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Development Of A Ph-Triggered Cargo Delivery System With Esxa Mutants

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WITH ESXA MUTANTS

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DEVELOPMENT OF A PH-TRIGGERED CARGO DELIVERY SYSTEM WITH ESXA MUTANTS

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Abstract

Tuberculosis (TB) is a communicable disease caused by *Mycobacterium tuberculosis* (Mtb). It was the leading cause of human death generated by a single infectious agent before the Coronavirus (COVID-19) pandemic. Mtb has a special mechanism to evade the host's immune system and utilize macrophages as reservoirs. Latent TB infections have been a threat to public health.

Liposome has been widely used for drug delivery. Immunoliposomes are special liposome carriers coated with ligands, such as antigens, targeting certain cells or organs to regulate the uptake of liposome. By constructing the liposome components, or surface modification, cargo release of liposome can be triggered with change of pH.

EsxA is a small protein secreted by the Esx-1 secretion system with its chaperon EsxB. EsxA has a special membrane permeabilizing activity (MPA). Under low pH, EsxA/B goes through conformational change while EsxA exposes more hydrophobic residues, interrupting phagosome maturation and helping Mtb evade host immune response. Moreover, EsxA also interacts with the host's immune system as a potent T-cell antigen and regulates host immune response through multiple pathways.

In this study, we modified the liposome with EsxA mutants through a maleimide reaction. EsxA with G10C, G45C, and G88C mutations were generated and purified from both *Escherichia coli* (*E. coli*) and *Mycobacterium smegmatis* (*M. smegmatis*). During our experiment, the modified liposome system remained stable under physical pH. All EsxA mutants were able to rupture the liposome and trigger cargo release with drop of pH. G45C mutants showed best efficiency with even higher cargo release rate than free protein. EsxA/B heterodimers were not as efficient as EsxA alone. However, central linkage on G45C still enhanced the membrane rupture compared

with free protein. This delivery system has potential for targeting delivery to bacteria reservoirs, and the enhancement of cargo release rate by central linkage may suggest a special role of central loop in membrane insertion.

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Chapter 1: Introduction

Mycobacterium tuberculosis (Mtb) is the main pathogen causing human Tuberculosis and was the leading cause of death as a single infectious agent before the Coronavirus (COVID-19) pandemic, ranking above the human immunodeficiency virus (HIV) (WHO, 2021). Besides active TB cases, an estimated one third of the world population carries latent TB infection (WHO, 2021).

1.1 Statistics and Recent Progress

Great efforts have been applied to fight TB worldwide and progress has been made during the 2015-2020 period. TB incidence rate has reduced by 11%, and the number of deaths has been reduced by 9.2%. However, TB is still a major challenge to the health of the world's population (WHO, 2021).

According to WHO, "an estimated 9.9 million people fell ill with TB worldwide during 2020, 1.5 million people died from TB, including 214,000 people with HIV" (WHO, 2021). There was a decrease in new incidence from 2019 both worldwide and in the U.S, which possibly came from the influence of the COVID-19 pandemic (Filardo et al., 2021). However, the pandemic influenced TB diagnosis and treatment, which was reflected in an increase of TB deaths. According to WHO (2021), the global number of deaths officially documented caused by TB was 1.3 million in 2020, which was almost twice as many as the number caused by HIV/AIDS (0.68 million).

A 6-month multi-drug therapy of four first-line drugs: isoniazid (INH), rifampicin (RIF), ethambutol (ETB), and pyrazinamide (PYR) can treat about 85% of TB disease successfully (WHO, 2021). The natural antibiotic streptomycin (STR) obtained from *Streptomyces griseus* was formerly widely used to treat Mtb infection and is sometimes still considered part of the "first line" therapy (Grotz et al., 2018).

For the first line treatment, drug resistance has become a severe problem. According to the definition provided by WHO, a multidrug-resistant TB (MDR-TB) refers to TB that does not respond to at least INH and RIF, the 2 most powerful anti-TB drugs. Rifampicin-resistant TB (RR-TB) is also documented. In 2020, 150,359 people were enrolled on treatment for MDR/RR-TB (WHO, 2021). Second line pharmacotherapy has been applied to fight the development of Mtb strain. They usually come with more side effects which limit their use in TB treatment. Despite the various options of second line treatment, extra drug resistant tuberculosis (XDR-TB, resistance to RIF, any fluoroquinolone and at least one of bedaquiline or linezolid) became a new challenge. Therefore, WHO recommends drug-susceptibility test for those patients requiring re-treatment for Mtb infection. (WHO, 2021).

1.2 Latent TB Infection and TB Immune Evasion

There is no "golden standard" for defining a latent tuberculosis infection (LTBI). According to the definition recognized by WHO, LTBI refers to "a state of persistent immune response to stimulation by Mtb antigens without evidence of clinical manifestations of active TB" (Kasambira et al., 2011). People with LTBI usually do not show any symptoms and are not infectious. However, upon change of physical condition, especially immune status (Kasambira et al., 2011), an average 5–10% lifetime risk of those with LTBI will eventually develop active TB disease usually within the first 5 years after initial infection (Biraro et al., 2016).

Both tuberculin skin test (TST) and interferon-gamma release assay (IGRA) can be used in LTBI diagnosis (WHO, 2018). Most LTBI can be treated with a lower dosage of first-line therapy, such as 6-month Isoniazid or Rifampicin plus Isoniazid daily for 3 months as preventive treatment for children living with a high TB incidence. However, considering the adverse health

effect of anti-TB drugs and the huge cost, WHO only recommends programmatic management of LTBI in high-risk and immune compromised groups.

The mechanism of LTBI relies on the strategy of Mtb to evade the host's immune response. The intracellular pathogen Mtb is usually recognized and engulfed by macrophage cells (Weiss & Schaible, 2015). With innate immune response, activated macrophages digest the pathogen with lysosome-phagosome fusion. At the same time, antigens are presented to dendritic cells, triggering t-cell response to eliminate the pathogen (Jee, 2020). Acidification of phagosome from neutral pH to acidic pH triggers the MPA of a small protein EsxA secreted by Mtb, resulting in membrane disruption and cytosolic translocation.

The failure to eradicate intracellular Mtb can result in an active TB infection or LTBI. In LTBI, various immune cells (macrophages, lymphocytes, and dendritic cells) recruit around a core of Mtb infected macrophage, forming a tissue structure known as a granuloma. The lymphocytes in the granuloma activate the secretion of IL-8, IL-12, and other proinflammatory cytokines by macrophage (Khabibullina et al., 2022). This latency state presents as a balance of phagocytes recruitment and necrosis of infected cells.

Considering its essential role in Mtb infection, macrophage reservoirs have been regarded as important delivery targets for efficient treatment as well as prevention of LTBI.

1.3 Nanotechnology in the Fight with Tuberculosis

Different nano-carriers have been studied to carry anti-TB compounds, such as liposomes (LPs), nanoparticles (NPs), polymeric micelles, polymersomes and niosomes (Grotz et al., 2018).

