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The Development of a System to Study the Prion Protein at the Membrane Surface

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by
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Abstract

The Development of a System to Study the Prion Protein at the Membrane Surface

Amy M. Freiberg

The Cellular Prion Protein (PrP^C), discovered by Nobel Laureate Dr. Stanley Prusiner, represents the causative agent of a class of transmissible neurodegenerative diseases known as prion diseases. Despite knowledge that PrP^C initiates early events of toxicity at the cell surface, limited research efforts and therapeutics have targeted the membrane environment. To meet this end, we are designing fully new and important ways of studying prion-induced toxicity at the membrane surface. Given that membrane-anchored PrP^C purified from mammalian cells have heterogeneous post-translational modifications that are not amenable to biophysical assays, it is essential to instead chemically modify recombinantly expressed PrP^C with a synthetic anchor. It is also important to prepare a simple representative membrane system that creates a controlled environment for studying the protein's molecular and structural mechanisms. We first determine that liposomes are an ideal membrane system given their similar morphology to the native membrane, their ability to incorporate peripheral membrane proteins and their amenability to various biophysical assays. We then test the expression of mPrP S230pAcPhe and subsequent modification with a representative membrane anchor. We produce a protein construct that can be expressed using a modified expression system that incorporates the unnatural amino acid para-acetyl phenylalanine. We identify a two-step scheme that will selectively modify the protein construct with a synthetic membrane anchor. We successfully modify the

protein with a dPEG linker that has aminooxy functionality, but struggled to further modify the construct with lipid tails. Alternatively, we successfully demonstrate that mutation of two residues near the extreme C-terminus of PrP^C to cysteines allows for selective modification with a synthetic maleimide-functionalized membrane anchor. The development of this system enables us to investigate PrP^C in the context of a membrane environment using biophysical approaches. Given that toxicity occurs at the membrane surface, such information will help identify interactions to target in the progression of prion diseases and other neurodegenerative diseases with similar pathology.

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My scientific journey started during my undergraduate career at the University of Central Florida. It was here that I joined my first research lab and had the opportunity to grow as a budding scientist. During this time, I had the pleasure of interacting with some amazing professors who challenged me, supported me, and encouraged me to pursue a graduate career. I am thankful for their guidance and patience while helping me navigate the graduate application process and for always seeing my potential to grow in any career that I chose to pursue. In particular, I would like to thank Dr. Robert Borgon. I first met Robert when I was a student in his Quantitative Biological Methods course during my sophomore year, and our relationship only deepened over the years. We established a wonderful rapport with each other. I always enjoyed hearing his words of wisdom and his life advice, which I still remember to this day. We continue to stay in touch with each other and it has been wonderful to update him throughout my graduate career.

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Chapter 1

Introduction

Introduction

Prion diseases represent a class of fatal neurodegenerative diseases [1]. Similar to other neurodegenerative diseases, individuals diagnosed with prion diseases suffer from deteriorating neurons, memory loss and impaired movement. Also consistent with other neurodegenerative diseases, prion diseases are caused by the templated misfolding and subsequent aggregation of protein in the brain [1,2]. Unique to prion diseases, however, these diseases can be transmitted from animal to human or even from human to human. Hence, this class of diseases is also called Transmissible Spongiform Encephalopathies (TSEs) [3,4].

The ability for prion diseases to infect both wildlife and human populations represents a significant threat to the agriculture industry and the safety of our food supply chain. Scrapie disease infected a large population of sheep in England and throughout Europe in the 1700s, creating a great disturbance to the wool industry [5]. The consumption of prion-contaminated cattle in the United Kingdom during the 1980s transmitted the disease into otherwise healthy individuals [6]. Such transmission of prion diseases are classified as acquired prion diseases. Other classes of prion diseases include sporadic [7,8] or genetic [9, 10].

The infectivity of prion diseases utilizes a self-templated misfolding mechanism, in which the pathogenic isoform of the prion protein promotes the misfolding of the native protein. The Cellular Prion Protein (PrP^{C}) is naturally expressed throughout the central nervous system of all mammals and avian species [11]. The accumulation of Scrapie Prion Protein (PrP^{Sc}) is found only in diseased individuals and is expressed as aggregates of beta-sheet rich protein [12].

Early studies that focused on PrP function and toxicity primarily utilized transgenic mice with expressed PrP. This foundational work identified that PrP-knockout mice do not exhibit symptoms of prion disease [13]. When these knockout mice are inoculated with PrP^{Sc}, the mice still do not display any symptoms of disease and do not reveal any PrP^{Sc} aggregates in the brain [14]. PrP^{C} expression is a requirement given that its presence is necessary for the conformational conversion to PrP^{Sc} and that it also serves as a signal transduction pathway for the toxicity driven by PrP^{Sc} [15].

Studying the structure and function of PrP^{C} provides important insights into prion-induced toxicity. Additionally, deletion of regions in PrP^{C} allows for the onset of rapid neurotoxic activity, without the need for infection and subsequent long incubation periods typical of prion diseases [16,17]. PrP^{C} contains a structured C-terminal domain that is primarily alpha-helical and a flexible N-terminal domain (Figure 1) [18-21]. Post-translational modification during biosynthesis removes the first 22 residues of PrP^{C}, yielding a mature protein that contains residues 23 - 231 and that has several functionally-relevant motifs.

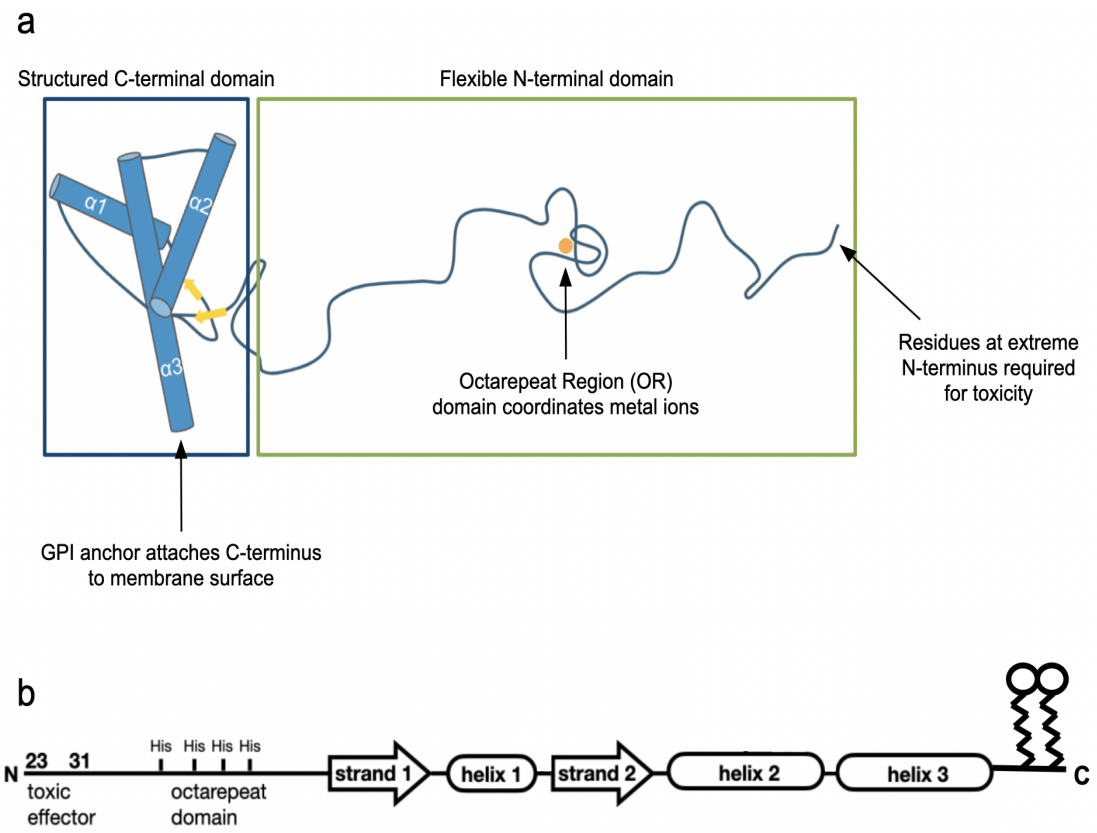


Figure 1: Structural and functional motifs for PrP^C. **a)** Schematic displays the structured alpha-helical C-terminal domain that expresses a GPI anchor to attach the protein to the membrane surface. Key motifs in the flexible N-terminal domain include the OR, which binds copper ions through its repeating histidines. PrP^C elicits toxicity through the extreme N-terminus (residues 23 - 31). **b)** Linear map indicates secondary structure and functional motifs.

A poly-basic region (residues 23 - 31) at the extreme N-terminus is required for prion toxicity and deletion of this motif suppresses neurotoxicity [22,23]. Transgenic mice (Delta 23 - 31) reveal diminished production of PrP^{Sc} [24]. Neuronal cells transfected with PrP^C constructs that previously revealed toxic transmembrane currents display reduced toxicity upon removal of the poly-basic region [22,23]. The transmembrane currents reflect fluctuations of cations across the lipid bilayer that are associated with toxic phenotype. We hypothesize that PrP^C mediates such toxicity at

the membrane surface via transient electrostatic interactions between the poly-basic motif and anionic lipid headgroups. Additionally, this segment interacts with other membrane proteins, including the AMPA receptor (AMPAR) and NMDA glutamate receptors [25,26].

PrP^C displays a nanomolar affinity for copper through a motif of repeating histidines in the octarepeat region OR (residues 59 - 90) [27-32]. The connection between PrP^C and copper has been well studied. PrP^C expression is influenced by the presence of copper, as copper binding to the *PRNP* promoter is correlated with increased protein expression levels [33,34]. Likewise, the absence of PrP^C in knockout mice is associated with decreased copper concentrations in the brain relative to wild type [27]. Copper also influences prion-induced toxicity, as the addition of copper alleviates neurotoxicity observed for toxic variants of PrP^C. Given that copper coordination to the OR drives tertiary contact between the N-terminal and C-terminal domains, it is likely that the interdomain interaction is neuroprotective. This *cis* interaction can provide regulation over the toxic N-terminus by positioning it in such a way that it cannot participate in aberrant interactions (Figure 2) [35-39].

Figure 2: Current model for regulation of PrP^C toxicity. **a)** During normal physiological states, the flexible N-terminus is positioned in such a way that it is not available to participate in aberrant interactions. The addition of copper promotes a neuroprotective interdomain interaction that re-orients the flexible N-terminus. **b)** Toxicity arises upon dysregulation of the N-terminus.

