The Role of Phosphoinositides in the Clustering and Assembly of Viral Proteins Quantified by Fluorescence Super-Resolution Microscopy

Prakash Raut
prakash.raut@maine.edu

Follow this and additional works at: https://digitalcommons.library.umaine.edu/etd

Recommended Citation

This Open-Access Dissertation is brought to you for free and open access by DigitalCommons@UMaine. It has been accepted for inclusion in Electronic Theses and Dissertations by an authorized administrator of DigitalCommons@UMaine. For more information, please contact um.library.technical.services@maine.edu.
THE ROLE OF PHOSPHOINOSITIDES IN THE CLUSTERING AND ASSEMBLY OF VIRAL PROTEINS
QUANTIFIED BY FLUORESCENCE SUPER-RESOLUTION MICROSCOPY

By

Prakash Raut

B.Sc. St. Xavier’s College, 2009
M.Sc. University of Delhi, 2012

A DISSERTATION
Submitted in Partial Fulfillment of the
Requirements for the Degree of
Doctor of Philosophy
(in Physics)

The Graduate School
The University of Maine
August 2021

Advisory Committee:
Samuel T. Hess, Professor of Physics, Advisor
R. Dean Astumian, Professor of Physics
James P. McClymer, Associate Professor of Physics
Julie A. Gosse, Associate Professor of Biochemistry
Melissa Maginnis, Associate Professor of Microbiology
Influenza A is a highly contagious and a pathogenic virus that causes serious respiratory illness, the complications from which can be fatal even to young and healthy adults. On average, approximately 250,000 to 500,000 people die each year from complications due to Influenza A (IAV) worldwide. Frequently occurring mutations can evade the vaccine-developed immune response, so the vaccines need to be updated continuously. Drug resistance to some of the existing drugs has already been established. Hence new antiviral therapeutics need to be explored in order to prevent further morbidity and mortality. To develop such a novel antiviral therapeutic, knowledge of the viral life cycle and interaction between the viral proteins and host cell lipids and proteins is crucial, as influenza viruses hijack these cellular components during infection.

IAV Hemagglutinin (HA) is the most abundant viral glycoprotein responsible for viral binding and entry. HA clusters at the host cell plasma membrane, and these clusters need to be high in density to catalyze membrane fusion for viral entry. However, the mechanism by which HA forms clusters remains unknown. We recently showed clustering of HA is modulated by phosphatidylinositol (4,5) bisphosphate (PIP2). Targeting this interaction could lead to possible alternative antiviral therapeutics. CPC (Cetylpyridinium Chloride) is a positively charged quaternary ammonium compound used in mouthwashes and personal care items. CPC has been previously shown to have antibacterial and antiviral properties. While both the antibacterial and antiviral properties of CPC are well understood at high
concentrations (millimolar), the effect of CPC on cell function at relatively low (micromolar) concentrations is not well understood.

In this study, we use the super-resolution microscopy technique FPALM to study the effect of CPC on PIP2-binding proteins, and to illuminate the mechanism of the antiviral properties of CPC at these much lower, non-cytotoxic micromolar concentrations in the cell model. Results show that CPC at these concentrations significantly modulates PIP2 clustering and HA clustering, and more importantly, reduces the HA density and the co-clustering of HA and PIP2. CPC also disrupts the assembly of HA and the IAV Matrix Protein 1 (M1). These results are important because dense HA clusters correlate with efficient viral entry and infectivity, and modulating PIP2 clusters reduces HA clustering and in turn disrupts the assembly of HA and M1. In addition, we also show CPC at micromolar concentrations can improve the survival of zebrafish infected with IAV. In addition, we also show for the first time that M1 colocalizes with PIP2 and demonstrate the influence of HA in M1 clustering at the plasma membrane, which might have an impactful role in the viral life cycle.
DEDICATION

To all my family members for the support and the encouragement which continuously inspired me through this journey.
ACKNOWLEDGEMENTS

First of all, I am extremely grateful to my Ph.D. advisor Dr. Samuel T. Hess whose continuous guidance, support, and extremely valuable advice made my Ph.D. journey and this dissertation possible. Dr. Hess’s endless patience, encouragement and teachings helped me grow as a scientist. His passion and motivation for research have been a source of inspiration for me in this journey and will continue to be in the future. I’d also like to thank my Ph.D. committee members Dr. R Dean Astumian, Dr. James P. McClymer, Dr. Melissa Maginnis for their valuable inputs which made my research more meaningful and especially to Dr. Julie A. Gosse who believed in me and provided me with the opportunity to collaborate with her lab. Collaboration with Dr. Gosse’s lab was extremely important for this dissertation to be possible.

My sincere gratitude to Matthew Valles who started training me in the lab and later by Dr. Matthew Parent. Dr. Parent’s hands-on training on the scope as well as on the data analysis was very crucial in this journey and I will always be grateful for his training. My sincere thanks to Komala Shivanna, Brandon Aho and David Winski from the Hess lab, Suraj Sangroula, Bright Obeng, Sasha Weller, Bailey West, Christian Potts, Marissa Kinney, Jack Burnell from the Gosse lab, Mary Astumian from the Henry lab, and Brandy Soos from the King lab for all their contributions in this dissertation.

I would also like to thank my parents Kamal B. Raut and Pramila Raut; my wife Nazima Khatri Raut and my sisters Srijana Raut and Joshna Raut for their love, support, and continuous encouragement throughout the years which kept me motivated.

Finally, I want to thank the Department of Physics and Astronomy, University of Maine for believing in me and providing me with the opportunity to pursue my Ph.D.
# TABLE OF CONTENTS

DEDICATION ........................................................................................................................................... v

ACKNOWLEDGEMENTS ................................................................................................................................. vi

LIST OF FIGURES .......................................................................................................................................... xii

LIST OF ABBREVIATIONS ............................................................................................................................ xiv

Chapter

1. INTRODUCTION ........................................................................................................................................ 1

1.1. Introduction ........................................................................................................................................... 1

1.2. Influenza A virus structure and subtypes ............................................................................................. 2

1.3. Influenza A viral life cycle ..................................................................................................................... 3

1.4. Hemagglutinin (HA) ............................................................................................................................... 5

1.5. Matrix Protein 1 (M1) ............................................................................................................................ 7

1.6. Actin ....................................................................................................................................................... 9

1.7. Phosphatidylinositol (4,5) bisphosphate (PIP2) .................................................................................... 10

1.7.1. Platforms .......................................................................................................................................... 11

1.7.2. Synthesis .......................................................................................................................................... 12

1.7.3. Megapool .......................................................................................................................................... 12

1.8. Diffraction limit ..................................................................................................................................... 13

1.9. Fluorescence .......................................................................................................................................... 14

1.9.1. Radiative Transition ............................................................................................................................ 14

1.9.1.1. Absorption .................................................................................................................................... 14

1.9.1.2. Emission ....................................................................................................................................... 15

1.9.2. Non-radiative transitions .................................................................................................................... 15

1.9.2.1. Vibrational Relaxation .................................................................................................................. 15
3.3.3. MARCKS Assay in RBL-2H3 Mast Cells ................................................................. 33
3.3.4. Confocal Microscopy ............................................................................................. 34
3.3.5. Automated Image Analysis .................................................................................. 34
3.3.6. CPC Cytotoxicity and Survivability in RBL-2H3 cells ....................................... 35
3.3.7. Fluorescence Photo Activation Localization (FPALM) Imaging and Processing .................................................. 36
3.3.8. FPALM Data Analysis ......................................................................................... 37
3.3.9. Zebrafish Care and Maintenance ....................................................................... 37
3.3.10. Microinjection of IAV ...................................................................................... 38
3.3.11. CPC treatment on Zebrafish ............................................................................ 38
3.3.12. IAV and Mortality Curves ................................................................................ 38
3.4. Results ...................................................................................................................... 39
  3.4.1. CPC Significantly displaces MARCKS from the plasma membrane into the cytoplasm after 30-minute incubation ................................................................. 39
3.4.2. CPC at concentrations ≤ 15 μM is not cytotoxic to RBL-2H3 cells during a short-term (1-hour) exposure ............................................................................................ 41
3.4.3. CPC disrupts PIP₂ clusters in NIH3T3 cells ....................................................... 43
3.4.4. CPC disrupts HA clusters in NIH3T3 cells ......................................................... 45
3.4.5. CPC reduces the co-clustering of HA and PIP2 in NIH3T3 cells ....................... 47
3.4.6. CPC treatment Reduces IAV infections and Increases survival in AB Zebrafish Embryos .......................................................... 49
3.5. Discussion ............................................................................................................... 51
  3.5.1. CPC Modulates the Cellular Distribution of PIP2-Binding Proteins .................. 51
3.5.2. PH-Domain Cluster Properties Compared to Published Values...................... 52
4. CHAPTER 4 CETYLPYRIDINIUM CHLORIDE (CPC) DISRUPTS THE ASSEMBLY OF INFLUENZA A HEMAGGLUTININ (HA) AND THE MATRIX PROTEIN 1 (M1) ........................................ 55

4.1. Preface ............................................................................................................ 55

4.2. Introduction .................................................................................................... 55

4.3. Methods ......................................................................................................... 57

4.3.1. Cell Culture, Transfection and Fixation ....................................................... 57

4.3.2. CPC solution preparation ........................................................................... 58

4.3.3. Two-color FPALM Imaging ..................................................................... 58

4.3.4. Data Analysis ............................................................................................. 59

4.3.5. Manders’ Colocalization Coefficient ....................................................... 60

4.3.6. Cluster Identification .................................................................................. 60

4.3.7. Co-clustering Analysis ............................................................................. 61

4.3.8. Statistical Analysis .................................................................................... 61

4.4. Results .......................................................................................................... 61

4.4.1. CPC disrupts PIP2 and M1 colocalization. ................................................ 61

4.4.2. CPC disrupts PIP2 clusters in NIH3T3 cells expressing M1 ..................... 64

4.4.3. CPC disrupts M1 clusters in NIH3T3 cells .............................................. 67

4.4.4. CPC disrupts the co-clustering of PIP2 and M1 in NIH3T3 cells ............. 69

4.4.5. M1 enhances PIP2 clustering ................................................................... 71

4.4.6. CPC disrupts the colocalization of HA and M1 expressed together in NIH3T3 cells ............................................................. 73

4.4.7. CPC disrupts HA clusters in NIH3T3 cells expressed together with M1 .......... 76

4.4.8. CPC disrupts M1 clusters in NIH3T3 cells expressed together with HA .......... 78
LIST OF FIGURES

Figure 1.1  A schematic diagram showing the structure of Influenza A along with the viral proteins and vRNP ................................................................. 3

Figure 1.2  A schematic of the IAV life cycle ........................................................................... 5

Figure 1.3  A simplistic picture of Jablonski diagram .............................................................. 16

Figure 1.4  Concept of FPALM ............................................................................................... 20

Figure 1.5  A schematic diagram showing Widefield versus TIRF illumination ...................... 21

Figure 2.1  Colocalization of HA and PH domain (PLC-δ) by confocal and super-resolution microscopy ........................................................................... 26

Figure 2.2  PIP2 and HA cluster properties as a function of enrichment each other ............... 28

Figure 3.1  CPC effects on plasma membrane (PM) versus cytoplasmic distribution of PIP2-binding protein MARCKS in RBL-2H3 mast cells ......................... 40

Figure 3.2  Measurement survivability of RBL-2H3 cells at different doses of CPC ............... 42

Figure 3.3  CPC disrupts PIP2 cluster properties in NIH3T3 cells ........................................ 44

Figure 3.4  CPC disrupts HA cluster properties in NIH3T3 cells ........................................... 46

Figure 3.5  Super-resolution images show colocalization of HA-Dendra2 and PAmKate-PH domain (PLC-δ) in fixed NIH3T3 cells ........................................... 47

Figure 3.6  CPC treatment reduces HA-PH co-clustering ........................................................ 48