Liposomes (LPs) are created by the formation of lipid bilayer with an aqueous core. LPs have high physical stability and are capable of loading hydrophilic compounds in the core as well

as hydrophobic compounds in the lipid layer (Abed & Couvreur, 2014). The usage of LPs with first line compound usually focuses on respiratory delivery such as inhaled RIF (Changsan et al., 2009; Letizia Manca et al., 2012), INH (Chimote & Banerjee, 2010), and PYR (El-Ridy et al., 2007) to bring down the side effects by directly delivering the drug to the infected site. For second line drugs such as moxifloxacin (MOX), application of NPs can minimize liver accumulation and significantly reduce hepatoxicity (Mustafa et al., 2016). Application of LPs also facilitates the anti-TB effect by carrying compounds with low solubility such as Clofazimine (Adams et al., 1999).

Other than applied as protective carrier of drug, NPs and LPs can be coated with specific ligands to make active targeting delivery through ligand-receptor interaction with host cells or tissues. Alveolar macrophages (AMs) failing to kill phagocytosed Mtb can act as reservoirs in latent Mtb infections (Rao et al., 2019). Mannose coating was found to enhance the alveolar macrophage (AM) uptake compared with mannose-free carriers (Song et al., 2015; Vieira et al., 2018) as well as free drug (Kumar et al., 2008). Hyaluronic acid (HA) receptor has also been studied to enhance AM uptake by HA modification on micelle surface (Gao et al., 2015).

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1.4 pH SENSITIVE DELIVERY SYSTEM

The pH sensitive delivery system is made up of structures formed by biomaterials capable of carrying various drugs for medical applications (Allen & Cullis, 2004; Fenton et al., 2018). The pH-sensitive biomaterials only perform conformational change or bind cleavage after exposure to certain acidic or alkaline environments. Thus, this type of system enhances the efficiency of treatment and minimizes side effects by optimizing the pharmacokinetics of medication. The acidic pH-sensitive delivery system has been mostly studied for the delivery of anti-cancer drugs because of the acidic environment (pH 6.5-6.9) in tumor tissue (Patel & Sant, 2016; Tao et al., 2021). It

has also been used for stomach drug release (pH 1.5-3.5) (Gottesmann et al., 2020) and lysosome-triggered drug release (pH 4.5-4.0) (Li et al., 2018; Maniganda et al., 2014).

The pH-sensitive system can be developed in different formats. The first choice is to chemically link the cargo molecules to a self-assembly monomer with pH-sensitive chemical bonds such as imine bonds, hydrazone bonds, oxime bonds, amide bonds, acetals and orthoesters (Zhuo et al., 2020). Upon acidification, the cargo molecule is released by cleaving from the carrier. Secondly, the system can be developed by pH-sensitive nanoparticles. Disruption of the structure of the carrier results in cargo release with pH change. A pH-sensitive liposome has been developed with pH-sensitive components (Chang et al., 2001). However, the possibility of modifying the liposome's surface with pH-sensitive molecules came with the best of our interest. The molecule chosen should disrupt the membrane structure under certain pH conditions.

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1.5 STRUCTURE AND FUNCTION OF ESXA

The structure of EsxA is formed by a helix-loop-helix hairpin structure with flexible arms on both C- and N- terminals. The EsxA protein, either purified from *E. coli* or Mtb culture supernatant, has MPA under low pH (de Jonge et al., 2007; de Leon et al., 2012). Upon acidification, EsxA forms a more folded structure, exposing more hydrophobic residues and placing the two helices into the membrane while flexible arms remain outside (de Leon et al., 2012). However, these flexible arms have been found essential for MPA (Ma et al., 2015). Computational models suggested that the flexible arms are involved in the oligomerization of EsxA during membrane insertion. The tetramer structure of EsxA has the lowest energy and can be the most possible structure of membrane pore formed by EsxA(Karki et al., 2020).

Different from EsxA, EsxB has not shown MPA in research. It also has a similar hairpin structure. The hydrophobic surfaces of EsxA/B helix allow two proteins naturally form heterodimer through van der Waals contacts. Meanwhile, salt bridges also form between the N-terminal helix-1 of EsxB to the C-terminal helix-2 of EsxA, and the C-terminal helix-2 of EsxB to N-terminal EsxA helix-1 (Renshaw et al., 2005). A study with the native heterodimer isolated from the culture filtrate suggested that EsxA/B is able to dissociate with the drop of pH, releasing EsxA to interact with lipid membranes (de Jonge et al., 2007). EsxB has also been found to stabilize EsxA to preserve its MPA at 37 ℃, which is the physical temperature (Vazquez Reyes et al., 2020). However, this dissociation was not found with EsxA purified from *E. coli*. EsxA produced in mycobacteria is finally secreted with acetylation at the Thr2 residue (Nα-acetylation), which was found to play an essential role in the dissociation of EsxA/B upon acidification (Aguilera et al., 2020). This finding was further supported in the paper with molecular dynamics (MD) simulations, in which the Nα-acetylated Thr2 makes frequent "bind-and-release" contacts with EsxB upon acidification, generating a force that pulls EsxB away from EsxA.

EsxA and its MPA play a key role in mycobacterium virulence. Compared with Mtb, BCG (commonly used attenuates stain as vaccine) lacks the region of difference 1 (RD1). RD1 is located in the esx-1 locus. The deletion of RD1/ESX-1 results in attenuation of mycobacterial virulence (Conrad et al., 2017; Hsu et al., 2003) by reducing their activity of phagosome disruption and cytosolic translocation (Houben et al., 2012; McDonough et al., 1993; Welin & Lerm, 2012). Non-pathogenic *M. smegmatis* also secretes an orthologous EsxA without MPA while pathogenic mycobacteria, such as Mtb and *Mycobacterium Marium* (Mm), secretes EsxA with MPA (de Leon et al., 2012).

EsxA also plays an essential role in host-pathogen interaction. Deletion of EsxA/B in Mm greatly reduced adherence on Raw264.7 cell, while complementation with EsxB/EsxA significantly restored the adherence (Zhang et al., 2016). Human A549 cells and THP-1 cells have laminin enriched on cell membrane. Purified EsxA interacts with the immobilized laminin protein and the binding occurs in a dose-dependent manner (Khan et al., 2020). When inhaled, EsxA shows activity in mediating Mtb entry into the M cells. Co-immunoprecipitation identifies scavenger B-1 as a possible surface receptor in this interaction (Khan et al., 2020). Purified EsxA also binds to the cell surface of RAW264.7 cells with participation of TLR2 (Pathak et al., 2007). With multiple receptors on the macrophage cell surface, EsxA would be a possible ligand for liposome modification.

1.6 LIPOSOME MODIFICATION WITH MALEIMIDE REACTION

Active targeting can be achieved by modifying the liposome surface with specific ligands such as antibodies, aptamers, or small molecules that can interact with receptors on target cells. PEG2000 is widely used to coat the liposome surface. “Stealth liposome” generally has longer blood circulation time. Modified PEG2000 is also used to link targeting ligands to liposomes (Liu et al., 2021). When lipids with PEG2000 are modified with a maleimide group, they can react with a reduced thiol group in cysteine and conjugate the protein to the liposome surface.

We chose G10C, G45C, and G88C mutants of EsxA. Since the flexible arms are essential for MPA and heterodimer dissociation, we hypothesized that the conjugation may have influence on the membrane lytic activity of EsxA as well as EsxA/B dissociation.

