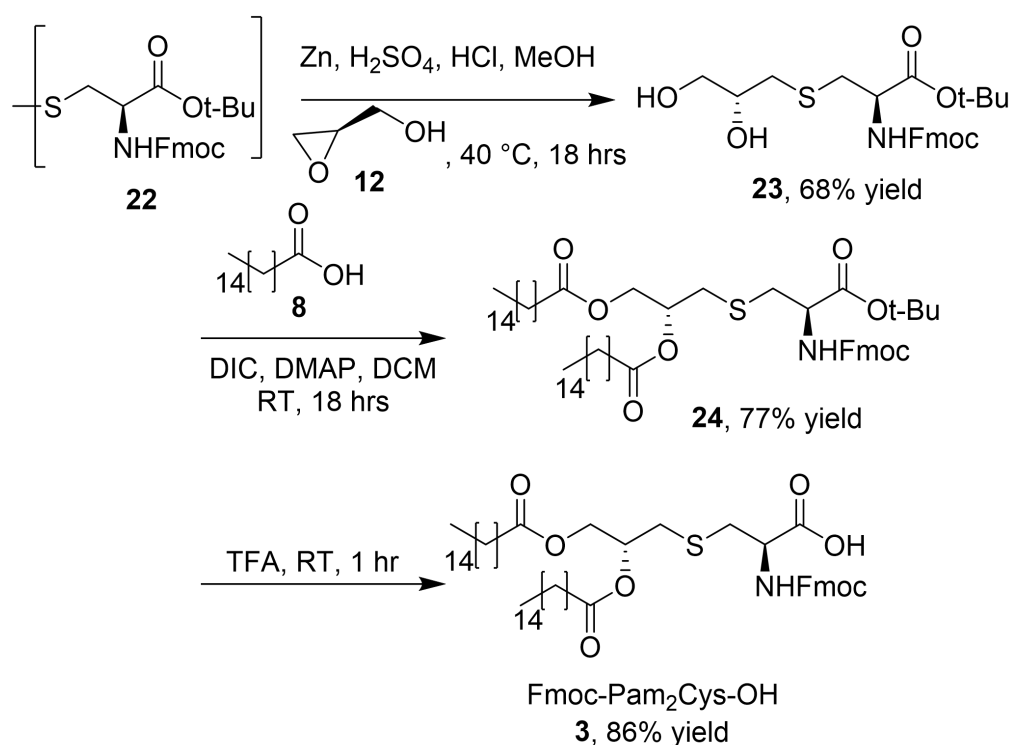
The Fmoc-Pam₂Cys-OH (**3**) synthesis requires several steps shown in Scheme 6; the first step is the synthesis of Fmoc-Cystine²⁰⁹ (**17**), in which an Fmoc protecting group is used to mask the amines. This reaction was performed using Fmoc-OSu to give crude Fmoc-cystine (**17**) in a high yield 95% after work up. The crude compound was then taken on to the next step; allyl bromide (**18**) was used to protect the carboxylic acid groups and purified using flash column chromatography, resulting in moderate yield (40%). The disulfide of compound (**19**) was reduced with zinc under acidic conditions at RT for 15 mins to afford a free thiol, which was then alkylated with (*R*)-glycidol (**12**) at 40 °C for

18 hrs to give the allyl-Fmoc-Cys-diol (**20**) which then purified to produce the desired compound in 72% yield. The hydroxyl groups in compound (**20**) were then palmitoylated by esterification with PA (**8**) using DIC and a catalytic amount of DMAP at RT for 18 hrs to give dipalmitoylated product (**21**) then purified resulting 82% yield. However, the ^1H NMR spectra of the product indicated that excess PA remained in the purified product, thus the yield is not an accurate reflection for this reaction there is an impurity of palmitic acid which affected the integral values of the peaks at 2.32, 1.60, 1.26 and 0.88 ppm. From the peak at 1.26 ppm if 48 of the integral value is coming from the compound (**21**) and the other 30 of the integral value coming for palmitic acid, that mean for every mole of the compound ($48/48 = 1$) [the integral value divided by the number of hydrogens in the compound belonging to the peak] there is 1.25 mole ($30/24 = 1.25$) palmitic acid in the sample. In weight percentage there 75 % compound palmitic acid. That is calculated from $1 \times \text{MW for the compound} + 1.25 \times \text{MW for palmitic acid} = 1254$. Then $934/1254 = 75\%$. The co-elution of compound (**21**) and PA made purification particularly challenging for this step, it was therefore decided to carry the semi-pure compound (**21**) through to the next step. Finally, to enable the coupling of Fmoc-Pam₂Cys to peptide on-resin the carboxylic acid group was deprotected via a Tsuji-Trost deallylation reaction at RT for 18 hrs to give Fmoc-Pam₂Cys (**3**).²¹⁰ However, the initial purification of compound (**3**) proved difficult due to co-elution of impurities, repurification was required, and the desired product was obtained in a poor yield (24%) compared to the yield reported by literatures (69%, 86%).^{195,211}

2.5 Synthesis of Fmoc-Pam₂Cys-OH (Method 2)

The overall yield of Fmoc-Pam₂Cys (**3**) synthesised by method 1 was 6%; this is too low considering the required amount for lipopeptide synthesis on the solid-phase. It was clearly important to find a strategy with fewer steps and higher overall yield. Therefore, an alternative method was attempted, Scheme 7, starting from (Fmoc-Cys-OtBu)₂ (**22**) which is considerably more expensive than cystine, however; starting from this

material shortens the synthesis to the final desired compound (**3**) by 2 steps which will increase the efficiency of the entire route.

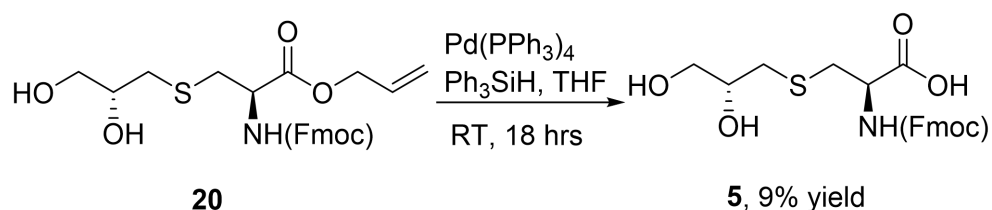


Scheme 7: Synthetic scheme **2** for the synthesis of Fmoc-Pam₂Cys-OH (**3**)²¹¹

This method started with reduction of the disulfide using similar conditions to method 1, compound (**22**) was then reacted with (*R*)-glycidol (**12**) at 40 °C for 18 hrs then purified to give the Fmoc-Cys-OtBu-diol (**23**) in 68% yield. This was followed by palmitoylation of the alcohol groups using PA in the presence of DIC and DMAP and purified to give dipalmitoylated-Fmoc-Cys-OtBu (**24**) in 77% yield. In the final step the carboxylic group was deprotected in TFA to produce Fmoc-Pam₂Cys-OH (**3**) which when purified, resulted a very good yield of 86%. This method involved fewer steps (including fewer purification steps via silica gel chromatography), resulting in pure and a higher overall yield of 45% relative to the initial route.

2.6 Synthesis Fmoc-diol-Cys (Method 1)

As discussed at the start of this chapter, we aimed to produce several building blocks to enable different approaches to the synthesis of palmitoylated peptides (CT antigens) on the solid phase. The original plan was to synthesis both building blocks (**3** & **5**) shown in Figure 28 starting with the same synthetic route, Scheme 8.

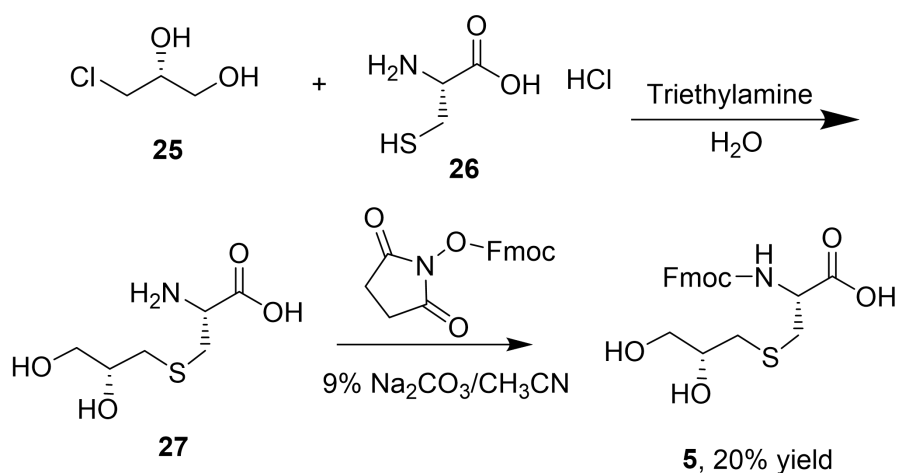


Scheme 8: Synthetic scheme **1** for the synthesis of Fmoc-diol-Cys (**5**).

Therefore, once allyl-Fmoc-diol-Cys (**20**) was successfully synthesised via method 1, the compound was split into two batches, half was taken through to compound **3** and then remain material deallylated using the conditions previously outlined to give compound (**5**), Scheme 8. However, purifying Fmoc-diol-Cys (**5**) using silica flash chromatography was challenging due to the polarity of the compound. Therefore, preparative HPLC was used to isolate the pure product. Purification of the compound was still challenging, and the desired compound (**5**) was isolated in 9% yield.

2.7 Synthesis Fmoc-diol-Cys (Method 2)

To form a library of lipidated antigens, larger-scale synthesis of Fmoc-diol-Cys (**5**) was required, and the low overall yield described above would not enable a significant amount of material to be synthesised. Therefore, an alternative 2-step synthesis was proposed, containing no silica gel chromatography, Scheme 9.



Scheme 9: Synthetic scheme **2** for the synthesis of Fmoc-diol-Cys (**5**).⁷⁷

To obtain the free Cys-diol (**27**), L-cystine hydrochloride (**26**) was coupled to (*R*)-3-chloro-1,2-propanediol (**25**) via the free thiol. The amine group was then protected using Fmoc-OSu and the purification of Fmoc-Cys-diol (**5**) was performed using prep. HPLC. Difficulties with the solubility in the HPLC solvents meant DMSO was required as a co-solvent followed by filtration, leading to a low yield (7%). The mass recovery showed product in both the filtrate and precipitate by MS. To improve the yield recrystallization was attempted by exploring several different solvent systems. Again, the main problem was the poor solubility of the material. With help of filtration the desired product was recrystallized from diethyl ether and ethyl acetate at -20 °C successfully to yield the desired pure compound (**5**) in 20% yield.

2.8 Conclusion

Developing an effective self-adjuvanting cancer vaccine based on a naturally occurring bacterial lipoprotein attached to peptide antigens is the main goal of this project. This chapter described the optimisation of several approaches towards the synthesis of the lipid-adjuvant component/building block. Synthesis of an iodinated lipid (Pam₂I, **2**) that could be conjugated to any protein/peptide carrying an N-terminal Cys

residue was the initial priority. Optimisation of the synthesis of this compound addressed several obstacles and pure compound **2** was obtained. The main difficulties encountered concerned the esterification step; palmitoyl chloride yielded the desired compound compared to esterification with PA. However, conjugating Pam₂I (**2**) to model peptide **15** gave an unidentified by-product and required high temperature and use of a high ratio of organic solvent. Therefore, the focus was shifted to the synthesis of building blocks that would allow the synthesis of the palmitoylated CTA on the solid phase. Two different methods were explored: one method involves the attachment of the whole Fmoc-Pam₂Cys-OH (**3**) amino acid to the peptide on resin. The second strategy concerns the attachment of Fmoc-diol-Cys (**5**) and on-resin esterification. The synthesis of the first method's building block, Fmoc-Pam₂Cys-OH (**3**) was achieved by two different strategies, the second route described starting from (Fmoc-Cys-OtBu)₂ (**22**) was more efficient as it saved time through fewer synthetic steps and produced pure product in higher overall yield. Fmoc-diol-Cys (**5**), the building block synthesised to explore the on-resin palmitoylation of the CTA peptides, was synthesised successfully through two alternative routes. Starting from (*R*)-3-chloro-1,2-propanediol (**25**) the second method discussed provided the desired product more efficiently in higher yield compared with the initial route. These building blocks will now be applied to synthesise self-adjuvanting vaccines on the solid phase.

Chapter 3: Synthesis and characterization of palmitoylated CTA peptides.

3.1 Introduction

CTA have been shown to produce high immunogenicity and due to their restricted expression are potential clinical candidates.²¹² A well-established method for boosting antigen-mediated immune response involves the co-administration of a lipid adjuvant. However, direct attachment of an adjuvant to antigenic material has been shown to induce a strong and very specific immune response.²¹³ This chapter will discuss the synthesis of CTA peptides that carry a Pam₂Cys adjuvant. These adjuvants will covalently be linked to the chosen CTA sequences to develop a self-adjuvanting, targeted vaccine, which may result in a safer, more effective immune response. Since the antigen is covalently bound to the adjuvant, any resulting action should be directed toward this sequence, limiting the side effects that are often associated with separately employed adjuvants.^{15,214,215}

Two CTA sequences will form the basis of our study: a fragment of the CTA NY-ESO-1₁₅₇₋₁₆₅ (**29**) that has been shown to produce a strong anticancer immune response^{216,217} and stimulate CD8⁺ T cell responses in humans restricted through the HLA class I allele HLA-A2²¹⁸ and the BAGE4 peptide²⁶ (**28**), an underexplored melanoma antigen. These peptides will be synthesized using Fmoc-SPPS and a Pam₂Cys residue will be installed using 3 different possible methods, Figure 31.

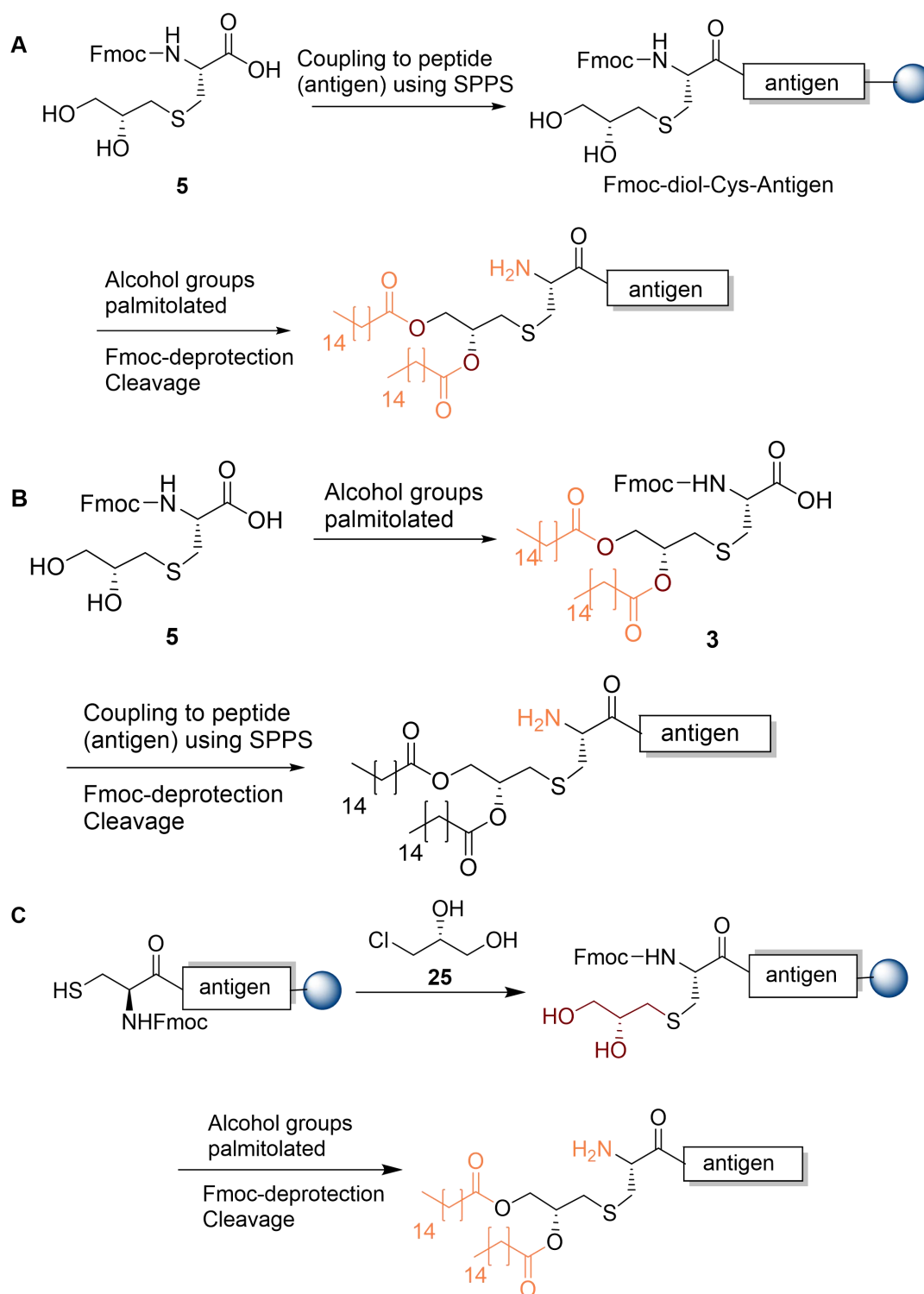


Figure 31: **A)** first strategy where the Fmoc-diol-Cys (**5**) couple to the peptide on resin first then palmitoylated by PA. **B)** Second strategy where the entire Fmoc-Pam₂Cys (**3**) couple to the peptide on resin using SPPS. **C)** Third strategy where free thiol Cys alkylated on resin using (*R*)-3-chloro-1,2-propanediol (**25**) then palmitoylated by PA using SPPS.

3.2 Synthesis of BAGE4₁₈₋₃₉ (**28**)

There are several families of CTA such as MAGE^{219,220}, GAGE^{221,222,223}, NY-ESO-1^{224,225,226}, and BAGE^{26,28}. This project will initially start with the native BAGE4 sequence (**28**), shown in Figure 32. As well as sharing same features as the other CTAs described previously, it is a relatively short sequence which makes it an ideal vaccine candidate as it should be easy to produce via chemical synthesis.



28

Figure 32: The amino acids sequence of BAGE4₁₈₋₃₉ (**28**).

The peptide was synthesised using standard Fmoc-SPPS protocols (see Experimental chapter for details), however; during the synthesis several difficulties were encountered. The first amino acid leucine (Leu) was loaded onto 2-CTC resin with only 46% loaded yield, (0.116 mmol, 0.3 mmol/g) which was lower than expected, this might be due to the resin haven taken up moisture and hydrolysing the 2-chlorotrityl chloride linker. Several modifications to the method were introduced, to improve the loading yield such as drying the resin in a desiccator for 2-4 hrs before swelling it for at least 1hr in dry DCM. Also, the equiv. of the first amino acid was increased to 1.5 equiv., leading to a very high loading yield of 93% (0.232 mmol, 0.6 mmol/g). However, above 80%, 0.5 mmol/g loading yield is not preferable for the synthesis of long sequences or difficult sequences with high aggregation tendencies leading to low overall yield; therefore, the resin with 46% loading yield was carried on to completion. Moreover, there were several points that should be considered during the synthesis of BAGE4. Firstly, a common side reaction that takes place when glycine (Gly) is followed by an aspartic acid (Asp) residue is the formation of aspartimide during the

Fmoc deprotection shown in Figure 33. Using a microwave reactor to assist the coupling steps could increase the possibility of this issue. So, it was important to synthesise the first 5 amino acid manually at RT before moving to automated synthesis. Also, there is another effective solution to overcome this problem, protecting the nitrogen of the preceding residue temporally which prevents it from attacking the carbonyl of the β -ester Figure 33.²²⁷ This could be achieved easily by incorporating (2-hydroxy-4-methoxybenzyl) Hmb or (2, 4-dimethoxybenzyl) Dmb protected amino acid prior to the problematic Asp. Therefore, substitution of Gly by (Dmb)Gly was necessary to overcome not only the aspartimide formation but to prevent aggregation during chain assembly, which will lead to faster and more predictable acylation and deprotection reactions. For coupling Asp, the follow amino acid, first Oxyma and DIC used as a coupling reagent, double coupling was performed, however; the Asp has not been coupled as shown in MS of mini cleavage. The MS shown (see Appendix, p. 253) is the mass of acetylation Gly which prevent the coupling of Asp. The Gly (Dmb) is a very sterically hindered secondary amine which make second amino acid coupling difficult. Therefore, a fresh new resin was used, and the sequence was resynthesised up to Gly using Oxyma and DIC and for Asp, an alternative coupling reagent (HATU & DIPEA) were used, and 'triple coupling' employed (first for 30 mins, then second and third coupling for 1 hr and 30 mins with fresh reagents). During the Asp coupling, a 'mini cleavage' (cleavage of a few beads for analysis) was performed before capping step to check for any unreacted peptide.

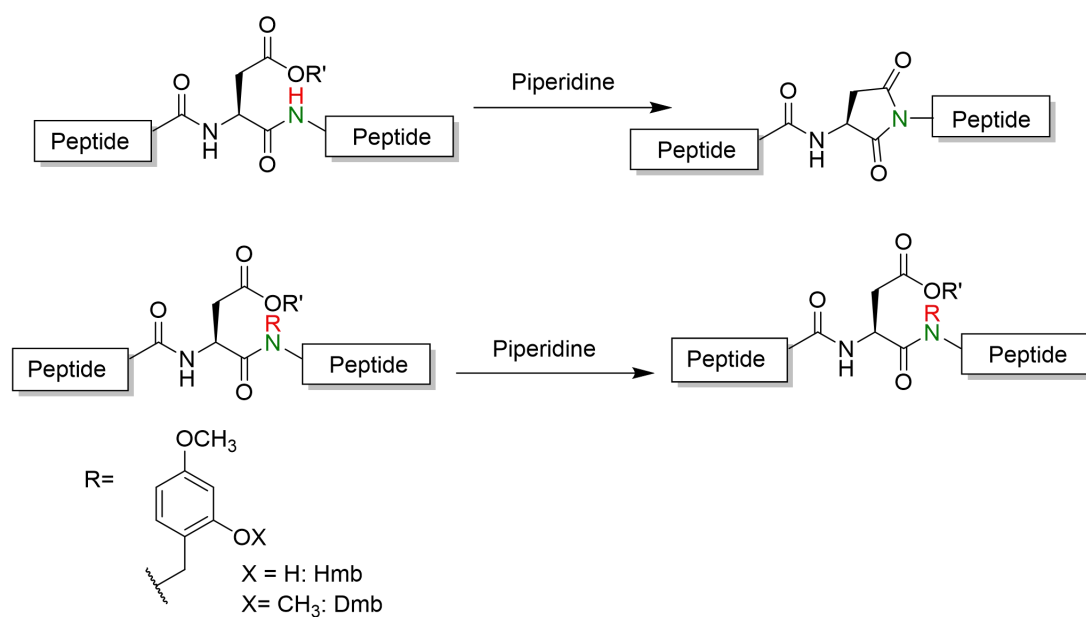


Figure 33: Prevention of aspartimide formation by backbone-amide protection.²²⁷

The other point that had to be considered is the hydrophobicity of the sequences, which can make the coupling difficult as peptides that are poorly solvated while attached to the solid support prevent complete deprotection and coupling steps²²⁸, therefore a double coupling was needed with a number of mini cleavages during the synthesis to ensure the completion of the coupling. However, the result after the second mini cleavage which took place after the coupling of Ser showed an increased mass of 88. This increased mass was due to the carbonation of the two Trp residues present, these can be removed by lyophilisation. The synthesis of BAGE4₁₈₋₃₉ was achieved successfully with high impurities as shown by HPLC Figure 34. The impurity' peak (eluted at 8.5 mins in analytical HPLC) was isolated and the MS shows several different unidentified species while peak eluted at 6.1 mins represented the desire peptide, Figure 35. During the synthesis, the capping step was performed after each amino acid coupling, terminating any unreacted amino groups thus leaving them unreactive towards the next step. Therefore, since these impurities should not be involved in any further reaction, this batch of synthesised BAGE4 was taken onto the final step

of adjuvant coupling.

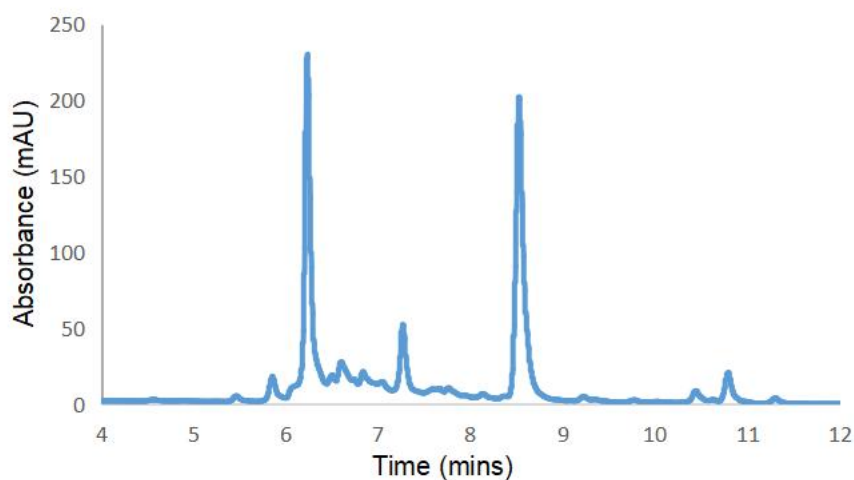


Figure 34: Analytical HPLC trace and MS of the crude BAGE4₁₈₋₃₉ antigen (**28**). The HPLC trace using (gradient: 2-95% solvent B) shows 2 main peaks, peak eluted at 6.1 mins present the desired product.

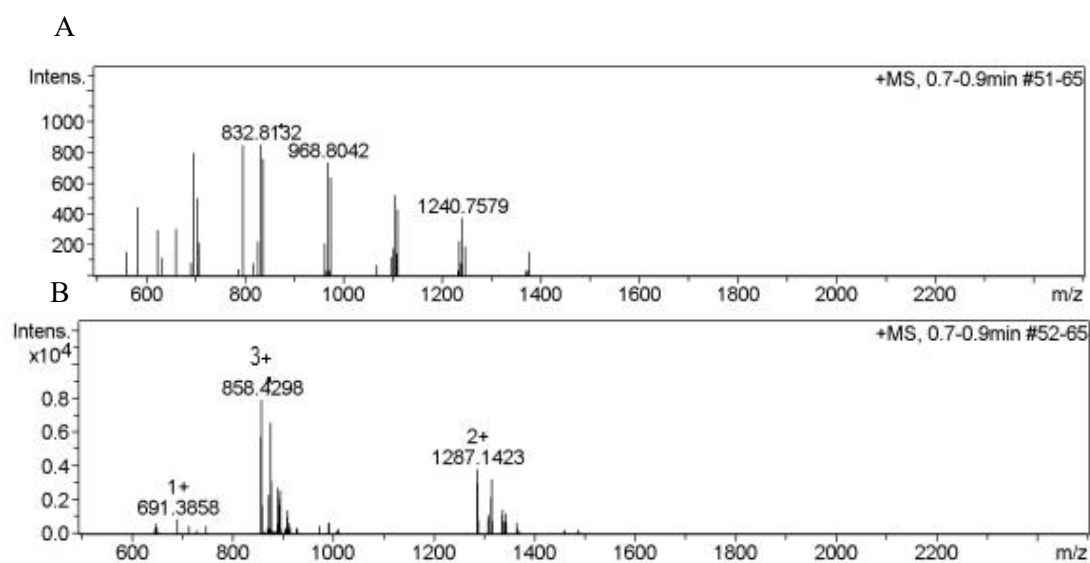


Figure 35: A) show the ESI⁺ MS spectra for the isolated impurity peak eluted at 8.5 mins while **B** show the ESI⁺ MS spectra for desired peptide BAGE4₁₈₋₃₉ eluted at 6.1 mins. ESI MS Calc. for C₁₁₆H₁₇₈N₂₈O₃₆S [M+2H]²⁺:1286.6339, [M+3H]³⁺:858.0893. Obs. [M+2H]²⁺:1287.1423, [M+3H]³⁺:858.4298.

3.3 Synthesis of NY-ESO-1₁₅₇₋₁₆₅ (**29**)



29

Figure 36: The amino acids sequence of NY-ESO-1₁₅₇₋₁₆₅ (**29**)

NY-ESO-1 is gaining significant attention as a potential cancer vaccine antigen; it has been evaluated in several clinic trials.²²⁹ Small peptide representing sections from the NY-ESO-1 protein have been studied in details^{216,230,231}; the SLLMWITQC₁₅₇₋₁₆₅ peptide (**29**) is one of the immunogenic fragments. It is very efficiently recognized by CD8⁺ T cells from HLA-A*0201 melanoma patients²¹⁶ which makes it a potentially efficient vaccine candidate. In the clinical trials for this peptide, an adjuvant was co-administered (as is standard practice).²³² It would, therefore, be interesting to lipidate this sequence with the Pam₂Cys adjuvant and compare the immune response with the reported NY-ESO-1₁₅₇₋₁₆₅ peptide (**29**) with co-administered adjuvant. It will also be interesting to compare these to engineered NY-ESO-1₁₅₇₋₁₆₅ (**29**) with a T helper epitope and the Pam₂Cys moiety. Therefore, the peptide was synthesised on Rink amide resin using the standard Fmoc-SPPS protocol. The synthesis was successful; the peptide on resin was then divided into 3 batches: one was used to directly couple on the Fmoc-Pam₂Cys-OH (**3**) residue; the second batch was cleaved and purified to afford the peptide antigen for control studies; the third batch was continued on to include a T-helper epitope sequence (discussed later in section 3.4) followed by coupling of Fmoc-Pam₂Cys-OH (**3**).

The purification of the NY-ESO-1₁₅₇₋₁₆₅ peptide (**29**), was problematic due to solubility issues related to the peptide sequence. A solution of 20% DMSO in water (0.1% TFA) was required to solubilise the crude material. At this % of DMSO, co-elution of desired peptide and impurities in the injection peak is a problem. The other challenge is the co-elution of the desired peptide and impurities at the same ratio of HPLC solvents. In

order to solve these issues, a second purification with a lower concentration of DMSO (10%) was required, and an isocratic method was designed to achieve better separation (30%-80% solvent B over 35 mins, isocratic @ 35% solvent B for 5 mins). HPLC data of the pure product shown on Experimental Chapter, section 7.4, p.181.

3.4 Synthesis of lipopeptide

General protocol, A

As discussed previously we considered several methods to palmitoylate the antigens. After exploring the conjugation of Pam₂I (**2**), we explored solid-phase strategies, starting with the coupling of Fmoc-diol-Cys (**5**) to the peptide (antigen) on-resin followed by coupling of PA to afford the final desired candidate Figure 37.

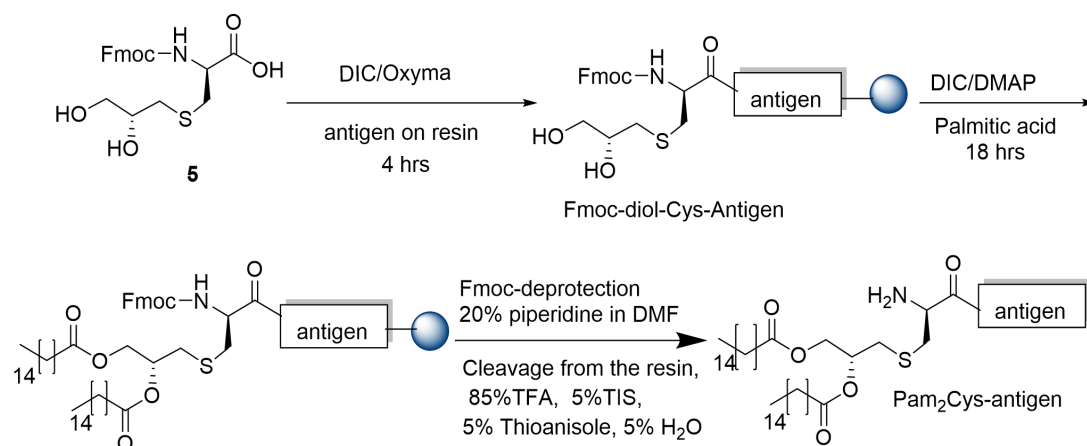


Figure 37: Synthetic scheme of general protocol, **A** for the synthesis of Pam₂Cys-antigen.

It was important to optimize the method first on a model peptide. For this purpose, H-HAKTKEA-NH₂, (**30**) was synthesised on-resin for general protocol (A & B) optimisation and comparison. This model peptide has been chosen from the CTA PAGE4 (residues 91-97).¹⁹³ It is a convenient peptide, easy to synthesise and has high aqueous solubility

(pI: 9.71). In this method, Fmoc-diol-Cys (**5**) was coupled to model peptide **30** on resin using standard Fmoc-SPPS conditions (DIC, 1.5 equiv. and Oxyma, 1.5 equiv.) in DMF (2 mL) at 0 °C for 5 mins with an extension in coupling to 4 hrs. This was followed by palmitoylation of the alcohol groups by PA using standard coupling conditions overnight at RT. The lipidated peptide was then cleaved from the resin after Fmoc-deprotection followed by purification via preparative HPLC to give the final product (**31**) with 9% yield for the final coupling step.

General protocol, B

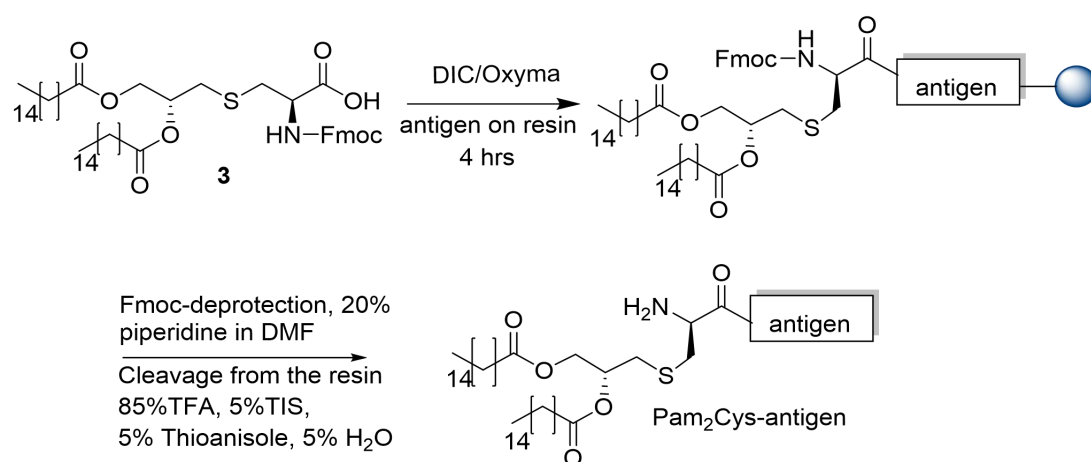


Figure 38: Synthetic scheme of general protocol, **B** for the synthesis of Pam₂Cys-antigen.

In the second method the entire pure Fmoc-Pam₂Cys-OH building block (**3**), synthesised as discussed in Chapter 2, Schemes 6 & 7, was coupled to model peptide H-HAKTKEA-NH₂, (**30**) on-resin using SPPS as in Figure 38. The lipidated peptide was then cleaved from the resin to give the final product (**31**), which was purified via prep HPLC to give 8% yield for the final coupling step. HPLC data of the pure product shown on Experimental Chapter, section 7.5, p192.

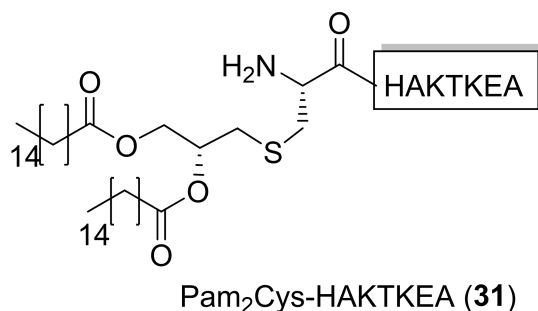


Figure 39: Structure of Pam₂Cys-HAKTKEA (**31**)

Synthesis of Pam₂Cys-BAGE4₁₈₋₃₉

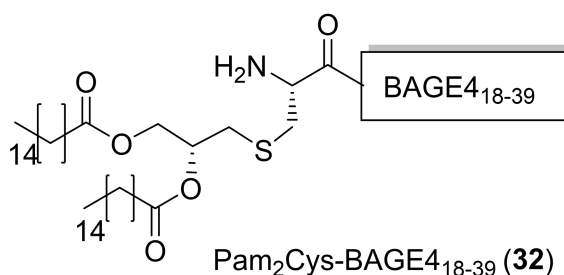


Figure 40: Structure of Pam₂Cys- BAGE4₁₈₋₃₉ (**32**).

Since the yields were comparable, it has been decided to choose first method, general protocol, **A** (Figure 37) as less purification steps involved, thus more time efficient. Therefore, it was applied to BAGE4₁₈₋₃₉ (**28**); however, BAGE4₁₈₋₃₉ (**28**) is considerably larger than the model peptide. This increases the possibility of aggregation or precipitation in conventional solvents; therefore, the synthesis may prove more challenging. The coupling of the Fmoc-diol-Cys (**5**) was successful as shown via mini-cleavage, however; palmitoylation of the alcohol groups was problematic. The analytical HPLC data for the reaction mixture showed the starting peptide- diol-Cys-BAGE4₁₈₋₃₉, as confirmed by MS, even with repetition of PA coupling Figure 41. The product with only one palmitoylated chain was also observed.

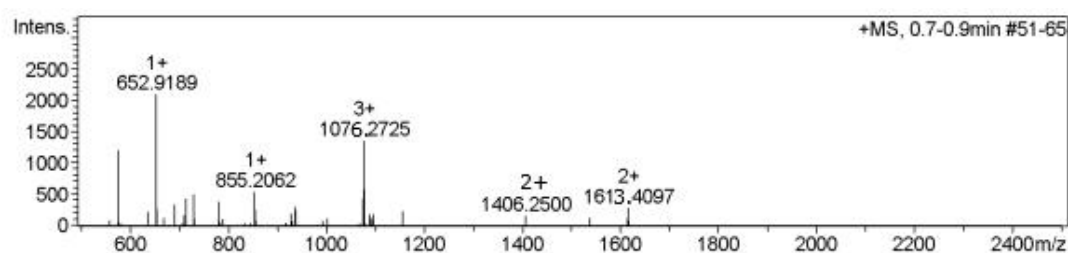
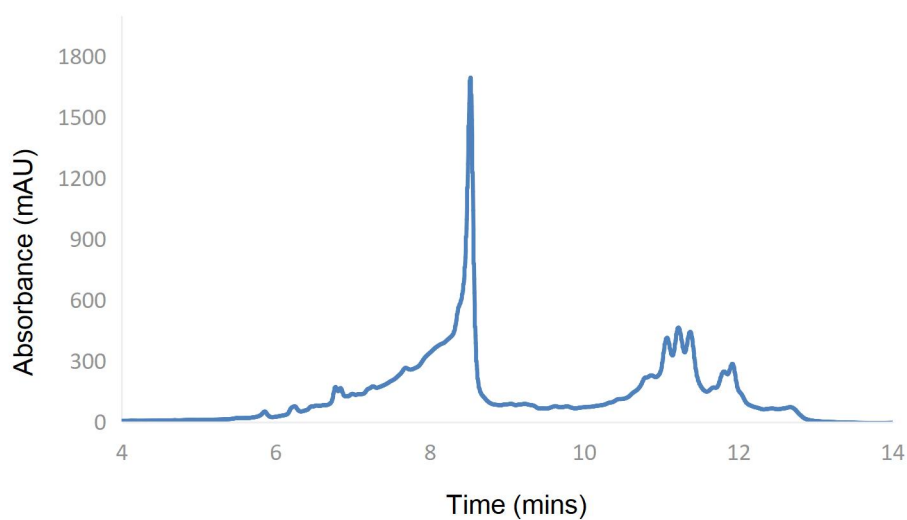


Figure 41: Analytical HPLC trace of the crude Pam₂Cys-BAGE₄₁₈₋₃₉. The HPLC trace (gradient: 2-95% solvent B, C18) shows the main eluted peptide at 8 mins (diol-Cys-BAGE₄₁₈₋₃₉) and the desired product eluted at approx. 11 mins. The crude mass spectra of the reaction mixture shows the Pam₂Cys-BAGE₄₁₈₋₃₉ (**32**), Calc. m/z for C₁₅₄H₂₄₉N₂₉O₄₁S₂ [M+2H]²⁺:1613.39, [M+3H]³⁺:1075.92. Obs. m/z [M+2H]²⁺:1613.41, [M+3H]³⁺:1076.27 and the starting peptide (diol-Cys-BAGE₄₁₈₋₃₉ as [M+Na+CO₂]²⁺:1406.25.

The synthesis was completed regardless of the presence of the starting material and mono-acetylated by-product; the material was cleaved from the resin and purified. The purification was challenging due to the high hydrophobicity of the product and by-products, affecting the solubility. Prep HPLC, C18 column used first, and the crude sample was dissolved in 100% DMSO, however; no peaks were observed at 280 nm. Replacing the C18 column with a C4 column was necessary to improve separation

of such hydrophobic molecules. Using this column, the pure product eluted as a broad peak, this makes the collection and separation of the pure product difficult as >10 fractions of 1 mL vol. were collected in each run. The Pam₂Cys-BAGE₄₁₈₋₃₉(**32**), was purified with a very low overall yield of 1 %, 1.24 μ mol (using BAGE₄₁₈₋₃₉ batch of initial loading of 0.116 mmol) and the HPLC data of the purified material indicated some impurities as shown in Figure 42. The correct molecular weight was confirmed by ESI-MS (section 7.5, p 193).

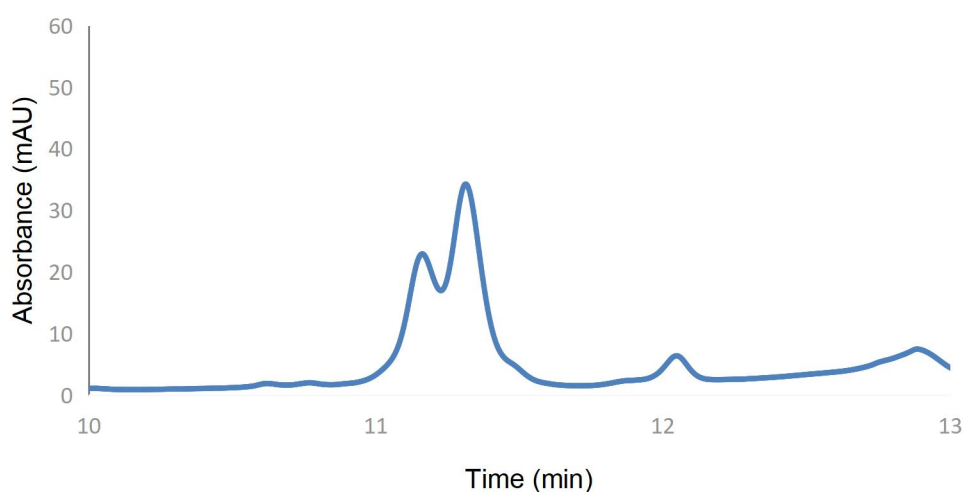


Figure 42: Analytical HPLC trace of the pure Pam₂Cys-BAGE₄₁₈₋₃₉ (**32**). The HPLC trace using (gradient: 2-95% solvent B, C4) shows one main peak with a shoulder, peak eluted at 11 mins present the desired product.

The HPLC shows 2 peaks eluting at similar retention times, both giving the desired mass with only one proton difference when the peaks were collected separately and analyzed. There is possibility of amino acid contamination, i.e., Glutamine (Gln) in place of Glutamic acid (Glu), or Asparagine (Asn) in place of Asp, that's causing a mass difference of 1, this would also explain the different retention times. Therefore, due to low yield further optimizations were carried out. A new batch of BAGE₄ was synthesised, 61% loaded yield (0.1525 mmol, 0.4 mmol/g), using fresh reagents (i.e., fresh Glu) to exclude the possibility of contamination. To the BAGE₄ sequence on resin was coupled Fmoc-diol-Cys (**5**). For the esterification step, several variables were explored to push the reaction to completion. Starting with increasing the equiv. of DIC from 20 to 25.

In another attempt, PA was replaced with palmitoyl chloride and DIPEA. In both repeats, the desired product could not be identified by MS or HPLC. Surprisingly, the starting peptide Fmoc-diol-Cys-BAGE4₁₈₋₃₉, was also not identified. This could indicate successful full conversion to the product which does not elute from the column under the gradient applied. Poor ionization of the lipid-peptide conjugate could account for the absence of the desired mass. Increasing the concentration of the MS sample caused precipitation of the material. Therefore, attention was directed to analysis by analytical HPLC. However, no absorbance was observed, even with alternative methods over an hour, an increase in the concentration of the injected material, and the use of a C4 column. It was decided to revert back to the use of PA, DIC (25 equiv.) with the application of heat (50 °C) during the palmitoylation step to ascertain if the heating improves the coupling efficiency. The result remained the same, no absorbance was detected at 280 nm by UV. Moving to an alternative analytical technique, MALDI MS was explored to analyse the crude material. The resulting MALDI spectra of the crude sample shows the desired molecular weight of the product, Figure 43.

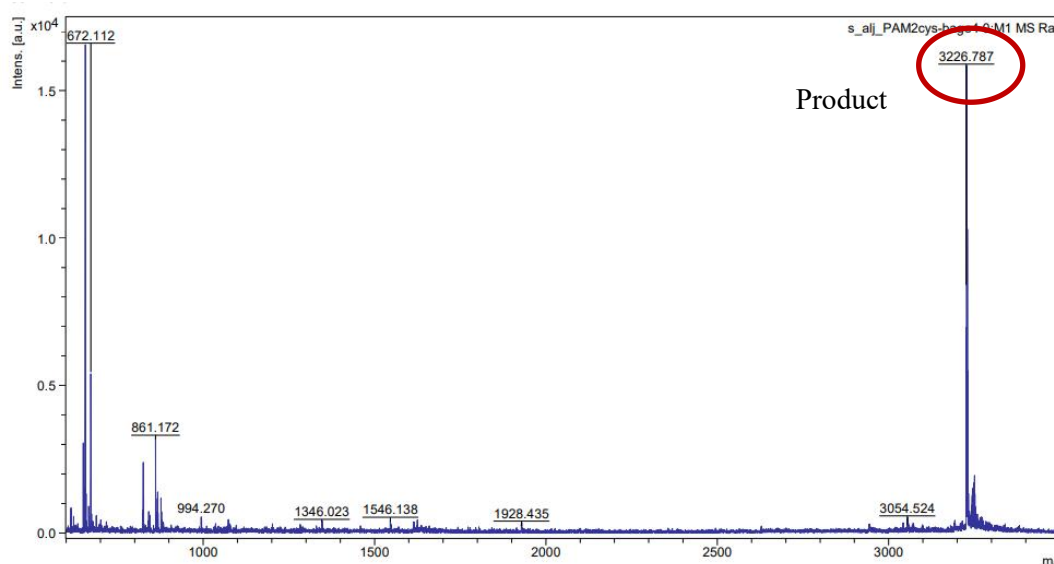


Figure 43: MALDI spectra shows the desired mass of crude Pam₂Cys-BAGE4₁₈₋₃₉ (**32**). Calculated Mass $[M+H]^+1$: 3226.77. Observed: $[M+H]^+1$: 3226.78.

This indicated that the reaction was successful, but the product clearly does not 'fly' well in ESI-MS. However, it was still important to elucidate the cause for the poor yield before repeating the reaction. Following

extensive investigation, including employing the ninhydrin test to confirm the completion of the coupling of the Fmoc-diol-Cys to BAGE4 on resin and mini-cleavage to confirm the full consumption of the diol-Cys-BAGE4 peptide, it was determined that the peptide itself was the issue. The purity of the cleaved crude peptide material of Pam₂Cys-BAGE4₁₈₋₃₉ (**32**) for previous batches was noticeably low which alerted us to the importance of knowing the yield after the final amino acid coupling (this can be determined by the calculation the absorbance of the deprotected Fmoc of the last amino acid). This gives us an indication of success of the peptide synthesis and allows us to save precious material. Thus, an accurate amount of peptide on resin can be calculated and couple to the Fmoc-diol-Cys (**5**) / Fmoc-Pam₂Cys (**3**) using general protocol A or B respectively. A fresh batch of BAGE4 using a new bottle of 2-CTC resin was used with an initial amino acid loading of 67% (0.337 mmol, 0.45 mmol/g) and a final yield of only 12% (0.042 mmol, 0.056 mmol/g) which is a low yield for a peptide of this size. The BAGE4 resin was divided into three equal batches, one was taken on to the lipopeptide synthesis and two batches were combined for cleavage to produce pure BAGE4 peptide. Using 0.018 mmol of BAGE4 on-resin, general protocol A was employed for the esterification step; coupling conditions were as follow (20 equiv. PA, 25 DIC, 2 DMAP @ RT), then a global cleavage was carried out and the material purified using prep HPLC (C4 column). The desired pure product, Pam₂Cys-BAGE4₁₈₋₃₉ (**32**) was obtained in 2.17 μ mol, 12% yield, calculated for last coupling step (lipidation). Interestingly, unlike the crude mixture, pure Pam₂Cys-BAGE4₁₈₋₃₉ (**32**) identified successfully by HRMS & HPLC. This could be because that the impurities in the crude mixture mask the HRMS signal of desired product.

Based on the above results, the solid-phase synthesis linker was switched from 2-CTC to Rink Amide, yielding a peptide with a C-terminal amide, to see if any improvement in yield could be gained. The synthesis was monitored every 5 amino acids to identify where the main drop in the yield occurred. Therefore, the coupling efficiency of each of the first five amino acids was determined by quantification of the Fmoc deprotection steps, as previously discussed. Starting from the initial

loading of 72%, 0.36 mmol loading yield, the second Fmoc quantification was 71% for Asp, then 98% for tryptophan (Trp), 86% for Pro and 86% for arginine (Arg), ended with the overall yield of 51%, 0.182 mmol. This resulted in around 40% increase in the yield of the final amino acid coupling compared with BAGE4 synthesised on 2-CTC resin. This could be because Rink amide resin is more stable while the 2-CTC more labile and the peptide can be cleaved easily under mildly acidic conditions or could be cleaved using the microwave radiation. The determined loading yield showed no dramatic drop at any point, though the first five amino acids were slightly low comparing with the rest of the sequence. In addition, it has been noticed that the significant impurities shown by HPLC occur within the first five amino acids. This was indicated by the HPLC of the crude sample from mini-cleavage, Figure 44. Therefore, it was decided to synthesise the first five amino acids using the microwave-assisted peptide synthesiser instead of manual to improve the coupling efficiency. Room temperature coupling was used to couple Asp to minimise the potential for aspartimide formation. The loading yield was calculated as 62%, which is slightly higher than previous batches, but more interestingly the impurity peak almost disappear using the microwave, as shown in the HPLC of the crude batch of BAGE4₁₈₋₃₉ (**28**), Figure 45.

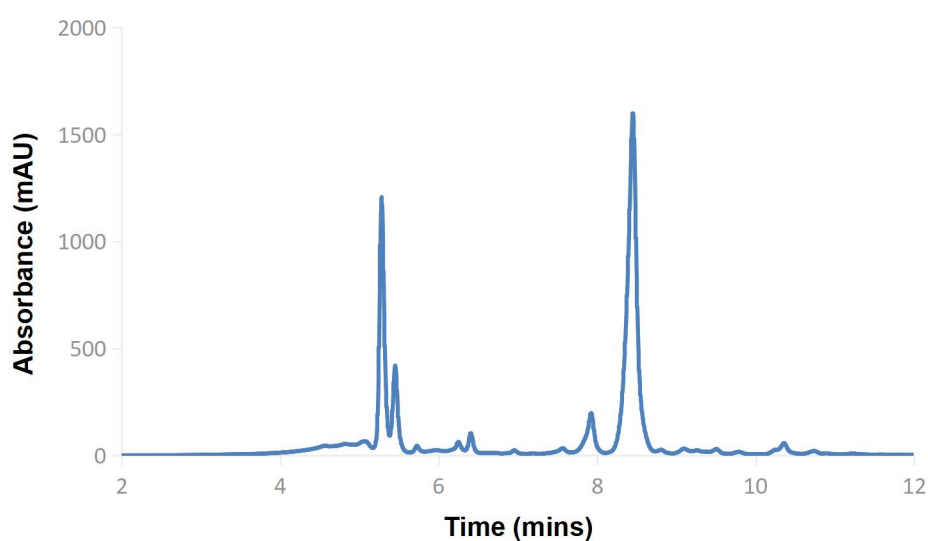


Figure 44: Analytical HPLC trace of the crude BAGE4₁₈₋₂₂ antigen in Rink amide resin. The HPLC trace (gradient: 2-95% solvent B) shows 2 main peaks, peak

eluted at 5.2 mins present as the desired product while the peak eluted at 8.2 mins shows the impurities.

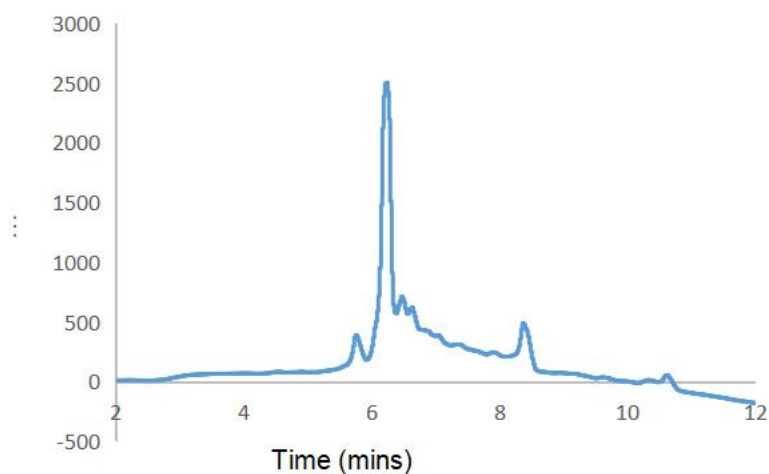


Figure 45: Analytical HPLC trace of the crude BAGE4₁₈₋₃₉ antigen using Rink amide resin and the microwave-assisted synthesiser. The HPLC trace (gradient: 2-95% solvent B) shows 1 main peak eluted at 6.2 mins presents the desired product.

