HIV-1 Vpu Oligomerization in Membrane Mimetics

by

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Abstract

HIV-1 is a pandemic virus that has a continuing impact on human society. As treatments for HIV-1 infection improve, new targets for viral inhibition have provided a means to reduce a once-lethal infection into a manageable chronic condition. Among other proteins, HIV-1 encodes the Vpu accessory protein which upregulates the process of viral release from infected cells, providing an enticing target for future anti-HIV-1 therapies. In this thesis, I outline a conserved AxxAxxxAxxxA motif responsible for the assembly of Vpu into its functional oligomeric form, probe the importance of conserved residues in the motif via mutagenesis, and assess the relative stability of each resulting Vpu complex. Utilizing dynamic light scattering experiments, I characterized the molecular size of the Vpu oligomer in sodium dodecyl sulfate (SDS) solutions. To examine the Vpu oligomeric complex in greater detail, I employed the use of explicit all-atom simulations of the Vpu transmembrane domain oligomer in a hydrated lipid bilayer, verified the importance of the conserved AxxAxxxAxxxA motif, and characterized the stability of several Vpu oligomeric arrangements both in the presence and absence of water channels. Finally, I utilized NMR techniques to probe the Vpu assembly in SDS, dodecyl phosphocholine, and lipid membrane mimetics, providing a comparison between these three environments, and propose further studies based on the results of solution state NMR and solid-state magic angle spinning
NMR experiments. The findings of this work are interpreted in the context of Vpu oligomerization relative to previous molecular dynamics simulations, the effect of membrane mimetic environment, and implications for Vpu interactions with host cell target proteins.
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Abbreviations

AcN – acetonitrile
AIDS – acquired immune deficiency syndrome
ART – anti-retroviral therapy
AUC – analytical ultracentrifugation
β-TrCP – beta-transducin repeat containing E3 ubiquitin protein ligase
BN PAGE – blue-native polyacrylamide gel electrophoresis
BST-2 – bone marrow stromal cell antigen 2
C14SB – 3-(N,N-dimethylmyristylammonio)-propanesulfonate
C8E5 – pentaethylene glycol monoctyl ether
CCR5 – C-C motif chemokine receptor 5
CD – circular dichroism
CD4 – cluster of differentiation 4
CHAPSO – 3-((3-cholamidopropyl)dimethylammonio)-2-hydroxy-1-propanesulfonate
CHARMM – chemistry at harvard macromolecular mechanics
CHI – CNS searching for helical interactions
CNS – crystallography and NMR suite
CXCR4 – C-X-C motif chemokine receptor 4
DARR – Dipolar-assisted rotational resonance
DCM – dichloromethane
DDM – n-Dodecyl-β-D-maltopyranoside
DDPC – 1,2-dimyristoylaminoo-1,2-deoxy-phosphatidylcholine
DHPC – 1,2-diheptanoyl-sn-glycero-3-phosphocholine
DLPC – 1,2-dilauroyl-sn-glycero-3-phosphocholine
DLS – Dynamic light scattering
DMA – 5-(N,N-dimethyl)amiloride
DMPC – 1,2-dimyristoyl-sn-glycero-3-phosphocholine
DPC – n-dodecylphosphocholine
DPPC – 1,2-dipalmitoyl-sn-glycero-3-phosphocholine
DSPC – 1,2-distearoyl-sn-glycero-3-phosphocholine
DTPC – 1,2-ditridecanoyl-sn-glycero-3-phosphocholine
ER – endoplasmic reticulum
FTIR – Fourier transform infrared spectroscopy
GPCR – G-protein-coupled receptor
GPI – glycosylphosphatidylinositol
HAART – highly active anti-retroviral therapy
HCV – Hepatitis C virus
HFIP – hexafluoro-2-propanol
HIV – human immunodeficiency virus
HMA – 5-(N,N-hexamethylene)amiloride
HPLC – high-performance liquid chromatography
HSQC – heteronuclear single-quantum correlation
IAV – Influenza A virus
IMB-LA – lapachol
INEPT – Insensitive nuclei enhanced by polarization transfer
IPTG – isopropyl β-D-1-thiogalactopyranoside
KcsA – pH-gated potassium channel
KSI – ketosteroid isomerase
LB – lysogeny broth
LCP – lipidic cubic phase
MALDI-TOF – matrix-assisted laser desorption/ionization time of flight
MALTS – multi-angle light scattering
MAS – magic angle spinning
MCP – methyl-accepting chemotaxis protein
MD – molecular dynamics
MHC-I – major histocompatibility complex class 1
MPRAP – membrane protein residue accessibility predictor
MSP – membrane scaffold protein
NAMD – not (just) another molecular dynamics program
NIH – national institutes of health
NMR – nuclear magnetic resonance
NTB-A – NK-, T-, and B-cell antigen
OG – n-octyl-β-D-glucoside
PAGE – polyacrylamide gel electrophoresis
PC – phosphatidylcholine
PE – phosphatidylethanolamine
PFG – pulsed-field gradient
PG – phosphatidylglycerol
PIV5 – parainfluenza virus 5
PME – particle-mesh Ewald
POPC – 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
POPE – 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine
POPG – 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylglycerol
PPM – parts per million
PS – phosphatidylinerine
PSF – protein structure file
RAD – RF-assisted diffusion
Rh – hydrodynamic radius
RMSD – root mean squared deviation
RMSF – root-mean squared fluctuations
SARS – severe acute respiratory syndrome
SDS – sodium dodecyl sulfate
SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC – size-exclusion chromatography
SIVcpz – Simian immunodeficiency virus, Chimpanzee
SMA – styrene/maleic acid copolymer
SMALPs – SMA-lipid particles
SMR – small multidrug resistance protein
SPC5 – super cycled post-C5
SPR – surface plasmon resonance
TASK-1 – tandem-pore acid-sensing potassium channel 1
TFA – trifluoroacetic acid
TFE – 2,2,2-Trifluoroethanol
TPPM – two-pulse, phase-modulated
TROSY – transverse-relaxation optimized spectroscopy


1 Introduction

1.1 Introduction overview

Human immunodeficiency virus (HIV) infection is a pandemic that continues to have a significant impact on human society. Throughout the past twenty years, improvements in treatment and management have meant that the expected lifetime for a North American person diagnosed with HIV has improved from 10.5 years in 1996 to between 22 and 31 years in 2000-2005 (Harrison et al. 2010; Samji et al. 2013). The improvement is largely attributed to the advent and use of highly active anti-retroviral therapy (HAART), which is typically a cocktail of antiretroviral drugs (Palella et al. 1998). Between 2000 and 2007, HAART efficacy improved dramatically as the results of newly discovered therapeutics were combined with early detection, and consequently expected lifespans have increased dramatically from 31 to 51 years post detection of HIV-1 infection (Samji et al. 2013; Farnham et al. 2013). However, while treatment regimes are delivering better results and longer lifetimes, there is opportunity for improvement in patient quality of life and in cost of treatment, particularly at a lifetime cost for HIV treatment between $253 000 and $402 000 (Farnham et al. 2013).

Current anti-HIV therapeutic treatments target the HIV viral fusion process, as well as HIV protease, reverse transcriptase, and integrase proteins, each of which play separate roles in the HIV lifecycle (De Clercq 2004; De Clercq 2005; De Clercq 2007; Greene et al. 2008; Torbett et al. 2015). However, the HIV-1 encoded integral membrane protein Vpu, an accessory protein that plays a key role in the budding of infectious viral particles from human CD4+ T cells, has not yet been targeted by anti-HIV-1 interventions (Strebel et al. 1988; Terwilliger et al. 1989; Klimkait et al. 1990). Moreover, some small molecules have been identified which inhibit the function of Vpu in vitro, but it is not currently known how the inhibitors function (D. Ewart 2002; Premkumar et al. 2004; Khoury et al. 2010; Sauter et al. 2014; Mi et al. 2015; Zhang et al. 2016). Thus, Vpu is a potentially interesting target for further biochemical study aimed at developing new drug interventions against HIV-1 infection.

1.2 Introduction to membrane proteins

Cells are bounded by membranes largely composed of a phospholipid bilayer; a hydrophobic barrier that also surrounds discrete organelles inside eukaryotic cells to compartmentalize
cellular functions and regulate cellular processes. Membranes are dynamic and complex systems modulated largely by proteins that can be transiently membrane-associated, anchored through covalent lipid anchors, or membrane-embedded via long stretches of residues spanning the membrane. Membrane proteins perform important roles in signal transduction, ion conductance, external sensing, and defining cellular scaffolding and morphology. While only an estimated 23% to 27% of the human proteome consists of membrane proteins (Uhlén et al. 2015; Almén et al. 2009), these proteins represent an estimated 60% of all drug targets (Overington et al. 2006) and as such understanding their function will enable new generations of small-molecule therapeutics.

Integral membrane proteins are anchored through membrane spanning segments, typically composed of long stretches of hydrophobic amino acids. While many soluble proteins contain hydrophobic residues, long stretches of hydrophobicity are required in some membrane proteins in order to provide a stable interaction with a largely organic membrane and are not easily solubilized in an aqueous phase. However, like their soluble counterparts, membrane protein structures are largely α-helical or β-sheet in character. In general, α-helical transmembrane proteins are found in eukaryotic organisms and inner membranes of mitochondria and bacteria, while β-strand membrane proteins are found in the outer membranes of mitochondria and bacteria (Wimley 2003) (Figure 1-1).

In either case, formation of these characteristic protein secondary structures is necessary to satisfy the hydrogen-bonding requirements of the polypeptide backbone. The low dielectric constant within the lipid bilayer does not provide polar groups to facilitate hydrogen-bonding interactions, and so they must be satisfied internally by the peptide, limiting the number of possible secondary structural elements found in transmembrane proteins and making random coil arrangements energetically unfavourable within the lipid bilayer (White & Wimley 1999). Protein folding, normally considered a fast and often a spontaneous phenomenon in aqueous proteins, can be challenging in membrane protein systems.
1.2.1  Folding of transmembrane segments

1.2.1.1  Arrangement of α-helical transmembrane segments

α-helices are the most common protein secondary structural element, and transmembrane α-helices can be predicted in a straightforward manner by analysis of hydrophobicity in sequential residues (Kyte & Doolittle 1982), or in greater detail by more modern tools such as TM finder (Deber et al. 2001) or membrane protein residue accessibility predictor (MPRAP) (Illergård et al. 2010). In the α-helical arrangement, the peptide backbone is coiled such that the peptide carbonyl C = O in position $i$ is hydrogen bonded to the amide proton of its $i+4$ counterpart, resulting in a right-handed helix with internally satisfied backbone hydrogen bonds, no empty space inside of the helix, and the sidechain of each residue facing outwards from the helix towards the solvent. α-helices have a screw pitch of 3.7 residues per turn, or a rise of approximately 1.50 Å per residue, therefore requiring approximately 20 to 30 residues to traverse the 30 Å lipid bilayer, depending on helical tilt within the membrane (Hildebrand et al. 2004). The side chains of transmembrane α-helices are largely hydrophobic; particularly enriched in Leu residues (Ulmschneider et al. 2005), and often feature either aromatic or charged residues at interfaces with the lipid headgroups followed by polar residues at locations where the transmembrane helix continues beyond the lipid bilayer into the aqueous milieu (Killian & von Heijne 2000). Frequently, there are conserved sequence motifs in transmembrane α-helices which are known to form dimer or higher-order oligomeric functional assemblies, resulting in a
two-stage folding model that has been described for the formation of functional membrane protein oligomeric complexes (Popot & Engelman 1990).

1.2.1.2 Arrangement of β-barrel transmembrane proteins

As for α-helical membrane proteins, β-barrel integral membrane proteins must also internally satisfy peptide backbone hydrogen bonding requirements in order to insert stably into the lipid bilayer. This is accomplished by the formation of β-sheet structure capable of hydrogen bonding backbone residues between adjacent strands to form the sheet, with the first strand in the sheet hydrogen bonded to the last, curling the sheet into a cylinder (Wimley 2003). The residues composing the sheet will typically have hydrophobic side chain groups at every other position, generating a hydrophobic external face which can be easily embedded into the lipid bilayer. β-barrel proteins form relatively stable structures due to rigid hydrogen bonding patterns, often featuring polar residues on the interior face of the pore to accommodate the transport of polar molecules through an otherwise hydrophobic lipid bilayer barrier, and often featuring Trp and Tyr residues at membrane-proximal regions of the protein (Tamm et al. 2004). Because of their extended backbone conformation, β-barrel proteins generally require strand lengths of 9 to 11 residues to span the lipid bilayer, accompanied by tight turns outside of the lipid membrane to form adjacent antiparallel strands, often at angles of 20° to 40° from the lipid membrane normal (Wimley 2003; Tamm et al. 2004).

1.3 Membrane protein interactions / oligomerization

1.3.1 Membrane protein folding

A two-step membrane protein folding model has been proposed to explain how multi-span proteins assemble, or how higher order membrane protein complexes can be formed within the lipid bilayer (Popot & Engelman 1990). The two-step membrane protein folding model was suggested after observing the spontaneous formation of bacteriorhodopsin complexes capable of binding chromophores from the 7 individual transmembrane helices after fusing together lipid vesicles containing each component (Popot & Engelman 1990). Since these initial observations, the two state model for transmembrane helix folding has been utilized, refined, and extended to explain transmembrane protein folding and spontaneous association into higher-order complexes (Popot & Engelman 2000; Engelman et al. 2003; White & Wimley 1999; Bowie 2005).
The first step of the folding process involves the formation of predominantly hydrophobic transmembrane helices across the lipid bilayer (Popot & Engelman 1990; Popot & Engelman 2000). In the second step of folding, the lipid-solubilized, transmembrane α-helices diffuse laterally in the membrane and interact with each other to form higher-order complexes (Figure 1-2).

Figure 1-2: Two-step α-helical membrane protein folding model. In step 1 (left arrow) the helical segment is inserted into the membrane bilayer. In step 2 (centre arrow), the protein can laterally diffuse within the membrane to interact with another helix.

In greater detail, peptide segments with sufficient hydrophobicity will spontaneously insert into lipid membranes and fold into an α-helical structure if they are of sufficient length to span the membrane (Liu et al. 1998). Helices as short as 14 residues may span a thin membrane featuring disordered acyl chain packing, or helices as long as 39 residues may span a thick membrane brought about by acyl chain ordering, in conjunction with helix tilt away from the bilayer normal (Killian & Nyholm 2006). An α-helical stretch of 26 Ala residues is roughly average.

In vivo, insertion of α-helical membrane proteins is accomplished by the translocon. In this process, transmembrane and secreted proteins are contained within the lumen of the translocon, and can either pass through the translocon (and the membrane) to be secreted into the endoplasmic reticulum, or be laterally released into the membrane bilayer directly for transmembrane domain insertion (Osborne et al. 2005; Pfeffer et al. 2016; Voorhees & Hegde 2016).
Once inserted into the bilayer, transmembrane α-helices can undergo a second stage of membrane protein folding. This requires that some of the favorable lipid-protein interactions that drive helix insertion into the bilayer be displaced by protein-protein interactions, allowing formation of higher-order tertiary and quaternary structures. This process is driven primarily by van der Waals interactions between sequence motifs on adjacent helices, burial of polar or charged groups within the final folded protein, and interhelical hydrogen bond formation. The sum of these interactions will also define the stability of helix assembly within the membrane environment (White & Wimley 1999). The study of transmembrane helix interaction motifs will therefore play a large role in defining membrane protein function and disease states. Knowledge of membrane protein sequence motifs is important for efforts to predict the organization of helix packing in multi-span or oligomeric membrane proteins.

1.3.2 Transmembrane α-helix interaction motifs

Optimal van der Waals packing contributes to helix-helix interactions via a knobs-and-holes type of packing arrangement between two helices, where the strongest arrangement will be the result of an entire helical face interacting with another helical face, generating a large contact area of many knobs interlocking strongly together with many holes. These helix-helix interaction motifs have largely been characterized via the extensive use of mutagenesis, defining ideal helical face packing arrangements by induced steric clash (Rath, Tulumello, et al. 2009), and providing an energetic advantage in forming helix-helix interactions versus helix-lipid interactions. Several protein sequence motifs have been identified which are sufficient to drive the interaction of helices in the lipid membrane by allowing the close approach and complementary fit between two helices. The simplest of these motifs is the addition of a polar residue to an otherwise hydrophobic transmembrane segment. A polar residue exposed to a hydrophobic bilayer will be poorly solubilized, and will drive helix-helix interaction in order to satisfy hydrogen bonds in an otherwise apolar solvent. As a result, as little as one polar residue per helix can be sufficient for assembly of two transmembrane helices via formation of a hydrogen bond: this has been demonstrated with the inclusion of a single Asn residue driving dimerization in a poly-Leu helix (Zhou et al. 2001; Gratkowski et al. 2001). Sequence motifs commonly associated with polar interactions are SxxSSxxT and SxxxSSxxT, where x represents any residue, generally a hydrophobic amino acid (Dawson et al. 2002). Although single Ser or Thr residues are insufficient to drive helix association alone, the mutation of any Ser or Thr residue within these
motifs to a hydrophobic residue is sufficient to disrupt the dimerization between helices, illustrating the specificity of the hydrogen bonding network (Dawson et al. 2002).

Another sequence motif found at transmembrane α-helix interfaces is the heptad repeat. These motifs have a seven-residue spacing consisting of hydrophobic residues that mediate left-handed helix-helix packing, stabilized by van der Waals interactions rather than hydrogen bonding (Lear et al. 2004). These motifs are typically composed of large residues such as Leu or Val. Phospholamban, a calcium-sensitive ATPase which forms an SDS-resistant left-handed pentamer, oligomerizes via a LxxIxxxLxxIxxxL motif, allowing the formation of a coiled-coil interaction between phospholamban helices (Oxenoid & Chou 2005).

The most characterized transmembrane helix interaction motif is the right-handed small-small or GG4 motif, composed of Gly or Ala residues separated by hydrophobic residues (X), in the form GXXXG. The spacing of this motif corresponds to a single turn of the transmembrane helix, resulting in two small residues forming a pocket on one face of the helix which provides an available van der Waals packing surface for another helix featuring this same motif (MacKenzie et al. 1997). GG4 motifs have been found in several well characterized TM homo-oligomers, including glycophorin A, methyl-accepting chemotaxis protein (MCP), F0F1-ATP synthase, and G-protein-coupled receptors (GPCRs) (MacKenzie et al. 1997; Melnyk et al. 2004; Senes et al. 2004).

1.4 A brief primer on the HIV-1 viral lifecycle

HIV is a lentiviral retrovirus, best known as the causative viral agent in acquired immune deficiency syndrome (AIDS). The viral lifecycle of HIV has several key stages: viral entry, viral fusion, viral uncoating, reverse transcription, nuclear import/integration, viral protein translation, viral budding, and proteolytic maturation (Figure 1-3).
Figure 1-3: Schematic of the HIV-1 life cycle, adapted from (Greene et al. 2008). The stages of the viral life cycle which have been targeted by therapeutics are indicated with underlined text. Some key proteins are indicated in blue at important stages of the viral life cycle.

HIV viral entry typically occurs as an infectious virion encounters a CD4+ T cell, monocyte, macrophage, or dendritic cell. HIV Env spikes found on the virion surface, composed of HIV gp120 and gp41, bind the CD4 receptor and a co-receptor (either CCR5 or CXCR4) (Wilen et al. 2012). Viral fusion is a result of structural changes in Env gp41, allowing fusion of the virion membrane with the target cell membrane. Several newer HIV therapies have targeted the fusion process, including the use of small molecules such as maraviroc, a CCR5 antagonist capable of blocking co-receptor mediated viral entry, small molecules targeting gp120 such as NBD-556 and BMS-806, and peptides targeting the gp41 heptad repeat (Mi et al. 2015; Leslie et al. 2016).
Following viral fusion and uncoating, the viral core can enter the target cell cytoplasm, and reverse transcription of viral RNA to DNA is accomplished in a process known as reverse transcription via viral reverse transcriptase protein. Reverse transcription has been targeted by nucleoside, nucleotide, and non-nucleoside inhibitor compounds with good success as part of HAART therapy (De Clercq 2004; De Clercq 2005; De Clercq 2007; Samji et al. 2013). In the next step of infection, the transcribed viral cDNA is transported through the nuclear pore complex into the nucleus of the host cell. Viral cDNA is integrated into the host genome to areas of high transcriptional activity by viral integrase protein, which is targeted by therapeutic inhibitor compounds such as raltegravir, elvitegravir, or second-generation dolutegravir (De Clercq 2007; Thierry et al. 2016). The next step in the process is known as viral transcription. Integrated viral cDNA is referred to as provirus, and can be transcribed after integration by the host cell machinery to generate viral mRNA transcripts from viral dsDNA, under the influence of viral Tat and Nef proteins. Subsequently, viral mRNA is translated into precursor proteins (viral translation). Viral precursor polyproteins are cleaved by viral proteases into functional viral proteins in the cytosol, such as viral Gag, GagPol, and most accessory proteins. Viral protease inhibitors can interfere with this process, often by competition within the viral protease active site, and are included with reverse transcriptase inhibitors as a core component of HAART therapies (De Clercq 2004; De Clercq 2007; Samji et al. 2013; Nozza et al. 2015).

HIV-1 Vpu and Env proteins are translated within the endoplasmic reticulum (ER). Env gp160 traffics via endoplasmic reticulum to the golgi apparatus, where it is cleaved to gp120 and gp41 and assembled into new Env spikes, and where Vpu plays a role in retention of host cell CD4 for degradation. In the process of viral assembly, new infectious virions nucleated by HIV Gag protein incorporate Env, Vpu, Tat, and Nef along with viral RNA transcripts and viral polymerase, allowing new infectious virions to be assembled (Ivanchenko et al. 2009). Maturation of viral particles requires cleavage with viral protease to liberate mature virions for further infection, and therefore can be disrupted by viral protease inhibitors.

Current HIV treatment usually involves a combination therapy known as HAART, in which three to four antiretroviral drugs are administered simultaneously to provide greater effect; if a mutation develops conferring resistance to one drug, the other drugs being administered should prevent the mutated virus from flourishing in the host. It has been observed that due to the low fidelity of the HIV-1 reverse transcriptase, each possible point mutation can occur in the HIV-1
genome on a daily basis (Bailey et al. 2004). Therefore, combinations of drugs can help slow viral infectivity by requiring several escape mutations to develop simultaneously before resistance to HAART is conferred. Currently, there are 30 drugs approved by the United States Food and Drug Administration for HIV treatment (Torbett et al. 2015), most of which act as viral protease inhibitors, nucleoside/nucleotide analogue inhibitors of the HIV-1 reverse transcriptase, or viral integrase inhibitors targeting gp41 or gp140. Five processes in the HIV-1 lifecycle are the targets of current antiviral therapies; however, viral uncoating, viral protein translation, and viral budding have not been successfully disrupted by anti-HIV therapeutics. HIV regulatory proteins enhance the infective capacity of the virus by increased translation of viral genes (Tat) and export viral RNA from the nucleus (Rev). HIV proteins, termed “accessory” proteins, are not strictly required for viral replication, but increase production of infective virions by counteracting the host cell immune response to HIV infection (Vpu, Vif, Nef, and Vpr). Since most anti-HIV drugs are active against only reverse transcriptase or viral protease, development of an unconventional drug against an untargeted HIV protein is an attractive option for further research.

1.4.1 Viroporin proteins

Viroporins are generally small, virally-encoded proteins which enhance the infectivity of viruses. Many pathogenic viral infections express viroporins, including hepatitis C (p7 protein), influenza A (M2 protein), coronaviruses (CoV E), and HIV-1 (Vpu) (Gonzalez & Carrasco 2003; Wilson et al. 2004; Torres et al. 2015). Currently known viroporin proteins are listed in Table 1.

As the name implies, the viroporin class of proteins have been proposed to perform their roles in viral infection by forming a pore or channel, which in turn enhances viral replication in vivo. Each of these proteins can perform diverse roles in the viral replication cycle, but all have been suggested to work by a mechanism which results from viroporin induced conductance of charged ions across inner and outer membranes of infected cells; brief reviews of known viroporin functions cover this topic well (Gonzalez & Carrasco 2003; Nieva et al. 2012; Sze & Tan 2015).

Viroporins are generally less than 120 amino acid residues in length and must oligomerize to form channels within the lipid bilayer; generally tetramers (IAV, SARS-CoV), but also including pentameric forms (HIV-1 Vpu) or hexameric assemblies (HCV P7) (Ouyang & Chou 2014). These ion channels have been characterized using single-channel measurements and experiments
with *Xenopus* oocytes, and in several cases have been shown to be specific for cations (ie. 6K protein and P7) (Wang et al. 2011; Ouyang & Chou 2014; Griffin 2003).

Table 1: List of known viroporin proteins; adapted from (Sze & Tan 2015).

<table>
<thead>
<tr>
<th>Virus</th>
<th>Viroporin</th>
<th>Function in Viral Life Cycle</th>
</tr>
</thead>
</table>
| IAV       | M2        | Genome uncoating  
Glycoprotein processing/trafficking  
Delay protein trafficking through TGN  
Viral release |
| HIV-1     | Vpu       | Degradation of CD4 and trafficking of Env proteins  
Viral release |
| HCV       | P7        | Viral morphogenesis  
Viral polyprotein processing |
| CoV       | E 3A      | Viral morphogenesis and assembly  
Viral release |
| Poliovirus| 2B 3A     | Blocks ER-Golgi traffic/host protein secretion |
| Alphavirus| 6K        | Viral release |
| Sindbis virus |         |                                       |
| Coxsakievirus| 2B        | Inhibit protein trafficking through Golgi  
Induce apoptosis for viral release |
| Rotavirus | NSP4      | Induce autophagy for viral protein transport |
| SV40      | VP2 VP3 VP4 | Translocation of DNA genome from ER to cytosol  
Viral release |

Perhaps the best studied viroporin is the M2 proton channel expressed by Influenza A. M2 is responsible for the acidification of viral particles and subsequent release of influenza virus from endosomes into the cytoplasm. The M2 protein is a single-pass transmembrane domain which forms a homo-tetrameric proton channel which is required for efficient infection by influenza A (Schnell & Chou 2008; Cady et al. 2009; Pielak & Chou 2011). M2 is the target of anti-influenza drugs amantadine and rimantidine, whose mechanism of action involves disruption of the proton-channel activity through occlusion of the channel, preventing uncoating of the virus and infection (Cady et al. 2009; Schnell & Chou 2008; Stouffer et al. 2008). The influenza M2/amantadine interaction is an excellent model system for other viroporins, as both the channel and an effective channel blocking compound can be studied in conjunction with each other.
1.5 Vpu of HIV-1

The HIV-1 accessory protein Vpu is expressed as a retroviral gene product. As an accessory protein, Vpu is not strictly required for HIV replication in vitro, but aids the production and budding of mature HIV virus particles (Klimkait et al. 1990; Neil et al. 2006). Vpu is translated within the endoplasmic reticulum (ER), which is unusual for an HIV-1 protein, and participates in the downregulation of host cell CD4 molecules as well as the host cell restriction factor known as CD317/Tetherin (Paul & Jabbar 1997; Levesque et al. 2003; Neil et al. 2006). Early studies of Vpu reported ion-channel conductance in lipid bilayers (Ewart et al. 1996; Schubert, Ferrer-Montiel, et al. 1996).

Vpu is not expressed in HIV-2 (Cohen et al. 1988). Vpu is unique to HIV-1 and one form of simian immunodeficiency virus found in chimpanzee hosts (SIV\textsubscript{cpz}), although the primary sequences of Vpu are not identical between these viruses (Schubert, Bour, et al. 1996; Gonzalez & Carrasco 2003). It has been shown that HIV-1 virus deficient for the Vpu gene exhibits impaired viral budding in several cell lines (Klimkait et al. 1990; Schubert, Bour, et al. 1996; D. Ewart 2002; Neil et al. 2006), and therefore has reduced viral transmissibility within the host.

1.5.1 Vpu structure

Vpu is an 82-residue, single-pass, \(\alpha\)-helical transmembrane protein. The N-terminal transmembrane domain of Vpu is highly hydrophobic, followed by an amphipathic second helix, and a soluble C-terminal helix (Figure 1-4).
Within an HIV-1 infected host cell, Vpu is found within the membranes of the endoplasmic reticulum, golgi (Klimkait et al. 1990; Schubert, Bour, et al. 1996), endosomes (Varthakavi et al. 2006), and cell plasma membrane (Terwilliger et al. 1989), with the N-terminus of each Vpu monomer embedded in the lipid bilayer and the more soluble helices of Vpu in the cell cytosol.

The structure of the Vpu transmembrane domain has been studied by molecular dynamics (MD) simulations (Grice et al. 1997; Kukol & Arkin 1999; Cordes et al. 2001; Lopez et al. 2002; Sramala et al. 2003), solid-state NMR (Marassi et al. 1999; Ma et al. 2002; Park et al. 2003; Park, Mrse, et al. 2006; Sharpe et al. 2006; Do et al. 2013) and FTIR experiments (Kukol & Arkin 1999; Park et al. 2003). The Vpu transmembrane domain has been shown to form a homo-oligomeric complex (Hussain et al. 2007) able to regulate virus release (Schubert, Bour, et al. 1996).

Vpu monomers are well-characterized, with a transmembrane domain encompassing residues 8 to 25, an amphipathic second helix encompassing residues 30 to 49 in DHPC (39 to 48 in DPC), and a soluble third helix encompassing residues 58 to 70 in DHPC (64 to 70 in DPC) (Park, De Angelis, et al. 2006; Ma et al. 2002; Wittlich et al. 2008). NMR data indicate the full-length Vpu monomer structure to be that of the transmembrane domain and cytoplasmic domains joined by a flexible linker containing conserved constituvely phosphorylated Ser residues responsible for the interaction of Vpu and CD4 (Schubert, Bour, et al. 1996; Paul & Jabbar 1997; Zhang et al. 2015). Structurally, phosphorylation has no great consequence on the cytoplasmic domain of monomeric Vpu, which features helices two and three folding back upon one another (Wittlich et al. 2008; Zhang et al. 2015). One potential arrangement of this structure is shown in Figure 1-5.