Chapter 2: Specific Aims

2.1 Build up the EsxA-liposome conjugation model.

Cysteine mutations were used to conjugate EsxA to liposome surface by maleimide reaction with DSPE-PEG2000-Maleimide. POPC/POPG (1:4) liposome have been found to generate better signal in liposome leakage assay with EsxA (Ray et al., 2019). However, in this study, we found that adding DSPE into the liposome affected stability of some composition. Different lipid compositions were tested for their stability to find the one we can use for following experiments.

2.2 Determine the influence of membrane lytic activity with EsxA conjugated to the liposome surface.

A. EsxA purified from E. coli were conjugated to liposome with G10C, G45C and G88C mutations and the membrane lytic activity was tested by a liposome leakage assay in Dr. Jianjun Sun's lab (Ray et al., 2019)

B. The results produced by EsxA from E. coli may be influenced with the C-terminus his-tag or the lack of post translational modification such as N-α acetylation. EsxA cloned into pMyNT plasmid and purified with *M. smegmatis* doesn't have these problems. The three mutations were cloned into EsxA on pMyNT plasmid and the MPA after protein-liposome conjugation was measured with liposome leakage assay.

C. With naturally formed heterodimer purified from *M. smegmatis*, the MPA was also tested with or without protein-liposome conjugation. Any influence on the activity could indicate that the binding site plays an important part in heterodimer interaction.

Chapter 3: materials and methods

3.1 Plasmids and mutations

For purification from *E. coli*, EsxA cloned into pET22b plasmid with c-terminus His-tag previously generated by Yue Ma (2015) in the lab were used. For amplification, primers were transformed into DH5-Alpha competent cells and extracted with Pure Yield Plasmid mini-prep system.

For purification from *M. smegmatis*, plasmids were generated with Agilent QuickChange site-directed mutagenesis protocol. Briefly, the pMyNT plasmid with EsxB/A inserted was used as a template (Aguilera et al., 2020). Primers were designed to include the G10C, G45C, and G88C mutation on EsxA (Table 1). DNA was amplified with PCR and digested with Dpn-1 restriction enzyme to remove methylated DNA, which was the original template without mutation. The digestion products were transformed into XL-1 gold super-competent cells and extracted with Pure Yield Plasmid mini-prep system.

Table 1 Primers used for site-directed mutagenesis

G10C F	5'- G CAG CAA TGG AAT TTC GCG TGT ATC GAG GCC GCG G-3'
G10C R	5'- CCG CGG CCT CGA TAC ACG CGA AAT TCC ATT GCT GC -3'
G45C F	5'- GCG GCC TGG GGC TGT AGC GGT TCG GAG GCG-3'
G45C R	5'- CGC CTC CGA ACC GCT ACA GCC CCA GGC CGC -3'
G88C F	5'- G GCA ATG GCT TCG ACC GAA TGT AAC GTC ACT GGG ATG TTC GC-3'
G88C R	5'- GCG AAC ATC CCA GTG ACG TTA CAT TCG GTC GAA GCC ATT GCC -3'

3.2 Protein expression and purification with *E. coli*

The C-terminus His-tagged EsxA on pET22b plasmid was transformed into BL21(DE3)-C41 competent cells. 100 μg/mL carbenicillin was applied to agar as well as liquid culture. A single colony was picked and grown overnight in 50mL of Luria-Bertani broth (LB) under 37°C

with 200 RPM shaking as a start culture. 10 mL of start culture was used to generate 1 L culture with LB. The culture was induced with 1 mM IPTG at an OD600 of 0.6-0.8. The culture was then incubated for 8 hours post induction and collected by 8000 RPM ultracentrifuge.

Protein was purified by a Ni-affinity on-column refolding protocol as previously described (de Leon et al., 2012). The harvested cells were lysed by sonication in the cell lysis buffer (20 mM Tris-HCl, 100 mM NaCl, 0.5 mM EDTA, and 100 μM PMSF, pH 7.4). Inclusion bodies containing EsxA protein were collected by centrifuging at 12,100 × g for 15 min at 4 °C and washed three times with the wash buffer (50 mM Tris-HCl, 10 mM EDTA, 0.5% Triton X-100, pH 8.0). The pellet was then resuspended in the denaturing buffer (8 M urea, 1 mM EDTA, and 100 μM PMSF, pH 7.4) by sonication, followed by centrifugation at 15,200 × g for 40 min at 4 °C. A Ni^{2+} charged Sepharose column was pre-equilibrated with the pre-equilibration buffer (20 mM Tris-HCl, 8 M urea, and 20% glycerol, pH 7.4). The denaturing urea was removed gradually with a linear gradient (0–88%) of the refolding buffer (20 mM Tris-HCl 500 mM NaCl, 20% glycerol, pH 7.4), which allowed EsxA to refold on the column. The refolded EsxA was then eluted with a linear gradient (10–100%) of 5000 mM imidazole supplemented refolding buffer.

The eluted protein was concentrated with 5000 MWCO viva spin and further purified with Superdex-75 size exclusion column in the gel filtration buffer (20 mM Tris-HCl, 100 mM NaCl, pH 7.4).

3.3 PROTEIN EXPRESSION AND PURIFICATION WITH *M. SMEGMATIS*

Genes in pMyNT plasmid were electroporated into *M. smegmatis*. 100 μg/mL hygromycin was applied to 7H10 agar as well as 7H9 broth. The start culture was generated with 7H9 broth with OADC enrichment for~1 day at 37℃ with 200 RPM shaking. 10mL start culture was used to generate 1L culture in 7H9 with glucose, glycerol, and tween-80 as enrichment instead of OADC.

The 1L culture was under 37°C with 200RPM shaking overnight and induced with 2g/L acetamide at an OD600 of 2.0. The induced culture was allowed to grow for another day and collected by 8000 RPM ultracentrifuge.

The EsxA/B heterodimer with His-tag on EsxB was purified by a Ni-affinity protocol as previously described (Reyes et al., 2020). Briefly, the harvested cells were lysed with sonication in soluble purification buffer A (500mM NaCl, 10mM imidazole, 50mM Tris-HCl, pH 7.4). Cell debris was removed by ultracentrifuge, and the supernatant containing EsxA/B was loaded on to pre-equilibrated Ni^{2+} charged Sepharose column. After column wash, the His-tagged protein was eluted with 10%-100% gradient of soluble purification buffer B (500mM NaCl, 500mM imidazole, 50mM Tris-HCl, pH 7.4).

The eluted protein was concentrated with 5000MWCO viva spin and further purified with Superdex-75 size exclusion column. From the purification, naturally formed heterodimer was collected and stored.

Part of the protein fraction was incubated with 6 M guanidine for at least 4 hours at 4 °C to separate heterodimer. The incubated protein was injected into a 5 mL Ni Histrap column. EsxA was washed off first with separation buffer (6 M guanidine, 25 mM NaH_2PO_4, 100 mM NaCl, 10 mM imidazole, pH 6.5). EsxB was eluted with 0-100% gradient of 500 mM imidazole. Guanidine was removed by dialysis against gel filtration buffer before storage.