BAGE4 has good water solubility with pI of 3.88, however, the attachment of the Pam₂Cys will dramatically decrease the overall solubility of the peptide. Therefore, increasing the solubility was the next priority to ensure that the vaccine can be delivered in a buffered aqueous solution. A solubility tag (SKKKK) was added to the N-terminus of the BAGE4 sequence. Then, the synthesised BAGE4-SK₄ was divided into 2 batches in preparation for the synthesis of Pam₂Cys-SK₄-BAGE4₁₈₋₃₉ (**33**) using both lipidation syntheses general protocol A & B. The synthesis of the desired product using both methods was successful, however, the purification of the general protocol, B product was less challenging. This could be due to the fact that it is a one-step reaction, which limits the potential impurities/by-products such as production of a diol-Cys-BAGE4 with only one acetyl chain, observed during the purification of Pam₂Cys-SK₄-BAGE4₁₈₋₃₉ (**33**), Figure 46, produced by general protocol, A. The desired pure product of Pam₂Cys-SK₄-BAGE4₁₈₋₃₉ (**33**) obtained in 22% yield of final lipidation step, general protocol, B, Figure 47.

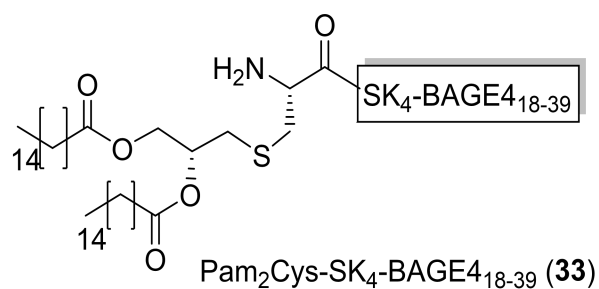


Figure 46: Structure of Pam₂Cys-SK₄-BAGE₄₁₈₋₃₉ (**33**).

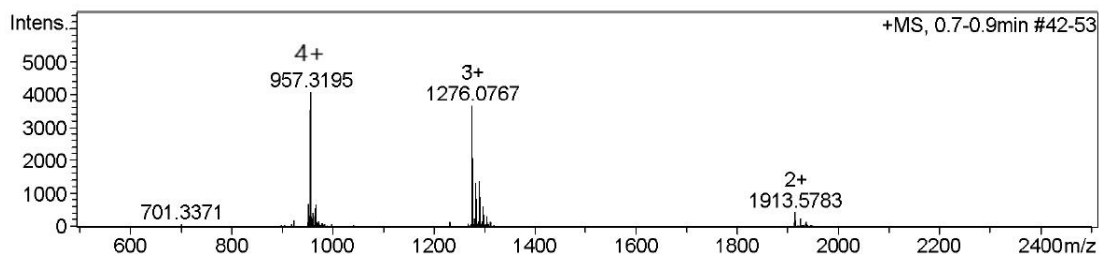
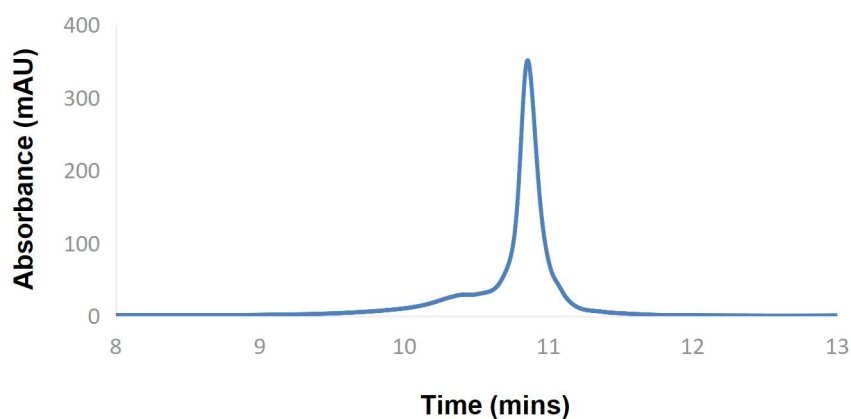


Figure 47: Analytical HPLC trace of pure Pam₂Cys-SK₄-BAGE₄₁₈₋₃₉ (**33**). Analytical gradient 2-100% B over 10 min, 280 nm. ESI⁺ MS Calc. m/z for C₁₈₁H₃₀₃N₃₉O₄₆S₂ [M+2H]²⁺:1912.88, [M+3H]³⁺:1275.74, [M+4H]⁴⁺:957.05. Obs. m/z [M+2H]²⁺:1913.57, [M+3H]³⁺:1276.07 [M+4H]⁴⁺:957.31.

Synthesis of Pam₂Cys-NY-ESO-1₁₅₇₋₁₆₅

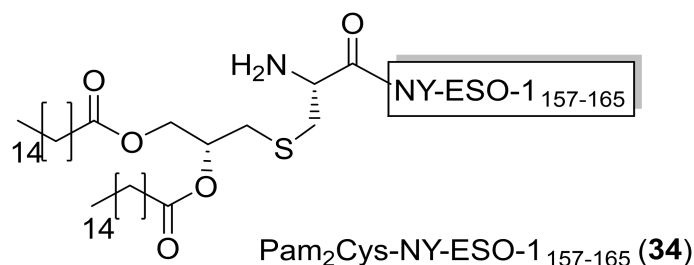


Figure 48: Structure of Pam₂Cys- NY-ESO-1₁₅₇₋₁₆₅ (**34**).

Since general protocol, B enables simpler purification due to the one-step lipidation, this method was chosen as the preferred synthetic strategy for the remaining vaccine candidates. Therefore, general protocol, B was applied to synthesise Pam₂Cys-NY-ESO-1₁₅₇₋₁₆₅, Figure 48, however; the initial analysis of the crude material by MS showed the presence of the starting peptide and desired product while only the starting peptide (**29**) could be detected by HPLC. Peptide (**29**) was not consumed despite the repetition of coupling, changing coupling reagents including HATU & DIPEA, Oxyma & DIC, DIC & DMAP, and DIC only, or application of heat (via the microwave reactor unit). Global cleavage was carried out on the resin-bound material, and we proceeded to the purification step.

This peptide sequence is poorly water soluble and attachment of the Pam₂Cys lipid has exacerbated the problem. This was expected, however, deviation from the native sequence of the peptide was undesirable to ensure direct comparison with the reported immune response to NY-ESO-1₁₅₇₋₁₆₅ (**29**). However, such poor solubility was a significant challenge and caused several issues. The purification of this vaccine was very challenging; the material was insoluble even in 60% DMSO in H₂O. The appearance of the material was like a gel, Figure 49.



Figure 49: Show the physical properties of Pam₂Cys-NY-ESO-1₁₅₇₋₁₆₅ (**34**).

Therefore, dilution was necessary; a small amount of the crude (approximately 2 mgs) was dissolved in 6 mL of 60% DMSO then injected using a semi-prep C4 HPLC column. However, only material in the injection peak absorbed in the UV. It could be that the solution was now too dilute, and the nature of the lipid caused the product to elute over a very broad retention time making it hard to identify and isolate. For the next purification attempt, the sample was concentrated slightly (2 mgs were dissolved in 4 mL of 60% DMSO) and small fractions (approx. 1 mL) were collected across the entire gradient. All of the samples were analysed by MS, only 2 of them contained the desired product which could not be seen clearly in the analytical HPLC. Therefore, it has decided to move on from this vaccine construct and resynthesise the product with inclusion of a solubility tag (see page 104).

Synthesis of Pam₂Cys-PADRE-NY-ESO-1₁₅₇₋₁₆₅

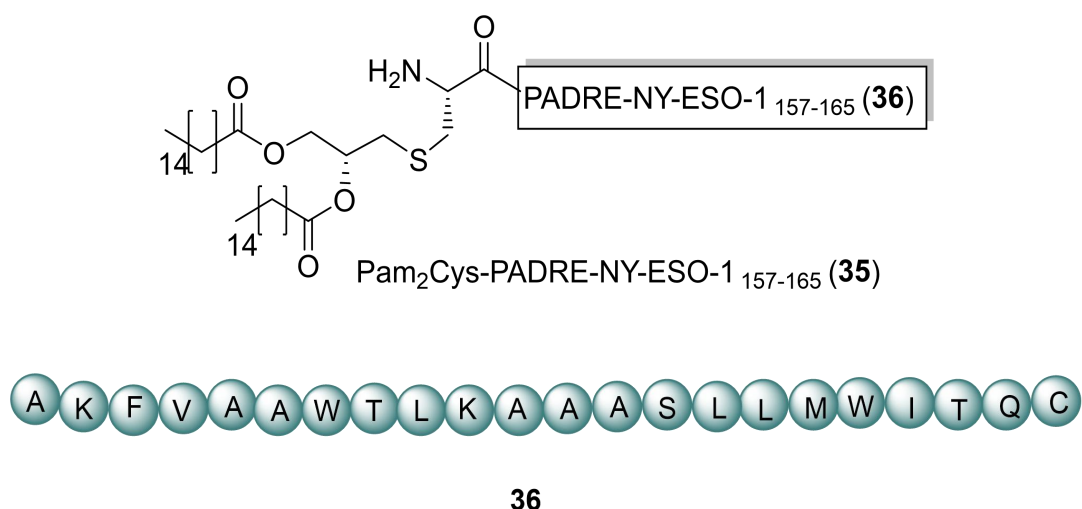


Figure 50: Shows structure of Pam₂Cys-PADRE-NY-ESO-1₁₅₇₋₁₆₅ (35) along with the amino acid sequences of PADRE-NY-ESO-1₁₅₇₋₁₆₅ (36).

In parallel with the synthesis of Pam₂Cys-NY-ESO-1₁₅₇₋₁₆₅ (34), Pam₂Cys-PADRE-NY-ESO-1₁₅₇₋₁₆₅ (35) was prepared, structure as Figure 50. To further enhance the immune response of the antigen, a T-helper epitope can be inserted into the Pam₂Cys-NY-ESO-1₁₅₇₋₁₆₅ (34) construct, Figure 48. The PADRE sequence (AKFVAAWTLKAAA) would be a suitable choice because it is a universally applied/investigated sequence which has been shown to trigger CTL responses and guarantee maximal MHC-II allele coverage. In addition to ensuring optimum efficiency and potency of subunit vaccinations, the PADRE sequence binds to a wide range of DR alleles.²³³ It also produces excellent CTL responses compared with vaccines without PADRE.²³⁴ Therefore, the PADRE sequence has been included at the N-terminus of the NY-ESO-1₁₅₇₋₁₆₅ (29) sequence. Once the peptide was prepared on resin, this longer sequence was palmitoylated using general protocol, B. The initial analysis showed only the starting peptide by analytical HPLC, but the mass of desired product showed in the HRMS as well as the starting peptide. Repeat coupling and analytical HPLC showed small peak at the end of the run @ 100% of solvent B that represented the desired product, Figure 51. Using PyBop and HOBT in present of DIPEA as a coupling reagent instead of DIC/Oxyma lead to slightly better consumption of starting material based

on reaction mixture initial analysis by analytical HPLC, Figure 52.

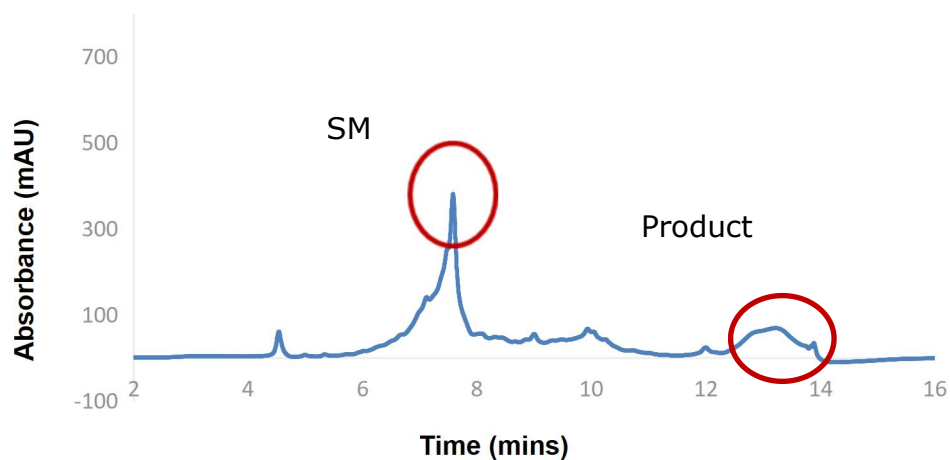
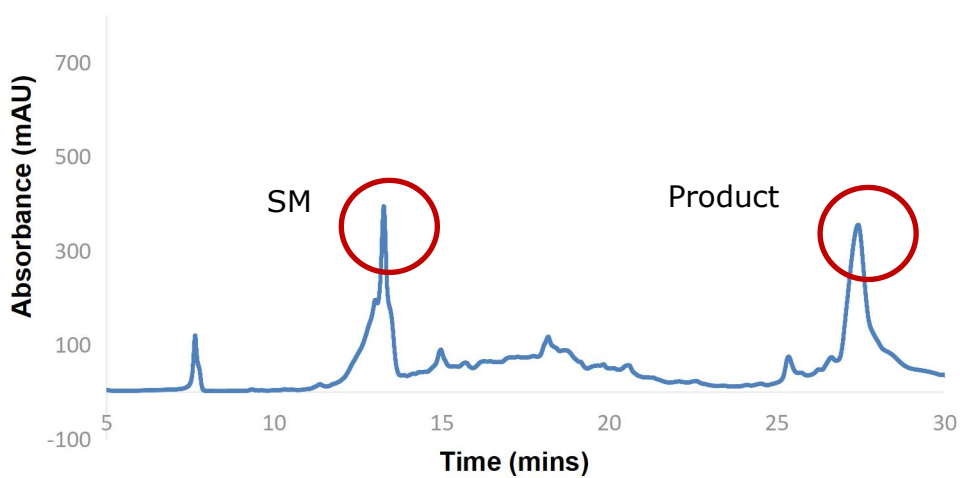


Figure 51: Analytical HPLC trace of the Pam₂Cys-PADRE-NY-ESO-1₁₅₇₋₁₆₅ (**35**). The HPLC trace using (gradient: 10-100% solvent B over 10 mins, 2 mins @ 100% B).



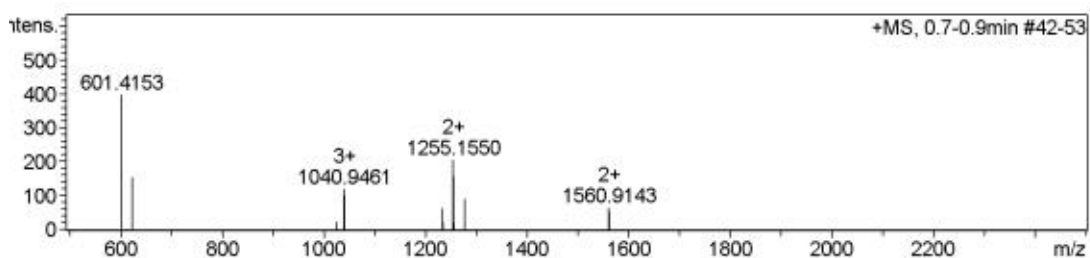


Figure 52: Analytical HPLC trace of the crude Pam₂Cys-PADRE-NY-ESO-1₁₅₇₋₁₆₅ (**35**). The HPLC trace using (gradient: 5-100% solvent B over 25 mins, 2 mins @ 100% B). The crude MS spectra for Pam₂Cys-PADRE-NY-ESO-1₁₅₇₋₁₆₅ (**35**) shows the mass of the starting peptide PADRE-NY-ESO-1₁₅₇₋₁₆₅ (**36**), ESI⁺ MS m/z Calc. for C₁₁₄H₁₈₀N₂₈O₂₆S₂ [M+2H]²⁺:1212.15. Obs. m/z [M+CO₂]²⁺: 1255.16. Also, it shows the mass of desired product (**35**). ESI⁺ MS Calc. for C₁₅₂H₂₅₁N₂₉O₃₁S₃ [M+Na]²⁺:1561.00, [M+3H]³⁺:1026.59. Obs. m/z [M+Na]²⁺:1560.91, [M+3H+CO₂]³⁺:1040.95.

The purification of Pam₂Cys-PADRE-NY-ESO-1₁₅₇₋₁₆₅ (**35**) was challenging. Similar to Pam₂Cys-NY-ESO-1₁₅₇₋₁₆₅ (**34**), the solubility and lack of UV absorbance were the main issues, despite the presence of two tryptophan residues in the sequence. Dissolving the crude in 1:1 DMSO: H₂O and running several methods starting with 50-100% solvent B over 30 mins did not result in a clear HPLC trace (no absorbance peaks were identified). Then a 60-100% over a 30 mins method was run and this time any peak with an absorbance above 0.1 @280 nm was collected. Of these, only 2 samples contained the desired product with some impurities as shown in the Experimental chapter, section 7.5, p 196. When this synthesis and purification was repeated, the max absorbance was 0.05 @280 nm which is extremely low, however, the material was collected regardless. Several fractions contained the desired product but with some impurities. Thus, the total yield of semi pure material was very low with only around 0.5% for the Fmoc-Pam₂Cys-OH (**3**) coupling step. At this scale, that represented isolation of 0.4 mg of material which was insufficient for in vivo testing. Such a low yield could be the result of both the incomplete reaction and the difficulties in purification. Consequently, it is clear that a solubility tag is required for all lipidated vaccine constructs.

Synthesis of Pam₂Cys-SK₄-NY-ESO-1₁₅₇₋₁₆₅

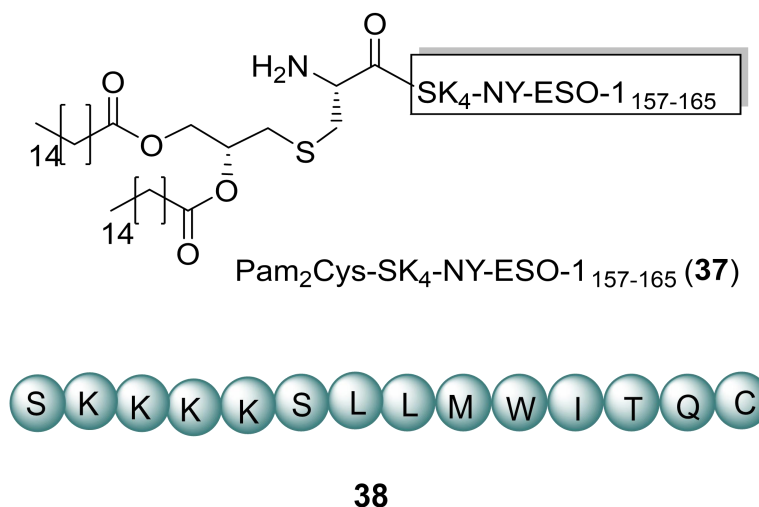


Figure 53: Structure of Pam₂Cys-SK₄-NY-ESO-1₁₅₇₋₁₆₅ (**37**) along with its amino acid sequences of SK₄-NY-ESO-1₁₅₇₋₁₆₅ (**38**).

The difficulties encountered regarding the synthesis of Pam₂Cys-NY-ESO-1₁₅₇₋₁₆₅ (**34**) indicated the necessary inclusion of the solubility tag SK₄. This cationic region should assist the purification by enhancing solubility of the construct in aqueous solution. Improved solubility in buffer will also ensure that solutions of the construct can be prepared for injection into in vivo models, making biological research more manageable. The tag has previously been explored for vaccine synthesis^{83,235,236} and should not have any direct impact regarding a targeted immune response. Once the peptide was synthesised on resin, general protocol B was employed using DIC/Oxyma as coupling reagent to attach the Fmoc-Pam₂Cys (**3**) to Ser at the N-terminus of NY-ESO-1₁₅₇₋₁₆₅-SK₄ (**38**), Figure 53, These conditions did not produce the desired product. Double coupling or an increase in the equiv. of the reagents did not help. However, using PyBop and HOBt in the presence of DIPEA lead to the desired product.¹⁹⁵ The purification showed improved peak resolution and the desired pure product of Pam₂Cys-SK₄-NY-ESO-1₁₅₇₋₁₆₅ (**37**), Figure 54, was obtained in 26 % yield of final lipidation step.

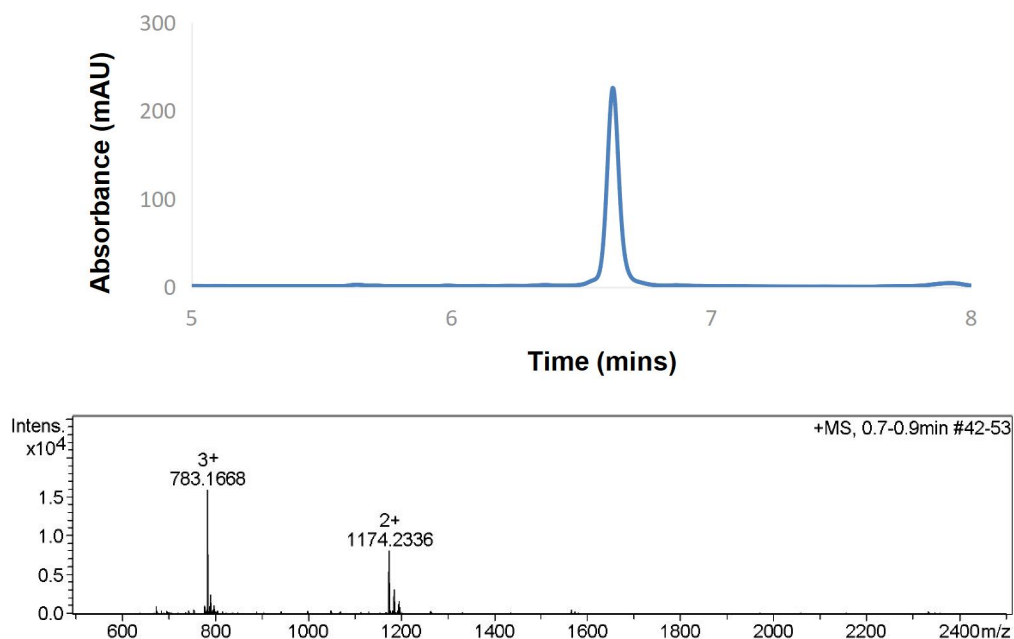


Figure 54: Analytical HPLC trace of pure Pam₂Cys-SK₄-NY-ESO-1₁₅₇₋₁₆₅ (**34**). Analytical gradient 10-100% B over 5 min, 280 nm. ESI⁺ MS Calc. m/z for C₁₁₄H₂₀₄N₂₂O₂₃S₃ [M+2H]²⁺:1173.74, [M+3H]³⁺:782.83. Obs. m/z [M+2H]²⁺:1174.23, [M+3H]³⁺:783.16.

Synthesis the entire candidate on resin using SPPS (Route C)

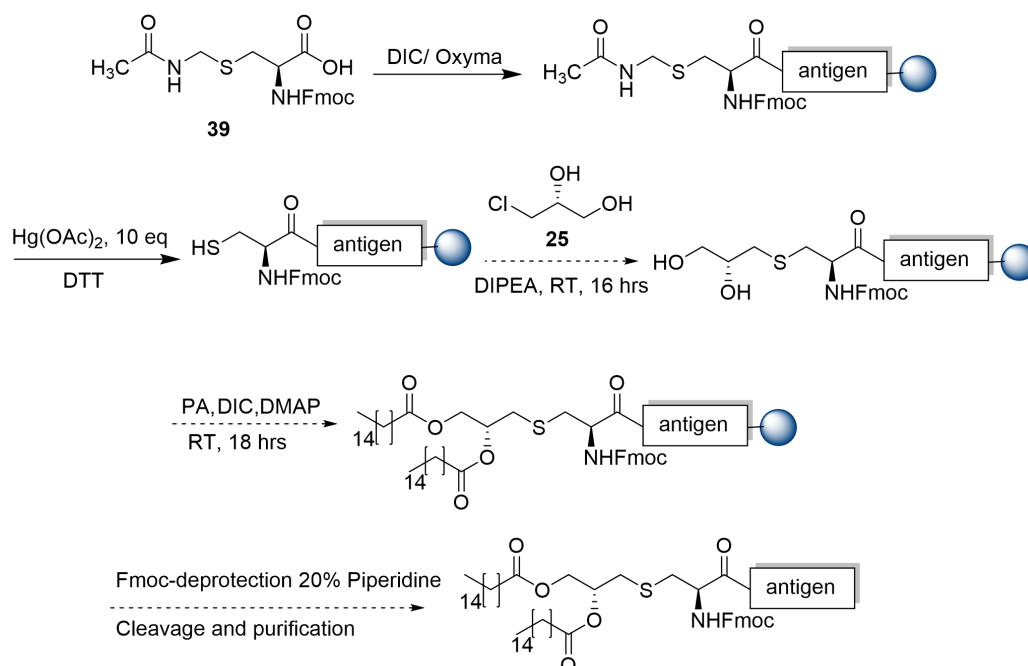


Figure 55: Virtual synthetic scheme for the synthesis of Pam₂Cys-peptide antigen.

Due to the difficulties faced during the synthesis of Pam₂Cys-BAGE₄¹⁸⁻³⁹ (**32**), we also explored a robust method for directly installing palmitoylated Cys into peptides via conjugation of compound *R*-3-chloro-1,2-propandiol (**25**). This strategy aims to synthesise the whole candidate on the resin starting with coupling Fmoc-Cys (Acm)-OH (**39**) to the model peptide. The thiol would then be selectively deprotected on-resin and *R*-3-chloro-1,2-propandiol (**25**) conjugated to the free thiol, followed by palmitoylation of the diol groups by PA and cleavage from the resin to afford the final vaccine construct as shown in Figure 55. Fmoc-Cys(Acm)-OH (**39**) was chosen as the conditions for removal of the Acm (Acetamidomethyl) PG are compatible with the 2-CTC and Rink Amide resins. First the protected Cys was coupled to the model peptide H-HAKTKEA-OH (**30**) on-resin which was confirmed by mini-cleavage. Then the removal of the Acm PG was attempted first by 10 equiv. of I₂ then by AgOAc with no success. Following this, the resin was treated with 10 equiv. of mercury acetate for 1 hr, filtered, washed with DCM/DMF, and then treated with 100 equiv. of DTT, to ensure reduction of thiol. The resin was further washed and analysed by mini cleavage. The MS confirmed the successful removal of the Acm group. The next step was to couple the *R*-3-chloro-1,2-propandiol (**25**) to the free thiol, however, a control reaction was first attempted with iodoacetamide (10 equiv., for 1 hr). The thiol was alkylated successfully, and desired product of alkylated Cys-model peptide obtained as confirmed by MS of mini-cleavage sample. Therefore, the *R*-3-chloro-1,2-propandiol (**25**) was employed to alkylate the peptide, starting with 5 equiv. at RT for 72 hrs, however; the coupling failed. Then 3 equiv. of DIPEA used with time limited to 16 hrs to avoid Fmoc-deprotection but that did not assist the conjugation. Therefore, the resin was treated first with DTT (10 equiv.) in DMF for 10 minutes, the reagent was plunged from the resin, and the resin washed with DMF to ensure reduction of the thiol. The conjugation was then repeated. Increasing the equiv. of *R*-3-chloro-1,2-propandiol (**25**) to 15 and then 25 equiv. with 5 equiv. of DIPEA and applying microwave irradiation for 30 mins @ 50 °C, did not lead to any progress.

Due to time constraints, the optimisation of this method was halted at this stage. Since the previous general protocol, A & B successfully yielded the desired vaccine constructs we were able to continue with the evaluation of the products.

3.5 Conclusion

To conclude, this chapter discussed routes towards several palmitoylated antigens. The first method involved the coupling of Fmoc-diol-Cys (**5**) to peptide on-resin, followed by palmitoylation by PA. The second route involved coupling of the entire Fmoc-Pam₂Cys building block (**3**) to the peptide on resin using SPPS conditions. Although both strategies were successful, the second route enabled clearer analysis and more efficient purification. There were critical issues in the analysis and purification steps, mainly involving the vaccines constructs without a solubility tag. Applying general protocol B the following vaccine constructs were successfully synthesised: Pam₂Cys-BAGE₄₁₈₋₃₉ (**32**), Pam₂Cys-SK₄-BAGE₄₁₈₋₃₉ (**33**), Pam₂Cys-SK₄-NY-ESO-1₁₅₇₋₁₆₅ (**37**), Pam₂Cys-PADRE-NY-ESO-1₁₅₇₋₁₆₅ (**35**).

Chapter 4: Formulation of liposome bearing CTA peptides.

4.1 Introduction

Liposomes (also known as lipid nanoparticles – LNPs) are small, synthetic vesicles that consist of one or more lipid bilayers composed of cholesterol and natural phospholipids, surrounding an aqueous solution core. The polar head groups of the lipids are directed to the interior and exterior aqueous phases. They can be used as a vehicle for aqueous or hydrophobic drugs and nucleic acids by encapsulating the water-soluble compounds within the aqueous internal hydrophilic space, while the lipophilic compounds such are integrated into the lipid bilayer. Liposomes can be also used as a vehicle for antigen display which can be integrated by covalent conjugation²³⁷ (discussed in Chapter 1). Liposomes can also provide adjuvant activity either by enhancing antigen delivery or by activating innate immune responses. The mechanism and level of severity of the immune response that may be induced by liposomes is mediated by several factors such as lipid composition, particle size, surface charge, nature and location of antigens and adjuvants within the liposome. High quality liposomes should enhance both humoral and cellular immunity to antigens²³⁸ as it can extend the half-life of antigens in circulation which makes the antigen exposure to APCs longer post-immunisation.²³⁹

The types and ratios of phospholipids (i.e., lipid tail and head-group charge) should be considered in liposome formulation as these affect the size of liposome which then determines the final liposomal structure. In general, smaller diameter liposomes with high positive charge are more effective; liposomes applied for medical use are usually 50-450 nm.¹¹⁵ Cationic liposomes are the most well-studied type of liposome, due to their enhanced interactions with the negatively charged phospholipid bilayer of cells; however, anionic liposomes can increase drug permeation, such as through the skin. Negative charge improves the differentiation between head groups and decrease the electrostatic

interactions that stabilise the formulation.²⁴⁰ As mentioned previously, cholesterol is one of the main components of liposomes and is used as a stabiliser, however, the ratio must be considered in the formulation. It has been shown that approximately 30-40% cholesterol content improves the integrity of vesicles by promoting phospholipid alkyl chain alignment, allowing for compact packing of the lipid bilayer.^{139,241,242} However, higher cholesterol levels have been correlated with reduced immune responses, as reported in the study by Kaur et al., in which reduced IgG was detected.²⁴³ Therefore, it is important to optimise the liposomal formulation carefully, considering all of the aspects mentioned above (size, charge, composition ratios, stability) to enable the preparation of high-quality liposomes. Using liposomes as a vaccine delivery system could, therefore, provide great potential for an enhanced immune response and reproducible antigen display.

The aim of this aspect of the project is to conjugate CTA to the external surface of liposomes, allowing the particles to be used as a delivery tool, an antigen display scaffold, and an adjuvant. Surface-exposed antigens are accessible for, and can stimulate, B-cells for antibody production as well as T-cell responses.²⁴⁴ It has been shown that liposomes exposing several epitopes from their external layer can produce a more effective immune response compared to encapsulating liposomes^{245,246} and this could be due to the high avidity provided by this loading method. This can be achieved by formulating liposomes with lipids that carry an appropriate group for conjugation of peptide material. In this chapter we explore the synthesis and application of a Mal-lipid (**1**) to enable conjugation of terminal Cys residues, Figure 56. This can be considered as a simple system but could be less effective since the mal-lipid-CTA conjugate will contain a linker that deviates from the native bacterial Pam₂Cys group which may affect the adjuvant ability of Pam₂Cys. Therefore, another formulation with native Pam₂Cys will be formulated for biological comparison. The Pam₂Cys-SK₄-BAGE₄₁₈₋₃₉ (**33**) construct which was prepared in the previous chapter will be incorporated into cationic liposomes to formulate liposome with native Pam₂Cys adjuvant. This will be then compared to the immune response of conjugated mal-

lipid-BAGE4₁₈₋₃₉ formulation.

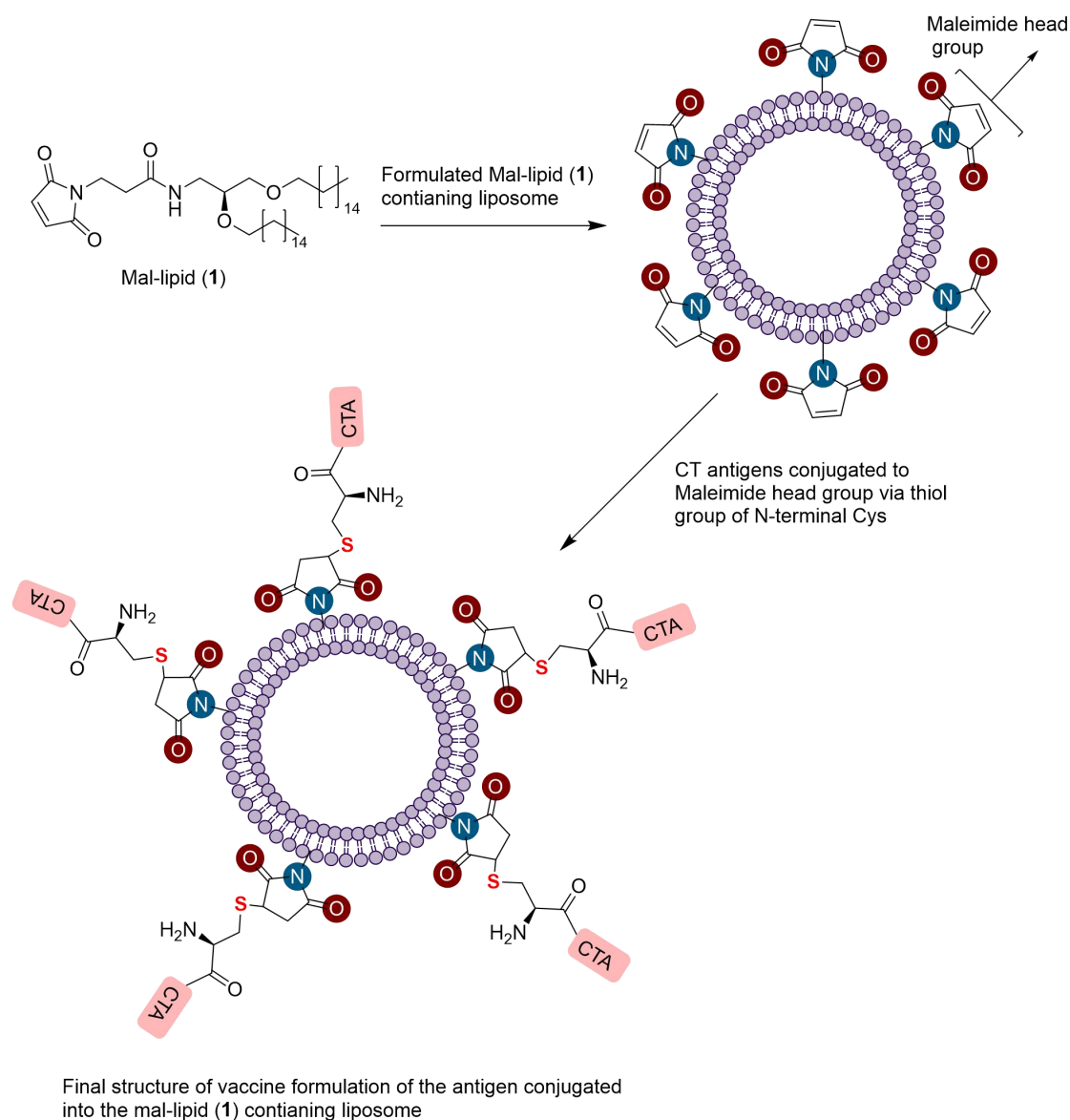


Figure 56: The pathway for formulation of a vaccine construct where the antigen is conjugated onto the maleimide linker on the liposome, mimicking the native presentation of lipoproteins.

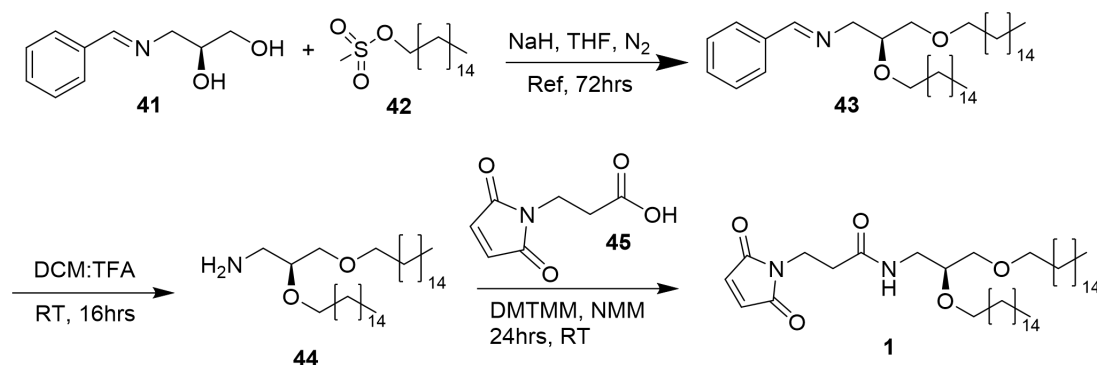
This chapter will describe the formulation of liposomes decorated with the BAGE4₁₈₋₃₉ antigen (**28**). The synthesis of a Mal-lipid (**1**) and Cys-BAGE4₁₈₋₃₉ (CRLMKKEESPVVSWWLEPEDGTAL, **40**), will be followed by optimisation of liposome formulation, and investigation/optimisation of peptide conjugation to the outer lipid envelope using model peptide H-

HAKTKEA, (**30**), as detailed in section 4.4. Furthermore, the formulation of liposomes with the Pam₂Cys-SK₄-BAGE₄₁₈₋₃₉ (**33**) (see Chapter 3 for synthetic route) will also be discussed in detail.

4.2 Synthesis of Mal-lipid (**1**)

Maleimides are considered to be the most popular thiol-reactive conjugation handles because they couple specifically and efficiently with *S*-alkyl thiols, resulting in high-yielding peptide conjugations.²⁴⁷ To exploit this technology, we designed a maleimide-containing lipid that links the maleimide group to palmitoyl chains via an amino diol head group. We opted for ether linkages, rather than ester, to enhance the stability of the lipid. While this lipid will be ideal for the conjugation and presentation of antigens on the surface of the liposome, it is unlikely to act as an effect adjuvant. However, Pam₂Cys adjuvant can be separately incorporated into the liposome formulation to make up for this disadvantage.

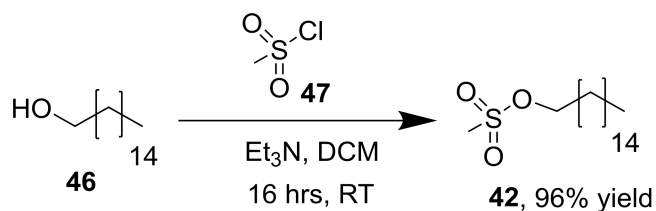
Following is the synthetic route to the Mal-lipid (**1**).²⁴⁸



Scheme 10: Mal-lipid (**1**) synthetic pathway.

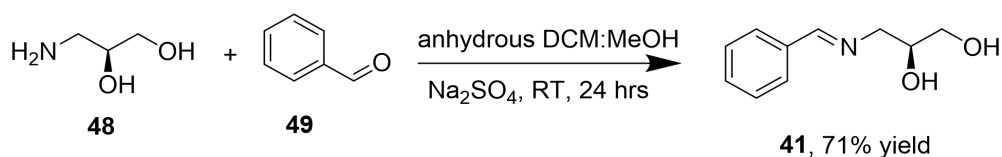
The synthesis began with alkylation of (*S*)-3-(benzylideneamino)propane-1,2-diol (**41**) with hexadecyl methanesulfonate (**42**), followed by the hydrolysis of imine **43** and coupling of the resulting amine to 3-maleimidopropionic acid (**45**) to produce the final desired product, Mal-

lipid (**1**), as shown in Scheme 10.



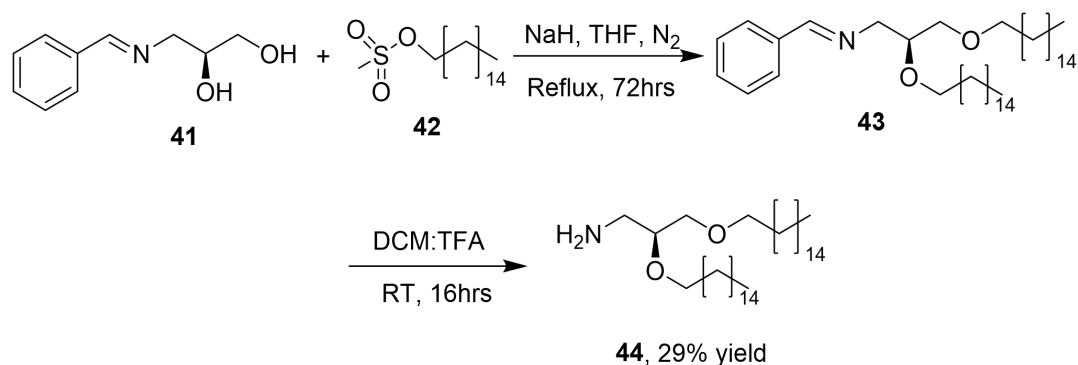
Scheme 11: Synthesis of hexadecyl methanesulfonate (**42**).

Hexadecyl methanesulfonate (**42**) is the first starting material required for lipid ether amine (**44**) synthesis; it was synthesised via the reaction of hexadecane-1-ol (**46**) in DCM in the presence of Et_3N and methanesulfonyl chloride (**47**), Scheme 11. The addition of methanesulfonyl chloride (**47**) was performed dropwise and on ice over 30 mins then allowed to warm to RT with stirring. After 16 hrs, the organic product was diluted with DCM and then extracted with a 1 M NaHCO_3 then washed with water 3x and brine 1x, then dried to give a yield of 96%. The product was dried under a very high vacuum, to ensure the complete removal of organic solvent and get an accurate crude yield to ensure the stoichiometry of the next step is accurate. The product was used in the next stage without purification. To synthesise (*S*)-3-(benzylideneamino) propane-1,2-diol (**41**), (*S*)-3-aminopropane-1,2-diol (**48**) was stirred with sodium sulfate in mixture of anhydrous $\text{DCM}:\text{MeOH}$ for 2 hrs prior to a drop-wise addition of benzaldehyde (**49**), the reaction mixture was stirred at RT for 24 hrs. Thin Layer Chromatography (TLC) was used to confirm the completion of reaction as well as HRMS. After workup, an oily residue was isolated which was dried further under high vac to be used crude, with a yield of 71%, in the next step.



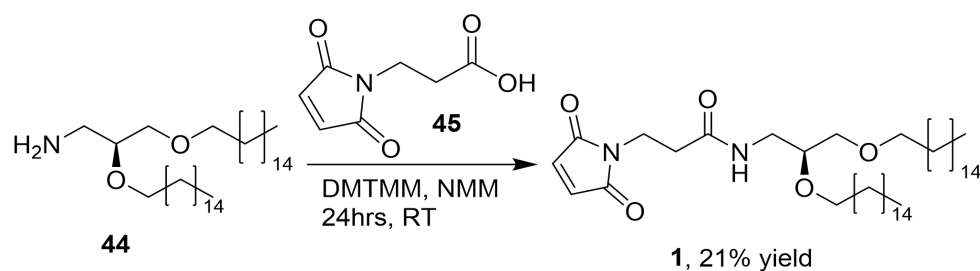
Scheme 12: Synthetic pathway of the (S)-3-(benzylideneamino) propane-1,2-diol (**41**).

In this step, the dried hexadecyl methanesulfonate (**42**) was reacted with (S)-3-(benzylideneamino) propane-1,2-diol (**41**) under anhydrous reflux conditions for 72 hrs to produced lipid ether amine (**44**), as in Scheme 13.



Scheme 13: Synthetic pathway of the lipid ether amine (**44**).

It is important to perform this reaction in an inert atmosphere under N₂ to prevent any contact of NaH with moisture. Dipalmitoylated product (**43**) was isolated and used crude in the next step, in which the imine was hydrolysed in DCM:TFA overnight. The reaction was monitored by TLC to confirm completion before the evaporation of the organic solvent, with the addition of toluene remove the TFA as an azeotrope; the resulting oil was further dried under high *vac* to produce brown oil product. This crude material was purified using flash column chromatography to obtain 29% of pure lipid ether amine (**44**). The last step in the maleimide synthetic pathway is to couple the maleimide head group to the pure lipid ether amine, Scheme 14.



Scheme 14: Synthetic pathway for Mal-lipid (**1**).

To obtain the final product, two different coupling agents were used. First, HATU was employed as the coupling agent, with DIPEA in DMF; 3-maleimidopropionic acid (**45**) was added to the reaction mixture and allowed to react for 5 mins before the lipid ether amine (**44**) was added. The reaction was completed over 16 hrs, and the product was obtained as a brown semi-solid, with impurities that remained even after multiple purification attempts (34% yield). The challenges associated with isolating this lipid at an appropriate purity level resulted in attempting to use a different coupling agent, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride (DMTMM) with N-Methylmorpholine (NMM) as a catalyst. This reaction produced a semi-white, pure product (**1**) after flash chromatography (21% yield), which was lyophilized for subsequent conjugations.

4.3 Liposome formulations and characterization

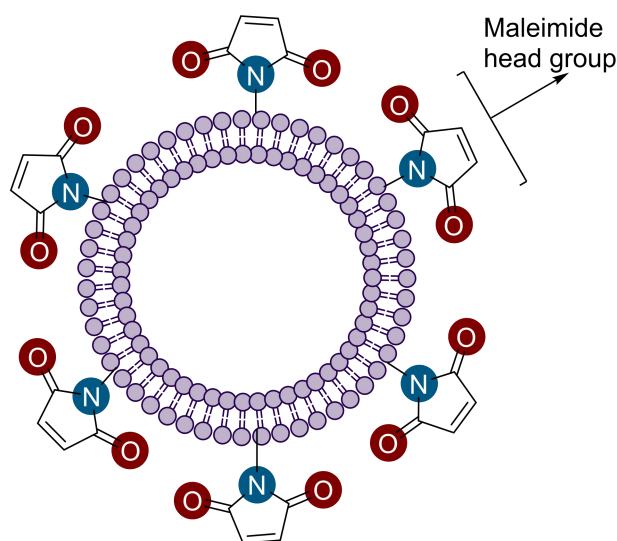


Figure 57: Structure of liposomes formulated with Mal-lipid (**1**).

As discussed, the overall aim of this chapter concerns the formulation of vaccine candidates into lipid nanoparticles. This can be achieved in several ways but in this project, the focus is on the following 2 methods. The first involves formulating the liposomes with Pam₂Cys-SK₄-BAGE₄₁₈₋₃₉ (**33**) (see Chapter 3). The second method is to prepare the liposome with the Mal-lipid (**1**) included in the formulation, see Figure 57, to enable conjugation of the antigen to the surface of the liposome. This second method allows us to display the antigen to the immune system, but this system is unlikely to work as an adjuvant. Therefore, this formulation will provide an appropriate negative control, along with delivery of the naked antigen peptide. However, if this formulation did result in an effective and targeted immune response, then it would represent a much simpler system to prepare compared to the challenging synthesis of Pam₂Cys-antigens which could also be applied to the display of protein antigens.

Initially, all liposomes were formulated using the lipid film hydration method.²⁴⁹ In this method, a 1 mM stock solution of each lipid was prepared in chloroform. Once prepared, the solutions of lipids were combined at a certain ratio (specific for each formulation), based on volume (see Experimental Chapter, section 7.6). The solvent from the combined solutions was removed under reduced pressure and the resultant film dried under high vacuum or by lyophilisation. Then PBS or distiller water (volume equal to the final volume of the organic solvent used each time) was added and the suspension formed was sonicated (using prob sonicator) on ice, Figure 58.

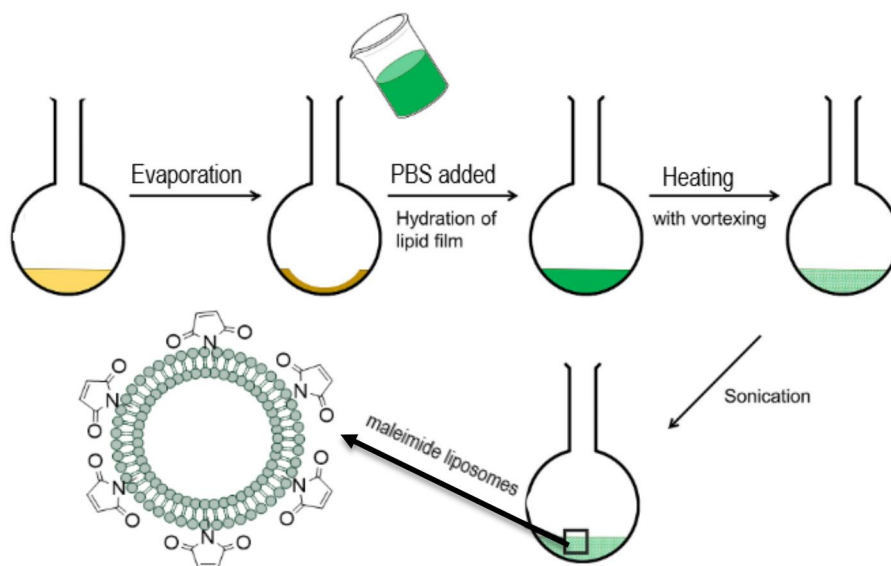
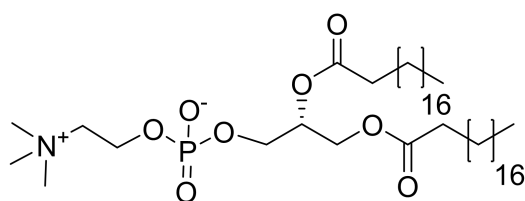


Figure 58: Liposome formulation using the lipid film hydration methods.²⁴⁹

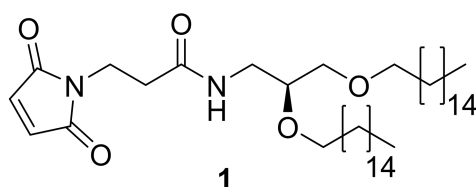
The formulation of the liposomes must be optimized to ensure reproducible particles of the desired diameter and polydispersity index (PdI). The goal is to formulate liposomes with sizes of around 100-200 nm and a size distribution less than 0.3 PI with good stability (i.e., $> \pm 10$ mV charge). Formulations were explored that would yield both positively and negatively charged particles to provide the option and to compare the formulations.

Liposomes were prepared as previously described.²⁴⁹ Starting with a formulation that would afford an anionic liposome, the vehicle was composed of the following lipids: 1,2-ditetradecanoyl-sn-glycero-3-

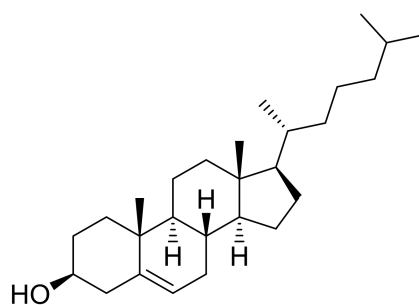
phospho-(1'-rac-glycerol) sodium salt (DMPG-Na), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), and cholesterol, Figure 59. Furthermore, our bespoke lipid carrying a peptide-conjugation linker (maleimide group) was included (Mal-lipid, **1**). The external liposomal lipid layer has been modelled on the outer phospholipid bilayer membrane of bacteria, which helps to anchor lipoproteins, facilitating the presentation of immune epitopes and enabling the host to dissociate the attached lipoproteins.²⁴⁴ The percentage of Mal-lipid (**1**) in the formulation will control the amount of antigen that can be conjugated to the outer envelope of the liposomes. The lipid structures making up the liposomal formulation are as follow:



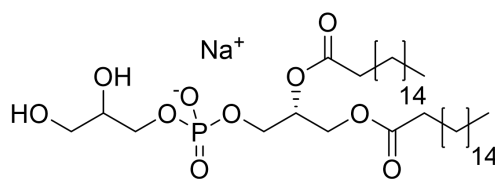
1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC)



Lipid ether maleimide (Mal-lipid)



Cholesterol



1,2-Distearoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt (DMPG-Na)

Figure 59: Lipid structures used in the liposome formulation (DSPC, Mal-lipid, cholesterol, and DMPG-Na).

The DMPG-Na, DSPC lipids and cholesterol are commercially available, and Mal-lipid (**1**) was chemically synthesized, as described. The formulation was optimised previously in the group, based on reports of stable formulations originally applied to Orally delivery; the optimised ratios are 25% of each DSPC & DMPG-Na, 10 % Mal-lipid (**1**) and 40% cholesterol, the stated % in each formulation represents mol%.²⁵⁰ It has been observed that the % cholesterol has a major effect on the shape and size of the formulated particles, due to its well-known structural role in biological systems; any deviations from the % cholesterol stated caused warping of the spherical shape of the liposomes.¹⁴⁰ The spherical structure of the nanoparticles is very important for *in vivo* applications, as antigens must be anchored around the surface of the sphere to mimic the native presentation of lipoproteins and trigger the maximum immunogenicity effects. Therefore, the formulation containing 40% cholesterol was used for further studies. For the % of Mal-lipid (**1**), since this component has not been tested *in vivo*, keeping a low % will minimize its effect on the properties of the liposome and limit any toxicity concerns; however, enough lipid should be included to effectively decorate the surface with antigen, thus formulations containing 10 mol% of Mal-lipid (**1**) were initially explored.

Two different liposomes were formulated, starting with **non-Mal-lipo -ve** for lipid ratio of 30 % DSPC, 30% DMPG-Na, and 40% of cholesterol. The second formulation is the **Mal-lipo -ve** with lipid ratio of 10% mal-lipid, 25% DSPC, 25% DMPG-Na, and 40% cholesterol. DLS analysis was

performed to ensure the quality requirements of size and population homogeneity. To achieve those, several formulations were explored, starting with non-mal first to save the synthetic mal-lipid material. The formulations described above were prepared by combining the relevant lipids in chloroform, removing the solvent under reduced pressure, and addition of PBS. The solution was sonicated using a probe sonicator (SONICS Vibra-Cell™, CPX 130) offset at 90% amplitude for 10 mins with no pulsing. The DLS results showed multiple populations with an average diameter of 200 nm and a large PdI: 0.438. To further optimize the formulation, alternative sonicators and probe sizes were trialed, but no clear effect was noted. Therefore, it was important to look at the other possible factors individually starting with gain setting on the sonicator i.e. the frequency of vibration of the probe, and amplitude percentage. Two identical batches of **non-Mal-lipo -ve** were formulated at different amplitude percentages of 30% and 90%. The 90% amplitude gives a more appropriate average size of 129.9 nm with homogeneity, PdI: 0.368 based on DLS results Figure 60, the DLS measurement for all the liposomes samples discussed in this chapter was repeated in triplicate.