The protein is naturally attached to the outer surface of cell membranes through a glycosylphosphatidylinositol (GPI) anchor at the extreme C-terminus [40,41]. The membrane-tethering is an important property of the protein, as the membrane surface represents a site for conversion to the Scrapie isoform [42-45]. While protein anchoring is a known requirement for conversion, the precise role of specific lipids involved remains to be fully determined. It is important to understand how different lipids affect protein structure and how PrP^C interacts with individual lipids. Does the N-terminus interact with select lipid groups? Also, are normal protein functions, such as copper-binding, affected in the presence of certain lipids?

Since biophysical techniques that study protein structure and function represent a viable approach to answering such questions, a pure sample is a requirement as biophysical experiments reveal poor signal in the presence of heterogeneous populations. Given that membrane-anchored PrP^C purified from mammalian cells have heterogeneous post-translational modifications, it is essential to instead chemically modify recombinantly expressed PrP^C with a synthetic anchor. Thus, it is also important to prepare a simple membrane environment that is representative of neuronal membranes.

The body of this thesis focuses on the design of a simple representative membrane system and the development of strategies for modifying recombinantly-expressed PrP^C with a synthetic membrane anchor. Chapter 2 of this thesis describes the considerations for the development of a representative membrane environment and details the protocol for the preparation of liposomes. The third chapter explores several strategies to modify recombinant PrP^C that make it amenable to downstream reaction with a representative membrane anchor. Lastly,

the fourth chapter describes the reaction between PrP^C and synthetic membrane anchors and the reconstitution into liposomes. In summary this work focuses on the design of a simplified system for studying membrane-anchored PrP^C.

Chapter 2

Liposomes are a Simple Representative Membrane Environment.

Introduction

The Cellular Prion Protein (PrP^C) is expressed in the central nervous system as a glycosylphosphatidylinositol (GPI)-anchored protein at the outer surface of neuronal cell membranes. Protein attachment to the membrane surface is recognized as an important process for the toxicity of prion diseases [42-45]. Alteration or deletion of the signal sequence that directs PrP^C to the membrane surface prevents conversion to the toxic isoform [43]. How exactly does membrane attachment cause toxicity? Neuronal cell models transfected with variants of PrP^C exhibit the generation of large, spontaneous inward cationic currents [46]. The nature of the ionic currents produced suggest that PrP^C itself permeabilizes the cellular membrane and forms membrane pores. Transgenic mice inoculated with the same protein variants display a neurodegenerative phenotype [16], suggesting that the observed transmembrane currents initiate toxicity.

The exact mechanistic and structural details for how PrP^C drives this toxicity remain unknown. Since the native membrane environment contains complex protein interactions [47], variable lipid concentrations [48], and endogenous ion channels [49], it is difficult to directly attribute functional significance to individual proteins. There is a need to develop a simple and controlled system that directly models PrP^C at the membrane surface and probes its physiological and pathological activities. Such a system should also be compatible for characterization with biophysical assays, such as NMR and EPR, to elucidate structural and functional information.

There are several membrane systems that can be prepared to create a representative environment for membrane proteins [50]. The model membrane used depends on various considerations, including whether the protein of interest is

integral vs peripheral and what types of assays the system will be exposed to [51]. For our purposes, we prepared liposomes to represent the native membrane environment given their similar morphology to actual membranes and their feasibility to prepare. Liposomes also allow flexibility for controlling the specific phospholipid components, which allows for selecting a membrane composition that is a representative membrane environment for mammalian neurons [52].

We design an optimized scheme for the preparation of liposomes and confirm successful preparation using Dynamic Light Scattering (DLS). We also test if the incorporation of different phospholipid components influences the proper formation of liposomes. We assess the preparation of homogenous liposomes that are of a single phospholipid type, as well as heterogeneous liposomes that contain several phospholipid components naturally expressed in mammalian cell membranes. Of interest, we examine the preparation of POPC (zwitterionic) and POPG (anionic) liposomes. We also examine the incorporation of an artificial lipid component that contains a functional head group for covalent modification with PrP^C. Our findings suggest that all liposome conditions comprise a homogenous population with a narrow size distribution.

Results

Liposomes are utilized for a variety of purposes ranging from drug-delivery systems to anchoring of membrane proteins and the preparation of such vesicles is dependent on the specific application [53-55]. Given that liposomes represented a new model system in our lab for investigating the prion protein (PrP^C), we tested different preparation schemes and optimized parameters to best suit our specific needs.

Our first consideration was selecting a liposome composition that would be representative of a neuronal membrane. There are a variety of lipids that are naturally expressed in cellular membranes and that can be used for the preparation of liposomes, including lipids that contain zwitterionic or anionic headgroups (Table 1) [56]. In order to test how PrP^C interacts with lipids that have such different chemical properties, we desired to prepare liposomes that contain only one or two lipid species at a time. Our rationale was that once we understand how the protein interacts with these specific lipids, then we can start to diversify the lipid composition to more accurately reflect the complexity of the native membrane environment. Additionally, we were curious if lipids with such chemical diversity could be prepared using the same preparation protocol.

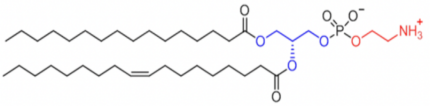
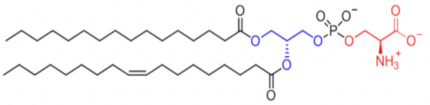
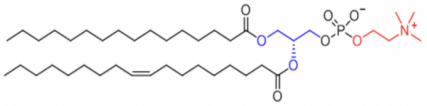
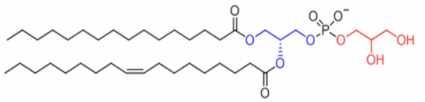
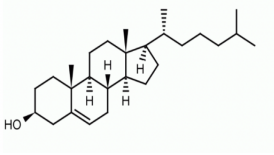
Abbreviation	Chemical Name	Structure
POPE	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine	
POPS	1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine	
POPC	1-palmitoyl-2-oleoyl-glycero-3-phosphocholine	
POPG*	1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol)	
Cholesterol		

Table 1: Lipid diversity expressed in the cellular membrane of mammalian neurons. Asterisks denote lipids that are not naturally expressed in the brain. Bold font indicates lipids that are used in this study. POPC (zwitterionic) and POPG (anionic) lipids are used in this work to develop a model membrane [57,58].

We previously determined that deletion of the poly-basic region (residues 23 - 31) rescues wild-type activity for toxic variants in neuronal cells [22]. We hypothesize that PrP^C mediates such toxicity at the membrane surface via electrostatic interactions between the poly-basic motif and anionic lipid headgroups. Thus, our first objective was to characterize how PrP^C interacts with anionic lipid headgroups. We also prepared liposomes composed of zwitterionic head groups as a control. When selecting phospholipids, we also considered the hydrocarbon length and unsaturation of the fatty acid tails. We opted to work with lipids that contained one palmitoyl chain and one oleoyl chain. The addition of a single double bond in the oleoyl chain decreases the phase transition temperature without introducing the oxidation that occurs for polyunsaturated fatty acids.

Unsaturated lipids are hygroscopic and we therefore ordered all lipids as a chloroform solution. We aliquoted phospholipids into glass vials layered with nitrogen and covered with a teflon lid before storing in the flammable -20C until ready to assemble liposomes. When preparing liposomes, it is necessary to make sure that there is a homogenous mixture of lipids. We gently stirred chloroform solutions that contained different phospholipids to allow all components to properly disperse.

The presence of chloroform can drastically alter the physical properties of liposomes [59]. Thus, we thoroughly removed chloroform from our samples by initial blow-down with a stream of nitrogen, followed by subjection to rotary evaporation and high-vacuum for one hour to eliminate any trace amounts of solvent. Addition of an aqueous buffer to the dried lipid film allows for swelling of the lipid bilayer from the round bottom flask. When considering what solvent would be appropriate for our system, we decided to use MOPS given that it is a buffer commonly used to study PrP^C and thus maintains the protein integrity. While the hydration process can be completed in one hour, we let our sample stand overnight as longer time periods allow for formation of a homogenous liposome population with a narrow size distribution [60]. We agitated the lipid bilayers to promote the formation of large multilamellar vesicles by performing five freeze/thaw cycles using liquid nitrogen and warm tap water. We noted that this process also allowed for complete detachment of the lipid cake from the glass wall of the round bottom flask.

We desired to obtain a final suspension of homogenous unilamellar vesicles. Liposome preparations vary in size, ranging from small unilamellar vesicles that are less than 100 nm to giant unilamellar vesicles that are greater than 1 um [61]. Sizing considerations for liposomes requires selecting an optimal size that is large enough

to be compatible with different assays yet small enough to not require an excessive amount of modifying material. For our purposes, we determined that it would be appropriate to work with liposomes that are 100 nm on average.

Sizing of liposomes can be achieved using either sonication or extrusion through a polycarbonate membrane [62]. Sonication disrupts vesicles using sound energy and produces small vesicles sized in the range of 15 - 50 nm [60]. We instead opted to size our liposomes by extrusion through a polycarbonate membrane of 100 nm. During this process, a sample is passed through a membrane a minimum of 21 times to achieve a homogenous population.

We utilized the above methodology to prepare different liposome compositions (Figure 1. The first batch of liposomes that we assembled were singly composed of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC). We verified the successful formation of the zwitterionic liposomes via Dynamic Light Scattering (DLS), which is a technique that is commonly used to characterize the mean size and polydispersity of particles. We found that the average diameter for POPC was 110.1 nm with a polydispersity index (PDI) of 0.068 (Figure 2). Such sizing features are consistent with extrusion through a membrane of 100 nm, which typically yields samples that have mean diameters between 120 - 140 nm [59]. Additionally, the PDI is below 0.1 and is indicative of a monodisperse population.

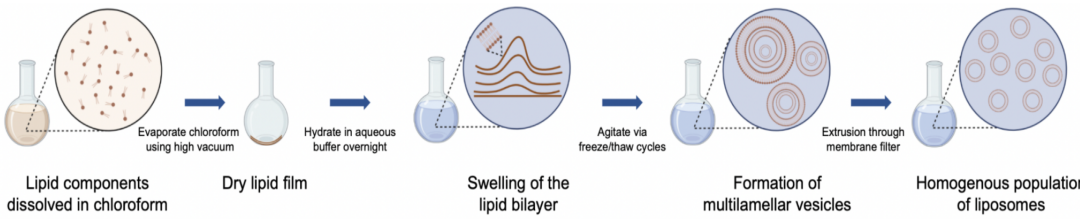


Figure 1: Scheme for the preparation of liposomes. Briefly, liposomes are prepared by initial solvation in chloroform, followed by organic solvent removal via vacuum and subsequent

hydration in aqueous buffer before final sizing steps.