3.4 PREPARATION OF LIPOSOME

The lipids used to generate liposomes are listed below:

DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine)

DSPE-PEG2000 (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N- [methoxy (polyethylene glycol)-2000], ammonium salt)

DSPE-PEG2000-Maleimide (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide (polyethylene glycol)-2000], ammonium salt)

DOPG (1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol), sodium salt)

POPC (1-palmitoyl-2-oleoyl-glycero-3-phosphocholine)

POPG (1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol), sodium salt)

For the liposome leakage assay, 10mg DOPC or the same molecular amount of lipid mix was dissolved in an organic solution with mix of methanol: chloroform=2:3 and dried to a thin layer with nitrogen gas. The dried lipid was then rehydrated with 1mL 5mM HEPES buffer containing 50mM fluorescent dye/quencher pair, 8-Aminonaphthalene-1,3,6-Trisulfonic Acid (ANTS), and p-Xylene-Bis-Pyridinium Bromide (DPX). The sample then went through 6 freeze-thaw cycles and passed through a 200 nm pore size membrane 22 times. A 5mL Hi-trap desalting column was used to remove the excess dye and exchange the liposome into gel filtration buffer. The fractions with liposomes were collected and stored at 4℃.

3.5 LIPOSOME STABILITY TEST

10% DSPE-PEG2000 was added to the lipid mix either containing DOPC, POPC, POPC/POPG=1:8, POPC: POPG=1:4. The liposomes were all prepared with ANTS/DPX encapsulated. The maximum signal intensity (excitation 380nm, emission 530nm, subtracting background signal) triggered with 2% triton was measured right after purification and after overnight storage at 4 ℃.

3.6 GENERATING LIPOSOME PROTEIN CONJUGATION

To test the pH-triggered lytic activity of conjugated protein, an ANTS/DPX liposome with DOPC and DSPE-PEG2000-Maleimide at 9:1 molar ratio was prepared. The protein mutants were reduced with 40X DTT for 30 min on ice. DTT was removed by a Hi-trap desalting column. The

reduced protein was then incubated with liposome at protein: maleimide=1:5, 4℃ overnight for EsxA (either purified from *E. coli* or *M. smegmatis*) and 4h for heterodimer purified from *M. smegmatis*. An SDS-PAGE was used to confirm the conjugation of protein with liposome.

For control of non-conjugated protein, DSPE-PEG2000 without maleimide group was used instead in the whole process.

To avoid the influence of intensity by lipid modification, the protein binding efficiency was calculated with unbound protein in reaction group and protein band intensity of the corresponding control group.

3.7 Liposome Leakage Assay

After the incubation, the reaction mix of protein and liposome was diluted into 1.35 mL with gel filtration buffer (150 mM NaCl, 20 mM tris, pH 7.4) to reach a protein concentration of 4.8μM. The signal intensity was measured with 380nm excitation and 530nm emission. 150uL 2% triton was used to identify the highest signal generated by liposome leakage. 150uL 1M sodium acetate (pH 4) was used to trigger the membrane insertion of EsxA. The signal intensity was presented as a percentage of triton-triggered intensity. The reaction rate was then calculated in GraphPad Prism with curve fitting plateau followed by a one-phase association Y= (X<X0, Y0, Plateau+(Y0-Plateau) *exp(-K*(X-X0))). A two-way ANOVA with a multiple comparison test was applied to compare the difference.

For EsxA/B heterodimer, the experiment was performed at 37 ℃ instead of room temperature in order to generate better signal. All samples were pre-warmed in 37 ℃ water baths for at least 5 min.

3.8 LIPOSOME PURIFICATION

To separate the liposome from unbound protein, the reaction mix was passed through a Sepharose CL-4B column with gel filtration buffer. To test the separation capability of the column, the liposomes and proteins were passed through the column separately. All experiments were done with the same flow rate.

Chapter 4: Results

4.1 Site-directed mutagenesis and protein purification

After site-directed mutagenesis of pMyNT plasmid containing EsxA/B, the mutations G10C, G45C, and G88C on EsxA were confirmed by sequencing.

```
  WT AATTTCGCGGGTATCGAGGCC
G10C AATTTCGCGTGTATCGAGGCC

  WT GCCTGGGGCGGTAGCGGTTCG
G45C GCCTGGGGCTGTAGCGGTTCG

  WT TCGACCGAAGGCAACGTCACT
G88C TCGACCGAATGTAACGTCACT
```

Figure 1 Sequence Align of WT EsxA and Mutants

Mutated EsxA on pET22b was purified from *E. coli* with on-column refolding protocol. EsxA/B on pMyNT were purified from *M. smegmatis* with Ni-affinity and separated with Guanidine. Proteins from either source were further purified using size exclusion chromatography. The final protein product had over 90% purity as shown on SDS-PAGE. The naturally formed EsxA/B heterodimer had 1:1 ratio of EsxA to EsxB.

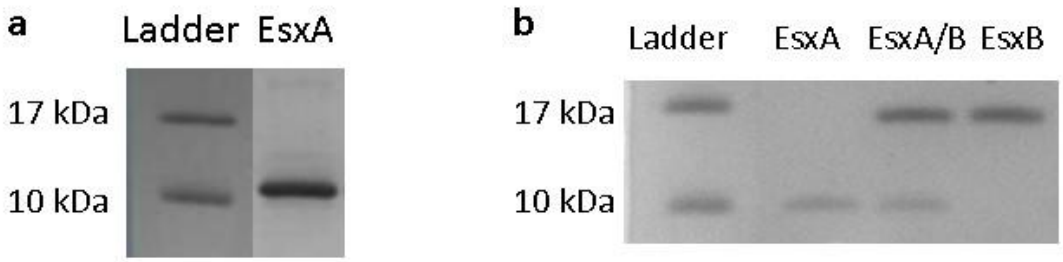

Figure 2 Reducing SDS-PAGE of Purified Protein.
a) *E. coli* derived EsxA. b) *M. smegmatis* derived EsxA, heterodimer, EsxB.

4.2 PROTEIN MUTANTS CAN BE SUCCESSFULLY CONJUGATED TO LIPOSOME

With overnight incubation, new bands were observed on reducing SDS-PAGE, while non-reacting wild type (WT) EsxA protein or mutants incubated with non-reacting liposome did not generate these bands, which indicated the conjugation was successful.

For heterodimer, the reaction was also confirmed by generation of new bands. There was no change on the EsxB band.