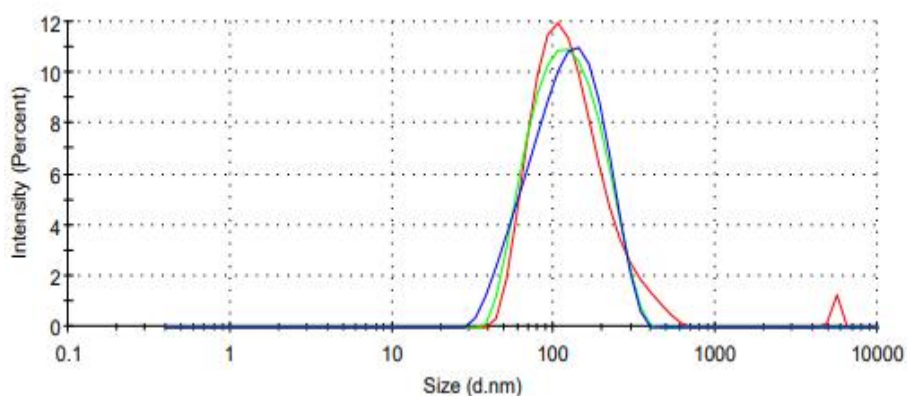


Figure 60: DLS results of non-mal-lipo -ve at 90% amplitude, different colour represents three repeats.

The exact method was then applied to the Mal-containing liposome, but this positive result was not replicated for this formulation. After a couple of repetitions, it was concluded that the thin film layer must be equally distributed around the surface of round flask rather than a thin band or small localisation. This was achieved easily with no effort in the previous

non-Mal-lipo -ve formulation. However, a thin film for the mal-lipo formulation, could be obtained by evaporating the chloroform under a flow N₂ or rotovap, starting slowly at first and then adjusted the angle and increasing the speed to maximum. If the required film was not formed, the lipids were re-dissolved in chloroform and the process repeated until the thin film was spread equally around the round bottom flask. Using this technique with the described settings (prob sonicator, 90% amplitude for 10 mins with no pulsing) to the mal-containing liposome formulation led to successful formulation with an average particle size of 114.6 d. nm, and PdI: 0.172, DLS shown in Figure 61. Summaries of both formulations with their characterizations are highlighted in Table 2.

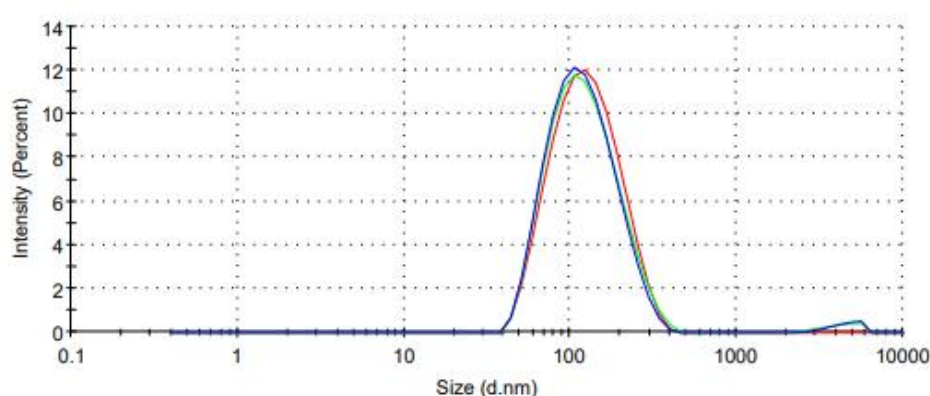


Figure 61: DLS results of **Mal-lipo -ve** at 90% amplitude. Different colour presents three repeats.

Table 2: Summaries the DLS data, size (average diameter, d. nm) and PdI @ 90% Amplitude of **Mal** & **non-Mal-lipo -ve** with their lipids composition %.

Anionic liposome formulation	Lipid composition %	Amplitude %	Z-average (d.nm)	PdI
Non-mal-lipo -ve	30 % DSPC, DMPG-Na, and 40% of cholesterol	90%	129.9 d. nm	0.368
Mal-lipo -ve	10% Mal-lipid (1), 25% DSPC, DMPG-Na, and 40% cholesterol	90%	114.6 d. nm	0.172

The stability of liposomes is determined by the physical and chemical properties of the particles/components. As discussed previously, by optimizing the size distribution, the lipid composition (including charge), as well as the addition of cholesterol, the stability of the liposomal formulations can be 'tuned'.²⁵¹ However, there are several factors affecting shelf-life stability, including temperature, pH and solvent medium. This could lead to coalescence of the formulation over the time which causes an increase on the size distribution.²⁵² Therefore, a stability study was carried out for both formulations, stored in a buffer at pH 7.4 and at around 4 °C.²⁵³ The results indicated a slight coalescence over 3 weeks which increases the average diameter by 20 nm in the **Mal-lipo -ve** and by 28 nm in **non-Mal-lipo -ve**. For the PdI, **Mal-lipo -ve** are more stable compared to **non-Mal-lipo -ve** which showed a dramatic increase of the PdI over 21 days, Tables 3 & 4.

Table 3: Summaries the DLS data, size (average diameter, d. nm) and PdI, of the **non-Mal-lipo -ve** over 3 weeks, stability study.

Time/day	Z-average (d. nm)	PdI
0	138.6	0.243
14	152.6	0.301
21	166.5	0.412

Table 4: Summaries the DLS data, size (average diameter, d. nm) and PdI, of the **Mal-lipo -ve** over 3 weeks, stability study.

Time/day	Z-average (d. nm)	PdI
0	114.6	0.172
7	115.3	0.194
21	134.2	0.217

Also, the effect of the concentration (@ 1 mM versus 2 mM) was also studied on **Mal-lipo -ve** and the DLS indicated similar size of (152.6 d. nm & 151.4 d.nm) and PdI (0.340 & 0.337) respectively for both formulations across these concentrations, Figure 62. This proved that

the liposome concentration does not play a role in the quality of the formulation up to 2 mM.

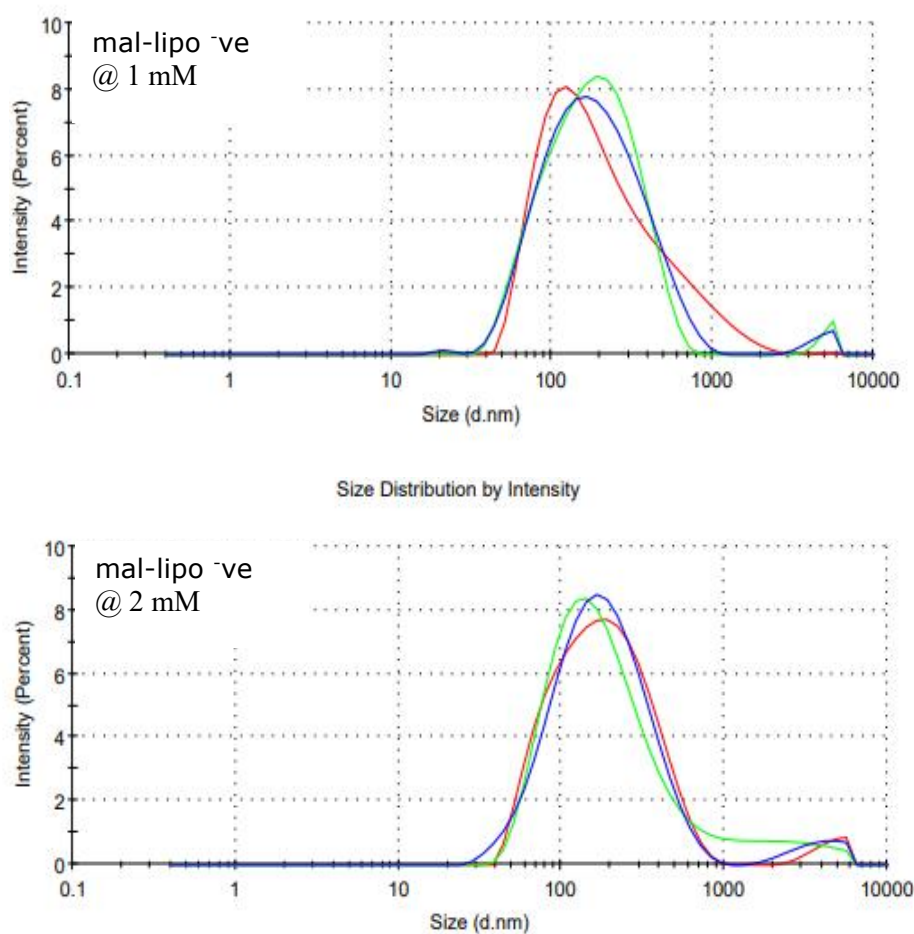
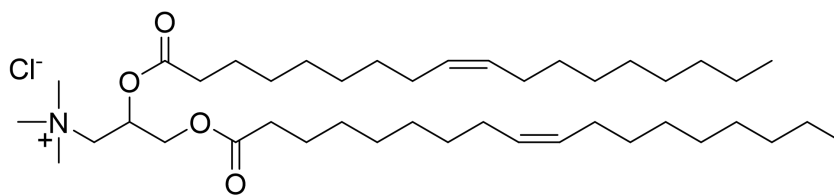


Figure 62: The DLS results showing the effect of the concentration (mM) on the **Mal-lipo -ve** formulation. The larger impurities could be lipid aggregates or particulate impurities. Different colour presents the repetition of analysis by

The cationic liposome formulation was prepared using the same method as described above, except DMPG-Na was replaced with 1,2-dioleoyl-3-trimethylammonium-propane-chloride salt (DOTAP), Figure 63.



1,2-dioleoyl-3-trimethylammonium-propane chloride salt (DOTAP)

Figure 63: Structure of cationic lipids DOTAP (racemic mixture of enantiomers).

Applying the same conditions used for the anionic liposomes with the same lipid ratio (40% cholesterol, 10% Mal-lipid (**1**), 25% DSPC, and 25% DOTAP) gave poor quality particles as stated in the DLS result quality report. This could mean that the particles were large or contained a high degree of polydispersity. Therefore, the ratio of lipids was optimized, avoiding any change to the Mal-lipid % and keeping the cholesterol % close to 40% to maintain bilayer integrity. Limiting the change in ratio to DOTAP & DSPC, starting with 5/95, 10/90, then 90/10 DOTAP: DSPC, (considering only lipid composition), the DLS results showed poor quality particles and large average diameter as per Table 5.

Table 5: Summaries the size (average diameter, d. nm) and PdI of the three different formulations of **Mal-lipo -ve** using three different lipids ratio of DOTAP: DSPC.

Ratio of DOTAP: DSPC	Z-average (d. nm)	PdI
50/50	1089	0.712
5/95	691.3	0.444
10/90	443.1	0.178
90/10	1060	0.615

The sonication time was extended to 30 mins at 90% amplitude with no pulsing but no improvement was recorded. This result was unexpected, as the anionic liposomes gave excellent results using the same settings. It had been noticed that, for the thin-film layer formed, it was a challenge to suspend the film into solution even with vortexing. The film formed a ring around the round bottomed flask, instead of spreading

across the flask in a thin even layer, Figure 64. This led to a necessary extension of the probe sonication time and also use of a bath sonicator to form the initial solution. However, both trials resulted in poor quality liposomes with a variety of populations. Finally, the application of heat was explored for 1-2 mins just prior to sonication with vortexing.²⁵⁴ The solution was heated to the transition temperature. This was determined based on the critical temperature of the main lipid used in the formulation with the higher ratio, which is DSPC, therefore, the liposome was heated up to its transition temperature of 55 °C.²⁵⁵ Also, we optimized the formulation using MilliQ water instead of PBS. Applying those recommendations to different ratio of lipids formulating **non-Mal-lipo +ve** gave high quality particles with a low PdI, summarized in Table 6.

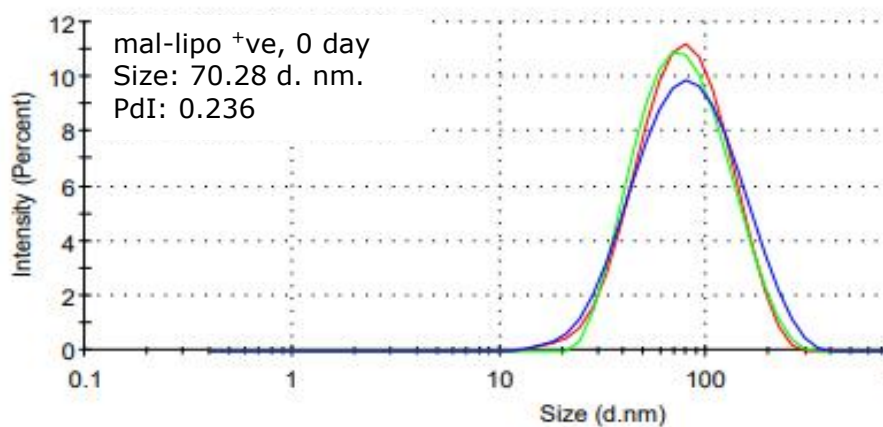


Figure 64: The ring layer formed with cationic **Mal-lipo +ve**.

Table 6: Summaries the size (average diameter, d. nm) and PdI of the two different formulations of **non-mal-lipo +ve** using two different lipids ratio of Cholesterol: DOTAP: DSPC.

Ratio of Cholesterol: DOTAP: DSPC	Z-average (d. nm)	PdI	Zeta potential mV
40/15/45	82.8	0.260	+ 34.5
25/15/60	80.06	0.239	+ 38.9

Application of the above conditions, i.e, heat and vortexing prior to sonication as well as using the same sonicator settings (90% amplitude, 10 mins, and no pulsing) to formulate **mal-lipo +** with following lipid ratio: **40% DSPC, 35% cholesterol, 15% DOTAP, and 10% Mal-lipid (1)**, resulted in high quality nanoparticles with a diameter of 70.28 d. nm; PdI: 0.236, zeta potential: + 30 mV, Figure 65. TEM imaging of the particles was carried out to confirm the DLS results, Figure 66. This **Mal-lipo +ve** recorded very high level of stability over 36 days at 4 °C, as the average diameter remained almost the same with a minor change of only 2 nm, and a 0.031 change in the PdI, see Figure 63. This formulation procedure was repeated several times to ensure reproducibility which indicates the importance of heating the formulations prior to sonication.



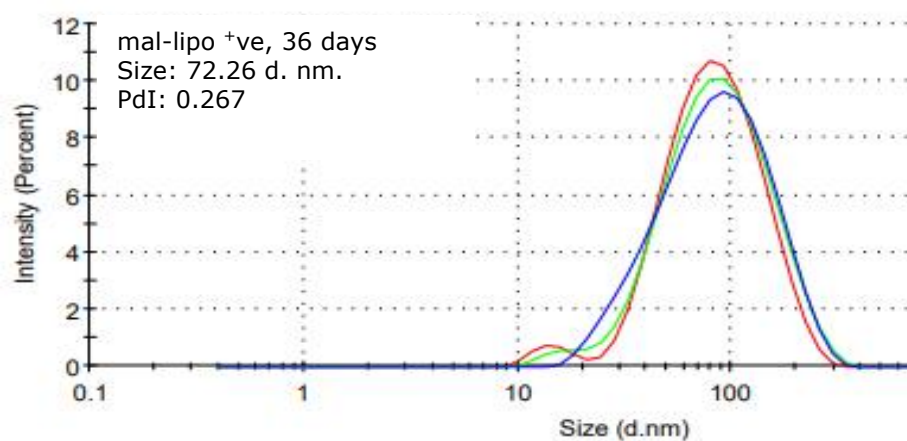


Figure 65: The DLS results of stability study for **Mal-lipo +ve** over 36 days. Different colour presents the repetition of analysis by 3.

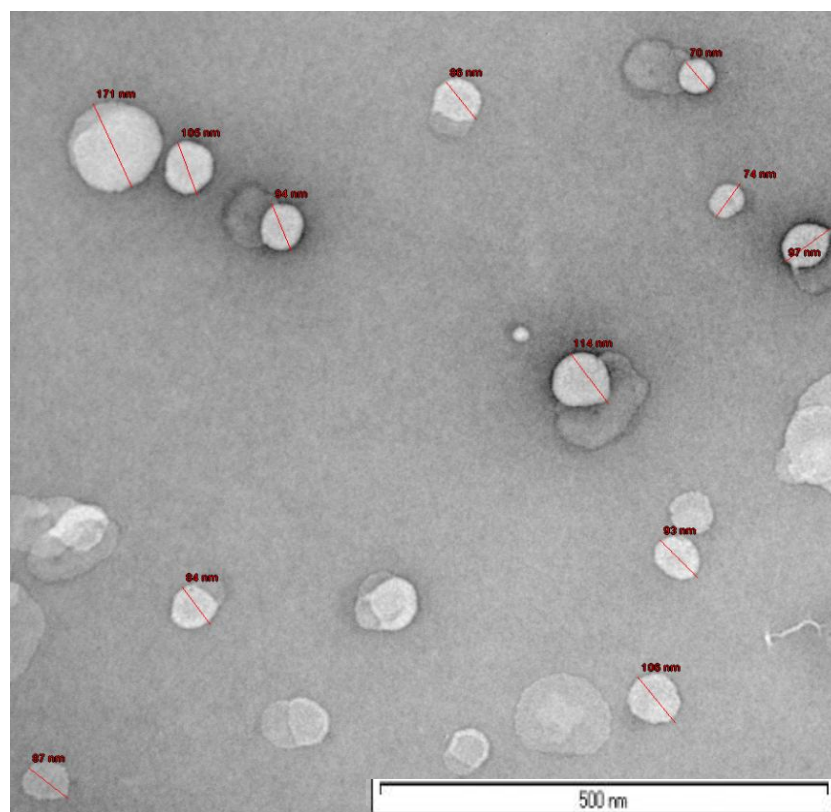
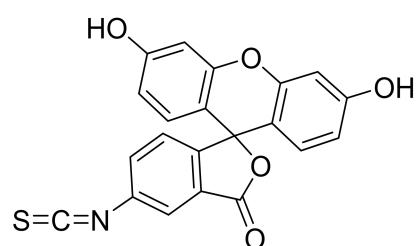


Figure 66: TEM for **Mal-lipo +ve**, the image capturing was operated at 43k.

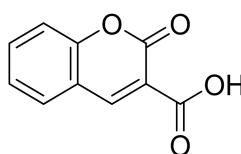
4.4 Synthesis of fluorescein model peptides (53, 54) and Extinction Coefficient calculation

To ensure the antigen could be successfully conjugated to the outer envelope of the liposomes containing the Mal-lipid (**1**), and to quantify the amount of antigen that could be 'loaded' onto the particles, we modified the previously synthesised model peptide H-HAKTKEA, (**30**) (see chapter 3) adding Cys at the N-terminus and Lys at the C terminus to prepare H-CHAKTKEAK-NH₂ (**50**) to enable attachment of a fluorescent tag, Figure 67.



51

Fluorescein isothiocyanate (FITC)



52

Coumarin carboxylic acid

Figure 67: Structure of the fluorescent tags; Fluorescein isothiocyanate (FITC), (**51**) and coumarin carboxylic acid (**52**).

A Lys carrying 4-Methyltrityl (Mtt) group protecting the sidechain amine was installed at the C-terminus of the peptide to enable orthogonal deprotection and on-resin addition of a fluorophore. The presence of an aromatic residue (His) aids in detection during HPLC analysis and purification. The sequence has a pI of 10.09 which should ensure solubility in aqueous solution. The N-terminus carried a Cys residue to enable conjugation with mal-liposome via the free thiol by Michael addition. The model peptide was synthesized successfully on Rink amide

resin; the Mtt group was cleaved selectively on-resin by a mixture of 1% TFA and 5% TIPS in DCM. FITC (**51**) was chosen as the initial fluorophore; FITC (3 eq) was dissolved in DMF and activated with DIPEA (6 eq) then added to model peptide on resin using SPPS successfully, Figure 68A; however, the purification of this peptide (**53**) was challenging, and the isolated yield was low (4%). A second peptide-fluorophore conjugate was also explored; coumarin carboxylic acid was coupled to the Lys on resin as described above, however; Oxyma (4 eq) & DIC (4 eq) were the activating agents. Since coumarin is a smaller conjugated system, the purification of this conjugate was expected to be easier. The coumarin model peptide (**54**) was synthesized in 15% overall yield, which is low but acceptable considering the many couplings and deprotection steps required to form the peptide (**54**), Figure 68B. The extinction coefficient (E.C.) of the coumarin-peptide conjugate was then calculated. This was done at 2 different wavelengths, 280 nm first and then at 300 nm as it was observed that the coumarin-peptide conjugate absorbed better at 300 nm (see the Experimental section for details). The E.C. value at 300 nm was $9018.9 \text{ M}^{-1} \text{ cm}^{-1}$ and this should facilitate the analysis further.

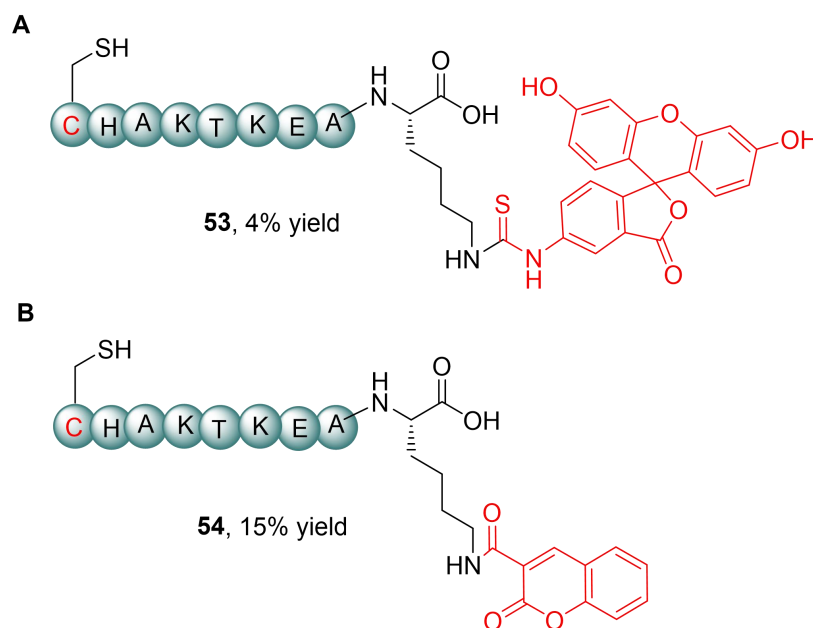
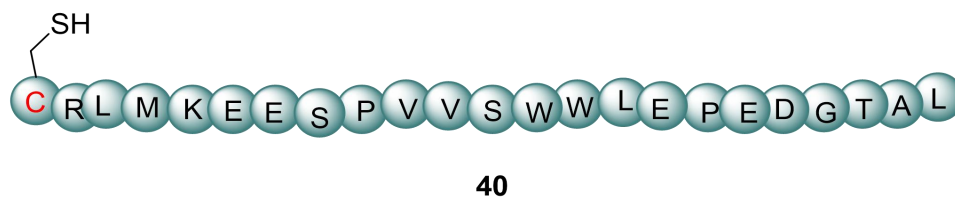


Figure 68: Structure of fluorescein model peptides; **A** is FITC- labelled model peptides (**53**), **B** is coumarin-labelled model peptides (**54**).

4.5 Synthesis of CysBAGE4₁₈₋₃₉



To enable conjugation to the outer envelope of the maleimide-containing liposomes, BAGE4₁₈₋₃₉ was slightly modified by adding Cys at the end of the sequence via the standard Fmoc-SPPS protocol. The synthesis was successful and the peptide (**40**) was purified using prep HPLC, resulting in 19% overall yield.

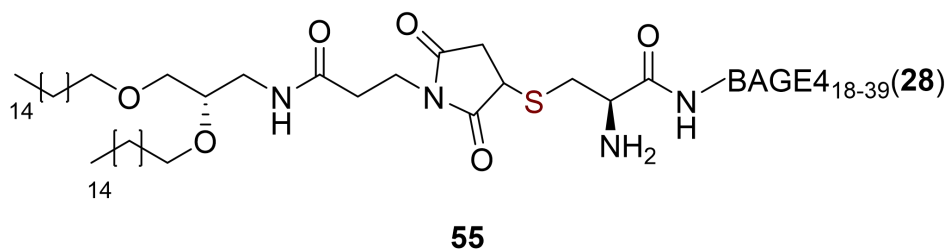
4.6 Conjugation Reaction

The conjugation reaction used is a Michael addition, which is a well-established and robust conjugation method. The structure of the Mal-lipid (**1**) contains hexadecyl chains (via ether linkages) and a maleimide head group, linked through a glyceryl moiety, Figure 59. The conjugation of the peptide to the maleimide groups protruding from the surface of the particles will occur via the N-terminal Cys residue to yield a thioether linkage. This reaction must be performed under neutral or slightly basic conditions to be specific. The maleimide group reacts chemoselectively with sulfhydryl groups when the pH of the reaction mixture is between 6.5 and 7.5, thus result is the formation of a stable thioether linkage that is not reversible, which means that the bond cannot be cleaved with reducing agents. In more alkaline conditions (pH >8.5), thiol chemoselectivity is lost and free primary amines may react competitively with thiols.

A free thiol is rarely available in proteins and those that are available tend to be biologically critical and should not be modified. This is because disulfide bridges formed between Cys residues in peptides and proteins are an important element for the molecular structure of proteins and peptides.²⁵⁶ Secondly, where a free thiol is present within the active site of a protein, it is likely to be integral to the bioactivity of the protein

and should not be modified. Because thiols are prone to oxidation, they often form disulfides, not only in proteins upon folding but also in synthetic peptides during synthesis. To revert disulfides into their sulfhydryl precursors, mild reducing agents, such as DTT and TCEP can be used.²⁵⁷ However, unlike DTT, the TCEP reducing agent does not have to be removed before reactions involving maleimide reagents. Therefore, TCEP was used during our thiol-maleimide conjugation.²⁵⁸ During the reaction it was important to keep the pH below 9 as above this, the TCEP will oxidise.

Initially, the Mal-lipid (**1**) was coupled to CysBAGE4 (**40**) and characterized to ensure the effectiveness of the reaction. A 1:1 mixture of mal-lipid: CysBAGE4 was conjugated at RT for 2 hrs using 20 equiv. of TCEP. Consumption of the starting peptide peak on HPLC and a significant shift in the retention time of the product indicated that the reaction was successful, as shown in Figure 69. HRMS analysis of the crude reaction confirms the presence of the desired mass, Figure 69.



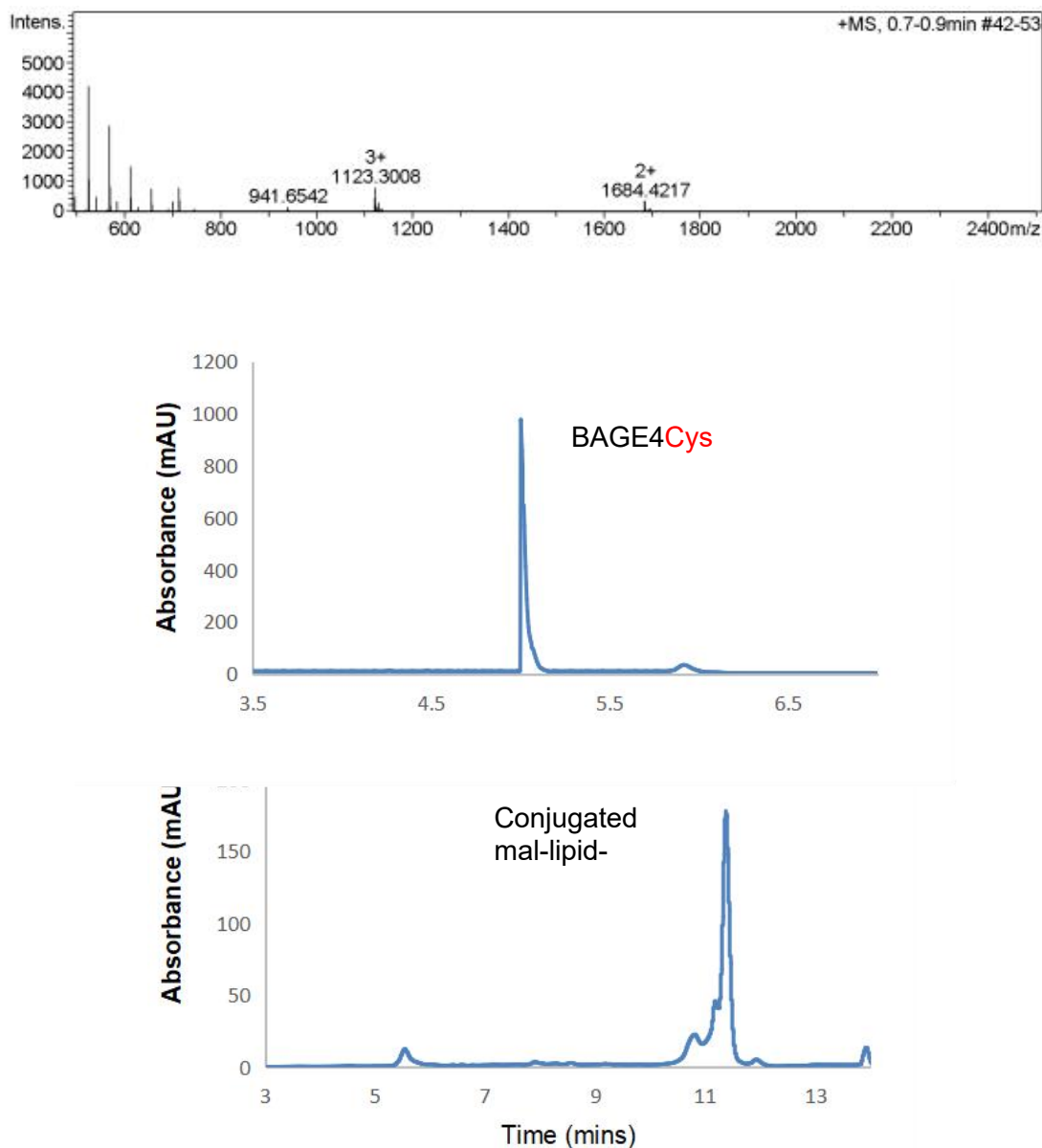


Figure 69: Analytical HPLC traces of pure CysBAGE4 (**40**) compared with conjugated mal-lipid-CysBAGE4 (**55**). Analytical gradient 10-100% B over 10 mins 0.6 mL/min, 280 nm. HRMS (ESI MS) Calc. for $C_{161}H_{263}N_{32}O_{41}S_2$ m/z $[M+2H]^{+2}$:1684.1160, $[M+3H]^{+3}$:1122.9307. Obs. m/z $[M+2H]^{+2}$:1684.4217, $[M+3H]^{+3}$:1123.3008.

4.7 Conjugation of coumarin-model peptide to mal-liposome and dialysis

The coumarin model peptide was conjugated to the **Mal-lipo -ve** formulation as well as the **non-Mal-lipo -ve** as a negative control (to assess electrostatic binding of the peptide to the non-mal liposomes), using the conditions optimized and described earlier. For the first

attempt, in an Eppendorf tube, 100 μ L of **Mal-lipo -ve** @ 2 mM (containing 0.2 mM of Mal-lipid (**1**), 0.02 μ mol) was added to 90 μ L, 5 equiv., 0.1 μ mol of the coumarin-model peptide in PBS with 10 μ L, 2 equiv. of TCEP @ pH 7.4. the negative control was identical with only the replacement of **Mal-lipo -ve** with a 100 μ L of **non-Mal-lipo -ve**. The reactions were vortexing overnight then each transferred to dialysis cassette, 3500 MW cut off, for purification. For the negative control there were small differences detected in the absorbance @ 300 nm between the blank (**non- Mal-lipo -ve**, 0.195) and the dialysed conjugated sample of coumarin-model peptide with **non-Mal-lipo -ve**, 0.437, i.e, a difference of 0.242 which could be due to electrostatic 'loading' (discussed below). However, for the **Mal-lipo -ve** conjugation reaction with coumarin-model peptide, an aggregation of the particles was noticed. This could be because the particles are electrostatically binding to the peptide and forming aggregates and this may only occur over longer time scales so a shorter reaction time followed by dialysis could solve this issue. Therefore, the reaction was re-conducted with extra care taken to ensure the pH of the solution remains at 7 and with close visual monitoring to ensure the reaction was dialysed before aggregation occurs. However, aggregation occurred just 15 mins from the start of the reaction. This system was initially employed to optimize the conjugation reaction for next step of CysBAGE4 conjugation reaction, however, due to the problematic aggregation observed, the investigation moved to the conjugation of the CysBAGE4 antigen using the mal liposomes.

4.8 Conjugation of CysBAGE4₁₈₋₃₉ peptide to mal-liposome and dialysis

One of the main goals of this chapter is to attach the synthesized antigen to the surface of formulated mal-liposome as per Figure 70.

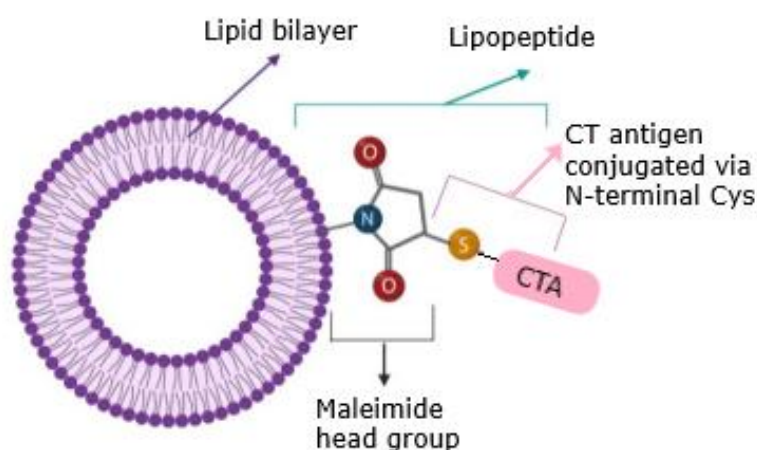


Figure 70: The final vaccine formulation of the antigen conjugated onto the maleimide linker on the liposome, mimicking the native presentation of lipoproteins.

Starting with the **Mal-lipo -ve**, the conditions detailed in the Experimental chapter were applied at 2mM conc. of liposomes (formulation containing 0.02 μmol of Mal-lipid (**1**)), 5 equiv. of CysBAGE4₁₈₋₃₉, and the reaction left for 3 hrs before loading into a dialysis cassette, 3500 MW cut off. The UV-Vis analysis showed the successful conjugation of the peptide with the liposomes. Comparing the UV absorbance @ 280 nm with the **Mal-lipo -ve** control solution, and using online tools (pepcalc.com) to calculate the E.C. of CysBAGE4₁₈₋₃₉, 11380 $\text{cm}^{-1} \text{M}^{-1}$, the amount of conjugated CysBAGE4₁₈₋₃₉ was calculated as shown in Table 7. However, the number of mmole of conjugated CysBAGE4₁₈₋₃₉ was higher than expected. This means that the absorbance value of the **BAGE4Cys-Mal-lipo -ve** sample represents not only CysBAGE4₁₈₋₃₉ that is covalently bound to Mal-lipid (**1**), but also peptide that is bound electrostatically to the surface of the particles. Therefore, it is important to conjugate the CysBAGE4₁₈₋₃₉ to **non- Mal-lipo -ve** using the same conditions to quantify the level of electrostatic 'loading'. The conjugation was repeated using the non-mal liposome formulation; no aggregation was observed using this peptide. After dialysis, the UV-vis absorbance of the solution was measured, and the presence of electrostatically bound peptide was confirmed. Therefore, it can be assumed that the difference in absorbance between the two samples accounts for covalent attachment. The calculation has been

carried out for both reactions as per Table 7 which suggests that covalently conjugated CysBAGE4₁₈₋₃₉ accounts for 0.017 μmol , i.e., 85% of the available mal sites, which is reasonable.

Table 7: The calculation of the differences in the yield of conjugated CysBAGE4₁₈₋₃₉ to anionic mal and non-mal-liposomes.

The liposome	Absorbance @ 280 nm ^[a]	E.C. CysBAGE4 ₁₈₋₃₉	The volume of sample ^[b]	The μmoles of conjugated CysBAGE4 ₁₈₋₃₉ ^[c]
mal-lipo ^{-ve}	0.467	11380 $\text{cm}^{-1} \text{M}^{-1}$	0.0008 L	0.033 μmol
non- mal-lipo ^{-ve}	0.231	11380 $\text{cm}^{-1} \text{M}^{-1}$	0.0008 L	0.016 μmol

[a]The blank' absorbance subscribed.

[b]The entire dialysed solution of mal-/non-mal-liposome with peptide reaction was containing in the 800 μL in the cuvette.

[c]Converting the concentration to mol using $\text{mol} = C \times V$.

Moving on to the cationic liposomes (**Mal-lipo ^{+ve}**), the CysBAGE4₁₈₋₃₉ was attached successfully using the same conditions as described for the anionic particles, expect that the PBS was replaced with MilliQ water (0.02 μmol mal-lipid, 5 equiv. CysBAGE4₁₈₋₃₉, and 3 hrs @ RT). The UV analysis showed much higher absorbance @ 280 nm compared of conjugated **BAGE4Cys-Mal-lipo ^{+ve}** with the blank **Mal-lipo ^{+ve}** and conjugated **BAGE4Cys-Mal-lipo ^{-ve}** sample as the amount of conjugated CysBAGE4₁₈₋₃₉ was calculated to be 0.06 μmol which is 3 times the amount of available μmol of Mal-lipid (**1**), see Table 8. This could be due to the negative charge of CysBAGE4₁₈₋₃₉ (-3 at pH 7). This cationic-conjugated formulation was analysed by TEM and shows small uniform particles, as per Figure 71.

Table 8: The calculation of the differences in the yield of conjugated CysBAGE4₁₈₋₃₉ to cationic Mal and non-Mal-liposomes.

The liposome	Absorbance @ 280 nm ^[a]	E.C. CysBAGE4 ₁₈₋₃₉	The volume of sample ^[b]	The μ moles of conjugated CysBAGE4 ₁₈₋₃₉ ^[c]
mal-lipo ^{+ve}	0.826	11380 cm ⁻¹ M ⁻¹	0.0008 L	0.06 μ mol
non-mal-lipo ^{+ve}	0.245	11380 cm ⁻¹ M ⁻¹	0.0008 L	0.02 μ mol

[a] The blank' absorbance subscribed.

[b] The entire dialysed solution of mal-/non-mal-liposome with peptide reaction was containing in the 800 μ L in the cuvette.

[c] Converting the concentration to mol using $\text{mol} = C \times V$.

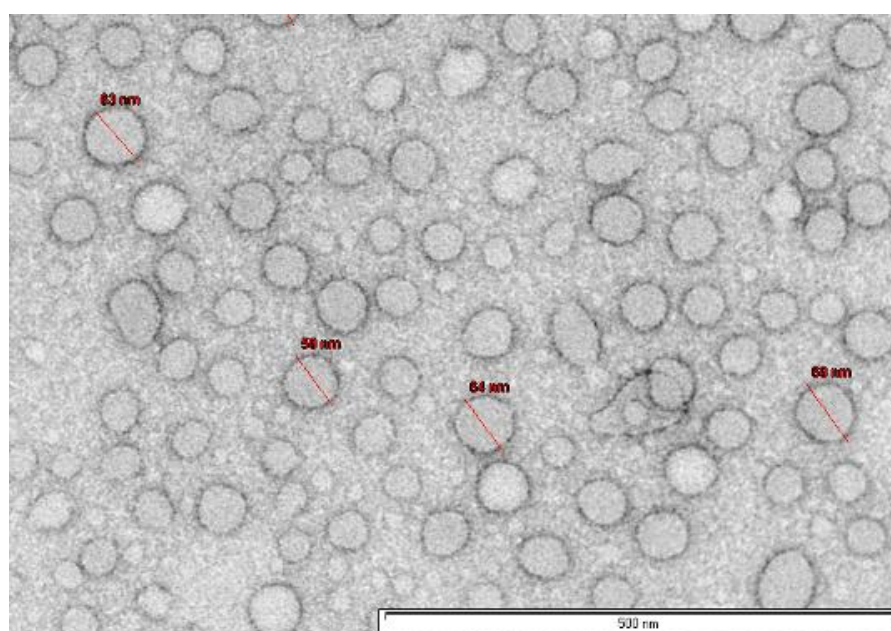


Figure 71: TEM for **BAGE4Cys- Mal-lipo ^{+ve}**. The image capturing was operated at 60k.

Table 9 shows the summary of the cationic and anionic liposome formulations conjugated to CysBAGE4₁₈₋₃₉

Table 9: Summary of the liposome peptide formulations

The liposome peptide formulations	Lipids components and ratio	The μ moles of conjugated CysBAGE4 ₁₈₋₃₉ [c]
BAGE4Cys-Mal-lipo^{+ve}	10% Mal-lipid (1), 35% Cholesterol, 15% DOTAP, 40% DSPC	0.06 μ mol
non-mal-lipo^{+ve}	40% Cholesterol, 15% DOTAP, 45% DSPC	0.02 μ mol
BAGE4Cys-Mal-lipo^{-ve}	10% Mal-lipid (1), 25% DSPC, DMPG-Na, and 40% cholesterol	0.033 μ mol
non- mal-lipo^{-ve}	30% DSPC, DMPG-Na, and 40% cholesterol	0.016 μ mol

4.9 Liposomes formulation for *in vivo* study

Once we had the palmitoylated antigens in hand and confirmed successful conjugations of a CTA to the surface of liposomal formulations, we could proceed with preparation of the formulations for *in vivo* study. For these animal studies, a cationic liposome formulation was selected. This is because when injected into the bloodstream, anionic liposomes are less stable than cationic liposomes. Indeed, anionic liposomes quickly engage with the biological system after becoming opsonized by circulating proteins such as complement.^{259,260} This results in a rapid uptake by the reticuloendothelial system (RES) and toxic effects such as pulmonary hypertension, dyspnea, and a drop in circulating platelets and leukocytes.²⁶¹ Formulation of cationic liposomes in MilliQ water affords liposomes with low PdI, and diameter size, however; for *in vivo* formulation, it is necessary to formulate in PBS. *In vivo* injection with

MilliQ water, while sterile and therefore safe, is not buffered and therefore tends to sting the animal. Therefore, the **Mal-lipo +ve** samples were formulated in PBS buffer, however, during the hydration process, it was noticed that the dry thin film did not suspend in the solution easily compared with MilliQ water (it takes around 40 mins instead of 1-2 mins). The DLS report stated that the sample does not meet the quality requirements, and this could be because of either large or polydisperse particles, so no accurate data of size or PdI could be obtained. Therefore, in order to overcome this problem, the liposomes were formulated in 90 vol.% MilliQ water then, after sonication and prior to DLS analysis, 10 vol.% of 10x PBS was added giving ideal particles as shown by DLS, 108.6 d. nm; PdI: 0.285, and zeta potential: + 34 mV. Based on this successful trial, a new batch of **Mal-lipo +ve** was formulated based on the number of moles requested for the *in vivo* experiments (see Chapter 5 & 6 for more details).

To explore the decoration of liposomes with the CTA *in vivo*, the liposome-peptide conjugation was repeated as discussed with some modifications to the volume of the reaction, which was increased to 2400 μ L, containing 0.24 μ mol of Mal-lipid (**1**) and 2 equiv. of CysBAGE4₁₈₋₃₉ instead of 5 equiv. to minimized loading via electrostatic association (see the Experimental chapter for detailed calculations). In parallel, **non-Mal-lipo +ve** was incubated with CysBAGE4₁₈₋₃₉ (**40**) to enable us to estimate the level of covalently bound CysBAGE4₁₈₋₃₉. UV analysis confirmed that almost 60% of the CysBAGE4₁₈₋₃₉ was attached covalently to **Mal-lipo +ve** to form **BAGE4Cys-Mal-lipo +ve** with the remaining peptide bound electrostatically, which is acceptable, less than what was shown previously using the anionic liposome (a summary of calculations shown in Table 10). This drop in covalent modification is attributed to the lower equiv. of CysBAGE4₁₈₋₃₉ (**40**) used.

Table 10: The calculation of the differences in the yield of conjugated CysBAGE4₁₈₋₃₉ to cationic Mal and non-Mal-liposomes for the in vivo samples.

The liposome	Absorbance @ 280 nm ^[a]	E.C. CysBAGE4 ₁₈₋₃₉	The volume of sample ^[b]	The μmoles of conjugated CysBAGE4 ₁₈₋₃₉ ^[c]
mal-lipo ^{+ve}	0.264	11380 cm ⁻¹ M ⁻¹	0.0005 L	0.23 μmol
non-mal-lipo ^{+ve}	0.210	11380 cm ⁻¹ M ⁻¹	0.0005 L	0.11 μmol

[a]The blank' absorbance subscribed.

[b]The entire dialysed solution of mal-/non-mal-liposome with peptide reaction was 0.001809/ 0.002065 L consequently, 20 μL from each diluted up to 500 μL for the UV analysis with 25 dilution factors.

[c]Converting the concentration to mol using mol = C x V.

4.10 Formulation of Pam₂Cys-SK₄-BAGE4₁₈₋₃₉ liposome

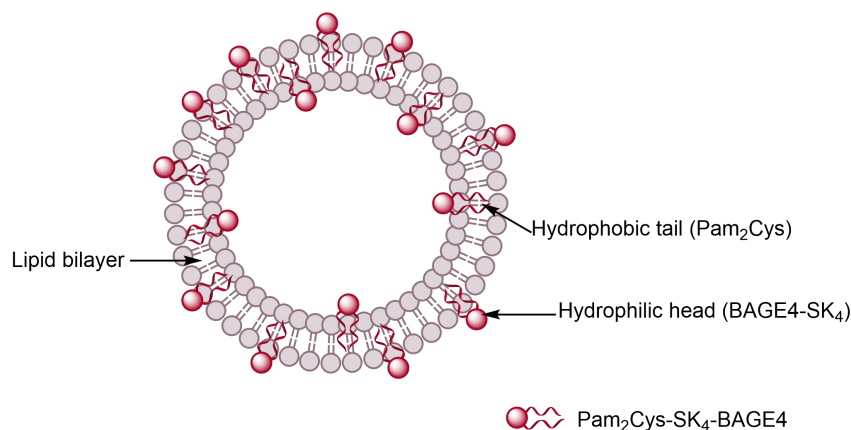


Figure 72: Structure of liposomes formulated with Pam₂Cys-SK₄-BAGE4 (33).

The second aspect in this chapter concerns the formulation of liposomes with CTA carrying the Pam₂Cys moiety (a native TLR2 agonist) incorporated into the bilayer to enhance the targeted immune response. Therefore, a cationic liposome was formulated as described, replacing the 10% of Mal-lipid (1) with 10% Pam₂Cys-SK₄-BAGE4₁₈₋₃₉ (33). For the initial attempt involved formulation in PBS buffer, the thin film layer

could not be suspended into the PBS even with incubation in a heating bath for 2 hrs. This led us to the use of a bath sonicator which resulted poor quality particles with a large average size of over 1000 d. nm. We thus moved on to formulation in MilliQ water with the addition of 10 vol.% of 10x PBS prior to analysis. However, once the PBS buffer was added, the clear solution became cloudy due to a precipitate. It is likely that an increase in the salt conc. is causing this precipitate due to mitigation of electric charge. Therefore, in order to save material considering the challenging synthesis of Pam₂Cys-SK₄-BAGE₄₁₈₋₃₉ (**33**) and due to time limitations, the liposomes were formulated using only MilliQ water, the pH was checked by the animal testing facility and the samples were considered to be acceptable. This allowed us to formulate cationic liposomes carry around 10 mol% of Pam₂Cys-SK₄-BAGE₄₁₈₋₃₉ (**33**), Figure 72. The DLS shows one population with average diameter of 171.5. nm; PdI: 0.234, and zeta potential: + 10 mV, Figure 73.

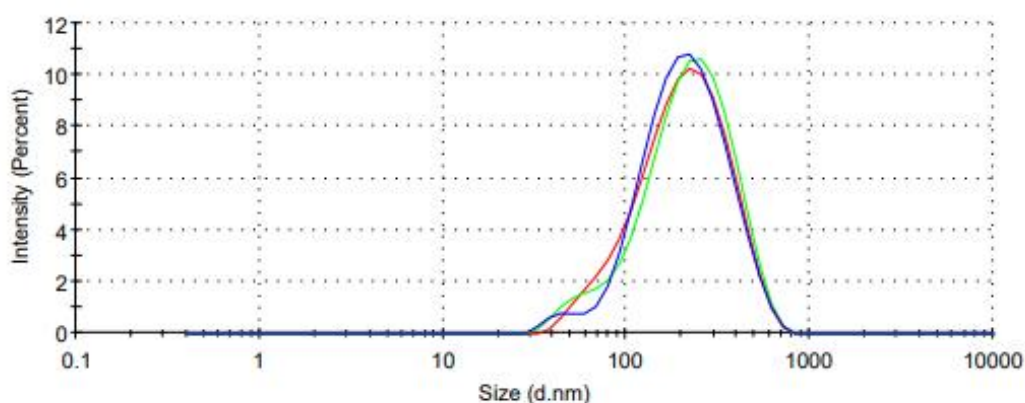


Figure 73: The DLS result for cationic Pam₂Cys-SK₄-BAGE₄ liposome. Different colour presents the repetition of analysis by 3.

4.11 Conclusion

To summarise this chapter, a reproducible formulation method has been achieved and applied successfully to formulate high quality anionic and cationic-mal-lipid containing liposomes. There were elements that affected the quality of particles formed other than the lipid ratio and the probe sonicator setting. These elements include the importance of an

even spread of thin film equally around the vessel and applying heat to detach the thin film from the internal surface of the flask to ensure suspension in the solution prior to the sonication process. Considering and addressing all these factors led to successful optimisation of the formulation to yield high-quality liposomes. Followed this, successful synthesis of a fluorescein model peptide enables optimisation of the conjugation reaction with mal-liposomes and characterisation; however, the reaction failed due to aggregation. Fortunately, this was not the case with our main focus, CysBAGE4₁₈₋₃₉ (**40**) which was successfully conjugated to the outer envelope of the particles in acceptable loading. This formulation will be used as a negative control for the Pam₂Cys-SK₄-BAGE4₁₈₋₃₉ (**33**) material which has been successfully incorporated into a liposomal formulation. However, if it transpires that the **BAGE4Cys-mal-lipo** ^{+ve} formulation does yield an effective immune response, this system will be a much simpler and cheaper alternative to formulating synthesised **Pam₂Cys-SK₄-BAGE4-lipo** ^{+ve}.

Chapter 5: Biological evaluation of synthesised CTA lipopeptides vaccine constructs.

5.1 Introduction

This chapter will discuss the biological evaluation of the synthesised vaccines: Pam₂Cys-SK₄-BAGE₄₁₈₋₃₉ (**33**), Pam₂Cys-SK₄-NY-ESO-1₁₅₇₋₁₆₅ (**37**), BAGE₄₁₈₋₃₉ antigen (**28**), and liposomal formulations prepared with Pam₂Cys-SK₄-BAGE₄₁₈₋₃₉ and conjugation of CysBAGE₄₁₈₋₃₉ (**40**) via the Mal-lipid (**1**). First, it is important to confirm that antibodies raised against the specific natural CTAs recognise the synthetic antigens and the palmitoylated antigens in order to go further into animal studies. This can be performed via several different binding assays such as western blot and ELISA (enzyme-linked immunosorbent assay). In this study, ELISA has been chosen as it is a simpler and faster procedure. It is a plates-based assay technique designed to detect and quantify soluble substances such as peptides, proteins, and antibodies. It relies on antibodies to detect a target antigen using highly specific antibody-antigen interactions.²⁶²

Additionally, it is essential to test the vaccines in a biological model to evaluate their ability to elicit a targeted immune response and demonstrate their efficacy. Healthy mice will be immunised at 3 time points with each vaccine formulation; once the dosing has been completed spleen-cells will be harvested from the models. For the initial *in vivo* experiments, the enzyme-linked immunospot (ELISpot) assay will be performed as it is a highly sensitive immunoassay that measures the frequency of cytokine-secreting cells at the single-cell level.²⁶³ In this assay, in the presence or absence of vaccine specific stimuli, cells are cultured on a surface coated with a specific capture antibody. Proteins secreted by cells, such as cytokines, will be captured by the specific antibodies on the surface. Following an appropriate incubation time, cells are washed off and the secreted molecule is detected using a detection antibody in a manner similar to that of the ELISA. The detection antibody is either biotinylated and then conjugated to an enzyme, or the

antibody is directly conjugated to an enzyme. The use of a precipitating rather than a soluble product as a substrate result in visible spots on the surface. Each dot represents a single cytokine-secreting cell.²⁶⁴ The ELISpot assay will enable us to quantify cytokine release from T-cells upon recognition of the antigen peptide. If the vaccine formulation has primed the model's immune system to recognise the antigen, a positive response will be recorded to the vaccine specific stimulation. The level of immune response is also quantified against non-specific stimulus (lipopolysaccharide – LPS) as a positive control for the assay.

5.2 ELISA for BAGE4₁₈₋₃₉

As described, an ELISA assay was performed to validate the binding of CT-specific antibody to each antigen peptide. The aim of this first run was to determine which concentration of antigen and antibody gives the best binding. For all the ELISA experiments, MaxiSorp plates were used as they have the widest breadth applications, designed to bind to the greatest range of substrate molecules/biomolecules that have hydrophilic/hydrophobic character in particular.²⁶⁵ Since the vaccines are palmitoylated these plates should be suitable. Following an indirect ELISA protocol²⁶⁶, 23 different concentrations of BAGE4₁₈₋₃₉ peptide antigen (**28**) with 8 different dilution series of anti-BAGE4 antibody (produced in rabbit) were used. The resulting data confirms that the antibody does bind to the BAGE4₁₈₋₃₉ antigen. The level of binding varies depending on the concentration of both the antibody and the antigen. It shows that when the concentration of BAGE4₁₈₋₃₉ antigen (**28**) increased above 10 ng/ml the binding affinity decreased Figure 75. These results demonstrate the “Hook” effect which is a phenomenon that causes binding to decrease at high concentrations of the antigen. This is because the ability of antibodies to form immunological complexes with the antigen on the plate is compromised by the high concentration of antigen in the bulk solution, Figure 74.²⁶⁷ Therefore, this preliminary binding assay shows that 10 ng/mL is the ideal concentration of antigen that give the highest binding affinity with 1:250 of the antibody. Thus, for the next ELISA experiments, concentration of 10 ng/mL BAGE4₁₈₋₃₉

antigen (**28**) with 1:250 antibody concentration used.