Figure 3.7  CPC treatment reduces IAV infections and increases survival in AB Zebrafish embryos ................................................................. 50

Figure 4.1  Super-resolution image showing the colocalization of PIP2 and M1 .................... 62

Figure 4.2  CPC reduces the Manders’ Colocalization Coefficient (MCC) of PIP2 and M1 in fixed NIH3T3 cells ................................................................. 63

Figure 4.3  CPC modulates the PIP2 clustering in fixed NIH3T3 cells .................................... 66
Figure 4.4. CPC modulates the M1 clustering in fixed NIH3T3 cells ............................... 68
Figure 4.5. CPC reduces the co-clustering of PIP2 and M1 .............................................. 70
Figure 4.6. M1 enhances PIP2 clustering .............................................................................. 72
Figure 4.7. Super-resolution image showing the colocalization of HA and M1 ................. 74
Figure 4.8. CPC reduces the Manders' Colocalization Coefficient (MCC) of HA and M1 in NIH3T3 cells ........................................................................................................... 75
Figure 4.9. CPC modulates HA clustering in NIH3T3 cells .................................................. 77
Figure 4.10. CPC modulates the M1 clustering in NIH3T3 cells .......................................... 79
Figure 4.11. CPC reduces the co-clustering of HA and M1 in NIH3T3 cells ....................... 81
Figure 4.12. HA does not modulate M1 association with the membrane .......................... 82
Figure 4.13. HA enhances the M1 clustering in NIH3T3 cells ............................................. 84
Figure A1. SARS-CoV-2 S protein colocalizes with PIP2 ................................................ 116
Figure A2. CPC modulates the PH clustering in NIH3T3 cells ......................................... 118
Figure A3. CPC modulates SARS-CoV-2 S protein clustering in NIH3T3 cells ................. 120
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CPC</td>
<td>Cetylpyridinium Chloride</td>
</tr>
<tr>
<td>CMC</td>
<td>Critical Micelle Concentration</td>
</tr>
<tr>
<td>CT</td>
<td>Cytoplasmic Tail</td>
</tr>
<tr>
<td>CTD</td>
<td>Cytoplasmic Tail Domain</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DRMs</td>
<td>Detergent-Resistant Membranes</td>
</tr>
<tr>
<td>EM</td>
<td>Electron Microscope</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>FPALM</td>
<td>Fluorescence PhotoActivation Localization Microscopy</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>IAV</td>
<td>Influenza A Virus</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol trisphosphate</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate Dehydrogenase</td>
</tr>
<tr>
<td>M1</td>
<td>Matrix Protein 1</td>
</tr>
<tr>
<td>M2</td>
<td>Matrix Protein 2</td>
</tr>
<tr>
<td>MCC</td>
<td>Manders’ Colocalization Coefficient</td>
</tr>
<tr>
<td>NA</td>
<td>Neuraminidase</td>
</tr>
<tr>
<td>NEP</td>
<td>Nuclear Export Protein</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear Localization Signal</td>
</tr>
<tr>
<td>NP</td>
<td>NucleoProtein</td>
</tr>
<tr>
<td>PA</td>
<td>Polymerase Acid</td>
</tr>
<tr>
<td>PB1</td>
<td>Polymerase Basic 1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>PB2</td>
<td>Polymerase Basic 2</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin Homology</td>
</tr>
<tr>
<td>PI4K</td>
<td>Phosphatidylinositol 4 Kinase</td>
</tr>
<tr>
<td>PI5K</td>
<td>Phosphatidylinositol 5 Kinase</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol (4,5) bisphosphate</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphatidylinositol (3,4,5) trisphosphate</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PM</td>
<td>Plasma Membrane</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylerine</td>
</tr>
<tr>
<td>PSF</td>
<td>Pont Spread Function</td>
</tr>
<tr>
<td>PtdIns</td>
<td>Phosphoinositides</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin Homolog</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of Interest</td>
</tr>
<tr>
<td>SLCA</td>
<td>Single Linkage Cluster Analysis</td>
</tr>
<tr>
<td>TIRF</td>
<td>Total Internal Reflection Fluorescence</td>
</tr>
<tr>
<td>VLP</td>
<td>Virus Like Particles</td>
</tr>
<tr>
<td>vRNA</td>
<td>viral RiboNucleic Acid</td>
</tr>
<tr>
<td>vRNP</td>
<td>viral RiboNucleo Protein</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

1.1. Introduction

Influenza A virus (IAV) infection is one of the leading causes of death around the world. IAV is highly contagious and its complications are responsible for serious illness of 3 to 5 million people and about 250,000 to 500,000 deaths per year worldwide (Fireman et al., 2009; Ginsberg et al., 2009). Influenza A epidemics can affect people of all age groups, but children aged under 5 years, elderly individuals, pregnant women and people with chronic lung conditions are at the greatest risk. IAV can cause severe respiratory distress, and the complications can be fatally dangerous to young and healthy adults as well. IAV has caused numerous pandemics. In 1918, the Spanish flu pandemic infected about 500 million people and was responsible for the mortality of an estimated 40 million people worldwide (Taubenberger et al., 2001). The strains arising due to frequently occurring mutations can lead to evasion of the protection resulting from vaccination. According to the World Health Organization (WHO), every year approximately 3-5 million people fall seriously ill and about 290,000-650,000 people die globally due to complications from IAV (Influenza (Seasonal), no date). While vaccines are major weapons in our arsenal to prevent influenza, the efficacy of the vaccines is not 100%. Furthermore, vaccines are prepared in advance by predicting the strains that might be dominant in the upcoming season, and unpredicted mutations can also lead to evasion of the vaccine, or can lower the efficacy of the vaccines (Vaccine Effectiveness: How Well Do the Flu Vaccines Work? / CDC, no date). The major drugs that are commonly used to treat influenza after the infection are neuraminidase inhibitors and M2 channel blockers. As the name suggests, M2 channel blockers prevent infection by blocking the M2 ion channels needed during viral entry. However, the effectiveness of such drugs has been limited, as the virus has been reported to rapidly develop resistance against them (Dharan et al., 2009). Neuraminidase inhibitors have been more effective than M2 channel blockers; however, the study of the 2009 pandemic showed a high level of
resistance to oseltamivir and a 100 times decrease in peramivir susceptibility (Vanderlinden and Naesens, 2014). Thus, it is important to investigate new classes of antiviral compounds. In order to identify such compounds, it is crucial to understand the viral life cycle and the resulting interactions between the virus and host cell, as viruses hijack the host cell signaling, gene expression, protein synthesis, and trafficking machinery, as well as (for enveloped viruses) host cell lipid components during many stages of their life cycle (Avalos, Yu and Nayak, 1997; Ali et al., 2000; Nayak, Ka-Wai Hui and Barman, 2004; Radtke, Döhner and Sodeik, 2006; Davey, Trave and Gibson, 2011; Rossman and Lamb, 2011; Zhao, Wang and Li, 2017).

1.2. Influenza A virus structure and subtypes

Influenza A virus (IAV) is a pleomorphic filamentous or spherical enveloped virus of approximately 100 nm in diameter (Fujiyoshi et al., 1994) belonging to orthomyxoviridae family (Lamb, Robert and Choppin, 1983). Although the laboratory produced strains are typically spherical in shape, virus isolated directly from the samples obtained from the upper respiratory tract shows a filamentous morphology (Chu, Dawson and Elford, 1949; Rossman and Lamb, 2011). The IAV genome consists of eight negative stranded RNA segments which code for 10 viral proteins (Lamb, Robert and Choppin, 1983; Enami, Fukuda and Ishihama, 1985), of which seven are structural proteins and three are nonstructural proteins. The viral envelope, which it derives from the plasma membrane of the host cell (Zhang, Pekosz and Lamb, 2000) contains three viral membrane proteins: Hemagglutinin (HA), Neuraminidase (NA), Matrix Protein 2 (M2). HA and NA protrude out of the extracellular side of the lipid bilayer, while M2 forms a transmembrane ion channel. Beneath the lipid bilayer, Matrix protein 1 (M1) forms a layer of scaffold for the virus particle. Nucleoprotein (NP), three polymerases (PB1, PB2, PA) together with eight negative stranded RNA form vRNP. vRNP along with a few Nuclear Export Protein (NEP) form the viral core of the virus. Influenza A is further divided into subtypes on the basis of HA and NA. According to the CDC, there are 18 known HA subtypes and 11 known NA subtypes. Hence many different combinations are possible. For example, influenza A H3N2 means IAV has HA protein subtype 3 and NA protein subtype 2. The naming convention
used for the influenza virus starts with the antigenic type (A/B/C/D), or virus type, followed by the host type (if the virus has not been isolated from a human), place of origin, strain number, and year of isolation. In the case of influenza A, the HA and NA antigen type inside the parentheses follow the virus type, e.g. avian influenza A/HongKong/156/97(H5N1). The nomenclature A/duck/Alberta/35/76(H1N1) is an example of the influenza A virus isolated from duck which originated in Alberta, with the strain number 35 in the year of (19)76 that had H1 and N1 antigen types for hemagglutinin and neuraminidase, respectively.

Figure 1.1: A schematic diagram showing the structure of Influenza A along with viral proteins and the vRNP.

1.3. Influenza A viral life cycle

The IAV life cycle begins with viral binding to receptors expressed in the epithelial cells of the respiratory tract containing certain sialic acids (Shinya et al., 2006). In humans, upper respiratory tissues such as in the lungs have α2-6 linked sialic acids in abundance. In contrast, in avians, α2-3 linked sialic acids are dominant in the upper respiratory tract (Sun and Whittaker, 2013). HA binds to receptors with
either α2-3 or α2-6 linked sialic acids, and then undergoes internalization by receptor mediated endocytosis (Wilson, Skehel and Wiley, 1981; Skehel and Wiley, 2000). Once inside, the host cell endosome acidifies, and at a pH of 5.0, enzymatically cleaved HA undergoes a dramatic conformational change to initiate the fusion process (Skehel and Wiley, 2000). Cleavage of HA is necessary for the fusogenic properties of HA (Hamilton, Whittaker and Daniel, 2012; Harrison, 2015). With the conformational change in HA, its fusion peptide is exposed and inserts itself into the endosomal membrane. The fusion peptide pulls the endosomal membrane toward the viral membrane, thus making a fusion pore through which vRNP is released into the cytoplasm. HA solely drives the fusion process by two sequential mechanisms (Pabis, Rawle and Kasson, 2020). During the acidification process, the virion core is also acidified through the M2 channel. Acidification of the virion core liberates vRNP bound to M1. The vRNP free of M1 makes its way into the nucleus of the host cell through the nuclear pores (Neumann, Hughes and Kawaoka, 2000). M1 bound to vRNP cannot enter the nucleus. Proteins need to have specific nuclear localization signals (NLS) in order to pass through the nuclear pores. vRNA bound with NP has an NLS, hence it is able to make its way into the nucleus. Inside the nucleus, vRNP hijacks the host cell protein synthesis system and initiates messenger RNA (mRNA) synthesis (transcription). Experiments suggest that two types of vRNPs are found inside the nucleus (Nayak, Ka-Wai Hui and Barman, 2004). One type of vRNP is transcriptionally active and is not exported out of the nucleus whereas the second type is transcriptionally inactive and is exported out of the nucleus (Nayak, Ka-Wai Hui and Barman, 2004). It was observed that in the absence of M1, vRNPs remained inside the nucleus (Bui et al., 2000). Hence we can infer that M1 plays a crucial role in the transport of vRNP out of the nucleus. mRNA makes its way to the ribosomes where it is translated to the polypeptide molecule (protein) encoded by the corresponding viral mRNA. The translated HA is packaged in the Golgi apparatus and delivered to the plasma membrane via actin comets (Guerriero et al., 2006). M1 and NP are synthesized in the ribosomes and are released into the cytoplasm. It is believed that actin microfilaments help in the transport of the M1, vRNA and NP
Once all the viral components are assembled at the plasma membrane, the budding process initiates. M1 is considered a major entity in bending the membrane and drives the budding process (Gómez-Puertas et al., 2000). As the bud is fully closed, the new virion is released. To prevent re-binding of the virion to the sialic acid present in the host cell membrane, NA cleaves the sialic acid from the surface of the cell and avoids the aggregation of the progeny virions (Palese et al., 1974; Murti and Webster, 1986; Calder et al., 2010).