![Figure 1-5: Chimeric model of Vpu. A model composed of the Vpu transmembrane domain structure (Sharpe et al. 2006) combined with Vpu cytoplasmic domain structure (Wittlich et al. 2009). Acidic residues are indicated in red, basic in blue. Side chains of aromatic residues have been illustrated in stick diagrams for visual reference.](image)

1.5.2 Vpu as a viroporin

Vpu can form oligomers via its conserved membrane spanning domain (Maldarelli et al. 1993). Early work characterized Vpu as having cation-selective ion channel activity in black lipid bilayers, similar to that reported previously for the M2 channel from influenza A (Ewart et al. 2004).
Further experiments indicated that Vpu ion channels were selective for monovalent cations in Xenopus oocytes, and that scrambling the Vpu transmembrane domain sequence prevented ion channel conductance as well as the ability to enhance virion release (Schubert, Ferrer-Montiel, et al. 1996).

Several years after the reported ion-channel conductance of Vpu and its classification as a viroporin protein, a known epithelial sodium channel blocker approved for human use (reviewed in (Teiwes & Toto 2007)), was tested against Vpu ion channel activity, leading to the discovery that amiloride derivatives, such as 5-(N,N-hexamethylene)amiloride (HMA) and 5-(N,N-dimethyl)amiloride (DMA) inhibit Vpu conductance, but amiloride does not (D. Ewart 2002). These molecules were also reported to inhibit viral budding in HeLa cell lines co-transfected with Vpu and Gag proteins from HIV-1 (D. Ewart 2002; Ewart et al. 2004). The structural and functional details of the HMA / DMA interaction with Vpu are not known at this time, but MD simulations suggest that the conserved Ser 23 in the Vpu transmembrane domain is important for small molecule binding (Lemaitre et al. 2004; Kim et al. 2006; Rosenberg et al. 2016). Currently, several other small molecules have been found to be effective inhibitors of HIV-1 Vpu including BIT225 (Khoury et al. 2010; Khoury et al. 2016), Genistein (Sauter et al. 2014), and SM-111 (Mwimanzi et al. 2016), all of which have been reported to inhibit Vpu ion channel function.

Based on the successful targeting of M2 with small molecule inhibitors of channel activity (Royle et al. 2015), Vpu has become an interesting potential target for innovative anti-HIV therapies, as disruption of its function is known to impede the progress of HIV-1 infection (Klimkait et al. 1990; D. Ewart 2002). Experiments which involved replacing the Vpu transmembrane domain with the M2 transmembrane domain from influenza A resulted in functional viral chimeras capable of enhancing viral release from cells (Hout et al. 2006). The correlation between enhancement of viral release and ion channel activity led to the conceptual model of ion-channel blocking small molecules being useful as a prospective anti-Vpu, and therefore anti-HIV, treatment paradigm.

However, some recent studies have shown that several mutations which block Vpu-mediated viral release have no effect on Vpu ion channel activity, casting doubt on the link between ion channel activity and enhancement of viral release (Skasko et al. 2011; Bolduan et al. 2011). Furthermore, high-throughput screening assays capable of assessing viroporin activity have not clarified any connection between these putative effects of Vpu (Herrero et al. 2013; Taube et al.
Consequently, it has been suggested that the reported ion channel function of Vpu may not be linked to Vpu-mediated enhancement of viral release from infected cells (Strebel 2014; Opella 2015).

1.5.3 Other functions of Vpu: interactions with membrane proteins

In addition to being an ion channel, Vpu has also been shown to interact with a number of host cell proteins, including CD4, MHC-I, β-TrCP, NTB-A, TASK-1, and others, leading to altered localization or degradation of its targets (reviewed in (González 2015)).

Vpu expression reduces the half-life of CD4 within cells by over twenty-fold (Schubert, Bour, et al. 1996). Degradation of host CD4 molecules upon Vpu binding occurs via a proteasome-dependent mechanism, without ubiquitination or degradation of Vpu (Margottin et al. 1998). This requires two constitutively phosphorylated Ser residues in the cytoplasmic domain of Vpu (Paul et al. 1998), eventually resulting in degradation of CD4 from the ER via the recruitment of a β-TrCP complex, and ubiquitination of CD4 in trans (Willey et al. 1992; Binette et al. 2007). In the absence of Vpu expression, host cell CD4 receptors accumulate at the cell surface and thereby are incorporated into HIV virions (Levesque et al. 2003). In the presence of Vpu, downregulation of CD4 prevents HIV Env protein binding CD4 in already infected cells, preventing nonfunctional gp120-CD4 complexes forming at the virion surface (Levesque et al. 2003). With downregulation of CD4 by Vpu in infected cells, free/unbound Env assembled in virions can interact with CD4 expressed on the surface of uninfected cells to promote further HIV infection.

Vpu can also target the cell surface protein tetherin for internalization and degradation (Douglas et al. 2009; Goffinet et al. 2009; Mangeat et al. 2009). Tetherin, also known as CD317 or BST-2, is selectively expressed in host cells in response to interferon-α signaling during HIV infection (Neil et al. 2008). Tetherin is a 30-36 kDa single-pass integral membrane protein which also contains a C-terminal glycosylphosphatidylinositol (GPI) anchor capable of tethering fully formed HIV virions to the cell surface, preventing their continuous egress from the infected cell and ultimately leading to the encapsulation and degradation of HIV viral particles (Neil et al. 2008; Van Damme et al. 2008; Perez-Caballero et al. 2009; Hammonds et al. 2010). Upon HIV-1 expression of Vpu, the capability for viral restriction by Tetherin is prevented via its internalization and degradation within the host cell, allowing HIV virions to properly bud from
the infected cell and complete the cycle of infection (Neil et al. 2008; Gupta et al. 2009; Mitchell et al. 2009). Therefore, Vpu appears to play a role in negating the host cell restriction of viral budding. The mechanistic basis for this restriction is currently not well understood, but a direct Tetherin-Vpu interaction via the transmembrane domain of each protein has been reported based upon NMR experiments, wherein the transmembrane domains of Vpu and Tetherin are proposed to form an anti-parallel helix-helix interaction within the lipid bilayer (Skasko et al. 2012).

Mutagenesis of tetherin residues Ile 34, Leu 37, and Leu 41 have revealed their importance in the tetherin-Vpu interaction, as has mutagenesis of Vpu transmembrane domain residues Ala 14, Trp 22, and to a lesser extent Ala 18 (Vigan & Neil 2010). Vpu directs the degradation of tetherin by ensuring tetherin recycling from the ER to endosomes, decreasing tetherin concentration at the cell surface (Mangeat et al. 2009; Dubé et al. 2011).

**Figure 1-6:** Functions of Vpu. Vpu (blue) is known to be trafficked through the ER and Golgi apparatus to the cell surface (A). Once at the cell surface, Vpu can interact with tetherin (purple), resulting in a change in its recycling pathway through endosomes, eventually resulting in Tetherin degradation (B). During its path through the ER/Golgi, Vpu may interact with cellular CD4 (red), resulting in proteasomal degradation and reduced cell surface levels of CD4 (C).
1.5.3.1 Vpu oligomers

A single monomer of Vpu has a molecular weight of 9167 Da. Since Vpu is a single-pass α-helical transmembrane protein, it would be necessary for Vpu to form a higher-order oligomeric complex to accommodate a charged ion within the hydrophobic bilayer. Several studies have explored potential homo-oligomeric arrangements of Vpu, largely by MD simulations (Krüger & Fischer 2008; Mehnert et al. 2008; Moore et al. 1998; Kim et al. 2006; Lin et al. 2016a). Vpu has been reported to be a cation-selective ion channel, with some preference for Na\(^+\) and K\(^+\) ions in lipid bilayers (Ewart et al. 1996; Schubert, Ferrer-Montiel, et al. 1996). Therefore, Vpu oligomer models have been proposed as tetrameric, pentameric, and hexameric arrangements of Vpu monomers based upon the size of a charged ion modelled within the putative viral pore. Several residues have been proposed to be important for ion channel function within the Vpu transmembrane domain, including Trp 22 and Ser 23, based upon single channel measurements of mutant Vpu transmembrane domain peptides (Mehnert et al. 2008). Despite these studies, no molecular basis for the importance of these residues has been demonstrated, leaving the mechanism of Vpu transmembrane channel assembly and activity largely unknown. MD models have largely focused on the transmembrane domain alone, as single-channel measurements have indicated that the transmembrane domain is necessary and sufficient for the ion channel activity of Vpu without inclusion of Vpu helix 2 or 3 (Schubert, Bour, et al. 1996; Ewart et al. 1996).

Biophysical characterization of the Vpu oligomer has been attempted via the application of SDS-PAGE crosslinking, MALDI-TOF, and AUC sedimentation velocity experiments (Maldarelli et al. 1993; Hussain et al. 2007; Lu et al. 2010; Chen et al. 2016). While not yielding a single discrete model for Vpu oligomer size, this work suggests that tetrameric to hexameric arrangements are favoured in equilibrium, although dimer and trimer arrangements are observed in some cases, and that any or all of these arrangements could be sampled in a membrane environment. If these results are interpreted to mean that Vpu oligomeric arrangements are not necessarily discrete, it follows that a single distinct channel arrangement of Vpu is unlikely to be elucidated.
1.5.4 Molecular dynamics models of Vpu oligomerization

There have been numerous MD studies of the structure, assembly and putative ion channel function of HIV-1 Vpu. Early MD studies of Vpu focused primarily on ion channel assembly, as initial studies of Vpu function suggested that channel conductance was of importance in the upregulation of viral budding (Klimkait et al. 1990; Ewart et al. 1996; Schubert, Ferrer-Montiel, et al. 1996; D. Ewart 2002). Restrained MD simulations have examined the stability and structure of Vpu tetramers, pentamers, and hexamers, assembled in vacuo without explicit hydrogen atoms over 100ps of simulation (Grice et al. 1997). These simulations concluded that trimeric assemblies of Vpu were too narrow for ions to pass through easily, while assemblies larger than hexamers could not account for the reported ion selectivity of Vpu. All models featured the conserved Ser residue 23 facing inside the Vpu transmembrane domain pore, forming a hydrogen bond with the backbone carbonyl group from the preceding helical turn. Based on these simulations, it seemed that the pentameric Vpu assembly was most likely to correspond to channel measurements (Grice et al. 1997).

These results were supported by a combined FTIR and MD study that yielded a pentamer model of Vpu (Kukol & Arkin 1999), based on the use of the CNS for helical interactions (CHI) suite previously utilized to generate pentameric models of phospholamban (Adams et al. 1995). Longer (1 ns) unrestrained MD simulations of Vpu pentamers in octane slabs found that the symmetry of the oligomeric assembly was lost and that oblong pentameric shapes were formed over the timeframe of the simulation (Moore et al. 1998). This resulted in expulsion of water from the pore and collapse of the Vpu oligomeric assembly, suggesting that the pentamer may not in fact be the most stable conformation of the Vpu channel, and that other arrangements could occur. Ser-inside oligomeric models of Vpu were found to form hydrogen bonds with the protein backbone in the same segment after 2 ns, featuring Trp residues at the interface with lipid headgroups, and reporting that water was critical to the simulation (Cordes et al. 2001). Further 3 ns simulations of Vpu in POPE resulted in pentamer models as the most stable arrangement (Lopez et al. 2002), while MD of Vpu monomer in POPC elucidated a salt bridge at the EYR motif beyond the transmembrane domain (Sramala et al. 2003), and in full-length models of Vpu helix 3 was observed to wrap back to the membrane near helix 2 (Lemaitre et al. 2006). By 2008, different lipid environments had been utilized in 10 ns simulations of Vpu transmembrane domain including DPPC, POPC, DTPC, and DDPC, concluding that Vpu ion channel gating may
depend on surrounding lipid, and by extension the surrounding cellular compartment (Krüger & Fischer 2008). Recent course-grained simulations in POPC bilayers have recorded the assembly of Vpu monomers into an oligomeric complex via the transmembrane domain: first as dimers, then larger patches of Vpu, followed by arrangement into channel-like configurations (Chen et al. 2016; Lin et al. 2016a).

MD simulations focused on the Vpu transmembrane domain interaction with small molecules have highlighted the binding of HMA, reporting conserved Ser 23 positioned inwards, with Trp 22 facing the lipid bilayer (Lemaitre et al. 2004). This work was extended using AUTODOCK to describe hydrogen bonding between Ser 23 and HMA in the pore (Kim et al. 2006).

The focus in many recent Vpu MD studies has moved away from ion channel activity, as Vpu has been reported to play a key role in suppressing a host-cell viral restriction system via a direct interaction with the Tetherin protein, previously identified as BST-2 (Neil et al. 2008). This interaction does not appear to require Vpu ion channel activity, and as described below, may explain why Vpu was observed to be required for efficient HIV-1 budding from some cell types and not others, as there is a direct correlation between the requirement for functional Vpu and the nascent expression of host cell Tetherin protein (Van Damme et al. 2008).

1.5.5 Effects of Vpu sequence on Vpu-Tetherin interactions

Vpu sequence effects can be correlated by comparing different HIV-1 subtypes. M-type Vpu bears the standard sequence utilized in this work, and differs from Cameroon N-type Vpu, a rare subtype with reduced function. Cameroon O- and P-type Vpu are least similar to M-type Vpu, and are more closely related to gorilla SIV: generally these are considered an ineffective type M Vpu (Sauter et al. 2012).

Changes to several key residues in the sequence motif found in the Vpu-Tetherin interaction (A₁₀xxxA₁₄xxxA₁₈) can be correlated to decreased Vpu function. The Ala 14 and Ala 18 residues of the Vpu transmembrane domain are conserved in M-type and N-type Vpu, but are not in O-type proteins. Conversion to Ala 14 and Ala 18 conferred anti-Tetherin effects to O-type Vpu in one study (Sauter et al. 2012), but the inclusion of these two residues was insufficient to generate anti-Tetherin activity when tested in O-type Vpu by another research group (Vigan & Neil
2011), who suggested that Vpu Lys 32 was also required for localization with and downregulation of Tetherin.

These reports highlight the difficulty of determining sequence effects in protein-protein interactions as multiple motifs may be required to facilitate protein-protein interactions. In a rapidly changing sequence context (ie. HIV, where the rate of mutation is high, such that subtypes and isolates may share limited sequence homology) it can be difficult to establish which residues and motifs are primarily responsible for a given cellular effect. Furthermore, the Vpu AxxxxAxxxxA motif has a less-defined characteristic helical interaction distance than other canonical motifs (ie. GxxxGxxxG) (Kleiger et al. 2002), suggesting structural plasticity may be possible with regards to conformation of the motif, obscuring direct conclusions about helix-helix interactions.

To this end, the role of the Vpu AxxxxA motif in Vpu-Tetherin interactions has been explored via NMR experiments (Skasko et al. 2012). Solubilization in diheptanoylphosphatidylcholine (DHPC) micelles and the addition of covalently-bonded paramagnetic relaxation enhancement agents indicated that Vpu and Tetherin transmembrane domains interact in opposing directions. When Ala residues in the Vpu AxxxxAxxxxA motif were mutated to FxxxxFxxxxF, Vpu colocalized with Tetherin but did not decrease Tetherin cell surface expression or enhance virion release (Skasko et al. 2012). Mutation of only A14F showed a similar result, as did mutation of A10F/W22A. The combined mutation of A10F/A14F/A18F in Vpu induced minimal chemical shift change (and therefore minimal conformational differences) in BST-2, taken as evidence that the triple-mutant co-localized well with Tetherin but did not form a strong or viable interaction with this binding partner. The precise mechanism of this interaction is unclear, but is assumed to be a direct protein-protein interaction.

1.5.6 Small molecule inhibitors of Vpu function

Vpu has been shown to interact with various small molecules; of these, HMA and DMA have been best characterized (D. Ewart 2002; Ewart et al. 2004). Building on this success, several other compounds have been identified as Vpu inhibitors in small molecule screens or through combinatorial chemistry starting with the amiloride backbone, including BIT-225 (Khoury et al. 2010), SM111 (Mwimanzi et al. 2016), and IMB-LA (Mi et al. 2015) (Figure 1-7).
Figure 1-7: Small molecules shown to interact with Vpu. Amiloride derivatives (A, B, C) originally shown to have effects on Vpu ion channel activity (D. Ewart 2002; Ewart et al. 2004), with more recent examples given for comparison (D, E, F) (Khoury et al. 2010; Mwimanzi et al. 2016; Mi et al. 2015).

Initial reports of HMA and DMA effects noted a loss of virus-like budding from cells expressing Gag and Vpu, leading to the hypothesis that Vpu exerts its cellular effects via ion channel activity (D. Ewart 2002). HMA has also been found to block ion channels formed by hepatitis C P7 protein (Premkumar et al. 2005), as well as Vpu transmembrane domain peptides within black lipid membranes (Römer et al. 2004). HMA and DMA have been reported to inhibit HIV-1 replication in cultured human-blood derived macrophages, cementing their potential as anti-HIV therapeutics (Ewart et al. 2004).

Recent studies have featured BIT225, a small molecule currently in phase 2 trials as an anti-HIV therapeutic, targeting HIV-1 Vpu based on ion channel measurements and cellular assays (Khoury et al. 2010). This molecule is an amiloride derivative with efficacy in late stages of HIV infection, is tolerable at high levels, and has efficacy against hepatitis C infection. Studies of BIT225 have demonstrated that the Vpu-Tetherin interaction is unaffected by BIT225 in cell
assays, suggesting that BIT225 is a selective inhibitor of viroporin function but not protein-protein interactions (Kuhl et al. 2011). The mechanism of action of this molecule is currently unknown.

Studies of other amiloride derivatives have explored the effects of IMB-LA, which has been shown to specifically inhibit the Vpu-mediated degradation of tetherin/BST-2 (Mi et al. 2015). IMB-LA did not prevent the interaction of Vpu and tetherin based upon cell assays, but did prevent the localization of tetherin to the lysosome. IMB-LA did not prevent CD4 downregulation by Vpu (Mi et al. 2015). A flavonoid small molecule, Genistein, has also been reported to prevent ion channel activity in Vpu based on Xenopus oocyte studies, but has not been tested for an effect on HIV-1 viral budding directly (Sauter et al. 2014). Finally, an acylguanidine-based molecule known as SM111 has been shown inhibit the influenza M2 proton channel as well as to inhibit replication of HIV-1 in vitro (Mwimanzi et al. 2016; Jalily et al. 2016). SM111 was shown to have lower toxicity than either HMA or BIT225, but also downregulated native tetherin and CD4 expression in uninfected cells (Mwimanzi et al. 2016). The effect of SM111 against Vpu ion channel activity has not been tested directly.

MD studies have identified a possible mode of HMA interaction with Vpu, demonstrating a simple stopper-like occlusion mechanism within the Vpu ion channel, featuring HMA interacting with conserved Vpu transmembrane domain Ser 23 (Lemaitre et al. 2004; Kim et al. 2006; Rosenberg et al. 2016). Vpu Ser 23 is implicated in the ion channel function of Vpu (Grice et al. 1997; Cordes et al. 2001; Cordes et al. 2002; Padhi et al. 2013; Padhi et al. 2014). This model is similar to the arrangement of the inhibitor amantadine bound within the influenza M2 proton channel (Hu et al. 2007; Stouffer et al. 2008; Cady et al. 2009).

Despite these studies, the structural and functional details of the HMA and DMA interactions with Vpu have not been conclusively determined, and the molecular stopper model is not the only possibility which could explain the inhibition of viral budding brought about by HMA and DMA. There is a distinct possibility that the mechanism of action of HMA is not in fact discrete channel/pore blocking, but may be an indirect effect of small molecule binding preventing a specific functional conformation of the protein. For instance, NMR has shown that influenza M2 protein binds amantadine at two different protein binding sites, one inside of the proton channel, and one outside (Schnell & Chou 2008; Jing et al. 2008). Indeed, study of the Vpu-HMA
interaction in lipid via the use of surface plasmon resonance (SPR) showed HMA binding to a Ser to Ala mutant of Vpu, indicating that the presence of Ser in the transmembrane domain is not a strict requirement for HMA interaction, which would obviate the requirement for a stopper-like mechanism (Rosenberg et al. 2016). Given the potential conformational plasticity in Vpu helix-helix interactions, it may be possible that alternate binding modes occur in the Vpu-HMA system; these configurations remain unexplored to date.

Another possibility is an interaction of small molecules with Vpu which prevents formation of an active Vpu complex. In such a scenario, a conformational change would be required between the Vpu configuration in cellular membranes to another conformation required for activity, be that via protein binding and degradation, or via ion channel / pore activity. This possibility may be envisioned as a situation in which small molecules could prevent a Vpu oligomeric rearrangement by means of stabilizing an active Tetherin-binding complex or by stabilizing an inactive arrangement of Vpu.

*S. cerevisiae* and *E. coli* assays have been developed to aid in screening drug libraries which may inhibit Vpu ion channel function, by means of assays sensitive to cation (specifically potassium) transport into these cells and the complementation of native transporters with Vpu constructs (Herrero et al. 2013; Taube et al. 2014). It is likely that utilizing these screening systems will provide greater insight into small molecule Vpu inhibitor mechanisms, as well as providing a suitable system for screening new small molecule Vpu inhibitors. However, it is clear that further insight into the functional significance of Vpu oligomerization will be required to differentiate between different mechanistic models of small molecule action.

### 1.6 Membrane mimetics for structural studies of membrane proteins

#### 1.6.1 Organic solvents for membrane protein solubilization

Membrane proteins exist in a very different environment than soluble proteins, and consequently some of the solvents and mimetics used to study membrane proteins are often unusual, such as Hexafluoroisopropanol (HFIP) for example. HFIP is often utilized by protein chemists who specialize in solid-phase peptide synthesis. Often used in conjunction with dichloromethane (DCM), HFIP can overcome peptide solubility problems in some organic solvents by disruption of β-aggregation of these peptides (Narita et al. 1988). A wash with these solvents can allow
solid-phase peptide coupling reactions to proceed at higher efficiency, as the application of HFIP can disrupt undesirable peptide aggregation due to disruption of β-sheet hydrogen bonding patterns (Milton & Milton 1990).

HFIP has also been shown to promote the cold denaturation of protein secondary structures; the hydrogen bonding normally occurring between protein and water is supplanted by interactions with HFIP, and in turn random coil secondary structure cannot be easily supported by the limited availability of hydrogen bonding with the solvent. In this manner, random coil secondary structure is destabilized relative to the self-contained hydrogen bonding associated with protein helical secondary structure after fluoroalcohol treatment (Andersen et al. 1996). Thus, it is possible to increase development of helical secondary structure by application of HFIP to membrane proteins and peptides in some cases; we have found this to be true in the Vpu system.

Similarly, Trifluoroethanol (TFE) features prominently in membrane protein purification schemes, often utilized in a blend of solvents to ensure the helicity of membrane protein segments. Like HFIP, trifluoroethanol has also been reported to stabilize α-helical secondary structure formation in proteins. This effect has been reported as early as 1963 in multimers of peptide derivatives measured by UV absorbance at 190nm (Goodman et al. 1963).

Some models propose that the presence of TFE changes the solvent shell surrounding a peptide, resulting in a stabilization of helical character in peptides which do not have strong β-sheet stabilization and packing (ie. random coil secondary structure) (Walgers et al. 1998). Other models suggest that 1-propanol and 2-propanol are as effective as TFE at inducing helical structure, but HFIP is more so (Kumaran & Roy 1999). The effects of these co-solvents on tertiary or quaternary structure are currently unknown, and may have a large impact on structures generated in solutions containing these fluoroalcohols (Opella 2015), as these solvents are clearly not a close approximation of the native environment for membrane proteins.

1.6.2 Use of liposomes for biophysical and structural studies of membrane proteins

Lipid bilayers are arguably the most relevant medium for the study of transmembrane peptides and proteins. The ideal choice of lipid membrane would be intact lipid bilayers containing the proteins of interest, but this can be confounding due to the presence of proteins and small molecules. Reducing a cell membrane to only its lipid components is a good compromise: the
model lipid can be chosen to mimic the relevant characteristics of the cell membrane which natively surrounds the protein of interest.

Important considerations in lipid selection include similarity in headgroup choice. Phosphatidylcholine (PC) is the most abundant headgroup in eukaryotic membranes, while phosphatidylethanolamine (PE) is most abundant in prokaryotic membranes; the choice of lipid for a given protein should reflect these concerns (Cullis et al. 1996; Warschawski et al. 2011). Membrane fluidity is another large concern: proteins expected to exist in fluid membranes may not function correctly in gel phase lipids, or vice versa.

In some cases, specific lipids are known to be required for protein function; for example, cytochrome c oxidase requires a bound cardiolipin molecule, and KcsA requires anionic phospholipids. In other cases, the choice of lipid can strongly affect the conformation of a protein of interest, such as in the case of PIV5 protein from paramyxovirus, which is α-helical in POPC/POPG mixtures, but β-sheet in conformation in neutral POPC and DMPC lipid bilayers (Yao & Hong 2013).

Lipid bilayers are large systems for observation by NMR techniques. Uni-lamellar and multi-lamellar vesicles are well beyond the size limit for fast tumbling sufficient to generate isotropic linewidths in NMR spectra at approximately 100 nm to 1000 nm in diameter (Reviewed in (Warschawski et al. 2011)), and as a consequence most NMR spectra observed are equivalent to dry-powder samples. This disadvantage can be overcome in some cases by use of magic angle spinning (MAS) NMR techniques (Castellani et al. 2002), but even the improved resolution offered by these techniques is often insufficient for characterization of protein structure when combined with the low sample volumes and concentrations afforded by lipid-bilayer reconstituted samples. Simply put, the signal to noise available in lipid-protein samples is insufficient for 3-dimensional NMR analysis on a reasonable experimental timescale for many membrane proteins, particularly in lipid bilayer environments.

1.6.3 SDS and DPC as membrane mimetics for aqueous media

Detergent micelles are often used as a substitute for lipid bilayers. Like lipids, detergents have polar head groups and acyl tails that segregate away from water. Hydrophobic transmembrane domains may interact with acyl tails of detergents similarly to the lipid bilayer, while the polar
regions of the protein remain exposed to solvent, and hydrogen bonds forming \( \alpha \)-helical structure can exist in detergent micelles. Therefore, transmembrane helix folding is thought to occur in a similar fashion in detergents as to a lipid bilayer system.

However, while detergents are similar to lipids, they can also be different in critical ways. Detergent micelles are commonly smaller than lipid vesicles, approximately 6 nm in diameter (Warschawski et al. 2011). Many common detergents used in biophysical characterization contain strongly charged or ionic headgroups, such as sodium dodecyl sulfate (SDS). Other detergents can possess headgroups often found in lipid bilayers such as the zwitterionic dodecylphosphocholine (DPC) group. Acyl tail length can vary comparably in detergent systems to lipid systems, resulting in a somewhat spherical micellar structure with a diameter of roughly two acyl tail lengths, comparable to a lipid bilayer in thickness, but markedly different from the uniform thickness presented by the nearly planar bilayer environment of native lipid. Furthermore, membrane protein complexes must usually be studied at sufficient detergent concentration to ensure a micellar system, which precludes the use of some milder detergents such as dodecylmaltoside (DDM) due to its high aggregation number; DDM micelles represent a ~60 kDa complex which will tumble slowly and broaden signals in NMR experiments, as well as decreasing the mass of protein relative to the detergent, interfering with high-resolution studies (Warschawski et al. 2011). Schematic diagrams of several popular membrane mimetic molecules are presented in Figure 1-8.

Two detergents are most commonly used in the solubilization and NMR study of transmembrane proteins: SDS and DPC (Strandberg & Ulrich 2004). SDS is a relatively harsh detergent capable of solubilizing membrane proteins and denaturing native structure in soluble proteins, and is best known for its use in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE has become a ubiquitous standard for characterization of a protein mixture. In this capacity, it is assumed that SDS will bind to proteins in a known ratio, approximately 1.4g SDS/1g protein (Reynolds & Tanford 1970), imbuing them with a uniform charge distribution relative to the mass of the polypeptide chain. In this manner, a mixture of proteins will all become consistently and regularly coated with SDS at high concentration, unfold from native conformation due to high anionic charge density, and migrate predictably in an electric field relative to their monomeric mass.
Figure 1-8: Some common membrane mimetics utilized for study of membrane proteins. Examples include (A) charged SDS, (B) zwitterionic DPC, (C) modified sugars, (D) short chain lipid, (E) saturated lipid, (F) unsaturated lipid.

In water-soluble proteins, the hydrophobic core may be adequately solubilized by the presence of detergents such that a linear “beads on a string” representation of interaction between SDS and the polypeptide chain may be a reasonable approximation of polypeptide configuration in SDS-PAGE. However, this is not necessarily true of the dissolution of membrane proteins in SDS (Rath, Glibowicka, et al. 2009). In the cases of membrane proteins, structural elements which would normally be embedded within the bilayer may remain intact within the hydrophobic center.
of a detergent micelle; in this way SDS dissolution can provide a useful system to study solubilized membrane protein complexes.

SDS has frequently been utilized to solubilize membrane proteins for NMR characterization, as has DPC, a milder detergent due to its zwitterionic rather than anionic headgroup, in addition to other less used detergents such as octyl glucoside (OG) and DHPC (Strandberg & Ulrich 2004; Warschawski et al. 2011). The applicability of detergents for NMR study must be carefully assessed by comparison with other membrane mimetics to avoid potential changes in protein structure. This has been well illustrated in the case of influenza M2 protein, which has been studied in lipid bilayers including DLPC, DMPC, and DOPC, as well as DOPC/DOPE blends, and DHPC micelles. These mimetics gave different results by solution NMR and solid-state NMR (Wang et al. 2001; Hu et al. 2007; Schnell & Chou 2008; Cady et al. 2009; Cross et al. 2011), as well as differences from X-ray crystallography studies in octyl glucoside (Stouffer et al. 2008). Detailed comparison has led to the understanding that high detergent micelle curvature and bias in crystal packing resulted in significant differences from the most relevant solid-state NMR structure of M2 protein (Cross et al. 2011).