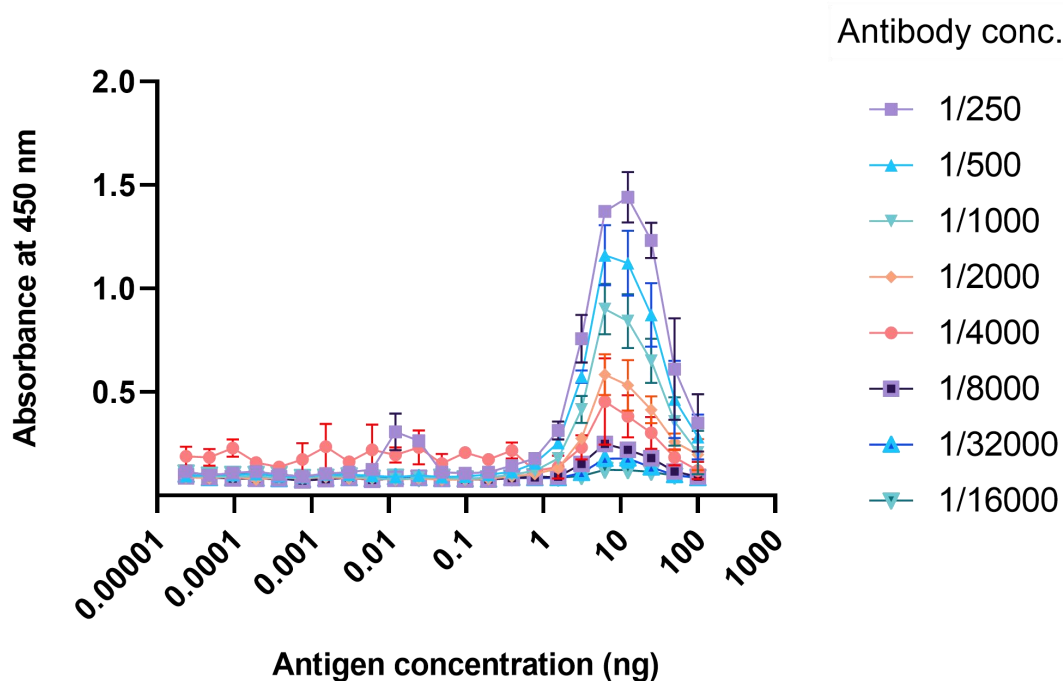


Figure 74: Graphical representation of ELISA binding curves of serial different concentrations of BAGE4₁₈₋₃₉ antigen (**28**) with different dilution series of antibody Anti-BAGE4 antibody, the absorbance at 450 nm. (The graph presents the mean with standard deviation of two repeats).

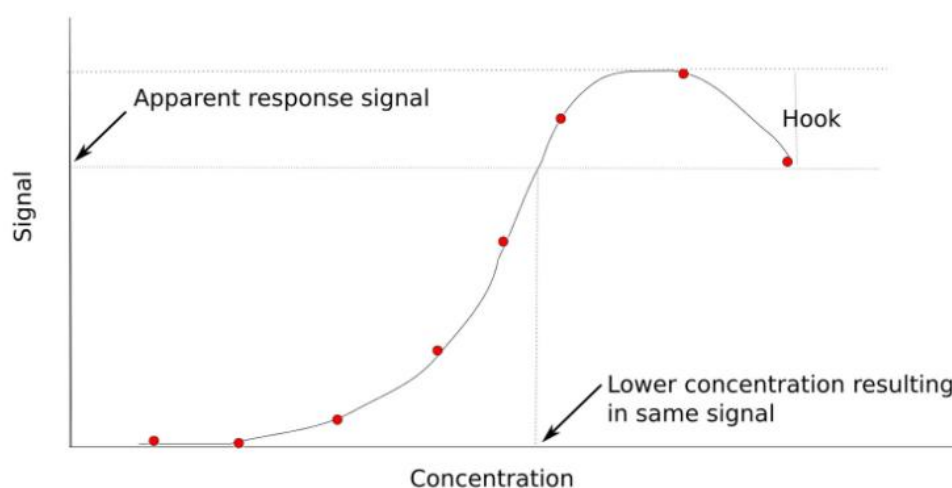


Figure 75: "HOOK" effect where at high concentrations of analyte, a lower signal is observed.²⁶⁸

5.3 Competitive ELISA for BAGE4₁₈₋₃₉ and BAGE4₁₈₋₃₉ Liposomal Conjugate Vaccines

Competitive ELISA is one of the most widely used techniques when only one antibody is available for the antigen of interest. This technique was used to assess whether the conjugated vaccine will bind to the antibody and if the binding abilities differed compared to the antigen alone. The primary antibody was incubated with the Pam₂Cys-SK₄-BAGE4₁₈₋₃₉ (**33**), BAGE4₁₈₋₃₉ (**28**) and the Cys-BAGE4₁₈₋₃₉ liposome conjugates at 11 different concentrations starting with 1000 ng/mL and end with 0.977 ng/mL. Antibody-antigen complexes were then added to 384 well plates which were pre-coated with 10 ng/mL of antigen. So, in general, the more antigen in the sample, the less antibody will be able to bind to the antigen in the well, i.e., a competition assay. The secondary antibody that is specific to the primary antibody and conjugated with horse radish peroxidase (HRP) enzyme is added, followed by TMB substrate for colour development. In a positive assay, no antibody is left to bind to the antigen in the well therefore the enzyme-conjugated antibody cannot bind which will be indicated by an absence of colour. However, in the negative results, the available antibody can bind to the antigen in the coated wells, followed by binding of the enzyme-conjugated antibody which then acts on the substrate to produce colour.

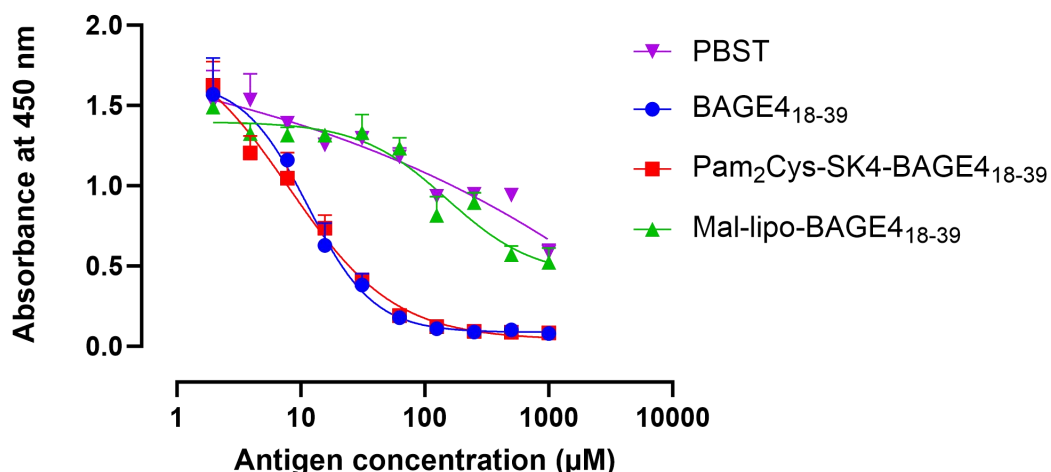


Figure 76: ELISA data to compare the binding affinity of BAGE4₁₈₋₃₉ antigen (**28**) IC₅₀ = 11.41 μM +/- 3.523, Pam₂Cys-SK4-BAGE4₁₈₋₃₉ (**33**) IC₅₀ = 8.037 μM +/- 6.035 Cys-BAGE4₁₈₋₃₉ liposome conjugate IC₅₀ = 151.4 μM, PBST buffer with 0.05% Tween 20 as negative control.

Figure 76 confirms that the antigen carried by the vaccines candidates binds to the primary antibody. The binding affinity of Pam₂Cys-SK4-BAGE4₁₈₋₃₉ (**33**) is similar to the antigen peptide alone; **33** gives a IC₅₀ value of 8.037 μM, while **28** gives an IC₅₀ value of 11.41 μM. However, the CysBAGE4₁₈₋₃₉ liposome conjugate shows reduced competition relative to the BAGE4₁₈₋₃₉ antigen (**28**) (IC₅₀ = 151.4 μM and IC₅₀ = 11.41 μM respectively) suggesting that less antigen is present for antibody binding on the surface of the liposomes. This could be because the presentation of the antigen to the bulk solution in the vaccine formulations is compromised by the formation of higher-order liposomal/micelle/vesicle structures (i.e., the antigen is buried within the lipid (bi)layers). Or potentially, antibodies bound to a single antigen on the surface of the liposome may still able to bind to the plate via their second binding region.

5.4 ELISA for NY-ESO-1₁₅₇₋₁₆₅

An ELISA assay was performed on the NY-ESO-1₁₅₇₋₁₆₅ antigen (**29**) to validate the binding of NY-ESO-1-specific antibody and to indicate at which concentration of antigen and antibody leads to the most effective binding affinity. Applying the indirect ELISA protocol²⁶⁶ with same conditions of concentration as described for the BAGE4 assay resulted in no binding occurring at any concentration, Figure 77. These results were expected as the NY-ESO-1₁₅₇₋₁₆₅ antigen (**29**) is a small peptide, comprised of only 9 amino acids while the primary antibody is designed to bind to the native NY-ESO-1 protein. Based on these results no further ELISA experiments for the conjugated NY-ESO-1₁₅₇₋₁₆₅ vaccine were attempted, however, due to the prior in vivo research carried out for this peptide antigen, confidence was still high for our planned in vivo experiments.

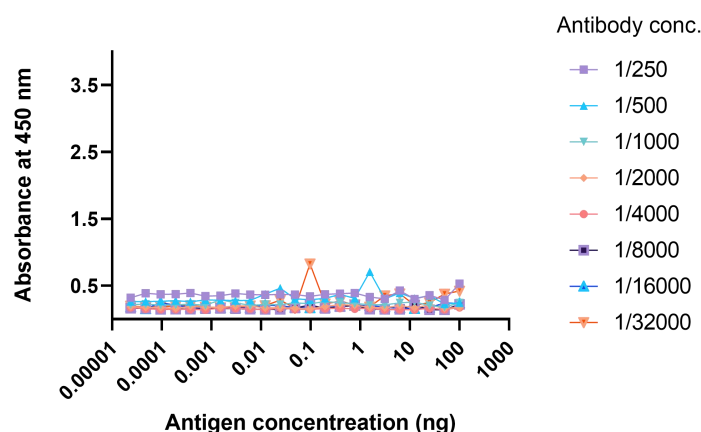


Figure 77: Graphical representation of ELISA binding curves of serial different concentrations of NY-ESO-1₁₅₇₋₁₆₅ antigen (**29**) with different dilution series of antibody Anti-NY-ESO-1 antibody, the absorbance at 450 nm.

5.5 The Enzyme-Linked Immunospot (ELISpot) assay

The ELISpot assay works using plate immobilised antibody to capture target cytokines secreted by cells cultured with specific immune stimulants.²⁶⁹ These are detected with additional detection antibodies and quantified by colour change of the substrate solution.²⁷⁰ This assay has been used to monitor T cell responses from pre-clinical animal studies.

5.5.1 ELISpot for Pam₂Cys-SK₄-NY-ESO-1₁₅₇₋₁₆₅

The aim of this study is to determine the T-cell responses induced by the Pam₂Cys-SK₄-NY-ESO-1₁₅₇₋₁₆₅ (**37**) candidate and to compare this with previous studies.^{271,272} Thus a group of 3 healthy HLA-A2 transgenic mice were immunized on three occasions (1, 8 and 15 days) with 10 nmol of Pam₂Cys-SK₄-NY-ESO-1₁₅₇₋₁₆₅ (**37**) via s.c injection (at a total concentration of 0.2 mM). The readout of this experiment was the frequency of peptide-specific IFN γ secreted by T-cells in ELISpot assay. The T-cells were obtained from spleen cells of the sacrificed injected mice (see Experimental Chapter, Section 7.8 for details). Summary of injection procedure has been shown in Table 11

Table 11: Summarise the injections plan (including dose concentration and number) for Pam₂Cys-SK₄-NY-ESO-1₁₅₇₋₁₆₅ (**37**) candidate using HHDII/DR1 Tg mice.

Group	Test article	Dose (μ g)/vol	Route	Dosing schedule	N	Termination	Assays at termination
1	NY-ESO-1 peptide/ Pam ₂ Cys conjugate	10 nmol in 50 μ l	SC	Day 1,8 & 15	3	Day 21	ELISpot on splenocytes

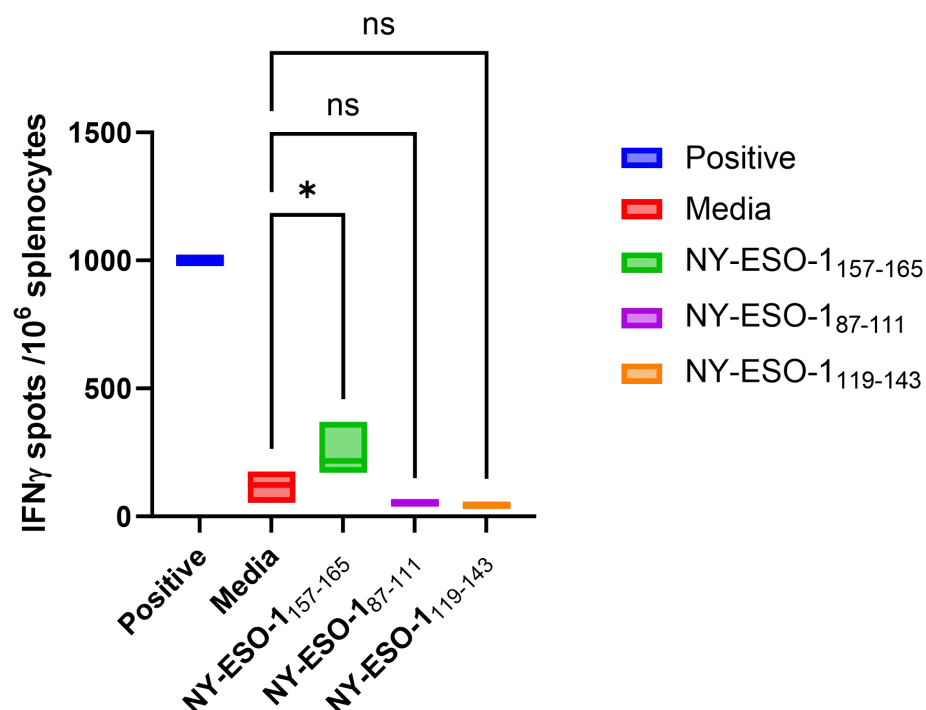


Figure 78: The mean of immune responses induced in HHDII/DR1 mice immunized with the Pam₂Cys-SK₄-NY-ESO-1₁₅₇₋₁₆₅ (**37**) vaccine candidate. Isolated splenocytes from the immunised mice tested against 3 peptides from the NY-ESO-1 protein, NY-ESO-1₁₅₇₋₁₆₅, NY-ESO-1₈₇₋₁₁₁ & NY-ESO-1₁₁₉₋₁₄₃, and measured in IFN γ Elispot assay.

The data for the T-cell responses via ELISpot is displayed per million splenocytes and this gives an estimate of the magnitude of the immune response. This data is shown in Figure 78. Each spot is one T-cell that has responded to the stimulus in the assay. In this assay the splenocytes from the immunised mice were tested against 3 peptides from NY-ESO-1 that are known to reactivate T-cell responses in this strain of mouse if they have been vaccinated against NY-ESO-1. One is the NY-ESO-1₁₅₇₋₁₆₅ peptide (**29**) that is used in vaccine conjugate (**37**) (blue bar). The other two peptides, NY-ESO-1₈₇₋₁₁₁ & NY-ESO-1₁₁₉₋₁₄₃ (red & green bars, provided by Prof. Lindy Durrant's group) act as negative controls for the conjugate. Strong responses were observed to the LPS positive control for the assay (purple bar) in all mice. The bar graph shows specific but weak responses detected by vaccine conjugate (**37**) over the media-only

negative control (yellow bar). A one-way anova statistical analysis has been carried out to assess the significance of the effect of Pam₂Cys-SK₄-NY-ESO-1₁₅₇₋₁₆₅ (**37**) conjugate on the immune response of mice. The analysis confirmed a significant effect for NY-ESO-1₁₅₇₋₁₆₅ ($p = <0.0229$, $q = 3.567$, $DF = 8$) while the data for both NY-ESO-1₈₇₋₁₁₁ ($p = 0.3280$, $q = 1.704$, $DF = 8$) and NY-ESO-1₁₁₉₋₁₄₃ ($p = 0.2293$, $q = 1.969$, $DF = 8$) were not significant. Therefore, there is evidence that this Pam₂Cys-SK₄-NY-ESO-1₁₅₇₋₁₆₅ (**37**) candidate is immunogenic but it does not produce a significantly strong enough response to take this candidate on to cancer models. Comparing this result with a previous study, by Stevenson's group in 2013, that measured the T-cell responses elicited by NY-ESO-1₁₅₇₋₁₆₅ with PADRE peptide and emulsified with Montanide, (median = 280) demonstrates that the quantity of T-cell response induced by our Pam₂Cys-SK₄-NY-ESO-1₁₅₇₋₁₆₅ (**37**) candidate (median = 250), Figure 78, is slightly lower.²⁷² Moreover, the average number of T-cell responses elicited is almost the same between this finding and the earlier study carried out by Durrant's group in 2016²⁷¹, where the HHDII mice were immunised with NY-ESO-1₁₅₇₋₁₆₅ peptide with complete/incomplete Freund's adjuvant combination. Reading these different results, we can conclude that NY-ESO-1₁₅₇₋₁₆₅ (**29**) is an immunogenic peptide, able to bind to HLA-A2 and expressed by a wide range of cancers.^{273,274,275} This is why it has been the focus of many different studies to induce immunity by range of applications, however; the level of T-cell response was not at the desired level compared with the native NY-ESO-1 protein which was at level of 1000.²⁷²

5.4.2 ELISpot for BAGE4₁₈₋₃₉ peptide/Cys-BAGE4₁₈₋₃₉ liposome conjugate

The aim of this study is to compare the T-cell and antibody responses induced by the Pam₂Cys-SK₄-BAGE4₁₈₋₃₉ candidate (**33**), CysBAGE4₁₈₋₃₉ liposome conjugate, Pam₂Cys-SK₄-BAGE4₁₈₋₃₉ liposome formulation, and BAGE4₁₈₋₃₉ peptide (**28**) mixed with standard adjuvant (incomplete Freund's). To do this, groups of healthy mice were immunized on three

occasions (1, 8, and 15 days) with 10 nmol of each conjugate via s.c injection (0.2 mM dose concentration). The readout would again be the frequency of peptide-specific IFN γ secreting T-cells by ELISpot assay. Since nothing is known about T cell responses to BAGE4 in conventional or HLA-A2 transgenic mice, conventional mice were initially selected. The H-2d haplotype (Balb/c) strain have been chosen for this study as the predictions for peptides that will bind to MHCI and II are the best, using the epitope prediction software (IEDB Analysis Resource) for BAGE4 protein. The same injection procedure used for the NY-ESO-1 peptide/Pam₂Cys conjugate (as detailed in Table 11) was followed for the four different vaccine candidates: Pam₂Cys-SK₄-BAGE4₁₈₋₃₉ (**33**), BAGE4₁₈₋₃₉Cys-Mal-liposome conjugate, Pam₂Cys-SK₄-BAGE4₁₈₋₃₉ liposome formulation, and BAGE4₁₈₋₃₉ peptide mixed with standard adjuvant (incomplete Freund's) using BALB/c mice.

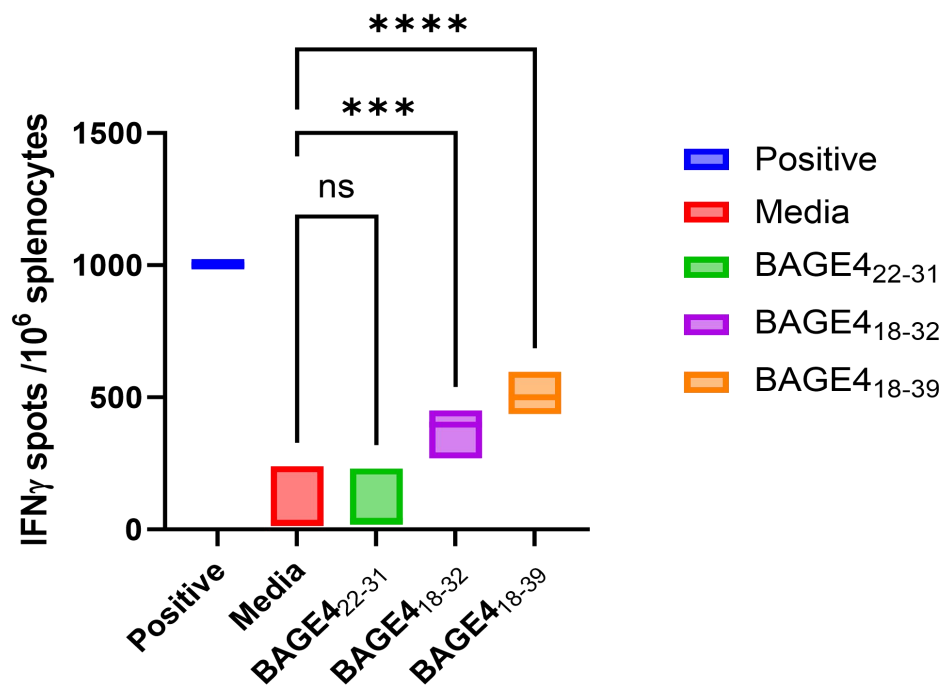


Figure 79: Immune responses induced in Balb/c mice immunized with BAGE4₁₈₋₃₉ peptide mixed with IFA. Isolated splenocytes from the immunised mice tested against 3 peptides from the BAGE4₁₈₋₃₉ protein and measured in IFN γ ELISpot assay.

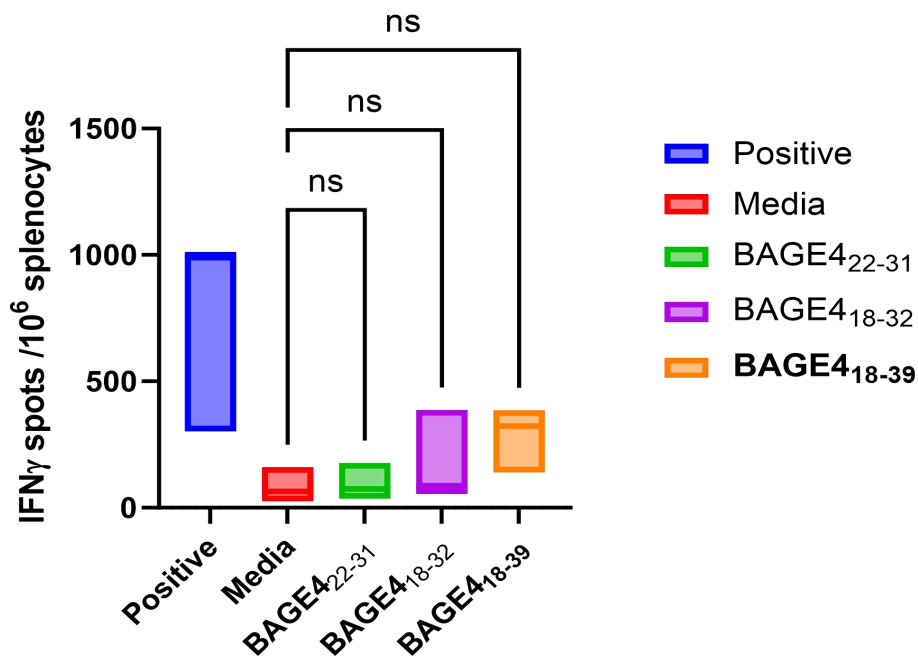


Figure 80: Immune responses induced in Balb/c mice immunized with BAGE4₁₈₋₃₉Cys-mal-liposome conjugate. Isolated splenocytes from the immunised mice tested against 3 peptides from the BAGE4₁₈₋₃₉ protein and measured in IFN γ ELISpot assay.

In this assay the splenocytes from the immunised mice were tested against 3 predicted peptides from BAGE4₁₈₋₃₉ which should reactivate T-cell responses in this strain of mice when immunised with BAGE4₁₈₋₃₉ vaccine candidates (based on the epitope prediction software for BAGE4 protein). One is the BAGE4₁₈₋₃₉ peptide (**28**), (blue bar) that is used in peptides, BAGE4₁₈₋₃₂ (**56**) & BAGE4₂₂₋₃₁ (**57**) (green & red bars) are smaller peptides from within the sequence used to immunise that were predicted by the epitope prediction software to be potential peptides that the T cells may be specific for and respond to. The data shows that the BAGE4₁₈₋₃₉ sequence (**28**) is immunogenic in conventional Balb/c mice and can stimulate T cell responses that recognise BAGE4 with good responses to the whole native BAGE4₁₈₋₃₉ sequence (median = 511) and slightly lower responses to the BAGE4₁₈₋₃₂ peptide. However, there is almost no response to BAGE4₂₂₋₃₁, Figure 79. A one-way anova statistical

test was carried out and confirmed a significant effect for BAGE4₁₈₋₃₉ ($p = <0.0001$, $q=9.206$, $DF=8$) & BAGE4₁₈₋₃₂ ($p= 0.0009$, $q=6.142$, $DF= 8$) while BAGE4₂₂₋₃₁ shows no significance ($p=>0.9999$, $q=0.02932$, $DF= 8$). This could support the results from NY-ESO-1 study; when the whole sequence of protein presented to the immune system the response can be better relative to shorter fragments. For the BAGE4₁₈₋₃₉Cys-mal-liposome conjugate, the immune response is not as effective, Figure 80, and somewhat varied (385, 139, 323, with median= 282). A one-way anova statistical test was carried out and confirmed no significant effect for BAGE4₁₈₋₃₉ ($p= 0.5013$, $q=1.359$, $DF=8$), BAGE4₁₈₋₃₂ ($p= 0.9165$, $q=0.6330$, $DF=8$) & BAGE4₂₂₋₃₁ ($p>0.9999$, $q=0.08425$, $DF=8$) due to high variability between repeats. This may suggest that the antigen is not presented as effectively to the immune system due to been buried within the multiple layers of the liposome.²⁷⁶ The other possible reason could be the chemical structure of Mal-lipid (**1**) as it has been reported that any change in the structure of the TLR2 agonist (Pam₂Cys) will affect its activity as an adjuvant¹⁹⁹, structures shown in Figure 81.

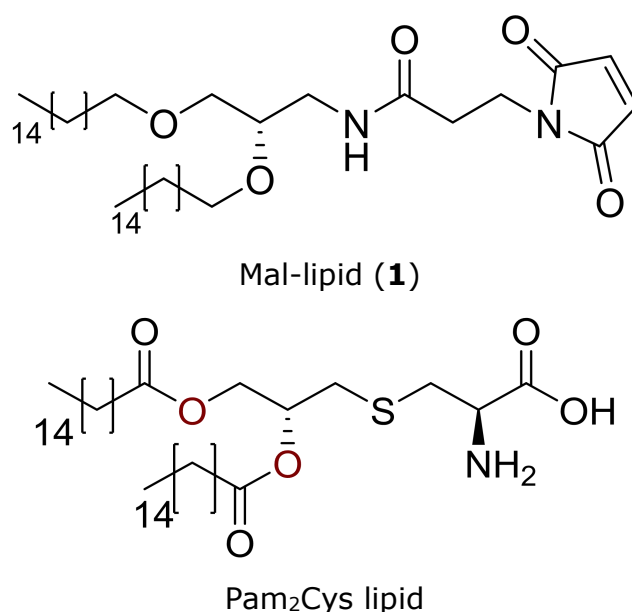


Figure 81: The structure of Mal-lipid (**1**) vs the Pam₂Cys.

The mice dosed with the Pam₂Cys-SK₄-BAGE₄₁₈₋₃₉ (**33**) candidate and the Pam₂Cys-SK₄-BAGE₄₁₈₋₃₉ formulated into liposomes reacted negatively to these vaccines and were culled. There was significant bleeding and darkening at the injection site and the liver appeared abnormal (pale and patchy coloured), Figure 82. Liver abnormality is often a sign of more systemic toxicity as most toxic components are cleared through the liver. Since the reaction was noticed within first 24 hrs it is more likely to be general toxicity rather than immune-mediated as immune-related toxicity takes longer than 24 hrs to manifest effects.^{277,278} The BAGE₄₁₈₋₃₉ antigen (**28**) alone does not show any toxicity and Pam₂Cys is a well-studied and safe adjuvant.^{279,280} Also, the Pam₂Cys-SK₄-NY-ESO-1₁₅₇₋₁₆₅ (**37**) candidate did not show any signs of toxicity. Therefore, an endotoxin test was conducted to exclude bacterial toxicity. The first run was positive which confirms that there was bacteria in the formulation that may be present in the buffer or transferred into the sample via pipette tips but not the vaccine candidates themselves as they are both fully synthetic. Therefore, a new sample was prepared under stricter sterile conditions, and the endotoxin test for this sample was negative. Thus the *in vivo* experiment was repeated using only Pam₂Cys-SK₄-BAGE₄₁₈₋₃₉ (**33**) and the concentration of the dose was 20-fold lower. However, the results remained the same; the mice reacted negatively and were culled within the first 24 hrs. This confirms that this palmitoylated BAGE₄ candidates were toxic. The reason behind this toxicity is currently unclear; further investigations are required to elucidate the mechanisms behind this toxicity.



Figure 82: Pictures of a mouse from each group showing the liver and injection site necropsy.

5.7 Conclusion

In conclusion, the biological evaluation of the synthesised vaccines Pam₂Cys-SK₄-NY-ESO-1₁₅₇₋₁₆₅ (**37**), BAGE₄₁₈₋₃₉ antigen (**28**), BAGE₄₁₈₋₃₉Cys-mal-liposome conjugate, Pam₂Cys-SK₄-BAGE₄₁₈₋₃₉ (**33**), and liposomal formulations prepared with Pam₂Cys-SK₄-BAGE₄₁₈₋₃₉, has been studied by readout of T-cell response using ELISpot assay. The results showed an immunogenic response elicited by Pam₂Cys-SK₄-NY-ESO-1₁₅₇₋₁₆₅ (**37**) conjugate; however, was not strong enough to be taken to cancer models. The data also, shows that the BAGE4 sequence is immunogenic as there are good responses to the whole native BAGE₄₁₈₋₃₉ sequence (**28**) with median of 511 spots per million splenocytes. For the BAGE₄₁₈₋₃₉Cys-mal-liposome conjugate, the immune response is not as effective and somewhat varied with mean of 282 spots per million

splenocytes. For the last two vaccine candidates containing Pam₂Cys-SK₄-BAGE₄₁₈₋₃₉ (**33**) either by itself or formulated with liposome, both candidates were toxic which led to sacrifice of the injected animals within the first 24 hrs, thus, study was concluded.

6. Final Conclusions and Future Research

This thesis describes the synthesis, formulation, and in vivo evaluation of tissue-restricted cancer antigens covalently attached to a lipid adjuvant. Chapter 2 and 3 describes efforts towards the preparation of the lipid building block (Pam₂Cys) and studies towards the conjugation of this component to the CTA peptides (BAGE4₁₈₋₃₉ & NY-ESO-1₁₅₇₋₁₆₅) to develop a self-adjuvanting peptide-based vaccine using two different synthetic routes discussed in Chapter 3. Further engineering of the peptide antigens to include a solubility tag was also undertaken. The adjuvant was conjugated to these versions of the CTAs to produce vaccine constructs with higher aqueous solubility.

Chapter 4 describes the successful formulation of liposomes decorated with the BAGE4₁₈₋₃₉ antigen via reaction with a maleimide linker. These particles can be used as a delivery tool, an antigen display scaffold, and (potentially) an adjuvant. The chapter initially describes the synthesis of a maleimide-containing lipid (Mal-lipid **1**) and formulation of this compound into liposomes. This is followed by the successful optimisation of Cys-BAGE4₁₈₋₃₉ conjugation, resulting a BAGE4Cys-maleimide-liposome formulation. This is considered as a simple system but less effective compared to the native linker of Pam₂Cys which may affect the adjuvant ability of Pam₂Cys. Therefore, a second formulation that incorporates the native Pam₂Cys linked to the BAGE4₁₈₋₃₉ was formulated for comparison.

Chapter 5 describes the biological evaluation of the vaccines described in the previous chapters: Pam₂Cys-SK₄-NY-ESO-1₁₅₇₋₁₆₅, BAGE4₁₈₋₃₉ antigen, BAGE4Cys-Mal-lipo formulation, Pam₂Cys-SK₄-BAGE4₁₈₋₃₉, and formulated Pam₂Cys-SK₄-BAGE4₁₈₋₃₉ liposome by readout of T-cell response using ELISpot assay. The results showed an immunogenic response was elicited by the Pam₂Cys-SK₄-NY-ESO-1₁₅₇₋₁₆₅ conjugate; however, it was not strong enough to be taken to cancer models. The data also shows that the BAGE4₁₈₋₃₉ sequence is immunogenic as there

are good responses to the native BAGE4₁₈₋₃₉ sequence. For BAGE4Cys-maleimide-liposome formulation, the immune response is not as effective and somewhat varied. Unfortunately, both of the last two vaccine candidates; Pam₂Cys-SK₄-BAGE4₁₈₋₃₉ administered alone and formulated into liposomes, were toxic to the model which led to sacrifice of the animals within the first 24 hrs, thus, study was stopped.

Future work based on Chapter 2 and 3 may involve insertion of a peptide solubility tag (SK₄) into the Pam₂Cys-PADRE-NY-ESO-1₁₅₇₋₁₆₅ (**35**) conjugation as this should simplify the purification of this vaccine construct. This construct may induce a more effective immune response compared to Pam₂Cys-SK₄-NY-ESO-1₁₅₇₋₁₆₅ (**37**) due to the presence of the T-helper epitope. The exploration of alternative CTA sequences such as PAGE4 or MAGE could also be considered. Further engineering of these antigens include insertion of different T helper epitopes sequence such as universal T-helper epitope human IL-1b peptide (VQGEESNDK, amino acids 163–171).^{281,282}

Future work based on Chapter 4: since the BAGE4₁₈₋₃₉ sequence (**28**) showed a positive and targeted immune response, further study and investigation should be considered. This could involve further engineering of the vaccine. Insertion of a T helper sequence into the Pam₂Cys-BAGE4 scaffold should be evaluated. Encapsulation of the BAGE4₁₈₋₃₉ sequence (**28**) into the liposomes or insertion of T helper epitopes into the BAGE4₁₈₋₃₉ sequence and conjugation to the mal-liposome could be considered. This approach could also be applied to different CTA peptides for comparison and evaluation. Also, the Pam₂Cys-SK₄-NY-ESO-1₁₅₇₋₁₆₅ (**37**) conjugate could be formulated into a liposome and the immune response compared to the conjugate alone.

Future research based on Chapter 5 could include further *in vivo* experiments to ascertain the toxicity of the Pam₂Cys-SK₄BAGE4, however, this work may be difficult to justify, ethically.

Table 12: Summary of the constructs that have been synthesised, the purpose of synthesis, the experiments involved and their outcomes.

Compound	The purpose of Synthesis	Experiment involved and outcomes
Lipid ether maleimide (1)	To be formulated as a liposome to enable the conjugation of CTA, via terminal Cys, to the external surface via the maleimide linker.	The synthesis of compound 1 was successful. Formulated successfully with in Mal-lipo -ve & Mal-lipo +ve Conjugated successfully to CysBAGE4 ₁₈₋₃₉ (40) Conjugation attempted to NY-ESO-1 ₁₅₇₋₁₆₅ (29), however, aggregation occurred. Coumarin- CHAKTKEAK (54), however, aggregation occurred.
(<i>R</i>)-3-iodopropane-1,2-diyl dipalmitate (2)	To be conjugated to H-CITGF-OH (15) as a simple model of a CTA.	The (<i>R</i>)-3-iodopropane-1,2-diyl dipalmitate (2) was synthesised successfully. Conjugation to H-CITGF-OH (15) was attempted under several conditions but was not successful.
Fmoc-Pam ₂ Cys (3)	To be conjugated to the CTA to synthesise a self-adjuvanting-CTA vaccine construct (Figure 28, route C)	The synthesis of compound 3 was successful. Compound 3 was conjugated to H-HAKTKEA-NH ₂ (30) for optimization then to BAGE4 ₁₈₋₃₉ (28), NY-ESO-1 ₁₅₇₋₁₆₅ SK ₄ (38) & PADRE-NY-ESO-1 ₁₅₇₋₁₆₅ (36) successfully.
H-CITGF-OH (15)	Model peptide sequence that carries a Cys at the N-terminus which makes it ideal for optimisation of conjugation conditions to iodo-lipid probe (2) as per Figure 28, route A.	Peptide 15 was synthesised successfully however, the conjugation reaction to iodo-lipid probe (2) was not successful.
BAGE4 ₁₈₋₃₉ (28)	A member of the CTA family and the central focus of this thesis. To be conjugated to Fmoc-Pam ₂ Cys (3) as shown in Figure 28, route C. To be injected into Balb/c mice and the frequency of peptide-specific IFN γ secreting T-cells quantified by	The synthesis of BAGE4 ₁₈₋₃₉ (28) was successful. The conjugation to compound 3 was successful with insertion of SK ₄ as a solubility tag to create the Pam ₂ Cys-SK ₄ -BAGE4 ₁₈₋₃₉ (33) construct. The ELISpot indicated that a good level of immunogenic specific response was elicited by BAGE4 ₁₈₋₃₉ (28) as shown in Figure 80A. It has been confirmed that BAGE4 ₁₈₋₃₉ (28) does bind to Anti-BAGE4

	ELISpot assay. ELISA assay to study the antigen- antibody binding.	antibody at a concentration of 10 ng/mL BAGE4 ₁₈₋₃₉ antigen (28) with 1:250 antibody concentration, Figure 75
NY-ESO-1 ₁₅₇₋₁₆₅ (29) & NY-ESO-1 ₁₅₇₋₁₆₅ SK ₄ (38)	A member of the CTA family with reported specific immune response. To be conjugated to Fmoc-Pam ₂ Cys (3) as shown in Figure 28, route C. ELISA assay to study the antigen- antibody binding.	The synthesis of NY-ESO-1 ₁₅₇₋₁₆₅ (29) was successful, however; purification was problematic due to poor solubility. Thus peptide (38) was synthesised with solubility tag SK ₄ . The conjugation to compound 3 was unsuccessful, however; it was successful with ESO-1 ₁₅₇₋₁₆₅ SK ₄ (38) producing the Pam ₂ Cys-SK ₄ -NY-ESO-1 ₁₅₇₋₁₆₅ (34) construct. No binding was recorded between NY-ESO-1 ₁₅₇₋₁₆₅ (29) and the antibody Anti-NY-ESO-1 antibody using ELISA assays as shown in Figure 77.
H-HAKTKEA-NH ₂ (30)	Model peptide sequence taken from one of the CTA peptides suitable for optimising the coupling conditions of peptide palmitoylation using protocol A & B shown in Figures 37 & 38. Optimisation of the conjugation to Mal-lipo with some modifications as per peptides 53 & 54 .	The synthesis of H-HAKTKEA-NH ₂ (30) was successful. The palmitoylation was successful using both protocols A & B resulting pure Pam ₂ Cys-HAKTKEA (31) construct. No further tested was required. Applying the necessary modifications to (30) (attachment of a fluorescent tag & adding a Cys terminus) was successful and resulted in the preparation of FITC-CHAKTKEAK (53) & coumarin-CHAKTKEAK (54), however; conjugate both to Mal-lipo was unsuccessful.
Pam ₂ Cys-SK ₄ -BAGE4 ₁₈₋₃₉ (33) & Pam ₂ Cys-SK ₄ -NY-ESO-1 ₁₅₇₋₁₆₅ (34)	The aim of this thesis is to develop library of self-adjuvanting CT antigens against cancer using constructs 33 and 34 .	Synthesis of Pam ₂ Cys-SK ₄ -BAGE4 ₁₈₋₃₉ (33) & Pam ₂ Cys-SK ₄ -NY-ESO-1 ₁₅₇₋₁₆₅ (34) was successful. The ELISpot indicated an immunogenic specific response was elicited by Pam ₂ Cys-SK ₄ -NY-ESO-1 ₁₅₇₋₁₆₅ (34); however; it was not strong enough for further <i>in vivo</i> testing, Figure 79. Pam ₂ Cys-SK ₄ -BAGE4 ₁₈₋₃₉ (33) construct was toxic which led to sacrifice of the injected animals; thus, study was concluded.
Pam ₂ Cys-PADRE-NYESO-1 ₁₅₇₋₁₆₅ (35)	A self-adjuvanting CT antigen construct against cancer. To determine if inclusion of a T-helper epitope, PADRE (34), could induce a	Synthesis of Pam ₂ Cys-PADRE-NYESO-1 ₁₅₇₋₁₆₅ (35) was successful, however; the purification was problematic and led to very poor yield. Not enough material was isolated for <i>in vivo</i> experiments. Due to time limitation no further

	stronger immune response compared to construct 34 .	optimisation was possible.
CysBAGE4 ₁₈₋₃₉ (40)	To be conjugated to Mal-lipo -ve & Mal-lipo +ve via the maleimide linker of compound 1 producing BAGE4 ₁₈₋₃₉ Cys-mal-liposome conjugate.	The conjugation to both Mal-lipo -ve & Mal-lipo +ve formulation was successful. ELISpot indicated that an immunogenic specific response was elicited by BAGE4 ₁₈₋₃₉ Cys-mal-liposome conjugate, Figure 80B, however; the response was lower by almost half compared to BAGE4 ₁₈₋₃₉ (28) response, Figure 80A.
BAGE4 ₁₈₋₃₂ (56) & BAGE4 ₂₃₋₃₁ (57)	Smaller peptides from within the sequence of BAGE4 ₁₈₋₃₉ (28) used for <i>in vivo</i> study. Predicted by the epitope prediction software to be potential peptides that the T cells may recognise and respond to, thus used as stimulator peptides in ELISpot assays for BAGE4 ₁₈₋₃₉ peptide/Cys-BAGE4 ₁₈₋₃₉ liposome conjugate.	The data shows that the BAGE4 ₂₂₋₃₁ sequence (57) can stimulate T cell responses that recognise BAGE4 with slightly lower responses compared to the complete native BAGE4 ₁₈₋₃₉ (28) sequence. Responses to BAGE4 ₁₈₋₃₂ (56) was low, Figure 80.

Chapter 7: Experimental

7.1 General Methods

NMR: NMR analysis was performed by dissolving 20 mg of product in the specified deuterated solvent. This sample was then analysed on a Bruker AVIII 400 NMR system. The frequency for ^1H -NMR was 400 MHz, and for ^{13}C -NMR was 100 MHz. Chemical shifts are reported in parts per million (ppm) and are referenced to solvent residual signals: CDCl_3 (δ 7.26 [^1H]; δ 77.16 [^{13}C]), $\text{DMSO}-d_6$ (δ 2.50 [^1H]; δ 39.52 [^{13}C]), CD_3OD (δ 3.31 [^1H]; δ 49.00 [^{13}C]), D_2O (δ 4.79 [^1H]). ^1H NMR data is reported as chemical shift (δ), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublets, ddd = doublet of doublet of doublets or combinations of these multiples; m = unassigned multiplet), relative integral, coupling constant (J Hz) and assignment where possible.

High resolution mass spectrometry (HRMS): Samples were dissolved in a suitable solvent, usually either water, acetonitrile or methanol. These were analysed on a Bruker micrOTOF focus II MS (ESI) operating in positive or negative ionisation mode.

Analytical HPLC: The instrument used was a Thermo Ultimate 3000 uHPLC. The instrument had a PDA detector, set to analyse at 210, 254, 280 and 450 nm. The column oven was set to 45 °C. Peptides were analysed using a Waters SunFire 5 μm , 2.1 x 150 mm column (C-18) at a flow rate of 0.6 $\text{mL}\cdot\text{min}^{-1}$. The mobile phase composed of 0.1% trifluoroacetic acid in H_2O (Solvent A) and 0.1% trifluoroacetic acid in acetonitrile (Solvent B). The analysis of the chromatograms was conducted using Chromeleon 7 software.

Preparative reverse-phase HPLC: A Waters 1525 binary pump HPLC was used. Peptides were purified on a Waters SunFire 5 μm (C-18) preparative column with 5- μm particle size, 19 x 150 mm, operating at a flow rate of 6 $\text{mL}\cdot\text{min}^{-1}$ using a mobile phase of 0.1% trifluoroacetic acid in water (Solvent A) and 0.1% trifluoroacetic acid in acetonitrile (Solvent B) using the gradient specified for each specific compound. Semi-

preparative reverse-phase HPLC was performed using the same HPLC and solvent system. Lipopeptides were purified on a Waters SunFire 5 μm (C-4) Semi-preparative column, 10 x 250 mm, operating at a flow rate of 5 $\text{mL}\cdot\text{min}^{-1}$ using the gradient specified for each specific compound. The dual wavelength detector was set to 210 and 280 nm. Fractions were collected manually. The gradients used are specified for each individual peptide/lipopeptide sequence later in this section.

Materials

Commercial materials were used as received unless otherwise noted. Amino acids, coupling reagents and resins were obtained from Novabiochem, Fluorochem or GL Biochem. Reagents that were not commercially available were synthesised as outlined for each specific compound. Solvents were obtained as reagent grade from Merck or Fisher.

Solid Phase Peptide Synthesis (SPPS)

Following are our protocols for manual peptide synthesis – fritted syringes (Torviq, USA) were used with a stainless-steel needle. The syringes were capped and agitated on a rotating table at room temperature during coupling. Syntheses were carried out on a 0.25 mmole scale unless otherwise stated; loading of the resin was 0.5 – 0.8 mmol/g.

Preloading Rink Amide resin: Rink amide resin was initially washed with DCM (5 $\times$ 3 mL) followed by removal of the Fmoc group by treatment with 20% piperidine/DMF (2 $\times$ 5 min). The resin was washed with DMF (5 $\times$ 3 mL), DCM (5 $\times$ 3 mL) and DMF (5 $\times$ 3 mL). Oxyma (4 eq.) and DIC (4 eq.) were added to a solution of Fmoc-AA-OH (4 eq.) in DMF. After 5 min of pre-activation, the mixture was added to the resin. After 2 h the resin was washed with DMF (5 $\times$ 3 mL), DCM (5 $\times$ 3 mL) and DMF (5 $\times$ 3 mL), capped with acetic anhydride/pyridine (1:9 v/v) (2 $\times$ 3 min) and washed with DMF (5 $\times$ 3 mL), DCM (5 $\times$ 3 mL) and DMF (5 $\times$ 3 mL).

Preloading 2-chlorotrityl chloride resin: 2-Chlorotrityl chloride resin was swollen in DCM for 30 min then washed with DCM (2 × 3 mL). A solution of Fmoc-AA-OH (0.5 eq. relative to resin functionalization) and *i*Pr₂NEt (2 eq. relative to resin functionalization) in DCM (final concentration 0.1 M of amino acid) was added and the resin shaken at rt for 16 h. The resin was washed with DMF (5 × 3 mL) and DCM (5 × 3 mL). The resin was treated with a solution of DCM/CH₃OH/*i*Pr₂NEt (17:2:1 v/v/v, 3 mL) for 1 h and washed with DMF (5 × 3 mL), DCM (5 × 3 mL), and DMF (5 × 3 mL). The resin was subsequently submitted to iterative peptide assembly (Fmoc-SPPS).

Estimation of amino acid loading: The resin was treated with 20% piperidine/DMF (2 × 3 mL, 3 min) and 20 µL of the combined deprotection solution was diluted to 10 mL using 20% piperidine/DMF in a volumetric flask. The UV absorbance of the resulting piperidine-fulvene adduct was measured ($\lambda = 301$ nm, $\epsilon = 7800$ M⁻¹ cm⁻¹) to determine the amount of amino acid loaded onto the resin.

Manual Iterative peptide assembly (Fmoc-SPPS)

General amino acid coupling: A pre-activated solution of protected amino acid (4 eq.), DIC (4 eq.) and Oxyma (4 eq.) in DMF (4 – 6 mL with final concentration 0.1 M) was added to the resin. After 1 h, the resin was washed with DMF (5 × 3 mL), DCM (5 × 3 mL) and DMF (5 × 3 mL). For precious amino acids, 2 eq. of all reagents were used, and the coupling left overnight (the coupling left overnight only applicable to DIC/Oxyma).

Capping: Acetic anhydride/pyridine (1:9 v/v) was added to the resin (3 mL). After 3 min the resin was washed with DMF (5 × 3 mL), DCM (5 × 3 mL) and DMF (5 × 3 mL).

Deprotection: The resin was treated with 20% piperidine /DMF (3 mL, 2 × 3 min) and washed with DMF (5 × 3 mL), DCM (5 × 3 mL) and DMF (5 × 3 mL). Final N-terminal deprotection: Once the sequence has been

completed, the N-terminal Fmoc group was removed by treatment with 20% piperidine/DMF (2 x 5 min). The resin then was washed with DMF (10 x) and DCM (10x) and then dried in *vacuo*.

Cleavage: A mixture of TFA, triisopropylsilane (TIS) and water (90:5:5 v/v/v) was added to the resin. For peptides containing cysteine or methionine, a mixture of TFA, triisopropylsilane (TIS), thioanisole and water (85:5:5:5 v/v/v/v) was used. After 2 hrs, the solution was plunged into a 50 mL falcon tube and evaporated under a stream of N₂ to < 5 mL (if required). Diethyl ether (approx. 30 mL) was added to precipitate the peptide and the solution centrifuged. The supernatant was discarded, and the pellet dissolved in 0.1% TFA water/MeCN for HPLC purification.

Automated solid-phase peptide synthesis

Automated Fmoc-SPPS was carried out on a Biotage Initiator⁺ Alstra microwave peptide synthesizer. General synthetic procedures for Fmoc-deprotection and capping were carried out in accordance with the manufacturer's specifications. Standardized amino acid couplings were performed for 15 mins at 50 °C under microwave irradiation in the presence of amino acid (0.5 M in DMF, 4 eq.), Oxyma (0.5 M in DMF, 4 eq.) and diisopropylcarbodiimide (0.5 M in DMF, 4 eq.). Peptide cleavage and work-up were carried out as described above for manual SPPS.

7.2 General Protocols

General procedure for coupling Pam₂Cys to antigens (Protocol, A)

The attachment of Fmoc-diol-Cys (**5**) was achieved by dissolving a mixture of Fmoc-diol-Cys (1.5 eq.), DIC (1.5 eq.) and Oxyma (1.5 eq.) in DMF (2 mL) at 0 °C for 5 mins. The activated species was then added to the resin and left to couple for 4 hrs, followed by a thorough wash with DMF and DCM. The S-glycerol-cysteine hydroxyl groups were then

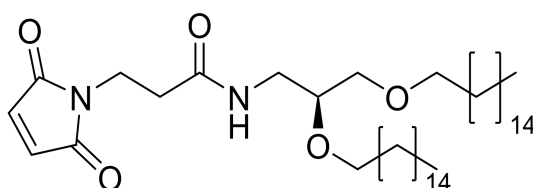
palmitoylated by addition of palmitic acid (20 eq.) activated with DIC (25 eq.) and 4-(dimethylamino) pyridine (DMAP; 2 eq.) in DCM. The reaction was left to complete overnight, and the resin subsequently washed with DCM. The Fmoc group was deprotected after this period using 20% piperidine and the resin cleaved using 85% TFA as detailed in the standard Fmoc-SPPS procedures above. The crude product was purified via preparative RP-HPLC using a C-4 semi-preparative column (see the general methods section for column details).

General procedure for coupling Pam₂Cys to antigens (Protocol, B)

1.2 eq of Fmoc-Pam₂Cys (**3**) was dissolved in DMF, and activated by DIC (1.8 eq.) and Oxyma (1.8 eq.) for 5 mins before addition to the resin and agitated overnight at RT. After this period, the resin was washed as described in the Fmoc-SPPS protocols, the Fmoc group was removed by treatment with 20% piperidine, and the resin cleaved by 85% TFA as detailed in the Fmoc-SPPS section, the crude product was purified via preparative RP-HPLC using a C-4 semi-preparative column (see the general methods section for column details).

7.3 Lipids Synthesis

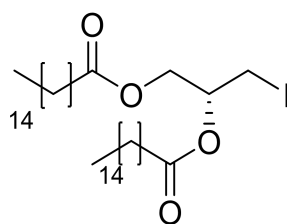
Synthesis of lipid ether maleimide (**1**) using DMTMM.



To a solution of 3-maleimidopropionic acid (**45**, 0.027 g, 0.16 mmol) in DMF (5 mL) was added DMTMM (0.044 g, 0.16 mmol) and NMM (0.032 g, 0.32 mmol). This solution was stirred for 5 minutes before the addition of lipid ether amine (**44**, 0.085 g, 0.16 mmol). The solution was left to stir for 24 hrs before being acidified with 1 M HCl and the aqueous extracted with EtOAc (3 x 50 mL). The combined organic layers were washed with

water (3 x 50 mL), brine (1 x 50 mL), dried over MgSO₄, filtered and concentrated *in vacuo*. The resultant oil was purified by flash column chromatography (DCM to DCM: MeOH (9:1) and dried under high *vacuo* to yield a creamy-solid (**1**, 0.023 g, 21% yield). HRMS *m/z* Calc. for C₄₂H₇₈N₂O₅ [M+Na⁺]: 713.5809. Obs. [M+Na⁺]: 713.5803 (error 0.85 ppm). **¹H NMR** (400 MHz, CDCl₃) δ 6.69 (s, 2H, CH=CH), 5.98 – 5.92 (m, 1H, -NH), 3.84 (t, *J* = 7.3 Hz, 2H, CH₂-CH₂-N), 3.60 – 3.50 (m, 2H, CH_aH_b-N and CH-O), 3.51 – 3.35 (m, 6H, CH₂-O), 3.31 – 3.23 (m, 1H, CH_aH_b-N), 2.50 (t, *J* = 7.3 Hz, 2H, CH₂-CH₂-N), 1.58 – 1.51 (m, 4H, CH₂-CH₂-O), 1.34 – 1.21 (m, 52H, -(CH₂)₁₃-), 0.88 (t, *J* = 6.7 Hz, 6H, -CH₃). **¹³C NMR** (100 MHz, CDCl₃) δ 170.9 (C), 169.9 (C), 134.6 (CH), 76.9 (CH), 72.3 (CH₂), 72.0 (CH₂), 70.6 (CH₂), 41.3 (CH), 35.16 (CH₂), 34.76 (CH₂), 32.5 (CH₂), 30.6 (CH₂), 30.3 (CH₂), 30.2 (CH₂), 30.1 (CH₂), 29.9 (CH₂), 26.7 (CH₂), 23.3 (CH₂), 14.7 (CH₃).