We wanted to test if the same preparation scheme could be applied to lipids with different chemical properties. We thus assessed the preparation of liposomes composed of 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (POPG), which contain a negatively-charged head group. We also explored the preparation of liposomes that contained a heterogeneous mixture of lipids and examined the formation of liposomes that incorporated both POPC and POPG. While POPC is naturally expressed in mammalian neurons, POPG is not. However, POPG is routinely used in research studies to represent anionic lipids found in membranes because it retains fluidity at room temperature. When considering what would be an appropriate ratio for POPC and POPG, we considered PrP^C's ability to coordinate copper in its native environment. We desired to develop a membrane system that would allow protein-copper interactions, even in the presence of metal chelators such as POPG. We selected a lipid profile of 70% POPC and 30% POPG, which is a lipid composition that had been previously characterized in the literature to promote such interactions.

We also explored the preparation of POPC liposomes that incorporate a maleimide-functionalized lipid. The maleimide moiety serves to react with site-specific functional groups on PrP^C, which covalently anchors the protein to the liposome surface. We determined that limiting incorporation of the maleimide-lipid to 1 molar % of the final lipid concentration prevents lipid aggregation, which is consistent with reports in the literature. The liposome composition revealed a homogenous population, which further reinforced the versatility of the liposome preparation scheme.

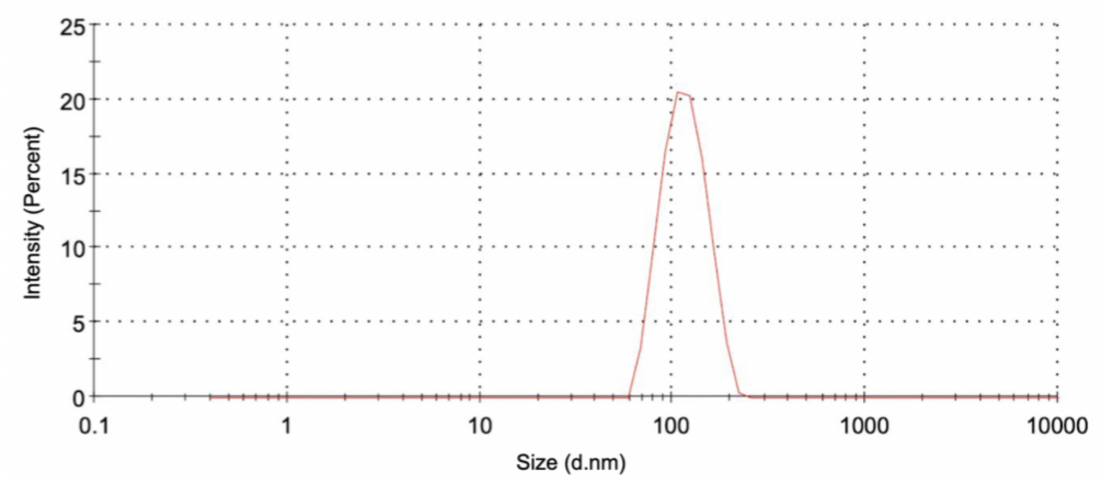


Figure 2: Dynamic Light Scattering analysis for POPC liposomes that are prepared in 50 mM MOPS pH 7.4. The liposomes comprise a homogenous population with a narrow size distribution. The liposomes have an average diameter of 110.1 nm with a polydispersity index (PDI) of 0.068.

Discussion

In this study we developed a general protocol for the preparation of liposomes that can be applied to the preparation of liposomes with varied chemical composition. The liposome compositions that we prepared are simplified systems that only contain select components of what is naturally expressed in neuronal cellular membranes. Given that we aim to explore how PrP^C interacts with membrane components, we selected zwitterionic or anionic lipid headgroups for our model membrane to establish a framework for such interactions.

Future directions will expand the scope of this work by studying how the protein interacts with other membrane components and proteins. An additional component of neuronal membranes includes cholesterol [56]. Interestingly, GPI-anchored proteins such as PrP^C localize at lipid rafts in the membrane that contain clusters of cholesterol [63,64]. We are interested in designing liposome compositions that consider such membrane diversity to characterize how components collectively influence protein-membrane interactions. PrP^C also interacts with a range of membrane proteins at the cell surface, including amyloid-beta that is responsible for Alzheimer's disease [65,67]. We anticipate that such protein-protein interactions influence PrP^C's conformation and subsequently affect how PrP^C interacts with the membrane. We believe that liposomes represent an ideal membrane environment for investigating the interactions between membrane proteins and the potential influence on protein-membrane interactions.

For our purposes, we characterized liposome integrity in terms of size and polydispersity using DLS. Liposomes are amenable to characterization using a range of assays that would provide more information about protein-membrane interactions.

For example, liposomes can be directly imaged using CryoEM which can provide critical information about liposome morphology and integrity. Other biophysical techniques that this system can be exposed to include HDX-MS, EPR and NMR.

Materials and Methods

Preparation of Liposomes

Lipids were delivered dissolved in chloroform solution inside of a glass ampoule at a concentration of 25 mg/mL (Avanti Polar Lipids). Upon arrival, lipids were aliquoted into 5 mg quantities into glass vials that were layered with nitrogen gas prior to sealing with a teflon-lined cap. Samples were stored long-term in the flammable -20 C freezer until ready for use.

Liposomes were typically assembled at a concentration of 5 mg/mL. To prepare liposomes, a 5 mg lipid aliquot was transferred to a 5-mL round bottom flask using a glass pasteur pipette. Bulk chloroform was evaporated using a stream of nitrogen gas for a few minutes. Any residual chloroform was removed using a rotary evaporator followed by high vacuum for at least 1 hour. The dried lipid film was hydrated overnight using 1 mL of 50 mM MOPS pH 7.4 at room temperature. The lipids were agitated using five freeze/thaw cycles, resulting in the formation of large multilamellar vesicles. A homogenous population of unilamellar liposomes were prepared by extruding the sample twenty-one times through a 100 nm membrane filter (Avanti Polar Lipid Extruder).

Dynamic Light Scattering (DLS)

1 mL of liposome solution was transferred to a plastic cuvette for analysis in the Malvern Zetasizer. The average size and polydispersity index (PDI) were output.

Chapter 3

Modification of Prion Protein to Render it Available for Reaction with Membrane Anchor

Introduction

The Cellular Prion Protein (PrP^C) is modified at the extreme C-terminus with a GPI anchor that serves to anchor the protein to the outer leaflet of the cellular membrane. In the native context, the final residue of PrP^C is a Serine at position 230. We sought to study the structural and functional effects of the membrane anchor in a controlled membrane environment. In order to study PrP^C in such an environment, it is necessary to obtain a homogeneous population of membrane-anchored protein. Given that membrane-anchored PrP^C purified from mammalian cells have heterogeneous post-translational modifications, it is essential to instead chemically modify recombinantly expressed PrP^C with a synthetic anchor.

To resolve this, we tested different methods to modify recombinantly expressed PrP^C to render the protein available for reaction with a representative membrane anchor. Following protein expression and purification, our goal is to chemically modify recombinantly expressed PrP^C via a synthetic anchor that has a functional head group (Chapter 4). Our approaches considered feasibility, biological representation and ease of production. Additionally, the use of recombinantly expressed protein allows for isotopic labeling that makes the protein amenable to structural studies such as NMR.

All approaches incorporate amino acids that are amenable to "click"-chemistry with functionalized membrane anchors. The amino acids of interest are either cysteine or an unnatural amino acid, para-acetyl phenylalanine (pAcPhe). The motivation for incorporating these particular residues is that they both have specific reactivities for functional groups that allow for covalent modification of the protein. This chapter will focus on the preparation of different protein constructs and

the following chapter will discuss the reaction between the mutated protein and the functionalized membrane anchor.

Our first approach relied on the site-specific incorporation of cysteine at the final residue of the protein sequence. We determined that this method was not the most viable option given the difficult protein purification and instead considered incorporating a photo-caged cysteine at the final residue. This approach would allow us to purify the protein using our standard protocol because we can mask the cysteine throughout the purification process and hence not interfere with the formation of the native disulfide. Given the difficulty of obtaining the necessary materials and the time commitment required for a new expression system, we ultimately decided to not move forward with this otherwise exciting possibility.

However, we were curious if instead incorporating two cysteines in close proximity to each other at the extreme C-terminus would resolve this issue. Our logic was that the native disulfide bridge would form without interference from the incorporated cysteines, as they would create their own disulfide bridge with each other. This strategy was successful and allowed for improved protein yield, despite the presence of additional cysteines.

We also tested the incorporation of pAcPhe at the final residue of PrP^C as a viable option. The genetic incorporation of such unnatural amino acids was first introduced by the Schultz lab at UC San Diego and the concept has since been extended to many applications [65]. The incorporation of pAcPhe into PrP^C has previously been applied to selectively modify the protein with artificial glycans by reaction with the ketone functional group [66]. Protein expression for this system requires supplementing the growth media with the materials necessary for

incorporation of pAcPhe. Otherwise, the protein purification is identical to our typical protocol. We demonstrate the successful incorporation of pAcPhe at the final residue of PrP^C and deem that it is a practical candidate for modification with membrane-anchor to model.

Ultimately, our work suggests that there are multiple viable protein constructs that can be prepared for future modification with a representative membrane anchor. For our purposes, we will only proceed forward with the approach that incorporates two cysteines and the approach that incorporates a single pAcPhe residue.

Results

The Cellular Prion Protein (PrP^C) follows an insoluble protein preparation that requires extraction of inclusion bodies and subsequent protein purification. Briefly, the purification protocol requires several successive chromatography steps (Figure 1). One critical purification step is the formation of a native disulfide bridge that is necessary for proper tertiary structure. We promote oxidative refolding between cysteines at positions 179 and 214 by increasing the pH of the solution to slightly basic conditions just above pH 8. While this general workflow is appropriate for most constructs of PrP^C, it is appropriate to recognize that some mutations can alter the expression or purification strategy. We sought to modify PrP^C in a way that would make the protein amenable to reaction with a functionalized membrane anchor and observed how such mutations influenced the canonical protocol.

Figure 1: Standard protein purification for prion protein. First, the protein is separated from other components given its strong affinity for the Ni2+ resin. The protein then forms its native disulfide under oxidative refolding conditions, followed by a buffer exchange to remove denaturing conditions. Lastly, properly folded protein is purified via HPLC and characterized via LC-MS.