1.6.4 Bicelles for NMR and biophysical studies of membrane proteins

Bilayer micelles, or bicelles, combine the physiological relevance of a lipid bilayer system with the convenient water solubility of a detergent system. Generally, bicelles are composed of planar lipid bilayer components, combined with a high curvature micellar component. Bicelles are generated by combining a short chain lipid/detergent with a longer chain lipid, although not all combinations will form bicelles. The most frequently used bicellar system is a mixture of DHPC (short chain) and DMPC (long chain). By varying the ratio of DHPC:DMPC (the q value of the bicelle) it is possible to vary the morphology of the resulting bicelles, affecting the viscosity of the system and the rate of molecular tumbling (Prosser et al. 1996; Sanders & Prosser 1998; Prosser et al. 2006; De Angelis & Opella 2007; Warschawski et al. 2011).

At low ratios of long chain versus short chain lipid (q=0.10 to q=1.0), these bicelles form coin-shaped objects, exhibiting isotropic chemical shift values much like detergent micelles. At high ratios of long chain versus short chain (q=2.8 to q=6.5), these components are thought to form large planar bilayer structures with short-chain induced toroidal defects, as lipid sheets containing holes, and are able to spontaneously align with the long-chain lipid normal
perpendicular to the external magnetic field (Prosser et al. 2006). This unique characteristic allows the determination of high resolution protein backbone structures via NMR, as well as providing information on helix tilt angles and rotation in the lipid bilayer (Prosser et al. 2006; Park, Mrse, et al. 2006; Warschawski et al. 2011). Oriented bicelles have been used extensively to characterize the tilt and backbone structure of the Vpu transmembrane domain revealing the presence of a characteristic kinked helix, which is likely dependent on the bilayer (Park, De Angelis, et al. 2006).

The major drawback of the bicellar system is the relatively limited selection of lipids which self-arrange into bicellar structures. Currently, bicelles can be constructed of DMPC long chain lipids and another amphiphile such as CHAPSO or DHPC (Prosser et al. 2006; De Angelis & Opella 2007; Warschawski et al. 2011). Phosphorus NMR studies have shown that some level of POPC can be tolerated without loss of bicellar structures, but it must be the minor component (Triba et al. 2006). Unfortunately, the use of DMPC is not appropriate for the functional reconstitution of all protein systems, which can be highly dependent on bilayer composition. One example is the structural elucidation of pentameric phospholamban. This protein yielded different structures in differing mimetics, such as DPC (Oxenoid & Chou 2005), POPC, POPC/POPG, and DOPE/DOPC (Karp et al. 2006; Traaseth et al. 2007; Traaseth et al. 2009). This issue demonstrates the importance of membrane mimetic choice, particularly in terms of the fluidity and thickness of the lipid mimetic chosen.

1.6.5 Scaffolded lipid particles for NMR characterization of membrane proteins

Nanodisc particles are a system which combine the benefits of bicellar particle size with lipid membrane characteristics. Nanodiscs are small (10nm) lipoprotein particles created by combining modified human apolipoprotein A known as membrane scaffold protein (MSP) along with a lipid bilayer component in a circular disc of planar bilayer (Bayburt & Sligar 2003). Nanodiscs provide the means to study membrane proteins in a homogeneous aqueous solution capable of rapid molecular tumbling due to their compact size. The system has been successfully implemented to study several proteins conventionally considered large, such as 7-transmembrane receptor bacteriorhodopsin (Bayburt & Sligar 2003), membrane bound cytochrome P450 (Denisov & Sligar 2011), and CD4 (Glück et al. 2009). However, the strong helical character of MSP in nanodiscs will overwhelm CD measurements conducted on the system, as well as
absorbance measurements at 280nm, making protein quantitation more involved (Viegas et al. 2016). Furthermore, there is a strong possibility that homo-oligomeric protein forms within nanodiscs may exist by virtue of being trapped within the confined lipid area contained within the disc, affecting assembly kinetics, lateral diffusion, and the possible formation of unfavourable oligomers (Viegas et al. 2016).

Recent research has demonstrated that a similar system composed of styrene/maleic acid copolymers (SMA) can form SMA-lipid particles (SMALPs) when combined with lipid bilayers. These small (12nm) particles are analogous to nanodisc particles, but utilize a polymer rather than a protein scaffold. In SMALPs, circular dichroism measurements are easily accomplished, and the SMA polymer can be synthesized cheaply and in abundant supply (Zhang et al. 2014). Like the nanodisc system, proteins can be reconstituted into lipid particles, including bacteriorhodopsin (Swainsbury et al. 2014). Particles can be generated simply by mixing a SMA solution with liposomal suspensions for several hours’ time with gentle heating, and are relatively stable (Scheidelaar et al. 2015; Lee & Pollock 2016). SMALPs can be constructed with many lipid moieties, including native membranes, and form particles of consistent sizes which can be tuned by adjustment of the ratio of styrene to maleic acid within the copolymer (Scheidelaar et al. 2015). The drawbacks to these systems have not been well established, but given the recent rise in their utilization, details will be rapidly emerging on the benefits and drawbacks of utilizing these particles for membrane protein study.

1.7 Hypothesis and thesis outline

Since a great deal of emphasis has been placed on the putative ion channel activity of Vpu, there is an implicit assumption that Vpu must form an oligomeric assembly to fulfil this role in vivo. Understanding how the oligomeric structure of Vpu is formed will correspondingly inform our knowledge of Vpu function. To explore the determinants of Vpu oligomerization and the structure of this protein, I have three goals, explored in this thesis:

1. To determine the helix-helix interaction face which will define the orientation of each monomer within the Vpu oligomer.
2. To apply molecular dynamics simulations in bilayer-embedded, fully hydrated all-atom models of Vpu transmembrane domain oligomers to verify the importance of the helix-helix interaction face as well as study the stability of Vpu water channels.

3. To gather structural details about the Vpu oligomeric assembly by conducting solution and solid-state NMR on full-length Vpu in various membrane mimetic environments.

1.7.1 The oligomeric state of Vpu

Based upon the assumption that Vpu must form an oligomeric assembly to fulfil its role in vivo, I explored important residues for forming Vpu oligomers in membrane mimetics such as SDS, described in Chapter 2. I disrupted Vpu oligomers by selective mutation of well-conserved transmembrane domain residues Ala 7, 10, 14, and 18, ultimately concluding that these residues play a role in Vpu oligomer formation in detergent systems. I examined the oligomeric size of the Vpu-SDS complex via the use of PFG-diffusion NMR experiments, but was only able to observe particles consistent with the expected size of protein monomers in detergent micelles. With the use of DLS experiments, I found a particle which corresponded well in size to a Vpu pentamer within a detergent micelle as well as a particle which corresponded well to the mass of empty micelles in aqueous solution, suggesting that the oligomeric Vpu-SDS particles were unobservable by solution NMR methods.

1.7.2 Molecular dynamics simulations of oligomeric Vpu in bilayer membranes

By applying the use of all-atom MD simulations to lipid bilayer embedded, fully-hydrated models of Vpu transmembrane domain oligomers, I conducted an investigation of the dynamics of the complex on the sub-millisecond timescale ranging from 0 to 150 ns, described in Chapter 3. By constructing models consistent with our conclusions from the mutagenesis experiments conducted in Chapter 2, I simulated oligomers containing 4, 5, and 6 monomers without experimental constraints applied to the system aside from the force field parameters of the simulation. These oligomeric complexes did not show spontaneous formation of hydrated channels, and resulted in the collapse of channels when repeating each of these simulations with pre-constructed hydrated arrangements. I observed that the Vpu AxxAxxxAxxxA interface remained intact throughout each simulation, and concluded that the Ala 10 and 14 positions formed the most frequent contacts in this motif, finding that the AxxAxxxAxxxA motif in the
Vpu transmembrane domain is important for Vpu homo-oligomer formation within the lipid bilayer. I further concluded that the formation of a hydrated Vpu oligomeric complex is unlikely to be seen on timescales represented by molecular-dynamics simulations.

1.7.3 Structural investigation of oligomeric Vpu

I attempted to generate detailed, atomic-level observations of Vpu via the use of traditional solution-state and solid-state protein NMR techniques in SDS and DPC detergents, as described in Chapter 4, after providing detailed information on the issues associated with production of recombinant Vpu in E. coli based systems. I utilized uniformly labelled $^{15}$N-$^{13}$C Vpu samples to perform 2-dimensional solution NMR experiments in SDS, but was unable to provide unequivocal, site-specific resolution sufficient to proceed with 3-dimensional experiments to determine protein structure. I concluded that final determination of a representative structure of oligomeric Vpu will require data combined from multiple experimental techniques, or substantially altered sample preparation, as the current work was unable to reveal atomic level detail in structural models. Using $^{15}$N-$^{13}$C Vpu samples, I provided a direct comparison between the SDS, DPC, and POPC membrane mimetic systems, concluding that the data previously collected in SDS is likely representative of Vpu in lipid bilayer systems.

Altogether, I have performed mutagenesis to characterize a potential oligomerization interface of Vpu, from which I demonstrated the importance of each residue within the motif via SDS-PAGE. Based upon the conclusions from these experiments, I generated molecular models of the Vpu oligomeric complex. I further utilized MD simulations to show that these models were stable within a POPC bilayer and incapable of maintaining an aqueous pore. Finally, I utilized NMR spectroscopy to demonstrate the similarity of Vpu configurations in SDS, DPC, and POPC environments, validating the use of these membrane mimetics for Vpu characterization.
2 The oligomeric state of Vpu

2.1 Introduction

HIV-1 Vpu is a helical, single-pass transmembrane protein, proposed to form oligomeric structures to act as a monovalent cation specific ion channel in cellular membranes. While the oligomeric state of Vpu has not been accurately determined, MD simulations of tetrameric, pentameric, and hexameric Vpu have identified several possible arrangements which are consistent with the reported channel conductance levels (Krüger & Fischer 2008; Mehnert et al. 2008; Moore et al. 1998; Kim et al. 2006). In this work, pulsed-field gradient NMR experiments, gel filtration, dynamic light scattering and targeted mutagenesis experiments were used to probe the oligomeric state of Vpu in model membranes and membrane mimetics.

2.1.1 A link between Vpu function and oligomerization

Vpu oligomerization has been examined by different methods, but most extensively using MD. Vpu has been modeled as tetrameric, pentameric, and hexameric in MD simulations of the Vpu transmembrane domain (Grice et al. 1997; Kukol & Arkin 1999; Cordes et al. 2001; Cordes et al. 2002; Kim et al. 2006; Mehnert et al. 2008; Krüger & Fischer 2008; Lin et al. 2016a). The most favoured arrangements have largely been pentameric Vpu assemblies (Moore et al. 1998; Kukol & Arkin 1999; Lopez et al. 2002; Kim et al. 2006; Padhi et al. 2013). While there is a reasonable expectation that oligomeric Vpu assemblies may be required to accommodate a charged ion within the lipid bilayer, there is no demonstrated requirement for oligomers of Vpu to facilitate interaction with other proteins. This situation is highlighted by mutations which inhibit ion channel activity but have no effect on viral release, such as Vpu residue Ser 23 to Ala, or mutations such as Vpu Ala 14 or Ala 18 to glutamic acid which inhibit both viral release and Vpu interaction with host cell tetherin protein, yet without affecting ion channel activity (Bolduan et al. 2011; Vigan & Neil 2010).

Vpu forms higher-order oligomeric assemblies spontaneously. However, data from chemical crosslinking, matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectroscopy, analytical ultracentrifugation (AUC), and size-exclusion chromatography (SEC) experiments were unable to determine the precise arrangement of Vpu oligomers and thus the structure has been represented by a number of models (Maldarelli et al. 1993; Hussain et al.
Furthermore, since Vpu interaction with host cell factors requires conserved sequence motifs adjacent to the Vpu transmembrane domain (Pacyniak et al. 2005; Ruiz et al. 2008; Dubé et al. 2009; Skasko et al. 2011; Douglas et al. 2013), there is a strong possibility that helix-helix interaction motifs both within and nearby the transmembrane region could play a role in the oligomerization of the protein. Therefore, studies of the full-length Vpu protein are at lower risk of omitting important sequence motifs responsible for the functional assembly of Vpu oligomers. We have investigated the stoichiometry of full-length Vpu homo-oligomeric assemblies and the molecular basis for interaction between Vpu monomers using SDS-PAGE, pulsed-field gradient diffusion NMR, and dynamic light scattering measurements.

2.1.2 A basis for oligomerization: examining conserved Ala/Val residues in the Vpu transmembrane domain

Several genetic studies have examined the conservation of Vpu sequence in the context of pandemic HIV-1 infection, demonstrating that extensive selection is evident for some portions of Vpu (Ngandu et al. 2008). We have focused on the transmembrane domain of Vpu for its role in oligomerization within the context of full-length protein, and attempted to identify residues which may play a role in helix-helix interactions. Primary sequence analysis was used to identify conserved sets of residues within the Vpu transmembrane domain which would be likely to play a role in the monomer-monomer interactions required to form a Vpu oligomer.

Based on reported small-small motifs known to play a role in helix-helix interactions, such as the GxxxG motif in Glycophorin A (Fleming & Engelman 2001), we investigated a conserved A7xxA10xxxA14xxxA18 motif in the Vpu transmembrane primary sequence (Figure 2-1).

N-terminus – MQPIQIAIV allocations AIVVV AlVVA IIIAIVVVWSIVIIEYRK –
– ILRQRKIDRLIDRLIERAEDSGNESEISISVALVELGVELGHHAPWDVDDL – C-terminus

**Figure 2-1:** Full length Vpu amino acid sequence. Sequence representation split to indicate Vpu residues 2-32 (transmembrane domain) separately from residues 33-82 (cytoplasmic domain). Underlines represent conserved Ala residues (7, 10, 14, and 18).
This sequence is well conserved in Vpu from HIV-1 type M subtype C (while showing a limited propensity for A7G and A18T variants), which is responsible for over 50% of HIV infections worldwide (Ariën et al. 2007; McCormick-Davis et al. 2000; Bell et al. 2007). Deletion of several Vpu residues containing either the first or last two Ala residues in this motif has a negative impact on oligomerization, visualized as a shift to lower molecular weight on blue-native (BN) PAGE and loss of a discrete single Vpu band (Lv et al. 2011). In a study of subtype C Vpu isolates, each active Vpu featured an intact Ala 10, Ala 14, and Ala 18 motif; in fact, the Ala 14 to Val mutation was found to reduce viral egress activity (Douglas et al. 2013). In a study of 851 Vpu sequences collected over 10 years in 14 individuals, several were found to be stronger tetherin antagonists than the NL4-3 clone; residues within the AxxAxxxAxxxA motif were also found to be highly conserved in these isolates (Pickering et al. 2014). More recent studies of Vpu in pandemic strains have also shown invariant Ala 14 and Ala 18 residues (Iwami et al. 2015; Rossenkhan et al. 2016).

Informed by these studies, we targeted the A7xxA10xxxA14xxxA18 motif in the Vpu transmembrane domain primary sequence, utilizing mutational analysis in order to assess relative importance for Vpu homo-oligomerization. In three-dimensional models of the Vpu transmembrane domain, these Ala residues compose one face of the transmembrane helix (Figure 2-2, panel A). Another potential helical interaction face can be observed by examining the alignment of Val residues in the Vpu transmembrane helix (Figure 2-2, panel B), or by visualizing the Vpu sequence via an ideal helical wheel diagram (Schiffer & Edmundson 1967). This second potential helical interaction face could allow a two-face packing arrangement, which would be necessary for assembly of a high order oligomeric Vpu complex (Figure 2-2, panel C).

We performed conservative mutagenesis of Ala to Val at each of the Ala residues depicted in Figure 2-2 (panel A) to add a steric interference effect to a possible homo-oligomeric helix-helix packing surface in Figure 2-2 (panel D), without grossly altering the charge or hydrophobicity of the Vpu transmembrane domain.
Figure 2-2: Putative conserved amino acid motifs for helix-helix packing in Vpu. Vpu transmembrane domain monomer structure based on dihedral angles calculated from MAS ssNMR data (Sharpe et al. 2006). Trp side chain bonds are shown in stick depiction for visual reference. (A) The conserved Ala residues are depicted in blue. (B) Val residues are depicted in green, and the Ala backbone atoms (same positions as (A)) are depicted in blue ribbon. (C) Helical wheel diagram of Vpu transmembrane domain monomer, highlighting positions of A7, A10, A14, A18 (blue) and corresponding Val face (green), with C-terminal axis coming out of page. (D) Vpu transmembrane domain pentamer model featuring Ala residues as one helix-helix interaction face, depicted in blue, and Val residues as a second helix-helix interaction face, depicted in green.

2.1.3 NMR pulsed-field gradient diffusion for size measurement

To probe the size of higher order assemblies of Vpu, we utilized pulsed-field gradient (PFG) NMR techniques to measure the translational diffusion of Vpu protein in solution. To accomplish diffusion measurements, nuclei of interest can be labeled by application of a magnetic field
gradient pulse along one direction of the solution, commonly along the Z axis. After some delay to allow free molecular diffusion in solution, the same gradient pulse is applied again in reverse bias, refocusing the magnetization, and a standard NMR acquisition can be performed to observe the intensity of NMR-active nuclei in solution. During the diffusion period of the experiment, any nucleus which has not moved through the solution during the diffusion delay will contribute a signal with the same intensity (minus spin-lattice relaxation effects) as a standard one-pulse experiment (Wu et al. 1995). Nuclei which have diffused a large distance through the solution will experience a different-than-initial magnetic field associated with the reverse-biased PFG, the coherence of this magnetization will be lost; and the signal will decrease in intensity corresponding to the distance it has travelled through solution according to the following relationship:

\[ I = I_0 e^{-D \gamma^2 g^2 \delta^2 \left( \Delta - \frac{\delta}{3} \frac{\tau}{2} \right)} \]

Where \( I \) is the observed signal intensity, \( I_0 \) is the reference signal intensity, \( D \) is the diffusion coefficient, \( \gamma \) is the gyromagnetic ratio of the observed nucleus, \( g \) is the gradient strength in G/cm (0.963 G/cm to 47.187 G/cm), \( \delta \) is the gradient duration (2.5 ms), \( \Delta \) is the diffusion time (100 ms), and \( \tau \) is the delay between gradient pulses for bipolar gradients (0.0002147 s) (Wu et al. 1995).

Therefore, factors which affect the ability of a molecule to freely diffuse through a solution must be considered in order to accurately model the translational diffusion of a molecule in solution; viscosity and temperature are two important factors to be considered in these measurements. Often, an internal control molecule of known size can be included in the experimental sample in order to calibrate measurements; two common examples are acetate with 2.26 Å radius of hydration (Wagner & Gruetzmann 2005) or dioxane with 2.12 Å radius of hydration (Wilkins et al. 1999).

### 2.2 Materials and methods

#### 2.2.1 Recombinant purification of Vpu in *E. coli*

Vpu was expressed in *E. coli* (BL21-DE3-pLysS) cells from a pET-32 vector (Novagen) encoding a fusion of thioredoxin and Vpu with a His\(_6\) tag between the two. Cells were induced
with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) in lysogeny broth (LB) growth media, while shaking at 250 RPM in Fernbach flasks for a total expression time of 20 h at 16°C. Cells were harvested by centrifugation for 20 minutes at 7000x g, and frozen for later purification. Cell pellets were resuspended in 50 mM Tris-HCl with 100 mM NaCl at pH 8.0 containing Complete protease inhibitor (Roche), lysed in a cell homogenizer (Avestin) at 20 000 psi, DNAse1 was added to the lysate to a final concentration of 1mg/mL. The cell extract was clarified by centrifugation at 10 000 x g for 20 minutes, and an equivalent volume of 6 M urea was added to clarified extract to reduce precipitation of the fusion protein during later purification steps. Vpu was purified by nickel affinity chromatography, loading in 50 mM Tris-HCl at pH 8.0 with 100 mM NaCl, 3M urea and 15 mM imidazole, then washed with 30 mM imidazole, and eluted in 150 mM imidazole. Approximately 80mL of eluted fusion protein was dialyzed against 3 x 4L water and freeze-dried. Removal of the thioredoxin and His fusion partners was accomplished via chemical cleavage with cyanogen bromide as reported previously by other groups (Ma et al. 2002), utilizing a 6 M guanidine hydrochloride solution for protein dissolution, and followed by dialysis against 3 x 4L water and freeze-drying. Final purification was accomplished by reverse-phase high performance liquid chromatography using a 10 x 300 mm C8 peptide column (Vydac) and a gradient of 10% acetonitrile (AcN) to 100% isopropanol, with 0.1% trifluoroacetic acid (TFA) in the mobile phase. Solvent was removed by rotary evaporation followed by freeze-drying, with a final yield of approximately 1 mg of Vpu per L of cell growth media. Final purity was confirmed by mass spectrometry.

2.2.2 Peptide purification of Vpu transmembrane domain

Peptides of Vpu transmembrane domain residues 2-32 and 2-40 were prepared by solid phase peptide synthesis using standard 9-fluorenylmethoxycarbonyl chemistry (APTC, Hospital for Sick Children). Peptides were purified by reverse-phase high performance liquid chromatography using a 10 x 300 mm C8 peptide column (Vydac) and a gradient of 10% AcN to 100% isopropanol, with 0.1% TFA in the mobile phase. Solvent was removed by rotary evaporation followed by freeze-drying. Final product purity was confirmed by mass spectrometry.
2.2.3 Sample preparation and SDS PAGE

Samples for SDS PAGE were dissolved from lyophilized powder in 50% 2,2,2-Trifluoroethanol (TFE) and assayed for concentration using optical absorbance at 280 nm. Samples were aliquoted, dried to a thin film by evaporation, and resuspended in 2x Laemmeli buffer to a final concentration of 10 μg in 10 μL of buffer. Samples were equilibrated for 24 hours at room temperature and were then loaded onto a precast 14% acrylamide Bis-tris/MES gel (Invitrogen) and electrophoresed at 150V for 90 minutes. Gels were rinsed in water and stained with Gelcode Blue (Thermo scientific).

2.2.4 Circular Dichroism

All CD measurements were performed with a Jasco J-810 spectropolarimeter, using a 1 mm path length quartz cuvette (Hellma Analytics). Spectra were measured from 250 nm to 190 nm at a scan rate of 100 nm/min; CD spectra were recorded for samples containing 0.25 mg/mL peptide in trifluoroethanol (TFE), 2% sodium dodecyl sulfate (SDS), 2% dodecylphosphocholine (DPC) or 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) (at a 4 mol % protein relative to lipid).

2.2.5 SDS-SEC HPLC

Samples of full-length Vpu for size-exclusion chromatography (SEC) HPLC were dissolved from lyophilized powder in 50% TFE and assayed for concentration by absorbance at 280 nm. Samples were aliquoted, dried to a thin film by evaporation, and resuspended in 50 mM phosphate buffer containing 2% SDS at pH 6.8 to desired final concentrations. Samples were equilibrated for 24 hours at room temperature before 100 μL of each sample was injected onto an analytical SEC-S200 gel filtration column equilibrated in matched buffer, and run at a flow rate of 1.0 mL per minute. Retention times were monitored via absorbance at 214 nm. Experiments were performed in triplicate.

2.2.6 NMR PFG-diffusion measurements

Solution NMR experiments were conducted at 25°C on samples of Vpu reconstituted in 2% w/v SDS and 2% w/v DPC buffered with 15 mM HEPES pH 7.4. PFG diffusion experiments were conducted on a Bruker Avance III Spectrometer with a 1H Larmor frequency of 600 MHz using a 1 mm TXI Probe equipped with a Z-axis gradient, with temperature regulated at 25°C. A stimulated echo pulse sequence with bipolar gradients (Wu et al. 1995) was used for all diffusion
measurements. Curve fitting was accomplished with Wolfram Mathematica through the following equation:

\[ I = I_0 e^{-D \gamma^2 g^2 \delta^2 (\Delta - \delta - \frac{\tau}{2})} \]

Where \( I \) is the observed signal intensity, \( I_0 \) is the reference signal intensity, \( D \) is the diffusion coefficient, \( \gamma \) is the gyromagnetic ratio of the observed nucleus, \( g \) is the gradient strength, \( \delta \) is the gradient duration, \( \Delta \) is the diffusion time, and \( \tau \) is the delay between gradient pulses for bipolar gradients. Experiments were performed with a 100 ms total diffusion time \( \Delta \), and 2.5 ms gradient pulse \( \delta \), varying in strength over 32 linear increments from 0.963 G/cm to 47.187 G/cm with \( \tau \) of 0.0002147 s. The resulting 1D spectra were processed in NIH NMRPipe software (Delaglio et al. 1995), and resulting peak intensity lists were exported for Wolfram Mathematica 9.0 software in order to determine the translational diffusion coefficient, \( D \), from signal intensity at each gradient strength, using the diffusion of both acetate and dioxane as internal standards.

Experiments conducted utilizing \(^{15}\)N filtering were performed as described for previous work (Choy et al. 2002) with data collection in 1D mode. Curve fitting was accomplished with Wolfram Mathematica through the following equation:

\[ I = I_0 e^{-D \gamma^2 g^2 \delta^2 (\Delta - \delta - \frac{\tau_1 + \tau_2}{2})} \]

Experiments were performed with a 404 ms total diffusion time \( \Delta \), and 4 ms gradient pulse \( \delta \), varying in strength over 32 linear increments from 0.963 G/cm to 47.187 G/cm with \( \tau_1 \) and \( \tau_2 \) of 0.0002726 s.

2.2.7 Molecular modeling for translational diffusion coefficient calculations

To generate a model of full-length Vpu, we combined previously published structures of the Vpu transmembrane domain determined by magic angle spinning solid state NMR (Sharpe et al. 2006), and of the Vpu cytoplasmic domain from solution state NMR in DPC (Wittlich et al. 2009). A fusion of the two structures was generated in UCSF Chimera (Pettersen et al. 2004) by inserting missing intervening residues 37-39 (QRK) using ideal \( \alpha \)-helical phi/psi torsion angles and the shortest possible length of coil. This initial model was duplicated and rotated manually to produce parallel Vpu oligomers of desired size without steric clashes. In order to simulate a solvation condition in SDS micelles, these models were then supplemented with detergent
molecules using the CHARMM-GUI Micelle Builder tool, using sets of 0, 62, and 124 SDS molecules corresponding to the aggregation number of detergent monomers per micelle for each embedded oligomer (Cheng et al. 2013). The resultant models were input into WinHydroPRO v10 (García de la Torre et al. 2000; Ortega et al. 2011) to calculate the translational diffusion coefficient of each protein-detergent complex for comparison to experimentally obtained values.

2.2.8 Dynamic light scattering

Dynamic light scattering (DLS) measurements were performed utilizing 4 µL of sample on a DynaPro NanoStar instrument (Wyatt Technology) at a wavelength of 662.17 nm and a temperature of 25°C, processed using DYNAMICS software. Protein samples contained 2% SDS, 5 mM HEPES pH 7.4 with 1mg/mL Vpu. Identical measurements were performed on a dialysis-matched buffer solution without Vpu as a control.

2.3 Results

2.3.1 Vpu secondary structure in SDS micelles

Initial SDS-PAGE experiments were run with samples of full-length Vpu (residues 2-82), Vpu transmembrane domain (residues 2-32), and an extended construct of the Vpu transmembrane domain (residues 2-40) (Figure 2-3). Vpu has a highly hydrophobic transmembrane domain which represents over 25% of the full-length protein, and constructs containing the Vpu transmembrane domain migrated on SDS-PAGE with electrophoretic mobility higher than their corresponding monomer molecular weights of 9 kDa (Full-length Vpu), 3.5 kDa (Vpu 2-32), 4.6 kDa (Vpu 2-40). SDS-PAGE migration of TM peptides at greater than approximately 2 fold their molecular weight is often taken as evidence of self-association (Rath, Glibowicka, et al. 2009); in this case, the full length Vpu migrates consistently with the expected mobility of a homo-oligomer composed of approximately five Vpu subunits. Transmembrane domain Vpu 2-32 peptide migrated in a manner consistent with approximately trimeric molecular weight, but the extended transmembrane domain Vpu 2-40 peptide did not form a discrete single band on SDS-PAGE; the visible band corresponding to Vpu 2-40 is a diffuse range from 10 kDa to 20 kDa. This result is similar but not identical to previous SDS-PAGE results run on this peptide, which contained a strong band at 20 kDa and weaker diffuse staining from 4.6 kDa to 20 kDa, potentially due to different electrophoresis conditions (2x lithium dodecyl sulfate buffer at pH
8.4 rather than the sodium dodecyl sulfate at pH 6.4 in this work, and 200V rather than 150V) (Sharpe et al. 2006). Purity of Vpu was verified via mass spectrometry (Figure 2-4).

**Figure 2-3:** SDS-PAGE of full-length and transmembrane domain Vpu constructs. Samples were run in Bis-Tris/MES buffer system on a 12% acrylamide gel. Arrows indicate the approximate monomer molecular weights expected for each protein: 9 kDa (Full-length Vpu), 3.4 kDa (Vpu 2-32), 4.6 kDa (Vpu 2-40).

**Figure 2-4:** Representative mass spectrometry of full-length Vpu after purification. Mass reconstruction from 104 scans of electrospray ionization time of flight mass spectrometry, with an expected Vpu mass of 8989 Da for purified, full length Vpu 2-82 after chemical cleavage of the N-terminal Met residue.
To confirm that the secondary structure of Vpu and peptides was consistent between SDS, DPC, and POPC liposomes, we utilized circular dichroism measurements in each of these membrane mimetics (Figure 2-5). Each of the CD traces in Figure 2-5 is representative of α-helical protein secondary structure, as characterized by the absorption minima at 208nm and 222nm (Tinoco et al. 1963; Chen et al. 1974). The similarity between full-length Vpu and transmembrane domain peptides of Vpu indicate that both share α-helical secondary structure in TFE, POPC, SDS, and DPC environments. Other work with α-helical coiled-coil peptides has indicated that CD signal observed at 222nm is sensitive to helical bundling (Lau et al. 1984), and the appearance of a ratio of 222nm:208nm larger than unity in cases of coiled-coil interactions in helices (Kwok & Hodges 2004). Our data appear to conform to this trend for Vpu transmembrane domain peptides reconstituted in POPC, DPC, and SDS, but not in TFE; this may indicate that Vpu oligomerization is disrupted in TFE. Additionally, full-length Vpu protein reconstituted in POPC, but not in TFE, SDS, or DPC, exhibits a 222nm:208nm ratio greater than one, possibly suggesting an increase in propensity or stability of helix-helix interactions in POPC, although the SDS PAGE results support oligomerization of Vpu in SDS micelles.