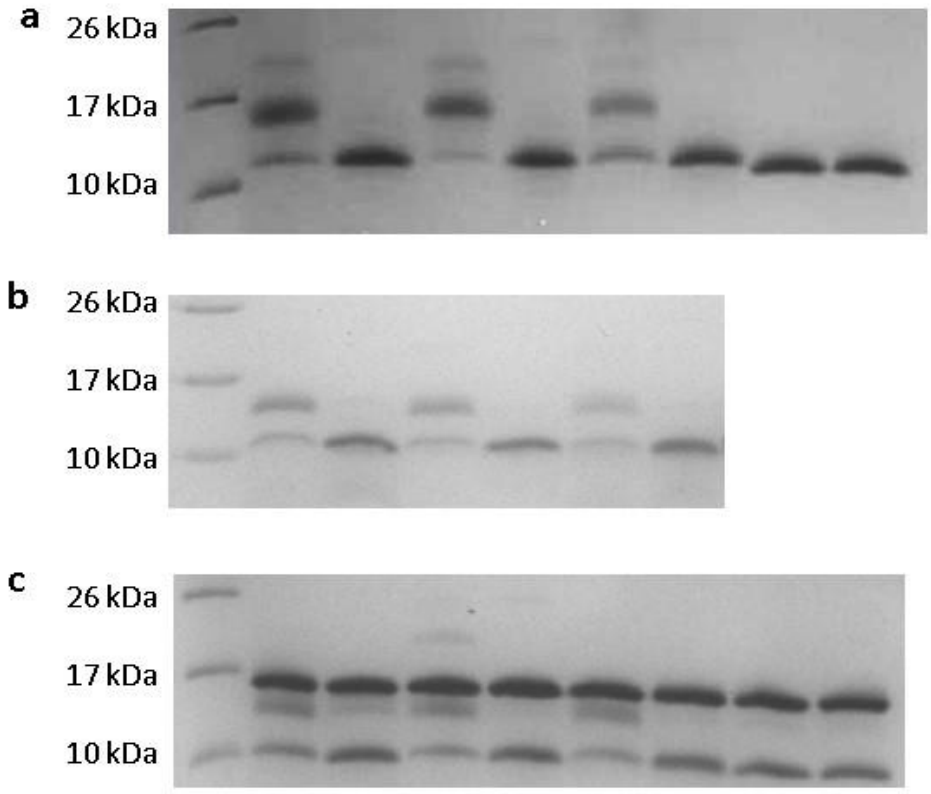

Figure 3 Conjugation Confirmed by Reducing SDS-PAGE.
a) *E. coli* derived EsxA. b) *M. smegmatis* derived EsxA. c) *M. smegmatis* derived heterodimer. From left to right: G10C with maleimide liposome, free G10C with non-reacting liposome, G45C with maleimide liposome, G45C with non-reacting liposome, G88C with maleimide liposome, G88C with non-reacting liposome, and wide type EsxA with both liposomes.

4.3 DOPC LIPOSOME WITH DSPE ADDED WAS STABLE ENOUGH FOR STORAGE

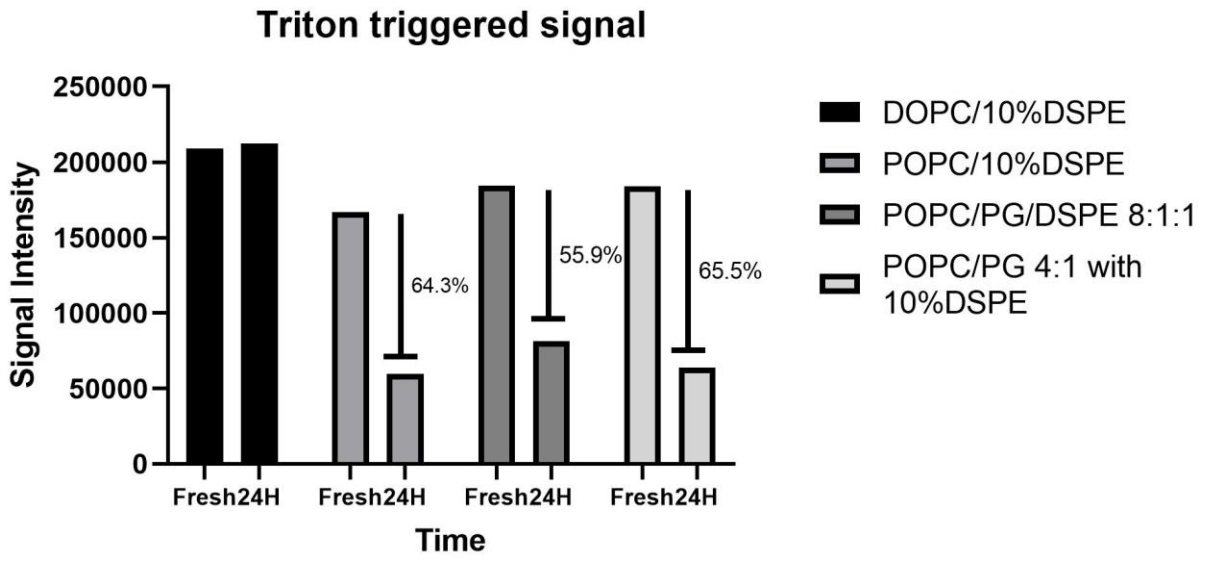

Figure 4 Stability of liposome with different lipid composition.

As previous research showed (Reyes et al., 2020), the POPC/POPG liposome generates better signal in pH-triggered membrane insertion. Therefore, the POPC/POPG model was first considered to build up the system. However, the POPC/POPG composition with DSPE was found unstable during storage under physical pH (gel filtration buffer, pH 7.4).

Therefore, we tested the stability of POPC, POPC/POPG, and the most commonly used DOPC composition, with 10% DSPE-PEG2000 added. After 24h storage at 4℃, only DOPC/DSPE liposomes remained stable, all others had a different level of leakage resulting in a loss of approximately 60% fluorescence signal intensity, which indicated cargo leakage during storage. Therefore, instead of using the POPC/POPG liposome, we changed back to the DOPC liposome for the following assay.