Our first approach was to mutate the final residue at the extreme C-terminus to a cysteine that could react with a maleimide-functionalized membrane anchor (Figure 2). We coined this construct mPrP S230C. We were concerned that incorporation of an additional cysteine would complicate the formation of the native disulfide bridge and result in misfolded protein that contains non-native intra- or intermolecular disulfide bonds. Our lab previously tested the substitution of cysteine at a position proximal to the native disulfide and determined that the protein yield

was compromised post-oxidative refolding [69]. HPLC is the final step of the protein purification protocol and separates protein based on protein fold. Whereas a pure population of protein reveals one main peak on the HPLC trace, the HPLC trace for this particular protein construct displayed several peaks suggestive of different disulfide connectivities.

Despite this, we remained hopeful that we could incorporate a cysteine at the final residue given that the position was distant from the native disulfide. However, the HPLC trace for the protein purification indeed contained several peaks that we collected as separate fractions. We reasoned that a reduction step post-HPLC could serve to selectively reduce any non-native disulfide connectivities while preserving the native disulfide that is less solvent-exposed. After running the reduction reaction, we loaded our sample onto the HPLC to separate out any residual misfolded protein and were disappointed to still observe the presence of several peaks. We were interested in characterizing the protein purity and mass using LC-MS and were discouraged to observe the presence of multiple protein species that were not of the correct mass (Figure 3). At this point, we determined that the incorporation of a single cysteine did not represent the most viable approach given the difficulty of protein purification with an odd number of cysteines.

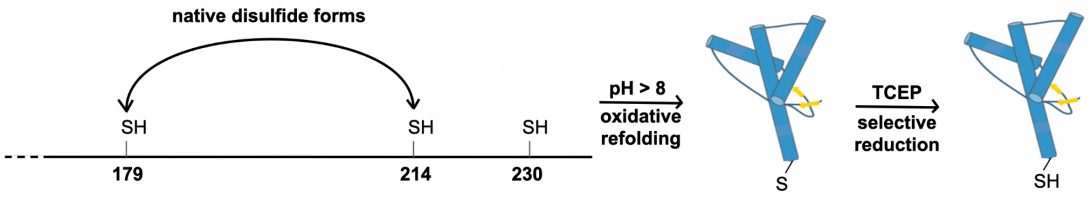


Figure 2: Scheme for the preparation of mPrP S230C. The protein construct is expressed and purified using the standard protocol, including the oxidative refolding step necessary for the formation of the native disulfide. An extra purification step selectively reduces the additional cysteine.

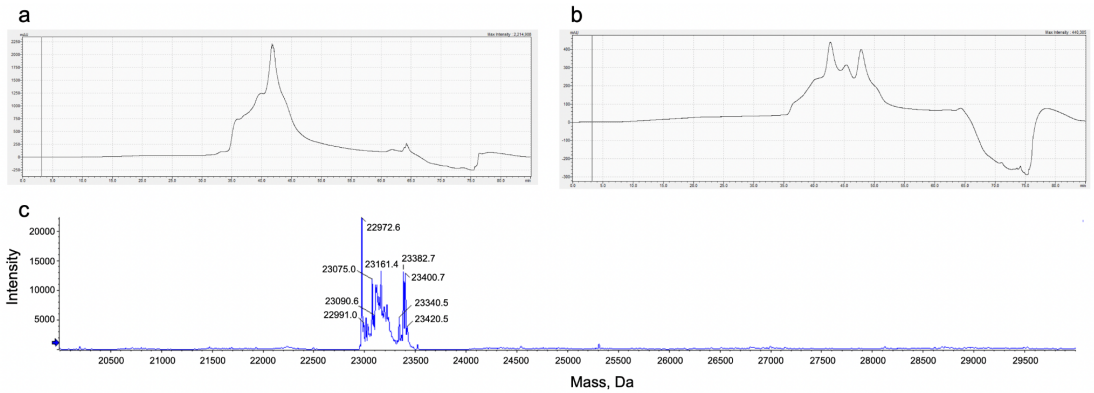


Figure 3: Protein purification and characterization for mPrP S230C. **a)** HPLC trace for the protein pre-TCEP reduction suggests that there are different disulfide connectivities. **b)** HPLC trace for the protein post-TCEP reduction indicates that there are still multiple protein species. **c)** LC-MS analysis confirms that this is not a pure sample prep and that the expected mass is not observed.

One alternative strategy that we considered was instead incorporating a photo-caged cysteine. Our rationale was that the photo-caged cysteine would not interfere with the purification process as the cysteine would be masked until removal of the cage by brief light exposure post-purification (Figure 4). Implementation of this preparation scheme would require access to the tRNA/aminoacyl-tRNA synthetase (aaRS) pair and synthesis of the photo-caged cysteine, amongst other considerations. Also, the development of new protein purification schemes are difficult and extremely time-consuming. Given the following considerations we decided to not attempt this strategy, even though we believe that it may represent a viable and exciting approach.

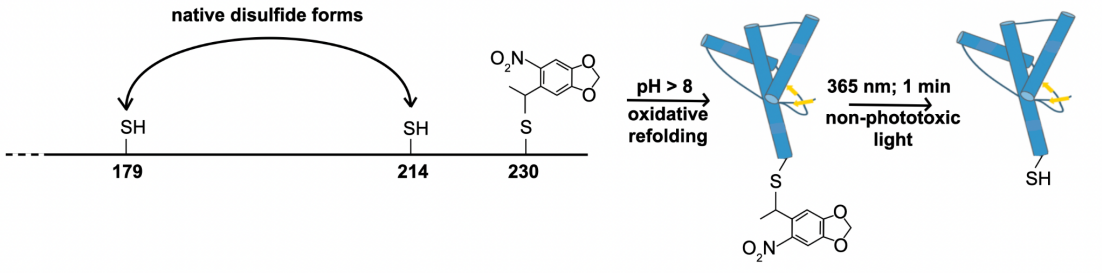


Figure 4: Scheme for the preparation of mPrP with a photocaged cysteine at the final residue. The protein construct is expressed and purified using the standard protocol, including the oxidative refolding step necessary for the formation of the native disulfide. An extra purification step utilizes non-phototoxic light to remove the caging and expose a reduced cysteine.

Instead, we reasoned that mutation of two residues at the extreme C-terminus to cysteines (mPrP G227C S230C or mPrP 2 Cys) may circumvent the difficulty of purifying a protein with an odd number of cysteines. We hypothesized that if the two additional cysteines form their own disulfide bond due to proximity that the native disulfide would still form and that the protein would be properly folded (Figure 5). Our workflow was similar to the approach used with the single cysteine, in which we performed the typical protein purification followed by a selective TCEP reduction. We observed that the first HPLC run revealed a trace that contained one main peak, indicative of a singly-folded protein species. We suspected that the peak contained properly folded protein with an additional disulfide linkage between the two additional cysteines. We thus ran a reduction reaction to selectively reduce the additional disulfide linkage followed by HPLC purification. Analysis of the HPLC trace indicated that there was only one main protein peak.

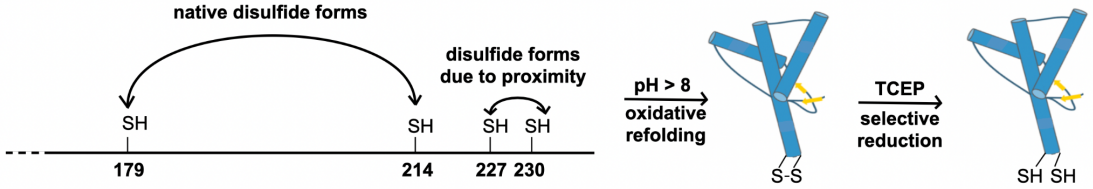


Figure 5: Scheme for the preparation of mPrP G227C S230C. The protein construct is expressed and purified using the standard protocol. The oxidative refolding step allows for the formation of both the native disulfide and the disulfide between the two cysteines at the extreme C-terminus. An extra purification step selectively reduces the additional cysteines.

Since the new protein construct contains four total cysteines, we wanted to characterize the protein to verify that the protein was properly folded and that the two solvent-exposed cysteines were reduced. We first assessed the protein purity and mass via LC-MS and determined that we had a population of pure protein with a mass of 23122.9 Da. We also tested that the protein was properly folded and that the incorporation of two additional cysteines did not disrupt the native protein fold. We observed that mPrP 2 Cys displayed secondary structure features that are characteristic of properly-folded WT PrP^C via Circular Dichroism (CD). Given that the protein was properly folded, we were confident that the native disulfide bridge formed. We next examined if the two non-native cysteines were indeed reduced. To test this, we reacted mPrP 2 Cys with N-ethylmaleimide (NEM), which has high selectivity for free thiols. We monitored the reaction via LC-MS and identified the formation of product with a mass of 23372.5 Da, which suggests that the modified protein contains two solvent-exposed cysteines as expected (Figure 7). We determined that this protein preparation scheme represented a feasible approach for preparing protein with functionality that is amenable to downstream reaction with a maleimide-functionalized synthetic membrane anchor.

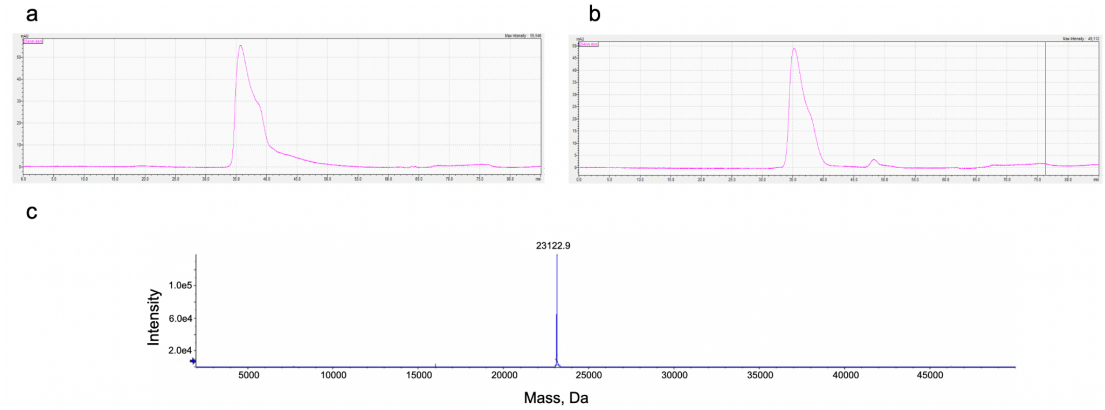


Figure 6: Protein purification and characterization for mPrP G227C S230C. **a)** HPLC trace for the protein pre-TCEP reduction suggests that there is a pure protein population. **b)** HPLC trace for the protein post-TCEP reduction indicates that there is still a pure population. **c)** LC-MS analysis confirms that this is a pure sample prep and that the expected mass is observed.