**Figure 2-5:** CD spectra of Vpu in different membrane mimetics. (A) Full-length Vpu. (B) Vpu peptide corresponding to transmembrane domain residues 2-32.

### 2.3.2 Vpu mutations disrupt oligomerization in SDS

After performing Ala to Val mutations at residues 7, 10, 14, and 18 of full-length Vpu, we subjected the purified mutant proteins to gel electrophoresis alongside wildtype Vpu for direct
comparison (Figure 2-6). Based on the electrophoretic mobility of the major bands, the wildtype protein could be directly compared to the single point mutants. The Vpu A10V, A14V, and A18V mutations had altered electrophoretic mobility versus wildtype Vpu, as the oligomeric protein band present in the wildtype was clearly disrupted in the mutant samples. In the case of each of these mutations, no clear single band was present on SDS-PAGE gels, and diffuse staining visible within each lane occurred at a lower molecular weight than the wildtype protein. However, the A7V mutation showed no difference in electrophoretic mobility versus the wildtype protein, with a stained band appearing at the same mobility and intensity as the wildtype Vpu sample. These experiments indicate that substitutions of Ala to Val residues at positions 10, 14, and 18 in the Vpu transmembrane domain have a disruptive effect on Vpu oligomer stability, while the same substitution at position 7 shows a similar stability to the wildtype Vpu.

![Figure 2-6: SDS-PAGE of full-length Vpu and single point mutations. This image is a composite of 4 SDS-PAGE gels, each run with protein markers, Vpu wildtype protein (1mg/mL) as a positive control, and a Vpu transmembrane domain Ala to Val mutant (1mg/mL). Arrow indicates the approximate Vpu monomer molecular weight expected (9.1 kDa).](image)

To confirm our SDS-PAGE data indicating the reconstitution of a Vpu oligomeric species in detergent micelles, we used DLS to estimate the hydrodynamic radius of the Vpu-SDS complex. The dynamic light scattering experiments conducted on Vpu-SDS protein and a dialysis-matched buffer solution containing 2% SDS in 5 mM HEPES at pH 7.4 yielded computed radius of hydration (Rh) values of 0.6nm and 1.7nm for buffer, corresponding well to a single SDS
monomer and a modeled empty SDS micelle (1.73nm). DLS of Vpu-SDS protein samples yielded a computed Rh of 3.17nm, falling between the radii expected for Vpu tetramer (modelled at 3.1nm) and Vpu pentamer (modelled at 3.25nm).

**Figure 2-7:** DLS of Vpu in SDS micelles. Plot represents a fit of radius of hydration with respect to total mass observed in solution. The weighted average (by mass) of these radii corresponds to a peak of radius 3.17nm accounting for 99.7% of total mass observed in the experiment.

To confirm the mutagenesis results from SDS-PAGE experiments and to obtain a more accurate size measurement of the Vpu oligomeric complex in SDS, we performed size exclusion chromatography in the presence of detergent under the same conditions as the previous SDS-PAGE experiments. The wildtype Vpu yielded chromatographic traces in which the monomeric and oligomeric Vpu forms could be resolved, varying in proportion based on protein concentration in the injectant (Figure 2-8).
Figure 2-8: Representative separation of Vpu on a SEC-S200 size exclusion column in 2% SDS, 50 mM Tris-HCl, pH 6.8. Representative elution profiles of oligomeric (Peak A) and monomeric (Peak B) Vpu indicated versus elution time. Solid line, 1000 µg/mL Vpu, dashed line, 10 µg/mL Vpu. Curves were normalized against maximum intensity at 214nm to correct peak heights for overall concentration differences.

Repeating each of these measurements over a range of protein concentrations and normalizing the observed peak intensity versus the injectant concentration, we determined relative ratios for the oligomeric propensity of wildtype Vpu and each mutant protein (Figure 2-9).
Figure 2-9: SEC-HPLC of full-length Vpu constructs in 2% SDS, 50 mM Tris-HCl, pH 6.8. The fraction of total protein in the monomeric (open bar) or oligomeric (hatched bar) peaks are shown for (A) wildtype Vpu, (B) A7V, (C) A10V, (D) A14V, (E) A18V mutants.

The relative ratios of Vpu monomer/oligomer in Figure 2-9 indicate that the wild-type protein has the largest oligomeric peak area of all samples tested, with oligomeric species first appearing
at an injectant concentration of 10 µg/mL protein. Vpu A7V and A10V mutants required an increased sample concentration of 100 µg/mL to generate detectable oligomeric species, while the Vpu A14V and A18V mutants did not show a measurable degree of oligomerization in the SDS-SEC HPLC experiments performed. This is largely consistent with the SDS-PAGE experiments illustrated in Figure 2-6, with some exceptions. In SDS-PAGE samples at 1 mg/mL protein, the Vpu A7V mutant migrated with a similar profile to wildtype Vpu, but in SEC experiments, both Vpu A7V and Vpu A10V mutations were similar to wildtype Vpu based on the proportion of total protein eluting in the oligomeric peak on SEC (Figure 2-9). This may indicate that the A14V and A18V mutations better disrupt Vpu oligomer formation than A7V or A10V mutations. However, it should be noted that all mutations tested showed a lower propensity to oligomerize than wildtype Vpu via SDS-SEC experiments.

2.3.3 PFG-diffusion measurements of Vpu in SDS and DPC

Results from SDS-PAGE and SEC experiments were insufficient to conclusively determine the size of the Vpu oligomeric complex in SDS. Attempts to couple SEC experiments with multi-angle light scattering (MALS) experiments in 2% SDS detergent solutions proved too noisy to be applicable for this system. Previous experiments on isolated Vpu cytoplasmic domain have been conducted in DPC solution (Wittlich et al. 2009), so we elected to utilize pulsed-field gradient (PFG) diffusion NMR experiments to determine the oligomeric size of full-length Vpu in SDS and DPC solutions. Vpu protein in SDS yielded signals in NMR experiments which were compatible with a PFG diffusion study; these 1H NMR spectra are illustrated in Figure 2-10.

Peaks visible in the 1H NMR spectrum (Figure 2-10) readily identified as single peaks not corresponding to internal standards or buffer components were fit to the PFG-diffusion model (a total of 6 peaks arising from protein signals), resulting in diffusion fits illustrated in Figure 2-11.
Figure 2-10: $^1$H NMR spectra of Vpu in SDS. (A) $^1$H-detected NMR spectrum of Vpu dissolved in 2% w/v SDS, 15 mM HEPES, pH 7.4 using Watergate solvent suppression at 14.1 T. (B) $^1$H NMR spectrum from (A) expanded to illustrate detail of the 0.7 to 2.0 ppm aliphatic region. Peaks corresponding to internal standards for diffusion measurements are visible in panel B within the boxed area, at 1.8 and 2 ppm. Representative peaks arising from protein signals are indicated with arrows.

These experiments were fit to the diffusion model, but the fit values did not correspond well with expected values based upon the higher-order oligomeric band represented in SDS-PAGE. Experimental data obtained by diffusion experiments (Figure 2-11) yielded a translational diffusion coefficient fit of $9.8 \times 10^{-7}$ cm$^2$ s$^{-1}$ for full-length Vpu, corresponding most closely to models containing only detergent micelles without embedded Vpu (Table 2). These data were not in the expected size range for the Vpu oligomeric complex, even though the errors of fitting were very small ($R^2 > 99\%$). Assuming that the detergent does not disrupt native protein structure, bound detergent will still necessarily contribute some mass to the protein particle of interest, as well as altering the viscosity of the bulk solution and complicating the measurement of protein particle motion through the solution in the case of diffusion measurements. Therefore we utilized visible signals from several molecules as internal standards, including dioxane, with a measured translational diffusion coefficient of $1.099 \times 10^{-5}$ cm$^2$ s$^{-1}$ in our experiments versus $1.091 \times 10^{-9}$ m$^2$ s$^{-1}$ reported in literature (Holz et al. 2000). Unexpectedly, Vpu transmembrane domain constructs had a smaller translational diffusion coefficient than full-length Vpu. All Vpu peaks were slower-translating than peaks measured from SDS controls without protein, indicating that the measurements were not fitting empty detergent micelle signals.
Figure 2-11: Diffusion coefficient fits of Vpu NMR peaks. NMR peak intensities at each gradient strength are indicated by circles, with fit to diffusion constant represented by solid line, with value given above each plot. $R^2$ of fit > 0.99 for each fit shown. (A) Dioxane standard. (B) Vpu full length protein in 2% SDS. (C) HEPES buffer from 2% SDS control sample. (D) Vpu transmembrane domain peptide in 2% SDS. (E) SDS control in no-protein sample. (F) Vpu transmembrane domain 2-40 peptide in 2% SDS.
2.3.4 Molecular modelling of the Vpu detergent micelle

To provide a basis for data analysis in NMR diffusion and light scattering data, we generated models of Vpu in detergent micelles as predictive estimates of protein/detergent particle size and translational diffusion coefficient. To assemble these models, we combined the published structures of Vpu soluble helices 2 and 3 solved by solution NMR (in DPC) with the published structure of the Vpu transmembrane domain solved by solid-state NMR (in POPC) into a chimeric construct. Arrangements of this chimera were generated to represent monomer, dimer, trimer, tetramer, pentamer, and hexamer arrangements of Vpu, and each structure was embedded into an SDS or DPC micelle utilizing the CHARMM-GUI Micelle builder web interface (Jo et al. 2008; Cheng et al. 2013). An example of the chimeric structure of the Vpu pentamer model is illustrated in Figure 2-12, with and without SDS molecules shown.

**Figure 2-12:** Vpu pentamer models bound to SDS. (A) Oligomeric model composed of the Vpu transmembrane domain structure (Sharpe et al. 2006) combined with Vpu cytoplasmic domain structure (Wittlich et al. 2009) arranged in a symmetric pentamer. (B) The model from (A) after conjugation with 64 SDS monomers to embed the transmembrane domain within an SDS micelle utilizing the CHARMM-GUI Micelle builder web interface (Jo et al. 2008; Cheng et al. 2013).
Each arrangement of the Vpu chimeric oligomer was then processed with the HydroNMR software package in order to calculate an estimated translational diffusion coefficient (D) for each model (García de la Torre et al. 2000). These values are given in Table 2. As expected, the calculated values for D decrease with increasing molecular size; however, doubling of the number of detergent monomers per particle has little effect on the expected diffusion rate of each particle versus inclusion of additional protein monomers. This is in good keeping with NMR diffusion measurements of diacylglycerol kinase, which showed no clear correlation between diffusion and the size of detergent micelles, finding instead that the protein component dictated the size of the complex (Vinogradova et al. 1998). Therefore, for simplicity we focused on the number of protein monomers composing the complex while holding detergent constant.

Table 2: Computed translational diffusion coefficients for Vpu oligomer models. Values given are in units of cm²s⁻¹.

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<thead>
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<tbody>
<tr>
<td>No Detergent</td>
<td>Monomers/Micelle Monomers/Micelle Monomers/Micelle</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>8.75x10⁻⁷</td>
<td>8.28x10⁻⁷</td>
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<tr>
<td>1</td>
<td>9.86x10⁻⁷</td>
<td>6.57x10⁻⁷</td>
</tr>
<tr>
<td>2</td>
<td>7.37x10⁻⁷</td>
<td>5.55x10⁻⁷</td>
</tr>
<tr>
<td>3</td>
<td>6.47x10⁻⁷</td>
<td>5.62x10⁻⁷</td>
</tr>
<tr>
<td>4</td>
<td>5.83x10⁻⁷</td>
<td>5.30x10⁻⁷</td>
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<tr>
<td>5</td>
<td>5.55x10⁻⁷</td>
<td>4.99x10⁻⁷</td>
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<tr>
<td>6</td>
<td>5.21x10⁻⁷</td>
<td>4.83x10⁻⁷</td>
</tr>
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To ensure that the peaks represented in Figure 2-10 and fit in Figure 2-11 were Vpu peaks, rather than another component in solution (such as free detergent, which also gives rise to signals in the aliphatic region of the proton spectrum), we utilized ¹⁵N and ¹³C insensitive nuclei enhancement by polarization transfer (INEPT)-based experiments. These experiments have the advantage of selecting only signals contributed by the protein sample, as the protein is uniformly labelled at each amino acid residue with ¹⁵N and ¹³C nuclei, which would only be observed at levels found in the natural abundance background within other molecules in the sample. We elected to utilize experiments which take advantage of the INEPT block for magnetization transfer from protons to lower-gamma nuclei as a signal-filtering step for further NMR PFG diffusion experiments as protein peaks were clearly apparent in HSQC experiments (data not shown). Experiments conducted utilizing ¹⁵N filtering were performed as described for previous work (Choy et al.
with data collection in 1D mode. Curve fitting was accomplished with Wolfram Mathematica through the following equation:

\[ I = I_0 e^{-D \gamma^2 g^2 \delta^2 \left( \frac{\Delta - \delta}{3 \frac{\tau_1}{\tau_2}} + \frac{\tau_2}{2} \right)} \]

Experiments were performed with a 404 ms total diffusion time \( \Delta \), and 4 ms gradient pulse \( \delta \), varying in strength over 32 linear increments from 0.963 G/cm to 47.187 G/cm with \( \tau_1 \) and \( \tau_2 \) of 0.000272 s. Utilizing selective filtering of signals using INEPT-based diffusion experiments, translational diffusion constants \( D \) were refit from \( ^{15} \text{N} \)-filtered NMR data (Figure 2-13). The \( ^{15} \text{N} \)-filtered data fits were in good agreement with the original \( ^1 \text{H} \) PFG diffusion NMR data fits, yielding translational diffusion coefficients from 8.93x10\(^{-7}\) cm\(^2\)s\(^{-1}\) to 9.50x10\(^{-7}\) cm\(^2\)s\(^{-1}\). This indicates that the original data collected arise from the diffusion rate of the Vpu complex in SDS.

![Figure 2-13](image)

**Figure 2-13:** PFG Diffusion fit of \( ^{15} \text{N} \) INEPT-transfer filtered NMR signals with \( R^2 = 0.99 \). Vpu full-length sample was isotopically labelled with \( ^{15} \text{N} \) measured in 2% SDS solution.

## 2.4 Discussion

### 2.4.1 Vpu has \( \alpha \)-helical secondary structure in membrane mimetics

To assess oligomerization of Vpu in a membrane mimetic environment, we ensured that the protein was pure by subjecting protein samples to mass spectrometry (Figure 2-4). The CD spectra of Vpu full-length protein (residues 2-82) and Vpu transmembrane domain 2-32 peptides illustrated in Figure 2-5 show minima at 208nm and 222nm indicative of \( \alpha \)-helical secondary structure, similar to other Vpu studies (Njengele et al. 2016). Based on these measurements and the reported \( \alpha \)-helical native secondary structure of Vpu transmembrane domain and full-length protein based on NMR experiments, we conclude that SDS is an acceptable membrane mimetic
for investigation of the Vpu oligomeric complex (Sharpe et al. 2006; Park et al. 2003; Wittlich et al. 2009; Zhang et al. 2015). The SDS-PAGE experiment in Figure 2-3 also indicates pure protein of homogenous composition in each of our SDS-solvated samples, but the single stained band corresponding to Vpu is not consistent with electrophoresis of monomeric Vpu at molecular weight 9.1 kDa. The original work published describing Vpu erroneously indicated a 16 kDa gene product based on SDS-PAGE results (Strebel et al. 1988), although the true molecular mass of the Vpu gene product is now known to be 9.1 kDa. Anomalous migration in SDS-PAGE experiments is a documented phenomenon associated with some membrane protein sequences, and can be a consequence of unusual detergent binding modes to hydrophobic segments (Rath, Glibowicka, et al. 2009). In many cases, this results in the appearance of a smaller than actual molecular weight. In cases of membrane protein oligomerization, however, larger molecular weight species can be observed in SDS-PAGE experiments corresponding to the migration of an intact protein oligomer bound to SDS; this has been observed for the KcsA potassium channel, for example (Spelbrink et al. 2005). The SDS-PAGE results observed in our experiments are consistent with the observation of oligomeric Vpu under SDS-PAGE conditions.

Although it is not possible to conclusively determine the molecular weight of the oligomeric Vpu-SDS complex via SDS-PAGE, it is apparent that significant populations of differing oligomeric size were not observed in the Vpu wildtype sample, a result which lay in contrast to other studies (Hussain et al. 2007; Lu et al. 2010; Chen et al. 2016). This difference is also apparent when comparing full length Vpu (Figure 2-3, lane 2) with Vpu 2-40 peptide (Figure 2-3, lane 4). The Vpu 2-40 peptide contains a series of charged residues (ILRQRKID) which have been largely omitted from studies of the shorter Vpu 2-32 peptide; and given that charged residue motifs (such as EYRKLL) at the C-terminal end of the Vpu transmembrane domain have been implicated in Vpu localization, CD4 downregulation, and Tetherin interaction (Pacyniak et al. 2005; Ruiz et al. 2008; Dubé et al. 2009), it seems likely that the ILRQRKID motif may also play a role in Vpu oligomerization. In the case of Vpu 2-40 peptides, no single discrete band exists despite using highly purified peptide. While a small increase in aggregated peptide at the top of the resolving gel was visible in Vpu 2-40 samples, CD measurements were consistent with properly folded helical protein (Figure 2-5). The lack of a single discrete band in SDS-PAGE may indicate the presence of either multiple oligomeric species in solution or the possibility of on/off exchange of monomers occurring on the timeframe of the SDS-PAGE experiment. This is
in some ways supported by previous biophysical characterization of the Vpu transmembrane domain, which suggests the possibilities of tetramer, pentamer, or hexamer arrangements (Maldarelli et al. 1993; Lu et al. 2010; Chen et al. 2016). However, in contrast to the Vpu 2-40 peptide, a strong single band was evident in our SDS-PAGE experiments for full-length Vpu (Figure 2-3), similar to gel permeation experiments which revealed a stable equilibrium between pentameric and monomeric Vpu in SDS solution (Hussain et al. 2007). Reviewing these considerations, we utilized full-length Vpu protein for further biophysical study to provide the most relevant context for assessing the contribution of the AxxAxxxAxxxA motif in Vpu oligomer formation.

While SEC coupled to MALS in detergent solution was not able to resolve the size of the Vpu oligomer due to background scattering effects, DLS measurements were consistent with oligomeric species (Figure 2-7, Figure 2-12), corresponding well to SDS-PAGE experiments. After modelling chimeric Vpu oligomer structures into detergent micelles, we found that the species observable by DLS were best represented by a Vpu pentamer model embedded in an SDS micelle. Utilizing a series of models, we calculated the expected translational diffusion coefficients for Vpu oligomers, given in Table 2. However, NMR PFG diffusion fits did not correspond well to the values generated by the predictive models; the translational diffusion coefficient fit (9.8x10^{-7} \text{ cm}^2 \text{s}^{-1}) for Vpu in SDS corresponded better to the model of empty SDS micelle (8.75x10^{-7} \text{ cm}^2 \text{s}^{-1}) than the model of Vpu pentamer in SDS (5.6x10^{-7} \text{ cm}^2 \text{s}^{-1}). We investigated further utilizing $^{15}$N-selective transfer of magnetization coupled with PFG diffusion NMR, resulting in translational diffusion coefficient fits which are in agreement with our initial results, and smaller than the predicted Vpu-SDS models. We conclude that the observed Vpu signals likely correspond to Vpu monomers embedded in SDS micelles. In contrast, our SDS-PAGE data and DLS data appear to contain the bulk of observed Vpu as larger, oligomeric species.

The difference in observed species could be due to the inadvertent molecular size selectivity of each experiment. SDS-PAGE experiments and DLS experiments produce results biased towards larger species in solution; in SDS-PAGE experiments, the monomeric Vpu component has a smaller mass and lower staining sensitivity than the corresponding oligomeric Vpu component. While monomer is not visible as a strong band in our SDS-PAGE results, there is faint staining at the threshold of noise at a molecular weight consistent with monomeric Vpu (Figure 2-6). Most
SDS-PAGE results in literature with evident staining of the monomer Vpu band utilize western blotting or pulse-chase radioisotopic detection techniques which are considerably more sensitive than Coomassie staining, although none of these studies observing monomer show oligomeric Vpu bands (Strebel et al. 1988; Ewart et al. 1996; Schubert, Ferrer-Montiel, et al. 1996; Ma et al. 2002; Nomaguchi et al. 2010; Lv et al. 2011; Rollason et al. 2013; Lv et al. 2013). Similarly to our results, a study which reports Vpu oligomerization via SDS-PAGE does not contain bands corresponding to monomeric Vpu (Hussain et al. 2007). Utilizing a stain with a lower limit of detection such as silver nitrate may more firmly indicate the concentration of monomer in these experiments (Chevallet et al. 2006), but alongside higher monomer sensitivity, the oligomeric Vpu band will appear more strongly than a monomeric band due to higher mass sensitivity.

DLS experiments have a similar caveat to SDS-PAGE experiments, as DLS has greater sensitivity to larger particles than smaller particles in solution; this situation may be mitigated somewhat by use of back-scattering DLS measurements (Stetefeld et al. 2016).

In contrast to DLS, solution NMR experiments have an upper bound of approximately 25 kDa to 30 kDa based on particle correlation time without the application of specialized techniques (Frueh et al. 2013; Huang & Kalodimos 2017). If the slower correlation time of larger Vpu-SDS oligomeric particles exceeds the limit of NMR observation, it is possible that we have encountered a situation wherein monomeric Vpu in SDS micelles are easily observable by NMR, while the larger oligomeric Vpu assemblies are not. Consequently, the differences in measured sizes could indicate selective sampling of a subpopulation of Vpu molecules such as monomers in the shorter-timescale NMR experiment, while sampling a different subpopulation of Vpu complexes such as oligomers in SDS-PAGE and DLS experiments.

To verify this explanation for our experimental results, we performed SDS-PAGE on the solution NMR Vpu sample. We observed SDS-PAGE results from NMR samples identical to those illustrated in Figure 2-3. Since the Vpu sample observed by NMR bore the same oligomeric composition of that in the SDS-PAGE experiment in bulk solution, we infer that the experimental samples of SDS-Vpu oligomers may simply be too large to generate sufficient NMR signal for adequate fitting of the larger oligomeric complex. To overcome these difficulties, NMR techniques such as perdeuteration (Mal et al. 1998), high-field instruments, and selective methyl-group isotope labelling are often utilized, combined with transverse-
relaxation optimized (TROSY) experiments (Fernández et al. 2001; Page et al. 2006; Rosenzweig & Kay 2014; Huang & Kalodimos 2017).

Here, we employed the use of size exclusion chromatography experiments in SDS to bridge the gap between the SDS-PAGE/DLS results and the results from NMR based PFG diffusion. The SEC experiments resolved both a monomeric and an oligomeric component of each sample, which varied in proportion based on sample concentration (Figure 2-8, Figure 2-9).

The general observation that the oligomeric complexes of wildtype Vpu and the A7V point mutation have greater stability in SDS than that of the A10V, A14V, and A18V mutants is clear from SDS-PAGE experiments (Figure 2-6). In SEC measurements, the oligomeric complexes formed by Vpu A7V and A10V mutations were less stable than wildtype Vpu, while the A14V and A18V mutations did not feature measurable oligomerization by SEC measurements (Figure 2-9). This information is useful in illustrating the concept that Vpu Ala residues 10, 14, and 18 are important determinants of Vpu homo-oligomer complex stability in SDS detergent, and these residues are therefore likely to play a role in other membrane-like environments as well. However, the apparent differences in importance of each residue based on SDS-PAGE data (Figure 2-6) and SEC-HPLC data (Figure 2-9) regarding the oligomeric complex is difficult to reconcile. In all cases, the stability of oligomers composed of wildtype Vpu showed the greatest apparent stability, and the oligomeric complexes composed of protein harboring the A14V or A18V mutants showed the least stability. This could be an effect of the conditions present within each experiment; for instance, the timescale probed by the SEC experiment is far removed from the SDS-PAGE experiment. While the SEC experiment does take place on a similar overall timescale to the SDS-PAGE experiment (~45 minutes), the observation of the Vpu monomeric and oligomeric peaks eluting from the S200 column used occurs in 6-8 minutes (Figure 2-8), effectively sampling a time scale differing by nearly an order of magnitude from that of the SDS-PAGE experiment, in addition to sampling a range of detergent concentration nearly 4 orders of magnitude larger in protein:detergent ratio, from 1000μg/mL to 1μg/mL (Figure 2-9).

2.4.2 Alanine mutations disrupt Vpu function

The measured impact of mutations within the Vpu AxxAxxxAxxxA motif were slightly different between the SDS-PAGE and SEC experiments. In SDS-PAGE, the A10V, A14V, and A18V mutations disrupted oligomerization, while the A7V mutation did not. In SEC experiments, all
mutations exhibited an effect on oligomerization propensity, but A14V and A18V mutations had a stronger influence than A7V or A10V mutations. This corresponds well to other reports suggesting that these residues are important for Vpu interactions with host cell proteins. For instance, three of the four residues (Ala 10, 14, and 18) are important for the Vpu-mediated downregulation of CD155 in viral infection (Bolduan et al. 2014). Furthermore, our SDS-SEC analysis shows that the influence of A14 and A18 residues is more critical to Vpu oligomerization than A7V or A10V. It has also been suggested that Vpu residue A18 may be important in helix-helix interaction (Sharpe et al. 2006), and similarly that Vpu residues A14 and A18 could be important in conformation of Vpu oligomers (Vigan & Neil 2010), and our work is in good agreement with these proposed models for Vpu oligomer formation. Studies conducted prior to the discovery of a Vpu-Tetherin interaction also support the importance of the AXXXAXXXA motif to Vpu homo-oligomer formation. Early mutagenesis work on Vpu featuring truncations of the N-terminus of the Vpu transmembrane domain up to Ile residue 8 but retaining Val 9 and Ala 10 were found capable of assembling hetero-oligomeric complexes with native Vpu (Paul et al. 1998). This is well in keeping with our experimental findings, particularly if oligomer formation is important to Vpu function.

In an applied context, studies considering viral mutation over lifetime infection of 18 people who had contracted HIV-1 showed few Vpu mutations in the conserved AXXAXXXAXXXA motif – and if present, mutations were largely changes of Ala to Val with accompanying Ile to Ala or Val to Ala in an adjacent residue (Gondim et al. 2012), conserving the overall form of the motif. This is strong evidence that this motif is important for Vpu function in vivo, complementing our biophysical experiments in membrane mimetics.

While much of the recent literature on Vpu function has featured its interaction with other host cell factors, it is difficult to disentangle studies of Vpu oligomer formation from studies of Vpu-protein interactions. For instance, in early Tetherin-Vpu interaction studies, mutation of Vpu residue Ala 14 to Leu resulted in a loss of Tetherin inhibition equivalent to total deletion of Vpu, although this mutation still has the ability to downregulate human CD4 protein (Vigan & Neil 2010). Interestingly, the authors report a continued ability of Vpu to oligomerize in cellular membranes, but the stability or configuration of that arrangement could not be determined. Studies have also implicated the importance of residues Ala 14 and Ala 18 for Tetherin interaction based on sequence comparison, predicated on the premise that “only pandemic group
M viruses evolved fully functional Vpus that degrade CD4 and counteract human tetherin with high efficiency” (Sauter et al. 2012). There is one known sequence of an N-type HIV Vpu which possesses anti-tetherin activity, and while this sequence does not contain the Ala 7 residue in the AxxAxxxAxxxA motif, Ala 10, Ala 14, and Ala 18 are all present (Delaugerre et al. 2011; Sauter et al. 2012). Further, reciprocal changes to generate a functional Vpu from a chimpanzee strain of HIV also required the conversion of equivalent E15A and V19A (a one residue number offset from type N Vpu) in order to antagonize human Tetherin protein (Sauter et al. 2012), highlighting the essential requirement for these residues in the Vpu-Tetherin interaction.

Because the functions of Vpu and the AxxAxxxAxxxA motif are closely related, identifying the potential oligomer-formation role of the motif separately from the Vpu-protein interaction role of the motif may not be a feasible objective. Taken together, our results agree with reports that these residues are key for Vpu function, under the assumption that the functional unit of Vpu is the oligomeric arrangement. However, unlike many previously reported works, our oligomeric assemblies seem to be primarily composed of a single oligomeric species. To explore the oligomeric assembly of Vpu in greater detail, we turned to the use of MD simulations for further investigation.
3 Molecular dynamics simulations of oligomeric Vpu in bilayer membranes

3.1 Introduction

Vpu can form oligomers via its conserved membrane spanning domain (Maldarelli et al. 1993). Early work characterized Vpu as having cation-selective ion channel activity (Ewart et al. 1996), leading to the classification of Vpu as a viroporin protein. This work also showed that scrambling the Vpu transmembrane domain sequence prevented ion channel conductance and enhancement of virion release (Schubert, Ferrer-Montiel, et al. 1996), leading to the model that Vpu ion channel activity is linked to enhancement of viral release.

Much of the detailed knowledge related to Vpu oligomerization, ion channel activity, and small molecule interaction has been generated with the utilization of MD. Early 100ps Vpu simulations featured Vpu tetramers, pentamers, and hexamers, assembled in vacuo and utilizing spatial restraints to maintain oligomers suggested a pentamer for the likely arrangement of Vpu ion channels (Grice et al. 1997). 1.0 ns simulations performed using an octane slab in place of the lipid bilayer resulted in expulsion of water from the pore of pentameric Vpu models, and provided an indication that these models lost symmetry and became oblong during the simulation (Moore et al. 1998). Based upon site-specific FTIR dichroism measurements of Vpu transmembrane domain helix tilt in mechanically aligned bilayers, pentameric models best fit Vpu (Kukol & Arkin 1999).