**Fig 1.2:** Schematic diagram showing the life cycle of Influenza A.

**1.4. Hemagglutinin (HA)**

HA, which forms about 80% of the protein in the viral lipid bilayer (Nayak et al., 2009), consists of the ectodomain, a globular head that attaches to the receptor of the host cell, a transmembrane domain that traverses the lipid bilayer, and a short cytoplasmic tail (CT). HA is both sufficient and necessary for fusion (Pabis, Rawle and Kasson, 2020). Increased HA expression has been observed to directly correlate with an increase in the rate of fusion (Ellens et al., 1990), and a minimum of three HA trimers are shown to be sufficient to induce the fusion (Danieli et al., 1996; Ivanovic et al., 2013), although this number
remains somewhat controversial. The precursor HA₀ is trimerized after translation in the endoplasmic reticulum (ER) and is delivered to the plasma membrane (Copeland et al., 1988; Skehel and Wiley, 2000). Precursor HA₀ is cleaved by host cell enzymes to a form with two subunits, HA₁ and HA₂ (Garten et al., 1981; Chen et al., 1998).

HA in the plasma membrane clusters spontaneously, even in the absence of other viral proteins (Hess et al., 2005, 2007; Gudheti et al., 2013; Curthoys et al., 2019). This clustering of HA is crucial for the viral life cycle (Takeda et al., 2003) and the density of these clusters has been previously shown to directly correlate with fusion and infectivity (Ellens et al., 1990). Lipid rafts are lipid domains enriched with cholesterol, glycosphingolipids, and protein receptors (Simons and Ikonen, 1997). It was observed that the association of HA with lipid raft is crucial for the virus life cycle (Takeda et al., 2003; Chen, Takeda and Lamb, 2005). Palmitoylation is also necessary for the association of HA with lipid rafts (Chen, Takeda and Lamb, 2005). HA clusters have been proposed to concentrate in the lipid raft domains by detergent resistant method (Takeda et al., 2003), however, studies have shown that detergent-resistant membranes (DRMs) are not the same as pre-existing rafts (Brown, 2006). More recently, non-invasive studies done using super-resolution microscopy and secondary ion mass spectrometry have refuted the association of HA clusters with the lipid rafts (Hess et al., 2007; Wilson et al., 2015). However, the mechanism by which HA clusters is unknown.

The CT of HA is highly conserved and contains two cysteines which are typically acylated with one palmitate each (Kordyukova et al., 2008; Veit, Serebryakova and Kordyukova, 2013). One cysteine which is present in the transmembrane domain just above the cytoplasmic tail (CT) of HA is also acylated either by palmitic acid or stearic acid (Kordyukova et al., 2005, 2008). Palmitoylation of the cysteines is crucial for assembly and contributes to the membrane curvature (Veit, Serebryakova and Kordyukova, 2013; Chlanda et al., 2017). Mutations to these sites disrupt the M1 associations needed for assembly and inhibit viral growth. Two arginines present in the CT of HA presumably provide a net charge of +2 for a HA and a
net charge of +6 for a trimer of HA. By the detergent resistance method, it was proposed that the CT tail of HA and NA can independently stimulate membrane association (Enami and Enami, 1996) which is required for the virus assembly and budding. CT of HA has been suspected to interact with the M1 (Ali et al., 2000; Barman et al., 2001). While the direct evidence of such interaction is yet to be seen, viruses lacking the CT of HA and NA resulted in deformed morphology and severe defects in vRNP incorporation (Jin et al., 1997; Zhang et al., 2000). Thus, HA plays a crucial role from binding to the budding of the influenza A virus.

1.5. Matrix Protein 1 (M1)

M1 is the most abundant viral protein encoded by the seventh segment of RNA (Lamb, Robert and Choppin, 1983). M1 is made up of 252 amino acids (Ito et al., 1991). Of the 252 amino acids, residues 1-165 (165 amino acids) make up the N-terminal domain, and residues 166-252 (87 amino acid) make up the C-terminal domain of each M1 monomer. The crystal structure of the N-terminal domain has been solved at both acidic and at neutral pH (Sha and Luo, 1997; Harris et al., 2001). The N-terminal domain consists of two 4-helix regions (H1-H4 and H6-H9) that are connected by another short helix (H5). Despite several attempts, the C-terminal domain of M1 has not yet been solved. M1 is shown to have a tendency to oligomerize (Bui et al., 2000). In a solution of acidic pH, M1 mostly existed in monomer form (Arzt et al., 2001; Shtykova et al., 2013), while at pH 6.0 and above, oligomerization was observed to increase rapidly and this self-assembly or oligomerization has been attributed to electrostatic interactions (Shtykova et al., 2017). The clustering of M1 beneath the lipid bilayer is thought to be crucial for the viral life cycle as M1 is responsible for the assembly of the vRNP with other viral components (reviewed in Nayak et al., 2004). The importance of M1 in the assembly of the vRNP could be understood from a study where the vRNP remained bound inside the nucleus in the absence of M1 (Bui et al., 2000). M1 is thought to be a major driving force in the virus budding and can produce virus-like particles (VLPs) even in the
absence of other viral proteins (Gómez-Puertas et al., 2000). M1 has been shown to produce membrane deformation by the multimerization in simpler model membranes (Dahmani, Ludwig and Chiantia, 2019).

M1 has been previously shown to possess a lipid-binding domain (Gregoriades, 1980). However, another study suggested that due to the lack of an inherent membrane targeting signal, M1 cannot form VLPs on its own and requires other viral glycoproteins to direct M1 to the membrane (Wang et al., 2010). Although M1 does not possess any inherent membrane targeting signal (Wang et al., 2010) a plethora of studies suggest M1 can bind to the plasma membrane primarily due to electrostatic interactions (Ruigrok et al., 2000; Thaa, Herrmann and Veit, 2009; Shilova et al., 2017). In both model membranes and in the cells, M1 can bind to the lipid bilayer even without the presence of any other viral protein (Kretzschmar, Bui and Rose, 1996; Gómez-Puertas et al., 2000; Hilsch et al., 2014; Dahmani, Ludwig and Chiantia, 2019). The multimerization of M1 in model membranes in the absence of other viral proteins occurs after it binds to the membrane (Hilsch et al., 2014). This multimerization of M1 can induce lipid membrane deformation (Dahmani, Ludwig and Chiantia, 2019) and this ability is crucial for the viral life cycle as M1 is generally thought to be responsible for the membrane bending during the budding process (Gómez-Puertas et al., 2000). Previous studies have shown the interaction of M1 with Phosphatidylserine (PS) in model membranes and in cells is thought to be of an electrostatic nature (Bobone et al., 2017). M1 is not known to have a specific lipid-binding domain as in the Gag protein of HIV or VP40 of the Ebola virus. Rather, the interaction between M1 and the lipid bilayer is well accepted as through electrostatic interactions (Ruigrok et al., 2000; Hilsch et al., 2014; Brevnov, Fedorova and Indenbom, 2016), and M1 is twice more likely to be absorbed onto negatively charged phospholipidic surfaces than onto neutral surfaces (Shishkov et al., 2009). However, interactions with other lipids that have a net negative charge greater than that of PS have not been fully explored.

Although no direct evidence of interaction between viral glycoprotein(s) and M1 has been shown, other studies have shown that the expression of viral glycoprotein(s) increases the membrane association
of M1 (Ali et al., 2000; Barman et al., 2001; Takeda et al., 2003). Also, it has been shown that viruses lacking the CT of HA and NA resulted in deformed morphology and severe defects in vRNP incorporation (Jin et al., 1997; Zhang et al., 2000). So it is hypothesized that the CTs of the glycoproteins HA and NA interact with M1 (Ali et al., 2000; Barman et al., 2001). Although many experiments have provided evidence suggesting the possible interaction between M1 and the glycoprotein(s), the role of the glycoprotein(s) in the clustering of the M1 beneath the plasma membrane has not been explored yet. Thus, the knowledge of the role that the glycoprotein(s) play in the clustering of M1 might be a very important piece in developing the model of the assembly and budding of IAV.

1.6. Actin

Actin is a multifunctional protein and a cytoskeletal component of the cell (Winder and Ayscough, 2005). Its functions range from cell migration, muscle contraction, cell division, cytokinesis, endocytosis, and vesicle movement, to signaling (Sun, Kwiatkowska and Yin, 1995; Winder and Ayscough, 2005). Actin present in the monomeric state is called globular actin or G actin and if present in the filamentous form it is referred to as filamentous actin or F-actin. F-actin has been shown to be involved in virus infection, and many viral proteins use the actin cytoskeleton for their movement (Lehmann et al., 2005; Guerriero et al., 2006; Taylor, Koyuncu and Enquist, 2011; Spear and Wu, 2014). Actin has been shown to mediate the nanoscale clustering of HA (Gudheti et al., 2013). HA associates with actin-rich regions and disruption of actin cause HA clusters to change in size and density (Gudheti et al., 2013). HA relies on actin comets to be delivered at the plasma membrane (Guerriero et al., 2006). Despite actin being very important for the delivery and the nanoscale organization of a HA, direct interaction between actin and HA is yet to be proven and the exact mechanism of HA clustering has not yet been elucidated, and no direct actin-binding domain within HA has ever been described. Proteins that have been extracted from the purified virus (Shaw et al., 2008) are known to interact with and regulate actin through lipids called phosphoinositides.
Thus we hypothesize that phosphoinositides could serve as a functional link between the actin cytoskeletal and HA clustering.

1.7. Phosphatidylinositol (4,5) bisphosphate (PIP2)

The phosphoinositide phosphatidylinositol (4,5) bisphosphate (PIP2) is the most common phosphoinositide (PtdIns) but overall constitutes a minor (~1%) component of lipids in the plasma membrane of mammalian cells (Czech, 2000; Hurley and Misra, 2000; Irvine and Schell, 2001). PIP2 can have a charge of -3, -4, or -5 depending upon the protein-induced displacement of $K^+$ and $H^+$ ions binding to the lipid (McLaughlin et al., 2002). Experiments quantifying electrophoretic mobility of PC/PIP2 suggest that PIP2 has a net charge of -3 at neutral pH (Toner et al., 1988). Seven different isoforms can be generated by phosphorylating the inositol ring of the phosphoinositide (Balla, 2013). As the name suggests, phosphatidylinositol (4,5) bisphosphate is generated by phosphorylating the 4th and 5th positions of the inositol ring. PIP2 can be produced by one of the following ways: if the 5th position of PI4P is phosphorylated by PI5K, if the 4th position of PI5P is phosphorylated by PI4K, or by dephosphorylation of PI(3,4,5)P$_3$ (PIP3) by the PTEN enzyme (Liu et al., 2018).

PIP2 plays an important role in carrying out necessary cellular functions, and most of them are carried out from the plasma membrane (Czech, 2000; Gamper and Shapiro, 2007). PIP2 is known to play an important role in endocytosis/exocytosis (Cremona et al., 1999; Martin, 2001), actin cytoskeleton regulation (Sun et al., 1999), and cytoskeleton plasma membrane adhesion (Raucher et al., 2000), among many others. More famously, it acts as a secondary messenger when a ligand binds to a G-protein coupled receptor, causing the membrane-associated enzyme phospholipase C (PLC) to become activated, which catalyzes the hydrolysis of PIP2. Due to this catalysis, the secondary messengers inositol 1,4,5 tri-phosphate (IP3) and diacylglycerol (DAG) are produced (Berridge, 1984). DAG remains in the cell membrane and activates protein kinase C (PKC), which then activates other proteins by phosphorylating them (Hurley and Misra, 2000). IP3 in the cytoplasm activates the IP3 receptors of the endoplasmic
reticulum (ER), which leads to the opening of calcium ion \( (\text{Ca}^{2+}) \) channels and the release of \( \text{Ca}^{2+} \) into the cytoplasm (Clapham, 1995; Berridge, Lipp and Bootman, 2000).