4.3 FOR ESXA PURIFIED FROM *E. COLI*, G45C MUTANT ACTED AS THE MOST SUCCESSFUL PH-INDUCED RELEASE TRIGGER

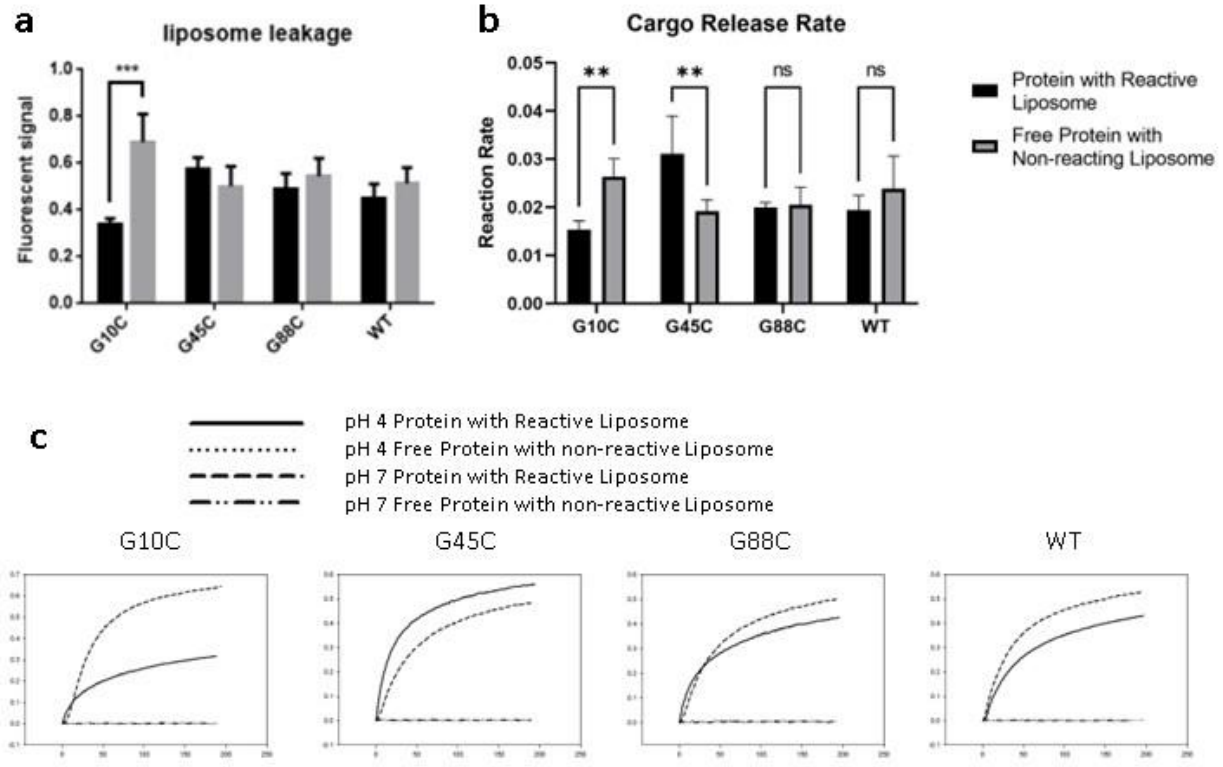

Figure 5. Signal intensity and reaction rate of *E. coli* derived protein.
a) Cargo release at intensity at 180s indicated by signal presented as percentage of triton triggered signal. b) Cargo release rate. c) Signal intensity measurement against time with a typical replication (P-value: 0 '***' 0.001 '**' 0.01 '*' 0.05)

In liposome leakage assay, all EsxA mutants showed membrane permeabilizing activity and triggered cargo release with the drop of pH while remaining stable under pH 7.4.

WT ExsA control indicated there was no significant difference between the two liposomes. For free protein, G10C showed higher MPA both in signal intensity and reaction rate. However, the conjugation influenced the activity of some mutants. With N-terminal conjugation on G10C to

liposome, significant inhibition was observed both on liposome leakage signal intensity and reaction rate. C-terminus conjugation on G88C did not generate significant difference in the MPA. The central conjugation of G45C was not observed to influence liposome leakage signal intensity but promoted the reaction rate which indicated a faster release. To summarize, G45C mutant would be the best cargo release trigger among *E. coli* purified EsxA.

This experiment did not rule out the influence of C-terminus His-tag as well as lack of post-translational modification during the purification from *E. coli*. Thus, the experiment was then repeated with protein purified from *M. smegmatis*, where EsxA and naturally formed EsxA/B heterodimers were purified.

4.4 For EsxA purified from *M. smegmatis*, liposome conjugation had no significant influence on membrane lytic activity. Central conjugation enhances membrane lytic reaction rate.

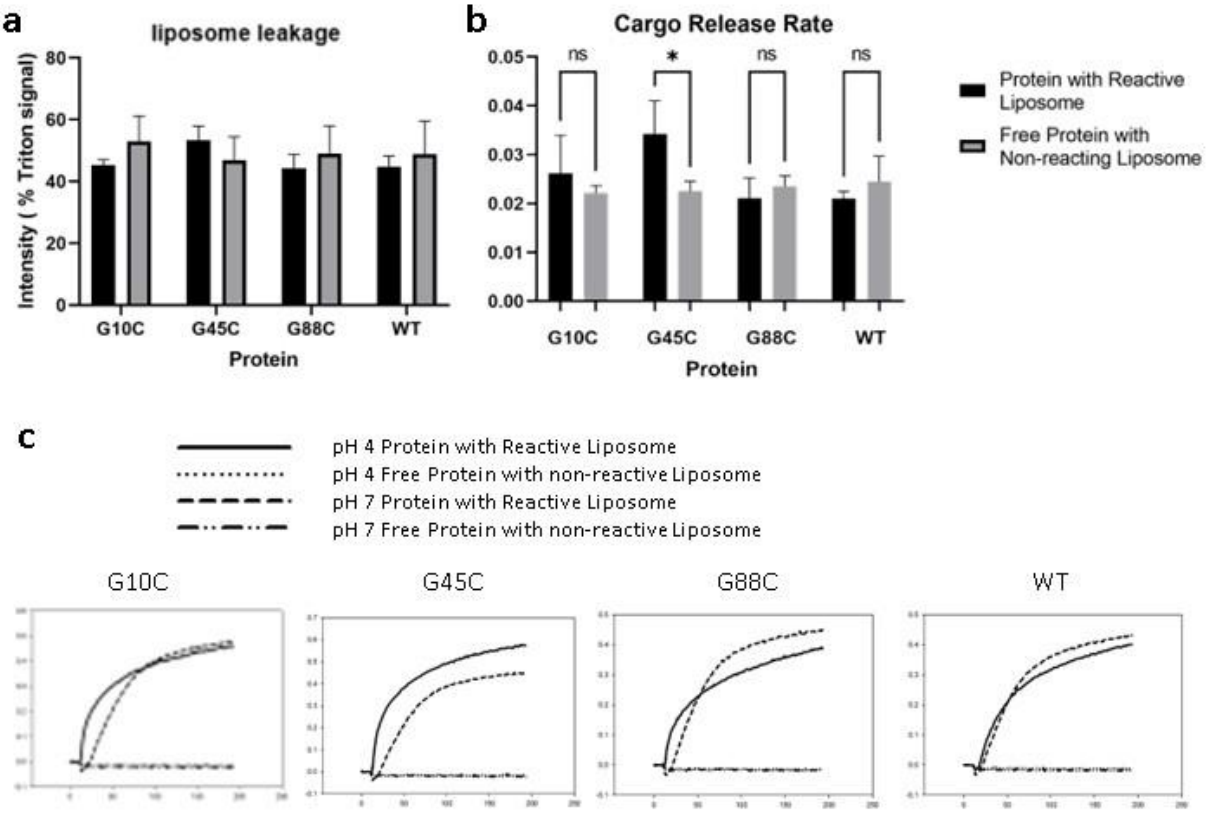

Figure 6 Signal intensity and reaction rate of *M. smegmatis* derived protein.
a) Cargo release at intensity at 180s indicated by signal presented as percentage of triton triggered signal. b) Cargo release rate. c) Signal intensity measurement against time with a typical replication (P-value: 0 '***' 0.001 '**' 0.01 '*' 0.05)

In liposome leakage assay, all EsxA mutants from *M. smegmatis* showed membrane triggered cargo release with the drop of pH while remaining stable under pH 7.4. No significant difference was found between the MPA of free protein. The difference of G10C from other mutants was not observed with *M. smegmatis* derived protein.

After conjugation, no significant difference in liposome leakage intensity was found either between different proteins or generated by protein-liposome conjugation. However, there was still a significant difference in the reaction rate of G45C, which was consistent from the result of *E. coli*-derived protein. This suggested that central conjugation of EsxA to the liposome facilitated the cargo release with an unknown mechanism.

EsxB has been proved to keep EsxA stability under 37 ℃. For the next step, we tested if heterodimer could act as sufficient pH-trigger in this system.