Following these studies, simulations were conducted in explicit lipid bilayer models; 2 ns of MD simulation time were reported with Ser residues located in the Vpu pentamer pore (Cordes et al. 2001), followed by 3 ns simulations indicating pentamer models as the most stable arrangement, featuring water expulsion from the assembly after 1.5 ns of simulated time (Lopez et al. 2002). Vpu pentamers simulated for 12 ns in the presence of HMA highlighted the potential importance of Ser 23, and the resulting models showed this residue to be oriented within the Vpu oligomer, while Trp 22 faced the lipid bilayer (Lemaitre et al. 2004). Subsequent small-molecule docking studies (using AUTODOCK) showed hydrogen bond formation between the Ser 23 hydroxyl group and HMA guanidine moiety within the pore (Kim et al. 2006). Steered MD simulations indicated that pentameric Vpu ion channels were weakly selective for cation conductance, associated with formation of a hydrated pore with a Ser-in configuration (Patargias et al. 2006).
All the above simulations have primarily focused on a pentameric Vpu oligomer, with particular emphasis on Ser 23 as a candidate for interactions with HMA and on the combination of Ser 23 and Trp 22 as possible sites for gating Vpu ion channel conductance. By contrast, the clearest measurements of ion channel behavior in Vpu transmembrane domain mutants show that conserved Trp 22 is not required for ion channel activity or gating, while Ser 23 was critical for ion channel activity (Mehnert et al. 2008). These studies also showed that Vpu is biased towards conducting cations (Li\(^+\), Na\(^+\), K\(^+\), Rb\(^+\), Cs\(^+\)), albeit not exclusively so, concluding that Vpu bundles exhibit ion conductance driven by diffusion, with significant pore characteristics, without gating by residues that mediate conductance (Mehnert et al. 2008). Expression of Vpu in *S. cerevisiae* and *E. coli* has been used as a means to permeabilize membranes to K\(^+\) ions by allowing the ions to move down a gradient (Herrero et al. 2013; Taube et al. 2014), which may suggest that Vpu is forming a poorly selective channel or perhaps a simpler pore. In this type of model, conductance measurements could be a byproduct of Vpu weakening the membrane and allowing sporadic breakdown of an ion potential across the membrane, rather than a specific gating event.

MD simulation work conducted after the discovery of the Vpu-tetherin interaction has featured different arrangements of Vpu monomers in the oligomeric complex. A study featuring 10 ns replica exchange experiments of Vpu transmembrane domains in POPC showed 4, 5, and 6-mer arrangements were all possible, with a pentameric model once again the most stable arrangement for Vpu oligomers (Padhi et al. 2013). Here, the proposed Tetherin-interacting Ala 14, Ala 18, and Trp 22 residues are facing outward from the helical bundle, available to interact with Vpu binding partners (Padhi et al. 2013). This study was expanded to include Vpu pentamer ion channel simulations: a remarkable 661 short 200ps simulations with Na\(^+\) and K\(^+\) ions inside the Vpu pore (Padhi et al. 2014), resulting in a model wherein a single ion could transit the Vpu pore by interacting with Ser residue 23. In this work, the ion retained its hydration shell within the pore, but many waters were stripped away from the ion during pore transit (Padhi et al. 2014).

Other recent MD work showed that Vpu Ala residues within the AxxAxxxAxxxA sequence are important for the sequential formation of dimer and trimer interfaces, wherein Ala residues packed against nearby neighbouring Val residues. Furthermore, to generate a circular bundle as featured in nearly all previous Vpu oligomer simulations many constraints were required (Li et al. 2013a). This study found that the previously prevailing “Ser-in” oligomeric arrangement of
Vpu was not the lowest energy structure, requiring instead a significant rotation of each monomer, and that a water column was not formed across the membrane even after 25 ns of simulation (Li et al. 2013b). Assembly of tetrameric and pentameric models around cations showed the lowest energy in tetrameric models, with AxxAxxxAxxxA facing inwards, and Ser 23 and Trp 22 facing out towards lipid (Li et al. 2013b). A follow-up coarse-grained simulation of Vpu monomers in POPC found Vpu oligomeric assemblies containing 2 to 6 Vpu monomers form spontaneously (Lin et al. 2016a). Only tetramers formed regular pore-like structures with Ser 23 facing inwards, while pentamers and hexamers were observed forming irregular patches within the bilayer simulations along with low concentrations of Vpu monomer; in systems containing more Vpu particles, these patches were capable of aggregating to a final size of 16 - 32 Vpu monomers (Lin et al. 2016a).

Given the inconsistencies between these previous MD studies of Vpu, and the irreconcilable differences between several of these Vpu oligomer models, we have performed unconstrained simulations of Vpu in multiple oligomeric arrangements in explicit all-atom simulations. Our MD models of Vpu oligomers were informed by our mutational analysis of important residues for Vpu oligomerization, as outlined in Chapter 2. We reduced bias by explicitly modelling all lipid, water, and salt molecules to solvate the Vpu transmembrane domain with no other simulation constraints and allowing the time evolution of the system under the CHARMM22 force-field system (MacKerell et al. 1998).

In previous studies, 100 ns was the longest simulation performed upon the Vpu transmembrane domain system, which in itself was three orders of magnitude longer than early simulations (Li et al. 2013a; Grice et al. 1997). At 150 ns, our current MD simulations are the longest all-atom trajectories obtained to date for the Vpu transmembrane domain. The results of these simulations support our previous observation that the conserved AxxAxxxAxxxA motif was important for Vpu oligomer stability, and counter to the results of Padhi et al., suggest that the proposed lumen of the Vpu pore rapidly lost internal water molecules and remained dehydrated for the remainder of the simulation. These data also support recent hypotheses that Vpu is in fact neither a channel nor a pore, but instead allows ion flux across the membrane due to local destabilization.
3.2 Materials and methods

3.2.1 Vpu transmembrane domain oligomer model generation

Vpu 2-32 peptides were chosen for molecular dynamics simulations to reduce computational complexity in all-atom simulations, with the knowledge that this portion of the Vpu sequence forms Vpu oligomers (Hussain et al. 2007; Lu et al. 2010), which have also been shown to supporting viral secretion (but not CD4 downregulation) and exhibit ion channel conductance (Schubert, Bour, et al. 1996; Schubert, Ferrer-Montiel, et al. 1996; Mehnert et al. 2008). Vpu2-32 model oligomers were generated using CNS helical interactions (CHI) scripts for CNS (Phillips et al. 2005; Adams et al. 1995). Parameters were used to generate homo-oligomeric pentamer models of α-helical Vpu transmembrane domain sequence in an environment of dielectric constant 2.0. Refinement was accomplished by symmetric rotation in 10° increments to a total of 360°, generating 4 structures per trial after energy minimization in each configuration. Structural clusters were defined with a minimum of 10 structures sharing a maximum backbone RMSD of 1.0 Å.

3.2.2 Vpu transmembrane domain oligomer model equilibration

The final Vpu2-32 model oligomers generated by CHI were subject to further refinement by all-atom simulations in Not (just) Another Molecular Dynamics program (NAMD) software (Phillips et al. 2005). This was accomplished by following published protocols freely available from the NAMD website for membrane protein insertion and equilibration (http://www.ks.uiuc.edu/Training/Tutorials/science/membrane/mem-tutorial.pdf). NAMD was developed by the Theoretical and Computational Biophysics Group in the Beckman Institute for Advanced Science and Technology at the University of Illinois at Urbana-Champaign (Phillips et al. 2005), available at http://www.ks.uiuc.edu/Research/namd/. In brief, model generation was accomplished utilizing the PDB coordinates of Vpu2-32 pentamer generated by CHI, which were used as a basis to generate a protein structure file (PSF) compatible with NAMD. All simulations performed in NAMD featured a full atomistic treatment of the model system composed of Vpu 2-32 oligomer, POPC bilayer (130-140 molecules), and water (8 200 molecules) containing 0.2M NaCl for a total of 40 000 to 50 000 atoms. All modeled components were simulated within an infinitely repeating periodic boundary configuration to avoid edge effects within the system. System construction was accomplished by steps; first, the Vpu 2-32 oligomer was
inserted into a crystalline POPC model lipid bilayer, and 500 ps of MD was performed at a
temperature of 310 K with restrained protein and water motion to equilibrate and melt lipid tails.
At this point, a further 500 ps of MD was performed under the same conditions, this time
allowing both the water and POPC to further equilibrate. Finally, 500 ps of MD was performed
with no atoms restrained within the modelled system to minimize and equilibrate the protein
molecules within the POPC/water solvent environment. In pre-hydrated models, the same initial
arrangement of monomers was utilized as a basis structure after insertion into the crystalline
POPC model lipid bilayer. In these models, the SOLVATE software written by Helmut
Grubmüller and Volker Groll at University of Munich (available at
http://www.mpibpc.mpg.de/grubmueller/solvate) was utilized to generate a column of fluid water
within the oligomeric central space and perform a local minimization of the water molecule
position. The water column within the oligomeric models was restrained along with the protein
until the final 500 ps of MD equilibration was performed prior to beginning production runs.

3.2.3 Vpu transmembrane domain oligomer molecular dynamics
simulations

Production runs performed on the Vpu oligomer assembly utilized the CHARMM22 force field
with TIP3P water model and the SETTLE algorithm (MacKerell et al. 1998), in NpT
configuration via the Nosé-Hoover method (Martyna et al. 1994) at a pressure of 1.01325 Bar
utilizing Langevin dynamics (Feller et al. 1995) with constant lipid area after completion of
minimization. These equilibrium production simulations were completed utilizing NAMD 2.9 on
the SciNet supercomputing cluster in parallel runs on 64 cores with InfiniBand connections
utilizing OpenMPI libraries, particle-mesh Ewald (PME) electrostatics (Darden et al. 1993) and
Lennard-Jones potentials, with increments of 2 fs computed time per step of simulation for a
total of 150 ns of simulated time. Simulations were conducted on model systems composed of
tetrameric, pentameric, and hexameric Vpu transmembrane domain assemblies both with and
without pre-constructed hydrated pores within the centre of the Vpu oligomer in each case, for a
total of 6 systems, each simulated for 150 ns of simulated time.

3.2.4 Analysis of molecular dynamics simulations on Vpu oligomers

Data analyses were performed utilizing the MDAnalysis toolkit (Michaud-Agrawal et al. 2011).
When utilizing MDAnalysis, Python scripts were written to analyze root-mean squared deviation
(RMSD) of backbone Cα residues utilizing minimization and alignment code included as an RMS module of MDAnalysis (Theobald 2005; Liu et al. 2009). Calculation of root-mean squared fluctuations (RMSF) of backbone Cα residues were also performed on a per-residue basis to provide information pertaining to the mobility of each residue within the Vpu transmembrane helix over the simulated time course. The number of waters in residence at each Vpu oligomeric pore was determined by measuring the number of unique water residues within a cylindrical volume 15 Å in radius, 14 Å height, centred upon the centre of mass of the Vpu transmembrane domain oligomeric bundle at residue 16. The calculation of Vpu monomer-monomer contacts was determined by observing the number of residues from a different monomer within 5 Å of each Ala of interest on each simulated monomer for each timestep of each simulation. The number of total intermolecular contacts per Ala residue of interest was recorded for each simulated system and plotted.

3.3 Results

3.3.1 Initial construction of Vpu oligomeric assemblies

A system of MD simulations was set up to explore water exposure to the Vpu transmembrane domain. Models were generated first using CHI software (CNS searching for helical interactions), an add-on module for the crystallography and NMR suite (CNS) (Adams et al. 1995). CHI searching of the pentamer Vpu transmembrane domain yielded 6 possible configurations; 3 left-handed helical bundles, and 3 right-handed helical bundles (Figure 3-1).
Figure 3-1: Modelled lowest-energy configurations of Vpu transmembrane domain output from CHI software. Averaged structures from CHI shown for each configuration. Vpu A7, A10, A14, and A18 residues coloured blue, W22 coloured gray in stick representation for visual reference. (A-C) left-handed pentamer arrangements. (D-F) right handed pentamer arrangements.

Of these CHI lowest-energy configurations, one left and one right handed bundle featured the Vpu Trp residue in the interhelical interface (Figure 3-1, B and F), while the other 2 left handed bundles had a Trp-in arrangement (Figure 3-1, A and C). The other 2 right handed bundles had a Trp-out arrangement relative to the central space between Vpu monomers (Figure 3-1, D and E). NMR studies of Vpu in rotationally aligned samples as well as MAS experiments have yielded data which are most consistent with a right-handed Trp-out arrangement (Park et al. 2003; Lu et al. 2010); the only arrangement to feature a Trp-out helical bundle where conserved Ala residues 7, 10, 14, and 18 were involved in the interhelical interface was right handed (Figure 3-1, D); this arrangement (Figure 3-2) was examined further using explicit all-atom simulations.
Figure 3-2: Vpu transmembrane domain CHI cluster 4 in ribbon style. Vpu A7, A10, A14, and A18 residues coloured blue, W22 coloured gray in stick representation for visual reference. (A, C) model averaged from the 17 lowest-RMSD structures in this arrangement generated by CHI modelling, next to (B, D) overlay of all structures in this arrangement.
3.3.2  MD simulations of Vpu pentamer models

The system was simulated for a total of 150 ns time, resulting in convergence of RMSD values after approximately 40 ns of simulated time (Figure 3-3). The simulation was completely unconstrained aside from supplying the initial conformation. In the final Vpu helical bundle arrangements at the end of simulated time, conserved Trp residues were found to face the lipid environment, similar to the initial orientation, and Ala residues 7, 10, 14, and 18 largely remained arranged at helix-helix interaction faces, packing closely to Val residues in each adjacent monomer within the complex, also similar to the initial orientation (Figure 3-4, supported by intermolecular distance analyses described below). The final arrangement of helices at time 150 ns shows an increase in the helical tilt angle of each monomer within the oligomer from 13.2° to 24.8° relative to the bilayer normal.
Figure 3-3: RMSD of Vpu pentamer peptide backbone residues. Deviation of backbone residues from original starting positions plotted against time for 150 ns of MD simulation for each monomer in the Vpu oligomeric assembly (coloured) and overall average (black).
**Figure 3-4**: Snapshots of Vpu pentamer Ala residues throughout 150 ns MD trajectory. Side views presented above top views in: (A, D) initial configuration at time 0 ns, (B, E) configuration after 15 ns of simulated time, (C, F) final model after 150 ns of simulated time. Phospholipid headgroups indicated in gold, with Vpu Ala side chains indicated in blue corresponding to residues 7, 10, 14, and 18, Val side chains indicated in green corresponding to residues 9, 12, 13 and 20, and Trp residue 22 in space filling representation for visual reference. Water molecules and lipid tails have been omitted for clarity.

### 3.3.3 Molecular dynamics simulations exclude water from the Vpu pentamer transmembrane domain

To better assess the possibility that the Vpu ion channel is a feature of the oligomeric complex that was not sampled during the simulated time course, we repeated the simulation from a modified starting condition which included a manually generated water channel through the centre of the Vpu transmembrane oligomer. This hydrated oligomeric arrangement was subject to the same conditions for the same simulated time duration as the original Vpu pentameric model. During the simulation time course, the system had once again converged to an equilibrated RMSD after the 55 ns timepoint relative to the starting model (Figure 3-5).
Figure 3-5: RMSD of Vpu pentamer channel peptide backbone residues. Deviation of backbone residues from original starting positions plotted against time for 150 ns of MD simulation for each monomer in the Vpu oligomeric assembly (coloured) and overall average (black).

A quantitative measure of the extent of hydration within the Vpu pentameric channel was determined by counting the number of water molecules present within the putative channel with respect to simulated time. This was accomplished by enumerating the water molecules wholly or partially present in a cylindrical volume of radius 15 Å and height 14 Å centered about the transmembrane segment’s oligomeric centre of mass, as illustrated in Figure 3-6.
Figure 3-6: Snapshot of pore water selection volume within Vpu oligomer models. To assess which Vpu models contained water within the putative channel domain, all water molecules within a cylinder of height 14 Å and radius 15 Å centred at the centre of mass of Vpu transmembrane domain at residue 16 was utilized. To illustrate radius of coverage, a narrow band is shown at (A) time 0 ns, and (B) time 150 ns superimposed upon the hydrated Vpu pentamer model. For a more complete representation, the total cylindrical volume utilized is illustrated for the hydrated Vpu pentamer at (C) 0 ns, and (D) 150 ns. Water is illustrated in in red/white space filling representation, with phosphate in gold for reference to lipid bilayer limits. Lipid tails have been omitted for clarity.

The number of water molecules found within the putative pore with respect to simulated time was determined by this method for both the pre-hydrated pentameric model and the non-hydrated helical bundle model for the full simulated time course of 150 ns (Figure 3-7).
Figure 3-7: RMSD of Vpu pentamer peptide backbone residues plotted alongside pore water occupancy. Deviation of backbone residues from original starting positions plotted against time for 150 ns of MD simulation for each monomer in the (A) Vpu pentamer model or (C) hydrated Vpu pentamer model for 150 ns of MD simulation. The number of water molecules residing within the (B) pentameric Vpu transmembrane domain (D) or hydrated pentameric Vpu transmembrane domain plotted against time for each model.

Examining the time course of the pentameric model, water within the Vpu oligomeric complex is sparse. In the first 10 ns of each simulation, expulsion of water was observed, coincident with a large change in backbone RMSD (Figure 3-7, panels C and D). Following this change, water occupancy within the Vpu oligomer remained below 2 molecules for the remainder of the simulation time course (Figure 3-7, panels B and D). Visualizing snapshots of the models of the Vpu oligomer over time, three distinct states can be observed (Figure 3-8).
Figure 3-8: Snapshots of water molecules within a pre-hydrated pentameric Vpu channel. (A, D) 0 ns / Initial model. (B, E) 15 ns simulated time. (C, F) 150 ns simulated time. Water is illustrated in red/white space filling representation, with phosphate in gold for reference to lipid bilayer limits. Lipid tails have been omitted for clarity, as have protein representations in panels D, E, F.

The structure of the hydrated Vpu pentamer model was relatively unchanged by the pre-equilibration procedure used to equilibrate the system prior to the MD simulation, and the water column was still present at the centre of the Vpu oligomer at the start of the production run (Figure 3-8, panels A, D). Because this model was specifically generated to test the stability of the hydrated Vpu channel assembly, it should be considered as intentionally biased towards the water-channel arrangement. Within 40 ns of simulated time, the water molecules had been expelled from the centre of the complex, and the Vpu monomers within the complex had formed a collapsed/dehydrated helical bundle (Figure 3-8, panels B, E). From this point until the end of the simulation, no further formation of a hydrated channel was observed, nor were any large-scale rearrangements within the Vpu helical bundle (Figure 3-8, panels C, F), with helix tilt angles increasing from 13.2° to 23.8°. At the final condition after 150 ns of simulation time a similar arrangement of helices was observed when compared to the pentamer simulation originally conducted without water (Figure 3-4), resulting in a more compact, flattened, dehydrated helical bundle relative to the symmetric starting configuration (Figure 3-9).
Figure 3-9: Snapshots of Vpu pentamer models. The pentamer (A) and hydrated pentamer (B) models are shown at starting, intermediate (15 ns), and final (150 ns) times in the MD simulations. The protein component of each simulation is illustrated in side-on and top-down views with Trp residue in space-filling style for reference, while omitting lipid, water, and ions for increased clarity.
Analysis of interhelical distance measurements within the Vpu pentamer models showed that while individual monomers had variable (9 Å to 12 Å) interhelical distances and total contacts (150 to 300) over time, the average interhelical distance remained largely stable between 10 Å and 11 Å, as did the average total interatomic contacts, between 200 and 250 (Figure 3-10).

The plots in Figure 3-10 represent the distance between the centre of mass of each individual Vpu transmembrane domain monomer to its nearest neighbor within the oligomeric bundle. Despite a steady average in the interhelical distances, there were notable departures from the average value; a large increase in interhelical distance was observed for the hydrated pentamer model to 15 Å for helix 1 (Figure 3-10, panel C) which was accompanied by a change in backbone RMSD (Figure 3-3) without a change in number of total interhelix contacts (Figure 3-10, panel D).

Overall, the pentameric models featured a shift from a highly symmetric toroidal starting model into a collapsed helical bundle over the first 20 ns of simulation time, but retained many other characteristics of the original model including average interhelical distance, average number of total interhelical contacts, and orientation of monomers within the bundle relative to the surrounding lipid (Figure 3-9 and Figure 3-10). After initial collapse and loss of geometric symmetry, the pentameric helical bundle remained largely unchanged for the remainder of the simulation.
Figure 3-10: Interhelical distances and total contacts between monomers of Vpu pentamer models with respect to time. Interhelical distance between adjacent monomers is plotted against time for (A) Vpu pentamer model or (C) hydrated Vpu pentamer model for 150 ns of MD simulation. The number of interhelical contacts measured between adjacent Vpu monomers is plotted against time for (B) Vpu pentamer model or (D) hydrated Vpu pentamer model. In all cases, the plots were averaged over a 5 ns window to improve clarity. Coloured lines represent measurements for individual monomers within the oligomer, while black lines indicate the average value for the oligomeric complex.
3.3.4 MD simulations of Vpu tetramer models

Like the pentameric Vpu model, MD simulations of tetrameric Vpu models exhibited a converged backbone RMSD after approximately 30 ns (Figure 3-11, panel A), and the convergence of the hydrated tetramer backbone RMSD also occurred after 30 ns of simulated time with the exception of a single-monomer rearrangement after approximately 60 ns (Figure 3-11, panel C). No more than 2 water molecules were found within the tetramer model transmembrane region during the 150 ns of simulation time (Figure 3-11, panel B), while helical tilt increased from 7.0° to 20.6°. In the hydrated tetramer model, the majority of water molecules were also excluded from the core of the complex by approximately 30 ns of simulated time (Figure 3-11, panel D) with the exception of two to three water molecules that were found to occupy the transmembrane region throughout the entire simulation. The plots of interhelical distance in tetrameric Vpu models showed the most stability with respect to time and an average interhelical distance of 10 Å to 12 Å (Figure 3-12).
Figure 3-11: RMSD of Vpu tetramer peptide backbone residues plotted alongside pore water occupancy. Deviation of backbone residues from original starting positions plotted against time for 150 ns of MD simulation for each monomer in the (A) Vpu tetramer model or (C) hydrated Vpu tetramer model for 150 ns of MD simulation. The number of water molecules residing within the (B) tetramer Vpu transmembrane domain (D) or hydrated tetramer Vpu transmembrane domain plotted against time for each model.
Figure 3-12: Interhelical distances and contacts between monomers of Vpu tetramer models with respect to time. Interhelical distance between adjacent monomers plotted against time for (A) Vpu tetramer model or (C) hydrated Vpu tetramer model for 150 ns of MD simulation. The number of total interhelical contacts measured between adjacent Vpu monomers is plotted against time for (B) Vpu tetramer model (D) or hydrated Vpu tetramer model. In all cases, the plots were averaged over a 5 ns window to improve clarity. Coloured lines represent measurements for individual monomers within the oligomer, while black lines indicate the average value for the oligomeric complex.

Several states from the hydrated Vpu tetramer model are presented in Figure 3-13; the initial hydrated model, as well as the trapped water molecules within the core of the model at later times. The final arrangement of the Vpu tetramer and hydrated tetramer models were largely devoid of water, and formed collapsed protein bundles of 4 Vpu monomers (Figure 3-14), with helical tilt increased from 7.0° to 13.2°.
Figure 3-13: Snapshots of water molecules within a pre-hydrated tetrameric Vpu channel. (A, D) 0 ns / Initial model. (B, E) 25 ns simulated time. (C, F) 150 ns simulated time. Water is illustrated in in red/white space filling representation, with phosphate in gold for reference to lipid bilayer limits. Lipid tails have been omitted for clarity, as have protein representations in panels D, E, F.

Close inspection of the hydrated tetramer model revealed a rotation of a single helix to a Trp-in configuration, which trapped 3 water molecules during the initial collapse of the helical bundle, explaining the appearance of these molecules in pore water plots (Figure 3-11, panel D) and snapshots (Figure 3-13).
Figure 3-14: Snapshots of Vpu tetramer models. Vpu helical bundles of (A) tetramer and (B) hydrated tetramer models illustrated at starting, intermediate, and finishing positions in MD simulations. The protein component of each simulation is illustrated in side-on and top-down views with Trp residue in space-filling style for reference, while omitting lipid, water, and ions for increased clarity.
3.3.5 MD simulations of Vpu hexamer models

Simulations featuring a hexamer Vpu arrangement converged to a consistent backbone RMSD within 10 ns of simulated time, although in this model we observed a higher water occupancy within the transmembrane region than all other models over the entire time course (Figure 3-15, panels A, B). Hexamer helical tilt angles increased from 13.4° to 18.2°, while interhelical distances averaged between 10 Å and 11 Å (Figure 3-16, panel A). The hexameric Vpu bundle featured the presence of up to 6 transient water molecules near the top of the helical bundle and lipid headgroups, close to residues Ser 23, Ile 24, and Val 25, resulting in the highest apparent water occupancy over time of all simulations. Despite these interactions, water molecules were not found to form a continuous channel during the simulation, and no water molecules were observed within the transmembrane region hydrophobic core.

The hydrated hexamer model converged to a largely steady RMSD after 40 ns of simulated time, along with the loss of most water molecules from within the transmembrane region (Figure 3-15, panels C, D). A steady average in the interhelical distance was observed for the hexamer model (Figure 3-16, panel A), while in the hydrated hexamer model two helices progressively departed from the average value with increased interhelical distances as time evolved (Figure 3-16, panel C). This increase in interhelical distances was accompanied by a corresponding loss in total interhelix contacts for one of these monomers, but did not result in a large change to the average number of interhelix contacts measured for the entire hydrated hexameric model (Figure 3-16, panel D). Helical tilt in the hydrated hexamer increased from 13.4° to 18.2°. A series of snapshots from the time course of the hydrated hexamer model can be visualized in Figure 3-17.
Figure 3-15: RMSD of Vpu hexamer peptide backbone residues plotted alongside pore water occupancy. Deviation of backbone residues from original starting positions plotted against time for 150 ns of MD simulation for each monomer in the (A) Vpu hexamer model or (C) hydrated Vpu hexamer model for 150 ns of MD simulation. The number of water molecules residing within the (B) hexamer Vpu transmembrane domain (D) or hydrated hexamer Vpu transmembrane domain plotted against time for each model.
Figure 3-16: Interhelical distances and contacts between monomers of Vpu hexamer models with respect to time. Interhelical distance between adjacent monomers plotted against time for (A) Vpu hexamer model or (C) hydrated Vpu hexamer model for 150 ns of MD simulation. The number of total interhelical contacts measured between adjacent Vpu monomers is plotted against time for (B) Vpu hexamer model (D) or hydrated Vpu hexamer model. In all cases, the plots were averaged over a 5 ns window to improve clarity. Coloured lines represent measurements for individual monomers within the oligomer, while black lines indicate the average value for the oligomeric complex.
**Figure 3-17:** Snapshots of water molecules within a pre-hydrated hexameric Vpu channel. (A, D) 0 ns / Initial model. (B, E) 25 ns simulated time. (C, F) 150 ns simulated time. Water is illustrated in red/white space filling representation, with phosphate in gold for reference to lipid bilayer limits. Lipid tails have been omitted for clarity, as have protein representations in panels D, E, F.
Figure 3-18: Snapshots of Vpu hexamer models. Vpu helical bundles of (A) hexamer and (B) hydrated hexamer models illustrated at starting, intermediate, and finishing positions in MD simulations. The protein component of each simulation is illustrated in side-on and top-down views with Trp residue in space-filling style for reference, while omitting lipid, water, and ions for increased clarity.
3.3.6 Summary: MD simulations of Vpu oligomer models

In total, 150 ns MD simulations were performed on tetrameric, pentameric, and hexameric bundles of Vpu monomers, each within a POPC bilayer in a fully hydrated system. In all simulations, the outcome was similar; the Vpu oligomer in the starting arrangement collapsed into a largely dehydrated helical bundle, regardless of whether or not a water column had been constructed in the centre of the oligomer prior to the start of simulation. All the models we described in this work shared many common structural features; the conserved Trp 22 residue facing outwards from the helical bundle, a high number of interhelical contacts observed between conserved Ala residues and neighbouring Vpu helices, and the exclusion of water from the core of the Vpu bundle. In all our models, Vpu oligomers began as symmetric circular arrangements, and in every case each arrangement formed a more compact bundle of Vpu helices (Figure 3-9, Figure 3-14, Figure 3-18). Additionally, despite high stability of each complex after initial collapse of each symmetric model into a helical bundle, the resulting bundles are not completely homogenous. In the case of the tetrameric hydrated model, a single Trp residue was observed to turn “inwards” to the core of the Vpu bundle, trapping two water molecules for the remainder of the simulation (Figure 3-11, Figure 3-13). In another case, the hydrated hexamer model features a slow “fraying” of a single Vpu monomer N-terminus from the outer edge of the flattened oligomeric bundle, most apparent in interhelix contact plots (Figure 3-16) and trajectory snapshots (Figure 3-18), with a concomitant increase in monomer backbone RMSD measurements (Figure 3-15). The Vpu hexamer arrangement which started without a hydrated channel best retained the symmetry of the initial model, but still showed an increase in interhelical distance and a reduction in interhelical contacts for one monomer (Figure 3-16). The hexamer simulation showed the greatest propensity for ingress of water, oscillating between 0 and 6 water molecules throughout the time course of the simulation (Figure 3-15).