Plasma membrane (PM) targeting motifs have been shown to possess polybasic domains that provide PM specificity and were observed to cluster with negatively charged lipids (Won et al., 2006). Clusters of PIP2 were observed by labeling with the pleckstrin homology (PH) domain of phospholipase C delta (PLCδ-PH) (Van Den Bogaart et al., 2011; Wang and Richards, 2012; Curthoys et al., 2019). Since the PH domain (PLC-δ) can bind to the PIP2 head with high affinity and specificity, PLCδ-PH is an excellent way of labeling PIP2 (Yagisawa et al., 1994; Lemmon et al., 1995; Kavran et al., 1998).

PIP2 is involved in different cellular functions, regulating membrane organizations (Balla, 2013), and is the primary target for protein-lipid interactions (Won et al., 2006). In order to understand how PIP2 carries out these functions, study of the nanoscale distribution of PIP2 in the plasma membrane is crucial. Multiple models by which PIP2 concentrates at the plasma membrane have been proposed, three of which are described in a recent review (Hammond, 2016): 1.Platforms, 2.Synthesis, and 3.Megapool. Because the distribution of PIP2 at the plasma membrane is very dynamic and its organization occurs at length scales well below the diffraction limit, live-cell super-resolution microscopy studies are crucial to understand these models at the nanoscale.

1.7.1. Platforms

This model envisions the heterogeneous distribution of the lipids (specifically PIP2) that leads to a spatially segregated ‘domain’ or ‘platform’. The author also doubts the viability of this model because PIP2 can diffuse rapidly with the diffusion coefficient ranging from 0.1 to 1 µm²/s (Yaradanakul and Hilgemann, 2007; Golebiewska et al., 2008; Hammond et al., 2009) so it can escape such a ‘domain’ or ‘platform’ without any corralling effect. However, the author suggests that cooperation with membrane proteins can overcome this constraint. Experiments present evidence in support and against this model.
According to this model, PIP2 is synthesized locally by the effector (a protein, probably a phosphoinositide kinase) which in turn recruits other effectors into the complex. While studies present evidence in support of this model (Di Paolo et al., 2002; Ling et al., 2002; Nakano-Kobayashi et al., 2007), the major constraint as forwarded by Hammond is the availability of PtdIns in the PM for the synthesis of PIP2. The availability of the PtdIns in the plasma membrane is much lower compared to its availability in other intracellular membrane compartments (Hammond, 2016) and hence there might not be enough PtdIns to convert it to PIP2.

1.7.3. Megapool

This model predicts the global availability of freely diffusing PIP2 is enough to carry out necessary PIP2-dependent functions. In this model, PIP2 is not pre-clustered, but the membrane proteins can form complexes that can recruit PIP2. It further postulates that the protein complexes are formed due to self-interaction rather than PIP2 acting as an anchor to recruit proteins and form a cluster of proteins. Little evidence is available which could confirm or refute this model.

Influenza virus has been previously shown to exploit PIP2-dependent pathways (Hale et al., 2006; Fujioka et al., 2013). HIV Gag protein and Ebola matrix protein VP40 have previously been shown computationally and experimentally to interact with PIP2 (Ono et al., 2005; Stahelin, 2014; GC, Gerstman and Chapagain, 2017; M., K. and R.V., 2017). Proteins with polybasic domains have been previously shown to sequester PIP2 (McLaughlin and Murray, 2005). In an HA cluster, each CT of HA presumably has a net charge of +2 (Veit, Serebryakova and Kordyukova, 2013) which makes a cluster of HA trimers highly polybasic. Also, HA relies on actin comets to be delivered at the plasma membrane together with the kinase PI4P5K which can make PIP2 from its precursor PI4P (Guerriero et al., 2006). Furthermore,
nаноскальная мембранная организация HA была ранее показана быть медиацію ее актином (Gudheti et al., 2013). Некоторые из белков, которые были извлечены из вируса гриппа, известны за их способность взаимодействовать с PIP2 (Shaw et al., 2008). Кроме того, недавно мы продемонстрировали, что свойства HA существенно различаются в присутствии PIP2 по сравнению с их отсутствием (Curthoys et al., 2019). Это исследование привело к гипотезе, что PIP2 может быть промежуточным звеном между плазматической мембраной, актиновым цитоскелетом, и HA, и что модулирование PIP2 могло бы, в конечном итоге, модулировать HA clustering.

1.8. Дифракционная лимитация

Многие внутриклеточные структуры и биологические процессы происходят на наномасштабном уровне, но к сожалению, дифракция ограничивает резолюцию в световом микроскопе. Поскольку свет действует как волна, его путь сильно изменяется при прохождении через узкое отверстие или через края. Этот процесс изгиба света называется дифракцией. Дифракционные эффекты наиболее ярко выражены, когда размер отверстия или размер препятствия сравним с длиной волны падающего света. Из-за этого свойства, свет от нанопризмы или отображаемый молекулярный сдвиг при помощи микроскопа рассеивается и создает Аироеву картину. Аироевая картина характеризуется центральным ярким пятном или максимумом с размытым краем и многочисленными окружающими яркими и темными концентрическими колцами. Диаметр первого максимума (~\( \frac{\lambda}{2} \)) намного больше диаметра нанопризмы (~1-10 нм). Откликом системы на точечный источник (т.е. изображение точечного источника при заданном наборе условий изображения) является функция распределения точки (PSF). Затуманивающийся тенденция из-за дифракции и интерференции световых лучей накладывает внутренний ограничение на резолюцию микроскопа или оптических приборов. Нижний предел для расстояния между двумя пространственно разнесенными объектами в порядке разрешения дан Рэлейным критерием. Согласно Рэлею, резолюционная способность светового микроскопа определяется

\[
r = \frac{0.61\lambda}{NA}
\]

где \( r \) — минимальное расстояние между двумя независимыми точечными источниками, которые должны быть разрешены, где NA — числовой апертурный диаметр объектива микроскопа или оптической системы, а \( \lambda \) — длина волны.
wavelength of the light emitted from the point-like source. For the mean wavelength of the visible spectrum and taking numerical aperture NA=1.5 which is near the highest commercially available value, the value of r comes out to be ≈ 220 nm. Hence at the nanoscale, conventional microscopy fails to resolve the molecules which are closer together than a distance r. Although electron microscopy (EM) can provide much better resolution (<1 nm) and has been used extensively in virus research, it cannot be used to image live cells because of its invasive nature. Also, EM can only be used to image static structure, while highly mobile molecules which are difficult to fix, such as lipids, are very difficult to image using EM.

1.9. Fluorescence

When a molecule absorbs a photon, the electrons of the molecule in the ground state jump to a higher energy state. If the excited electron is spin allowed to return to the ground state, it can lose its energy by emitting electromagnetic radiation called fluorescence (Lakowicz, 2006; Albrecht, 2008). The fluorescence lifetime is the time (on average) that a molecule will remain in the excited state before decaying to the ground state. The fluorescence lifetime is an intrinsic property of the fluorophore and can range from 100 picoseconds to 100s of ns (Berezin and Achilefu, 2010). Alternatively, the excited molecule can undergo a loss of energy through a non-radiative process on its way down to the lowest excited singlet state. Absorption of the radiation and loss of energy by radiation are radiative processes, so radiative transitions are solely responsible for fluorescence.

1.9.1. Radiative transition

1.9.1.1. Absorption

Absorption of electromagnetic radiation occurs when the energy of the radiation is equal to the energy difference between the ground state and the excited state. On absorption, electron(s) in the ground state molecule is excited to a higher energy state.
1.9.1.2. Emission

Electron(s) in the excited state spontaneously relax to the ground state by emitting a photon of energy equal to the difference between the excited state and the ground state.

1.9.2. Non-Radiative Transitions

1.9.2.1. Vibrational Relaxation

Electrons in the excited state molecule can lose their excess vibrational energy within the same molecule or to neighboring molecules until the excited molecule reaches its lowest vibrational energy level (Fujisaki and Straub, 2005). This is the most common and the fastest transition among other transitions and can occur on timescales of $10^{-12}$ to $10^{-10}$ s.

1.9.2.2. Internal Conversion

Internal conversion is the process by which the electron(s) in a higher energy state molecule decay to a lower energy state of the same spin state (Bixon and Jortner, 1968). Internal conversion is followed by vibrational relaxation to the lowest vibrational energy level. The time scale for the internal conversion is $10^{-11}$ to $10^{-9}$ s.

1.9.2.3. Intersystem Crossing

Intersystem crossing is a non-radiative transition where electron(s) from a molecule in a singlet state pass into a triplet state. This transition is generally forbidden due to spin conservation, but there is a low (non-zero) rate for such transitions due to spin-orbit coupling. The time scale of this transition is $10^{-8}$ to $10^3$ s and is one of the slowest transitions (Kovac, 1998).
Figure 1.3: A simplistic picture of a Jablonski diagram. An electron in the ground state \( (S_0) \) jumps to an excited state \( (S_1) \) on absorbing the energy equal to the energy difference between the excited state and the ground state. The excited electron undergoes a non-radiative transition before relaxing to the ground state by the radiative transition. In molecules, multiple electrons can participate in the process.

1.10. Fluorescence Microscopy

Fluorescence microscopy uses the fluorescence principle to capture the signal from the fluorophores in the biological specimen (Lakowicz, 2006). Fluorescence microscopy has been very popular and is being extensively used by biomedical researchers as a non-invasive probe to understand the
underlying mechanisms of biological systems (Albrecht, 2008; Masters, 2008). This popularity was further enhanced by the discovery of the green fluorescent protein (GFP) (O, FH and Y, 1962) which was later awarded the Nobel Prize in chemistry in 2008 (Zimmer, 2009). In Fluorescence Microscopy, a fluorescence (signal) emitted from a molecule of interest tagged with the fluorophore is captured and imaged using a photodetector or camera (Lakowicz, 2006; Albrecht, 2008). In the case of a protein, the molecule of interest is tagged by a fluorophore either by genetically encoding a fusion of the protein of interest together with a fluorescent protein or by tagging the protein of interest with a fluorescently-tagged antibody (or a fluorescently-tagged secondary antibody which labels the primary antibody which binds to the molecule of interest) (Curthoys et al., 2019). Fluorescent antibody labeling of a molecule of interest is called immunostaining (Donaldson, 2001). A laser illuminates all or a portion of the sample, causing the fluorophores to be excited and fluoresce. Fluorescence collected by the objective passes through a dichroic mirror, which separates the laser from the fluorescence, and is then filtered by an optical emission filter before ultimately reaching the detector (Lakowicz, 2006; Albrecht, 2008). Signals captured by the photomultiplier tube consist of the fluorescence from the large number of fluorophores that have been excited by the laser simultaneously (Lakowicz, 2006). Conventional fluorescence microscopes, such as confocal microscopes, are generally diffraction-limited and cannot resolve the spatial organization of the cellular structures that are on length scales smaller than the diffraction limit (Masters, 2008).