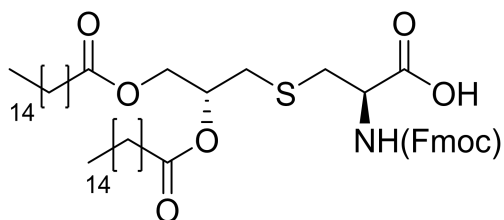
Synthesis of ®-3-iodopropane-1,2-diyl dipalmitate (**2**)



(*R*)-3-iodopropane-1,2-diyl dipalmitate (**4**, 0.140 g, 0.69 mmol) and pyridine (0.11 g, 1.39 mmol) were dissolved in DCM (10 mL). Freshly distilled palmitoyl chloride (0.39 g, 1.4 mmol) was added dropwise on ice over 30 mins. The stoppered flask was kept at RT for 12 hrs. Pyridinium chloride was removed by filtration. The filtrate was evaporated at 50°C under *vacuo*. Acetone (5 mL) was added to the residual oil. The mixture was kept in the refrigerator for crystallization. The crystals were collected by filtration and recrystallized from acetone to give the desired product (**2**, 0.135 g, 29% yield) which was stored at 0°C in the dark. HRMS *m/z* Calc. for C₃₅H₆₇IO₄ [M+Na⁺]: 701.3979. Obs. [M +Na]⁺: 701.3972 (error 1.0 ppm). **¹H NMR** (400 MHz, CDCl₃) δ 5.06 – 4.99 (m, 1H, CH-O), 4.33 (dd, *J* = 11.9, 4.2 Hz, 1H, CH_aH_b-O), 4.22 (dd, *J* = 11.9, 5.7 Hz, 1H, CH_aH_b-O), 3.37 (dd, *J* = 10.6, 5.8 Hz, 1H, CH_aH_b-I), 3.30 (dd,

$J = 10.6, 5.7 \text{ Hz}$, 1H, $\text{CH}_a\text{H}_b\text{-I}$), 2.40 – 2.30 (m, 4H, $-\text{CH}_2\text{-COO}$), 1.70 – 1.58 (m, 4H, $-\text{CH}_2\text{-CH}_2\text{-COO}$), 1.40-1.20 (m, 48H, $-(\text{CH}_2-)_{12}$), 0.90 (t, $J = 6.9 \text{ Hz}$, 6H, $-\text{CH}_3$). **^{13}C NMR** (100 MHz, CDCl_3) δ 173.4 (C), 172.9 (C), 70.1 (CH), 64.3 (CH_2), 34.4 (CH_2), 29.9 (CH_2), 29.8 (CH_2), 29.6 (CH_2), 29.5 (CH_2), 29.4 (CH_2), 29.3 (CH_2), 25.1 (CH_2), 22.8 (CH_2), 14.3 (CH_3), 2.6 (CH_2).

Synthesis of Fmoc-Pam₂Cys (**3**) (Method 1)



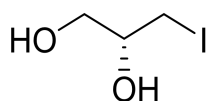
Allyl-fmoc-Pam₂Cys (**21**, 300 mg, 0.3215 mmol, 1 eq.) was dissolved in dry THF (9 mL) and placed under an atmosphere of nitrogen before the addition of *triphenylsilane* (277 mg, 2.2508 mmol, 7 eq.) and *tetrakis*(triphenylphosphine) palladium(0) (37.15 mg, 0.0321 mmol, 0.1 eq.). The RM was stirred at RT for 18 hrs. Purification was carried out via column chromatography (eluent: DCM MeOH/ AcOH 95:5:1) followed by repurification via column chromatography (eluent: DCM → DCM/ MeOH 95:5) to give the product as a light brown solid (70 mg, 0.078 mmol, 24%).

Method 2²¹¹

TFA (130.7 mmol, 14.9 g, 9.1 mL) was added to compound **24** (1.2 mmol, 1.1 g) and the solution was stirred for 1hr at RT. Co-evaporation of TFA with toluene (3 x 10 mL) resulted in a crude compound. Purification of the crude by column chromatography (15%-20% EtOAc in PE/ 1% AcOH) yielded the final building block **3**, (*R*)-Fmoc-Pam₂Cys-OH (1.03 mmol, 0.9195 g, 86%). HRMS *m/z* Calc. for C₅₃H₈₃NO₈S [M+Na]⁺: 916.5737. Obs. [M+Na]⁺: 916.5729 (error 0.87 ppm). **¹H NMR** (400 MHz, CDCl₃) δ 7.78 (d, *J* = 7.5 Hz, 2H, Ar*H*), 7.63 (d, *J* = 7.5 Hz, 2H, Ar*H*), 7.42 (t, *J* = 7.4 Hz, 2H, Ar*H*), 7.34 (t, *J* = 7.4 Hz, 2H, Ar*H*), 5.80 (d, *J* = 7.8 Hz, 1H, -NH), 5.24 – 5.14 (m, 1H, CH-OH), 4.71 – 4.62 (m, 1H, CH-N), 4.43 (d, *J* = 7.2 Hz, 2H, Fmoc-CH₂-O), 4.37 (dd, *J* = 11.8, 3.3 Hz, 1H, CH_aH_b-O), 4.26 (t, *J* = 7.0 Hz, 1H, Fmoc-CH-), 4.20 – 4.16 (m, 1H, CH_aH_b-O), 3. – 3 – 3.04 (m, 2H, C(N)-CH₂-S), 2.82 – 2.27 (m, 2H, C(O)-CH₂-S), 2.38 – 2.28 (m, 4H, -CH₂-COO), 1.68 – 1.55 (m, 4H, -CH₂-CH₂-COO), 1.37 – 1.20 (m, 48H, -(CH₂)₁₂), 0.90 (t, *J* = 6.7 Hz, 6H, -CH₃). **¹³C NMR** (100 MHz, CDCl₃) δ 173.4 (C), 173.3 (C), 172.1 (C),

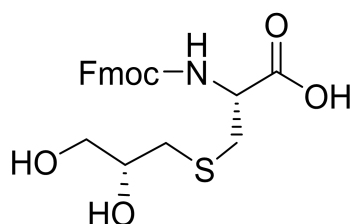
155.7 (C), 143.5 (C), 141.1 (C), 127.6 (CH), 126.9 (CH), 125.0 (CH), 119.8 (CH), 70.1 (CH), 67.2 (CH₂), 63.4 (CH₂), 50.9 (CH), 46.9 (CH), 34.5 (CH₂), 34.1 (CH₂), 33.9 (CH₂), 32.8 (CH₂), 31.8 (CH₂), 29.6 (CH₂), 29.5 (CH₂), 29.5 (CH₂), 29.3 (CH₂), 29.2 (CH₂), 29.1 (CH₂), 29.0 (CH₂), 28.9 (CH₂), 24.7 (CH₂), 24.6 (CH₂), 22.5 (CH₂), 14.0 (CH₃).

Synthesis of (*R*)-3-iodopropane-1,2-diol (**4**)



To an ice-cold solution of 4-iodomethyl-2,2-dimethyl-1,3- dioxolane (**7**, 0.1 g, 0.42 mmol) in acetone (0.5 mL), was added 2 M HCl (0.5 mL, 0.83 mmol). The reaction mixture was stirred at 60 °C for 4 hrs. After this time, the mixture was neutralized with a saturated solution of aq. NaHCO₃ and extracted with ethyl acetate. The organic layer was dried over Na₂SO₄. The solvent was then removed under *vacuo*. The product was isolated as a yellow solid (**4**, 0.064 g, 312 μmol, 75%); no purification was performed.

Synthesis of Fmoc-*R*-(2,3-dihydroxypropyl) Cysteine (**5**) (Method 1)



Allyl-fmoc-diol-Cys (**20**, 210 mg, 0.4595 mmol, 1 eq.) was dissolved in dry THF (4 mL) and placed under an atmosphere of nitrogen before the addition of *triphenylsilane* (837.7 mg, 3.2 mmol, 7 eq.) and *tetrakis*(triphenylphosphine) palladium(0) (52.5 mg, 0.046 mmol, 0.1 eq.). The RM was stirred at RT for 18 hrs. The crude product was purified via preparative RP-HPLC using a C-18 column with a 30-50% solvent B gradient over 30 min to yield Fmoc-diol-Cys (**5**) (16.5 mg, 0.0396 mmol, 9%). HRMS m/z Calc. for $C_{21}H_{23}NO_6S$ $[M - H]^+$: 416.1168, $[2M - H]^+$: 833.2492 Obs. $[M - H]^+$: 416.1181, $[2M - H]^+$: 833.2392 (error 3.12 ppm). **^1H NMR** (400 MHz, $CDCl_3$) δ 7.67 (d, J = 7.6 Hz, 2H, ArH), 7.56 – 7.48 (m, 2H, ArH), 7.31 (t, J = 7.5 Hz, 2H, ArH), 7.22 (t, J = 7.4 Hz, 2H, ArH), 6.28 – 6.21 (m, 1H, -NH), 4.62 – 4.54 (m, 1H, CH-N), 4.38 – 4.18 (m, 1H, -OH), 4.35 – 4.25 (m, 2H, Fmoc- CH_2 -O), 4.12 (t, J = 6.8 Hz, 1H, Fmoc-CH-), 3.90 – 3.72 (m, 1H, CH-OH), 3.72 – 3.58 (m, 1H, CH_aH_b -O), 3.58 – 3.44 (m, 1H, CH_aH_b -O), 3.08 – 2.92 (m, 2H, C(N)- CH_2 -S), 2.70 – 2.56 (m, 2H, C(O)- CH_2 -S). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 173.6 (C), 156.3 (C), 143.7 (C), 143.50 (C), 141.2 (C), 127.8 (CH), 127.1 (CH), 125.1 (CH), 120.0 (CH), 71.2 (CH), 67.4 (CH_2), 64.9 (CH_2), 53.8 (CH), 46.9 (CH), 37.0 (CH_2), 35.0 (CH_2).

Method 2⁷⁸

A solution of fluorenylmethoxycarbonyl-*N*-hydroxysuccinimide (Fmoc-OSu) (6.1 g, 18.2 mmol) in acetonitrile (20 mL) was added to a solution of *R*-(2,3-dihydroxypropyl) cysteine (**27**, 3.5 g, 18.2 mmol) in 9% sodium carbonate (20 mL). The reaction mixture was stirred at room temperature for 4 hrs. After this time, water (50 mL) was added, and the solution was acidified to pH 2 with concentrated hydrochloric acid and then extracted with ethyl acetate (3 × 50 mL). The combined organic layers were washed with water (2 × 50 mL) and brine (2 × 50 mL), dried over anhydrous MgSO₄ and evaporated *in vacuo* to give sticky off white solid. Recrystallization from ether and ethyl acetate at -20 °C yielded a white powder (**5**, 0.87 g, 3.6 mmol, 20%). HRMS *m/z* Calc. for C₂₁H₂₃NO₆S [M - H]⁺: 416.1168, [2M - H]⁺: 833.2492 Obs. [M - H]⁺: 416.1181, [2M - H]⁺: 833.2392 (error 3.12 ppm).

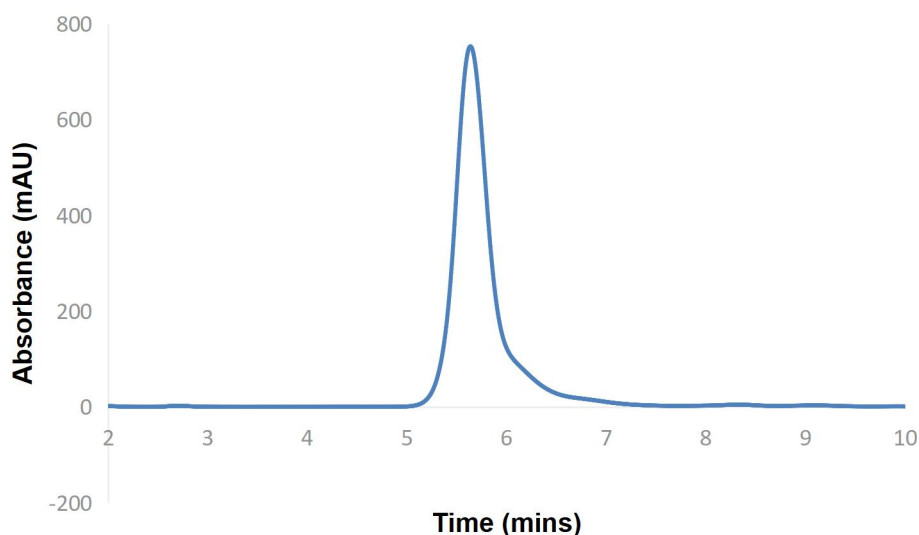
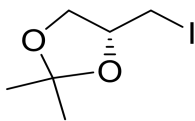


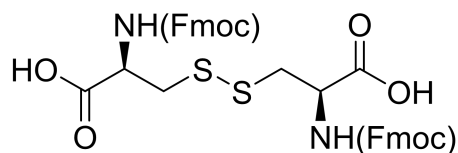
Figure 83: Analytical HPLC trace of Fmoc-*R*-(2,3-dihydroxypropyl) Cysteine (**5**). Analytical gradient 30% isocratic for 14 min; 0.6 ml/min, 280 nm.

Synthesis of Iodomethyl-2,2-dimethyl-1,3-dioxolane (**7**)



To a solution of 2,2-dimethyl-1,3-dioxolan-4-yl-methanol (**6**, 1 g, 7.57 mmol) in toluene (20.4 mL) was added triphenylphosphine (5 g, 19.0 mmol), imidazole (0.769 g, 11.3 mmol), and iodine (5 g, 19.6 mmol). The reaction mixture was stirred at 90 °C for 5 hrs, under N₂, in a dark. After removal of toluene under reduced pressure, the residue was dissolved in DCM, washed with saturated Na₂S₂O₃, brine and dried over Na₂SO₄. The solvent was then removed under *vacuo* and the residue was purified by silica gel flash column chromatography (hexane/ethyl acetate, 9.8:0.2) to give the desired compound as a colourless liquid (**7**, 0.95 g, 3.9 mmol, 52%). **¹H NMR** (400 MHz, CDCl₃) δ 4.32 – 4.26 (m, 1H, CH-O), 4.16 (dd, *J* = 8.6, 6.1 Hz, 1H, CH_aH_b-O), 3.80 (dd, *J* = 8.6, 5.4 Hz, 1H, CH_aH_b-O), 3.27 (dd, *J* = 9.8, 4.6 Hz, 1H, CH_aH_b-I), 3.15 (dd, *J* = 9.8, 8.4 Hz 1H, CH_aH_b-I), 1.47 (s, 3H, -CH₃), 1.36 (s, 3H, -CH₃). **¹³C NMR** (101 MHz, CDCl₃) δ 110.3 (C), 75.5 (CH), 69.4 (CH₂), 27.0 (CH₃), 25.4 (CH₃), 6.7 (CH₂).

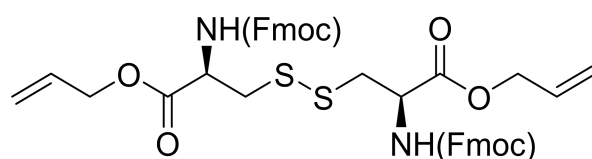
Synthesis of Fmoc-Cystine (**17**)



A suspension of Fmoc-OSu (8.4 g, 25.0 mmol, 2.4 eq.) in MeCN (35 mL) was added to a stirred solution of L-cystine (2.5 g, 10.4 mmol, 1 eq.) and NEt₃ (6 mL, 41.5 mmol, 4 eq.) in water (60 mL) at 0 °C. The RM was stirred at RT for 16 hrs. HCl (1 M, 50 mL) and EtOAc (ca. 150 mL) were added to the RM and the organic phase was washed with HCl (1 M, 150

mL), brine (2 x 150 mL) and water (150 mL). The organic phase was dried over MgSO_4 , filtered and the solvent was removed under reduced pressure. Crude product obtained as a white solid (6.7 g, 9.79 mmol, 94%). HRMS m/z Calc. for $\text{C}_{36}\text{H}_{32}\text{N}_2\text{O}_8\text{S}_2$ $[\text{M} + \text{H}]^+$: 685.1678, Obs. $[\text{M} + \text{H}]^+$: 685.1677 (error 0.15 ppm). The crude material was taken on to next step without purification.

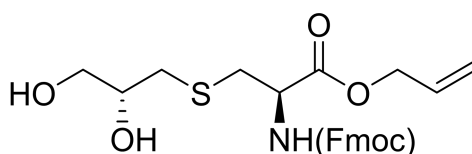
Synthesis of allyl-Fmoc-Cystine (**19**)²⁰⁷



To a stirred solution of Fmoc-cystine (**17**, 6.7 g, 10 mmol, 1 eq.) in DMF (65 mL) at 0 °C, DIPEA (7.25 mL, 41.6 mmol, 4 eq.) was added, followed by allyl bromide (2.8 mL, 31.2 mmol, 3 eq.). The RM was stirred at RT for 3 hrs before being cooled to 0 °C for the further addition of DIPEA (3.6 mL, 20.5 mmol, 2 eq.) and allyl bromide (0.9 mL, 10.4 mmol, 1 eq.). The reaction mixture was stirred at RT for 17 hrs. After this time, the RM was diluted in EtOAc (130 mL) and washed with HCl (1 M, 100 mL), water (5 x 100 mL) then brine (100 mL). The organic phase was dried over MgSO_4 , filtered and the solvent was removed under reduced pressure. Purification was carried out via column chromatography on 5.25 g out of 6.3 g. Eluent: Pentane → Pentane / EtOAc 1:1. Product obtained as a pale-yellow solid (2.5 g, 3.3 mmol, 40 %). **¹H NMR** (400 MHz, CDCl_3) δ 7.75 (d, J = 7.5 Hz, 4H, ArH), 7.50 – 7.58 (m, 4H, ArH), 7.39 (t, J = 7.4 Hz, 4H, ArH), 7.30 (t, J = 7.4 Hz, 4H, ArH), 5.97 – 5.85 (m, 2H, $\text{CH}_2=\text{CH}$), 5.75 (d, J = 7.9 Hz, 2H, -NH), 5.34 (d, J = 17.6 Hz, 2H, $\text{CH}_a\text{H}_b=\text{CH}$), 5.26 (d, J = 12.4 Hz, 2H, $\text{CH}_a\text{H}_b=\text{CH}$), 4.75 – 4.59 (m, 6H, CH-N and =CH- CH_2 -O), 4.45 – 4.35 (m, 4H, Fmoc- CH_2 -O), 4.21 (t, J = 7.0 Hz, 2H, Fmoc-CH-), 3.21 (d, J = 5.2 Hz, 4H, - CH_2 -S). **¹³C NMR** (100 MHz, CDCl_3) δ 169.8 (C), 155.5 (C), 143.5 (C), 141.1 (C), 131.0 (CH), 127.6 (C), 126.9 (C), 124.9 (C), 119.8

(CH), 119.1 (CH₂), 67.1 (CH₂), 66.4 (CH₂), 53.2 (CH), 46.9 (CH), 41.0 (CH₂).

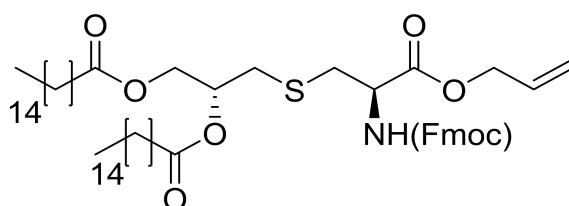
Synthesis of allyl- Fmoc-*R*-(2,3-dihydroxypropyl) Cysteine (20)²⁰⁸



Allyl-fmoc-cystine (**19**, 1.2 g, 1.57 mmol, 1 eq.) was dissolved in DCM (15 mL) and cooled to 0 °C before the addition of zinc dust (0.718 g, 10.99 mmol, 7 eq.) was added to the stirred RM followed by H₂SO₄/HCl/MeOH (5.2 mL, 1/7/100). The RM was stirred at RT for 15 mins. (*R*)-Glycidol (1.057 mL, 15.7 mmol, 10 eq.) was added to the RM which was stirred at 40 °C for 18 hrs. The RM was filtered before being concentrated under reduced pressure. The residue was dissolved in EtOAc (150 mL) and washed with H₂O (150 mL) and brine (150 mL). The aqueous phase was extracted with EtOAc (3 x 200 mL) before the combined organics were dried over MgSO₄, filtered and the solvent was removed under reduced pressure. Purification was carried out via column chromatography (eluent: Pentane/ EtOAc 5:1 → Pentane/ EtOAc 1:3). Product obtained as a white solid (520 mg, 1.14 mmol, 72 %) HRMS *m/z* Calc. for C₂₄H₂₇NO₆S [M+H]⁺: 458.1637. Obs. [M+H]⁺: 458.1635. ¹H NMR (400 MHz, DMSO-D₆) δ 7.76 (d, *J* = 7.4 Hz, 2H, ArH), 7.61 (d, *J* = 7.2 Hz, 2H, ArH), 7.40 (t, *J* = 7.4 Hz, 2H, ArH), 7.32 (t, *J* = 7.4 Hz, 2H, ArH), 5.98 – 5.85 (m, 2H, CH₂=CH- and -NH), 5.35 (d, *J* = 17.1 Hz, 1H, CH_aH_b=CH-), 5.28 (d, *J* = 10.4 Hz, 1H, CH_aH_b=CH-), 4.71 – 4.62 (m, 3H, =CH-CH₂-O and CH-N), 4.41 (d, *J* = 7.1 Hz, 2H, Fmoc-CH₂-O), 4.23 (t, *J* = 7.0 Hz, 1H, Fmoc-CH-), 3.94 – 3.87 (m, impurity), 3.82 – 3.72 (m, 2H, CH-OH and -OH), 3.71 – 3.64 (m, 2H, CH_aH_bOH and -OH), 3.64 – 3.55 (m, impurity), 3.52 (dd, *J* = 11.3, 6.1 Hz, 1H, CH_aH_bOH), 3.07 (dd, *J* = 14.1, 4.7 Hz, 1H, C(N)-CH_aH_b-S), 2.99 (dd, *J* = 14.1, 6.7 Hz, 1H, C(N)-CH_aH_b-S), 2.77 (dd, *J* = 14.0, 4.0 Hz, 1H, C(O)-CH_aH_b-S), 2.67 –

2.58 (m, overlap, 1H, C(O)-CH_aH_b-S). **¹³C NMR** (101 MHz, CDCl₃) δ 170.6 (C), 156.2 (C), 143.8 (C), 141.4 (C), 131.3 (CH), 127.9 (CH), 127.2 (CH), 125.2 (CH), 120.2 (CH), 119.6 (CH₂), 71.8 (CH), 67.4 (CH₂), 66.7(CH₂), 65.4 (CH₂), 54.2 (CH), 46.1 (CH), 41.0 (CH₂), 36.7 (CH₂). The impurity peaks observed at 3.9 and 3.6 ppm seem to originate from a single compound, which is Glycerol.

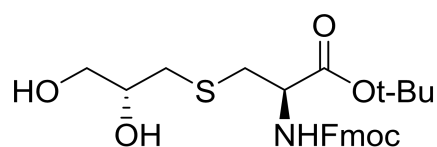
Synthesis of Allyl-Fmoc-Pam₂Cys (**21**)²⁰⁸



Allyl-Fmoc-S-((*R*)-2,3-dihydroxypropyl)-cysteine (**20**, 100 mg, 0.219 mmol, 1 eq.) was dissolved in dry THF (6 mL) before the addition of palmitic acid (224.5 mg, 0.875 mmol, 4 eq.), DMAP (10.69 mg, 0.0875 mmol, 0.4 eq.) and DIC (0.132 mL, 0.853 mmol, 3.9 eq.). The reaction mixture was stirred at RT under an atmosphere of argon for 18 hrs before being quenched by the addition of glacial acetic acid (0.2 mL). The reaction mixture was stirred at RT for 5 mins before the solvent was removed under reduced pressure. Purification was carried out via column chromatography (eluent: pentane/EtOAc 90:10). The desired product was obtained as a white solid (168 mg, 0.179 mmol, 82 %). HRMS *m/z* Calc. for C₅₆H₈₇NO₈S [M+H]⁺: 934.6230, Obs. [M+H]: 934.6216. Allyl-Fmoc-S-((*R*)-2,3-dihydroxypropyl)-cysteine (**20**, 100 mg, 0.219 mmol, 1 eq.) was dissolved in dry THF (6 mL) before the addition of palmitic acid (224.5 mg, 0.875 mmol, 4 eq.), DMAP (10.69 mg, 0.0875 mmol, 0.4 eq.) and DIC (0.132 mL, 0.853 mmol, 3.9 eq.). The reaction mixture was stirred at RT under an atmosphere of argon for 18 hrs before being quenched by the addition of glacial acetic acid (0.2 mL). The reaction mixture was stirred at RT for 5 mins before the solvent was removed under reduced pressure. Purification was carried out via column chromatography (eluent: pentane/EtOAc 90:10). The desired product

was obtained as a white solid (168 mg, 0.179 mmol, 82 %). HRMS m/z Calc. for $C_{56}H_{87}NO_8S$ $[M+H]^+$: 934.6230, Obs. $[M+H]^+$: 934.6216. **^1H NMR** (400 MHz, $CDCl_3$) δ 7.77 (d, J = 7.5 Hz, 2H, ArH), 7.61 (d, J = 7.5 Hz, 2H, ArH), 7.40 (t, J = 7.4 Hz, 2H, ArH), 7.32 (t, J = 7.4 Hz, 2H, ArH), 5.98 – 5.85 (m, 1H, $CH_2=CH-$), 5.70 (d, J = 8.0 Hz, 1H, -NH), 5.35 (d, J = 17.1 Hz, 1H, $CH_aH_b=CH-$), 5.28 (d, J = 10.5 Hz, 1H, $CH_aH_b=CH-$), 5.25 – 5.19 (m, impurity), 5.18 – 5.11 (m, 1H, CH-O), 4.72-4.60 (m, 3H, $=CH-CH_2-O$ and CH-N), 4.40 (d, J = 7.0 Hz, 2H, Fmoc- CH_2-O), 4.37 – 4.28 (m, 1H, CH_aH_b-O), 4.24 (t, J = 7.0 Hz, 1H, Fmoc-CH-), 4.19 – 4.08 (m, 1H, CH_aH_b-O), 3.72 – 3.60 (m, impurity), 3.16 – 3.00 (m, 2H, C(N)- CH_2-S), 2.75 (d, J = 6.5 Hz, 2H, C(O)- CH_2-S), 2.38 – 2.25 (m, 4H, - CH_2-COO), 1.68 – 1.54 (m, 4H, - CH_2-CH_2-COO), 1.37 – 1.20 (m, 48H, - $(CH_2-)_{12}$), 0.90 (t, J = 6.7 Hz, 6H, - CH_3). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 173.8 (C), 173.5 (C), 173.3 (C), 156.1 (C), 144.2 (C), 141.8 (C), 131.7 (CH), 128.2 (CH), 127.6 (CH), 125.6 (CH), 120.5 (CH), 119.8 (CH_2), 70.7 (CH), 67.8 (CH_2), 67.0 (CH_2), 63.9 (CH_2), 54.3 (CH), 47.6 (CH), 35.5 (CH_2), 34.7 (CH_2), 34.5 (CH_2), 33.6 (CH_2), 32.4 (CH_2), 30.3 (CH_2), 30.1 (CH_2), 30.1 (CH_2), 30.1 (CH_2), 30.0 (CH_2), 29.9 (CH_2), 29.83 (CH_2), 29.8 (CH_2), 29.6 (CH_2), 29.6 (CH_2), 25.4 (CH_2), 23.2 (CH_2), 14.6 (CH_3). The impurity peaks observed at 5.22 and 3.65 ppm seem to originate from a single compound, which is a derivative of Glycerol. Also there is an impurity of palmitic acid which affected the integral values of the peaks at 2.32, 1.60, 1.26 and 0.88 ppm.

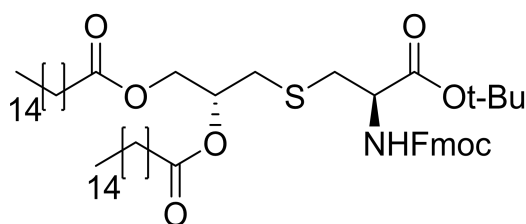
Synthesis of tert-butyl- Fmoc-*R*-(2,3-dihydroxypropyl) Cysteine (**23**)²¹¹



(Fmoc-Cys-OtBu)₂ (**22**, 1.25 mmol, 1 g) was dissolved in DCM (10 mL). Zinc dust (8.75 mmol, 0.572 g) and $H_2SO_4/HCl/MeOH$ (4 mL, 1/7/100) were added and the reaction mixture was stirred at RT. After 15 mins, (*R*)-glycidol (12.5 mmol, 0.841 mL) was added, the resulting mixture

was stirred for 5 hrs at 40 °C. The reaction mixture was filtered and then concentrated under *vacuo* until half of the volume. The crude was diluted (EtOAc) and washed (2M HCl). The aqueous layer was back extracted with EtOAc three times. The combined organic layers were dried over MgSO₄, filtered and concentrated. Silica gel column chromatography (50%-70% EtOAc in Pentane) yielded the product as a white solid (0.84 mmol, 0.4 g, 68%). HRMS *m/z* Calc. for C₂₅H₃₁NO₆S [M+H]⁺: 474.1950. Obs. [M+H]⁺: 474.1933. **¹H NMR** (400 MHz, CDCl₃) δ 7.76 (d, *J* = 7.5 Hz, 2H, *ArH*), 7.61 (d, *J* = 7.5 Hz, 2H, *ArH*), 7.40 (t, *J* = 7.4 Hz, 2H, *ArH*), 7.31 (t, *J* = 7.4 Hz, 2H, *ArH*), 5.85 (d, *J* = 8.0 Hz, 1H, -NH), 4.54 – 4.48 (m, 1H, CH-N), 4.40 (d, *J* = 7.0 Hz, 2H, Fmoc-CH₂-O), 4.23 (t, *J* = 7.0 Hz, 1H, Fmoc-CH-), 3.95 – 3.86 (m, impurity), 3.82-3.78 (m, 1H, -OH), 3.75 (dd, *J* = 11.3, 3.9 Hz, 1H, CH_aH_b-O), 3.71 – 3.65 (m, 1H, -CH-OH), 3.64 – 3.55 (m, impurity), 3.52 (dd, *J* = 11.3, 6.0 Hz, 1H, CH_aH_b-O), 3.03 – 2.90 (m, 2H, C(N)-CH_aH_b-S, C(N)-CH_aH_b-S), 2.83 – 2.74 (m, 1H, C(O)-CH_aH_b-S), 2.68 – 2.57 (m, overlap, 1H, C(O)-CH_aH_b-S), 1.49 (s, 9H, -CH₃). **¹³C NMR** (100 MHz, CDCl₃) δ 171.1 (C), 157.5 (C), 145.2 (C), 145.1 (C), 142.7 (C), 129.2 (CH), 128.5 (CH), 126.5 (CH), 121.4 (CH), 84.6 (C), 72.0 (CH), 68.6 (CH₂), 66.6 (CH₂), 55.9 (CH), 47.4 (CH), 38.1 (CH₂), 37.1 (CH₂), 29.4 (CH₃).

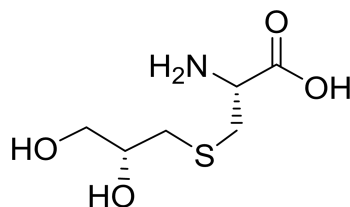
Synthesis of tert-butyl-Fmoc-Pam₂Cys (**24**)²¹¹



Compound **23** (11.5 mmol, 5.44 g) was dissolved in dry DCM (150 mL) under argon. Consecutively, palmitic acid (36.8 mmol, 9.42 g), DIC (44.85 mmol, 5.65 g, 7 mL) and DMAP (4.6 mmol, 0.56 g) were added. The reaction mixture was stirred over 72 hrs at RT. Glacial acetic acid (86.25 mmol, 5.2 g, 5 mL) was added and the solution was stirred for 15

min, then filtered and concentrated. Crystallization in DCM/MeOH (1/19) yielded compound **24** (8.9 mmol, 8.46 g, 77%). HRMS m/z Calc. for $C_{57}H_{91}NO_8S$ $[M+Na]^+$: 972.6363. Obs. $[M+Na]^+$: 972.6389. **^1H NMR** (400 MHz, $CDCl_3$) δ 7.79 (d, J = 7.5 Hz, 2H, ArH), 7.64 (d, J = 7.5 Hz, 2H, ArH), 7.42 (t, J = 7.4 Hz, 2H, ArH), 7.34 (t, J = 7.4 Hz, 2H, ArH), 5.73 (d, J = 7.6 Hz, 1H, -NH), 5.20 – 5.14 (m, 1H, CH-OH), 4.55 – 4.51 (m, 1H, CH-N), 4.43 (m, 1H, CH_aH_b -O), 4.38-4.32 (m, 2H, Fmoc- CH_2 -O), 4.26 (t, J = 7.1 Hz, 1H, Fmoc-CH-), 4.20-4.13 (m, 1H, CH_aH_b -O), 3.88-3.78 (m, impurity), 3.72-3.61 (m, impurity), 3.5 (s, impurity, Methanol), 3.13 – 3.00 (m, 2H, C(N)- CH_2 -S), 2.82 – 2.72 (m, 2H, C(O)- CH_2 -S), 2.39 – 2.24 (m, 4H, - CH_2 -COO with PA as impurity), 1.68 – 1.55 (m, 4H, - CH_2 - CH_2 -COO with PA as impurity), 1.54 – 1.45 (m, 9H, - CH_3 x 3), 1.37-1.20 (m, 48H, -(CH_2)₁₂ with PA as impurity), 0.90 (t, J = 6.7 Hz, 6H, - CH_3 with PA as impurity). The spectra of the analysed sample is a mixture of the (Compound 24) with the starting compound PA (palmitic acid) which affected the integral values of the peaks at 2.3, 1.6, 1.3 and 0.88 ppm. **^{13}C NMR** (100 MHz, $CDCl_3$) δ 173.4 (C), 173.3 (C), 169.5 (C), 155.7 (C), 143.8 (C), 141.3 (C), 127.7 (CH), 127.1 (CH), 125.1 (CH), 120.0 (CH), 83.0 (C), 70.2 (CH), 67.2 (CH₂), 63.5 (CH₂), 50.9 (CH), 47.1 (CH), 34.3 (CH₂), 34.1 (CH₂), 33.3 (CH₂), 31.9 (CH₂), 29.7 (CH₂), 29.6 (CH₂), 29.5 (CH₂), 29.4 (CH₃), 29.3 (CH₂), 29.2 (CH₂), 29.1 (CH₂), 28.0 (CH₂), 24.9 (CH₂), 24.8 (CH₂), 22.7 (CH₂), 14.0 (CH₃).

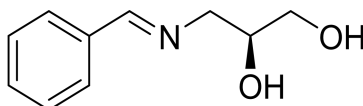
Synthesis of *R*-(2,3-dihydroxypropyl) Cysteine (**27**)⁷⁸



A mixture of L-cysteine hydrochloride (**26**, 3.34g, 19 mmol), (*R*)-3-chloro-1,2-propanediol (**25**, 3 g, 2.4 mL, 28.5 mmol) and triethylamine (5.8 g, 8 mL, 58 mmol) in water (5 mL) was covered and protected from light with aluminium foil and stirred for 72 hrs. The reaction mixture was evaporated *in vacuo* and the residue was washed with acetone (3 x 5 mL) and dried to give *R*-(2,3-dihydroxypropyl) cysteine as an off white

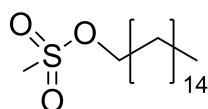
solid (**27**, 3.5 g, 18.2 mmol, 95%). HRMS m/z Calc. for $C_6H_{13}NO_4S$ $[M+H]^+$: 196.0643. Obs. $[M+H]^+$: 196.0638 (error 2.55 ppm). Crude taken on to next step without purification.

Synthesis of *S*-3-(benzylideneamino) propane-1,2-diol (**41**)



A solution of (*S*)-3-amino-1,2-propanediol (**48**, 0.2 g, 2.2 mmol) in anhydrous DCM: MeOH (10:1, 25 ml) was stirred over Na_2SO_4 (1.56 g, 11 mmol) for 2 hrs before the drop-wise addition of benzaldehyde (**49**, 0.27 g, 2.2 mmol). This was stirred at RT for 24 hrs before filtering away the solid and concentrating the solution *in vacuo*. The resultant oil was dried under high *vacuo* for 6 hrs before being used directly in the next stage (**41**, 0.28g, 1.56 mmol, 71%). HRMS m/z Calc. for $C_{10}H_{13}NO_2$ $[M+H]^+$: 180.1024, Obs. $[M+H]^+$: 180.1033 (error 5.00 ppm).

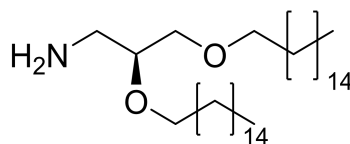
Synthesis of cetyl alcohol mesylate (**42**)



A solution of cetyl alcohol (**46**, 2.5 g, 10.30 mmol) and Et_3N (2.08 g, 20.6 mmol) in DCM (60 ml) was cooled to 0 °C before mesyl chloride (1.42 g, 12.40 mmol) was added dropwise. The solution was allowed to warm to RT and stirred for 16 hrs before being diluted with DCM (100 ml) and extracted with a 1 M $NaHCO_3$ (3 x 50 ml). The aqueous layer was extracted with DCM (3 x 50 ml), then the combined organic extracts washed with water (3 x 50 ml), brine (1 x 50 ml), dried over $MgSO_4$, filtered and concentrated in *vacuo*. The resultant yellow solid was dried under high *vacuo* for 4 hrs, yielding pale yellow flakes (**42**, 3.17 g, 9.89 mmol, 96% yield). HRMS m/z Calc. for $C_{17}H_{36}O_3S$ $[M+Na]^+$: 343.2283. Obs. $[M+Na]^+$: 343.2273 (error 2.91 ppm). The product was used in the

next stage without further purification.

Synthesis of lipid ether amine (**44**)²⁴⁸



To an over dried and argon flushed round bottom was added NaH (0.18 g, 7.5 mmol, 60% dispersion in mineral oil). To this was added a solution of compound (**41**) (0.28 g, 1.6 mmol) in anhydrous THF under an argon atmosphere. This was stirred at RT for 1 hr before a solution of crude cetyl mesylate (**42**, 2.57 g, 7.5 mmol) in anhydrous THF was added dropwise. Once added, the RM was heated to reflux and left for 72 hrs. The reaction was quenched by the addition of water (50 mL) and the aqueous layer extracted with DCM (3 x 30 mL). The combined organic extracts were washed with water (1 x 30 mL), brine (1 x 30 mL), dried over MgSO₄, filtered and concentrated *in vacuo*. The resultant oil was dissolved in wet DCM: TFA (1:1, 50 mL) and stirred at RT for 16 hrs to hydrolyse the imine. Reaction progress was monitored by TLC. The RM was concentrated *in vacuo* and the TFA co-evaporated with toluene (3 x 50 mL). The resultant oil was dried under high *vacuo* to yield a brown oil. This was purified by flash column chromatography (DCM to DCM: MeOH (8:2), yielding a brown solid (**44**, 0.170 g, 0.315 mmol, 29% yield). HRMS *m/z* Calc. for C₃₅H₇₃NO₂ [M+H⁺]⁺: 540.5719. Obs. [M+H⁺]⁺: 540.5743 (error 4.44 ppm). **¹H NMR** (400 MHz, CDCl₃) δ 4.85 – 4.54 (b, 2H, -NH), 3.62 – 3.55 (m, 1H, CH-O), 3.54 – 3.46 (m, 4H, CH₂-O), 3.43 (t, J = 6.7 Hz, 2H, CH₂-O), 3.08 (dd, J = 13.0, 4.3 Hz, 1H, CH_aH_b-N), 2.97 (dd, J = 13.0, 6.6 Hz, 1H, CH_aH_b-N), 1.61 – 1.50 (m, 4H, CH₂-CH₂-O), 1.33 – 1.20 (m, 52H, -(CH₂)₁₃-), 0.88 (t, J = 6.8 Hz, 6H, -CH₃). **¹³C NMR** (100 MHz, CDCl₃) δ 75.7 (CH), 71.8 (CH₂), 70.7 (CH₂), 70.2 (CH₂), 42.1 (CH₂), 31.8 (CH₂), 29.8 (CH₂), 29.6 (CH₂), 29.5 (CH₂), 29.5 (CH₂), 29.5 (CH₂), 29.4 (CH₂), 29.3 (CH₂), 29.3 (CH₂), 29.2 (CH₂), 25.9 (CH₂), 22.5 (CH₂), 14.0 (CH₂).

7.4 Peptide Synthesis

The following peptides were synthesised using standard Fmoc-SPPS techniques as described in the General Methods section. Their sequences calculated and observed mass values, and pure HPLC traces with analytical/preparative solvent gradients are shown below.

Synthesis of model peptide (15)

Model peptide sequence is H-CITGF-OH synthesised on 2CTC resin (300 mg, 0.25 mmol). The crude peptide was cleaved and lyophilized to produce the desired peptide with no further purification necessary (121 mg, 0.22 mmol, 88% yield).

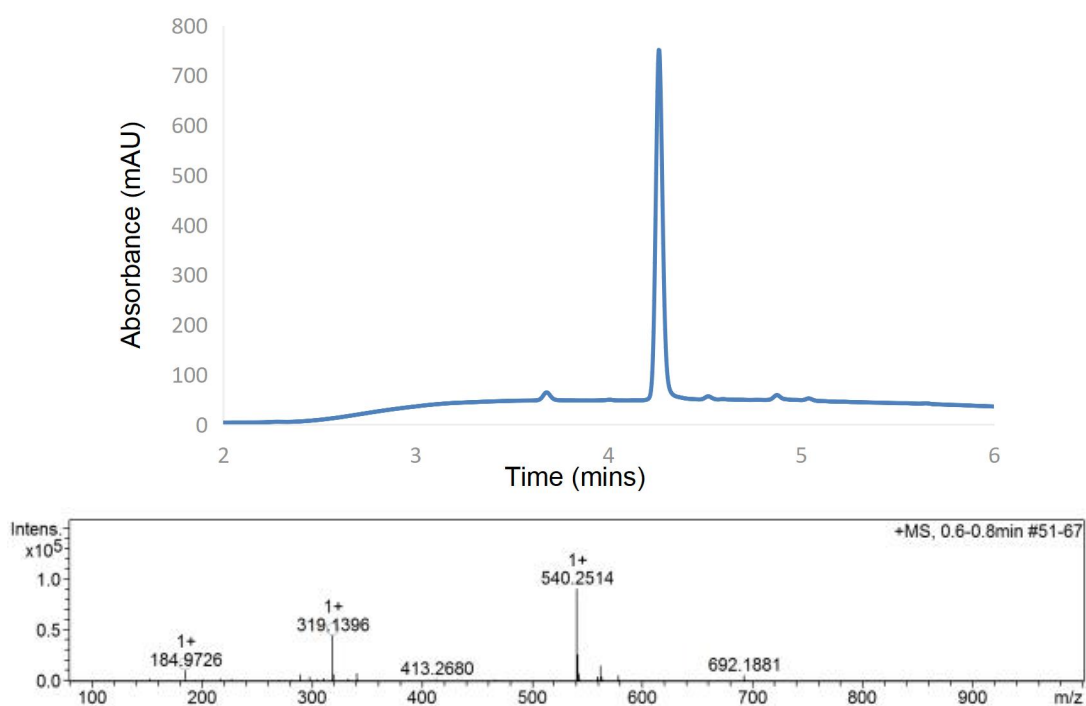


Figure 84: Analytical HPLC trace of crude model peptide (**15**). Analytical gradient 10-60% B over 5 mins 0.6 ml/min, 210 nm. HRMS m/z Calc. for C₂₄H₃₇N₅O₇S [M+1H]⁺:540.2492. Obs. [M+1H]⁺:540.2514 (error 4.07 ppm).

Synthesis of BAGE4₁₈₋₃₉ (**28**) on 2-CTC resin

BAGE4₁₈₋₃₉ sequence is H-RLMKEESPVVSWWLEPEDGTAL-OH synthesised on 2-CTC resin, 0.22 mmol. The crude peptide was cleaved and 0.05 mmol was purified by preparative HPLC (20 to 80% B over 30 mins) and lyophilized to produce the desired peptide (20 mg, 0.0077 mmol, 15% yield).

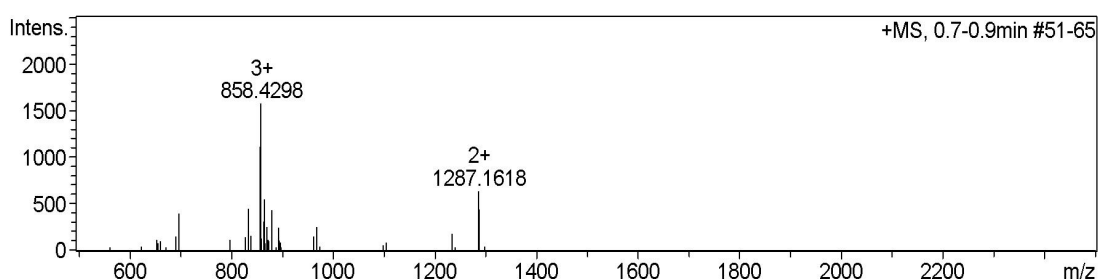
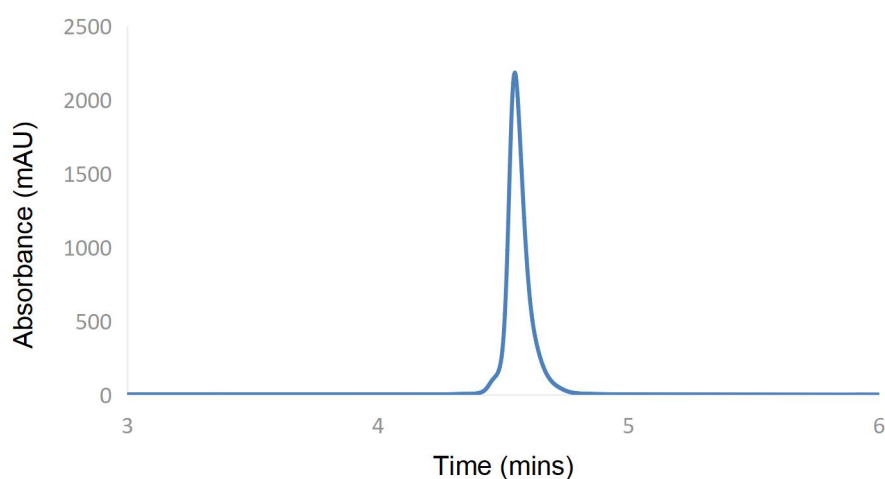


Figure 85: Analytical HPLC trace of pure BAGE4₁₈₋₃₉ (**28**). Analytical gradient 2-95% B over 5 mins 0.6 ml/min, 280 nm. ESI⁺ MS Calc. for C₁₁₆H₁₇₈N₂₈O₃₆S [M+2H]²⁺:1286.64, [M+3H]³⁺:858.08. Obs. [M+2H]²⁺:1287.16, [M+3H]³⁺:858.43.

Synthesis of BAGE4₁₈₋₃₉ (**28**) on Rink amide resin

BAGE4₁₈₋₃₉ sequence is H-RLMKEESPVVSWWLEPEDGTAL-NH₂, on Rink amide resin, 0.18 mmol. The crude peptide was cleaved and 0.06 mmol was purified by preparative RP-HPLC (20 to 80% B over 30 mins) and lyophilized to produce the desired peptide (30 mg, 0.0117 mmol, 20% yield).

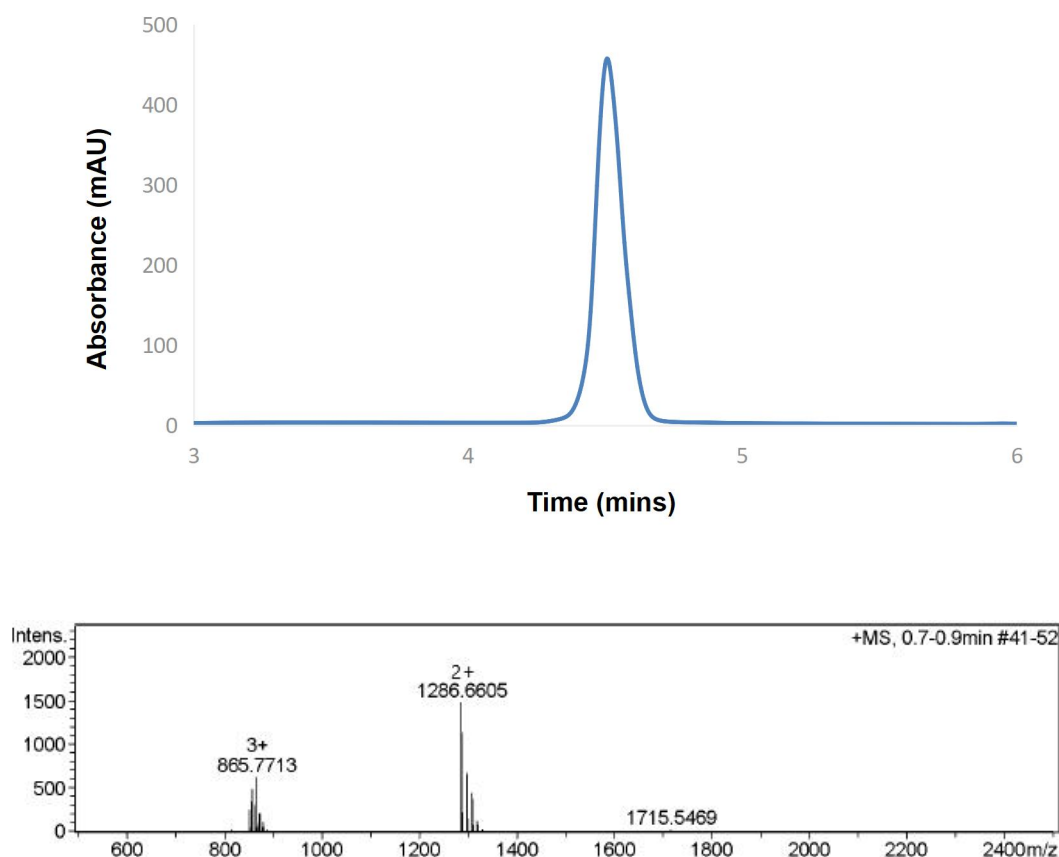


Figure 86: Analytical HPLC trace of pure BAGE4₁₈₋₃₉ (**28**). Analytical gradient 2-95% B over 5 mins 0.6 ml/min, 280 nm. ESI⁺ MS Calc. for C₁₁₆H₁₇₉N₂₉O₃₅S [M+2H]²⁺:1286.14, [M+Na]³⁺:865.43. Obs. [M+2H]²⁺:1286.66, [M+Na]³⁺:865.77.

Synthesis of NY-ESO-1₁₅₇₋₁₆₅ (**29**)

NY-ESO-1₁₅₇₋₁₆₅ sequence is H-SLLMWITQC-NH₂ synthesised on Rink amide resin, 0.05 mmol. The crude peptide was cleaved and purified by preparative RP-HPLC (30%-80% solvent B over 35 mins, isocratic @ 35% solvent B for 5 mins) and lyophilized to produce the desired peptide (11 mg, 0.0101 mmol, 20% yield).

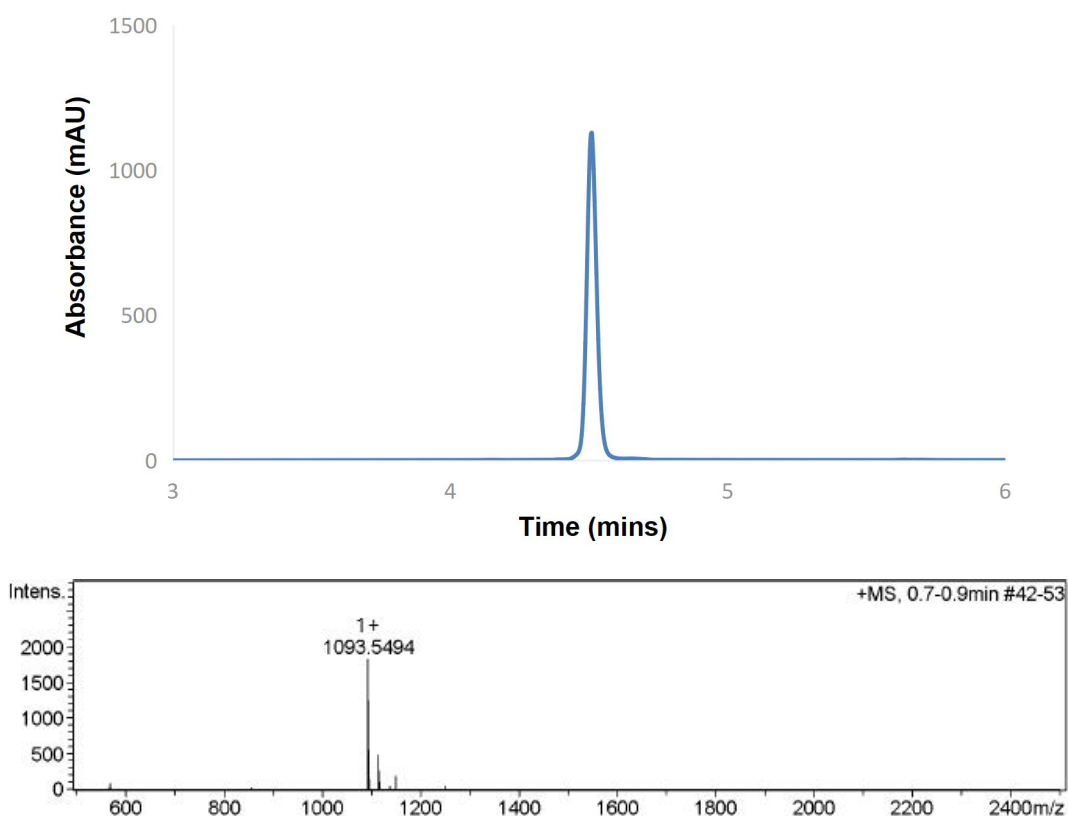


Figure 87: Analytical HPLC trace of pure NY-ESO-1₁₅₇₋₁₆₅ (**29**). Analytical gradient 10-100% B over 5 mins 0.6 ml/min, 280 nm. HRMS Calc. for C₄₉H₈₀N₁₂O₁₂S₂ [M+H]⁺:1093.5387. Obs: [M+H]⁺:1093.5494 (error 9.78 ppm).

Synthesis of model peptide (30)

Model peptide sequence is H-HAKTKEA-NH₂, synthesised on Rink amide resin (367 mg, 0.25 mmol).