3.3.7 Analysis of inter-helical contacts involving the conserved alanine residues in the Vpu TMD

The relative importance of Vpu residues 7, 10, 14, and 18 in maintaining oligomer assemblies of the transmembrane domain was assessed by examining the interhelical contacts formed by each Ala residue throughout the duration of the Vpu oligomer MD simulations (Figure 3-19). While
the interhelical contacts presented above (Figure 3-10, Figure 3-12, and Figure 3-16) provide information on monomer-monomer interactions within the Vpu bundles at a general level, they do not do so with sufficient resolution to allow identification of interaction faces between monomers. The time evolution of Ala-specific interhelical interaction presented in Figure 3-19 provides detailed information; none of the Ala residues within the AxxAxxxAxxxA motif were involved in fewer than two interhelix contacts at any point in time, and the majority of Ala residues formed an average of between four and ten contacts throughout the simulation. The fewest contacts observed overall in the AxxAxxxAxxxA motif involved the Ala 7 residue, while the greatest number of contacts overall involved the Ala 10 and Ala 14 residues. Most AxxAxxxAxxxA motif Ala residues averaged a consistent number of interhelix contacts after model equilibration, with two notable exceptions: the hydrated tetramer model, which experienced an increasing trend in number of contacts with time, and the hexameric model which experienced a slight decrease in the number of contacts as time evolved.
Figure 3-19: The number of interhelix contacts made by Ala residues within the Vpu TMD as a function of time. Average number of contacts plotted versus time for models of Vpu (A) tetramer, (B) hydrated tetramer, (C) pentamer, (D) hydrated pentamer, (E) hexamer, and (F) hydrated hexamer models. In all cases, the plots were smoothed with a 5 ns rolling average to improve clarity. Coloured lines represent individual Ala residues within the AxxAxxxAxxxA motif.
3.4 Discussion

Initially, this study was intended to simply determine whether Vpu oligomer models featuring the AxxAxxxAxxxA motif as an interhelical interface would form a stable arrangement over the course of extended MD simulations in an explicit bilayer environment. Using the same approach, we extended the work to test the capacity of Vpu oligomers to retain a hydrated channel, which may be required for ion conductance across the membrane. In our work, the MD simulation was conducted for 150 ns, which is fifty per cent longer than previously reported work performed on Vpu in atomistic detail (Li et al. 2013a).

The context for Vpu oligomer formation could represent two possible contexts in light of our MD results. First, there is the possibility that Vpu oligomer formation is specific and mediated by the AxxAxxxAxxxA motif we have studied by mutagenesis; in this case the residues within the motif would be important for assembly and function of Vpu oligomers, resulting in well-ordered oligomeric structures of Vpu. Alternatively, there is the possibility that Vpu oligomer formation is mediated simply by lateral pressure within the membrane bilayer, wherein non-specific interactions define monomer-monomer contacts that are more consistent with protein aggregation than ordered oligomeric structure. In aggregates, highly variable interaction faces between Vpu monomers would be expected rather than consistent contacts between monomers.

In our MD simulations, all Vpu models began as symmetric arrangements of Vpu monomers around a geometric centre, and in every case this arrangement collapsed into a compacted bundle of Vpu helices. Despite similar characteristics, the models are inhomogeneous in small details. In the case of the hydrated tetramer model, a single tryptophan residue was observed to turn inwards to the bundle, trapping two water molecules within the complex for the remainder of the simulation. However, this was not observed in the hydrated Vpu pentamer arrangement which retained much of the symmetry of the initial model, albeit with a more flattened than toroidal arrangement. In the hydrated hexamer model, a slow “fraying” of a single Vpu monomer from the outer edge of the flattened oligomeric bundle was observed, unlike our other models. These results demonstrate the fact that the suite of simulations performed show variability in the conformation of individual components, providing some support for a mechanism of non-specific interactions between monomers in Vpu oligomer formation.
To determine whether our oligomer models represented aggregates of non-specific helical bundles, we examined interhelix distance measurements as well as total interhelical contacts. Compared to literature values, the interhelical distances we observed between Vpu monomers in each model (averaging 10 Å to 11 Å) corresponded well to recent reports of replica exchange ensembles of Vpu tetramer, pentamer, and hexamer models, with distributions centred between 10 Å and 11.5 Å, and which also observed flattened Vpu oligomeric bundles (Padhi et al. 2013). The measured helical tilt angles at the end of our simulations, averaging 5.9°, 13.2° and 24.7° also corresponded well to reported replica exchange distributions centred at 6°, 14°, and 24° (Padhi et al. 2013), as well as FTIR derived helical tilt of 6.5° (Kukol & Arkin 1999), and the NMR derived measurement of 28° versus the bilayer normal based on PISA wheel fitting (Skasko et al. 2012).

In our MD simulations, the configuration of individual monomers within Vpu oligomers changed relative to the starting models in each case, but in tetramer and pentamer models the overall Vpu oligomeric assembly remained completely intact, while in both hexamer Vpu models it appeared likely that loss of a single monomer could occur provided a long enough MD simulation (Figure 3-16). This expulsion of a single helix from the hexameric arrangement has previously been reported in 10 ns explicit bilayer and 2 ns octane slab simulations of Vpu (Padhi et al. 2013; Lopez et al. 2002), and indicates that tetramer and pentamer configurations may be more stable than hexamer assemblies, consistent with our results.

In a situation where lateral pressure was the major force contributing to the formation of Vpu monomer-monomer bundles without specificity, we would have expected that the relative number of contacts formed by each residue within the AxxAxxxAxxxA motif would have varied in proportion with the number of overall monomer contacts. However, this is not consistent with the results of our MD simulations. In the case of the Vpu hexamer model, for example, the Ala 10 and 18 residues formed more contacts as time progressed, while the Ala 7 residue lost contacts with time (Figure 3-19). Given that these residues are aligned along the same helical face of the Vpu monomer, this implies specificity in the contacts formed by Ala 10 and 18 residues, unlike what would be expected for a nonspecific aggregative model of Vpu oligomerization.
In further support of a model where Vpu interactions were specifically mediated by the AxxAxxxAxxxA motif, we observed that helix-helix interaction interfaces composed of Ala 7, 10, 14 and 18 residues were not grossly disrupted during the simulations, contrary to what might have been expected given the wide variety of Vpu oligomer configurations reported by other MD work (Grice et al. 1997; Kukol & Arkin 1999; Moore et al. 1998; Cordes et al. 2001; Lopez et al. 2002; Lemaitre et al. 2004). We have inferred the relative importance of each of these Ala residues based upon the observation of intermolecular contacts in our simulations. Vpu residues Ala 10 and 14 featured a greater number of intermolecular contacts than Ala 7 in the AxxAxxxAxxxA motif in every model tested, while Ala 18 featured more intermolecular contacts than Ala 7 in the majority of models tested (Figure 3-19). The lower number of intermolecular contacts formed by Ala 7 in comparison to other residues within the AxxAxxxAxxxA motif indicates this residue is less important in stabilizing the Vpu oligomer, a finding consistent with our mutagenesis data, which indicated disruption of the Vpu oligomer in SDS-PAGE conditions when Ala 10, 14, or 18 was mutated to Val, but not Ala 7 (Figure 2-6).

In fact, in our simulations the higher number of contacts formed by Ala 10 and Ala 14 residues could indicate that these residues conferred a greater degree of stability to the complex than Ala 7 or Ala 18. A difference in stabilization between Ala 10, 14 and 18 was not a feature that could be discerned by our previous experiments on full-length Vpu in detergent (Figure 2-6). It is difficult to determine whether this differential outcome was an artefact of the SDS-PAGE experiment such as poor staining sensitivity, or simply a different outcome due to the membrane mimetic utilized (detergents in the case of SDS-PAGE experiments, versus lipid in the case of MD simulations).

The configuration of monomers within the oligomeric Vpu models we have described in this work feature the conserved Tryptophan residues in the Vpu transmembrane domain facing outwards from the Vpu oligomeric centre, towards the surrounding lipids. These models disagree with early models of the Vpu oligomer, which often featured Trp 22 facing into the centre of the oligomeric complex in order to facilitate gating of the putative Vpu ion channel (Moore et al. 1998; Cordes et al. 2001). However, more recent Vpu studies featuring channel measurements have indicated that mutation of residue Trp 22 to Leu does not prevent ion conduction in Vpu, suggesting that a Trp-in configuration is not required for Vpu channel activity (Mehnert et al. 2008). Additionally, the Trp 22 to Leu mutation has been reported not to impact the ability of
Vpu to bind HMA based on SPR measurements, which also suggests an exposed and therefore lipid-facing orientation of Vpu Trp 22 (Rosenberg et al. 2016). In a general case, NMR studies have reported that aromatic Tryptophan side chains form interactions with lipid carbonyl moieties at the membrane interface in α-helical transmembrane proteins (de Plaqué et al. 2003). More specifically, models based on NMR studies of Vpu in rotationally aligned samples (Park et al. 2003) and MAS NMR experiments (Lu et al. 2010) also indicate a Trp-out conformation of Vpu monomers. Therefore, the Trp-out configuration of monomers we have described in this work are consistent with recent findings, as well as recent MD studies (Padhi et al. 2013; Padhi et al. 2014; Li et al. 2013a; Li et al. 2013b; Lin et al. 2016a).

One notable exception to the Trp-out configuration was observed in our simulations, as the hydrated Vpu tetramer model underwent rearrangement of a single Vpu monomer early in the simulation, turning one Trp residue towards the centre of the oligomeric complex. As a consequence of this rearrangement, two water molecules between Vpu Ala residues 7 and 10 and the inwards-facing Trp residue at position 22 were trapped by the compaction of the complex, and resulted in a tetramer which retained these two water molecules throughout the simulated time course. This result is unique among the oligomeric models examined in this work and represents the only helix-turning event that was observed in any of the simulated models, as well as the only connection to Trp-gated models of Vpu function. Despite this rearrangement, we did not observe any evidence for the formation of hydrated Vpu oligomeric assemblies consistent with ion channels in the POPC bilayer. Furthermore, repeating the same simulations with a pre-formed water column in the core of Vpu oligomer models resulted in most or all water molecules being excluded from each arrangement (Figure 3-7, Figure 3-11, Figure 3-15). We infer from these results that this oligomeric Vpu arrangement is not likely capable of forming or sustaining a hydrated channel or pore for ion conduction. Our finding is consistent with other shorter MD simulations of Vpu which did not form an ion channel in either 1.5 ns or 20 ns of simulated time (Lopez et al. 2002; Li et al. 2013a). The lack of channel formation in Vpu oligomers in our models sits in disagreement with other recent simulation studies focused on Vpu ion channel activity, which describe the formation of a water channel upon forcing ions through the Vpu bundle utilizing steered MD (Patargias et al. 2009; Padhi et al. 2014). These Vpu ion channel studies stipulate a high energetic cost for ion conductance, estimated between 150 and 190 kJ/mol, much higher than those expected from experimental conductance measurements.
Our methods do not utilize steered MD or umbrella sampling as featured in the aforementioned work, but the expulsion of water molecules from each of our helical bundles suggests to us that the spontaneous formation of a hydrated channel is unlikely while maintaining the configuration of helices found in our models.

The configuration of oligomeric Vpu bundles in our MD simulations squarely aligns our models with other recent models featuring the conserved Vpu Ser residue facing away from the oligomeric centre. In other Vpu oligomer models featuring a charged ion within the central Vpu pore, the ion interacts with the conserved Vpu Ser residue, and remains hydrated within the pore (Padhi et al. 2014). Much like Trp-in Vpu models, Ser-in configurations have historically predominated structural models of the Vpu oligomer, particularly with regard to models of small-molecule inhibitors of Vpu mediated viral budding (Lemaitre et al. 2004; Kim et al. 2006). However, more recent MD studies of Vpu oligomers built around ions do not; these models must “force” Ser-in models into a higher energy configuration, while the lowest-energy structures have the Ser and Trp residues facing outwards (Li et al. 2013b). Coarse-grained simulations of full-length Vpu protein feature assembly of large-order Vpu oligomeric complexes within the lipid bilayer, wherein only the tetramer was observed in a Ser-in conformation, while trimer, pentamer, hexamer, 9-mer, and 12-mer configurations were not, nor were higher-order Vpu patches within the bilayer (Lin et al. 2016a). Ser-in mechanisms for interaction with water are incompatible with our Vpu oligomeric arrangement: we conclude that our MD work cannot provide support for mechanistic models featuring ion channel function as a necessity for Vpu activity if the pore is mediated by Ser residues.

Our results agree well with coarse-grained MD simulations, which indicate that Vpu models do not necessarily form pore-like structures, but rather irregular patches of Vpu monomers (Lin et al. 2016a). Follow-up coarse-grained studies also determined that oligomerization rate of Vpu was driven by the transmembrane domain of Vpu, while maximum degree of oligomerization was defined by electrostatic repulsion in residues Ser 52 and Ser 56 (Chen et al. 2016). While we did not simulate Ser 52 and Ser 56, these studies are otherwise in good agreement with our work, as we observed Vpu bundles with consistent averages of total interhelical contacts and interhelical distances, without observing ordered pore-like structures. Given the discerning power of our all-atom simulations versus other coarse-grained models, we were afforded the ability to establish the AxxAxxxAxxxA motif as a component of Vpu monomer-monomer
interactions. In terms of viable Vpu oligomers, MD work has previously posited that Vpu transmembrane oligomer models are unstable with more than 5 monomers, and our data also indicate that tetramer and pentamer are preferred to hexamer models, which may reduce to pentamer assemblies, a finding in good agreement with prior MD work (Lopez et al. 2002; Padhi et al. 2013).

All the Vpu models examined in our work contained common structural elements, such as a dehydrated helical bundle and conserved interhelical contacts within the AxxAxxxAxxxA motif, despite containing differing numbers of Vpu monomers per oligomeric complex. While we cannot conclude that our simulations represent the only viable models of Vpu oligomerization in lipids, we can conclude that Vpu does not necessarily exist in a single static oligomeric conformation, but more likely as an ensemble of states within an oligomer which share common structural features. Given that we have not seen evidence for dissociation of our tetrameric and pentameric models in MD simulations, multiple oligomeric arrangements of Vpu may also exist within the POPC bilayer; this finding is in agreement with other studies utilizing MD and biophysical measurements on peptides of Vpu (Li et al. 2013a; Maldarelli et al. 1993; Hussain et al. 2007; Lu et al. 2010; Chen et al. 2016).

Our molecular dynamics simulations demonstrate the ability of the AxxAxxxAxxxA motif to form contacts within a Vpu oligomer model as a feature of homo-oligomerization; additionally, Vpu residues Ala 14, Ala 18, and Trp 22 have been shown by mutagenesis to be important for the transmembrane-domain mediated interaction with Tetherin residues Ile 34, Leu 37, and Leu 41 (Vigan & Neil 2010). As a consequence of this finding, MD models have emphasized the availability of these residues for hetero-oligomeric interactions (Padhi et al. 2013; Li et al. 2013a). NMR experiments have shown chemical shift perturbations in the Vpu AxxAxxxAxxxA region upon the addition of Tetherin, supporting a direct-interaction model for this Vpu motif with Tetherin (Skasko et al. 2012). Given that we have demonstrated the importance of these residues in Vpu homo oligomer formation, a question will need to be addressed: which role do these residues play in vivo? These residues are assumed to interact directly (Vigan & Neil 2010; Skasko et al. 2012; McNatt et al. 2013). In light of our results, this leads to the further question: does a Vpu-Vpu interaction need to be disrupted in order to facilitate a Vpu-Tetherin interaction? Since the AxxAxxxAxxxA motif appears to be important for Vpu oligomerization, formation of these homo-oligomeric contacts will have the effect of sequestering these residues away from the
surrounding lipid bilayer, rendering them inaccessible to direct contact with potential binding partner proteins. Interactions mediated by this motif should reduce the capability of Vpu to interact with Tetherin. This is a difficult problem to study, but it is clear from colocalization experiments that the triple mutation of Ala 10, 14, and 18 to Phe abolishes the ability of Vpu to displace Tetherin from viral assembly sites in HeLa cells (Lewinski et al. 2015). However, it is not clear if this is a result of Vpu homo-oligomer disruption, or the disruption of the Vpu-Tetherin binding interface, or a combination of the two.

To accommodate a direct interaction between Ala 14, Ala 18, and Trp 22 on Vpu with Tetherin, the Vpu complex in our models must dissociate at least one monomer, which may present a case for the apparent behavior of Vpu hexamer assemblies, illustrating the potential for partially-ordered oligomeric assemblies to play a role in the presentation of Vpu to host cell binding partner proteins. However, such a model would be difficult to differentiate from a direct-interaction model with monomeric Vpu, and is beyond the scope of the current work.

In this work, we have observed simulations consistent with our Vpu mutagenesis data, showing the contribution of Ala 10, Ala 14, and Ala 18 to the stability of the oligomeric complex as observed by SDS-PAGE experiments. Our MD simulations are also consistent with lateral pressure and non-specific interactions playing a role in the interaction of Vpu monomers, as evidenced by the collapse of symmetric oligomer assemblies into flattened bundles early in the simulations, which are non-identical but also largely unchanging with respect to time.

In short, the simulations provide good insight into the Vpu oligomeric system, but still demonstrate a considerable degree of structural heterogeneity when conducting direct comparisons between individual Vpu residues in time. Overall, our data are consistent with a situation in which Vpu oligomers have characteristics closer to a bundle of helices defined by repeating interaction interfaces, rather than a discrete and uniform symmetry, and this may be the best descriptor of the Vpu oligomeric system. Our tetrameric and pentameric oligomeric bundles are stable despite macroscopic internal differences, featuring the strengthening of binding interfaces via the AxxAxxxAxxxA motif, but containing the structural plasticity required to undergo internal rearrangements, and perhaps allowing greater variability of exposed surface elements to facilitate protein-protein interactions with binding partners.
3.5 Conclusion

Our unrestrained 150 ns equilibrium MD simulations of Vpu tetramer, pentamer, and hexamer models are consistent with a Vpu complex that does not support a hydrated channel or pore within the lipid membrane. This supports recent proposals that ion conductance may not be a critical activity of Vpu in the HIV lifecycle. Our simulations, which are the longest to date for all-atom simulations of Vpu, show the importance of contacts between monomers in the conserved Vpu transmembrane domain AxxAxxxAxxxA motif and are consistent with our previous work conducted by mutagenesis in SDS-PAGE experiments on Vpu homo-oligomers (Chapter 2) and other NMR experiments on Vpu-Tetherin hetero-oligomers (Skasko et al. 2012). Our data provide an indication that monomers within each oligomeric complex could be composed of multiple internal arrangements but depend on the AxxAxxxAxxxA motif for stability, where tetramer and pentamer arrangements of Vpu exhibit greater stability than hexamer arrangements. The apparent tendency of Vpu hexamer arrangements to liberate monomer and revert to pentamer configurations provides ample motivation to actively explore the consequence of oligomeric state on the interaction of Vpu with other proteins. Since this oligomeric complex will likely be strongly affected by the surrounding environment, attempts will be required to characterize the effect of membranes and membrane mimetics on the formation of Vpu oligomer complexes to determine a relevant condition for studying Vpu.
4 Structural investigation of oligomeric Vpu

4.1 Introduction

This chapter describes efforts undertaken in the expression, purification and structural characterization of recombinant Vpu in a variety of membrane-mimetic environments. Previous experimental results, as presented in Chapter 2, have been obtained by solubilization of Vpu in SDS detergent micelles, however it may be important to compare results obtained in multiple solubilizing conditions to ensure that they reflect the native structure. We have explored use of phospholipid liposomes for solid state NMR of Vpu and detergent micelles for solution NMR of Vpu. While ultimately, we were unable to achieve the resolution required for complete resonance assignment and structure determination by NMR, a qualitative comparison of structural properties of the Vpu protein in lipid liposomes versus detergent micelles was obtained.

4.1.1 Lipid mimetics for membrane proteins

Lipids are arguably the most physiologically relevant membrane mimetics for transmembrane proteins, as biological membranes are composed largely of lipid molecules. If the goal of studying a membrane protein is to understand its structure and function, the most likely medium to retain native structure and function will be the native environment of the membrane protein.

The choice of lipid can have a significant impact on the structure of membrane proteins which interact with it. For instance, the parainfluenza virus 5 (PIV5) viral fusion peptide, studied by solid-state NMR, exhibited α-helical structure in anionic lipids containing POPG, and β-sheet structure in neutral POPC (Yao & Hong 2013). A similar example is the PG-1 antimicrobial peptide, which can form β-barrels in POPE/POPG membranes but smaller β-strand tetramers in POPC/cholesterol (Mani et al. 2006). Even simple single-span membrane proteins can be impacted by the lipid environment; the structure of Vpu is also affected by lipid composition, with its transmembrane domain forming an angle of 13° with the bilayer normal. This tilt is dependent on lipid composition, varying from 18° in 18:0 lipids to 35° in 12:0 lipids, as has been shown by oriented-sample NMR experiments and associated with the observation of a characteristic but variable kink in the Vpu transmembrane domain helix at residue Ile17 in DOPC/DOPG bilayers (Park et al. 2003; Sang & Opella 2005; Park, Mrse, et al. 2006).
Some membrane mimetic systems can be blends with unique properties meant to combine the physiological relevance of lipid membrane-like environments with the convenience of detergent solubilization for aqueous-phase characterization of proteins. One such example is the bicelle system which is utilized in several solution and solid-state NMR studies, composed of a short chain detergent-like lipid such as DHPC combined with a longer-chain lipid such as DMPC, and reviewed in Section 1.6.4. These systems are believed to form an arrangement whereby membrane proteins can be embedded in DMPC bilayers and yet solubilized into smaller domains of lipid/protein complexes by short-chained DHPC interactions forming coin-like segments of lipid, or swiss cheese-like bilayers containing holes that allow free diffusion of solutes to both sides of the lipid bilayer (Warschawski et al. 2011). Regardless of mechanism, these systems provide a valuable tool to study membrane proteins embedded in bilayers, which behave much like detergent micelle solubilized proteins.

4.1.2 Characterization of membrane proteins by NMR

Due to the intrinsic interactions between a membrane protein and its membrane environment, membrane protein solubilization conditions must often contain either lipids or detergent membrane mimetics to accommodate the hydrophobic character of transmembrane segments in order to present a folded, functional protein for structural study.

Hydrophobic segments in membrane proteins make characterization by conventional techniques in structural biology difficult. While X-ray crystallography has been the most successful technique for determining membrane protein structures, use of this technique is challenging in membrane proteins, favouring large complexes which exhibit a high ratio of molecular volume to hydrophobic surface area (Privé 2007). As such, there are some systems that will simply not be capable of forming relevant crystal assemblies. In order to avoid some of the common problems in protein crystal formation, lipidic cubic phase (LCP) techniques have been utilized to surround membrane proteins with a more natural lipid mimetic than the short chain detergents previously required, better stabilizing the protein in order to form crystal contacts (Hardy et al. 2016). Notably, this method has resulted in elucidation of over 200 structures (Caffrey 2015), including the β-adrenergic receptor-Gs protein complex (Rasmussen et al. 2011). However, many proteins do transition through multiple conformations to perform their function, and in cases where the observation of multiple conformations is important, X-ray crystallography techniques necessarily
require multiple crystals. This in turn often requires the mutagenesis or truncation of the protein to be studied, leading to questions of relevance versus native structure and the specificity of protein interactions.

Structural biology techniques such as electron microscopy can overcome some of these difficulties for membrane protein structural determination. This can be accomplished by use of heavy-atom staining techniques, allowing high contrast at the cost of lower resolution due to the size of stain particles and flattening artefacts from vacuum exposure (Binshtein & Ohi 2015). Alternatively, single-particle techniques with low contrast and high susceptibility to radiation damage have begun leveraging algorithms capable of discerning and sorting images into classes of conformations, and averaging each class to achieve high resolution (Binshtein & Ohi 2015). These techniques can provide valuable information on oligomerization or differing conformations of a given protein, but often see greatest success in the case of large complexes (>1MDa) at higher resolutions (~3 Å) (Subramaniam et al. 2016). The technique is extensible to membrane proteins in lipid bilayers, such as the BK potassium channel, which was the first such example solved in 2009, at 1.7nm to 2.0nm resolution (Wang & Sigworth 2009). A more recent example is the 3.4 Å resolution structure of a functional 630-residue construct of TRPV1 ion channel, solved in amphipol mimetics (Liao et al. 2013). Alternately, electron crystallography on large 2-dimensional planar protein crystals can be used to generate high-resolution structural models of membrane proteins in a near-native state, at the cost of losing dynamics information about the protein of interest, similarly to drawbacks associated with X-ray crystallography.

NMR techniques can provide high-resolution structures, information about proteins in multiple conformations, and dynamics information. However, traditional solution-state NMR techniques have a fairly low molecular size limitation in many cases, usually below 30 kDa but nearly always below 100 kDa (Warschawski et al. 2011). Furthermore, the inclusion of micellar or lipid environments often increases the size of protein-mimetic complexes to a degree that prevents the fast isotropic tumbling required for conventional solution NMR structural determination. Solution NMR comes with the advantages of sharply resolved mobile components, whilst solid state NMR can capture useful data on slow-moving or relatively immobile components. However, both solution and solid-state NMR depend on similar experimental timeframes, and any motion occurring on these timeframes will be averaged away over the course of the NMR experiment. For example, the Insensitive Nuclei Enhanced by Polarization Transfer (INEPT)
scalar coupling-based block in a solution NMR HSQC experiment is utilized to transfer magnetization between nuclei on a timescale of order ~2.5 ms, and typically these pulses will be applied approximately 10 or more times in a pulse sequence (Morris & Freeman 1979). Therefore, any motion occurring on the 1-10 ms timescale will be averaged in these experiments, and transfer is best achieved by large motions which average away dipolar interactions. In the opposite extreme, MAS solid-state NMR experiments generally utilize a coherence transfer via the dipolar interaction utilizing cross-polarization: this block is 0.1 to 1.5 ms in duration, and similarly motional averaging occurring on a timescale shorter than milliseconds can yield a loss of coherence in magnetization, while slower motions will remain visible (Hartmann & Hahn 1962; Pines et al. 1973). The utilization of MAS NMR techniques has yielded some impressive outcomes in specialized cases, such as the structure of the GPCR CXCR1 in DMPC using rotationally aligned NMR techniques (Park et al. 2012).

Many solid state NMR techniques are less limited by water solubility or molecular size limitations when compared to solution NMR experiments, in particular when magic angle spinning techniques are used to minimize chemical shift anisotropy and dipolar couplings between nuclei. However, solid state NMR experiments often suffer from problems associated with short T2 relaxation times, resulting in comparatively wide spectral linewidths, and therefore high spectral overlap combined with limited signal intensity (Tycko 1996; Tycko 2015). The increased resolution generally available with the application of solution-state NMR is often preferable for structural determination, and is therefore often the first attempt at structural determination of membrane proteins and membrane protein segments (Chill & Naider 2011). Those systems deemed not to be amenable for solution NMR characterization can be probed by solid-state NMR to gain complementary information and structural restraints in order to generate a high-resolution model (Brown & Ladizhansky 2015; Park et al. 2010). Assessment of appropriate techniques and data quality is often only achievable by the acquisition of experimental data, an unfortunate consequence of the widely varying nature of membrane protein composition, structural arrangement, size, and interactions with the surrounding milieu.

Given that no structure of Vpu has been solved to date in POPC bilayers, our goal was to attempt the structural determination of Vpu in this membrane mimetic system utilizing NMR techniques.
4.2 Materials and methods

4.2.1 Recombinant purification of Vpu in *E. coli*

Vpu was expressed and purified as described in Section 2.2.1, with the exception of utilizing minimal growth media with $^{13}$C-glucose (> 99 atom % $^{13}$C) as the sole carbon source in the media (Sigma-Aldrich), and $^{15}$N-ammonium chloride (> 98 atom % $^{15}$N) as the sole nitrogen source in the media (Sigma-Aldrich).

4.2.2 Solubilization of purified Vpu for SDS PAGE

Samples for SDS PAGE were prepared as described in Section 2.2.3.

4.2.3 Reconstitution of Vpu in membrane mimetics

To prepare Vpu samples, dried Vpu was weighed out and deposited into a glass vial, HFIP pipetted into the vial, and the mixture was bath sonicated for 10-15 minutes at room temperature, resulting in a fine suspension of undissolved Vpu. HFIP was evaporated away under dry N$_2$ gas, leaving a Vpu film. HFIP-treated Vpu residue was then dissolved in 50% v/v TFE/water with 10 minutes of bath sonication. The result was a homogeneous solution containing no film or precipitate, which was then co-dissolved with POPC at 4 mol percent ratio in a chloroform/methanol/trifluoroethanol/water mixture. Once a clear and transparent solution was obtained, the protein/lipid mixture was dried to a thin film under N$_2$ and lyophilized to remove solvent. To remove any residual solvent, the dry film was bath sonicated in deionized water, then lyophilized once more. The final lyophilized powder was resuspended in 10 mM HEPES buffer at pH 7.4. Samples were packed into a 3.2 mm Varian MAS rotor for solid state NMR experiments containing a final mass of 10 mg of Vpu protein.

Vpu for detergent experiments was dissolved from a dry film with 2% SDS or 2% DPC in 10 mM HEPES buffer at pH 7.4 with bath sonication to a final protein concentration of 1 mM Vpu. Once a clear and transparent solution was obtained, the protein/detergent mixture was lyophilized, then resuspended in water. Vpu-detergent samples were equilibrated for 48 h at room temperature, then centrifuged at 21 000 x g to clarify the solution. Final Vpu samples remained free of visible aggregation for more than three years’ time, indicating Vpu stability in SDS and DPC.
4.2.4 Solid-state NMR experiments

$^{13}$C, $^{15}$N, and $^{31}$P spectra were obtained on a narrow bore Varian VNMRS spectrometer operating with a $^1$H frequency of 499.78 MHz. Experiments were performed using a Varian triple-resonance 3.2 mm T3 MAS probe in a dual-channel configuration. Samples packed into 36 μL 3.2 mm MAS rotors were checked for adequate hydration by observation of static $^{31}$P lineshapes by direct-pulse experiments performed at room temperature. All other experiments were performed at a probe temperature of 25°C, or -22°C by delivery of high flow-rates of -35°C dry air as signal to noise ratio was improved observed in MAS NMR experiments at these temperatures versus 25°C. Spectra were referenced with respect to 38.56 ppm via observation of the downfield $^{13}$C resonance peak of adamantane (Morcombe & Zilm 2003).

Cross-polarization (Hartmann & Hahn 1962; Pines et al. 1973) was accomplished using a linear RF ramp on the $^1$H channel between 50-80 kHz with simultaneous application of 40-60 kHz RF fields on $^{13}$C for a contact time of 1.0 to 1.5 ms. $^{13}$C pulses applied during all experiments were 50 kHz while $^1$H pulses were applied at 100 kHz, with 100 kHz TPPM (Bennett et al. 1995) $^1$H decoupling during acquisition and a 3.0 s recycle delay between successive scans.