1.11. Localization Microscopy

As many subcellular structures and biological processes occur on length scales smaller than the diffraction limit, there is a need for microscopes that break the diffraction limit, which is fulfilled by localization-based microscopy methods (Betzig et al., 2006; Hess, Girirajan and Mason, 2006; Rust, Bates and Zhuang, 2006). In localization microscopy, molecules of interest are either tagged with a special type of fluorophore which is photoactivatable and only a small subset of molecules are randomly activated and fluoresce by the appropriate laser, or with a conventional fluorophore like Alexa647 whose fluorescence
is quenched so that only a small subset of molecules fluoresce at a time, making it compatible for localization-based microscopy (Van De Linde and Sauer, 2014). Within each camera frame, typically after the acquisition, the PSF of each visible molecule is localized, and a map of the locations of localized molecules (the image) is created which shows the spatial distribution of the molecule of interest. Localization microscopy can be used to image one protein or other molecular species (single color) or multiple species simultaneously (Gunewardene et al., 2011; Curthoys et al., 2013). Localization-based microscopy can also be customized to identify the molecular orientation (Gould et al., 2008, 2009) or structure of the cellular compartments in 3D (Huang et al., 2008; Juette et al., 2008; Sangroula et al., 2020)

1.12. Fluorescence PhotoActivation Localization Microscopy (FPALM)

Fluorescence Photoactivation Localization Microscopy (FPALM) is a localization-based super-resolution microscopy method where PSFs from individual fluorophores are imaged in the time sequence and a final map of the molecules is generated by plotting each localized fluorophore in the given time sequence. In FPALM, an activation laser activates a stochastic subset of the fluorophores, which are then excited by the readout laser and imaged with the camera. Excited fluorophores emit fluorescence until they reversibly or irreversibly photobleach. The PSF from the visible fluorophores is recorded by the camera. After each subset is imaged, another subset of the molecules is activated and imaged by the readout laser, respectively, before photobleaching. This process is continued until all the fluorophores are photobleached, or enough PSFs are recorded to obtain a clear picture of the structure or the distribution of the molecule of interest. Since only a small subset of the PSFs from the fluorophores are captured at a time, it is possible to localize the molecules and resolve them. Each PSF recorded in the time sequence is fitted with a 2D Gaussian or other appropriate function in order to localize the molecule. The precision of such a fit is given by the equation (Thompson, Larson and Webb, 2002; Mortensen et al., 2010)

\[ x^2 = \frac{s^2 + \frac{a^2}{12}}{N} + \frac{8\pi r^4 b^2}{a^2N^2} \]

Equation 1.2
where \( x \) is the localization uncertainty, \( s \) is the standard deviation of the pixel value of the PSF, \( a \) is the pixel size of the camera, \( N \) is the number of photons detected and \( b \) is the background noise.
Figure 1.4: Concept of FPALM. A small subset of photoactivatable fluorophores tagging the molecule of interest is stochastically activated by the activation laser which is then read out by the readout laser in each frame. The PSF from the fluorophores is recorded and localized. This process is repeated over and over again in order to get a final super-resolved image of the spatial distribution of the protein of interest. After subsequent acquisition and localization, a clear super-resolved image of the coordinates of the molecules of interest is obtained.
1.13. Illumination

FPALM is fairly non-invasive and compatible with fixed cell, living cell, and in vivo imaging (Hess, Girirajan and Mason, 2006; Hess et al., 2007; Gabor et al., 2015). FPALM imaging can be carried out in widefield illumination or total internal reflection (TIRF) illumination.

1.13.1. Widefield illumination

In FPALM using widefield illumination, the whole specimen is illuminated by the light source. This illumination method is used to image cytosolic proteins of interest as well as the plasma membrane, or other intracellular structures (Combs, 2010). Since the whole specimen is illuminated, fluorescence background from the out-of-focus planes in the sample is also captured, resulting in a higher background which can interfere with molecular identification and localization, degrading overall image resolution.

1.13.2. TIRF illumination

In this case, the illumination light source undergoes total internal reflection at the coverslip-sample interface, making it ideal for plasma membrane imaging. Fluorescence is generated only from the thin depth into which the evanescent electric field penetrates, so the background is much less compared to widefield imaging (Stout and Axelrod, 1989).

Figure 1.5: A schematic diagram showing the Widefield versus TIRF illumination.
1.14. Research Overview

HA clusters spontaneously at the plasma membrane at various length scales (Hess et al., 2007) and not all clusters can be resolved by traditional diffraction-limited microscopy. The mechanism behind the spontaneous clustering of HA is not well understood; however, we previously showed that the HA cluster distribution is significantly different in low and high PIP2 enriched regions of the plasma membrane (Curthoys et al., 2019). This finding is important because HA clusters can be disrupted by modulating PIP2 and PIP2 clusters. Targeting this interaction could yield novel approaches to reduce the infectivity of the virus since the density of HA clusters directly correlates with the infectivity of the virus (Ellens et al., 1990).

Cetylpyridinium chloride (CPC) is a positively charged quaternary ammonium compound that is well known to possess antibacterial and antiviral properties (Hwang et al., 2013; Popkin et al., 2017) at higher concentrations (mM), but which is also toxic to the cell at those concentrations. In the studies that follow (Chapters 3 and 4), we show that CPC at non-cytotoxic (µm) concentrations affects PIP2-binding-protein clustering by both the diffraction-limited microscopy (confocal) and super-resolution microscopy (FPALM). We explore the relationship between viral proteins and phosphoinositides, and explain a mechanism for the antiviral properties of CPC at non-cytotoxic concentrations, demonstrating for the first time that CPC affects the assembly of HA and M1. We also quantify the effect of M1 in PIP2 clustering and of HA in M1 clustering.
CHAPTER 2
EVIDENCE FOR HA-PIP2 INTERACTIONS

2.1. Preface

The figures in this chapter were adapted from the paper “Influenza Hemagglutinin Modulates Phosphatidylinositol 4,5-Bisphosphate Membrane Clustering” by authors Nikki M. Curthoys, Michael J. Mlodzianoski, Matthew Parent, Michael B. Butler, Prakash Raut, Jaqlin Wallace, Jennifer Lilieholm, Kashif Mehmood, Melissa S. Maginnis, Hang Waters, Brad Busse, Joshua Zimmerberg and Samuel T. Hess, published in Biophysical Journal, Vol. 116, 893-909, March 5, 2019.

2.2. Introduction

The animal cell plasma membrane is a complex organization of many varieties of lipids and membrane proteins, and the interactions between the membrane proteins and lipids are crucial to carry out necessary cellular functions. However, there is significant disagreement in the mechanism of such interactions, and specifically of clustering of lipids and membrane proteins (Viola and Gupta, 2007; Kusumi et al., 2011; Simons and Sampaio, 2011).

Phosphatidylinositol (4,5) bisphosphate (PIP2) is a significant and prominent member of the phosphoinositide family. While only comprising ~1% of the total lipid within the plasma membrane (Czech, 2000), PIP2 plays a crucial role in carrying out a range of cellular functions like signaling, membrane trafficking, and cytoskeletal dynamics (Takenawa and Itoh, 2001; Wenk and De Camilli, 2004). PIP2 has been observed to cluster at the plasma membrane (Van Den Bogaart et al., 2011; Curthoys et al., 2019). Several distinct models have been proposed to explain the underlying mechanisms of clustering of PIP2 (Hammond, 2016); however, these models need to be tested experimentally. It has been well-established in the virology community that the HA clusters at the plasma membrane (Hess et al., 2007; Gudheti et al., 2013; Curthoys et al., 2019) and this clustering of HA is crucial for the viral life cycle (Takeda et al., 2003). However, there is a considerable lack of mechanistic understanding of this spontaneous clustering of HA.
The cytoplasmic tail (CT) of a HA consists of 10-11 amino acids, including two positively charged arginine and palmitoylation sites that are known to play role in membrane association and interaction with PIP2 (Gambhir et al., 2004; McLaughlin and Murray, 2005; Won et al., 2006). After the translation of HA in the Golgi apparatus, HA is transported to the plasma membrane in vesicles propelled by actin comets which are dependent on PIP2 (Rozelle et al., 2000; Guerriero et al., 2006). Actin has been previously shown to mediate the nanoscale clustering of HA (Gudheti et al., 2013). Actin is regulated by actin-binding proteins and PIP2 has been known to interact with the many actin-binding proteins, several of which are found in purified influenza virus (Shaw et al., 2008). Thus we hypothesize that PIP2 might serve as a functional link between HA and the actin cytoskeleton interaction.

2.3. Results

Certain proteins domains such as the Pleckstrin Homology (PH), PX, and FYVE domains have been identified to bind to head the group of PIP2 (Hurley and Meyer, 2001). We used a fluorescent-protein (Dendra2)-tagged PH domain to label PIP2; such methods have been previously used and are well known to provide useful information about the spatial distribution of PIP2 (Gambhir et al., 2004; Hammond and Balla, 2015). First, we wanted to find the spatial correlation between HA and PIP2 expressed at the plasma membrane. CFP-PLC-δ was transfected in NIH3T3 cells stably transfected (HAb2) with HA. HA (A/Japan/305/57) in the cells was immunostained first by a monoclonal primary antibody which was further labeled by a secondary antibody with Alexa 647. Diffraction-limited images (Figure 2.1 A-H) obtained by confocal microscopy showed a high degree of colocalization. After this observation, the spatial correlation between HA and PIP2 was studied at the nanoscale using super-resolution microscopy (FPALM) (Hess, Girirajan and Mason, 2006). Cells stably expressing HA were transfected with Dendra2-PH, and HA was immunostained with anti-HA primary antibody (FC125) and Alexa 647 tagged secondary antibody. The plasma membrane of the cells was imaged under TIRF illumination. Spatial correlation between HA and PIP2 was observed at the nanoscale (Figure 2.1 I). In order to further test our hypothesis,
NIH3T3 cells were transfected with a fusion protein of Dendra2-HA (X-31B, Puerto Rico/8/1934-Aichi/2/1968) and PH-PAmKate in NIH3T3 cells. Plasma membranes of the cells expressing Dendra2-HA and PH-PAmKate were imaged under TIRF illumination. Dendra2-HA and PH-PAmKate were observed to spatially correlate at the nanoscale again (Figure 2.1 J). We then quantified the level of colocalization by using the Pearson Correlation Coefficient. Localizations were binned in three different sets of bins with dimensions of 20nmX20nm, 50nmX50nm, and 100nmX100nm. A small but positive colocalization coefficient value was obtained in each case (Figure 2.1 K).
**CONFOCAL MICROSCOPY**

Figure 2.1: Colocalization of HA and PH domain (PLC-δ) by confocal and super-resolution microscopy.

Colocalization between HA and PIP2 both by diffraction-limited and super-resolution microscopy. (A-D and E-H) Diffraction limited images of NIH3T3 cells stably expressing HA labeled with monoclonal primary antibody and then secondary antibody with Alexa 680 (red) was transfected with CFP-PLCδ-PH (green) show colocalization of HA and PIP2. (I) FPALM image of HA labeled by FC125 (green) and Dendra2-PH (magenta) in the HAb2 cells. (J) FPALM image of Dendra2-HA (green) and PH-PAmKate(magenta). Blue triangles with white outline point towards colocalization (white). (K) Quantitative analysis of the colocalization by Pearson Correlation Coefficient as a function of bin size. Each point represents the mean of 20 cells imaged in three separate replicates. Error bars represent the standard error of the mean.
Next, we studied the cluster properties of PIP2 and HA as both clusters at the plasma membrane (Hess et al., 2005, 2007; Wang and Richards, 2012; Gudheti et al., 2013). Clusters of both species were identified using single-linkage cluster analysis (SLCA) (Sneath, 1957). Wide ranges of areas and densities spanning nearly two and three orders of magnitude, respectively, were observed; however, most cluster areas were within the zone of <0.04 μm$^2$ and relative density <10X cell average. We also observed that PIP2 clustered in areas with low HA (relative HA density 0.05 times the cell average). However, PIP2 cluster area and density both increased significantly (p<0.0001) by two-tailed Mann-Whitney U test in the regions of high HA (relative HA density 5 times the cell average). A total of 1065 PIP2 clusters were identified with low HA regions whose mean cluster area was observed to be 0.0145±0.0020 μm$^2$ and relative mean cluster density of 6.1±0.6 compared to cell average. A total of 666 PIP2 clusters were identified with high HA whose mean cluster area was observed to be 0.0257±0.0060 μm$^2$ and relative mean cluster density of 7.6±0.8 compared to cell average. We also analyzed the HA cluster properties. First, we quantified the HA cluster properties without considering local PIP2 levels. We considered a cluster to have a minimum of 200 HA localizations. The mean area of a HA cluster was obtained 0.150±0.016 μm$^2$ which is in excellent agreement with our previous result (Gudheti et al., 2013). After this, we quantified HA clusters as a function of local PIP2 levels. We considered a cluster to have a minimum of only a minimum of 10 localizations to be considered as a cluster. The mean area of an HA cluster in regions of low PIP2 was observed 0.0105± 0.0005 μm$^2$ which increased significantly (p<0.05, two-tailed Mann Whitney test) to 0.0231± 0.0014 μm$^2$. Due to large variation, the density of an average HA cluster was not significantly different between low PIP2 regions and high regions using a two-tailed Mann Whitney U-test, but using a one-tailed Wilcoxon test, the HA density as a function of low and high PIP2 levels were significantly different.
Figure 2.2: PIP2 and HA cluster properties as a function enrichment of each other. NIH3T3 cells were transfected with Dendra2-HA and PamKate-PH (PLCδ). Plasma membrane of the transfected cells was imaged with the TIRF illumination by using FPALM. Localizations after assigning to either channel were quantified for cluster properties. A scatter plot of PIP2 cluster area vs density as a function of (A) low and (B) high levels of local HA. A scatter plot of HA cluster area vs density as a function of (C) low and (D) high levels of local PIP2.
2.4. Discussion