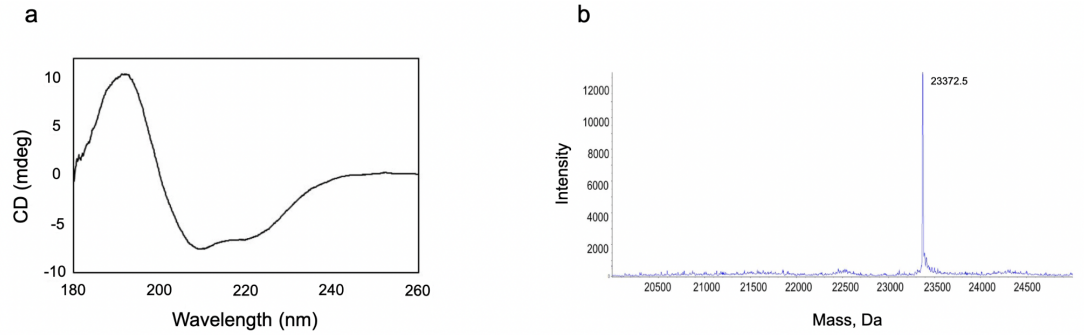


Figure 7: Characterization for mPrP G227C S230C. **a)** Circular Dichroism (CD) spectrum displays prominent features at wavelengths 208 nm and 222 nm that are characteristic of alpha-helical content for properly-folded PrP. **b)** LC-MS analysis for reaction between mPrP G227C S230C and N-ethyl maleimide (NEM). The observed mass suggests that the protein contains two solvent-exposed reduced cysteines, as expected.

While we were satisfied that we were able to successfully prepare a new protein construct that was amenable to bioconjugation, we were still stuck wondering if we could prepare a protein construct that only required modification of a single residue. We designed another approach that mutates the final residue of the protein sequence to an unnatural amino acid, para-acetyl phenylalanine (pAcPhe), that has functionality amenable to downstream bioconjugation (Figure 8). We coined this construct mPrP S230pAcPhe. The expression of the unnatural amino acid requires supplementing the growth media with the tRNA/aminoacyl-tRNA synthetase (aaRS) pair and para-acetyl phenylalanine. The protein purification steps are identical to our standard protocol. The HPLC trace displayed one main peak and subsequent LC-MS analysis revealed a pure population with a mass of 23162.9 Da, consistent with incorporation of the unnatural amino acid (Figure 9). We reason that preparation of this protein construct also demonstrates a viable strategy for the preparation of functionalized PrP^C that is available for bioconjugation.

In summary we introduced two new methods for modifying recombinant-expressed protein that make it available for modification with a functionalized synthetic membrane anchor.

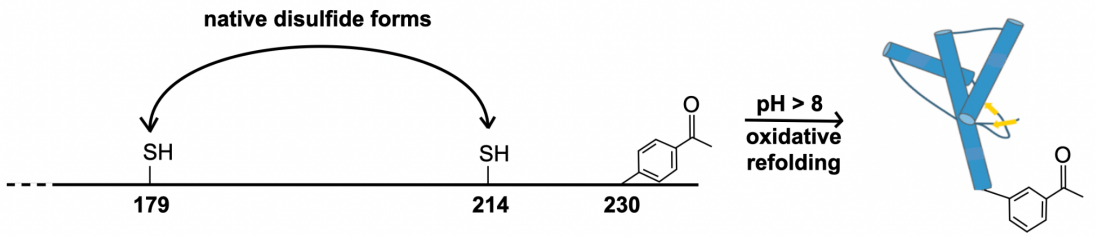


Figure 8: Scheme for the preparation of mPrP S230pAcPhe. The protein construct is purified using the standard protocol. The oxidative refolding step allows for the formation of both the native disulfide. The incorporation of pAcPhe does not interfere with the formation of the native disulfide and thus no additional purification steps are required.

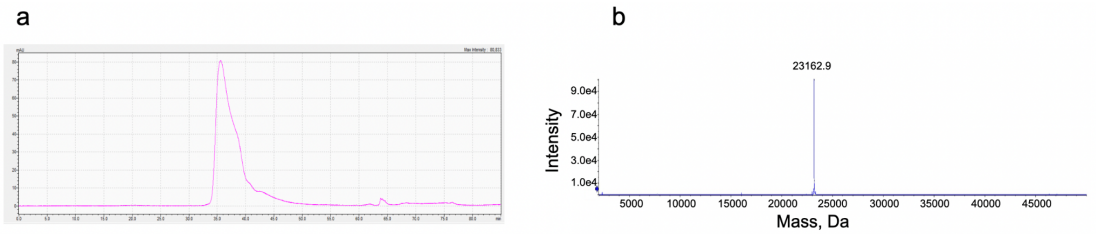


Figure 9: Protein purification and characterization for mPrP S230pAcPhe. **a)** HPLC trace for the protein at the final purification step suggests that there is a pure protein population. **b)** LC-MS analysis confirms that this is a pure sample prep and that the expected mass is observed.

Discussion

In this study we determine that there are several strategies to modify PrP^C to render it available for downstream bioconjugation. Our main consideration for mutating PrP^C was the functionality of the amino acid side chain, as we wanted to incorporate a residue that is amenable to covalent modification. We thus chose to exclusively mutate the protein with cysteine or pAcPhe given that both of these amino acids have functional side chains that can participate in covalent linkages. We also considered where we should incorporate the new amino acid in the protein sequence, while considering protein structure and biological representation. We reasoned that it was most appropriate to modify PrP^C at (or near) the final residue at the extreme C-terminus given that it is the position of the native GPI anchor and that it is an unstructured region.

We show successful incorporation of pAcPhe at the final residue using a modified expression system and for two proximal cysteines at the extreme C-terminus using an optimized purification protocol. Given that both protein preps had considerable and comparable yield, we considered other aspects to determine if one approach was more appropriate than the other. We first thought about the number of modifications present in each protein construct. Given that the ideal system would contain the minimum required modifications, we reasoned that mPrP S230pAcPhe may be preferred as it contains only a single mutation. Yet, we also recognized that pAcPhe is not naturally expressed in nature and is thus not a biologically-relevant residue. Meanwhile, mPrP 2 Cys incorporates natural amino acids but contains two mutations.

We also considered how compatible each protein construct would be with downstream applications that typically require protein labeling. Again, such labeling is typically done via incorporation of cysteine or pAcPhe into the protein sequence. However, due to the difficulty of purification with a single additional cysteine, we instead only consider the incorporation of pAcPhe as a viable option. We surmise that incorporation of pAcPhe and subsequent labeling will be feasible for the double cysteine mutant. We reason that we could also incorporate an additional pAcPhe into the protein construct that contains an existing pAcPhe residue. However, we believe that subsequent labeling would be tricky given that there would be two pAcPhe residues with separate labeling requirements.

We reason that each protein preparation has unique pros and cons and determine that both options should still be considered, given that the downstream modification with membrane anchor may present challenges.

Materials and Methods

Plasmids

PrP^C constructs were encoded in the pJ414 vector (DNA 2.0). Single point mutations were incorporated using primers designed for standard site-directed mutagenesis via PCR amplification. PCR product was transformed into *E. coli* (DH5-alpha(DH3) Invitrogen) cells and DNA was extracted (Qiagen mini prep kits) and subsequently sequenced to verify the PrP^C constructs of interest.

Standard Protein Expression and Purification for mPrP

PrP^C constructs were transformed into *E. coli* (BL21 (DE3) Invitrogen) cells. Cells were grown in 2 L of LB media containing ampicillin (100 ug/mL) at 37 C. Protein expression was induced when the cells reached an optical density (OD) of approximately 1.0 - 1.2 using 1 mM isopropyl-1-thio-D-galactopyranoside (IPTG). The cells continued to shake overnight at room temperature.

Cells were harvested and subsequently lysed by dissolving in lysis buffer and disrupting via tip sonicator. The lysis buffer contained 100 mM Tris HCl, 10 mM NaOAc, and 5 mM EDTA (pH 8) and the tip sonicator was operated at 40% power for 3 minutes with 15 seconds on and 15 seconds off. Lysed cells were centrifuged and the pellet was collected. Inclusion bodies were purified from the pellet by two washes in Wash Buffer A, followed by two washes in Wash Buffer B. Wash Buffer A components are 130.0 mM Tris/15.0 mM NaOAc, 7.0 mM EDTA, 5.0 mM DTT, 1.0 mM PMSF, pH 8. Wash Buffer B contains 100.0 mM Tris HCl/10.0 mM NaOAc.

Proteins were extracted from inclusion bodies at room temperature using extraction buffer (8 M guanidium chloride (GdnHCl), 100 mM Tris, 100 mM NaOAc (pH 8)). Extracted protein was purified via several successive chromatography steps. Extracted protein was first purified using Ni2+ immobilized metal-ion chromatography (IMAC), followed by oxidation of the native disulfide bridge. Oxidative refolding was performed overnight in denaturing conditions (5 M GdnHCl, 100 mM Tris/NaOAc) at pH 8. The oxidized protein was transferred into 50 mM KoAC (pH 4.5) using a desalt column. The protein was lastly purified via reverse-phase HPLC by loading onto a C4 column (Grace) using a gradient of water/acetonitrile with 0.1% trifluoroacetic acid. The purity and mass of purified protein were confirmed via LC-MS prior to lyophilization.

Modified Protein Expression for mPrP S230pAcPhe

To incorporate para-acetyl phenylalanine (pAcPhe) into *E. coli*-expressed protein, the codon at the site of pAcPhe incorporation was mutated to TAG using site-directed mutagenesis. The PrP^{C} construct and pEVOL-pAcPhe were co-transformed into *E. coli* (B-95.AAAfabR) cells. The cells were grown in 2 L of LB media supplemented with ampicillin (70 ug/mL) and chloramphenicol (70 ug/mL). Arabinose (0.02% w/v) and pAcPhe (~2 mM) were added when the cells reached an OD = 0.2. Protein expression was induced using IPTG (1 mM) when the cells reached an OD ~ 1.0 - 1.2. The cells continued to shake overnight at room temperature. Downstream protein purification followed the standard protocol previously outlined.