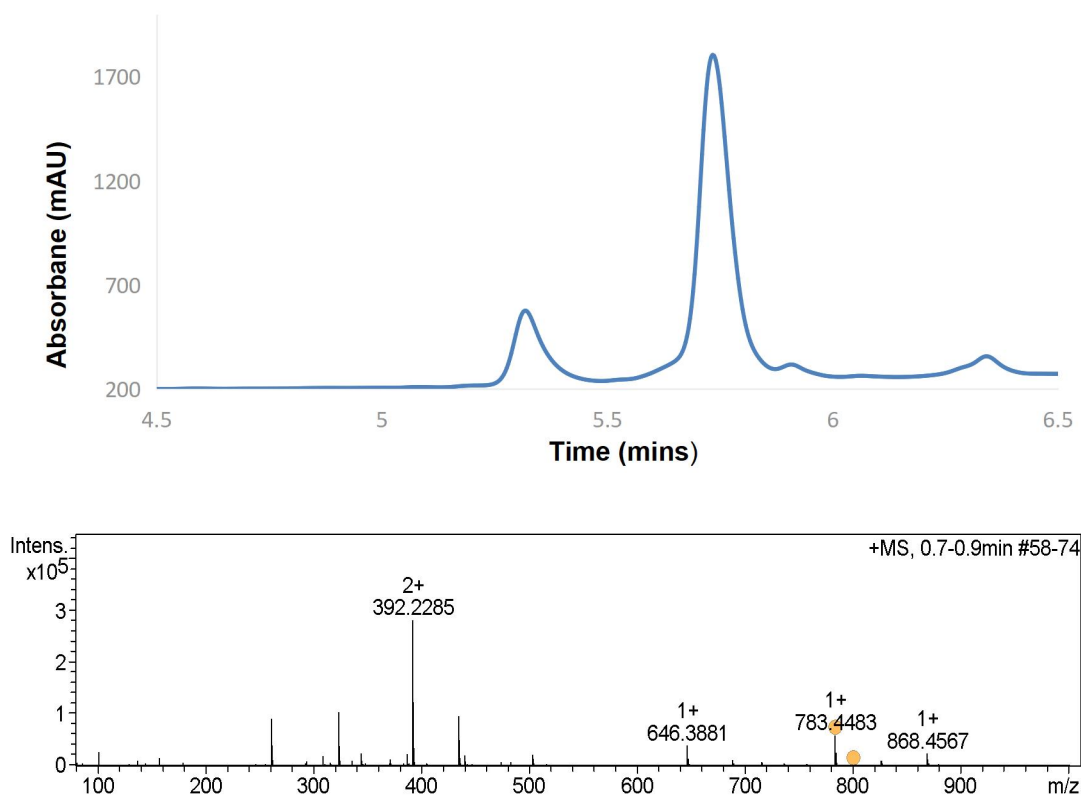


Figure 88: Analytical HPLC trace and MS of the crude model peptide (**30**). Analytical gradient 2-95% B over 10 mins 0.6 ml/min, 210 nm. ESI⁺ MS Calc. for C₃₃H₅₈N₁₂O₁₀ [M+H]⁺: 783.43, [M+2H]²⁺: 392.21. Obs. [M+H]⁺: 783.45, [M+2H]²⁺= 392.23.

Synthesis of PADRE-NY-ESO-1₁₅₇₋₁₆₅ (36)

PADRE-NY-ESO-1₁₅₇₋₁₆₅ sequences is H-AKFVAAWTLKAAASLLMWITQC-NH₂ synthesised on Rink amide resin, 0.19 mmol. The crude peptide was cleaved and 0.05 mmol was purified by preparative RP-HPLC (50%-100% solvent B over 30 mins) and lyophilized to produce the desired peptide (3.5 mg, 0.00145 mmol, 3% yield).

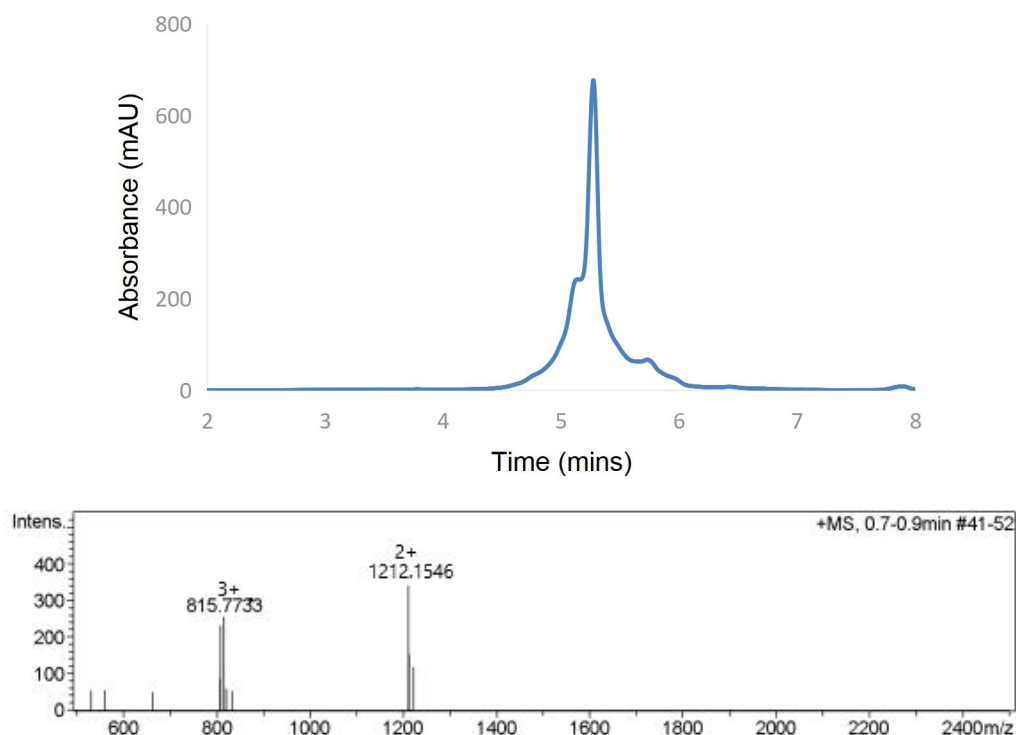


Figure 89: Analytical HPLC trace of pure NY-ESO-1₁₅₇₋₁₆₅ PADRE (**36**). Analytical gradient 2-95% B over 5 mins 0.6 ml/min, 280 nm. HRMS Calc. for C₁₁₄H₁₈₀N₂₈O₂₆S₂ [M+2H]²⁺:1212.1549, [M+Na]³⁺:815.1033. Obs. [M+2H]²⁺:1212.1546, [M+Na]³⁺:815.7733 (error 0.25 ppm).

Synthesis of NY-ESO-1₁₅₇₋₁₆₅ SK₄ (**38**)

NY-ESO-1₁₅₇₋₁₆₅ SK₄ sequence is H- H-SKKKKSLLMWITQC-NH₂ synthesised on Rink amide resin, 0.05 mmol. The crude peptide was cleaved and purified by preparative RP-HPLC (20%-70% solvent B over 30 mins) and lyophilized to produce the desired peptide (24 mg, 0.0142 mmol, 28% yield).

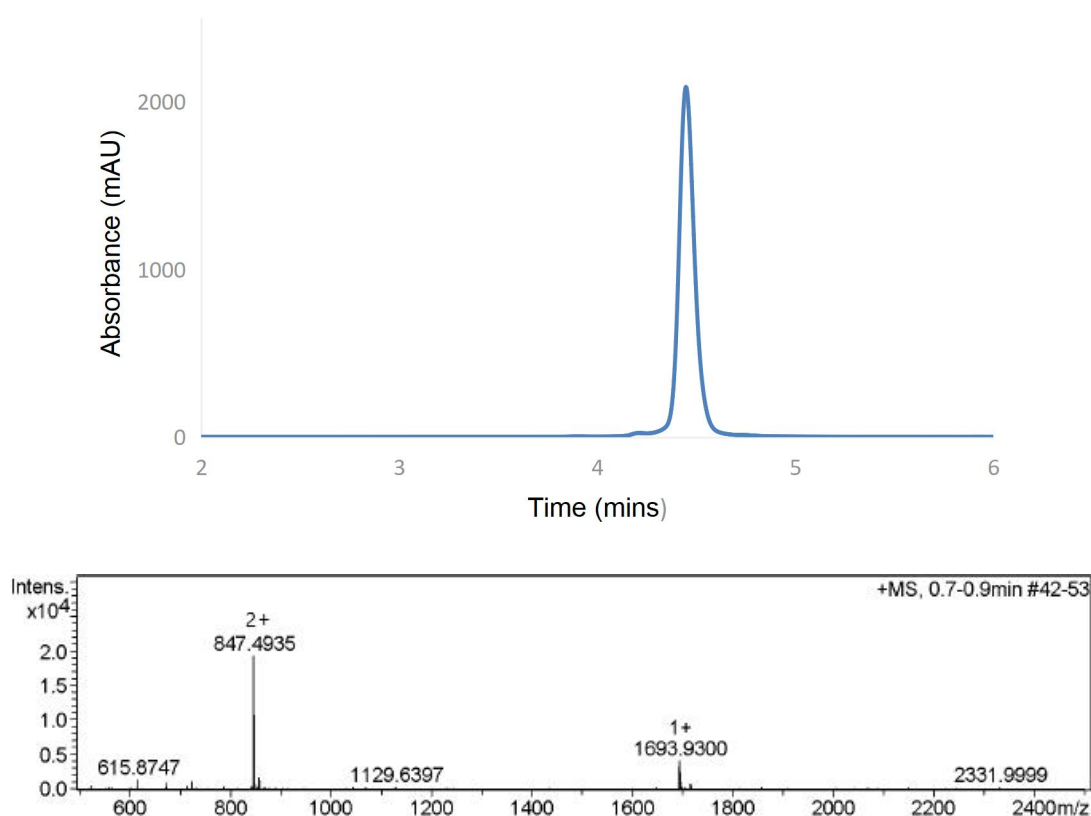


Figure 90: Analytical HPLC trace of pure NY-ESO-1₁₅₇₋₁₆₅ SK₄ (**38**). Analytical gradient 2-95% B over 5 mins 0.6 ml/min, 280 nm. ESI⁺ MS Calc. for C₇₆H₁₃₃N₂₁O₁₈S₂ [M+H]⁺:1693.70, [M+2H]²⁺:847.09. Obs. [M+H]⁺:1693.93, [M+2H]²⁺:847.49.

Synthesis of CysBAGE₄₁₈₋₃₉ (**40**)

CysBAGE₄₁₈₋₃₉ sequence is H-CRLMKEESPVVSWWLEPEDGTAL-NH₂ synthesised on Rink amide resin, 0.1 mmol. The crude peptide was cleaved and 0.05 mmol was purified by preparative RP-HPLC (20 to 80% B over 30 mins) and lyophilized to produce the desired peptide (25.5 mg, 0.0095 mmol, 19% yield).

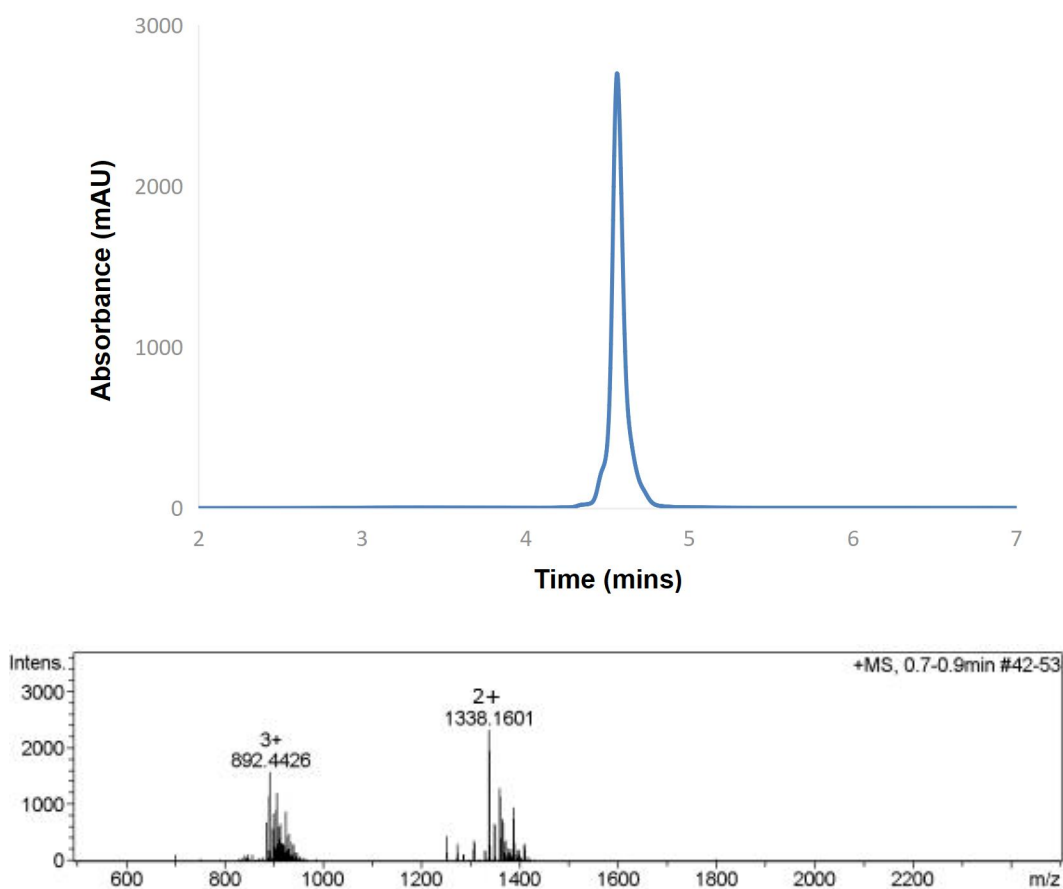
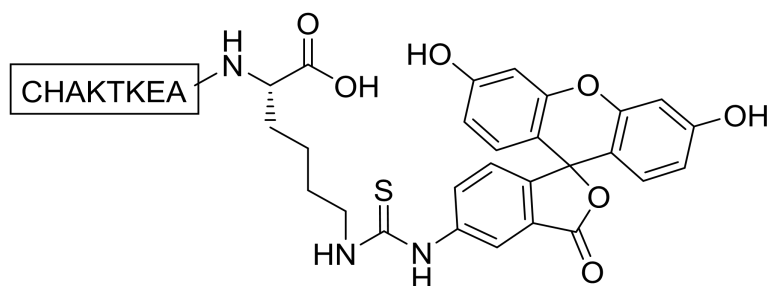


Figure 91: Analytical HPLC trace of pure CysBAGE₄₁₈₋₃₉ (**40**). Analytical gradient 2-95% B over 5 mins 0.6 ml/min, 280 nm. ESI⁺ MS Calc. for C₁₁₉H₁₈₄N₃₀O₃₆S₂ [M+2H]²⁺:1337.95, [M+3H]³⁺:892.10. Obs. [M+2H]²⁺:1338.16, [M+3H]³⁺:892.44.

Synthesis of FITC-model peptide (53)



Model peptide Fmoc-CHAKTKEAK(mtt) synthesised using the general Fmoc-SPPS method, on Rink amide resin, 0.0625 mmol. The Mtt group was deprotected on resin by 1% TFA and 5% TIPS in DCM (25x 5 mins) then washed with 5x DCM & 5x DMF. FITC (3 eq) was dissolved in DMF and activated with DIPEA (6 eq) then added to the resin and incubate at RT, dark, 4 hrs with agitation then washed with 5x DMF & 5x DCM. The Fmoc group was deprotected by 20% piperidine/DMF. The crude peptide was cleaved and 0.02 mmol was purified by preparative RP-HPLC (2 to 95% B over 30 mins) and lyophilised to produce the desired modified peptide (1.2 mg, 0.0007 mmol, 4% yield).

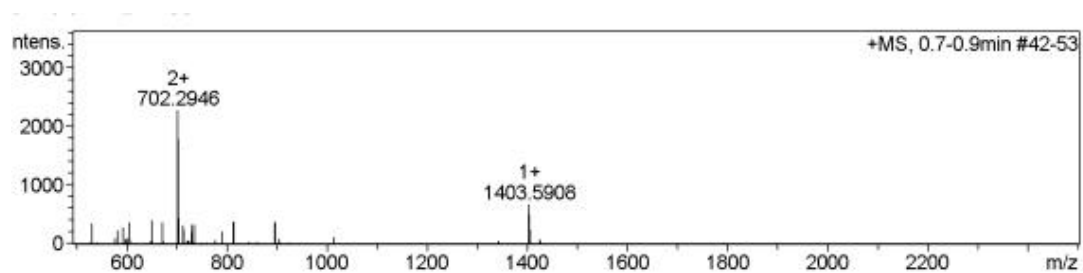
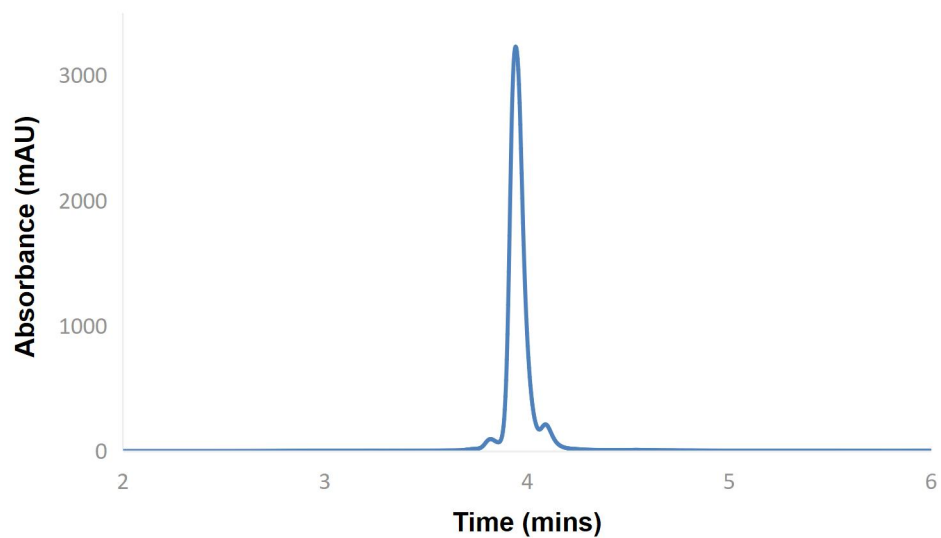
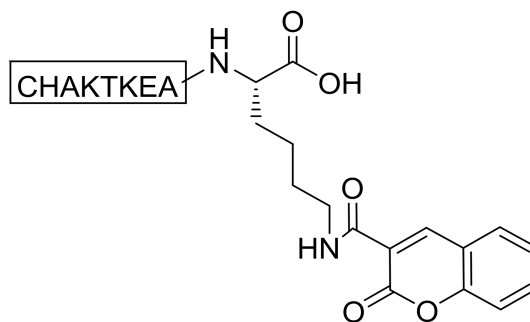


Figure 92: Analytical HPLC trace of pure FITC-model peptide (**53**). Analytical gradient 2-95% B over 5 mins 0.6 ml/min, 280 nm. HRMS Calc. for $C_{63}H_{85}N_{15}O_{18}S_2$ $[M+H]^+1:1403.5816$, $[M+2H]^+2:702.2897$. Obs. $[M+H]^+1:1403.5908$, $[M+2H]^+2:702.2946$ (error 6.55 ppm).

Synthesis of Coumarin-model peptide (54)



Model peptide Fmoc-CHAKTKEAK(Mtt) synthesised using the general Fmoc-SPPS method, on Rink amide resin, 0.00625 mmol. The Mtt group was deprotected on resin by 1% TFA and 5% TIPS in DCM (25x 5mins) then washed with 5x DCM & 5x DMF. Coumarin-3-carboxylic acid (4 eq) dissolved in DMF and activated with Oxyma (4 eq) & DIC (4 eq) then added to the resin and incubate at RT, 1.5 hrs with agitation then washed with 5x DMF & 5x DCM. The Fmoc group was deprotected by 20% piperidine/DMF. The crude peptide was cleaved and 0.02 mmol was purified by preparative RP-HPLC (2 to 30% B over 15 mins then 30% B iso over 15 mins) and lyophilised to produce the desired modified peptide (4 mg, 0.003 mmol, 15% yield).

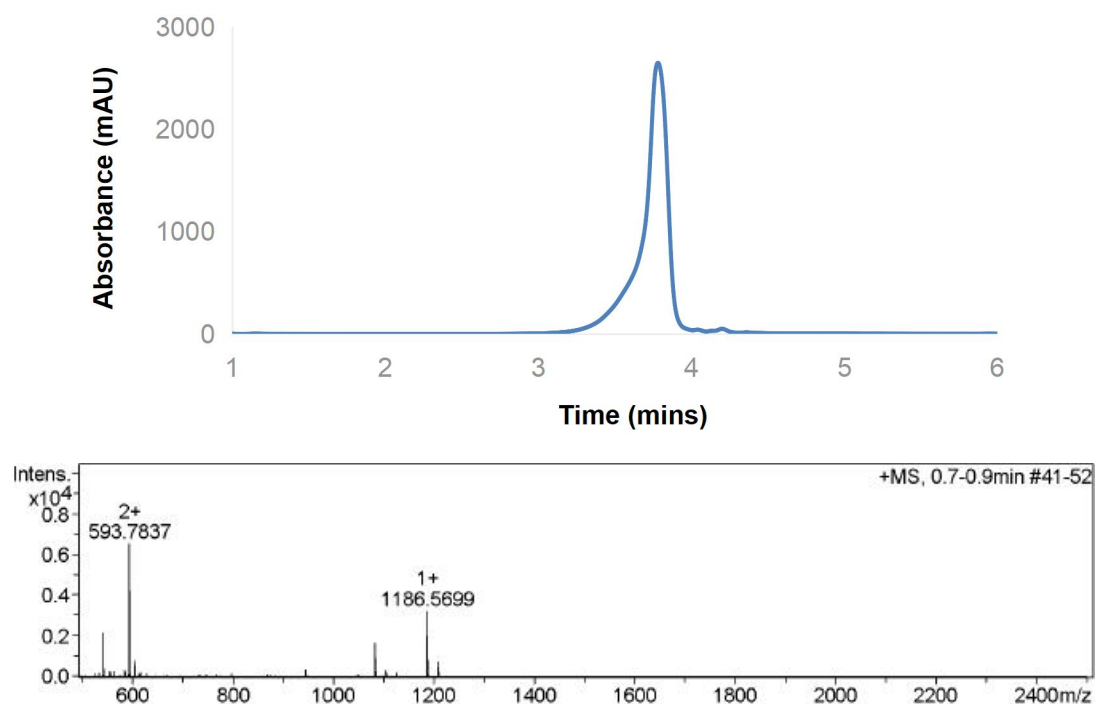


Figure 93: Analytical HPLC trace of pure Coumarin-model peptide (**54**). Analytical gradient 2-95% B over 5 mins 0.6 ml/min, 280 nm. HRMS Calc. for $C_{52}H_{79}N_{15}O_{15}S$ $[M+H]^+1:1186.5679$, $[M+2H]^+2:593.7839$. Obs. $[M+H]^+1:1186.5699$, $[M+2H]^+2:593.7837$ (error 1.69 ppm).

Synthesis of BAGE4₁₈₋₃₂ (**56**)

BAGE4₁₈₋₃₂ sequence is H-RLMKEESPVVSWWLE-NH₂ synthesised on Rink amide resin, 0.167 mmol. The crude peptide was cleaved and 0.023 mmol was purified by preparative RP-HPLC (20 to 60% B over 30 mins) and lyophilized to produce the desired peptide (11 mg, 0.006 mmol, 26% yield).

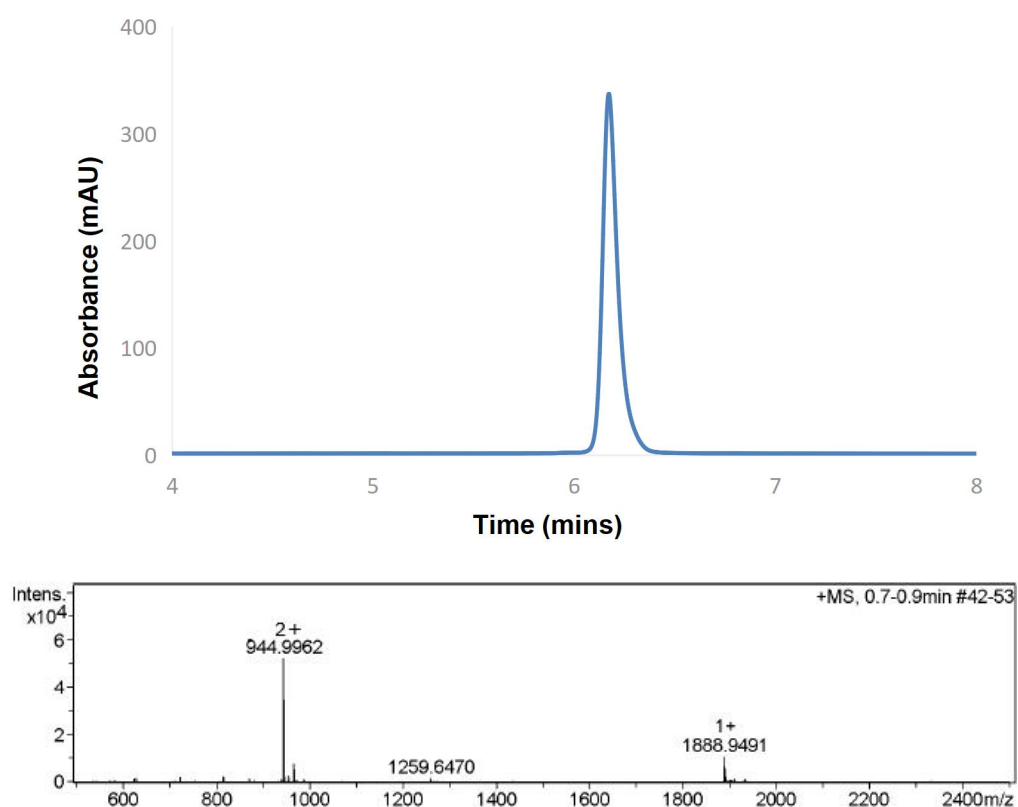


Figure 94: Analytical HPLC trace of pure BAGE4₁₈₋₃₂(**56**). Analytical gradient 2-95% B over 10 mins 0.6 ml/min, 280 nm. ESI⁺ MS Calc. for C₈₇H₁₃₄N₂₂O₂₃S [M+H]⁺:1887.97, [M+2H]⁺:944.79. Obs. [M+H]⁺:1888.95, [M+2H]⁺:945.00.

Synthesis of BAGE4₂₃₋₃₁ (**57**)

BAGE4₍₂₃₋₃₁₎ sequence is H-ESPVVSWWL-NH₂ synthesised on Rink amide resin, 0.16 mmol. The crude peptide was cleaved and 0.03 mmol was purified by preparative RP-HPLC (40 to 80% B over 30 mins) and lyophilized to produce the desired peptide (7 mg, 0.006 mmol, 20% yield).

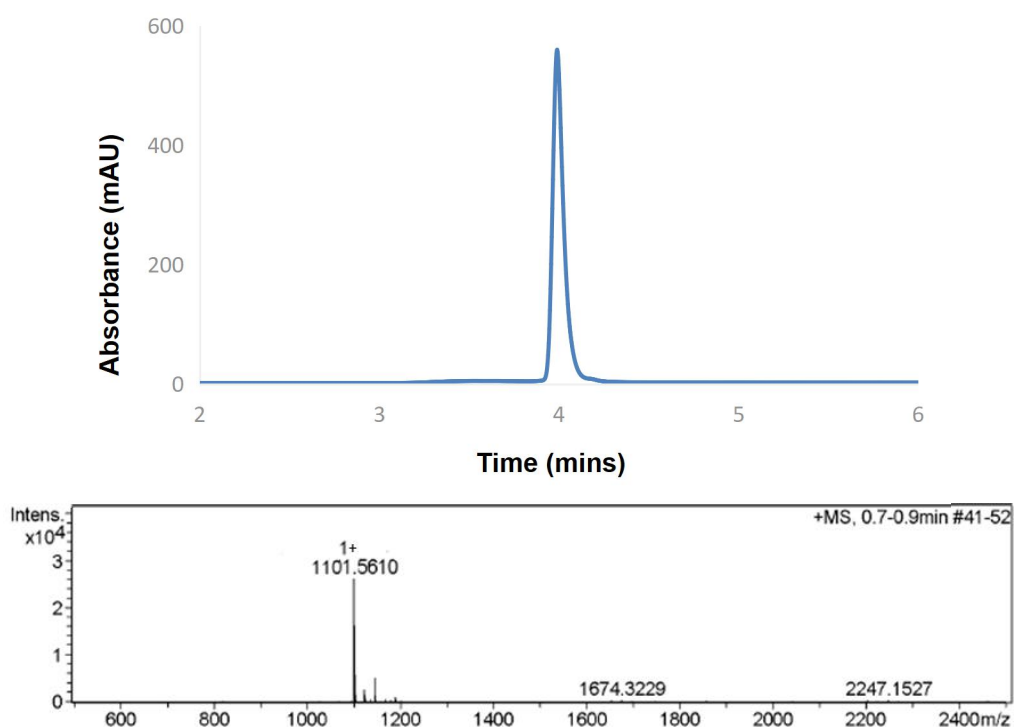


Figure 95: Analytical HPLC trace of pure BAGE4₂₃₋₃₁ (**57**). Analytical gradient 2% then 60% B over 10 mins 0.6 ml/min, 280 nm. HRMS Calc. for C₅₄H₇₆N₁₂O₁₃ [M+H]⁺:1101.5655. Obs. [M+H]⁺:1101.5610 (error 4.09 ppm).

7.5 Lipopeptide Synthesis

Synthesis of Pam₂Cys-HAKTKEA (31)

Synthesised using general protocols A and B, 0.025 mmol. The crude product was purified with a preparative RP-HPLC on C-18 column using (30-100% solvent B gradient over 30 min/ 10 min hold) giving Pam₂Cys-model peptide with (0.0023 mmol, 9%), (0.002 mmol, 8%) yield for the last step respectively.

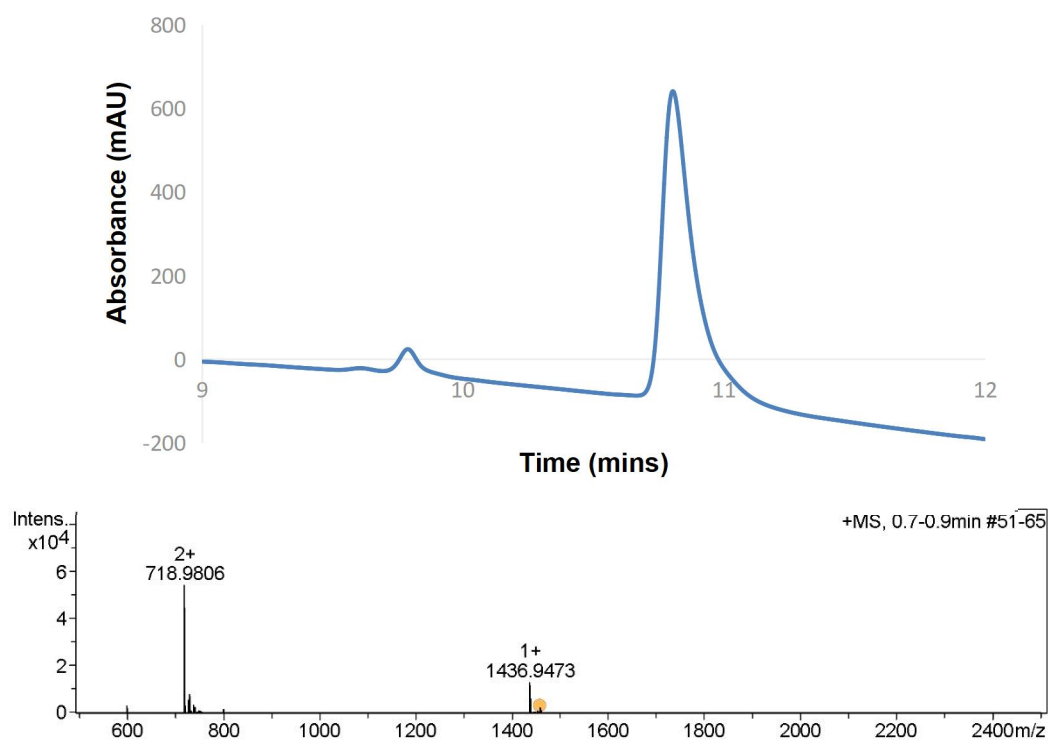


Figure 96: Analytical HPLC trace and MS of the pure Pam₂Cys- HAKTKEA (**31**). Analytical gradient 2-95% B over 10 min, 210 nm. HRMS Calc. for C₇₁H₁₂₉N₁₃O₁₅S [M+H]⁺:1436.9452, [M+2H]²⁺:718.9726. Obs. [M+H]⁺:1436.9473, [M+2H]²⁺:718.9806 (error 1.46 ppm).

Synthesis of Pam₂Cys-BAGE4₁₈₋₃₉ (**32**)

Synthesised using general protocol, B using BAGE4₁₈₋₃₉ on 2CTC resin (300 mg, 0.018 mmol). The crude peptide was cleaved and purified by semi-preparative RP-HPLC (40 to 80% B over 60 mins) and lyophilized to produce the desired Pam₂Cys-BAGE4 (7mg, 0.00217 mmol, 12% yield).

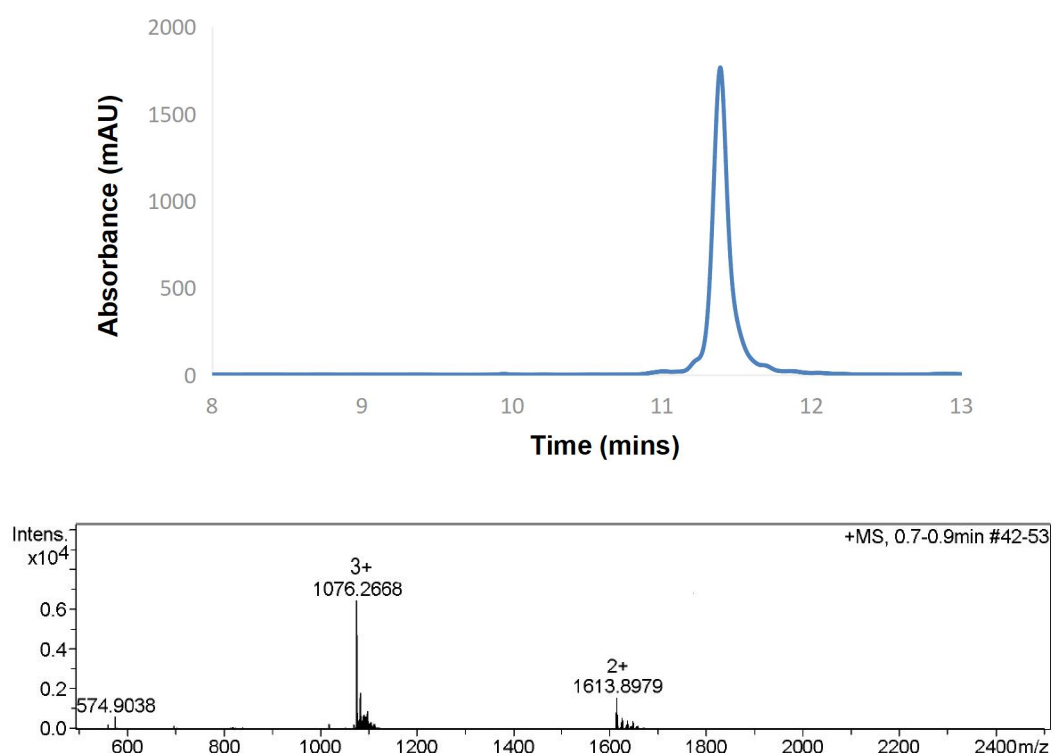


Figure 97: Analytical HPLC trace of pure Pam₂Cys-BAGE4 (**32**). Analytical gradient 2- 100% B over 10 min, 280 nm. ESI⁺ MS Calc. For C₁₅₄H₂₄₉N₂₉O₄₁S₂ [M+2H]²⁺:1613.39, [M+3H]³⁺:1075.92. Obs. [M+2H]²⁺:1613.90, [M+3H]³⁺:1076.27.

Synthesis of Pam₂Cys-SK₄-BAGE4₁₈₋₃₉ (**33**)

Synthesised using general protocol, B using BAGE4₁₈₋₃₉-SK₄ on Rink amide resin (210 mg, 0.025 mmol). 0.0125 mmol of the crude peptide was purified by preparative RP-HPLC (40 to 80% B over 60 mins) and lyophilized to produce the desired Pam₂Cys-SK₄-BAGE4 (9 mg, 0.0028mmol, 22.3% yield), calculation made based on amount of crude purified.

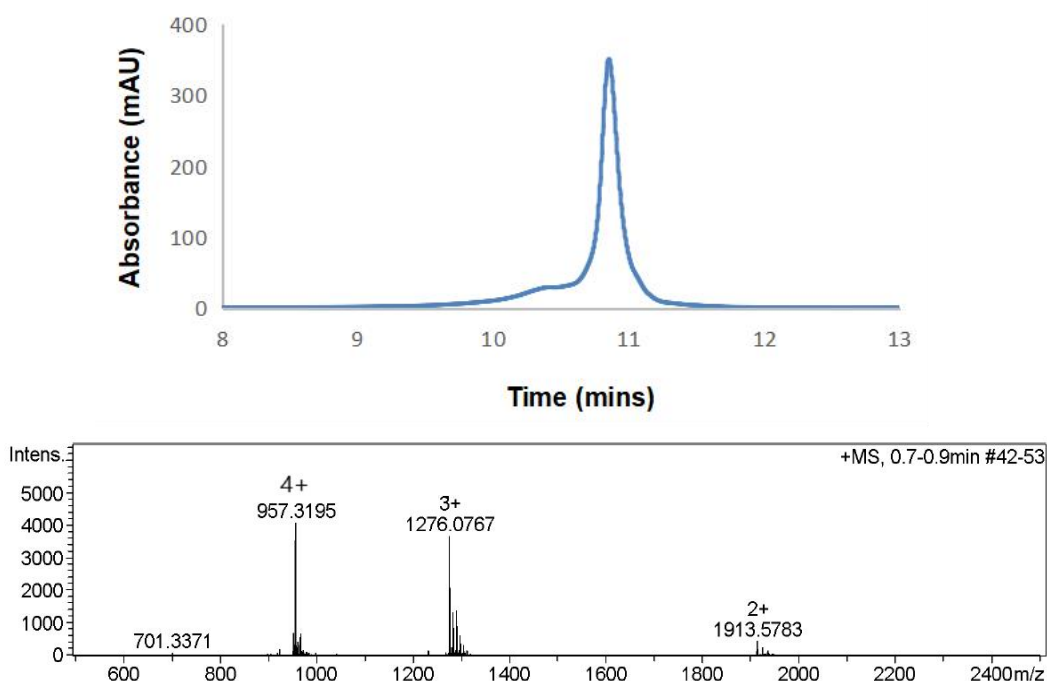


Figure 98: Analytical HPLC trace of pure Pam₂Cys-SK₄-BAGE4₁₈₋₃₉ (**33**). Analytical gradient 2-100% B over 10 min, 280 nm. ESI⁺ MS Calc. For C₁₈₁H₃₀₃N₃₉O₄₆S₂ [M+2H]²⁺:1912.90, [M+3H]³⁺:1275.74, [M+4H]⁴⁺:957.05. Obs. [M+2H]²⁺:1913.58, [M+3H]³⁺:1276.08 [M+4H]⁴⁺:957.32

Synthesis of Pam₂Cys-SK₄-NY-ESO-1₁₅₇₋₁₆₅ (**34**)

Synthesised using general protocol B using NY-ESO-1₁₅₇₋₁₆₅-SK₄ on Rink amide resin (185 mg, 0.03 mmol). For the coupling reagent DIC/Oxyma were replaced with 1.3 eq of each PyBop and HOBt in present of 2.7 eq of DIPEA. 0.015 mmol of the crude cleaved peptide was purified by semi-preparative RP-HPLC (40 to 80% B over 60 mins) and lyophilized to produce the desired Pam₂Cys-SK₄-NY-ESO-1₍₁₅₇₋₁₆₅₎ (9.4 mg, 0.004 mmol, 26 % yield).

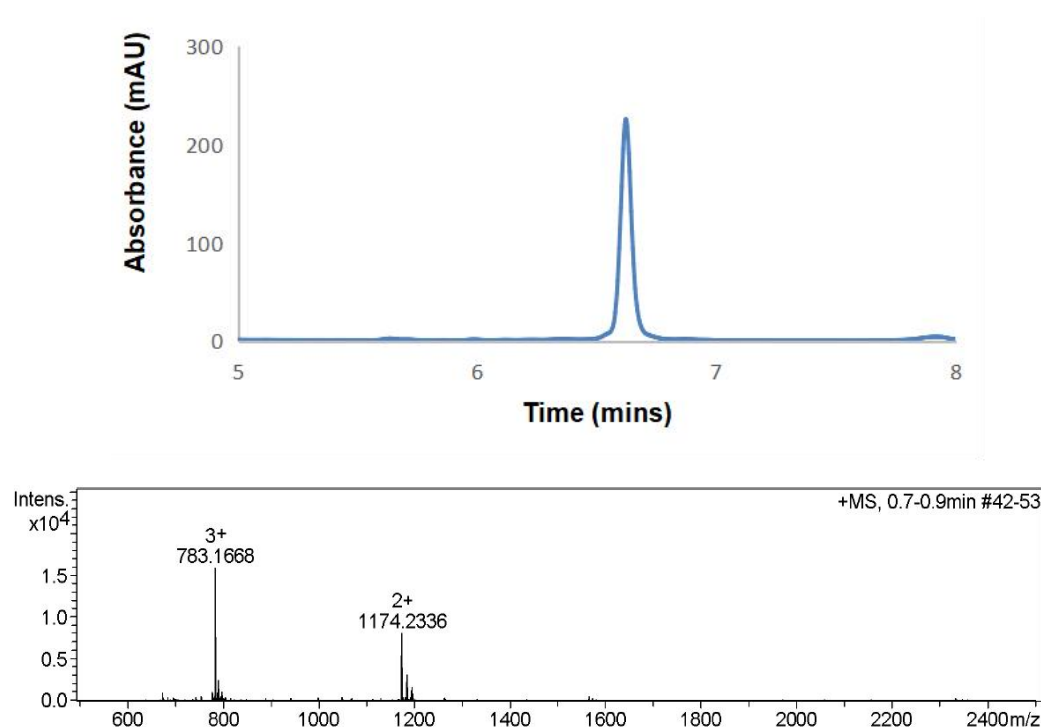


Figure 99: Analytical HPLC trace of pure Pam₂Cys-SK₄-NY-ESO-1₁₅₇₋₁₆₅ (**34**). Analytical gradient 10-100% B over 5 min, 280 nm. ESI⁺ MS Calc. for C₁₁₄H₂₀₄N₂₂O₂₃S₃ [M+2H]²⁺:1173.74, [M+3H]³⁺:782.83. Obs.[M+2H]²⁺:1174.23, [M+3H]³⁺:783.17.

Synthesis of Pam₂Cys-PADRE-NYESO-1₁₅₇₋₁₆₅ (**35**)

Synthesised using general protocol B using PADRE-NYESO-1₁₅₇₋₁₆₅ on Rink amide resin (465mg, 0.025 mmol). For the coupling reagent DIC/Oxyma were replaced with 1.3 eq of each PyBop and HOBT in present of 2.7 eq of DIPEA. Purified by semi-preparative RP-HPLC (60 to 100% B over 30 mins) and lyophilized to produce the desired Pam₂Cys-PADRE -NY-ESO-1₍₁₅₇₋₁₆₅₎ (0.4 mg, 0.00013 mmol, 0.5 % yield).

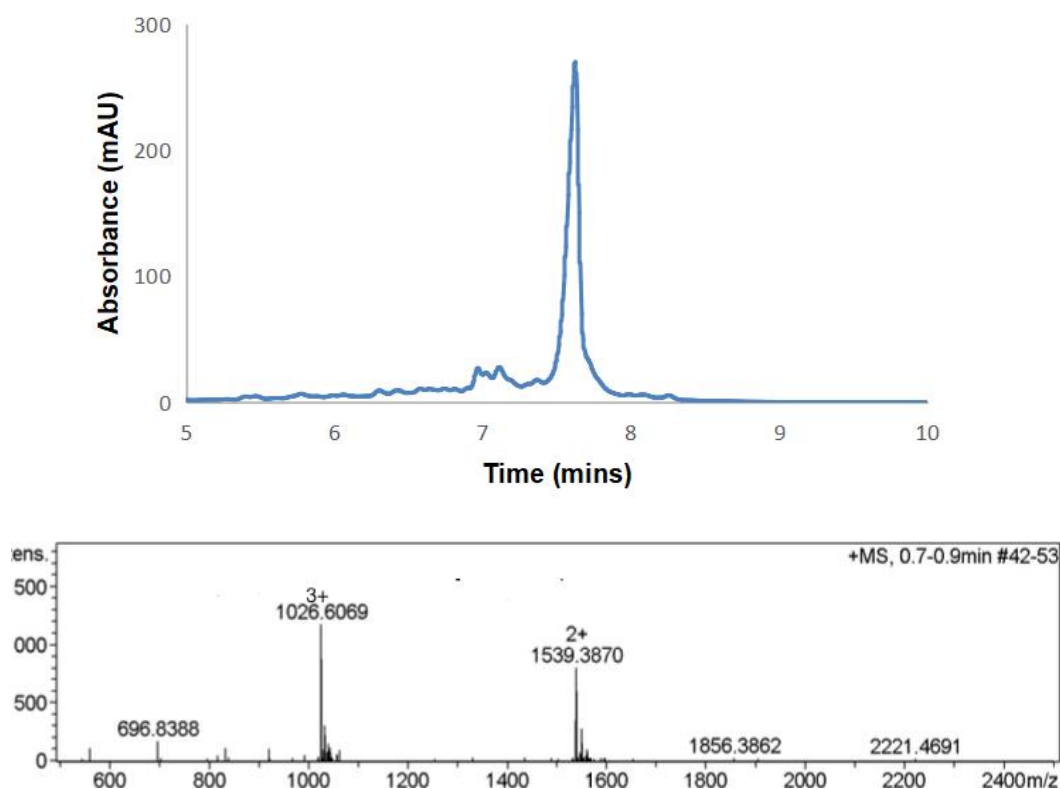


Figure 100: Analytical HPLC trace of pure Pam₂Cys-PADRE-NY-ESO-1₁₅₇₋₁₆₅ (**35**). Analytical gradient 40-100% B over 5 mins 100% for 3mins 0.6 ml/min, 280 nm. ESI⁺ MS Calc. for C₁₅₂H₂₅₁N₂₉O₃₁S₃ [M+2H]²⁺:1539.11, [M+3H]³⁺:1026.42. Obs. [M+2H]²⁺:1539.39, [M+3H]³⁺:1026.61.

7.6 Liposome Formulation

Anionic & cationic liposomes were formulated using the lipid film hydration method.²⁴⁹ Briefly, all lipids (DSPC, mal-lipid, cholesterol, and DMPG-Na or DOTAP) were dissolved in chloroform, details shown in Tables 10-13. For each formulation (5 mL or 2.5 mL), lipids were added to the mixture according to the ratios described in relevant table. Chloroform was slowly evaporated under slightly reduced pressure with the help of rotatory evaporator to make a continuous lipid film, which was dried under high *vacuo* for 4 hrs. PBS (5 mL or 2.5 mL) was added to hydrate the film with vortexing at 55 °C for 1-2 mins. The resulting mixture was sonicated for 10 minutes, no pulsing, amplitude@ 90%, and using ice bath resulting in a 2 mM liposome solution.

Table 13: Summary of the reagents, mass, and volume used to formulate non-mal-lipo -ve (2 mM, 5 mL).

Reagents	mg	mmol	MW	Volume (mL)	%
DSPC	2.37	0.003	790.145	1.5	30
DMPG-Na	2.06	0.003	688.845	1.5	30
Cholesterol	1.55	0.004	386.65	2	40

Table 14: Summary of the reagents, mass, and volume used to formulate non-mal-lipo -ve (2 mM, 5 ml).

Reagents	mg	mmol	MW	Volume (mL)	%
DSPC	1.97	0.0025	790.145	1.25	25
DMPG-Na	1.72	0.0025	688.845	1.25	25
Cholesterol	1.55	0.004	386.65	2.0	40
Mal	0.69	0.001	690	0.5	10

Table 15: Summary of the reagents, mass, and volume used to formulate non-mal-lipo +ve (2 mM, 2.5 mL).

Reagents	mg	mmol	MW	Volume (mL)	%
DSPC	1.82	0.0023	790.145	1.125	45
DOTAP	0.524	0.00075	698.54	0.375	15
Cholesterol	0.773	0.002	386.65	1.0	40

Table 16: Summary of the reagents, mass, and volume used to formulate mal-lipo +ve (2 mM, 2.5 mL).

Reagents	mg	mmol	MW	Volume (mL)	%
DSPC	1.58	0.002	790.145	1.0	40
DOTAP	0.524	0.00075	698.54	0.375	15
Cholesterol	0.676	0.00175	386.65	0.875	35
Mal	0.345	0.0005	690	0.25	10

Formulation of cationic Pam₂Cys-Sk₄-BAGE₄₁₈₋₃₉ liposome

All lipids (DSPC, DOTAP and cholesterol) were dissolved in chloroform and Pam₂Cys-Sk₄-BAGE₄₁₈₋₃₉ was dissolved in methanol prior to formulation, Table 16. Solvents were evaporated slowly in *vacuo* to make a continuous lipid film, which was lyophilized overnight. Milli-Q water (1 mL) was added to hydrate the film with vortexing at 55 °C. The resulting mixture was sonicated for 10 minutes, no pulsing, amplitude@ 90%, and using ice bath resulting in formulated Pam₂Cys-Sk₄-BAGE₄₁₈₋₃₉ vaccine construct.

Table 17: Summary of the reagents, mass, and volume used to formulate Pam₂Cys-Sk4-BAGE4₁₈₋₃₉ lipo +ve (2 mM, 1 mL).

Reagents	mg	mmol	MW	Volume (μ l)	%
DSPC	0.632	0.0008	790.145	400	40
DOTAP	0.209	0.0003	698.54	150	15
Cholesterol	0.271	0.0007	386.65	350	35
Pam ₂ Cys-Sk4- BAGE4 ₍₁₈₋₃₉₎	0.859	0.0002	3825	100	10

Liposomal Characterization

All liposomes (Tables 10-14) were analysed using DLS to measure the particle size and PDI. All samples were diluted 10 times with Milli-Q water and transferred to disposable cuvettes before measurement at 25 °C with a 173° light scattering angle. Non-mal-lipo +ve, mal-lipo +ve, and BAGE4Cys-mal-lipo +ve formulations were characterized with Transmission Electron Microscopy (TEM) to check the particle size and shape. All samples were diluted in Milli-Q water and applied to glow-discharged carbon-coated copper 200 mesh grids and negative-stained with 2% uranyl acetate.

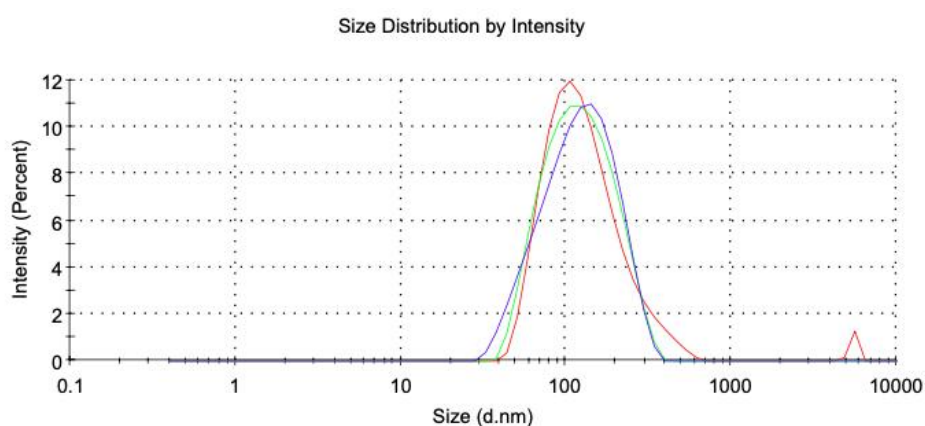


Figure 101: DLS spectra of particle non-mal-lipo –ve size distributions by intensity. Z-Average (d.nm): 129.9, PdI : 0.36.

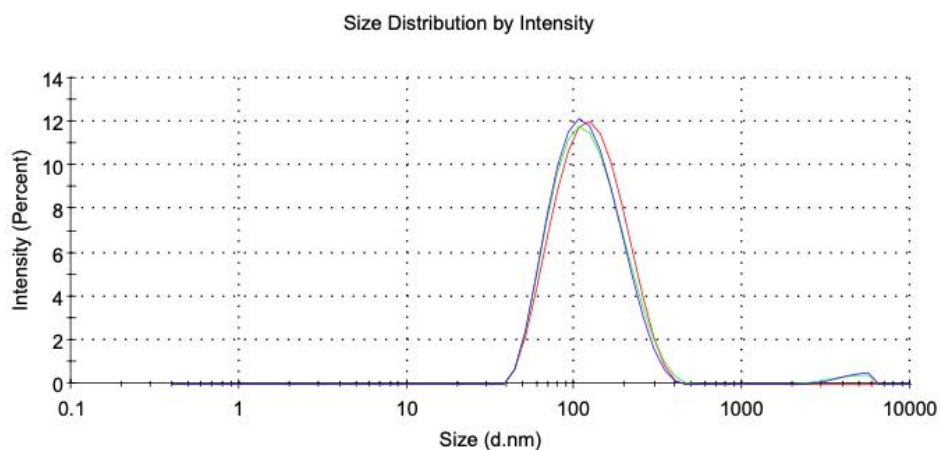


Figure 102: DLS spectra of particle mal-lipo -ve size distributions by intensity. Z-Average (d.nm): 114.6, PdI : 0.194.

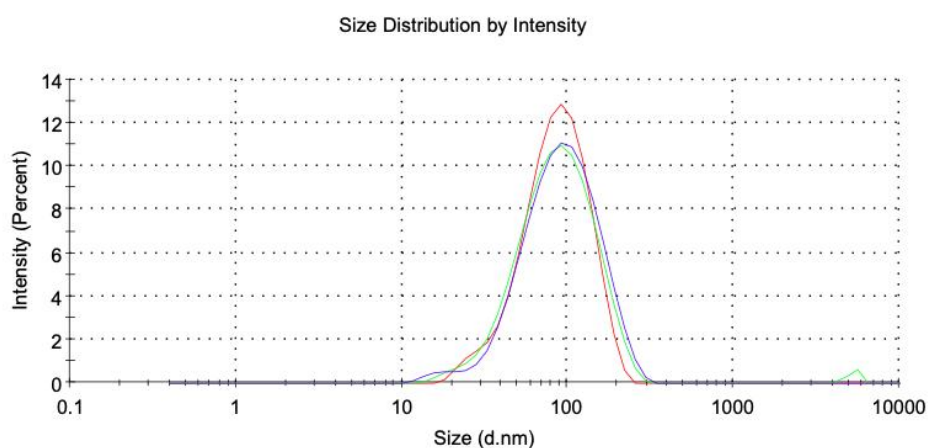


Figure 103: DLS spectra of particle non-mal-lipo +ve size distributions by intensity. Z-Average (d.nm): 80.06, PdI: 0.239.