Membrane proteins and lipids are asymmetrically distributed in the plasma membrane of the cell, and lipid-protein interactions are crucial to carry out necessary cellular functions (Lee, 2003; Wenk and De Camilli, 2004; Corradi et al., 2019). Lipids either have a binding site for a membrane protein to bind (Ono et al., 2005; Stahelin, 2014) or can have non-specific or transient binding with the membrane protein. Our results show the colocalization (Figure 2.1 A, E, I, and J) of HA and PIP2 at the plasma membrane of the NIH3T3 cell by both diffraction-limited and super-resolution microscopy (FPALM) indicating possible interaction between HA and PIP2. Diffraction-limited microscopy showed a high degree of colocalization. At the nanoscale, the degree of colocalization is reduced and the Pearson correlation coefficient remained positive at different bin sizes (Figure 2.1 K). Clusters of PIP2 and HA were observed at the plasma membrane in line with previous observations (Won et al., 2006; Hess et al., 2007; Wang and Richards, 2012; Gudheti et al., 2013). However, clusters of PIP2 were obtained with and without of local enrichment of HA, indicating PIP2 can cluster without HA as well. We observed a mean cluster diameter of PIP2 (from mean cluster area considering spherical shape) ~135±9 nm and 182±21 nm in regions with low HA and high HA, respectively. PIP2 cluster diameters of 0.073±0.042 μm and 0.064±0.027 μm have been previously reported in PC12 membrane sheets and PC12 whole cells (Van Den Bogaart et al., 2011; Wang and Richards, 2012). The differences between our observed PIP2 cluster diameter and the previously published results cannot be attributed to a single factor only. Differences in the cell line, techniques used to identify the clusters, effective resolution of the microscopy technique (Hess, Girirajan and Mason, 2006), and localization density (Pennacchietti, Gould and Hess, 2017) all may be contributing collectively towards the discrepancy. We observed a small upward trend with the density vs area of a PIP2 cluster indicating the denser PIP2 clusters are bigger as well. On quantification of PIP2 clusters, we observed that the PIP2 clustering was significantly different in regions of low and high HA indicating an effect of HA on PIP2 clustering.
We also quantified the HA cluster properties as a function of enrichment of PIP2 clusters. Due to large variation, HA cluster density was barely insignificant ($p = 0.06$); however, the mean area of HA clusters was observed to be significantly greater in regions with high PIP2 levels compared to those with low PIP2 levels. This shows the HA and PIP2 clusters are interdependent. Dense HA clusters have been previously shown to correlate with infectivity; modulating PIP2 clusters could be a novel therapeutic approach to influenza infection. Proteins with the polybasic domain and acylation sites are known to be dependent on phosphoinositides (Won et al., 2006). As each cytoplasmic tail (CT) of an HA monomer contains 2 positive amino acids and three acylation sites (Veit et al., 1991; Veit, Serebryakova and Kordyukova, 2013), and HAs are found as trimers in the plasma membrane, we can infer that clusters of HA trimers (like we observe) would create a highly basic (positively charged) region underneath the plasma membrane. With all the acylation sites and multiple basic amino acid residues underneath the HA clusters (close to the cytoplasmic leaflet of the plasma membrane), there is great potential for interaction between the PIP2 head and the CT of HA, although this does not necessarily demonstrate any kind of direct interaction between HA and the PIP2. The co-dependency of clustering between PIP2 clusters and HA clusters and the possible electrostatic interaction between HA and PIP2 could be a drug target for the novel therapeutic approach for influenza A because dense HA clusters correlate with the infectivity of the virus and disruption of dense HA cluster can be achieved by modulating the PIP2 clusters.
CHAPTER 3

ANTIMICROBIAL AGENT CETYLPYRIDINIUM CHLORIDE INTERFERES WITH PHOSPHATIDYLINOSITOL 4,5 BISPHOSPHATE PROTEIN INTERACTIONS IN INFLUENZA INFECTION FIBROBLAST MODEL AND IN MAST CELLS

3.1. Preface

Parts of this chapter are adapted from the manuscript in preparation “Antimicrobial agent cetylpyridinium chloride interferes with phosphatidylinositol 4,5-bisphosphate-protein interactions in influenza infection fibroblast model and in mast cells.” by authors Prakash Raut, Sasha Weller, Bright Obeng, Bailey West, Christian Potts, Suraj Sangroula, Marissa Kinney, Jack Burnell, Brandy Soos, Benjamin King, Julie A. Gosse, and Samuel T. Hess

3.2. Introduction

Influenza A virus (IAV) is highly contagious and can cause life-threatening illnesses and complications. IAV belongs to orthomyxovirade family of viruses. The bilayer consists of three viral proteins Hemagglutinin (HA) and Neuraminidase (NA) sticking out of the bilayer and the transmembrane ion channel Matrix Protein 2 (M2). Underneath the bilayer Matrix Protein (M1) forms a layer of a scaffold and bridges between the bilayer and the viral core. The viral core consists of three polymerases PA, PA1, and PB2, 8 negative strands of RNA with nucleoprotein (NP), and three nuclear export proteins (NEP).

HA is the most abundant viral glycoprotein responsible for attaching to the host cell receptor and infecting the healthy cell by catalyzing the membrane barrier of the host cell. HA rely on actin comets for the delivery to the plasma membrane (Rozelle et al., 2000; Guerriero et al., 2006) and has been observed to colocalize with the actin-rich regions (Gudheti et al., 2013). HA delivered at the plasma membrane spontaneously clusters at various length scales (Hess et al., 2007; Gudheti et al., 2013; Curthoys et al., 2019). Dense HA clusters have been correlated with the infectivity of the virus (Ellens et al., 1990). No clear mechanism has been explained for the spontaneous clustering of HA however our previous study
showed HA colocalizes with PIP2 and HA distribution was observed significantly different in the presence of low and high regions of PIP2 (Curthoys et al., 2019). PIP2 is a negatively charged signaling lipid involved in a number of cellular functions like regulation of cell motility, actin reorganization, endocytosis, exocytosis (McLaughlin and Murray, 2005; Catimel et al., 2008; Balla, 2013). Thus the involvement of PIP2 in the actin comets regulation, in the modulation of HA clustering, and the positioning of the PIP2 in the bilayer are suggestive of the possible charge interaction between the PIP2 head and the cytoplasmic tail (CT) of HA. CT tail of a HA has two positively charged arginines (Simpson and Lamb, 1992), two highly conserved acylations (Veit et al., 1991; Kordyukova et al., 2008; Veit, Serebryakova and Kordyukova, 2013), and one acylation in the transmembrane domain of a HA. We hypothesize that disrupting this possible charge interaction between PIP2 and HA can modulate HA clustering which in turn can reduce the infectivity of the virus (Ellens et al., 1990).

Cetylpyridinium chloride (CPC) is a positively charged quaternary ammonium compound that has been previously shown to possess antibacterial and antiviral properties in the range of higher orders of µm (Hwang et al., 2013; Popkin et al., 2017; Baker et al., 2020). At higher concentrations CPC can disrupt the integrity of the membrane however there is very much less knowledge on the effects of CPC in the cellular functions at non-cytotoxic concentrations. In this study, we show the effect of CPC in two PIP2 binding proteins and explain a mechanism of the antiviral property of the CPC at non-cytotoxic concentrations and further we also extend our study in the animal model and show CPC at non-cytotoxic concentration rescues the zebrafish from mild to moderate IAV infections.

3.3. Methods

3.3.1. Chemical and Reagents

Cetylpyridinium chloride (CPC; 99% purity, VWR; CAS no. 123-03-5) was prepared in a pre-warmed Tyrodes buffer by sonication (Branson 1200 ultrasonic cleaner; Branson Ultrasonics, Danbury, CT, USA) at 150 µM and 37°C for 20 minutes, protected from light. Following dilution in pre-warmed Tyrodes buffer,
exact concentrations were determined using UV-Vis spectrophotometry (Weatherly et al., 2013) and the Beer-Lambert equation \( A_{260} = \varepsilon_{260} \ell c \), using an \( \varepsilon_{260} \) of 4389 M\(^{-1}\) cm\(^{-1}\) (Bernauer, 2015). Bovine serum albumin (BSA) was added to create a final solution of CPC in BT (Tyrodes buffer containing BSA) (Hutchinson et al., 2011).

3.3.2. Cell Culture

3.3.2.1. RBL-2H3 Cell Culture

RBL-2H3 mast cells were cultured as described in (Hutchinson et al., 2011).

3.3.2.2. NIH-3T3 Cell Culture

NIH-3T3 mouse fibroblast cells (ATCC, CRL-1658) were cultured in T25 NuncTM flasks (ThermoFisher Scientific) as in (Curthoys et al., 2019). Cells were incubated and suspended in growth media (DMEM with Glucose and L-Glutamine, Lonza, 12-604F) with 10% Calf Bovine Serum (30-2030, ATCC) and antibiotics (Penicillin Streptomycin, 100µg/ml) at 37 °C and 5% CO\(_2\). For sample preparation, cells were seeded in growth media (DMEM with Glucose without phenol red, Lonza, 12-917F) with 10% Calf Bovine Serum (30-2030, ATCC) and antibiotics (Penicillin Streptomycin, 100µg/ml) at concentration 35,000 cells/ml overnight in petri dishes (MatTek, P35G-1.5-20-C). After 20-24 hours, cells were transfected using Lipofectamine 3000 (L3000008, Invitrogen) with 2 µg of total DNA (1µg of HA-Dendra2 and 1µg of PAmKate-PH) and treated with Control (0 µm CPC + Tyrodes- BSA vehicle), 5µM and 10 µM CPC for 1 hour in the incubator under same conditions. After one hour cells were washed twice with phosphate-buffered saline (PBS) (Sigma-Aldrich, D8537), fixed at room temperature in 4% paraformaldehyde (PFA) (Alfa Aesar, J61899AK) for 10-15 minutes, and then washed again with PBS two times.

3.3.3. MARCKS Assay in RBL-2H3 Mast Cells

RBL-2H3 mast cells were transiently transfected with monomeric red fluorescent protein (mRFP)-MARCKS-ED plasmid (a gift from Dr. Barbara Baird and Dr. David Holowka, Cornell University; “MARCKS”,
(Gadi et al., 2011) via electroporation using Amaxa Nucleofector kit T (Lonza) as described in (Weatherly et al., 2018). After electroporation cells were plated at 1.7 x 10^5 cells per well (eight-well ibidi plate) in a phenol red-free media and incubated overnight at 5% CO_2 / 37°C. The next day, the spent media was tossed away and cells were incubated with 200 µl of 5 or 10 µM CPC and 200 µl of BT for 30 minutes in experimental and control groups respectively. Following this incubation, cells were washed in BT, 200 µl BT added to each well, and images were taken immediately using confocal microscopy. See “Confocal Microscopy” below for imaging details.