4.5 For EsxA/B heterodimer conjugated liposome, only G45C heterodimer triggered the fastest and strongest leakage.

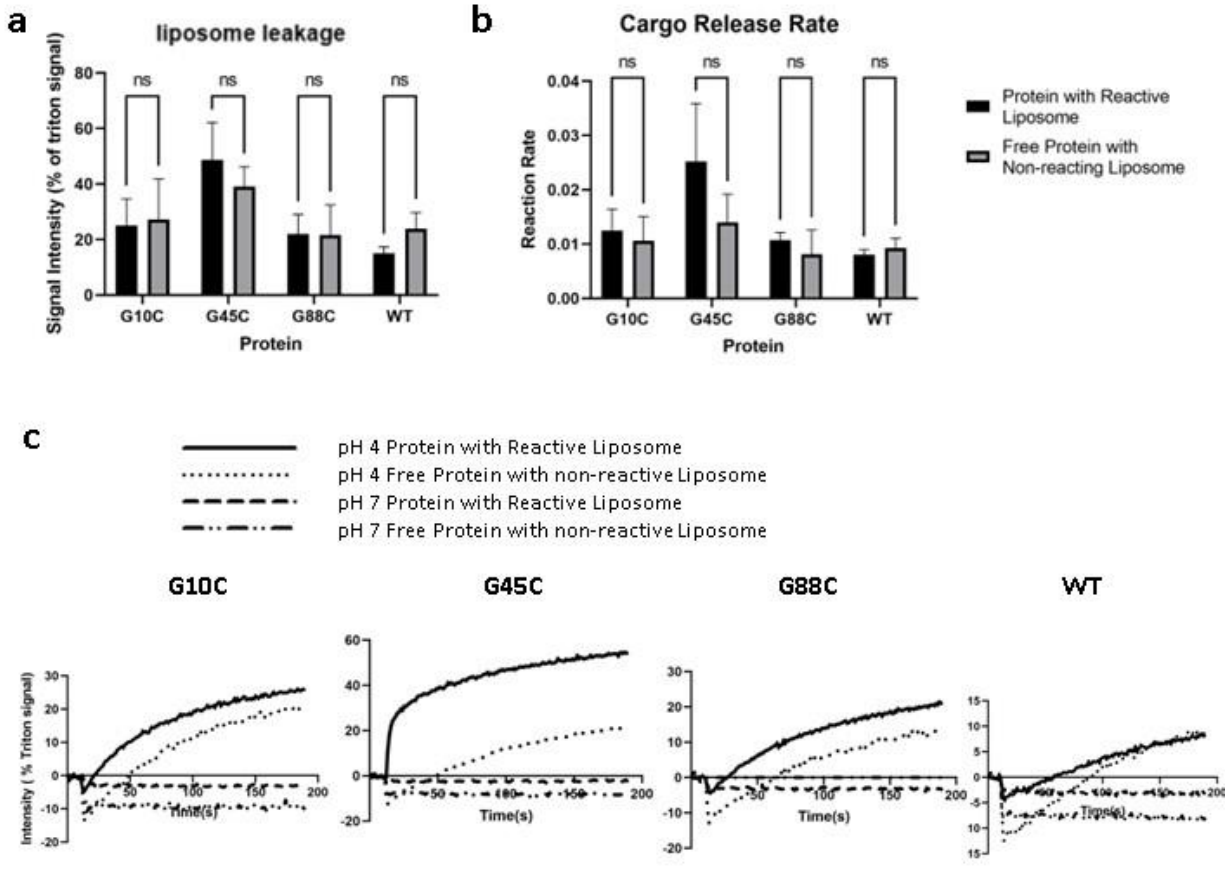

Figure 7 Signal intensity and reaction rate of *M. smegmatis* derived EsxA/B heterodimer. a) Cargo release at intensity at 180s indicated by signal presented as percentage of triton triggered signal. b) Cargo release rate. c) Signal intensity measurement against time with a typical replication

Under pH 7.4, the system was stable.

In liposome leakage assay, the liposome lytic activity was based on the original MPA of heterodimer. As shown in the figure above, there was no statistical difference on conjugated heterodimer from their corresponding free protein. If we compare among different proteins, only G45C EsxA with its chaperon EsxB triggered the highest (P=0.0019) and fastest (P=0.0062) cargo release. The conjugated EsxA-G45C heterodimer is also the only one triggered up to 48.8% of

triton signal (full release) at 180s, reaching the monomer level which was 40-60% triton signal, suggesting this could be used as a pH sensitive cargo carrier.

For other mutants as well as the WT protein, EsxA/B heterodimer from *M. smegmatis* showed very low membrane lytic activity with either conjugation or free protein.

The reason why G45C mutation on EsxA enhanced the membrane lytic activity is still unknown. It was believed that EsxA/B forms heterodimer before acidification. With the drop of pH, EsxA is released and inserts into the membrane. A possible explanation could be that G45C mutation or conjugation onto liposome interferes more with the interaction between EsxA and EsxB, making it easier for EsxA to be released into the membrane lytic process.

4.6 With CL-4B column, liposome separation was not successful

Firstly, free protein and free liposomes, same as the reaction required, were loaded separately onto a packed Sepharose CL-4B column. As shown in the following figure, the column was capable of separating the free protein from the liposome.

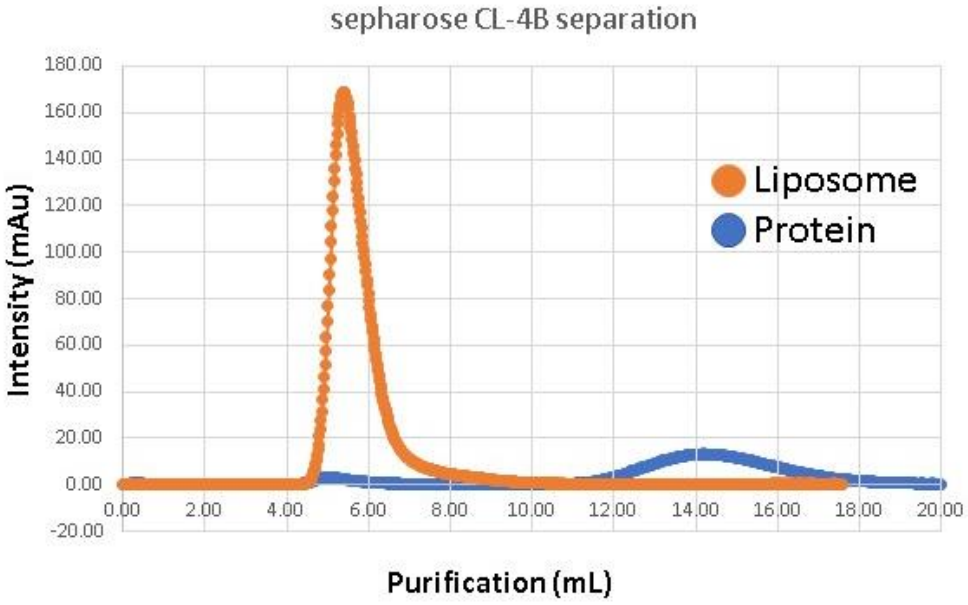

Figure 8 Liposome and free protein generated different peaks on Sepharose CL-4B column

However, when the reaction mix was passed through the column, only G10C had 2 visible peaks. For G45C and G88C, the liposome peak was very low, and nothing was detectable on SDS-PAGE. After the experiment, the column resin cleaved for an unknown reason and was repacked. The problem could be that EsxA itself had membrane interacting activity which interfered with liposome solution and caused aggregation of liposome. The aggregated liposome became stuck in the column; therefore, the liposome peak was very low.