Modified Protein Purification for mPrP Containing Additional Cysteines

The protein expression and purification for such protein constructs were performed in accordance with standard protocol, followed by an additional purification step to selectively reduce the additional residue(s). Reduction reactions were performed using 100 uM protein and 500 uM TCEP in 5 mM KoAc (pH 4.5) for one hour at room temperature. Purification was conducted with a reverse-phase HPLC C4 column. Reduction of the cysteine was verified by reaction with N-ethylmaleimide (NEM) and analysis via LC-MS. Protein structure was investigated via circular dichroism (CD). Pure protein was lyophilized.

Chapter 4

Reaction between Prion Protein and Functionalized Membrane Anchor

Introduction

In the previous chapter we discuss methods to prepare recombinantly-expressed PrP^C that is amenable to bioconjugation. We determine that incorporation of two cysteines at the extreme C-terminus and introduction of an unnatural amino acid para-acetyl phenylalanine are both suitable protein modifications. The goal of this chapter is to test bioconjugation strategies that will covalently modify the protein constructs with a synthetic membrane anchor. We implement a different approach for each of the two protein constructs. Both approaches will utilize a membrane anchor composed of two palmitoyl moieties, glycerol and phosphate. The main difference lies in the reactivity of the functional group on the surface of the membrane anchor that couples to PrP^C.

The first membrane mimic that we designed incorporates two separate components that are covalently linked together using “click”-chemistry. The constituents are a dPEG linker for water solubility and two alkyl chains for insertion into the lipid bilayer. The dPEG linker contains an aminooxy functional group at one end that has specificity for ketones. For this, we prepared PrP^C with p-acetyl-phenylalanine at the final residue (mPrP S230pAcPhe), which is an unnatural amino acid that contains a ketone functional group for reaction coupling.

The other membrane mimic contains a maleimide functional group, which has specific reactivity towards cysteines. To react with this, we mutated two residues at the extreme C-terminus of PrP^C to cysteines (mPrP G227C S230C). Maleimide reactions can be run in aqueous solution between pH 6.5 to pH 7.5 [70]. We considered how pH, varying lipid and protein concentrations and different ratios of

lipid to protein influence the reaction efficiency. Additionally, we explore the maleimide-thiol reaction both at the liposome surface and prior to the addition of liposomes and evaluate the downstream purification for the strategies.

We find that mPrP G227C S230C can be modified with a maleimide-functionalized membrane anchor and thus represents a viable model for membrane-anchored protein. We reason that mPrP S230pAcPhe can also likely be modified with the proposed membrane components given a more comprehensive analysis. For the purposes of developing a new system for membrane-anchored protein, we focused on the success of membrane anchoring for mPrP G227C S230C.

Results

We first set out to covalently modify mPrP S230pAcPhe with a membrane anchor with “click”-chemistry via the functionality of the ketone-bearing unnatural amino acid at the final residue. We sought out to find a membrane mimic that was commercially available due to the ease of accessibility. Our search requirements included finding a molecule with a functional group that has specificity for ketones, a discrete polyethylene glycol (dPEG) unit for solubility and lipophilic tails that are representative of a biological membrane. While satisfying all such conditions was not feasible with any single commercially-available product, we instead developed an approach that incorporates two separate components that collectively represent a complete membrane anchor. The two constituents can be conjugated together using various “click”-chemistries and we therefore designed an approach to modify the protein that included two successive reactions.

The first membrane component is a dPEG linker that has aminooxy functionality at one terminus and an azide group at the other end. The aminooxy has strong specificity for ketones and serves to conjugate the molecule to the protein via the functionality of the unnatural amino acid. Given that the azide can participate in alkyne-azide “click” chemistry, we selected lipid chains that contained an alkyne group at the surface to covalently link the various components together. Our strategy was to first modify the protein with the aminooxy-functionalized dPEG and then subsequently react the pegylated protein to the lipid tails (Figure 1).

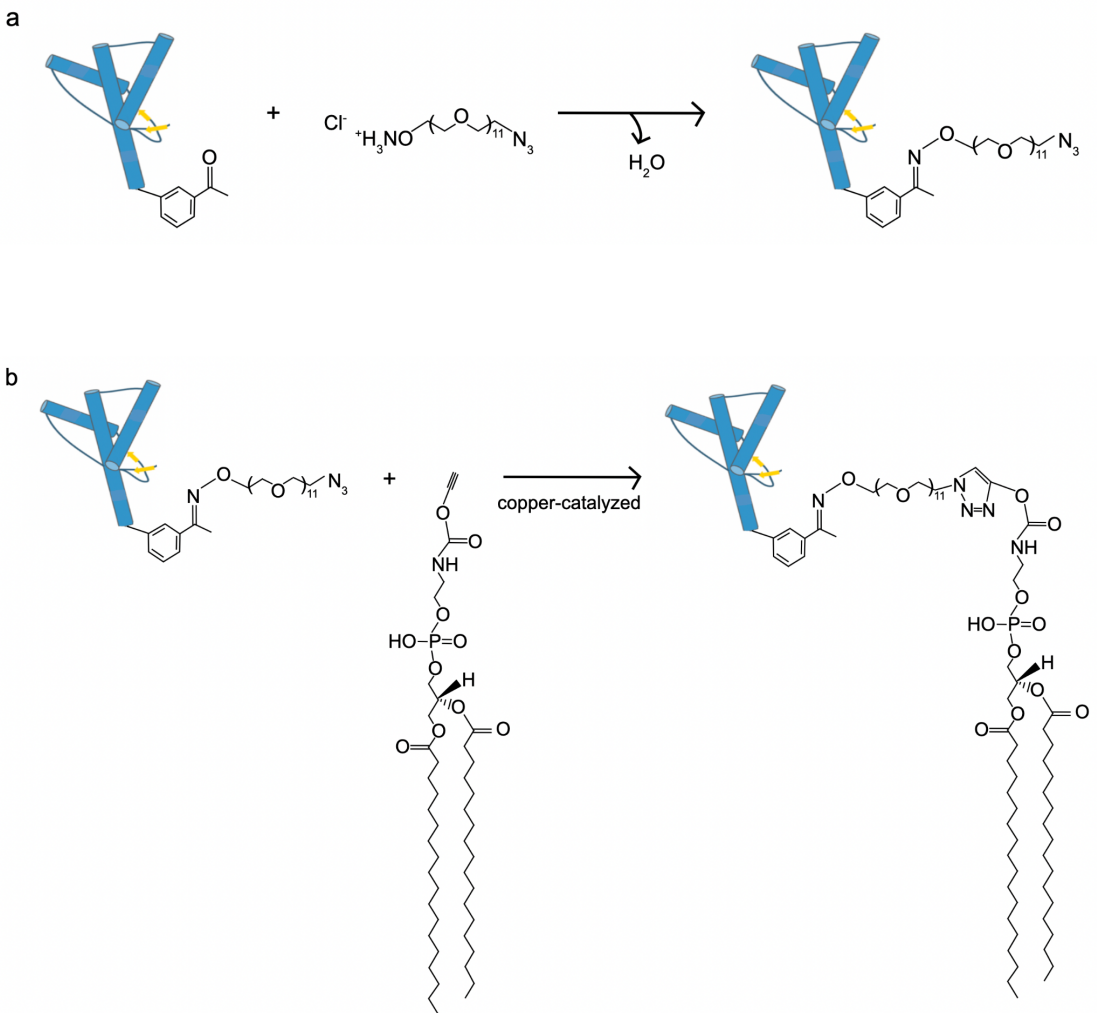


Figure 1: Scheme for the modification of mPrP S230pAcPhe. **a)** The protein reacts with aminooxy-dPEG$_{11}$-azide. HCl in 100 mM potassium acetate buffer pH 4.5. The bioconjugation proceeds forward and results in the loss of a water molecule. **b)** The azide functional group on mPrP S230pAcPhe reacts with the exposed alkyne at the surface of the lipid tails via copper-catalyzed chemistry. The two-step reaction results in membrane-anchored mPrP S230pAcPhe.

We performed an overnight reaction between mPrP S230pAcPhe and the aminooxy-functionalized dPEG in de-gassed potassium acetate buffer at pH 4.5 (Figure 2). We monitored the reaction completion via LC-MS and purified the reaction via HPLC. The final product had a mass of 23733.68 Da (as expected) and the final protein yield was 32.0%. Despite our initial concern about the protein yield, we recognized that purification via chromatography typically results in protein loss and that our actual reaction efficiency was likely higher.

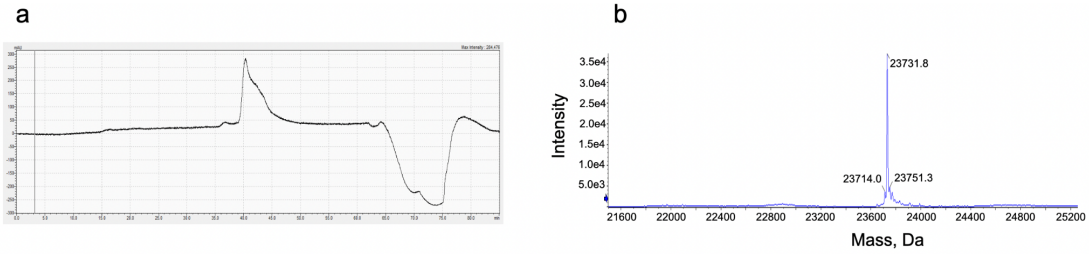


Figure 2: Protein purification and characterization for mPrP S230pAcPhe reacted with aminooxy-functionalized $dPEG_{11}$. **a)** HPLC trace for the reacted protein suggests that there is a pure population of reacted protein. **b)** LC-MS analysis confirms that this is a pure sample and that the expected mass for reacted protein is observed.

We proceeded to then react the pegylated protein with the lipid tails via alkyne-azide “click”-chemistry. Such reactions are typically run using a copper-catalyst and thus require the addition of copper, a ligand, and a reducing agent. We were optimistic that the reaction would be simple and robust given the nature of “click”-chemistry reactions. However, we were disappointed to observe the different solubility requirements for constituents in the reaction that thus hindered the success of the reaction.

We also considered the possibility of first reacting the two constituents of the membrane anchor prior to the addition of protein, with the hope that solubility would be improved. Our rationale was that by reacting in the absence of protein that the solubility requirements would be more feasible to satisfy. However, the removal of protein from reaction conditions did not simplify the complex solubility requirements. We still struggled to get the reaction to proceed and did not observe any product formation. At this point, we determined that it was most appropriate to try other approaches for anchoring the protein to a synthetic membrane anchor. We hypothesized that if we could instead apply a similar approach that did not require the addition of a catalyst, then solubility would not be a concern since there would be fewer constituents.