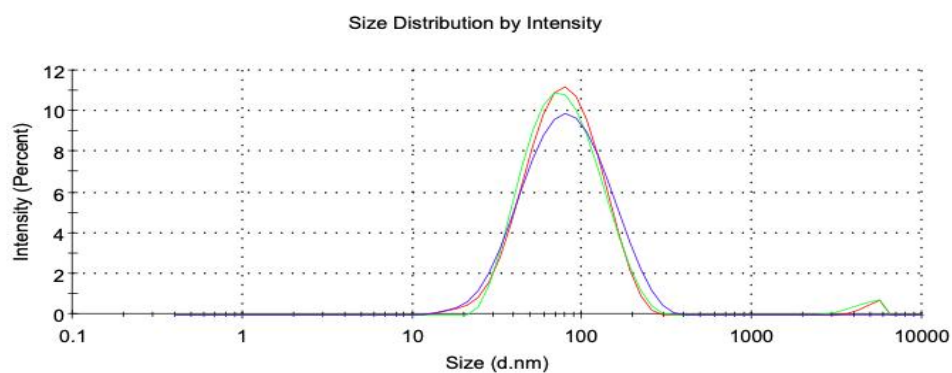


Figure 104: DLS spectra of particle mal-lipo +ve size distributions by intensity. Z-Average (d.nm): 70.28, PdI: 0.236.

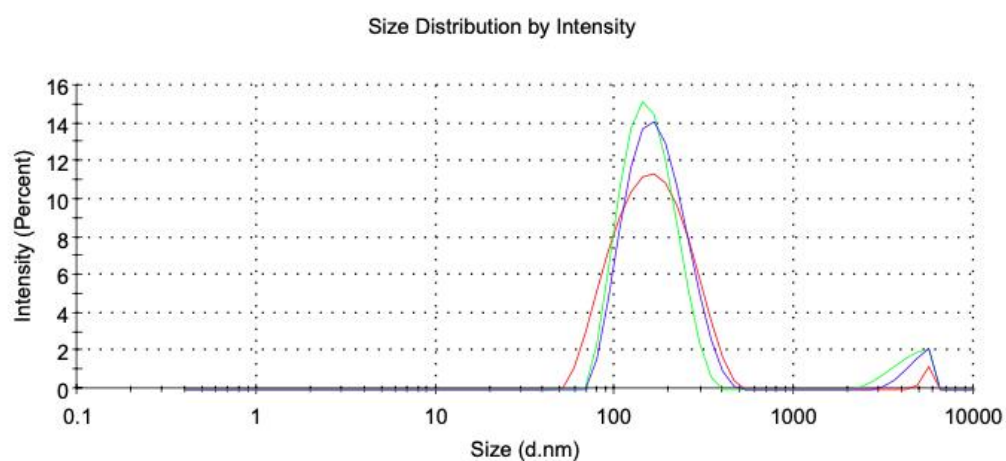


Figure 105: DLS spectra of particle Pam₂Cys-Sk₄-BAGE₄₁₈₋₃₉ Liposome +ve size distributions by intensity. Z-Average (d.nm): 164.5, PdI : 0.304.

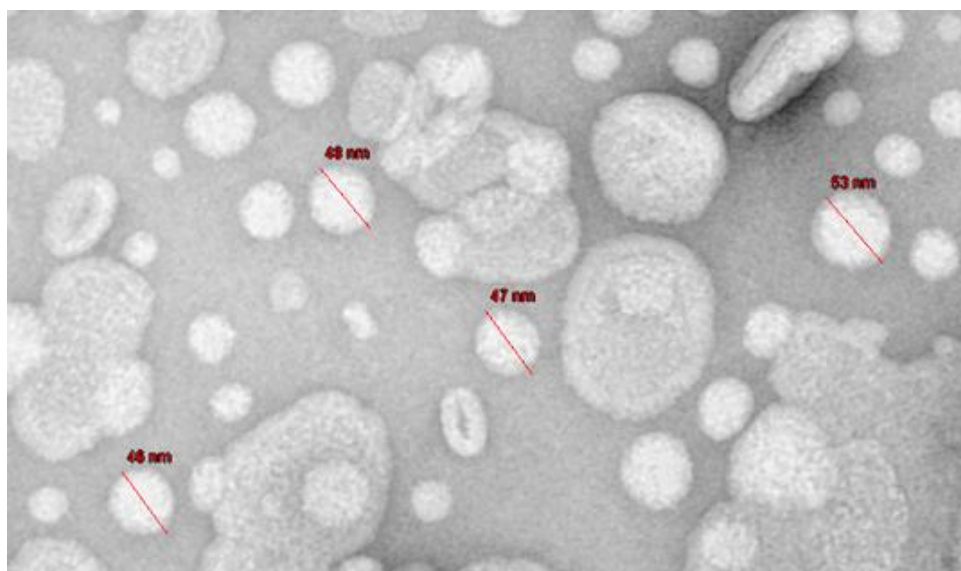


Figure 106: TEM for non-mal-liposome +ve, the image capturing was operated at 87k

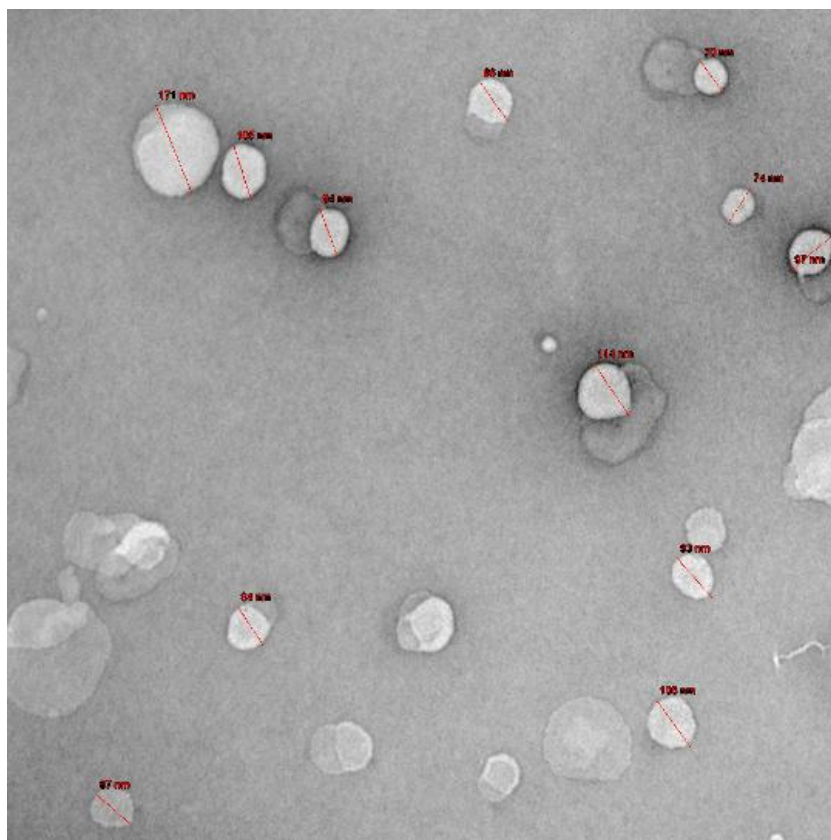


Figure 107: TEM for **Mal-lipo +ve**, the image capturing was operated at 43k.

Conjugation reaction and dialysis

1200 μL of 2 mM mal-liposome formulation (containing 0.24 μmol mal-lipid) was conjugated to (2 eq, 0.48 μmol) of CysBAGE₄₁₈₋₃₉ in the presence of TCEP, at pH 7. The reaction mixture was vortexed for 3 hrs at RT. The sample was loaded into Slide-A-Lyzer Dialysis Cassette (10k MWCO) then floated in dialysate buffer (PBS), 500 times the volume of loaded sample. The dialysate buffer was changed after 2 hrs, 3 times, the last for a overnight period. The sample was then recovered and analyzed by UV-vis @ 280 nm.

Measurement of extinction coefficient for Coumarin-model peptide

A series of concentrations (7 samples) were prepared starting from 0.125 mM to 0.00195 mM of coumيران model peptide (**54**) in PBS buffer. The absorbance measurement was taken for each sample using UV-Vis @ 300 nm. The data was plotted and the extinction coefficient calculated as $9018.9 \text{ M}^{-1} \text{ cm}^{-1}$ @ 300 nm.

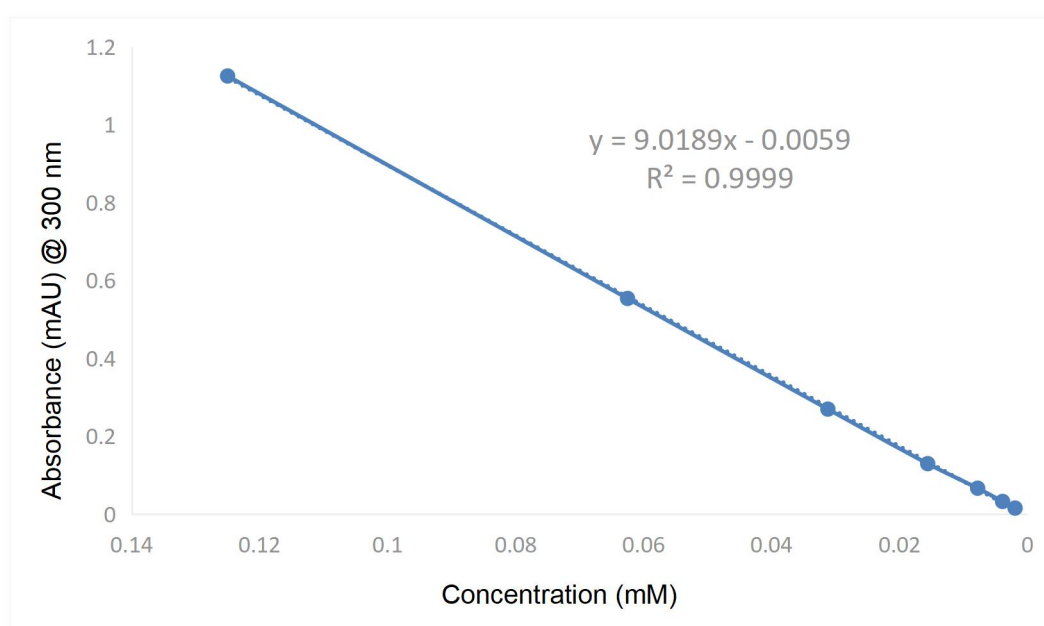


Figure 108: The absorbance values @ 300 nm for Coumarin-model peptide (**54**) at different concentration (mM).

7.7 Vaccination preparation and procedure

Animals studies were performed by Scancell Ltd at Nottingham Trent University animal facility under Home Office approved project licence PP2706800. The following tables are summarising the details of the vaccine candidates and vaccination procedure including (dose time points).

Table 18: Summarise the injections plan (including dose concentration and number) for Pam₂Cys-SK₄-NY-ESO-1₁₅₇₋₁₆₅ (**37**) candidate using HHDII/DR1 Tg mice.

Group	Test article	Dose (µg)/vol	Route	Dosing schedule	N	Terminal	Assays at termination
1	NY-ESO-1 peptide/ Pam ₂ Cys conjugate	10 nmol in 50 µl	SC	Day 1,8 & 15	3	Day 21	ELISpot on splenocytes

Table 19: Summarise the injections plan (including dose concentration and number) for the two different vaccine candidates, BAGE₄₁₈₋₃₉ (**28**) candidate, BAGE₄Cys-Mal-lipo⁺ve conjugate using BALB/c mice.

Group	Test article	Dose (µg)/vol	Route	Dosing schedule	N	Terminal	Assays at termination
1	BAGE ₄₁₈₋₃₉ peptide mixed with standard adjuvant (Incomplete Freund's) conjugate	10 nmol in 50 µl	SC	Day 1,8 & 15	3	Day 21	ELISpot on splenocytes ELISA on sera samples
2	BAGE ₄ Cys-Mal-lipo ⁺ ve conjugate	10 nmol in 50 µl	SC				

7.8 The enzyme-linked immunospot (ELISpot) assay general procedure

Preparation of media and buffers and steps performed on days 1 and 2 of the ELISpot assay should be performed using a laminar flow cabinet and aseptic techniques. This is to ensure the sterility of media, reagents and plates at stages before development of the ELISpot. Development of the ELISpot on day 4 can be performed on the laboratory bench.

Day 1: Coating of plates with capture antibody

a) Add 20 μL 35% ethanol/well. Flick off, using a multi-channel pipette wash each plate 4 times with 200 μL of sterile distilled water per well. Repeat for each plate.

b) Dilute the capture antibody (mAb AN 18, 1 mg/mL) to 10 $\mu\text{g/mL}$ (1:100) in sterile 1 x PBS.

Number of plates	1	2	3	4	5	6
Volume of capture antibody	50 μL	100 μL	150 μL	200 μL	250 μL	300 μL
Volume of 1 x PBS	5 mL	10 mL	15 mL	20 mL	25 mL	30 mL

c) Add 50 μL diluted capture antibody per well to MAIP multiscreen ELISpot plate(s) using a multichannel pipette. Ensure that the bottom of the wells are completely covered with coating antibody solution.

d) Cover each plate with lid and incubate at 4°C overnight.

Day 2: Blocking plates

a) Flick off the capture antibody solution, using a multi-channel pipette wash each plate 4 times with 200 μL of sterile PBS per well.

- b) Flick off the PBS, add 100 μ L Complete Medium to each well using a multi-channel pipette.
- c) Incubate at room temperature for at least 1 hour to 24 hours.

Preparation of effector cells from spleens

Kill mouse by cervical dislocation (Note that this was performed by the licenced animal facility).

Note: After this step sterile conditions are required, all further procedures are carried out in a class 2 microbiological safety cabinet.

- a) Lay the mouse on its right side, on a paper towel, in the hood. Wet the skin of the mouse with 70% ethanol. This step minimizes the risk of bacterial contamination.
- b) Use large forceps to grasp and lift the skin about 1 cm below the rib cage on the left side of the mouse. This will be over the spleen region. Use large scissors to cut the skin away.
- c) Use small forceps to grasp the peritoneum and small scissors to cut open the cavity exposing the spleen.
- d) With small forceps lift the spleen high and cut, with small scissors, to release it.
- e) Transfer the spleen to a petri dish containing 10 mL complete medium, warmed to 37°C.
- f) Draw 10 mL complete medium into a 20 mL syringe with a 21-gauge needle. Hold the spleen to the dish with large forceps. Puncture the outer capsule of the spleen with the needle and flush the splenocytes out of the spleen. Gently squeeze the spleen capsule with forceps to remove remaining splenocytes. Transfer cells to a sterile, 25 mL conical Universal tube.
- g) Allow clumps to settle for 5 minutes then decant cell suspension into

a fresh 25 mL conical Universal tube. Centrifuge 1,000 rpm for 10 minutes in a benchtop centrifuge. Resuspend cell pellet in 10 mL complete medium containing 2-mercaptoethanol.

h) Count viable cells by mixing equal volumes (50 μ L) of cell suspension and 0.4% Trypan blue and transferring 9 μ L to a FastRead disposable haemocytometer.

Note: Trypan blue is taken up by nonviable but not by viable cells. Nonviable cells appear blue while viable cells remain colourless.

i) Resuspend cells to a concentration of 5×10^6 cells/mL in complete medium containing 2- mercaptoethanol.

Addition of splenocytes and peptide to ELISpot plates

a) Flick media off blocked ELISpot plate(s).

b) Add cells from splenocyte harvest at 100 μ L/well (5×10^5 cells) in Complete Medium plus Mercaptoethanol (5×10^{-6} M). Assay performed in quadruplicate

c) Add peptides diluted appropriately in Complete Media plus Mercaptoethanol (10 μ g/mL to 0.01 μ g/mL range) to relevant wells in ELISpot plate

d) Include media control (no peptide) as a negative control and LPS as (at 5 μ g/mL) as a positive control

e) Incubate at 37°C for approx. 40 hours.

Day 4: Development of ELISpot

Note: At this step conditions are non-sterile and all further procedures can be carried out on the bench.

a) Flick off cells from ELISpot plate and wash 5 times with 200 μ L/well

PBS 0.05% Tween.

b) Add 50 μ L/well biotinylated detection antibody (monoclonal antibody yellow top vial from ELISpot kit) diluted 1:1000 in PBS.

c) Incubate at room temp for 2.5 hours (2-4 hours).

d) Flick off antibody and wash 5 times with 200 μ L/well PBS 0.05% Tween

e) Add 50 μ L/well streptavidin Alkaline Phosphatase (white top vial from ELISpot kit) diluted 1:1000 in PBS.

f) Incubate for 1.5 hours (1-2 hours) at room temp.

g) Flick off and wash 6 times with 200 μ L/well PBS 0.05% Tween.

h) Add 50 μ L/well development solution and leave in dark at room temp for 5 minutes to develop.

Development solution (for 1 plate) in this order:

4.8 mL dH₂O

200 μ L Development buffer (mix well)

50 μ L reagent A	}	Alkaline Phosphatase substrate kit
50 μ L reagent B		

Mix well and use immediately.

Number of plates	2	3	4	5	6
Volume of distilled water	9.6 mL	14.4 mL	19.2 mL	24 mL	28.8 mL
Volume 25 x buffer	0.4 mL	0.6 mL	0.8 mL	1.0 mL	1.2 mL
Volume of reagent A	100 μ L	150 μ L	200 μ L	250 μ L	300 μ L
Volume of reagent B	100 μ L	150 μ L	200 μ L	250 μ L	300 μ L

i) Once you can see spots wash plate under tap to stop the reaction. Remove the plastic plate back and wash the back of the wells too.

j) Leave to dry at room temp with the back off.

k) Count spots on ELISpot reader.

Enzyme-linked immunosorbent assay (ELISA)

Coating antigen

The concentration of stock samples used for ELISA for BAG₄₁₈₋₃₉ and Pam₂Cys-SK₄-BAGE₄₁₈₋₃₉ is 1 mM

a) Dilute the antigen to a final concentration of 100 ng/ml in carbonate bicarbonate buffer. Coat the wells of Maxisoup 384 well plate with the antigen by pipeting 60 μ L of the antigen dilution in the first column wells of the plate. Dilute down the plate as required (23 dilutions were prepared).

b) Cover the plate with a foil tape and incubate at 4°C overnight.

c) Remove the coating solution and wash the plate three times by filling the wells with 200 μ L PBST. The solutions or washes are removed by flicking the plate over a sink. The remaining drops are removed by patting the plate on a paper towel.

Blocking

a) Block the remaining protein-binding sites in the coated wells by adding 40 μ L blocking buffer, 3% whey, 0.05% NaN_3 , 0.1% EDTA in PBS, per well. Alternative blocking Intercept blocking buffer TBS.

b) Cover the plate with a foil tape and incubate for at least 2 hrs at room temperature or, if more convenient, overnight at 4°C.

c) Wash the plate three with PBS.

Incubation with primary and secondary antibody

a) In 96 Abgene well plate, prepared 8 different dilutions of primary antibody in PBST buffer starting with 1:250 in the whole top well then by 2 folds down the plate.

b) Add 20 μ L of each dilution of primary antibody to coated 384 well in duplicate.

c) Cover the plate and incubate for 1 h at room temperature.

d) Wash the plate four times with PBST.

e) Add 20 μ L of secondary antibody- HRP, diluted at the optimal concentration (according to the manufacturer) in PBST buffer immediately before use.

f) Cover the plate and incubate for 1 hr at room temperature.

j) Wash the plate four times with PBS.

Detection

Add 40 µL of 3,3',5,5'-tetramethylbenzidine (TMB) solution to each well, incubate for 20-30 min, add equal volume of stopping solution (2 M H₂SO₄) and read the optical density at 450 nm.²⁶⁶

Competitive ELISA

For competitive ELISA the steps of coating antigen, blocking, and detection are the same of ELISA describe above. For the incubation step details as below.

Incubation with primary and secondary antibody

In 96 Abgene well plate, add 60 µl of BAGE4 starting at concentration of 1000 ng/mL (1µM) on the first 2 top well of first column then diluted down the plate except column No.12. The same with lipopeptide (Pam₂Cys-Sk₄-BAGE4) for next 2 lines of well then for BAGE4Cys-mal-liposome. Nothing for last 2 rows of the plate.

a) Add 20 µL of primary antibody at the optimized concentration at 1:250 and incubate for 1 hr.

b) Upload the incubated BAGE4, Pam₂Cys-Sk₄-BAGE4, and BAGE4Cys-mal-liposome with primary antibody to washed coated plate with antigen (BAGE4) and incubate for 1 hr.

c) Wash the plate four times with PBST.

d) Add 20 µl of secondary antibody- HRP (Polyclonal swine Anti-Rabbit Immungic- HRP), diluted 1: 4000 (according to the manufacturer) in PBST buffer immediately before use and incubate for 1 hr. Wash the plate four times with PBS

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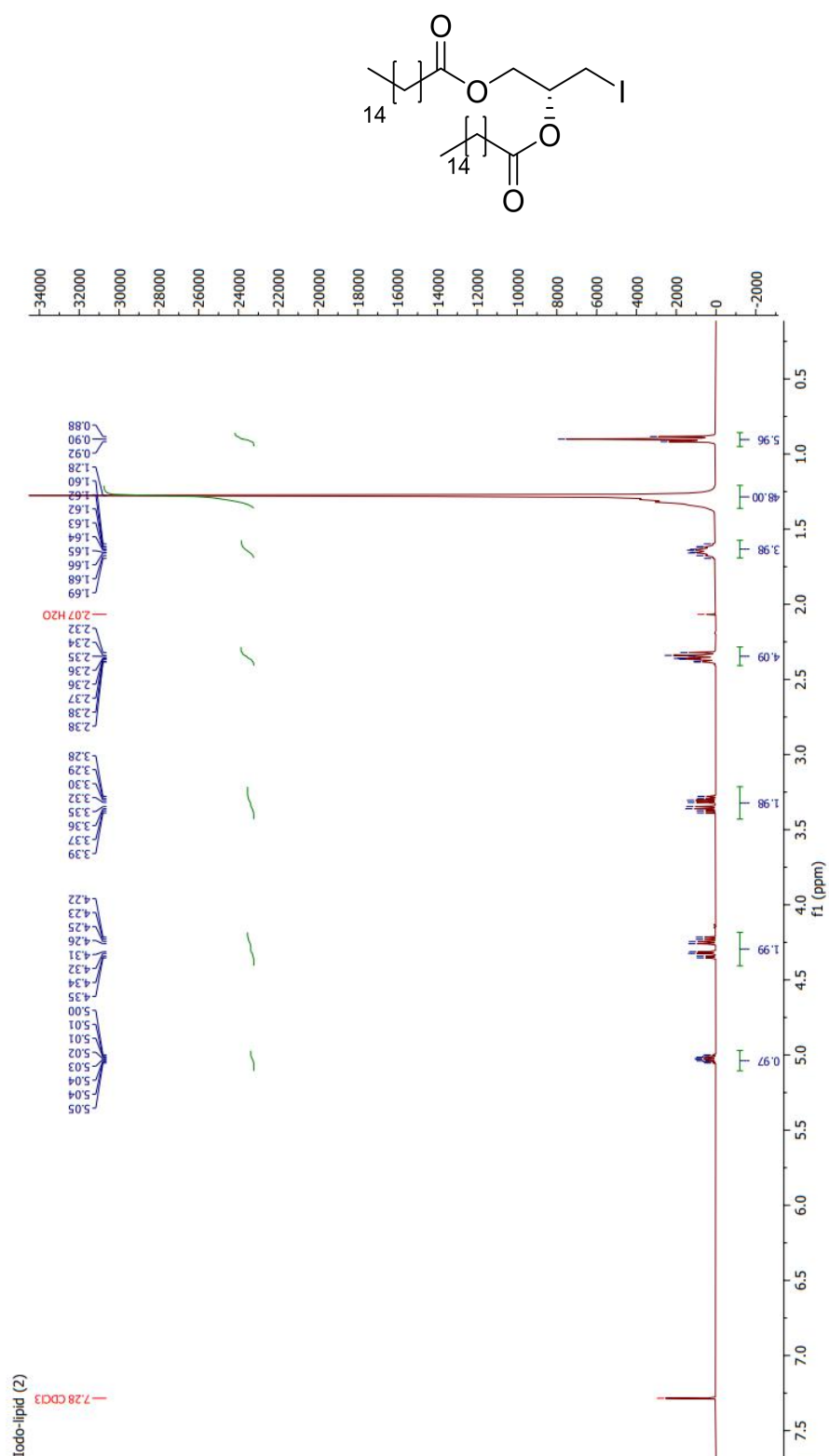
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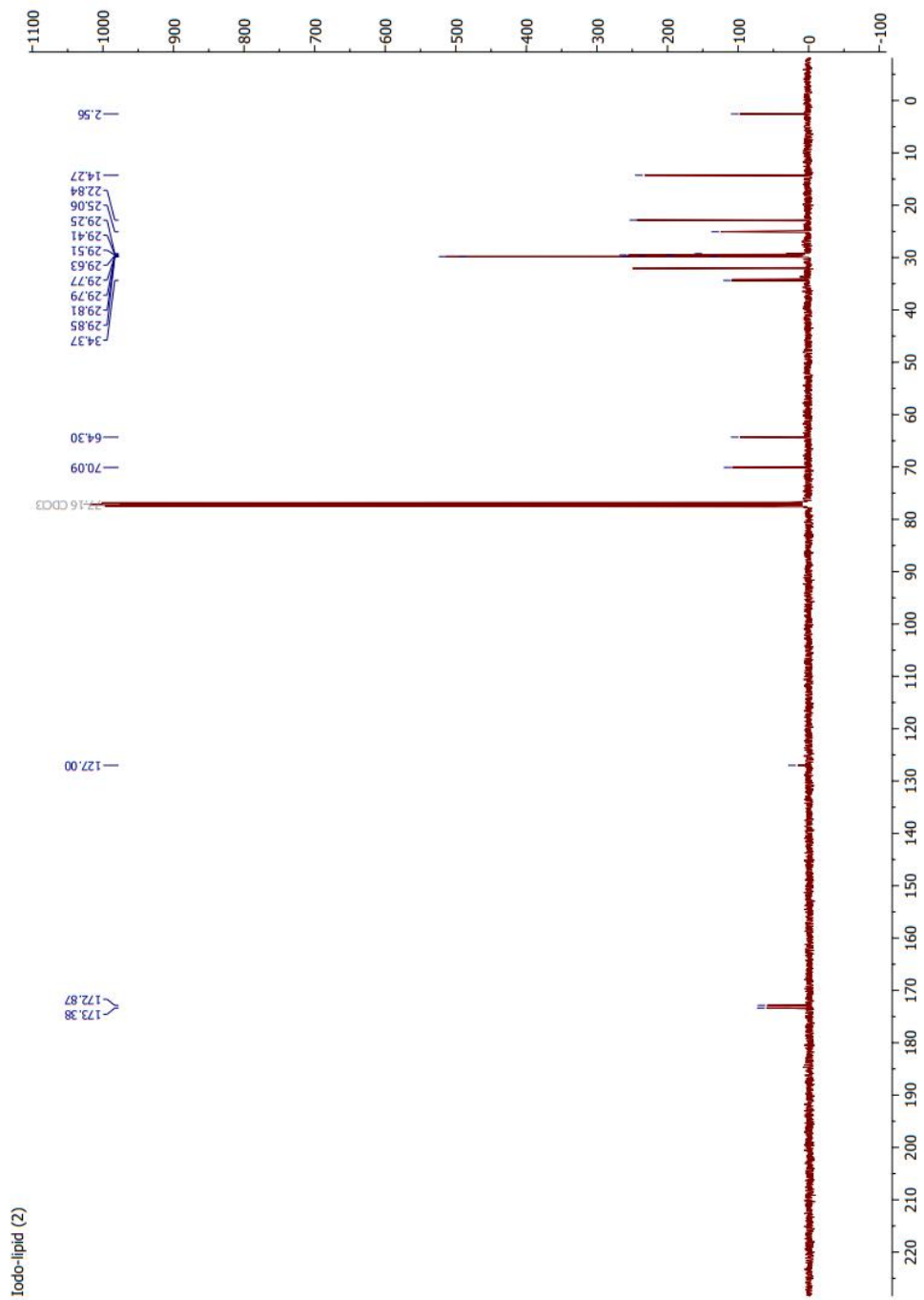
Appendix

Iodo-lipid ^1H & ^{13}C NMR spectra

Iodo-lipid (2)

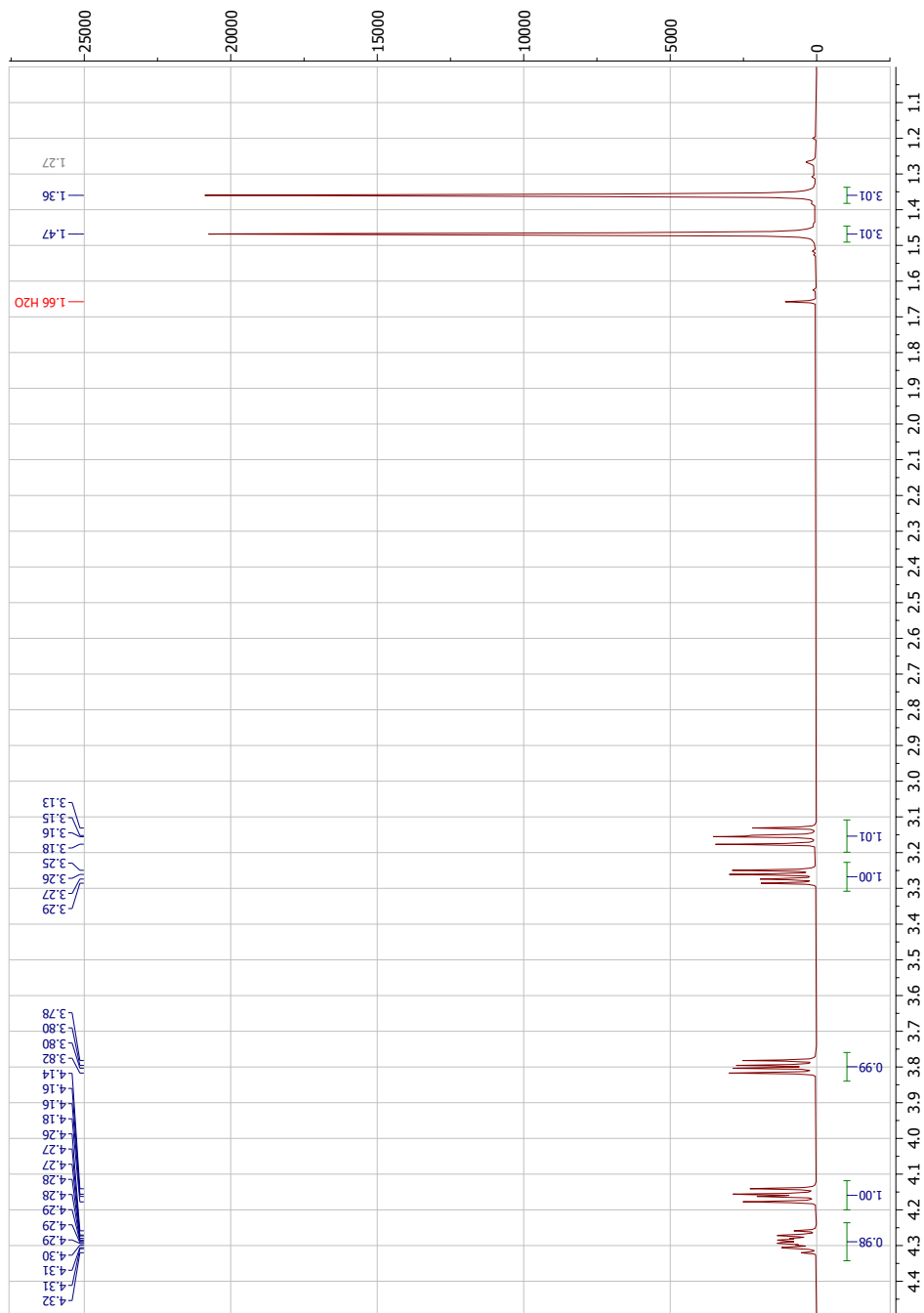
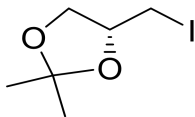
^1H NMR (400 MHz, CDCl_3) & ^{13}C NMR (101 MHz, CDCl_3)

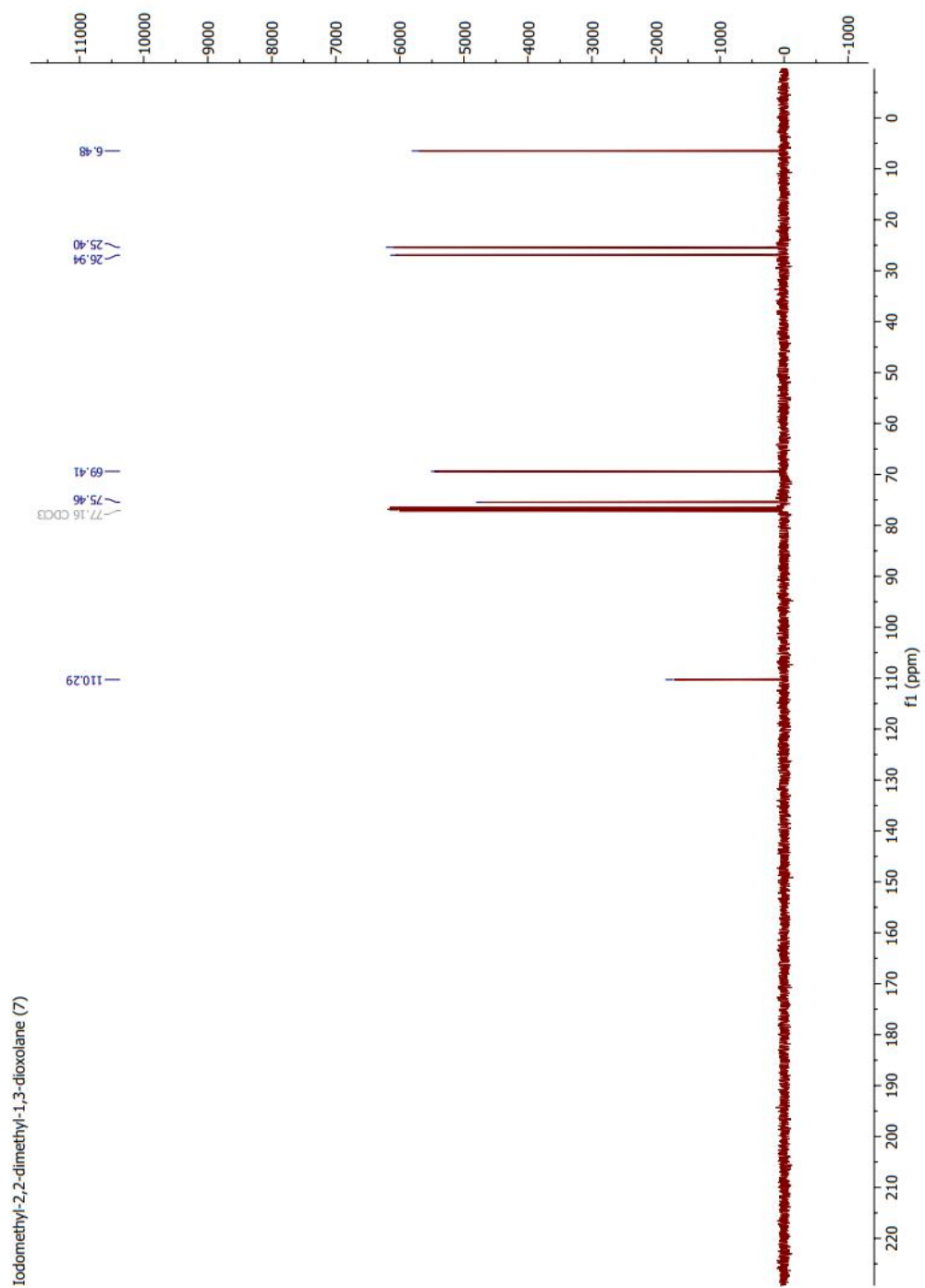




Iodomethyl-2,2-dimethyl-1,3-dioxolane (7)

^1H NMR (400 MHz, CDCl_3) & ^{13}C NMR (101 MHz, CDCl_3)

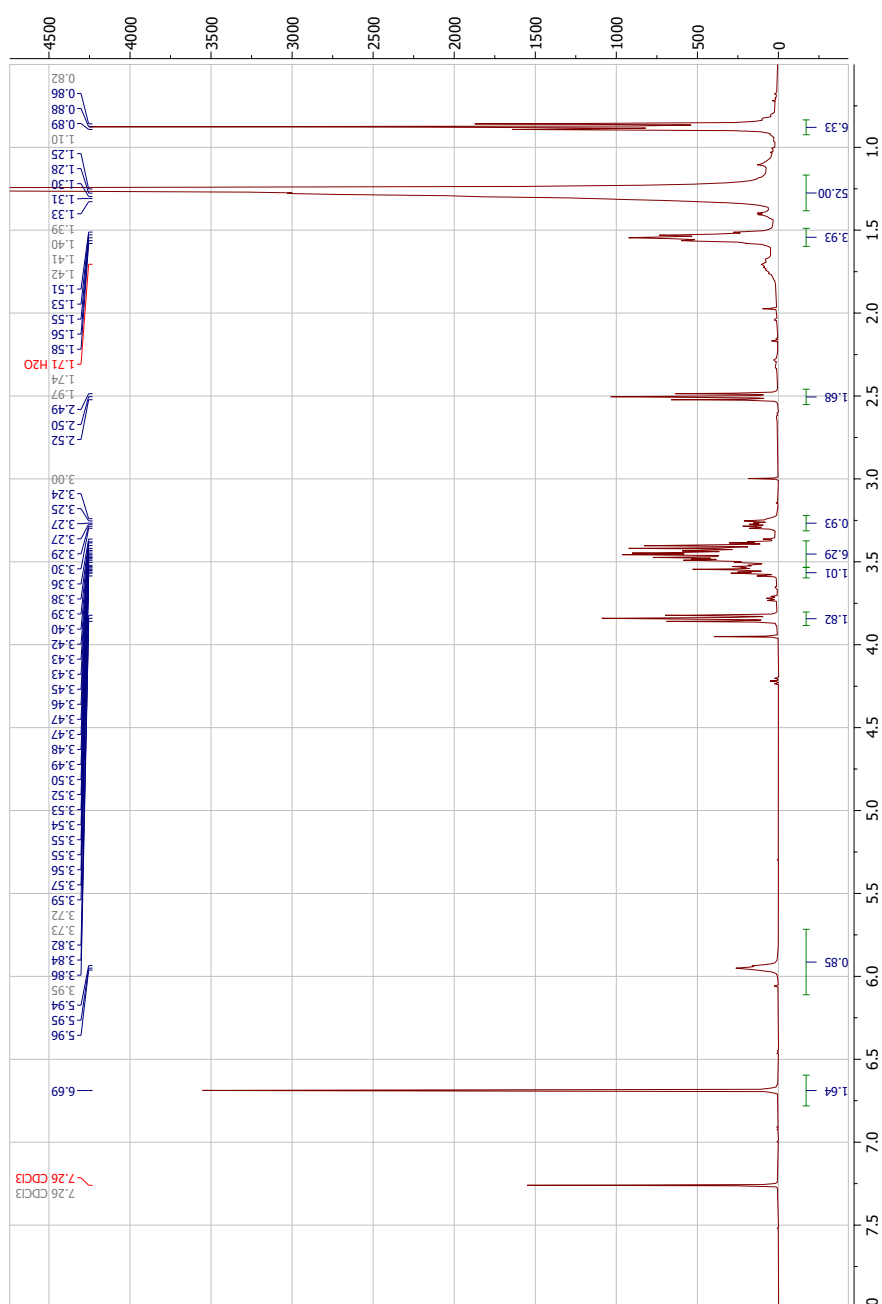
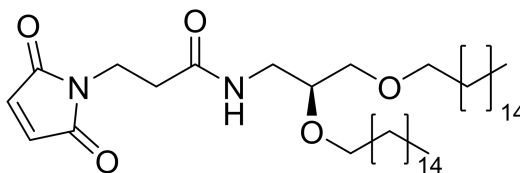


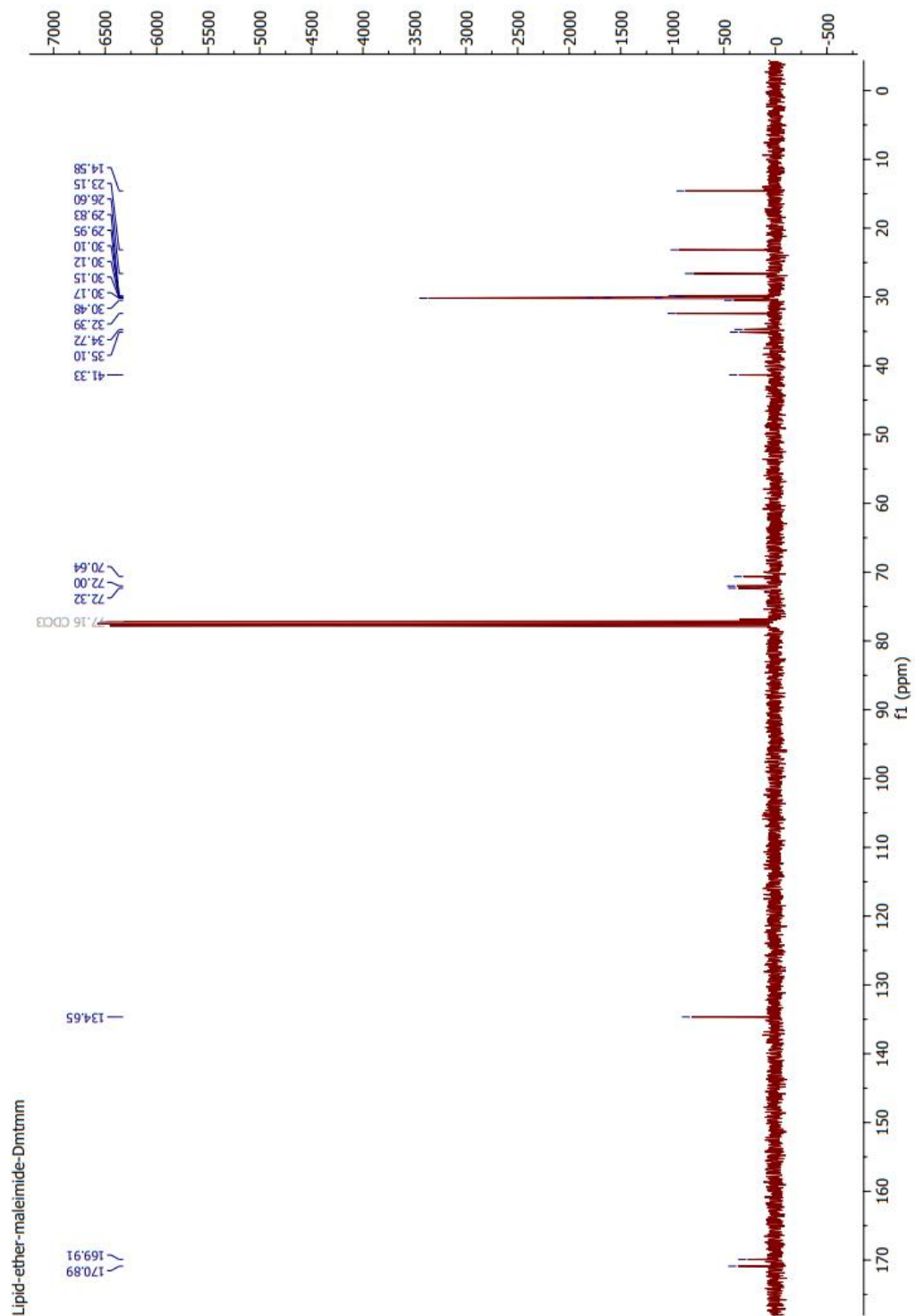


lipid ether maleimide ^1H & ^{13}C NMR spectra

lipid ether maleimide (1).

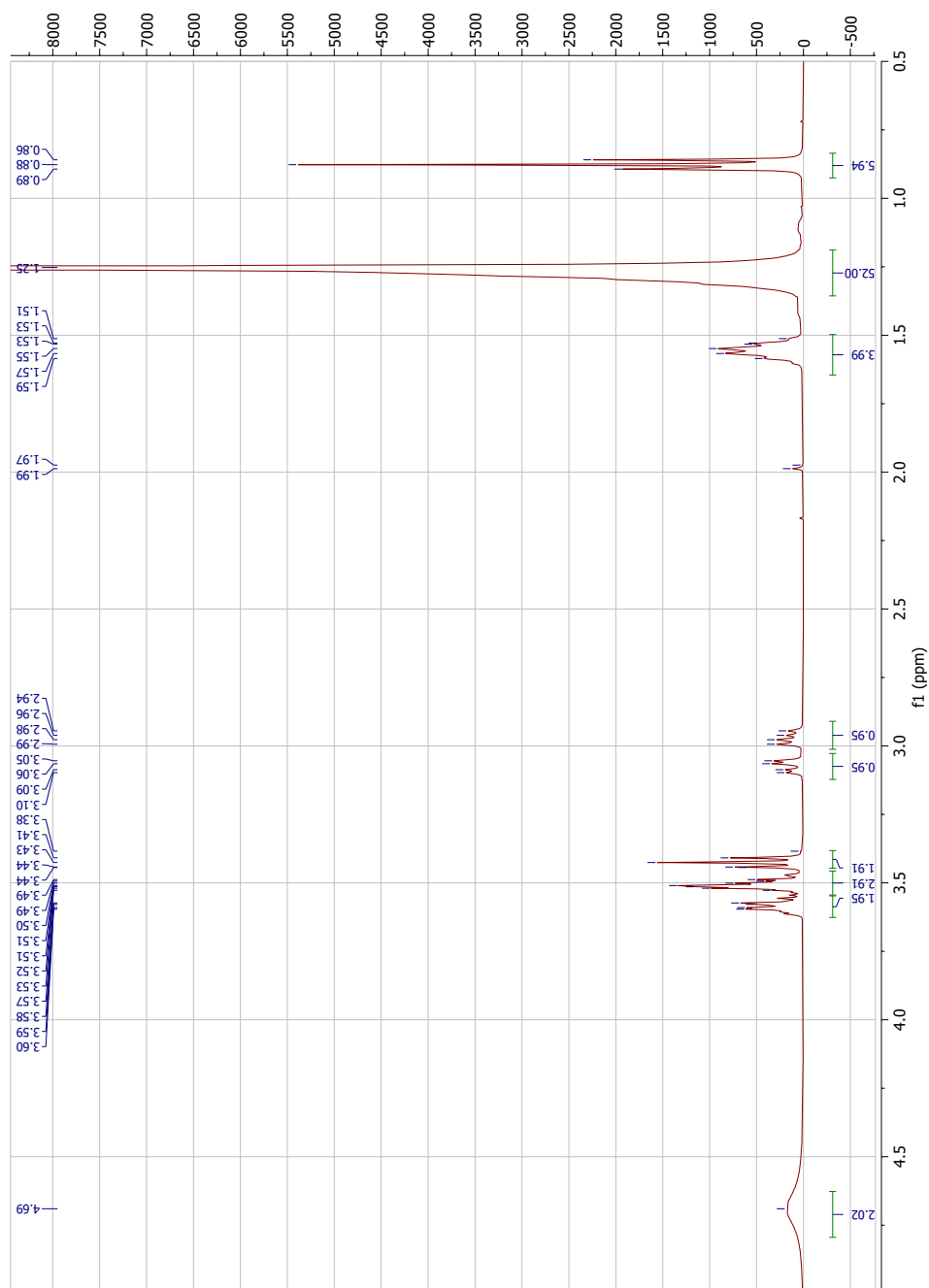
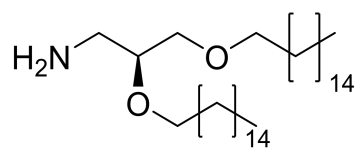
^1H NMR (400 MHz, CDCl_3) & ^{13}C NMR (101 MHz, CDCl_3)

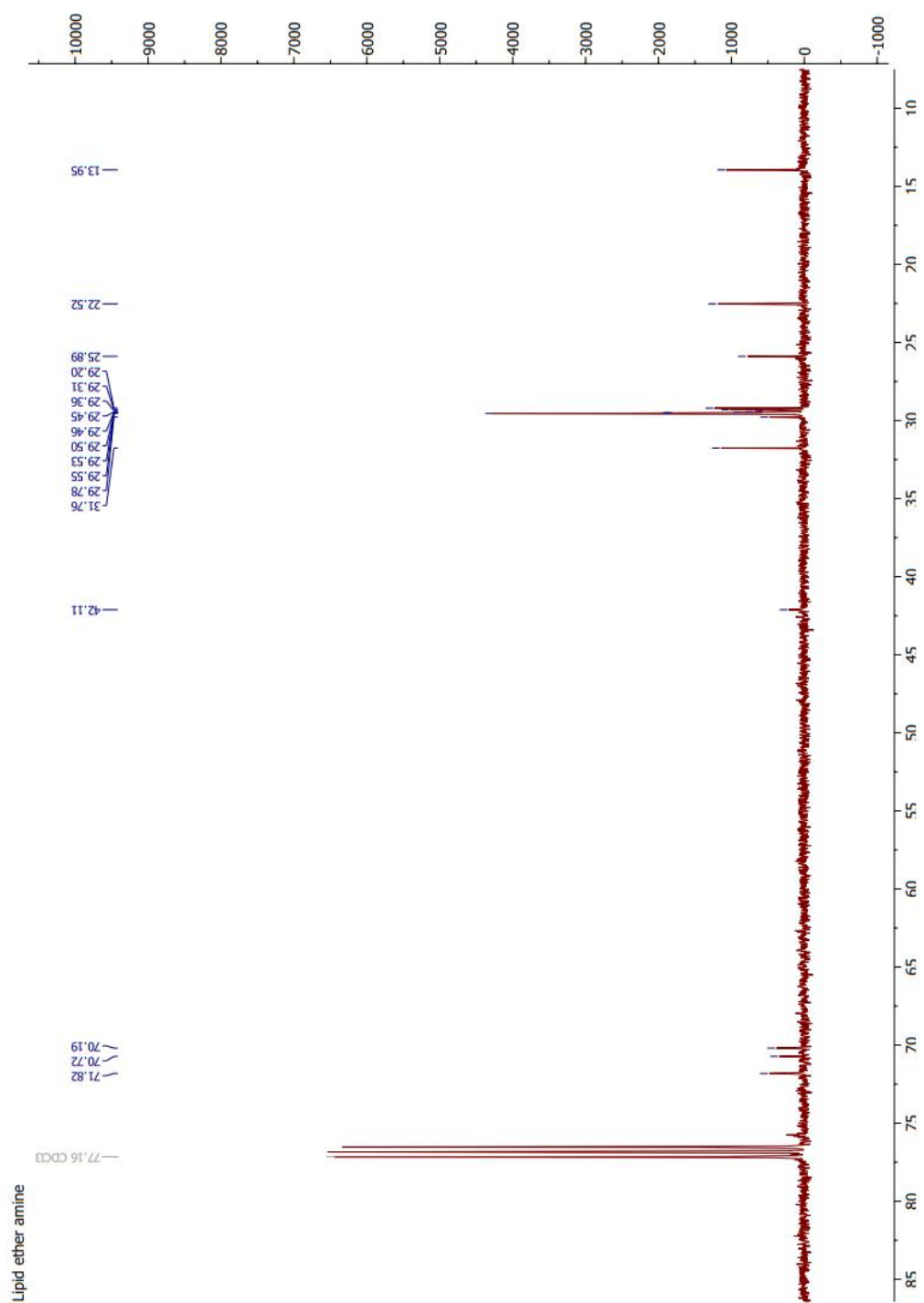




Lipid ether amine (44)

^1H NMR (400 MHz, CDCl_3) & ^{13}C NMR (101 MHz, CDCl_3)

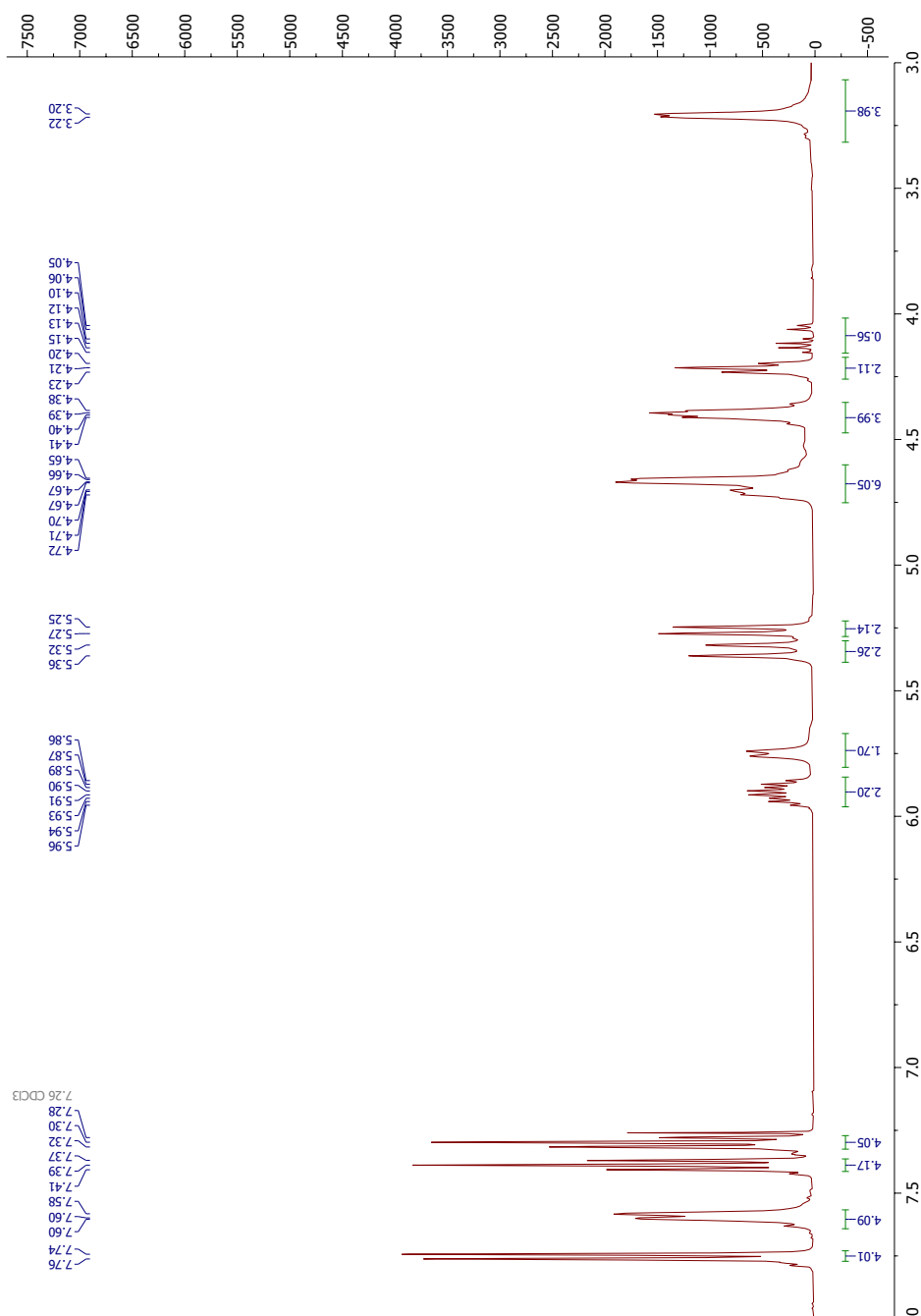
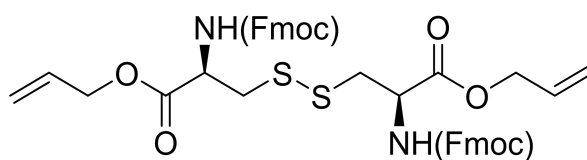