3.3.4. Confocal Microscopy

For RBL-2H3 cells transiently transfected with monomeric red fluorescent protein (mRFP)-MARCKS-ED plasmid, an Olympus FV-1000 Confocal microscope, with a 1-mW HeNe-Green laser (543 nm excitation and 560-660 nm emission filter) was used to collect images. An oil immersion 100 x objective with NA 1.4 and image acquisition speed of 2 µs/pixel were used to collect the images. Using the ibidi plate heating system, imaging was conducted at 37°C.

3.3.5. Automated Image Analysis

Fiji ImageJ software was used to analyze confocal microscopy images of RBL-2H3 cells transiently transfected with monomeric red fluorescent protein (mRFP)-MARCKS-ED. Images were converted to 8-bit images, channels split, and further analysis was done using the fluorescent channels. Pseudo flatfield correction was used to subtract background, appropriate threshold applied, and binary masks generated for the whole cell and the cytoplasm of a given transfected cell. The masks of the whole cell and cytoplasm were applied to the given transfected cell in the fluorescent channel to generate a region of interest (ROI) for the whole cell and the cytoplasm respectively. Next, the area, integrated density, and mean fluorescence per pixel of the whole cell and the cytoplasm were measured from the ROI. The mean fluorescence per pixel of the plasma membrane (PM) was generated by dividing the difference between the integrated density of the whole cell and cytoplasm by the difference between the area of the whole
cell and the cytoplasm. The PM/cytoplasm mean fluorescence per pixel was calculated by dividing the mean fluorescence of the PM by the mean fluorescence of cytoplasm and results were analyzed in Graphpad.

3.3.6. CPC Cytotoxicity and Survivability on RBL-2H3 cells

Trypan blue exclusion and lactate dehydrogenase (LDH) cytotoxicity assays were used to assess cytotoxicity and survivability on RBL-2H3 cells exposed to 30 μM CPC. In the trypan blue exclusion assay, RBL-2H3 cells were plated at 1.2 x 10⁶ cells per well in six-well Grenier CELLSTAR 6 well plates (Item no. 657 160) tissue culture plates and were incubated overnight at 37°C and 5% CO₂. Each treatment group was performed in experimental triplicate, with three total wells of cells per treatment per experiment. The following day, CPC dilutions were prepared with Tyrodes-BSA (BT) before removal of spent media from incubated cells and subsequent 1-hour exposure to 2 mL of CPC dilution, incubating at 37°C and 5% CO₂. This buffer was removed with a wash of 2 mL per well BT. The cells were next dislodged using first a 1 mL per well trypsin wash, which was similarly discarded, followed by further addition of 1 mL per well trypsin incubation for 10 minutes at 37°C. The trypsin was neutralized by the addition of RBL media to each sample. Surviving cells were exposed to trypan blue stain and counted using a hemocytometer.

LDH activity was measured using a cytotoxicity detection assay kit per instructions (Sigma, Catalog #474492601) as indicated by red fluorescence and membrane rupture. Components of the kit were aliquoted and stored at -20°C prior to the day of experimentation by which they were thawed at 37°C and protected from light. RBL-2H3 cells were harvested and prepared at 0.1 x 10⁶ cells mL⁻¹ concentration in RBL media to achieve a total cell count of 10,000 cells when 100 μL of the solution was plated. Clear, flat-bottom 96-well plates (Grenier CELLSTAR® Catalog # 655 180) were utilized and were incubated overnight at 37°C, 5% CO₂. Cells were exposed to CPC samples for 45 minutes at 37°C. Absorbance values were read using a spectrophotometric microplate reader (Synergy 2; Biotek) at 490 and 690 nm.
3.3.7. Fluorescence Photoactivation Localization Microscopy (FPALM) Imaging and Processing

Two-color FPALM was performed by previously published methods (Hess, Girirajan and Mason, 2006; Gunewardene et al., 2011; Curthoys et al., 2019; Sangroula et al., 2020). Briefly, lasers with wavelengths $\lambda = 405$ nm (CrystaLaser, 5mW) for the activation of Dendra2 molecule (Gurskaya et al., 2006) and $\lambda = 558$ nm for the readout (CrystaLaser, 100 mW) were focused in the back aperture plane of an oil immersion objective (Olympus 60X 1.45NA) using a second lens ($f = 350$mm, Thorlabs) at one focal length away from the back aperture. To match the polarization of the readout laser, the activation laser was passed through a half-wave plate (Newport, 10RP42-1) and a linear polarizer (Newport, 5511). For better activation and readout, both lasers were then converted to elliptical (approximately circular) polarization by passing them through a quarter-wave plate (Newport, 10RP54-1B). Laser power was recorded by the power meter (Thor labs) to be $\approx 13$ mW for the readout laser and about $\approx 75$ µW for the activation laser after passing straight through the objective lens. The angle of the beam was then changed for TIRF Imaging. Fluorescence emission was collected by the objective and filtered through a quad-band dichroic (Di01 R405/488/561/635-25x36) and a 561 notch filter (Semrock: NF03-561E-25). After the dichroic and notch filter, fluorescence emitted through the tube lens was magnified $\approx 2X$ using successive achromatic lenses with focal lengths +20 mm and +40 mm. Fluorescence then reached a second dichroic (Semrock, FF580-FDi02-t3) within the multi-color detection module, which reflected $\lambda < 595$ nm and transmitted $\lambda > 595$ nm, thus producing two wavelength ranges which were simultaneously imaged onto adjacent regions of the camera sensor. Fluorescence from the transmitted channel passed through an ET630/92 filter (Semrock, FF01-630/69-25) and fluorescence from the reflected channel passed through a 585/40 filter (Semrock, FF01-585/40-25) before reaching the camera (iXon+ DU897DCS-BV, Andor Scientific, Dublin, Ireland). Typically, ten thousand frames were recorded at $\approx 32$ Hz and EM gain of 200.
3.3.8. FPALM Data Analysis

Point Spread Functions recorded in the raw images were background subtracted (Sternberg, 1983) and localized by fitting into the 2D Gaussian function (Hess, Girirajan and Mason, 2006). After localization, images were further analyzed for drift subtraction and bleed-through correction (Kim et al., 2013). Localizations were assigned to either of two channels according to their alpha values (Gunewardene et al., 2011; Curthoys et al., 2013) and further processed through custom-built Matlab code for the quantitative analysis of clusters. We used single linkage cluster analysis (SLCA) (Sneath, 1957; Greenfield et al., 2009; Gudheti et al., 2013; Curthoys et al., 2019; Sangroula et al., 2020) for cluster identification. This technique detects molecules within \( d_{\text{max}} \) of each other and considers them as a cluster if total molecules within the distance of \( d_{\text{max}} \) pass a certain minimum threshold. For this analysis, \( d_{\text{max}} = 35 \) nm. To be considered for analysis, a threshold for the minimum number of molecules per cluster was required to be \( N_{\text{min}} \geq 50 \). Grid Pixel Sum analysis was performed by binning the localized molecules within a grid dimension of 35 nm X 35 nm, after separation of their color according to their alpha values. Pixels (Grid) were identified as a cluster for the first species which contained five or more molecules. After this identification, all the localizations of the second species were summed over only those pixels that were previously identified to have a cluster of first species and then averaged over all the cells.

3.3.9. Zebrafish Care and Maintenance

Ab Zebrafish (Danio rerio) used in the study were grown in accordance with the recommendations in the Guide of the Care and Use of Laboratory Animals of the National Institutes of health. The protocols utilized in this study were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Maine (Protocol Number: A2018-01-01). Zebrafish were maintained in the Zebrafish Facility at the University of Maine, Orono by Mark Nilan. The facility was maintained according to the IACUC standards. IACUC approved guidelines for zebrafish. IACUC approved guidelines for zebrafish care followed the standard procedures (www.zfin.org) of 14-hour light, 10-hour dark cycle at 28 °C. Embryos
were obtained through the natural spawning of adult AB zebrafish. AB zebrafish were crossed from varying sets of females and males. Fertilized eggs were collected as a pool from ~20 females and 15 males per experiment. Embryos were raised in sterilized egg water (60 μg/ml Instant Ocean sea salts; Aquarium Systems, Mentor, OH) at 33°C.

### 3.3.10. Microinjection of IAV

Embryos were manually dechorionated at 48 hpf with DuMont no. 5 forceps. Prior to injections, fish were anesthetized in tricaine solution. The fish were then lined up on a 2% agarose gel in a Petri dish coated with 3% methyl cellulose. The embryos were injected into the DC with 1.0 nl (~ 8.7 ×10² EID50) of APR8 IAV or HBSS (Gibco Hank’s Balanced Salt Solution, part no. 14170120). Needles containing IAV were changed every hour to guarantee the virus remained viable. Following injection, zebrafish were sorted into plates of 45 and maintained at 33°C. The egg water was changed daily. Microinjections were controlled by an MPPI-2 pressure microinjector (Applied Scientific Instruments). Pulled microcapillary pipettes (Sutter Instruments, Novato, CA) were used to inject either the virus or HBSS.

### 3.3.11. CPC Treatment on Zebrafish

CPC was administered to infect and control zebrafish at a concentration of 0.1 µM in 50 mL of egg water. Selection of the plates to receive CPC treatment was random. The fish were treated for 1 hour at 33°C under light-sensitive conditions. The fish were then rinsed in egg water to remove all traces of CPC and placed into fresh egg water for survivability experiments. Standard CPC treatment occurred at 6 hpi. Specialized treatments were administered 24, 48, and 72 hpi. Each timed CPC experiment was compared to standard CPC treated zebrafish and to untreated zebrafish for both IAV and HBSS injected zebrafish.

### 3.3.12. IAV and Mortality Curves

The influenza viral strains H1N1 (APR8/34, part no. 490710) were purchased from Charles River Laboratories (North Franklin, CT). The virus strains were stored at −80°C, thawed at room temperature, and diluted in cold sterile HBSS in a ratio of 87% virus to 13% diluent. Wild-type zebrafish embryos were
injected and maintained as described above. For survivability experiments, water was changed daily and mortality was monitored and counted from 24-120 hpi.

3.4. Results

3.4.1. CPC significantly displaces MARCKS from the plasma membrane into the cytoplasm after 30-minute incubation

To investigate the effect of CPC on the plasma membrane (PM) and cytosolic distribution of the PIP$_2$-binding protein MARCKS, RBL-2H3 cells were transiently transfected with monomeric red fluorescent protein (mRFP)-MARCKS-ED (Gadi et al., 2011) and incubated overnight. On the next day, the cells were incubated with 5 µM CPC, 10 µM CPC or BT (control) for 30 minutes, images taken with confocal microscopy; we then quantified R$_{pc}$, the ratio of the mean fluorescence of (mRFP)-MARCKS-ED at the plasma membrane to the mean fluorescence of (mRFP)-MARCKS-ED in the cytoplasm. The average R$_{pc}$ for cells exposed to 10 µM CPC was 1.75± 0.10 (n = 65), 2.42 ± 0.11 (n = 90) for cells exposed to 5 µM CPC, and 4.85 ± 0.17 (n = 143) for control as shown in figure 1B (error bars denote SEM). A comparison of R$_{pc}$ in CPC treated and control cells shows that CPC statistically and significantly displaces MARCKS, the PIP$_2$ binding protein, from the plasma membrane into the cytosol (Figure 1A). These data indicate that CPC displaces MARCKS from the plasma membrane.
**Figure 3.1:** CPC effects on plasma membrane (PM) versus cytoplasmic distribution of PIP$_2$-binding protein MARCKS in RBL-2H3 mast cells. (A) Representative live-cell confocal microscopy images of RBL-2H3 mast cells transiently transfected with mRFP-MARCKS-ED plasmid overnight, washed in BT (Tyrodes-BSA), and pre-exposed to BT (Control), 5 µM CPC and 10 µM CPC for 30 minutes. (B) RBL-2H3 mast cells were transiently transfected with mRFP-MARCKS-ED plasmid overnight, washed in BT, pre-exposed to BT (Control), 5 µM CPC, 10 µM CPC for 30 minutes, and confocal images were taken after washing off the CPC with BT. Mean fluorescence per pixel of MARCKS at the plasma membrane (PM) and cytoplasm was calculated using Fiji ImageJ. Values represent mean ± SEM of three independent experiments that were derived from analysis of $n = 143$ cells for BT (Control), $n = 90$ cells for 5 µM CPC and $n = 65$ cells for 10 µM CPC. Statistically significant results, as compared to the appropriate control (BT), are represented by ***$p < 0.001$, as determined by one-way ANOVA followed by Tukey’s post-hoc test.
3.4.2. CPC at concentrations $\leq 15 \, \mu\text{M}$ is not cytotoxic to RBL-2H3 cells during a short-term (1-hour) exposure

Two cytotoxicity assays were conducted to assess the survivability of RBL-2H3 cells when exposed to a range of CPC doses for 1 hour. The first assay performed was a trypan blue-exclusion assay. In this assay, cells underwent the desired exposure and then were stained with trypan blue. Cells were counted as healthy based on their ability to exclude the stain. The figure 2A shows a statistically significant cytotoxic effect only in cells exposed to 30 $\mu\text{M}$ CPC, while concentrations $\leq 15 \, \mu\text{M}$ were not cytotoxic as quantified by the assay.