We were interested in modifying mPrP G227C S230C via bioconjugation with a synthetic membrane anchor. The membrane anchor that we selected for this modification contains two alkyl chains, a dPEG linker, and a maleimide functional group at the surface that has strong specificity for cysteines (Figure 3). We expected that the maleimide-functionalized membrane anchor would modify the protein at two sites because the protein has two solvent-exposed cysteines. Even though the protein only expresses one anchor in its native environment, we reasoned that modification with two anchors would be acceptable because all protein modification would occur at the extreme C-terminus and likely wouldn't interfere with structure or function.

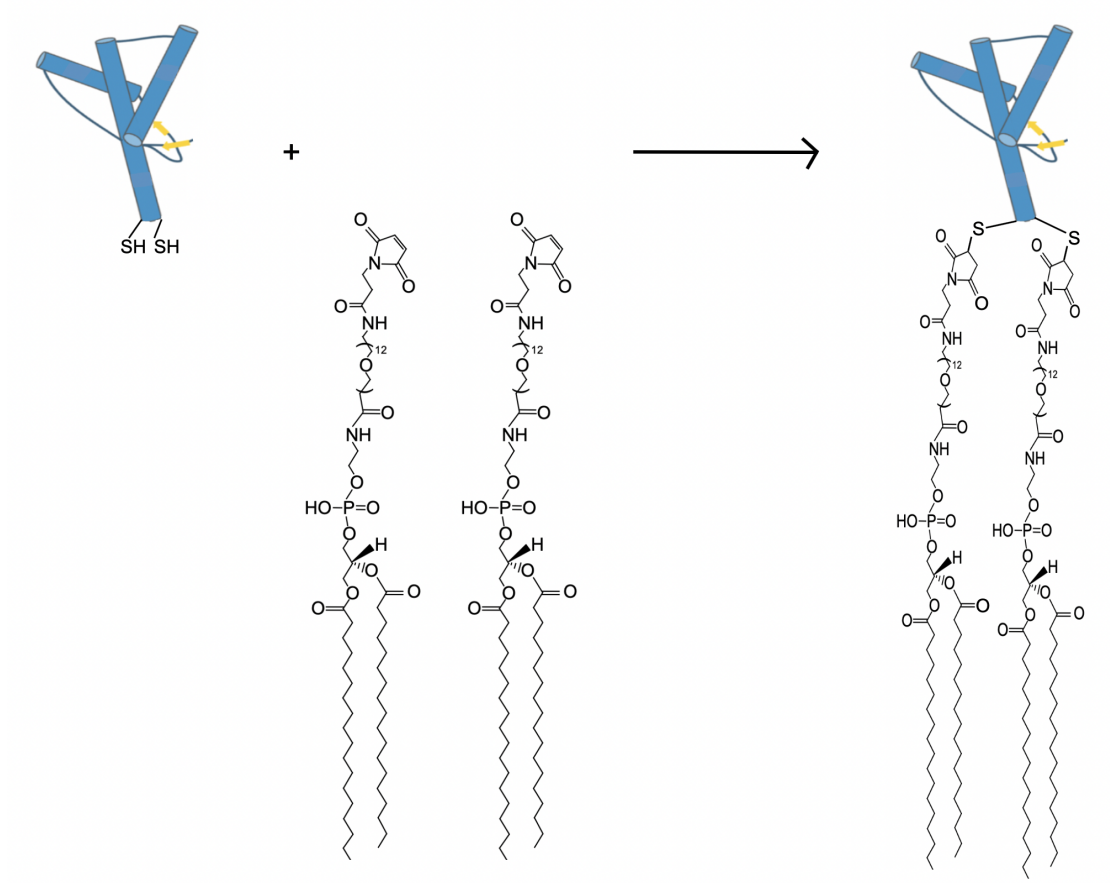


Figure 3: Scheme for the modification of mPrP G227C S230C. The two exposed cysteines react with the maleimide-functional group at the surface of the lipid tails. The bioconjugation proceeds forward and results in the formation of membrane-anchored protein. Note that the bond

Maleimide reactions are a type of “click”-chemistry that do not require the addition of a catalyst. Additionally, the maleimide moiety displays strong specificity for cysteines between pH 6.5 to pH 7.5 [70]. Given that each protein molecule contained two cysteines, we decided to run the reaction with an excess of maleimide to allow for complete labeling. Briefly, we ran the maleimide reaction in MOPS buffer with the addition of guanidine to aid solubility and observed successful product formation (Figure 4).

We were curious about how to reconstitute the membrane-anchored protein into liposomes. We reasoned that we could either react the protein at the surface of maleimide-functionalized liposomes or react the protein with the functionalized membrane anchor prior to the addition of liposomes. The motivation for trying both approaches was to consider any differences in the ease of purification and the final protein concentration on the liposome surface.

We first attempted to perform the maleimide-thiol reaction at the liposome surface. To do this, we prepared POPC liposomes that contained one molar percent of maleimide-functionalized lipid. We determined that this amount of maleimide-functionalized lipid was appropriate for anchoring protein molecules at the liposome surface without causing aggregation. However, the requirement for excess maleimide to sufficiently label the protein presents a limitation for how much protein can be incorporated on the liposome surface. Additionally, since reaction efficiency is never perfect, we were concerned about achieving a sufficient protein concentration for biophysical assays that typically require a few hundred micromolar of protein. At a minimum, attempting this method would guide our understanding of maleimide-thiol reactions in a membrane environment and the system would at least be amenable to select assays.

Despite these concerns, we were inclined to try this approach because only small amounts of protein are required. We tested a variety of reaction conditions that considered varying concentrations of protein and lipid, different ratios of protein to active lipid, and varying buffer solutions. We ran our initial test reaction at pH 7.4 and observed product formation via SDS-PAGE and LC-MS. Even though we were enthusiastic about the success of this pilot reaction, we believed that we could

improve the reaction efficiency. We first considered the effect of pH on reaction success. While maleimide-thiol reactions can be run in a pH range from pH 6.5 to pH 7.5, the reaction displays the strongest specificity for thiols at lower pH. We thus reasoned that it may be more appropriate to run the reaction at a lower pH, even though we initially selected pH 7.4 given that it is the most physiologically-relevant pH.

We ran a variety of test reactions at pH 6.5 that contained varying concentrations of protein and lipid and different ratios of protein to active lipid. We collected time points for each reaction condition at 15 minutes, 1 hour, 2 hours and overnight. The maleimide-thiol is quite robust and the reaction reached completion by 15 minutes, as demonstrated by the comparison between the 15 minute and 1 hour time points shown in Figure 5. While the qualitative analysis via SDS-PAGE does not accurately measure reaction efficiency, it provides a crude approach for determining optimal reaction conditions. Analysis via SDS-PAGE suggested that reaction efficiency was optimized for various conditions, with the greatest improvement observed for 5 mg/mL POPC and a 25:1 active lipid-to-protein ratio.

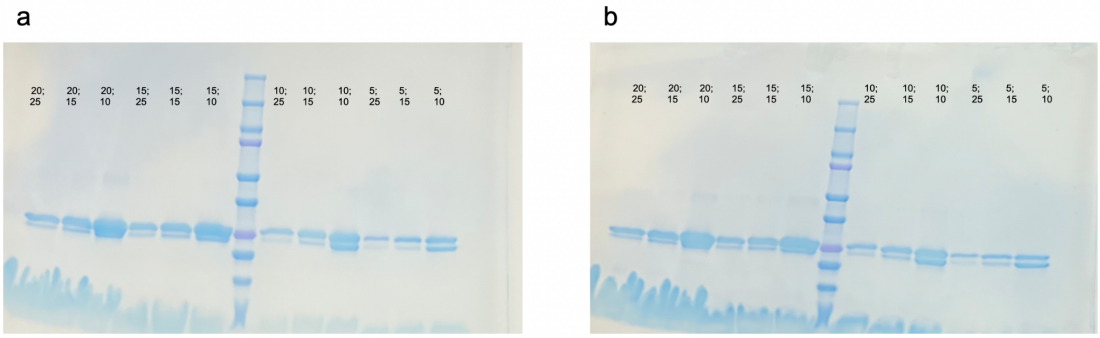


Figure 4: SDS-PAGE analysis for mPrP G227C S230C reacted with maleimide-lipid at the liposome surface. Reaction after **a)** 15 minutes and **b)** 1 hour. Each lane represents different concentrations of POPC (top label) and ratios of mal:PrP^C (bottom label).

We considered a few ideas for removing any unreacted protein from the proteoliposome solution. Ultracentrifugation is commonly applied for this purpose, in which unreacted protein is found in the supernatant and reacted protein forms a pellet with the liposomes. We tried this method and achieved proper separation; however, we were concerned about the integrity of the liposomes due to the high g-force applied and the tight packing in the pellet. We verified that the average size and dispersity of the liposomes were maintained post-ultracentrifugation by DLS. Ideally, we would have also investigated the liposome morphology and integrity using CryoEM.

We reasoned that purification via a gravity column may represent a “softer” separation technique. In this approach, the solution of proteoliposomes is loaded onto the column and reacted protein elutes first followed by later elution of unreacted protein. We first loaded “empty” liposomes onto the column as samples tend to stick to the resin, which decreases final yield. We then loaded our sample of interest and collected each fraction separately in wells on a 96-well plate. We aimed to detect the fractions that contained unreacted or reacted protein via the Bradford assay, but we unfortunately were not able to identify any protein. We speculated that either our sample still stuck to the resin despite pre-loading “empty” liposomes or that the eluted sample was so dilute that the Bradford assay was not a sensitive enough detection assay. We determined that mass spectrometry could represent a more sensitive characterization of eluted fractions, but loading and analyzing that many fractions would be a tedious process.

We also attempted to react the maleimide-functionalized lipid with the available cysteines on the protein prior to the addition of preformed liposomes. In this

approach, we would purify the modified protein prior to incorporation into the liposomes and thus avoid any downstream purification in the presence of liposomes. We ran the maleimide-thiol reaction in MOPS buffer at pH 7.4 and purified via HPLC, followed by characterization by LC-MS (Figure 8). The area under the curve on the HPLC trace can be analyzed to determine the relative reaction efficiency. We plan to test additional reaction conditions to determine if there are more optimal reaction parameters. We are especially interested in analyzing reaction efficiency at pH 6.5 given the stronger specificity for thiols at lower pH. Once ideal reaction conditions are identified, then reacted protein will be incubated with pre-formed liposomes at 37 C for a minimum one hour to allow for membrane-anchored protein to incorporate into the membrane. The advantage of this approach is that no downstream purification is required since the unreacted protein has already been removed.