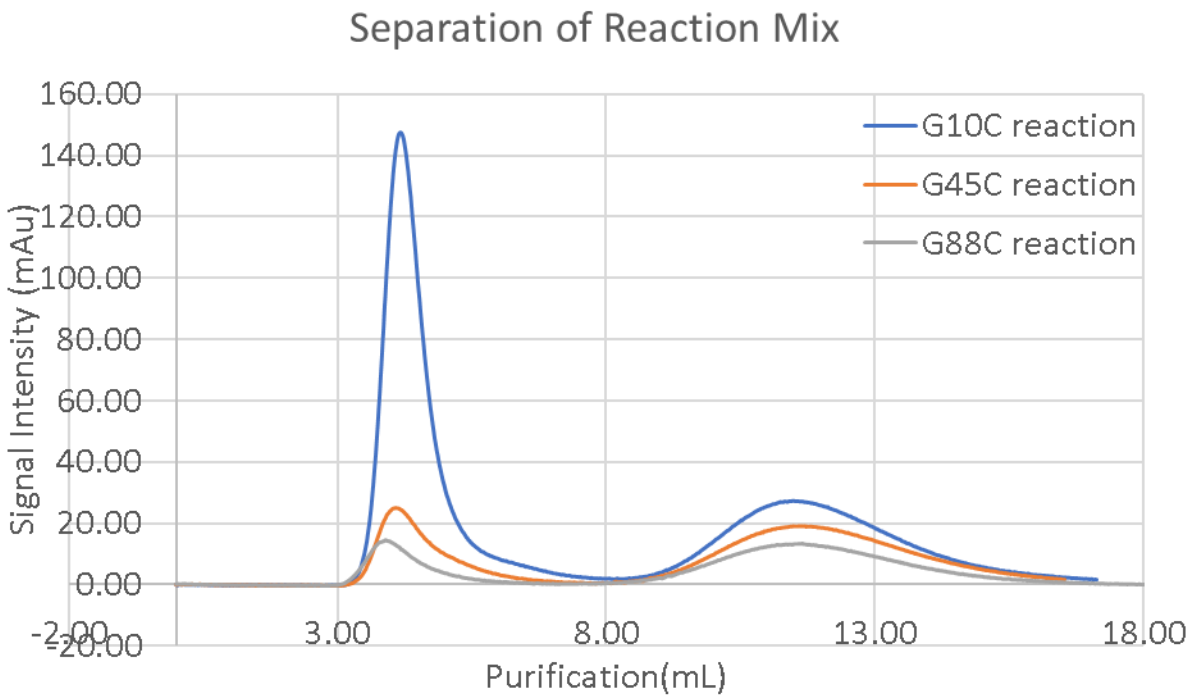

Figure 9 Separation of the reaction mix was not successful

Chapter 5: Discussion

EsxA has shown multiple rules in host-pathogen interaction. Several cell surface receptors on macrophage have been found to interact with purified EsxA(Bao et al., 2021). It also shows the MPA with a drop of pH (Bao et al., 2021). With this potent to be a ligand for active delivery, there have not been any studies that reported using ExsA for liposome modification. In this study, we decided to conjugate EsxA mutants to liposome surface with maleimide reaction as a pH-induced cargo release trigger.

DOPC-DSPE liposome has been widely used in drug delivery systems. With overnight incubation, conjugation could be observed on SDS-PAGE. However. Even with an excessive amount of the maleimide group, there was still a visible amount of unbound protein. According to literature, Sepharose CL-4B columns were used in an attempt of liposome purification. However, we were not able to get the liposome peak of G45C and G88C conjugated protein. Column clog also occurred during purification. As the unpurified liposome had high binding efficiency, with good signal in the following experiment, we chose to use the reaction mix directly in the following experiment.

With the liposome leakage results, EsxA derived from both *E. coli* and *M. smegmatis* can be used as pH-induced cargo release trigger on liposome surface. *E. coli* derived G10C was less sufficient, possibly because of the C-terminus His-tag (Chen et al., 2015; Ledent et al., 1997; Thielges et al., 2011; Zhao & Huang, 2016). This extra structure promoted membrane leakage in free protein but acted as inhibition when N-terminus of protein was linked to the membrane surface confirming the essential role of both N-terminus and C-terminus in membrane disruption.

EsxB stabilizes EsxA at physical temperature (Vazquez Reyes et al., 2020) suggesting its potential as protective chaperon during the delivery. However, in the experiment, the

heterodimer exhibited low efficiency in cargo release with trigger of acidic pH except EsxA-G45C heterodimer. Previous results suggested EsxA/B heterodimer conformation change is influenced with lipid components of liposome, and it works better with the POPC/PG formular (Ray et al., 2019). In future experiments, cholesterol stabilization could be added to the POPC formular. With a stable POPC liposome, EsxA/B heterodimer can still be used as pH- trigger.

Other than the function as a cargo release trigger, the influence on protein MPA by the linkage was also interesting. According to previous research, EsxA inserts into the membrane with two helical structures, while the flexible arms remain outside, and the central loop dives into the aqueous core of the liposome. With this model, it was hypothesized the conjugation of the central loop to the liposome surface should inhibit membrane insertion of protein. However, in our experiment, G45C conjugation resulted in a higher reaction rate for EsxA both from *E. coli* as well as *M. smegmatis*. The effective monomer length of the PEG molecule is 0.35 nm (Suk et al., 2016). The full length of the PEG2000 (molecular weight 2kDa, average polymer chain length 45.45) chain is calculated to be 15.91 nm (Chithrani, 2014), which would be sufficient to cross the membrane bilayer after pore formation. According to previous research, addition of PEG to liposome facilitated membrane insertion of purified EsxA (Ma, 2014), which could partially explain the reaction rate observed in our experiment.

5.1 FUTURE DIRECTION

In the experiment, we developed a target delivery model with EsxA as cargo release trigger. EsxA purified from both *E. coli* and *M. smegmatis* was able to disrupt the carrier with a drop of pH. However, more factors should be considered for in vivo delivery. The first to consider is toxicity. Although EsxA is a toxin secreted by Mtb, it has been studied as potential

vaccine (Brandt et al., 2000) or tuberculin (Aggerbeck & Madsen, 2006). Cytotoxicity of the delivery system is needed to apply this system to cell culture. Furthermore, we chose EsxA not only for its MPA, but also for its interaction with receptors on cell surface. Whether different binding sites would influence the recognition of EsxA and phagocytosis still needs to be tested. In the next step of cell experiment, fluorescent labelling of liposome would be sufficient to track the delivery pathway and colocalization with bacterial infection. If cargo release can be triggered near bacteria reservoir, this modified lipid model may also be used as active-targeting drug delivery carrier in the future.

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