$^{13}$C-$^{13}$C recoupling was accomplished via a RAD/DARR sequence (Takegoshi 2001; Morcombe et al. 2004) with recouping times of 50 ms or 500 ms at a MAS frequency of 8 kHz. Two dimensional spectra were collected with 1024 points in the direct dimension and a spectral width of 35.7 kHz, while the second dimension was collected in 160 points with a window of 25 kHz, averaging 64 scans of each increment.

4.2.5 Solution NMR experiments

Solution NMR experiments were conducted at 25°C on samples of Vpu reconstituted in 2% w/v SDS and 2% w/v DPC buffered with 15 mM HEPES pH 7.4. $^1$H 1D, 2D $^1$H-$^{15}$N HSQC, and 2D $^1$H-$^{13}$C HSQC experiments were conducted on a Bruker Avance III Spectrometer with a $^1$H Larmor frequency of 600.4 MHz using a 5 mm TXI Probe equipped with a Z-axis gradient.

Two dimensional $^1$H-$^{15}$N HSQC spectra were collected with 1024 points in the direct dimension and a spectral width of 9.6 kHz with the $^1$H transmitter centred at 4.7 ppm, while the second dimension was collected in 100 points with a window of 2.4 kHz with the $^{15}$N transmitter centred at 120 ppm. The trosetytopf3gpsi pulse sequence provided with TopSpin 3.2 was utilized (Czisch

Two dimensional $^1$H-$^1$H HSQC spectra were collected with 1024 points in the direct dimension and a spectral width of 9.6 kHz with the $^1$H transmitter centred at 4.7 ppm, while the second dimension was collected in 120 points with a window of 13.5 kHz with the $^{13}$C transmitter centred at 43 ppm. The hsqcetfpgpsi2 pulse sequence provided with TopSpin 3.2 was utilized (Palmer et al. 1991; Kay et al. 1992; Schleucher et al. 1994; Grzesiek & Bax 1993).

4.3 Results

4.3.1 Recombinant expression and purification of Vpu for structural studies

Prior to developing the purification scheme that is briefly described in Chapter 2, we initially attempted to use methods described in literature to produce full-length Vpu (residues 2-82). The previously reported methods have utilized the fusion between Vpu and ketosteroid isomerase (KSI) protein for high-level protein expression and direction to inclusion bodies for further purification (Park et al. 2003; Park, De Angelis, et al. 2006; Kuliopulos & Walsh 1994). The use of ketosteroid isomerase is important for high target protein yield: reported values are between 20% and 40% of total E. coli protein production for the construct of interest. In order to separate fusion proteins from desired sequences, previous protocols utilized chemical cleavage of the peptide backbone via the use of cyanogen bromide reaction under acidic conditions (Lawson et al. 1961; Gross & Witkop 1961). For Vpu this required the mutation of two internal methionine residues to Leu at positions 66 and 70 as per previous studies (Marassi et al. 1999; Ma et al. 2002).

Following fusion construct expression to inclusion bodies and subsequent purification, chemical cleavage with cyanogen bromide was utilized to cleave the fusion polypeptide and attempt to liberate Vpu from the insoluble KSI fusion partner protein, shown in Figure 4-1, panel A (Gross & Witkop 1961; Marassi et al. 1999). Protocols were duplicated, then further adapted from published methods of Vpu peptide purification (Marassi et al. 1999; Ma et al. 2002). We were unable to reproduce the published results, and were unable to reliably separate the KSI-Vpu fusion partners after cyanogen bromide cleavage by means of differential solubility in organic solvents or alcohols, nor by reverse-phase HPLC methods. For example, we had the greatest
success utilizing DCM to attempt selective solubilization of either cleaved fusion partner from the mixture of the two (Figure 4-1, panel B) as per published methods (Ma et al. 2002). When Vpu was separated from KSI, SDS-PAGE gels showed relatively pure protein (Figure 4-1, panel C). However, results were not repeatable: yields of purified product would vary despite similar starting masses of crude mixture, and purity varied with each preparation; this led us to pursue other strategies in Vpu expression and purification despite high expression levels of impure full-length KSI-Vpu fusion.

![Figure 4-1: Purification of KSI-Vpu fusion utilizing treatment with DCM and HFIP. (A) Vpu-KSI fusion protein fragments after cleavage with CNBr in 0.1 N HCl / 8 M Guanidine HCl. (B) the same Vpu sample after one hour of sonication with 5 parts DCM and 1 part HFIP. (C) several successive attempts to repeat the separation of Vpu from KSI fusion partner using the same method in DCM and HFIP. Upper arrow: Vpu oligomer. Lower arrow: Uncleaved KSI.]

We therefore reattempted expression of Vpu from the soluble fraction of E. coli whole cell extract after fusing Vpu to Thioredoxin. While expression levels were lower than that reported for a KSI fusion, the Thioredoxin fusion did yield a construct which successfully expressed and purified for further work, yielding approximately 1mg of purified protein per liter of E. coli growth media (Mass spectrometry illustrated in Figure 2-4). After dissolving Vpu in organic solution, only mixtures of acetonitrile-isopropanol-water prevented precipitation of Vpu. Use of
these solvents in gradients on reverse-phase C8 columns gave suitable purification for further work (Figure 4-2).

![HPLC Chromatogram](image)

**Figure 4-2:** (A) HPLC Chromatogram of Vpu purification from soluble expression of Thioredoxin-Vpu construct expressed in *E. coli* after cleavage with cyanogen bromide. (B) HPLC fractions indicated were subject to lyophilization followed by SDS-PAGE to demonstrate sample purity.

We have found that solvent history is an important consideration to obtain homogenous samples for downstream experiments. Generally, an acceptable workflow for any purified Vpu has been sonication in HFIP and drying to a thin film, followed by solubilization of the protein by sonication in 50% TFE/water and drying to a thin film, and finally reconstitution in the final buffer, solvent, or detergent desired. To observe oligomerization on SDS-PAGE (Figure 2-3) and correct helical folding in CD spectra (Figure 2-5), purified samples must be treated with HFIP, dried, then dissolved in 50% TFE prior to reconstitution in lipid or detergents. This critical step in the process was found to be necessary to obtain reproducibly soluble Vpu samples, even in SDS environments. Without treatment with HFIP and TFE, samples were prone to aggregation in SDS-PAGE and CD experiments indicating the presence of misfolded Vpu aggregates.

### 4.3.2 NMR of Vpu in POPC liposomes

Solid-state 2D MAS NMR spectra of Vpu in POPC membranes clearly demonstrate the presence of properly folded, membrane-inserted Vpu (Figure 4-3, Figure 4-4) and these spectra are typical
of those obtained in our studies of full-length Vpu. While low temperatures can often result in line broadening, MAS NMR experiments conducted on the sample between -24°C and 45°C were best resolved at low temperatures, presumably due to freezing out molecular motion within the protein itself as well as that of the POPC bilayer. Many resonances in these low-temperature 2D $^{13}$C-$^{13}$C correlation spectra are identifiable by residue type based on chemical shifts, while signal to noise ratio was poor at higher temperatures, likely due to unfavourable dynamics in fluid bilayers.

![Figure 4-3: $^{13}$C-$^{13}$C PDSD of uniformly labelled $^{13}$C/$^{15}$N Vpu in POPC. Aromatic region cut away to highlight carbonyl region (left) and corresponding aliphatic region (right). Labels indicated for prominent spin systems, with Ile spin system traced by dashed line. 50 ms PDSD mixing performed under 11 kHz MAS following $^{13}$C excitation by 550 µs $^{1}$H-$^{13}$C ramped cross polarization at 11.7 T at -22°C. Acquisition consisted of 1024 points with 100 kHz TPPM $^{1}$H decoupling, averaged over 64 transients. Second dimension measured in 160 indirect points with application of 50 Hz Gaussian line broadening applied to each dimension.](image)

The low number of chemical shifts in the data sets we obtained were insufficient for the unique assignment of each Vpu residue, even by comparison to previously published chemical shift values for Vpu (Sharpe et al. 2006; Park et al. 2003; Zhang et al. 2015). Degeneracy was also somewhat pronounced: for example, a single subset of peaks observed corresponded to the expected chemical shift of Ile resonances, but the Vpu sequence contains 16 individual Ile
residues. In order to achieve sufficient assignment of resonances to determine the three-dimensional structure of the protein, it must be possible to uniquely identify each amino acid residue in the NMR spectra, with an estimated resolution requirement of 1 ppm or less for 30 residue uniformly labeled proteins (Tycko 1996), or 0.2 ppm to 0.4 ppm for 166-residue proteins (Tycko 2015). Despite strong signals in the data collected, 2D MAS NMR experiments will require greater resolution to generate a structural model of full-length Vpu in POPC, given our observed linewidths of between 1.8 ppm and 2.3 ppm.

While other reports have identified resonances with narrower lines, they also incorporate Vpu in membranes of differing thickness and higher detergent concentration, allowing for faster molecular tumbling in solution (Park et al. 2003; Wittlich et al. 2009; Zhang et al. 2015). With the use of pure POPC as a membrane mimetic, we attempted to increase the resolution of individual Vpu signals by performing a double-quantum single-quantum correlation experiment (Figure 4-4, panel B). In double-quantum single-quantum correlation experiments such as the super cycled post-C5 (SPC5)-filtered experiment (Hohwy et al. 1999) illustrated in Figure 4-4 panel B, each peak on the y-axis represents the sum of chemical shift values for two peaks at each position in the x-axis dimension. We utilized this experiment for Vpu/POPC samples to increase the separation between peaks with similar but non-identical chemical shift values (Figure 4-4). Issues with spectral overlap were greatly reduced in this experiment, allowing approximately 60 peaks to be uniquely discerned, but retaining linewidths between 1.7 ppm and 2.3 ppm. However, given that each residue contains a minimum of 2 carbon atoms, and each double-quantum single quantum correlation should generate 2 spectral peaks, for each residue to be uniquely represented in the protein we would expect at least 320 peaks to be observable in this spectrum. The low number of peaks observed in this experiment could be related to broadening of signals, which are generally correlated to lower temperatures such as were used in this experiment, on the order of ~2 ppm in this data. Furthermore, protein dynamics could also be an important factor in the observation of fewer peaks; it is possible that protein motion on the timescale of the NMR experiment itself could render some signals invisible. In either case, the spectra observed were inadequate for the structural determination of Vpu due to the absence of complete sets of NMR peaks; for example, no peaks were observed with chemical shifts corresponding to charged residues, Trp, or Ser, which are predominantly found in the Vpu cytoplasmic domain, and few resonances were visible in the case of Arg (Figure 4-5). The
signals with these resonances could be decreased due to the presence of molecular motion on the timescale of the experiment, which may be linked to possible protein oligomerization, or the NMR decoupling frequency utilized (Saitô et al. 2003; Saitô 2004; Yamamoto et al. 2006; Saitô et al. 2010).

Given the limitations of NMR spectra obtained of full-length Vpu in POPC, we considered the NMR study of Vpu in detergents. Since SDS-solubilized full length Vpu yielded a single band in SDS-PAGE corresponding to an oligomeric complex (Figure 2-3), and circular dichroism measurements indicated α-helical secondary structure (Figure 2-5), we attempted the use of many common detergents to attempt solubilizing Vpu, including SDS, DPC, DHPC, C8E5, and C14SB. In the case of Vpu in SDS and DPC solutions, we have shown solubility and folding to be comparable to that of Vpu in POPC via SDS-PAGE experiments (Figure 2-3) and CD measurements (Figure 2-5). Therefore, we felt that Vpu in SDS and DPC had shown sufficient promise for the structural characterization of Vpu, and each remained in solution with no visible precipitate formation for up to 3 years’ time, indicating good general stability and solubility in these detergents.
Figure 4-4: 2-dimensional MAS solid-state NMR of Vpu in POPC. (A) $^{13}$C-$^{13}$C proton-driven spin diffusion (PDSD) spectra of Vpu embedded in POPC under 8 kHz MAS at -22°C sample temperature with 50 ms of mixing time at 11.7 T. (B) $^{13}$C-$^{13}$C SPC5 double-quantum filtered correlation spectra of Vpu embedded in POPC under 10987 Hz MAS at -22°C sample temperature with 50 ms of mixing time at 11.7 T.
Vpu detergent samples were screened for suitability by 1D solution NMR, as previously reported in $^1$H PFG diffusion measurements (Figure 2-10). These spectra are representative of all 3 major components of solution; water (slightly negative signal at 4.7 ppm after water suppression), SDS, and amide protons associated with the backbone resonances of the Vpu amino acid chain (from 6 ppm to 9 ppm). The Vpu amide resonance peaks in these spectra are small and broad relative to the observed aliphatic resonances. To explore the possibility of utilizing solution NMR for further structural determination of Vpu, we employed two-dimensional spectroscopy to better resolve overlapping signals.

Samples containing Vpu and detergent generated peaks in 2D experiments are commonly used for protein structural work, such as Heteronuclear Single-Quantum Correlation (HSQC) experiments. To resolve individual resonances in the amide region, Transverse Relaxation Optimized Spectroscopy (TROSY) variants of conventional HSQC experiments were utilized (Figure 4-5). Solution NMR spectra of Vpu dissolved in SDS such as the spectrum illustrated in Figure 4-5 show identifiable peaks characteristic of specific amino acid residue types. In the $^1$H-$^{13}$C HSQC of Vpu (Figure 4-5, panel B), peaks were better resolved than the TROSY $^1$H-$^{15}$N HSQC experiment (Figure 4-5, panel A), but still exhibited substantial line broadening. Line broadening in this case could be due to the size of the oligomer-micelle complex, estimated to be from ~27.5 kDa for a Vpu monomer in an SDS micelle up to ~63.5 kDa for a pentamer in an SDS micelle, or alternatively due to structural heterogeneity within the samples.
Figure 4-5: 2-D solution NMR of Vpu in 2% dSDS solution. (A) TROSY $^1$H-$^{15}$N HSQC of Vpu at 14.1 T with spectral window fit to emphasize amide backbone region of $^1$H chemical shift. (B) $^1$H-$^{13}$C HSQC of Vpu showing increased peak resolution versus $^{15}$N experiment. Experiments were performed at 25°C at 14.1 T.
To generate a complete suite of data for further analysis, we acquired $^{13}$C-$^{13}$C solid-state MAS spectra of Vpu in 2% perdeuterated SDS (Figure 4-6).

**Figure 4-6:** $^{13}$C-$^{13}$C PDSD of uniformly labelled $^{13}$C/$^{15}$N Vpu solubilized in dSDS. Aromatic region cut away to highlight carbonyl region (left) and corresponding aliphatic region (right). 50 ms PDSD mixing performed under 11 kHz MAS following $^{13}$C excitation by 550 µs $^1$H-$^{13}$C ramped cross polarization at 11.7 T at -22°C. Acquisition consisted of 1024 points with 100 kHz TPPM $^1$H decoupling, averaged over 64 transients. Second dimension measured in 160 indirect points with application of 50 Hz Gaussian line broadening applied to each dimension.

Solid-state NMR spectra also displayed identifiable and characteristic resonances for several amino-acid types within Vpu, but had much lower resolution than the solution NMR experiments already attempted – peaks seen in Figure 4-6 are less numerous and less well-resolved than in solution NMR experiments (Figure 4-5). Given differences in the anionic SDS detergent environment versus the zwitterionic POPC bilayer, spectra were collected to allow a direct comparison of Vpu in dSDS (Figure 4-6) to Vpu in zwitterionic dDPC, to Vpu in POPC (Figure 4-3). The three sample spectra are overlaid in Figure 4-7. In these spectra, spectral linewidths and chemical shifts were comparable between SDS, DPC, and POPC environments, however more peaks are visible albeit with broader lineshapes (such as Leu Cα-Cβ) in the sample reconstituted in POPC versus detergent samples, although all were collected under the same conditions. This outcome is likely to be a function of the differences in dynamics of molecular
motion between lipid and detergent. In the case of lipid, Vpu residues are held in a more immobile configuration by the bilayer itself than the residues solubilized by detergent micelles. The timescale of membrane undulation has been reported to be approximately 1 ms in the lipid bilayer (Rommel et al. 1988; Triba et al. 2006), which is on par with the cross-polarization contact time (~1200 µs) in our solid-state MAS NMR experiments. Therefore, the simplest solution to avoid these intermediate-timescale difficulties is by altering the motion of the nuclei to be observed with the application of sample heating or cooling. With this concept in mind, the spectra illustrated in Figure 4-3, Figure 4-4, Figure 4-6, and Figure 4-7 were obtained at -22°C (the lowest sustainable temperatures for our equipment) in order to decrease the effect of intermediate timescale motion, and increase observable signal (Sharpe et al. 2006).

**Figure 4-7:** $^{13}$C-$^{13}$C PDSD of uniformly labelled $^{13}$C/$^{15}$N Vpu solubilized in membrane mimetics. Vpu was reconstituted in POPC (blue), dSDS (purple), or dDPC (green) to compare chemical shifts in each membrane mimetic environment as per Figure 4-6 and Figure 4-3. 50 ms PDSD mixing performed under 11 kHz MAS following $^{13}$C excitation by 550 µs $^1$H-$^{13}$C ramped cross polarization at 11.7 T at -22°C. Acquisition consisted of 1024 points with 100 kHz TPPM $^1$H decoupling, averaged over 64 transients. Second dimension measured in 160 indirect points with application of 50 Hz Gaussian line broadening applied to each dimension.

To clarify the distinction between mobile and immobile regions of Vpu, we conducted Total through Bond Correlation Spectroscopy (TOBSY) experiments on the same Vpu samples (Hardy
Where PDSD experiments had the greatest observable signal when conducted at low temperatures, observable signal in TOBSY experiments had greatest signal at 22°C (Figure 4-8). In TOBSY experiments, Vpu resonances which did not appear in PDSD spectra were well-resolved, including Asp, Glu, Asn, Gly, and Ser. These residues are associated with more mobile regions of Vpu, including the loop region between the Vpu transmembrane domain and Vpu helix 2. The lineshapes in the TOBSY experiment were considerably narrower than those observed in the PDSD experiments, ranging from 0.8 ppm to 1.1 ppm. More peaks were visible in the TOBSY spectra of detergent-containing samples than the POPC-reconstituted Vpu sample. Some resonances were visible in both PDSD and TOBSY experiments, such as those belonging to Ile spin system side chains, which appear in the Vpu transmembrane domain, Vpu helix 2, and the Vpu C-terminal tail. While the number of residues observable in the combination of PDSD and TOBSY experiments is still insufficient for structural characterization, each experiment recorded different NMR signals within different regions of Vpu.

**Figure 4-8:** $^{13}$C-$^{13}$C TOBSY of uniformly labelled $^{13}$C/$^{15}$N Vpu solubilized in membrane mimetics. Vpu was reconstituted in POPC (blue), dSDS (purple), or dDPC (green) to compare chemical shifts in each membrane mimetic environment as per Figure 4-6 and Figure 4-3. 12.5 ms TOBSY mixing performed under 10987Hz MAS at 11.7 T at 22°C. Acquisition consisted of 1024 points with 100 kHz TPPM $^1$H decoupling, averaged over 64 transients. Second dimension measured in 160 indirect points with application of 25 Hz Gaussian line broadening.
4.4 Discussion

4.4.1 Vpu purification from the soluble fraction of *E. coli* cell extracts

We have developed new methods for the expression and purification of Vpu from the soluble cell extract fraction with high purity (Figure 4-2), after encountering problems which prevented successful purification of protein of high purity from the insoluble fraction of *E. coli* cell extracts (Figure 4-1). The lesser yield of Vpu-Thioredoxin fusion protein from the *E. coli* soluble cell extract is likely a byproduct of having correctly folded Vpu within living *E. coli* cells; in a context where properly folded functional Vpu could be capable of enhancing ion conductance, the loss of ion homeostasis is almost certainly detrimental to the host organism, likely resulting in low cell densities during Vpu overexpression. Recent work by other groups on the construction of ion-conduction assays utilizing Vpu in *E. coli* showed significant growth impairment of the bacteria with induction of Vpu expression in a maltose binding protein (MBP) fusion construct (Taube et al. 2014). Lending support to this hypothesis, we have observed the purified Vpu produced from the soluble *E. coli* fraction to spontaneously form homo-oligomeric assemblies in SDS solution, a result which has only been reported by one other *in vitro* study (Hussain et al. 2007). Uniquely, our method utilizes fluoroalcohol treatment in order to consistently generate Vpu oligomers of uniform size; an outcome that has not been reported by any previous study to date and can be observed as a single stained band in migration of Vpu on SDS-PAGE (Figure 2-3, Figure 4-2). Qualitatively, the omission of HFIP treatment during the process of Vpu purification results in a large fraction of total Vpu in SDS-PAGE experiments forming large molecular weight aggregates despite purity in mass spectrometry experiments (data not shown). Protein disaggregation is consistent with previously reported effects of HFIP in disaggregating peptides (Narita et al. 1988; Milton & Milton 1990), this effect has not previously been leveraged in Vpu literature to our knowledge.

4.4.2 NMR of Vpu for structural characterization

We have attempted to study Vpu by utilizing solid-state NMR under MAS conditions to select the most relevant bilayer mimetic possible, and to overcome the obstacle presented by slow-tumbling particles in solution. While initially promising, our solid-state NMR experiments are insufficient for structural determination by this method due in part to degeneracy associated with...
overlapping peaks in Vpu samples. This outcome has since been reported by another study, which found only 2 uniquely identifiable Vpu residues in a similar experiment (Do et al. 2013). This problem may be exacerbated by line broadening due to unfavourable protein dynamics at higher temperatures, or possibly at lower temperatures by structural heterogeneity as suggested by our MD simulation data presented in Chapter 3, as well as some previous studies of Vpu oligomerization (Lu et al. 2010; Lin et al. 2016a). NMR experiments can often be extended to a third dimension in order to further resolve individual resonances; in this case a 2-dimensional acquisition of sufficient signal/noise could only be completed in 6h of experimental time, and extrapolating this value to a (minimum) of 64 real increments (in a 3-dimensional experiment) would yield an experimental time of 32 days in order to acquire data for a single N-Cα-Cx experiment, and the same period for a matched N-CO-Cx experiment. Since this timeframe for data acquisition was not feasible, we attempted to obtain further resolution by performing double-quantum single-quantum correlation experiments (Figure 4-4, panel B). While these experiments visibly improved the spectral resolution of Vpu in POPC, the data were insufficient for high-resolution structure determination.

The relatively slow molecular tumbling rate of the oligomeric Vpu/detergent complex in aqueous solution likely represents the largest handicap in obtaining the requisite sharp lines for further work. We attempted to reduce the effects of the slow molecular tumbling of Vpu-detergent complex via the use of transverse relaxation optimized spectroscopy (TROSY) techniques with partial success. The SDS-solubilized Vpu oligomer samples show some discrete and well-resolved resonances which correspond to mobile regions of the protein, but the bulk of the signal present in 2-dimensional experiments arises from a portion of the spectrum with a high degree of spectral overlap (Figure 4-5, Figure 4-6). Given that direct comparisons between detergents have shown that unambiguous assignment of at least 90% of total resonances is required for successful structural determination (Page et al. 2006), our solution NMR spectra are not of sufficient resolution to proceed with the atomic-level structural determination of Vpu. One option which can be utilized in situations featuring poor correlation times is whole-protein perdeuteration to remove the effects of proton couplings and reduce the associated T2 relaxation effects upon the NMR spectrum (Gardner & Kay 1998), but due to the relatively poor chemical shift dispersion and broad linewidths observed in spectra such as Figure 4-5, this avenue was not pursued in our work.
Solid-state NMR experiments were conducted on frozen samples of Vpu in POPC, SDS, and DPC membrane mimetics to determine differences in chemical environment, assessed by changes in the chemical shift of observable resonances (Figure 4-7). Utilizing the knowledge that SDS-solubilized Vpu is a good representation of lipid-reconstituted Vpu, and that these samples are α-helical and oligomerize (Figure 2-5), we are confident that our solution NMR experiments on SDS-solubilized and DPC-solubilized Vpu are likely representative of Vpu in lipid. Overall, the lack of distinct changes in chemical shifts between samples reconstituted in SDS, DPC, and POPC indicates that the sites observed in the ssNMR experiment have similar chemical environments between the 3 sample conditions, and therefore a similar protein fold between the 3 environments. We conclude that the use of DPC and SDS as membrane mimetics for full-length Vpu are acceptable substitutes for a POPC lipid system. This is an important outcome, given that other membrane proteins such as SMR have been noted to form non-native structures in membrane mimetics such as DPC when compared to lipidic DMPC bicelles (Poget & Girvin 2007). The Vpu sample embedded in POPC bilayers exhibits more resonances in the PDSD experiment than the other membrane mimetics tested, but fewer resonances than SDS or DPC in the TOBSY experiment. This strongly indicates that differences exist in the degree of molecular motion occurring between lipid and detergents on the NMR timescale. The more rigid POPC lipid medium for protein insertion likely reduces the motion of some residues such as Ala, Ile, and Val residues primarily associated with the transmembrane domain of Vpu from that of the NMR timescale to something slower under cold sample conditions. This would serve to increase the visibility of these residues in CP-based solid-state NMR experiments, consistent with findings reported for full-length Vpu in DMPC lipids, wherein Vpu was found to be static below 15°C (Zhang et al. 2015). The charged residues found in the soluble Vpu cytoplasmic domain are better observed in the scalar-coupling mediated TOBSY experiment, and are associated with higher molecular motion and better resolution in NMR experiments when reconstituted in detergents such as DPC (Wittlich et al. 2009; Zhang et al. 2015).

The sharper lines observed in TOBSY experiments relative to PDSD experiments is indicative of an improvement in protein dynamics for NMR data collection, particularly given the appearance of residues in the more mobile extracellular region of Vpu; however, these linewidths fall short of those observed for the structural characterization of Vpu in DHPC micelles by solution NMR (Zhang et al. 2015), despite our use of SDS and DPC micellar systems. Therefore, we infer that
other factors are likely also playing a role contributing to the broad lines observed in our Vpu experiments.

4.4.3 Structure of Vpu monomers versus oligomers by NMR

We have shown NMR data acquired from Vpu samples reconstituted in a variety of membrane mimetic environments. In SDS and DPC, the full-length Vpu yields low signal to noise in solution NMR experiments (Figure 4-5), due to broad linewidths; lack of peak separation in $^1$H-$^{15}$N HSQC experiments (Figure 4-5) render these spectra unsuitable for further structural determination. Diffusion measurements of these samples indicate the prevalence of protein particles migrating at a similar rate to that of empty detergent micelles (Figure 2-11, Figure 2-13) despite the clear prevalence of these samples forming an oligomeric conformation based on data obtained by SDS-PAGE (Figure 2-3), size exclusion (Figure 2-8), and dynamic light scattering (Figure 2-7). We infer that full-length oligomeric Vpu is not amenable to structural determination by conventional solution state NMR techniques because of the large size, and therefore slow autocorrelation, of Vpu-detergent particles in aqueous solution. High-resolution NMR structural information reported for Vpu protein has largely been acquired utilizing solution NMR experiments with truncated Vpu protein in detergent solution (Wittlich et al. 2008; Wittlich et al. 2009; Zhang et al. 2015).

Recent work from the Opella laboratory has presented a structural model for monomeric, full-length Vpu (Zhang et al. 2015). These structures were determined by a combination of solution NMR of the Vpu cytoplasmic domain in DHPC and rotationally aligned, oriented sample solid state NMR of the full protein in DMPC. By combining data from highly mobile regions in DHPC with relatively immobile regions in DMPC, a structure of Vpu was determined, consistent with an N-terminal transmembrane domain aligned approximately with the bilayer normal, and two cytoplasmic helices close to perpendicular to the bilayer normal, available in the PDB as 2N28 and 2N29. Slight differences were observed in the cytoplasmic domain between DHPC and DMPC reconstituted samples, which were in turn slightly variant from previously reported data on this domain in DPC (Zhang et al. 2015; Wittlich et al. 2009).

The common approach utilized by these studies wherein the Vpu cytoplasmic domain was expressed and studied independently from the Vpu transmembrane domain yields an interesting corollary; that most high-resolution (solution) NMR data collected on Vpu is almost certainly
representative of a monomeric arrangement rather than an oligomeric assembly of Vpu, which has been a successful approach in determining the high-resolution structure of a Vpu monomer. While our preparations of Vpu show evidence for helical bundling via a shift in dichroism at 208nm/222nm, measurements are only presented for the Vpu cytoplasmic domain in other reports (Zhang et al. 2015). Although CD measurements do indicate α-helical Vpu protein in both cases, a direct comparison between our results and the high-resolution structures is not possible via these measurements. It is notable that the structures of other viroporin proteins such as the influenza M2 proton channel have been obtained in a similar manner to that utilized for Vpu, but direct comparison between detergent reconstituted and lipid reconstituted protein have yielded significant differences in protein structure (Cross et al. 2011).

In the approach we have taken, studying full-length Vpu by solid state and solution state NMR, the data we obtained were not sufficient to determine an atomic-level high resolution structure of Vpu in POPC, SDS, or DPC membrane mimetics, and we observed NMR lines which were broader than those reported in previous studies. However, we have also observed a likely oligomeric Vpu complex by SDS-PAGE, DLS, and SEC, while we observe only probable monomer species via solution NMR experiments. This may not be completely unexpected given the data reported in Chapter 3; the oligomeric models generated by MD simulations were heterogeneous, despite having well-conserved structural features. In NMR experiments, broadened lines are not necessarily indicative of poor solubilization, as observed NMR signal represents the average of many individual sites. Therefore, structural plasticity or a range of slightly different conformations represent a significant impediment to the high signal-to-noise ratio required for high-resolution structural determination by NMR methods. While the linewidth of MAS NMR spectra can be affected by the effects of dynamic motions on the T2 relaxation rates of the protein resonances, sample homogeneity in relatively rigid samples plays an important role in determining the success of NMR structural determination, particularly in the case of solid-state NMR experiments. Perfect sample homogeneity cannot be achieved in all cases, however; in some membrane proteins, structural heterogeneity is important for function. The pH gated KcsA potassium channel has recently been found to exhibit a degree of structural plasticity, consistent with slow interconversion between open and closed states required for channel function when measured in POPC/DHPC bicelles via NMR (Kim et al. 2016). Perhaps more relevant to viroporin studies, the influenza M2 proton channel has been examined by
oriented-sample NMR experiments, revealing that conformational heterogeneity contributes strongly to broadened linewidths despite only a small amount of structural variation (Li et al. 2007). Further investigations by MAS NMR techniques of pore-facing residues have characterized the configuration of M2 into three basis states, which are influenced by lipid composition, pH, and small molecule binding (Hu et al. 2011). The basis state structures described for M2 are not rigid, and show a degree of conformational plasticity even within each subset, resulting in larger spectral line widths for some basis structures important for proton conduction (Hu et al. 2011).