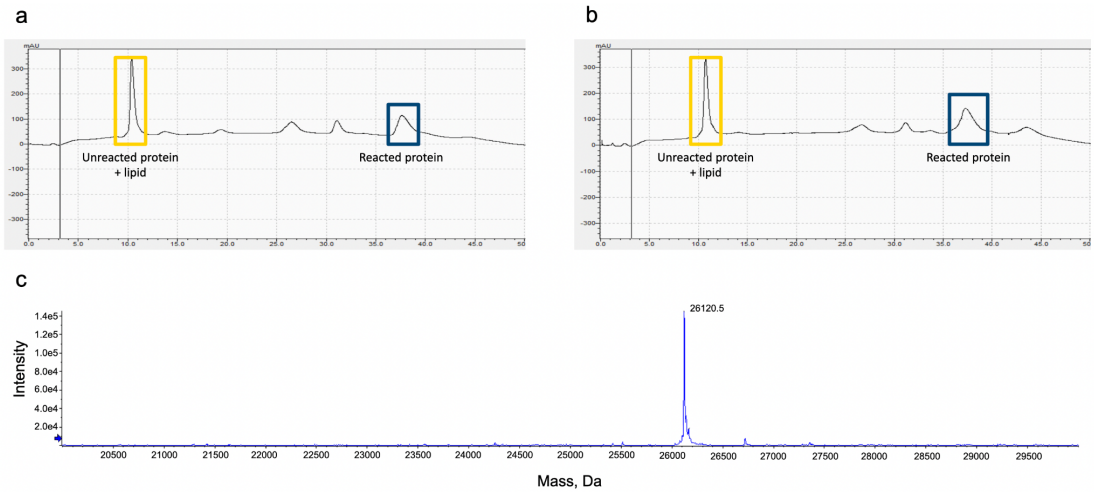


Figure 5: Protein purification and characterization for mPrP G227C S230C reacted with maleimide-functionalized lipid prior to the addition of liposomes. **a)** HPLC trace for the reacted protein after reaction for **a)** 1 hour and **b)** overnight. The traces suggest that the reaction reaches maximum efficiency after 1 hour and that there is a pure population of reacted protein. **b)** LC-MS analysis confirms that this is a pure sample and that the expected mass for reacted protein is observed.

Discussion

In this study we present several strategies to attach a synthetic membrane anchor to PrP^C. We explored protein modification using two different membrane anchors that are both representative of the native GPI anchor. Consistent with the GPI anchor, both membrane mimics contain fatty acid tails, phosphate and glycerol. The GPI anchor contains a conserved core sugar backbone that is not present in our membrane mimics. Instead of a sugar backbone, we incorporated a PEG linker for the core of our representative membrane anchors. We also opted to use a class of commercially-available discrete PEG (dPEG) linkers that contain a homogenous population of single molecular weight, versus the dispersed populations characteristic of traditional PEGs. Despite any differences in the core composition, the main goal of anchoring the protein to the surface was achieved.

We demonstrate that mPrP S230pAcPhe can be modified with a dPEG linker that has aminooxy functionality. However, subsequent modification with lipid tails via alkyne-azide chemistry was challenging and we did not obtain lipidated protein. Given that the scope of this work requires a strong chemistry background and that our skillset is mostly biophysical, we admittedly attribute part of this failure to an inadequate chemistry foundation. We believe that a deeper level of troubleshooting and analysis, perhaps in collaboration with chemistry-based labs, would yield a successful reaction.

One consideration would be to use an alternative alkyne-azide chemistry approach that does not require the use of a copper catalyst and thus alleviates the diverse solubility requirements. The strain-promoted alkyne-azide cycloaddition (SPAAC) requires a strained cyclooctyne, which is not a component of the current

alkyne-functionalized lipid. We could consider the feasibility of chemically modifying the alkyne-lipid that would allow for incorporation of the strained cyclooctyne. Alternatively, we could consider synthesizing our own functionalized membrane anchor that incorporates all necessary components to modify the protein in one single step. We believe that we have established a framework for the preparation of membrane-anchored PrP^C and that future modification would allow this system to serve as a model of lipidated PrP^C.

We also present the modification of mPrP 2 Cys with a membrane anchor that has maleimide-functionality. Given that the construct contains two cysteines that are available for reaction with the maleimide-lipid, we observed that protein was labeled with two membrane anchors. While it is not biologically-representative for PrP^C to be labeled with two membrane anchors, we recognize that this is simply a small caveat of our system. Our main goals were to position the cysteines near the extreme C-terminus since that is where the native GPI anchor is located and to ensure that such modification would not interfere with protein structure. We imagine that this system can be applied to study membrane-anchored PrP^C and that it can be extended to the study of other GPI-anchored proteins. We encourage others to modify reaction parameters as needed to determine conditions that are best suited to their specific system. We would like to note that maleimide reactions can be run in the presence of TCEP. We tried to run our reaction with the addition of TCEP but noticed sample crash out. Since we had previously reduced the cysteines prior to reaction, we opted to run the reaction without the addition of TCEP and still observed adequate reaction efficiency.

For the scope of this work we determined reaction efficiency by qualitative analysis via SDS-PAGE. We initially tried to quantitate reacted protein using the NanoDrop at an absorbance of 280 nm which is our standard approach for determining protein concentration, but observed excess scattering. Given that we were primarily interested in the development of a system for membrane-anchored protein, we were satisfied with a qualitative approach with SDS-PAGE. However, downstream application would require a more direct quantitative method. While there are quantitative assays available, we did not test them.

Materials and Methods

Reaction Between mPrP S230pAcPhe and Membrane Components

To couple the dPEG linker to the protein, 250 uM of mPrP S230pAcPhe and 10 mM aminooxy-dPEG$_{11}$-azide.HCl (Quanta BioDesign) were added to 100 mM KoAC (pH 4.5). The sample was degassed and the reaction proceeded overnight at 37 C. Reaction progress and completion were monitored via LC-MS and purification was performed on a reverse-phase HPLC C4 column. The azide-functionalized protein was available for reaction with DSPE-Alkyne (Nanosoft Polymers) via alkyne-azide chemistry. Briefly, 100 uM azide-functionalized protein, 50 uM DSPE-Alkyne, 100 uM Copper (II) Sulfate, 500 uM THPTA, and 5 mM Sodium Ascorbate were dissolved in solvent. Different solvent conditions were tested, including both aqueous and organic solvents (more details in Results section).

Reaction between mPrP G227C S230C and MAL-dPEG$_{12}$-DSPE at the Liposome Surface

Liposomes containing one molar percent MAL-dPEG$_{12}$-DSPE were prepared using the standard liposome preparation protocol. Maleimide-functionalized liposomes were incubated (in excess) to mPrP G227C S230C at room temperature. Various reaction conditions were tested, including different lipid:protein ratios and buffers (more details in the Results section). Removal of unreacted protein was achieved via ultracentrifugation with a TLA100 rotor at 77 K rpm for 30 minutes at 4 C. Unreacted protein was in the supernatant, whereas reacted protein pelleted with liposomes.

Reaction between mPrP G227C S230C and MAL-dPEG$_{12}$-DSPE prior to the Liposome Surface

MAL-dPEG$_{12}$-DSPE was incubated in excess of mPrP G227C S230C in aqueous buffer at room temperature. Briefly, the reaction was done by dissolving 60 uM protein and 300 uM mal-dPEG DSPE in 500 uL of 6 M Gdn HCl and 50 mM MOPS pH 7.4. Purification was performed via reverse-phase HPLC on an analytical C4 column (Grace Vydac 214MSC4 150 X 4.6 mm), followed by lyophilization.

Chapter 5

Conclusions

Conclusions

In this dissertation we develop a simple representative membrane system and design strategies for modifying recombinantly-expressed PrP^C with a synthetic membrane anchor.

We determine that liposomes are an ideal membrane system given their similar morphology to the native membrane, their ability to incorporate peripheral membrane proteins and their amenability to various biophysical assays. Liposomes have been assembled for a variety of applications and in this work we develop a liposome preparation protocol that yields reproducible samples for our purposes.

We next consider how we can modify PrP^C so that it is amenable to reaction with a functionalized membrane anchor. We explore four different protein modification schemes and determine that two of them are most feasible. Mutation of two residues near the extreme C-terminus to cysteines allows for protein purification with an additional purification step that selectively reduces the extra cysteines. The modification does not disrupt formation of the native disulfide bridge or overall protein structure. A single mutation of the final residue to the unnatural amino acid para-acetyl phenylalanine requires an adapted expression scheme but allows for purification using the standard purification protocol.

We perform the reaction between PrP^C and synthetic membrane anchors and the reconstitution into liposomes. We demonstrate that mPrP S230pAcPhe can be modified with a dPEG linker that has aminooxy functionality. However, subsequent modification with lipid tails via alkyne-azide chemistry was challenging and we did not obtain lipidated protein. We also present the modification of mPrP 2 Cys with a membrane anchor that has maleimide-functionality. Given that the construct contains

two cysteines that are available for reaction with the maleimide-lipid, we observed that protein was labeled with two membrane anchors. We also test the maleimide-thiol reaction both at the liposome surface and prior to the addition of preformed liposomes.

The development of this system enables us to investigate PrP^C in the context of a membrane environment using biophysical approaches. Questions that can be addressed include how does wild-type and biologically-relevant mutations influence membrane disruption and how is copper coordination influenced at the membrane surface under varying conditions. Additionally, does protein structure for wild-type and protein variants change at the membrane surface? Thus far, we have preliminary results (not shown) that demonstrate the suitability of this system with biochemical assays and biophysical techniques. We set up a fluorescent calcein release assay that could investigate if variants of mPrP destabilize the membrane. We explored the copper coordination environment for unanchored mPrP in a membrane environment using Continuous-Wave (CW) Electron Paramagnetic Resonance (EPR). We also determined if we could probe this system by solution Nuclear Magnetic Resonance (NMR). We ran a sample of unanchored mPrP in the presence of POPC liposomes and observed cross peaks. Additional controls remain to be done for the above experiments.

The system that we developed has the potential to provide pertinent information about structure and dynamics in a membrane environment. The addition of binding partners, such as divalent metals and other proteins, that PrP^C has strong affinity for will expand our understanding of the diverse activities and functions for PrP^C at the membrane surface. Given that toxicity occurs at the membrane surface,

such information will help identify interactions to target in the progression of prion diseases and other neurodegenerative diseases with similar pathology.

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