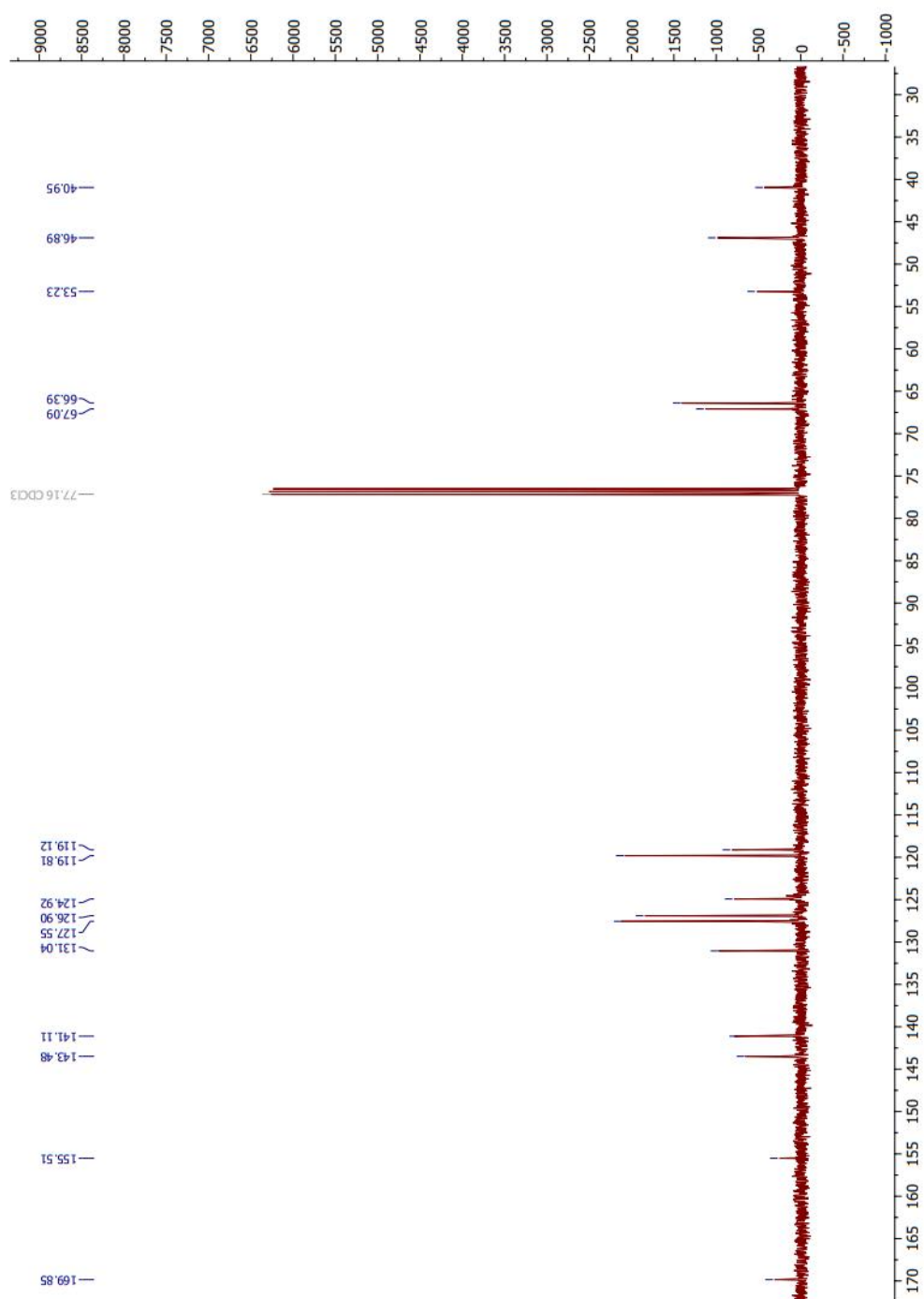


Fmoc-Pam₂Cys ¹H & ¹³C NMR spectra

Allyl-Fmoc-cystine (19)

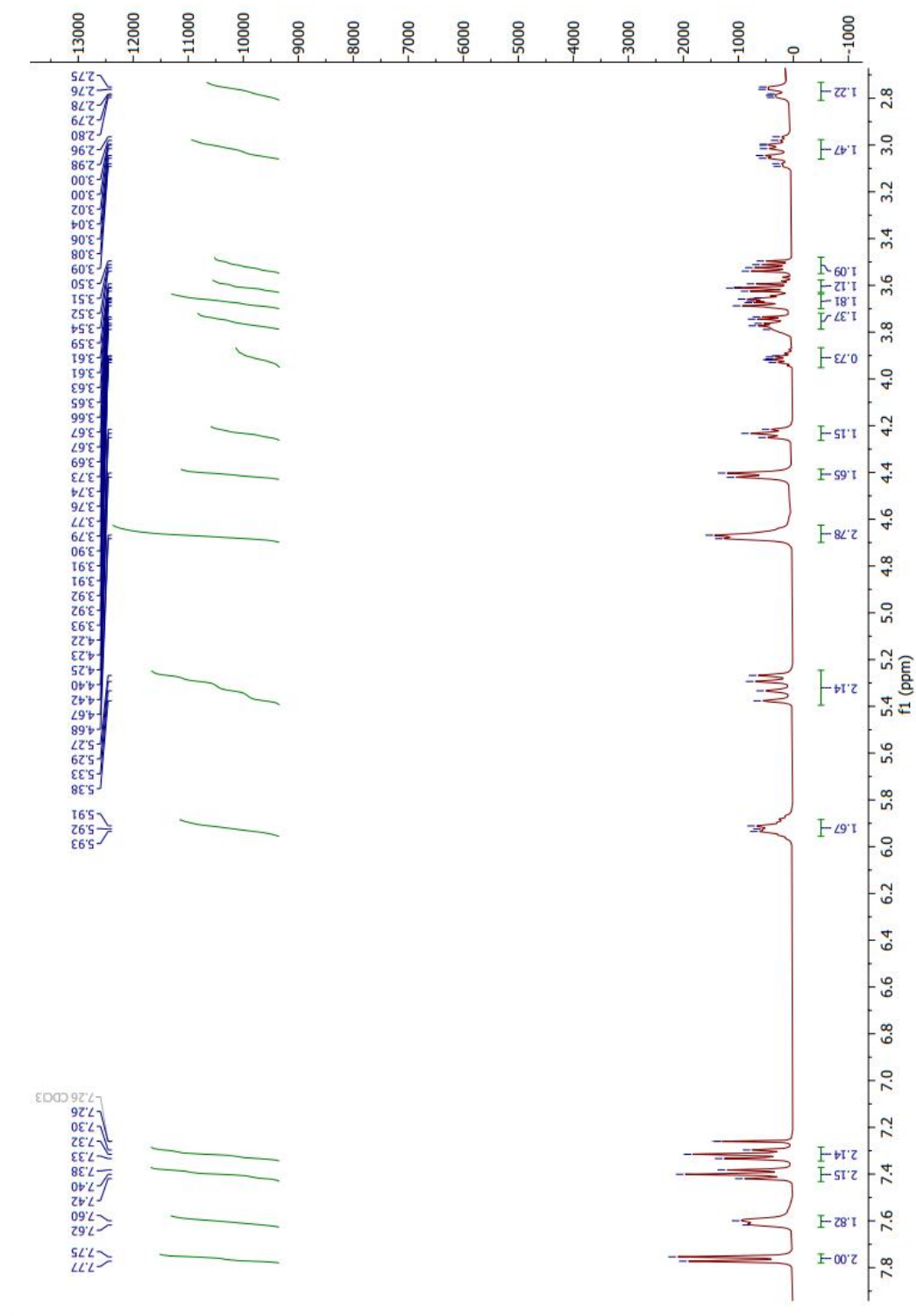
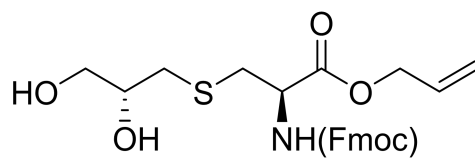
¹H NMR (400 MHz, CDCl₃) & ¹³C NMR (101 MHz, CDCl₃)

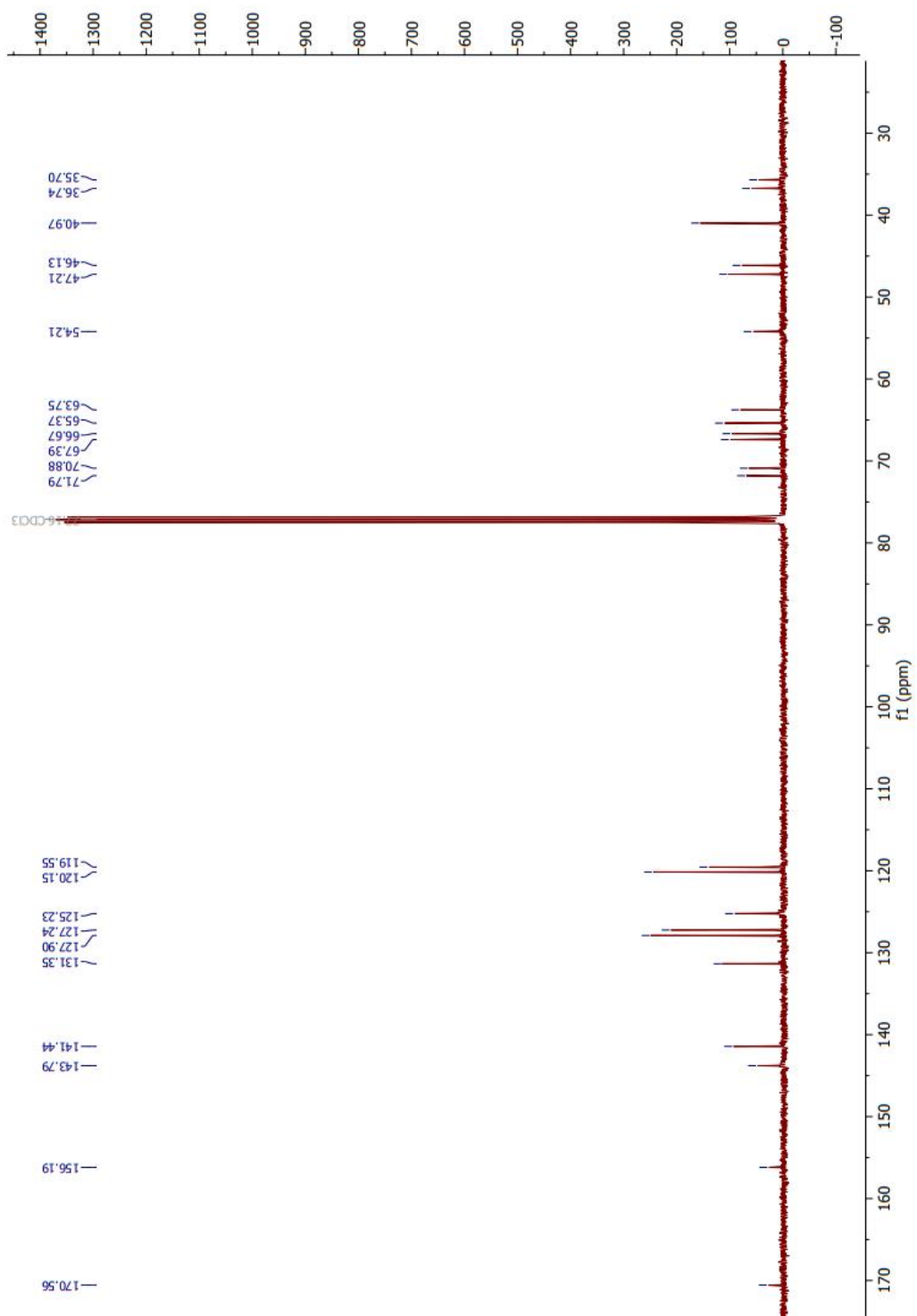




Allyl- Fmoc-*R*-(2,3-dihydroxypropyl) Cysteine (20)

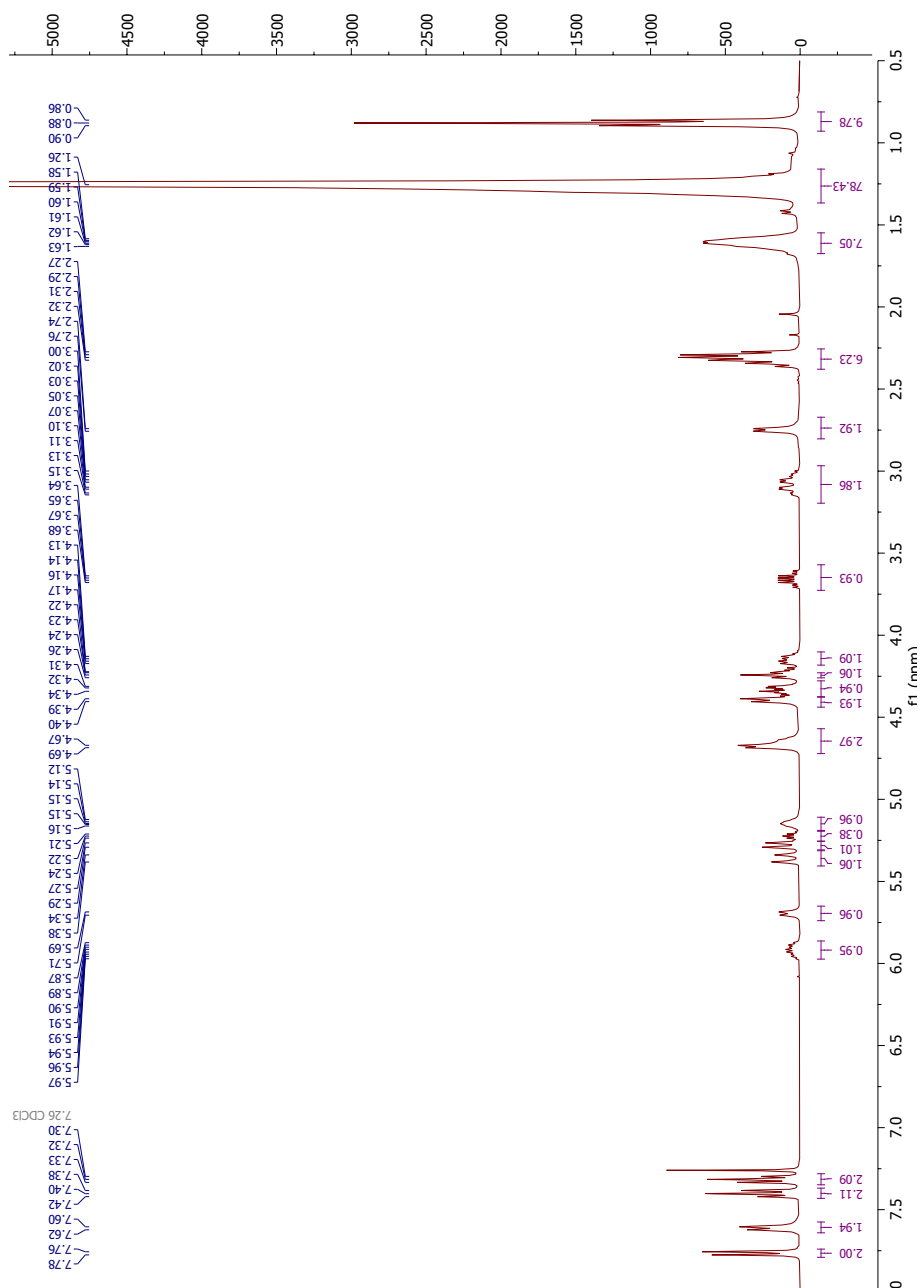
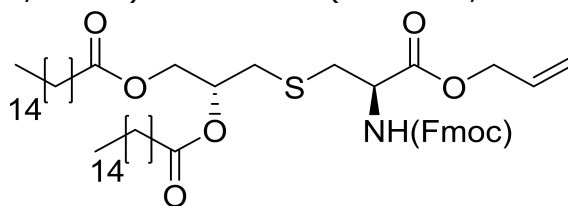
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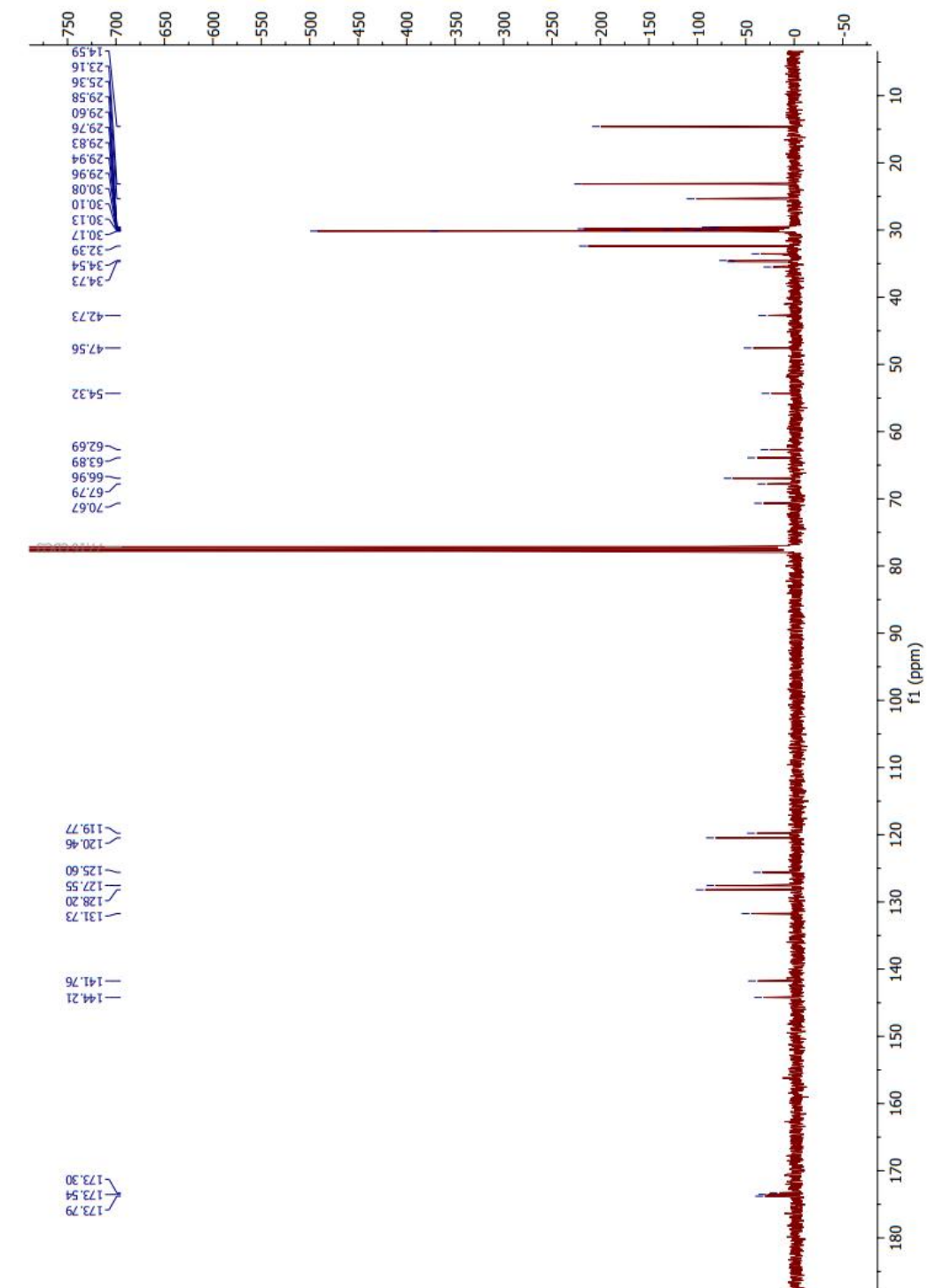




Allyl-Fmoc-Pam₂Cys (21)

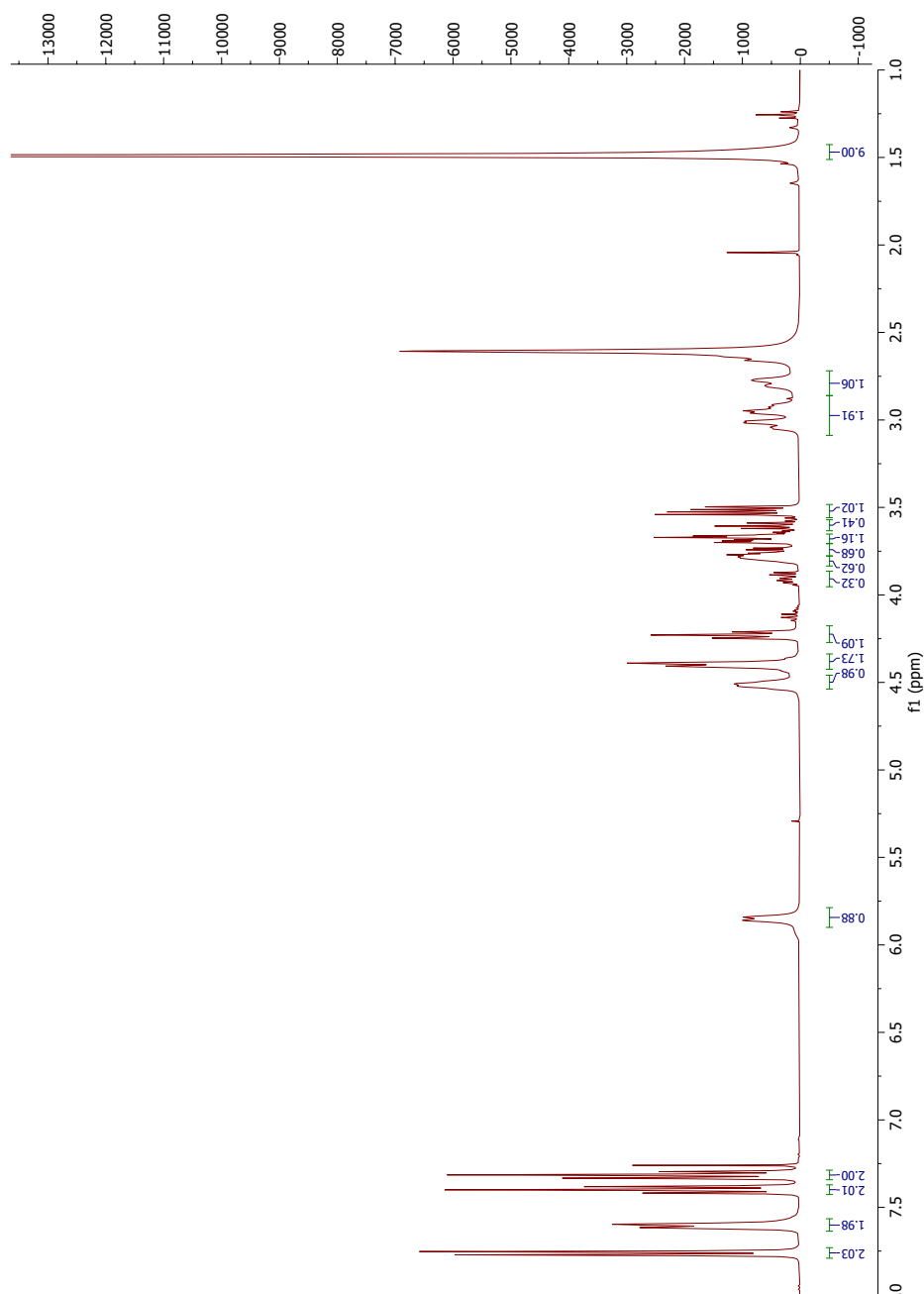
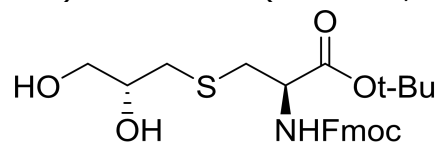
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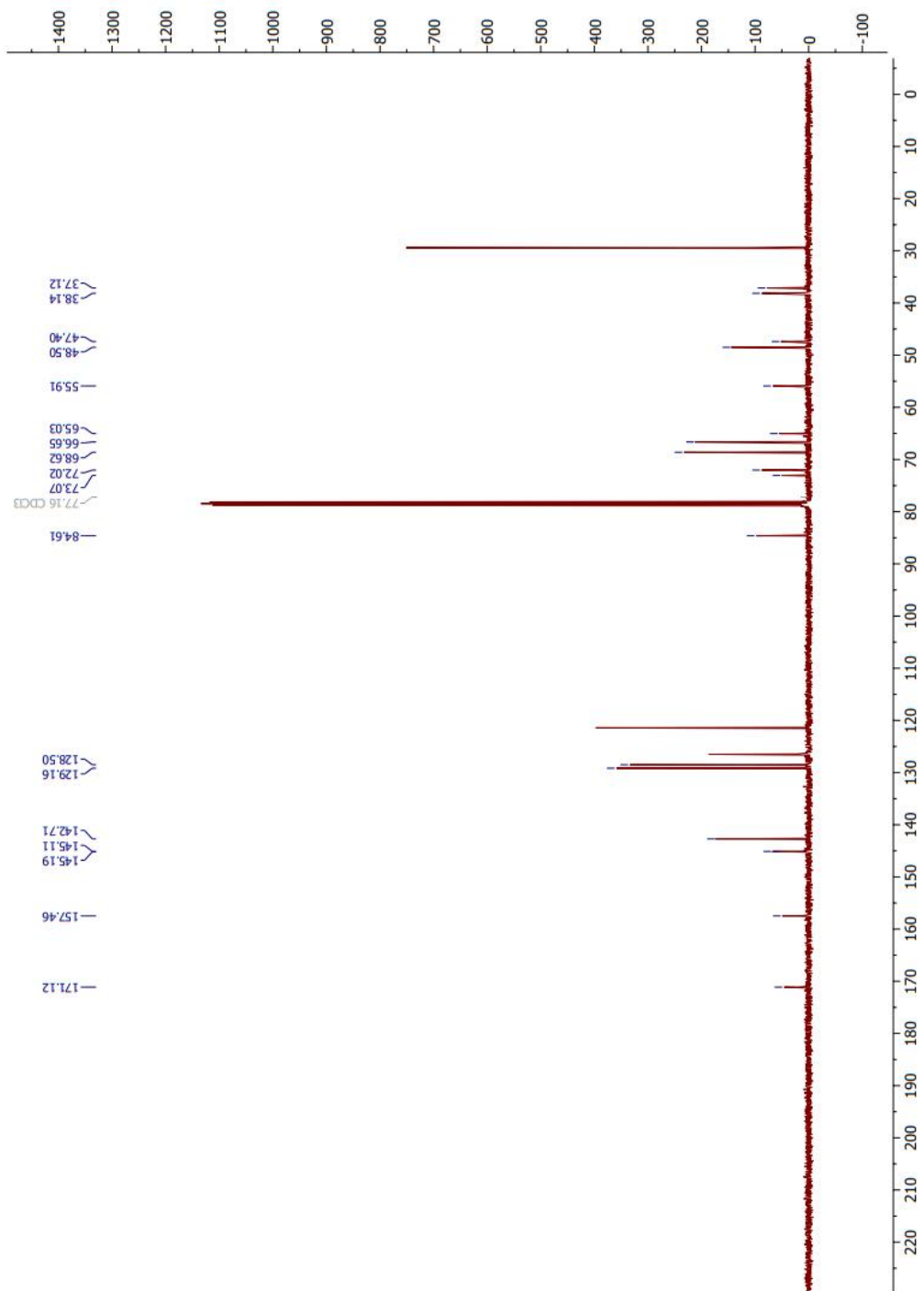




Tert-butyl- Fmoc-*R*-(2,3-dihydroxypropyl) Cysteine (23)

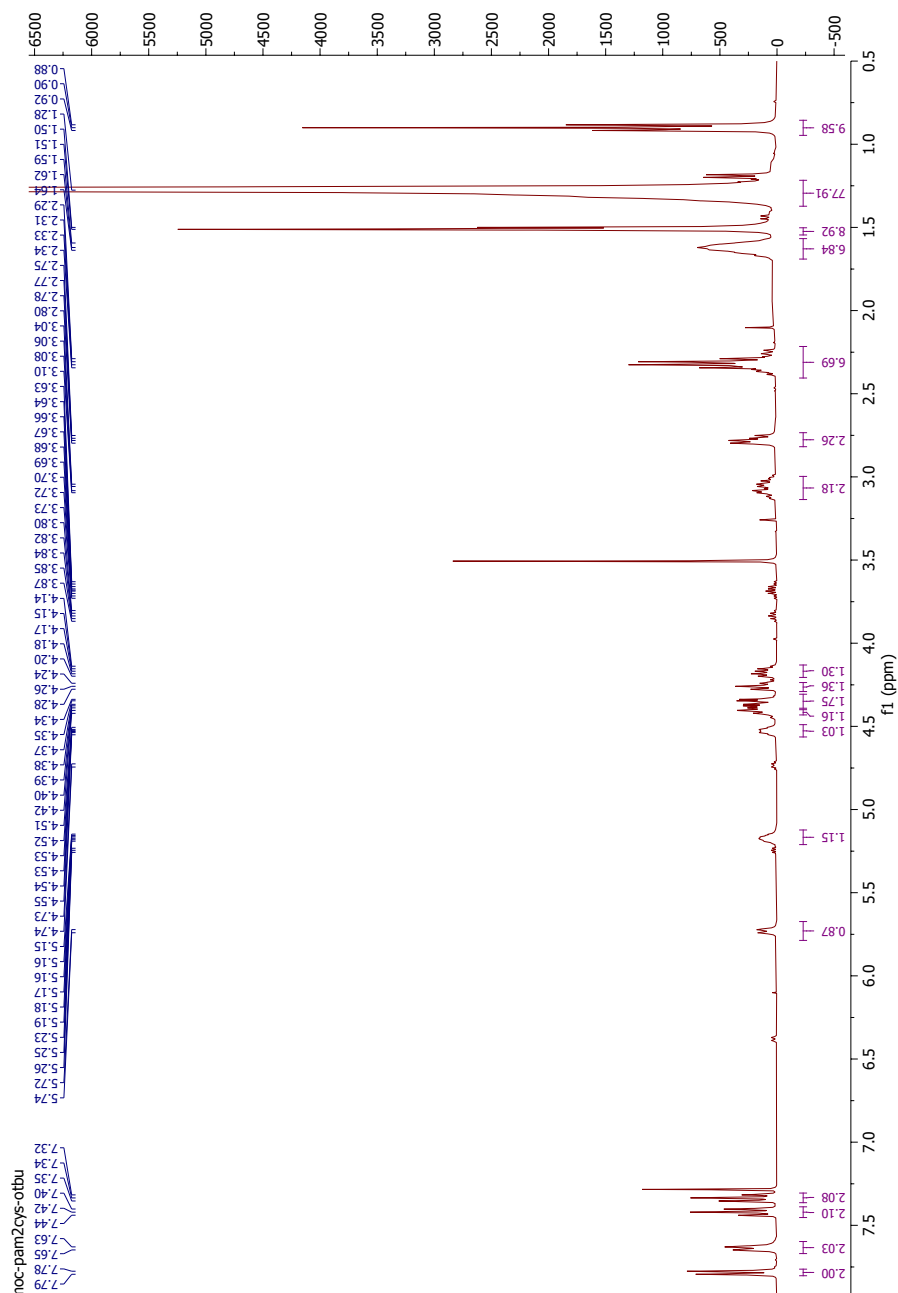
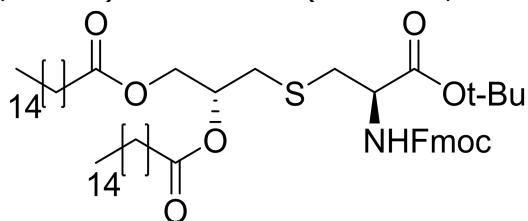
^1H NMR (400 MHz, CDCl_3) & ^{13}C NMR (101 MHz, CDCl_3)

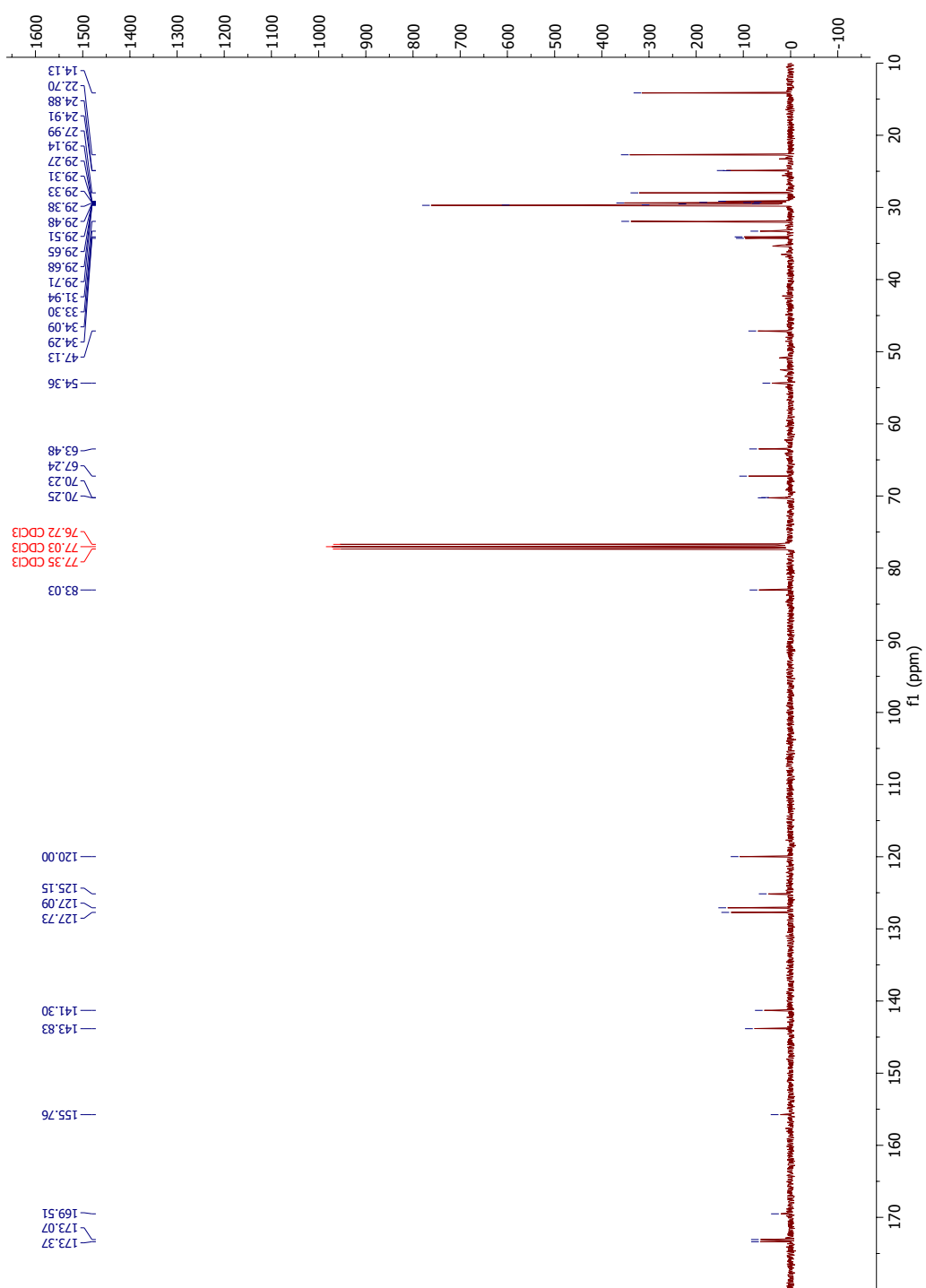




Tert-butyl-Fmoc-Pam₂Cys (24)

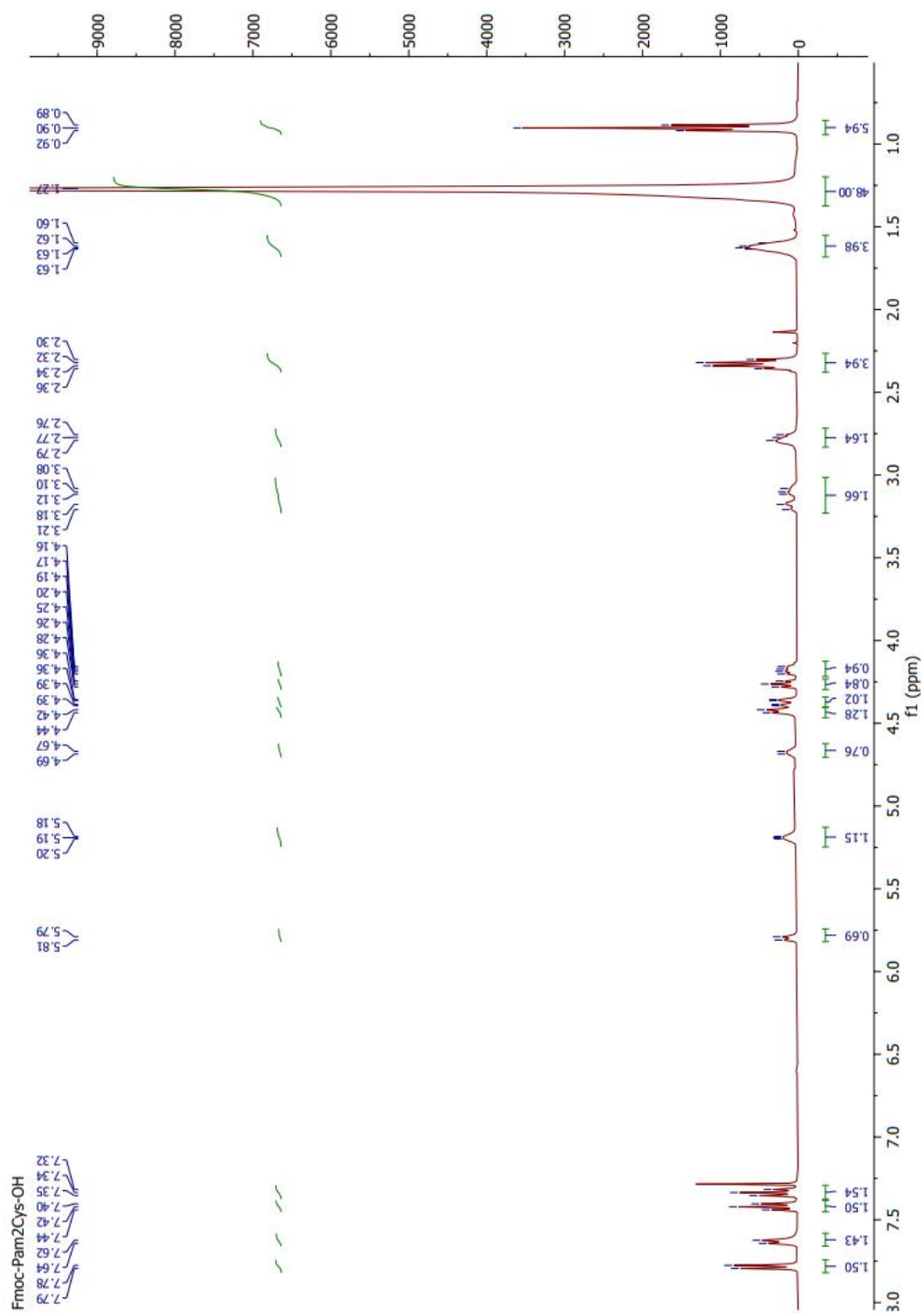
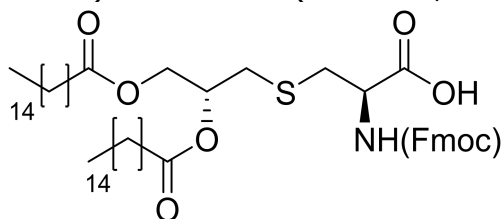
¹H NMR (400 MHz, CDCl₃) & ¹³C NMR (101 MHz, CDCl₃)

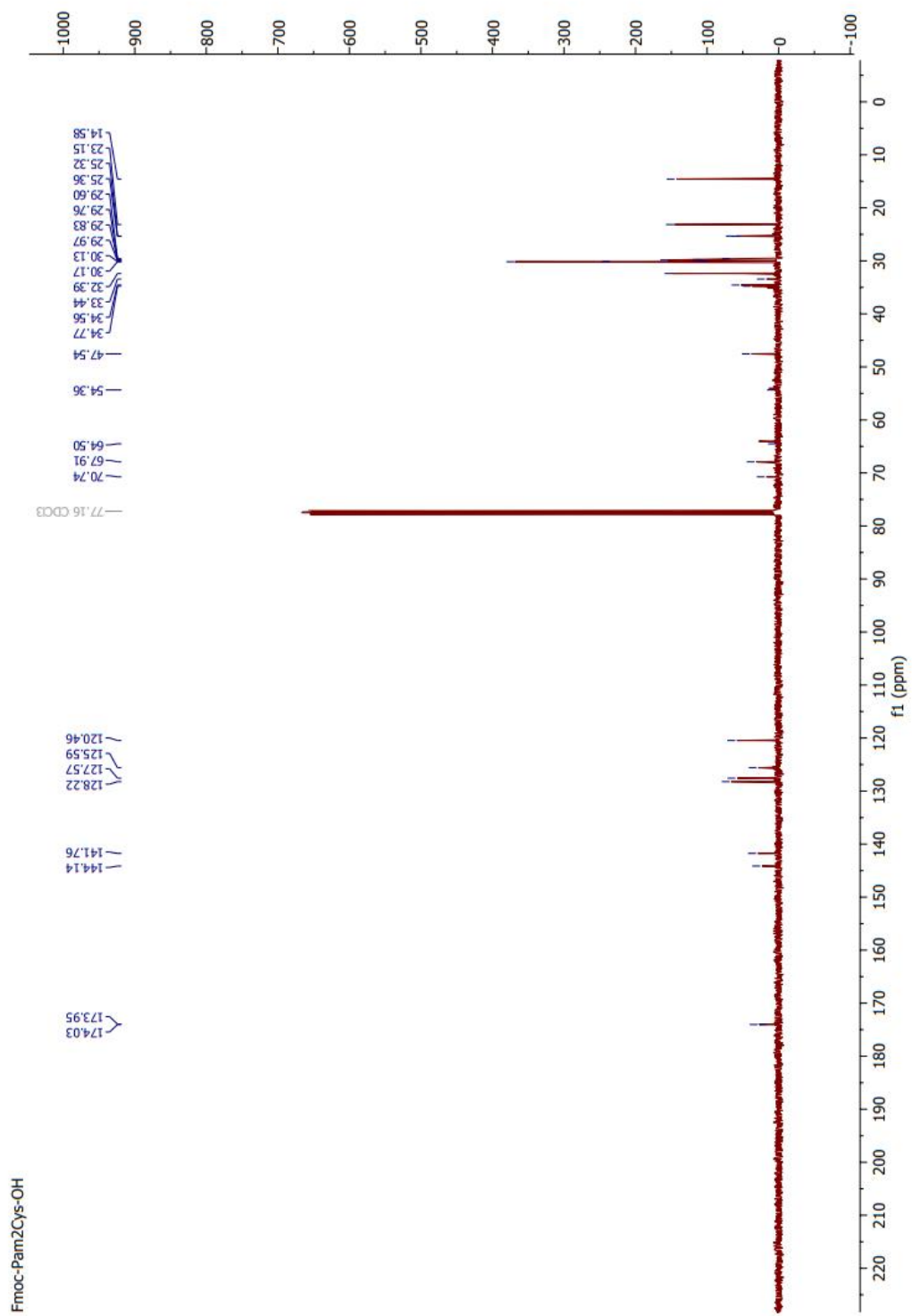




Fmoc-Pam₂Cys-OH (3)

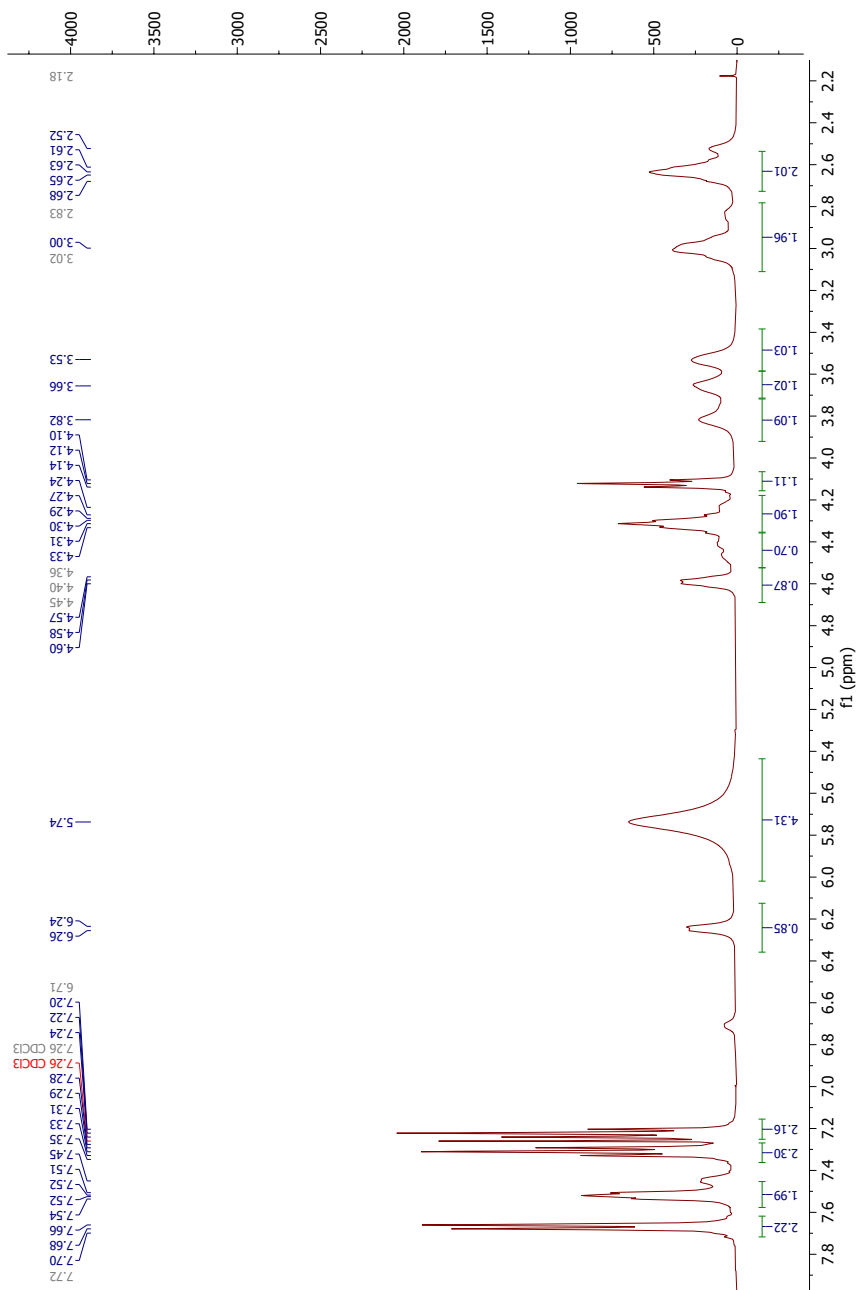
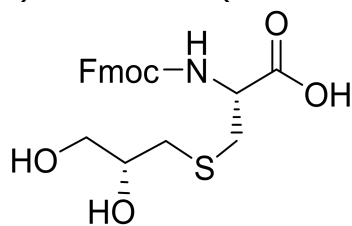
¹H NMR (400 MHz, CDCl₃) & ¹³C NMR (101 MHz, CDCl₃)

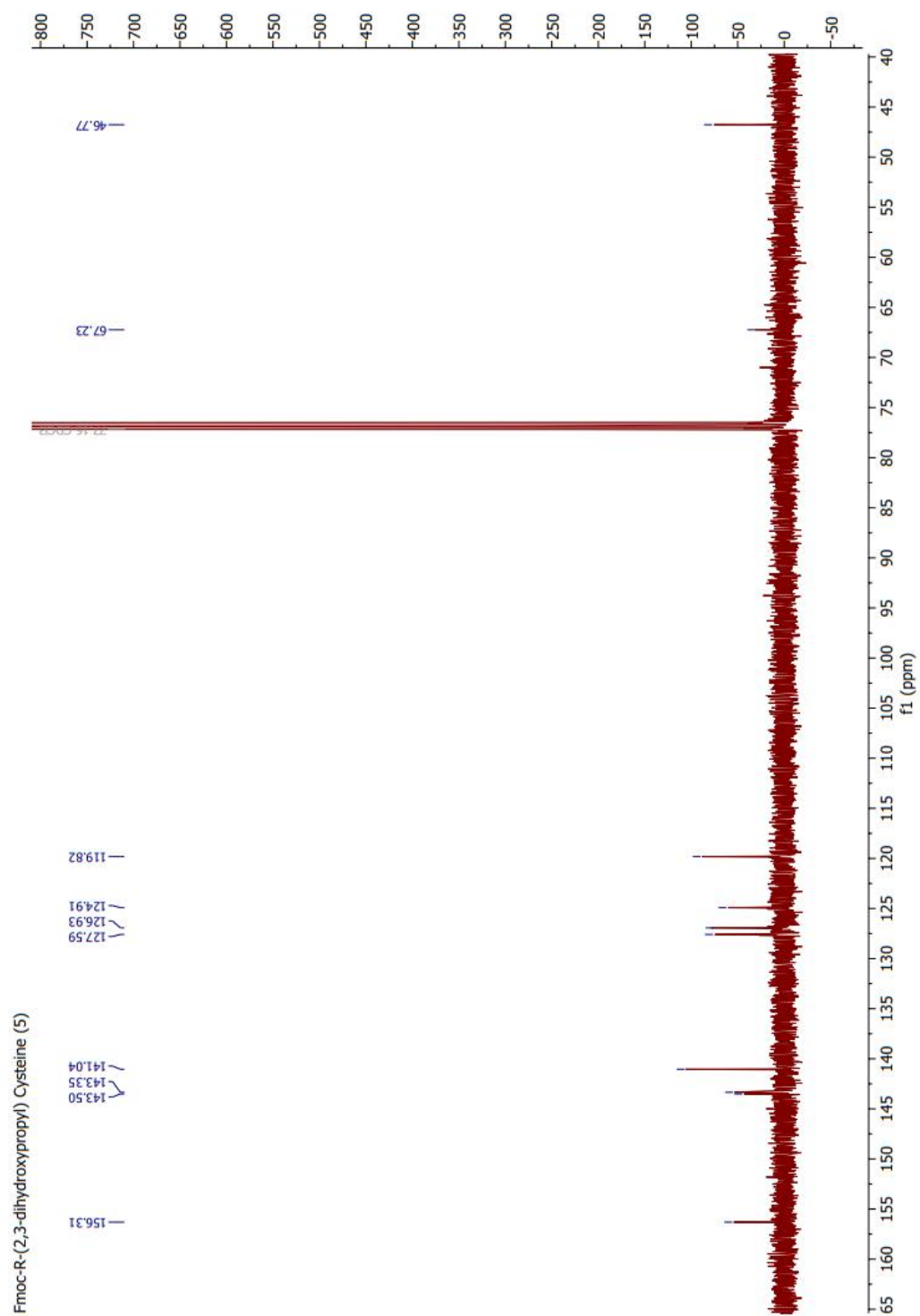




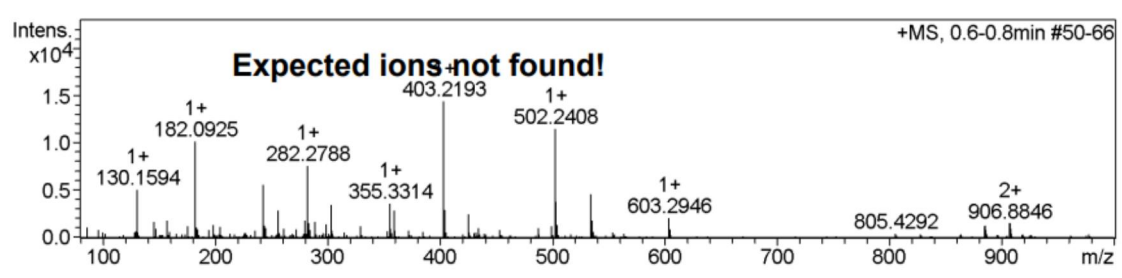
Fmoc-*R*-(2,3-dihydroxypropyl) Cysteine (5)

^1H NMR (400 MHz, CDCl_3) & ^{13}C NMR (101 MHz, CDCl_3)





The MS spectra of BAGE4₁₈₋₂₁.



The crude mass spectra of the reaction mixture of BAGE4₁₈₋₂₁, up to Gly. Calc. m/z for C₁₅H₂₈N₄O₆ [M+H]⁺: 360.40. However, acetylated BAGE4₁₈₋₂₁ was founded, Calc. m/z for C₁₅H₃₀N₄O₇ [M+H]⁺: 403.44. Obs. m/z [M+H]⁺: 403.22.