In light of these examples, it may be necessary to thoroughly examine variations in arrangements of Vpu in order to completely describe the structural ensemble required for function. Our MD data have indicated the presence of conformational heterogeneity even in the presence of persistent monomer-monomer contacts, supporting this hypothesis, while our NMR measurements of Vpu feature broad line shapes in conditions where Vpu oligomer species were observed by SDS-PAGE, DLS, and SEC experiments. In some cases, the inclusion of cholesterol can increase the rigidity of a membrane (Brown & Nevzorov 1999); this addition could a viable avenue to pursue higher signal to noise ratio in our NMR experiments, given the improvements provided by the decrease in molecular motion associated with low-temperature conditions.

Therefore, we conclude that an examination of the monomeric/oligomeric propensity of full-length Vpu will need to be undertaken in each membrane mimetic to understand whether the current high-resolution structures of Vpu monomers are also representative of Vpu oligomers. Our finding that NMR signal of full-length Vpu is observable in SDS, DPC, and POPC membrane mimetics will provide a valuable tool for the future refinement of oligomeric models.
4.5 Conclusion

In this Chapter, we have outlined our attempts to elucidate the structure of full-length Vpu in a lipid bilayer environment. While our work provides a clear demonstration that 2-dimensional HSQC spectra could be obtained of Vpu solubilized in SDS and DPC detergents, this did not allow unambiguous assignment of chemical shifts, as required for high resolution structural determination, which may be a consequence of Vpu oligomer formation.

Full-length Vpu was studied by solid-state NMR methods, and the data found to be comparable between detergent and lipid systems, and showing differences in mobility between residues within the transmembrane domain and the cytoplasmic domain. The similarity of NMR spectra between lipid and detergent systems provides reinforcement of our mutagenesis work in detergent systems, as these conditions are likely representative of lipid-solubilized Vpu, and may provide a future avenue for the exploration of water exposure and small molecule interactions with Vpu oligomeric assemblies.

Due to the intrinsic limitations on NMR measurements of Vpu samples outlined in this chapter, we expect that further work on Vpu oligomers will require alternate techniques as well as membrane mimetic systems capable of providing more solution-like characteristics. However, a careful comparison of Vpu spectra will need to be undertaken to validate the consistency of Vpu oligomerization in alternate mimetics.
5 Discussion

Vpu is a homo-oligomeric accessory protein from the HIV-1 virus which has been demonstrated to enhance the release of viral particles from infected cells. Vpu has been hypothesized to exert its effects *in vivo* via multiple mechanisms, either by ion channel formation or via interactions with host cell proteins such as CD4 and Tetherin. The importance of Vpu homo-oligomer formation is clear in the context of ion channel function, but less so with respect to protein-protein interaction; my goal in this body of work was to better understand the nature of Vpu homo-oligomers. I have characterized the oligomeric unit of HIV-1 Vpu, both in terms of the molecular basis for higher-order oligomer assembly, and through an examination of the physical size of that oligomer. This information will inform future protein-protein or protein-small molecule studies of Vpu, in the likely case that disruption or stabilization of the Vpu oligomer could have an impact on its physiological function.

5.1 Summary of findings

Based upon the assumption that Vpu must form an oligomeric assembly to fulfil its role in vivo, in Chapter 2 we demonstrated formation of Vpu oligomers in membrane mimetics such as SDS and the disruption of assembled oligomers by selective mutation. We hypothesized that residues Ala 7, 10, 14, and 18, falling in line upon one face of the transmembrane domain, are important in the formation of higher-order Vpu oligomeric complexes. We purified recombinant Vpu for SDS-PAGE experiments to assess the oligomeric state of Vpu solubilized in SDS, demonstrating that Ala 10, 14, and 18 contribute to Vpu oligomer formation in detergent systems, while Ala 7 played a lesser role than the other residues in the conserved AxxAxxxAxxxA motif. We examined the oligomeric size of the Vpu-SDS complex via the use of PFG-diffusion NMR experiments, but were only able to observe particles likely representing protein monomers within detergent micelles via this technique. With the use of DLS experiments, we observed particles which corresponded well in size to a Vpu pentamer within a detergent micelle. These data support the hypothesis that the large oligomeric Vpu-SDS particles were unobservable by solution NMR methods.

In Chapter 3, we explored the molecular basis for the interaction of Vpu monomers in greater detail. By applying the use of all-atom MD simulations to lipid bilayer embedded, fully-hydrated models of Vpu transmembrane domain oligomers, we conducted an investigation of the
dynamics of the complex on the sub-millisecond timescale from 0 to 150 ns. By constructing models consistent with conclusions from the mutagenesis experiments conducted in Chapter 2, we explored the configuration of Vpu oligomers containing 4, 5, and 6 monomers during the course of 150 ns simulations without experimental constraints applied to the system, aside from physical constants provided by the force field parameters of the simulation. These oligomeric complexes did not exhibit spontaneous formation of hydrated channels; furthermore, repeating each of these simulations with pre-constructed hydrated channels but otherwise identical conditions generally resulted in the collapse to a dehydrated complex in each case long before the end of each simulation run. Overall, we conclude that the formation of a hydrated Vpu oligomeric complex is unlikely to be seen on timescales accessible to all-atom molecular-dynamics simulations; an outcome which may explain the disagreement in literature between MD studies of Vpu channel function and ion channel conductance measurements.

We analyzed the simulation data in order to assess the importance of each of the Ala residues in the Vpu AxxAxxxxAxxxA motif, finding that the interface remained relatively intact throughout the time course of each simulation, and concluding that the Ala 10 and 14 positions formed more intermolecular contacts over the time course of the simulation than the Ala 18 position, which in turn formed more average contacts than the Ala 7 position. Based on these simulations we concluded that the AxxAxxxxAxxxA motif in the Vpu transmembrane domain is important for Vpu homo-oligomer formation within the lipid bilayer, and that the A10 and A14 residues are most critical of these residues for the stability of the Vpu homo-oligomer; this level of discerning power was previously unattainable by mutagenesis and SDS-PAGE experiments.

Finally, in Chapter 4, we provided detailed information on the production of recombinant Vpu in E. coli based systems utilized for structural study by NMR, generating uniformly $^{15}$N-$^{13}$C labelled protein. We attempted to generate atomic-level observations via the use of traditional solution-state and solid-state protein NMR techniques in SDS and DPC detergents, utilizing 2-dimensional $^1$H-$^{15}$N TROSY-HSQC experiments to observe signals associated with the amide backbone of Vpu, as well as 2-dimensional $^1$H-$^{13}$C HSQC experiments with slightly better resolution. Neither of these experiments was able to provide unequivocal, site-specific resolution for 3-dimensional experiments to determine protein structure. We were able to utilize uniformly labelled $^{15}$N-$^{13}$C Vpu samples to provide a direct comparison between the SDS, DPC, and POPC membrane mimetic systems, and observe differences in mobility of residues between the
transmembrane and cytoplasmic domains of Vpu. In solid-state NMR experiments, we observed largely transmembrane domain resonances, with best signal to noise ratio at lower temperatures in POPC, corresponding to increased membrane stiffness. These resonances can largely be classified as lower mobility versus the Vpu soluble domain, with wider linewidths than the resonances observed via scalar coupling mediated TOBSY experiments or solution NMR experiments, and featured the appearance of charged residues within the more mobile Vpu soluble domain, particularly at increased temperatures. After observing that the chemical shifts evident in these spectra were similar in SDS, DPC, and POPC mimetics, we conclude that the data collected in SDS are likely representative of Vpu in lipid bilayer systems. Overall, we conclude that final determination of a representative structure of oligomeric Vpu will require data combined from multiple experimental techniques, in order to observe residues with different dynamic regimes.

5.1.1 Vpu oligomerization in membrane mimetics

Our investigation into Vpu oligomerization began with an inspection of protein primary sequence, leading to several interesting possibilities for the arrangement of Vpu within the lipid bilayer. Based upon the requirement for approximately 20 to 30 residues to traverse the 30 Å lipid bilayer (Hildebrand et al. 2004) and largely hydrophobic side chains of transmembrane α-helices (Ulmschneider et al. 2005), featuring either aromatic or charged residues at interfaces with the lipid head groups (Killian & von Heijne 2000), the Vpu transmembrane domain is a strong example of common features in membrane protein primary sequence. Vpu contains a stretch of 20 hydrophobic residues in its transmembrane domain, with charged residues on each side of the lipid bilayer and an aromatic Trp residue within the transmembrane region (Figure 1-4).

We identified a sequence of residues within the Vpu transmembrane domain consisting of an AxxAxxxAxxxA cluster, interspersed with hydrophobic Val, Leu, and Ile residues reminiscent of a Phospholamban LxxIxxxLxxIxxxL oligomerization motif (Oxenoid & Chou 2005) or a glycophorin A small-small GxxxG or GG4 motif (MacKenzie et al. 1997). Each of these known motifs are involved in helix-helix packing; therefore, we undertook an effort to probe the requirement for this sequence in the assembly of a Vpu oligomeric complex. A Vpu oligomer has been proposed to explain the putative ion-channel assembly of Vpu (Ewart et al. 1996), with MD
simulations reporting a pentamer as the most likely arrangement for Vpu (Grice et al. 1997), and followed by further MD (Krüger & Fischer 2008; Mehnert et al. 2008; Moore et al. 1998; Kim et al. 2006) supporting the possibilities of tetrameric, pentameric, and hexameric Vpu oligomer arrangements.

Our results differ from several previous reported oligomeric Vpu studies in the observation of only a single oligomeric species via SDS-PAGE experiments (Figure 2-6). Despite disagreement with chemical crosslinking work conducted on the Vpu 1 to 40 peptide in bilayers suggesting the presence of multiple oligomeric populations (Lu et al. 2010), our current results may be a more representative data set because it describes the full length 82-residue Vpu rather than truncated peptides of the Vpu transmembrane domain. Our current observations are also in good agreement with an in vitro transcription/translation expressed full-length Vpu reported to contain a single oligomeric species by gel permeation chromatography (Hussain et al. 2007). It is notable that this previous study contained a significant amount of aggregated Vpu in SDS-PAGE experiments; the HFIP treatment of Vpu in our current work has improved upon these Vpu aggregation issues, at least in the case of the wildtype protein, and has allowed further characterization of the Vpu oligomer.

### 5.1.2 Vpu oligomers by molecular dynamics

We have attempted to model individual Vpu oligomeric complexes via the utilization of MD simulations. In this work, we have found that our models of the Vpu oligomeric complex represent a two-faces-per monomer bundle of Vpu rather than a less specific aggregative assembly. Since we have observed patterns in the number of contacts featured at each position in the Vpu AxxAxxxAxxxA motif, this situation seems likely to be a loosely organized oligomeric assembly driven by van der Waals forces, rather than demonstrating a near-crystalline consistency in structural arrangement.

However, we do not interpret this interaction model to mean that Vpu should be able to form oligomers of unlimited size; this is evidently not the case, as observed in hexameric complexes after the end of 150 ns simulations. In MD of hexameric Vpu complexes, we observed a single helix begin to “fray” off from the larger bundle (Figure 3-17, far right), a situation which is not evident in tetramer (Figure 3-13) or pentamer (Figure 3-8) arrangements. We infer that tetramer and pentamer Vpu configurations are more stable than hexamer arrangements, and indeed
perhaps the tetramer exhibits the greatest stability as it retains the greatest similarity with the MD starting structures. In all Vpu oligomeric models, Vpu helical bundles collapsed early in the simulation and become at least partially geometrically flattened, raising difficult issues classifying these bundles as a stable conformation. However, these tetrameric and pentameric oligomers are largely unchanged with respect to time after their initial collapse, and despite being conformationally heterogeneous, the continuous presence of the AxxAxxxAxxxA motif binding interface is observed to remain largely intact despite flattening of the initial arrangements. Therefore, our MD simulations are less consistent with a model in which Vpu oligomers form discrete and uniform symmetry, and more consistent with a bundle of Vpu helices defined by repeating interaction interfaces which also contain the structural plasticity required to undergo internal rearrangements. Such a population heterogeneity or mild asymmetry would be expected to give rise to the broader and more difficult to assign NMR data, as observed in our solid-state MAS experiments in Chapter 4.

Our MD results differ in some details from other reported oligomeric configurations, which claim a single discrete pentameric structure of Vpu (Kukol & Arkin 1999). MD studies featuring umbrella sampling techniques also claim a single pentameric Vpu assembly, with the caveat that the energetics are suitable for formation of tetramer, pentamer, and hexamer assemblies (Padhi et al. 2013). The basis for that conclusion: only the pentameric model forms a structure which will be an ion channel, and that the Vpu hexamer expels a monomer in 1 of 2 10 ns trials (Padhi et al. 2013). Indirectly, this assortment of Vpu oligomers is the closest published MD result to our own simulations. However, we did not observe the formation of a channel structure nor a hydrated channel in any oligomeric arrangement in a 15-fold longer 150 ns simulated time frame.

Other simulated Vpu systems indicate that residues composing the AxxAxxxAxxxA motif are important in dimer and trimer interfaces, and that Trp or purely hydrophobic residues face the centre of the oligomeric bundle on a 100 ns timescale (Li et al. 2013a). Furthermore, even in systems constructed featuring in a pore-like configuration with Ser facing inwards, no water column formation was reported in 25 ns simulations, and Ser hydrogen bonding was coordinated with the next carbonyl group in the same helix (Li et al. 2013a). Our MD work is in agreement with each of these results.
It is unclear if our modeled arrangements will be maintained on a millisecond timescale due to computational limitations, making it challenging to compare this work directly with discrete physical measurements of ion channel activity, as numerous studies have reported ion channel activity in Vpu (Maldarelli et al. 1993; Ewart et al. 1996; Schubert, Ferrer-Montiel, et al. 1996). Vpu has been described to exhibit cation-selective ion channel activity in black lipid bilayers (Ewart et al. 1996), specificity for monovalent cations in *Xenopus* oocytes experiments, and work has been presented to demonstrate that scrambling the Vpu transmembrane domain prevented ion channel conductance (Schubert, Ferrer-Montiel, et al. 1996). Due to high hydrophobicity, the Vpu transmembrane domain has virtually no way to interact with charged or polar molecules other than via a single Ser residue at position 23 (Figure 2-1).

This result raises another related issue: past models of Vpu oligomeric ion channels have suggested gating of a putative channel by the physical occlusion of the pore via the single conserved Trp residue (position 22), while other channel-pore measurements have demonstrated that the conserved Vpu Trp 22 residue previously assumed to act as the gating residue within the pore can be mutated with no effect on ion conductance (Mehnert et al. 2008). Our Vpu oligomeric assemblies feature both Ser 23 and Trp 22 facing away from the centre of the Vpu bundle in most cases.

In our MD models of Vpu Ser 23 is not facing into the centre of the helical bundle, making our model incompatible with previous ion channel models; this is also true of other recent models (Li et al. 2013a). It should be noted that in our proposed Vpu assembly, the models represent right-handed helical bundles, which are opposite to other accepted models of well-defined ion channels such as pentameric Phospholamban (Oxenoid & Chou 2005), but in agreement with recent Vpu oligomeric models which conclude that this arrangement may explain the weak selectivity of a putative Vpu ion channel (Li et al. 2013a; Li et al. 2013b). Overall, our results are in agreement with recent studies that have suggested that Vpu exhibits a “channel-pore dualism”, noting that Vpu channel-like activity is selective for neither anions nor cations, rendering ion conductance more of a pore effect than a channel effect (Mehnert et al. 2008). Furthermore, the conserved Ser 23 residue in the Vpu transmembrane domain is reported to be necessary but not sufficient to define ion conductance (Mehnert et al. 2008). Although our simulations do not feature Ser 23 within the Vpu bundle, they do not explicitly exclude the possibility that a Vpu helical monomer could exist in an alternate configuration in order to act as an ion channel. While
we have not directly observed a hydrated channel within our models, we did observe a single Vpu transmembrane helix with Ser turning into the oligomeric centre during the simulation of the Vpu hydrated tetramer, which resulted in the disruption of the pre-formed water channel. While ion conductance was not observed (presumably because the MD simulations did not contain an ion gradient or charge potential across the lipid bilayer), this result indicates that further investigation may be warranted in order to determine the frequency of single-monomer configurational changes.

Even if the rotation of Ser 23 into a correct position can occur, when Vpu is forming an extensive network of intermolecular contacts within the oligomer it is likely to be energetically unfavourable to break these contacts, overcome the lateral pressure within the lipid environment to dissociate a Vpu monomer from the complex, rotate the monomer to a Trp-in configuration, and reform the assembly without the energetic benefit of the AxxAxxxAxxxA packing interaction. This type of large-scale rearrangement was not observed during the 150 ns simulated timescale sampled by our simulations, but our data cannot categorically exclude the possibility of such an occurrence on much larger timescales which are only accessible by coarse-grained MD simulations. While one study has been conducted to explore the free diffusion of Vpu in lipid on a 10 µs timescale, the Vpu oligomers which were observed to form did not feature a Trp-in arrangement (Lin et al. 2016b).

In summary, our 150 ns all-atom MD simulations of Vpu homo-oligomers were not capable of forming nor maintaining a hydrated channel in tetrameric, pentameric, or hexameric arrangements, so we conclude that it is unlikely that the formation of Vpu ion channels is the main method of action by which Vpu increases viral budding. This conclusion is complemented by experimental work and editorial commentary from the Opella laboratory at UCSF, noting that as time has passed, research has passed from an interest in putative ion channel activity to other functions of these proteins, suggesting a reclassification from “viroporins” to “viral membrane-spanning miniproteins” (Opella 2015). Our work supports this sentiment.

5.1.3 Residues involved in Vpu homo-oligomer formation are implicated in Vpu interactions with host cell proteins

In our experiments, Vpu constructs migrated on SDS-PAGE with electrophoretic mobility higher than their corresponding monomer molecular weights of 9137 Da, and migrating consistently
with the expected mobility of a homo-oligomer composed of approximately five Vpu subunits. SDS-PAGE migration of TM peptides at greater than approximately 2 fold their molecular weight is often taken as evidence of self-association (Rath, Glibowicka, et al. 2009). Mutating Vpu Ala residues 10, 14, or 18 to Val had a noticeable effect on the migration of oligomeric Vpu in SDS-PAGE experiments, while mutation of Ala residue 7 did not.

Similarly, our MD simulations indicate that Ala residues 10, 14 and 18 formed a greater number of homo-oligomeric contacts during the time evolution of the simulation than Ala residue 7. These same Vpu residues have been implicated in other studies as important for Vpu interactions with host cell proteins.

In targeted Vpu-Tetherin interaction studies, mutation of Vpu Ala 14 to Leu was equivalent to total deletion of Vpu based upon viral particle release assays, but the mutant still retained the ability to downregulate human CD4 protein (Vigan & Neil 2010). The authors reported a continued ability of Vpu to oligomerize in cellular membranes, although the stability or configuration of that arrangement could not be determined. Similarly, viral release experiments have demonstrated that Vpu Ala residues 14 and 18 were of clear importance to the Vpu-Tetherin interaction, and could be important in the conformation of Vpu oligomers (Vigan & Neil 2010; Douglas et al. 2013). Vpu Ala 10 and 14 have been reported to be critical for functional interaction between Vpu and Tetherin/CD317 protein via viral particle release assays, without any effect on ion channel measurements (Bolduan et al. 2011). Furthermore, three of the four residues (Ala 10, 14, and 18) are critical for the formation of Vpu-Tetherin contacts based on NMR titration experiments (Skasko et al. 2012), and for the Vpu-mediated downregulation of host cell CD155 protein in viral infection (Bolduan et al. 2014). The reverse format of mutational analysis with non-pandemic HIV strains has also confirmed that conversion of glutamic acid 14 and Val 18 to Ala residues could confer efficient Vpu interaction with human Tetherin to these strains (Sauter et al. 2012).

Taken as a whole, these studies have demonstrated that Vpu Ala 10, 14, and 18 are all important for interaction of Vpu with Tetherin. While we have not directly tested the effects of Vpu oligomerization on its capability to interact with Tetherin, it is clear that the same residues play a key role in Vpu homo-oligomerization and interaction with binding partner proteins. Logically then, the question must be raised about interaction versus function: is the Vpu-Tetherin
interaction an important property defined by the Vpu AxxAxxxAxxxA motif, or is Vpu oligomer formation required for the effective interaction with Tetherin?

In what appears to be a definitive answer, Skasko et al. have utilized purified Vpu and Tetherin transmembrane domain peptides to perform NMR titration experiments to study the formation of Vpu-Tetherin contacts, showing a perturbation of chemical shifts as evidence that a direct interaction between the two proteins occurs (Skasko et al. 2012). Furthermore, Vpu transmembrane domain triple mutants in which Ala 10, 14 and 18 are substituted with Phe colocalize with Tetherin within endosomes, but these Vpu mutants were unable to remove Tetherin from the cell surface, implying a functional role for these residues in the Vpu-Tetherin interaction. The mutation of these same residues induced only minimal chemical shift changes in Tetherin transmembrane domain peptides, and therefore by both counts this Vpu mutant does not interact strongly with Tetherin (Skasko et al. 2012). A direct interaction is a logical conclusion to draw, since mutation of these Vpu Ala residues abrogates the Vpu-Tetherin interaction.

However, another crosslinking study designed to elucidate interaction between Vpu and Tetherin proteins shows a differing outcome. When Vpu Ala 10, 14, or 18 were mutated to Cys in scanning mutagenesis, crosslinking between Vpu and Tetherin was impaired, but HIV-1 virion generation and Tetherin downregulation were unimpaired (McNatt et al. 2013). The authors of this work conclude that these Vpu residues may not contact Tetherin directly, or that the oxidation reagent could not penetrate the cell membrane (McNatt et al. 2013). If these two studies are examined from a perspective focused upon Vpu homo-oligomerization, our current data may reconcile the two differing reports. Our Ala to Val mutations represent a larger steric change than the nonperturbing Ala to Cys mutations utilized by McNatt, but a smaller steric change than the perturbing Ala to Phe mutations utilized by Skasko. Given that Ala to Cys mutations did not disrupt Vpu-Tetherin function, and Ala to Phe mutations were disruptive to Vpu-Tetherin function, the effect of Ala to Val mutations at these same residues are a middle ground. These data would be worthwhile to collect to elucidate the potential role of the Vpu oligomer; if the Ala 10, 14, and 18 residues are important in formation of a Vpu oligomer, it will be interesting to determine whether Vpu oligomer formation is also required for proper Vpu interaction with binding partners. While we have directly established that these residues are important for Vpu-Vpu interaction, and others have shown their importance for Vpu-Tetherin interactions, indirect effects of these mutations may actually be the causative agent for reported
changes in Vpu-Tetherin interactions. With a large body of evidence pointing to the involvement of the residues within the Vpu AxxAxxxAxxA motif in Vpu-protein interactions, additional studies will ultimately be required to clarify the role or roles of the Vpu oligomer in interactions with host cell proteins.

An extension of this concept could examine the potential role of localization to lipid raft microdomains common to Tetherin/BST-2 (Dubé et al. 2009; Masuyama et al. 2009) and Vpu, which can be modulated by mutation of the Vpu transmembrane domain (Ruiz et al. 2010). Studies utilizing Amphotericin B methyl ester, known to localize to cholesterol-rich regions of membrane, indicate that Vpu-mediated viral budding is inhibited and Vpu-Tetherin interaction is downmodulated by the presence of Amphotericin B methyl ester in a manner distinct from that of simple cholesterol depletion (Waheed et al. 2008). Mutations found to abolish Vpu lipid-raft association include mutation of the conserved Trp 22 residue in the Vpu transmembrane domain, as well as Val to Ala mutations in the Trp proximal region (Ruiz et al. 2010). This region is a part of the putative interaction interface for Vpu Ala 18, found to be a modulator of homo-oligomerization in this work. In this scenario, the question that could be raised is one of membrane composition: could the presence of cholesterol within the bilayer have a strong effect on the homo-oligomerization of Vpu? This unexplored relationship could be relevant when considering the notion that cholesterol lipid rafts were originally identified as detergent-resistant membrane fractions, and we have demonstrated the capability of Vpu to oligomerize even in detergent conditions. Therein, a model could be envisioned whereby membranes containing low cholesterol such as the endoplasmic reticulum could prevent Vpu contact with potential binding partners in non-productive locales. Upon translocation to the cholesterol-enriched plasma membranes, which are the sites of viral particle assembly (via HIV Gag and Env proteins) and Tetherin localization (Ono & Freed 2001; Ruiz et al. 2010), Vpu could potentially adopt a productive configuration, allowing an interaction with Tetherin. Some evidence has been presented which could corroborate such a model; lipid raft fractions are specifically depleted in Tetherin in cells where Vpu is expressed, and an increased proportion of Tetherin molecules in these cases are found en route to endosomes for degradation (Rollason et al. 2013). Furthermore, Vpu oligomerization has been reported to be specific to subcellular compartments: based on FRET measurements, oligomerization occurs in the Golgi or intracellular vesicles, but not the endoplasmic reticulum (Hussain et al. 2007). An exploration of this theoretical model is well
beyond the scope of the work presented here, but would almost certainly be a worthwhile endeavour. However, the challenges with the characterization of Vpu within lipid membranes are manifest and have been explored in this work, particularly with respect to the resolution of NMR studies, where construction of a cholesterol-rich liposomal system would preclude the use of high-resolution solution NMR techniques. It is interesting to note that different lipid environments have been utilized in 10 ns simulations of Vpu monomer including DPPC, POPC, DTPC, and DDPC, showing that Vpu monomer is highly flexible, and therefore configuration may depend on surrounding lipid, and by extension the surrounding cellular compartment (Krüger & Fischer 2008). Simulation work has also suggested that lipid rafts may be required to allow structural rearrangements necessary to form ion channel activity within the membrane (Li et al. 2013a).

It is important to note, however, that none of these models are mutually exclusive. It may be true that important Vpu interacting residues elucidated by previous studies as being a Vpu-Tetherin interface are experiencing a different chemical environment, and therefore showing different chemical shifts, upon Tetherin binding. It may also be true that the same residues are involved in Vpu homo-oligomerization and are therefore important for this interaction in other specific contexts. Further experiments and more data will be required to corroborate these models, and to clarify the possible requirement for Vpu homo-oligomer disruption as a prerequisite for the formation of a Vpu heterodimer with other binding partner proteins.

5.2 Conclusions

Our data, taken together within the context of the larger field of Vpu and Tetherin knowledge, confirm the importance of the AxxAxxxAxxxA motif in the Vpu transmembrane domain by demonstrating the importance of this sequence for Vpu homo-oligomer formation. Furthermore, we have established that the interaction between Vpu monomers is stable enough to occur in SDS micelles, and that NMR chemical shifts are similar between Vpu solubilized in SDS, DPC, and POPC membrane mimetics. To our knowledge, our study is the first to date which characterizes the molecular basis for the formation of Vpu oligomers, and the first study to provide a demonstrated means to disrupt the oligomerization of Vpu. Moving forward, it is our hope that the mechanism will be determined more fully, allowing a detailed understanding of
Vpu oligomer stability and assembly in order to answer questions related to the role of the oligomer in the physiological function of Vpu.

Our MD studies of Vpu provide structural data on potential modes of assembly of Vpu homo-oligomers with models derived from physical observations of secondary structure and mutational analysis, and our simulated trajectories were used to confirm the interface mediated by AxxAxxxAxxxA motif residues via observation of these oligomers throughout 150 ns timescales. We conclude that critical factors influencing the stability of Vpu oligomeric species also likely play a role in the formation of Vpu interactions with other binding partners. The connection between the homo-oligomerization and the function of Vpu is not clear, but we hope these concepts and the data that we have accrued may become an important factor in developing new strategies to combat HIV infection.

5.3 Final concluding remarks

The work presented in this thesis showed that Vpu oligomerization likely plays a large role in the function of this protein. The propensity of Vpu monomers to form a stable oligomeric structure appears to be robust enough to occur in multiple membrane mimetics, and may represent a mechanism for switching between functional states of Vpu depending on environment. The molecular basis for the interaction of Vpu monomers has been probed via mutagenesis and can therefore be controlled for further work studies of viral infectivity. Models have been generated which represent likely configurations of Vpu oligomeric complexes which support the role of the strongly conserved AxxAxxxAxxxA motif in Vpu oligomer formation. While high-resolution NMR structures could not be determined, combined evidence from mutational analysis, SDS-PAGE experiments, MD simulations and NMR experiments suggests that Vpu may feature a heterogeneous conformation within oligomeric complexes, mediated by conserved residues within the AxxAxxxAxxxA motif.
Bibliography


Requirements for the selective degradation of CD4 receptor molecules by the human immunodeficiency virus type 1 Vpu protein in the endoplasmic reticulum. *Retrovirology*, 4, p.75.


Ivanchenko, S., Godinez, W.J., Lampe, M., Kräusslich, H.-G., Eils, R., Rohr, K., Bräuchle, C.,


agents and chemotherapy, 54(2), pp.835–45.


Kukol, A. & Arkin, I.T., 1999. vpu transmembrane peptide structure obtained by site-specific


MacKerell, A.D., Bashford, D., Bellott, M., Dunbrack, R.L., Evanseck, J.D., Field, M.J., Fischer,


Mitchell, R.S., Katsura, C., Skasko, M.A., Fitzpatrick, K., Lau, D., Ruiz, A., Stephens, E.B.,


phospholipid bicelles. *Biophysical journal*, 91(8), pp.3032–42.


Journal, 84(5), pp.3276–84.


Taube, R., Alhadeff, R., Assa, D., Krugliak, M. & Arkin, I.T., 2014. Bacteria-based analysis of


Triba, M.N., Devaux, P.F. & Warschawski, D.E., 2006. Effects of lipid chain length and


Yao, H. & Hong, M., 2013. Membrane-dependent conformation, dynamics, and lipid interactions