To further assess the survivability of RBL-2H3 cells under various CPC exposures, we next performed an LDH release assay. LDH is an intracellular enzyme released upon cell damage or death. Figure 2B shows a statistically significant rise in cytotoxicity upon exposure to 30 $\mu\text{M}$; concentrations $\leq 15 \, \mu\text{M}$ were not cytotoxic. The results from these two assays strongly suggest that CPC is not cytotoxic to RBL-2H3 cells at concentrations up to 15 $\mu\text{M}$ within a 1-hour exposure.
Figure 3.2: Measurement survivability of RBL-2H3 cells at different doses of CPC. (A) lactate dehydrogenase (LDH) assay and (B) Effects of CPC on RBL-2H3 cell survivability were assessed by a trypan blue-exclusion cytotoxicity assay. Values presented are means ± SEM of three independent experiments. Statistical significance, compared to the 0 μM CPC control, is represented by **p < 0.01, determined by one-way ANOVA followed by Tukey’s post-hoc test.
3.4.3. CPC disrupts PIP₂ clusters in NIH3T3 cells

We also studied the effect of CPC in PIP₂ clusters along with the HA, as PIP₂ has also been previously reported to cluster at the plasma membrane (Van Den Bogaart et al., 2011; Wang and Richards, 2012). In order to visualize the nanoscale distribution of PIP₂, we used FPALM to image a PAmKate-tagged version of the PIP₂-binding Pleckstrin Homology (PH-) domain from PLC-δ (Gambhir et al., 2004; Curthoys et al., 2019). Cluster analysis was performed using SLCA (see methods section). The mean area of a PH domain cluster (control) was found to be 0.105 ± 0.009 µm², which decreased to 0.081 ± 0.007 µm² and 0.085 ± 0.006 µm² for 5 µM CPC and 10 µM CPC treated cells, respectively (Figure 3.3C). Although the decrement in the mean area of a PIP₂ cluster is approximately about 23% and 19% respectively, no statistical significance was observed by one-way ANOVA followed by Dunnett’s multiple comparison test against control. Kruskal-Wallis test on the area of PIP₂ cluster resulted being barely insignificant (p = 0.0503). The mean density of a PH domain (PLC-δ) cluster decreased from 2700 ± 450 µm⁻² for control to 2430 ± 320 µm⁻² for 5 µM and 2110 ± 140 µm⁻² for 10 µM CPC treated cells, which is roughly a 10% and 20% decrease, respectively (Figure 3.3A). The mean perimeter of PH domain clusters also decreased to 1.44 ± 0.10µm for 5 µM and 1.53 ± 0.08 µm for 10 µM as compared to control, whose perimeter was found to be 1.7 ± 0.11 µm (Figure 3.3D). Both the mean density and mean perimeter of a PH domain cluster were statistically insignificant (one-way ANOVA with Dunnett’s post-test). Although the mean area and mean density of PIP₂ clusters remained insignificant, the number of molecules forming a PH domain cluster binding PIP₂ decreased significantly (p < 0.01). On average, 268 ± 30 molecules formed a PH domain cluster (control) while only 165 ± 13 and 193 ± 21 PH domain molecules on average were found in a cluster for 5 µM and 10 µM CPC treated cells, respectively (Figure 3.3 B).
**Figure 3.3:** CPC disrupts PIP$_2$ cluster properties in NIH3T3 cells. Two-color TIRF FPALM was used to obtain images from fixed NIH3T3 cells expressing HA-Dendra2 and PAmKate-PH domain (PLC-δ). NIH3T3 cells were exposed to Control (0 µM CPC + Tyrodes-BSA vehicle), 5 µM, and 10 µM CPC for 1 hour at 37°C and fixed with 4% Paraformaldehyde (PFA). PIP$_2$ cluster properties (A) Mean density of a PIP$_2$ cluster (B) Mean number of PIP$_2$ molecules forming a PIP$_2$ cluster (C) Mean Area of a PIP$_2$ cluster (D) Mean perimeter of a PIP$_2$ cluster for Control, 5 µM and 10 µM CPC treated cells were quantified using Single Linkage Cluster Analysis (SLCA). Values presented are mean ± SEM for a total of 80 cells in three independent experiments. Statistically significant results are represented by **p < 0.01 as compared to the Control by one-way ANOVA followed by Dunnett’s multiple comparison test against the Control.
3.4.4. CPC disrupts HA clusters in NIH3T3 cells

Considering the recently identified relationship between PIP\textsubscript{2} and the influenza viral protein hemagglutinin (HA) (Curthoys \textit{et al.}, 2019), and the reported antiviral properties of CPC (Popkin \textit{et al.}, 2017), we used super-resolution microscopy to test whether CPC could affect HA clustering, which is crucial for viral entry through membrane fusion (Takeda \textit{et al.}, 2003). We used FPALM (Hess, Girirajan and Mason, 2006) with TIRF excitation to image the plasma membrane of NIH3T3 cells expressing HA-Dendra2 and PAmKate–PH Domain (PLC-δ), which binds to and labels PIP\textsubscript{2} (Gambhir \textit{et al.}, 2004; Curthoys \textit{et al.}, 2019). Clusters were identified using SLCA (see methods). Quantification of HA clusters showed a wide range of density with the mean value of 12700 ± 4300 HA/µm\textsuperscript{2} for control (Figure 3.3(A)). This wide variation of density in HA cluster was absent in cells treated with CPC: the mean HA density in CPC treated cells decreased significantly by ~79% when compared against the control, with the mean HA density of 2700 ± 420 HA/µm\textsuperscript{2} for 5 µM and 2660 ± 360 HA/µm\textsuperscript{2} for 10 µM CPC treated cells (Figure 3.3(B)). A similar result was obtained for the number of HA molecules in a cluster that reduced significantly. On average, 340 ± 110 HA molecules were found in an HA cluster which decreased significantly by ~74% and ~76% to 88 ± 9 HA molecules and 81 ± 13 HA molecules for 5 µM and 10 µM CPC treated cells respectively (Figure 3C). Mean area of an HA cluster was observed 0.041 ± 0.004 µm\textsuperscript{2} for Control, 0.044 ± 0.006 µm\textsuperscript{2} for 5 µM and 0.039 ± 0.003 µm\textsuperscript{2} for 10 µM CPC and mean perimeter of an HA cluster was observed 0.90 ± 0.08 µm for control 0.97 ± 0.10 µm for 5 µM and 0.093 ± 0.06 µm for 10 µM (Figure 3.3D). Both the mean area and mean perimeter of an HA cluster were fairly similar as a function of CPC treatment and statistically insignificant by one-way ANOVA followed by Dunnett’s multiple comparison test against the control.
Figure 3.4: CPC disrupts HA cluster properties in NIH3T3 cells. Two-color TIRF FPALM was used to obtain images from fixed NIH3T3 cells expressing HA-Dendra2 and PAmKate-PH domain (PLC-δ). NIH3T3 cells were exposed to Control (0 µM CPC + Tyrodes-BSA vehicle), 5 µM, and 10 µM CPC for 1 hour at 37°C and fixed with 4% Paraformaldehyde (PFA). HA cluster properties (A) Mean density of an HA cluster (B) Mean number of HA molecules forming an HA cluster (C) Mean Area of an HA cluster (D) Mean Perimeter of an HA cluster for Control, 5 µM and 10 µM CPC treated cells were quantified using Single Linkage Cluster Analysis (SLCA). Values presented are mean ± SEM for a total of 66 cells in three independent experiments. Statistically significant results are represented by *p < 0.05, as compared to Control by one-way ANOVA followed by Dunnett’s multiple comparison test against the Control.
3.4.5. CPC reduces the co-clustering of HA and PIP2 in NIH3T3 cells.

In order to quantify the effect of CPC on HA and PH domain co-clustering, the mean molecule number per grid pixel (see methods) for HA within PH clusters ($N_{HA-PH}$) and PH within HA clusters ($N_{PH-HA}$) was determined from two-color FPALM results. The analysis shows a significant decrease in $N_{PH-HA}$ (mean number of PH molecules within HA clusters) in 10 µM CPC treated cells, decreasing by approximately 71% as compared to control, which was significant by unpaired t-test (Figure 3.6A). A similar result was obtained for the number of HA localizations within PH domain clusters, where $N_{HA-PH}$ dropped approximately by 61% compared to control, again significant by unpaired t-test (Figure 3.6B).

![Figure 3.5](image)

**Figure 3.5:** Super-resolution images show colocalization of HA-Dendra2 and PAmKate-PH domain (PLC-δ) in fixed NIH3T3 cells. Two-color FPALM images of fixed NIH3T3 cells expressing HA-Dendra2 (green) and PAmKate-PH (magenta) of (A) Control and (B) 10 µM CPC treated cells. Arrows (red with yellow outline) point to the areas of colocalization (white). Note that there are fewer colocalization areas in 10 µM CPC treated cells.
Figure 3.6: CPC treatment reduces HA-PH co-clustering. HA-PH co-clustering was quantified from the distribution of Dendra2-HA and PAmKate-PH molecules localized within a grid of 35 nm x 35 nm pixels, after bleed-through correction, in fixed NIH3T3 cells. (A) Pixels identified as HA clusters (at least 5 HA localizations) were selected. The number of PH domain (PIP_2) localizations was then summed over all pixels within the selection, and then averaged over all cells, and is plotted as “mean pixel sum” as a function of CPC treatment (10 µM). (B) Pixels were identified as PH domain clusters (at least 5 PH domain localizations) were selected. The number of HA localizations was then summed over all pixels within the selection and then averaged over all cells and is plotted as “mean pixel sum” as a function of CPC treatment (10 µM). Values presented are mean ± SEM for a total of 61 cells in three independent experiments. Statistically significant results are represented by *p < 0.05 as compared to the control, determined by unpaired t-test.
3.4.6. CPC treatment Reduces IAV infections and Increases survival in AB Zebrafish Embryos.

Virus and mortality curves revealed CPC treatment significantly enhanced survival in an in vivo Danio rerio (zebrafish) influenza virus infection (IAV) model while reducing IAV from 24-120 hpi. Specifically, CPC treatment in mild to moderate IAV infections was shown to significantly increase the chance of survival up to 27.5% if administered within the first 48 hpi.
Figure 3.7: CPC treatment reduces IAV infections and increases survival in AB Zebrafish embryos. (A) Survival curve between 0.1 µM CPC treated and untreated IAV infected zebrafish with CPC treatment administered at 6 hours post-infection (hpi). By 5 days post-infection (dpf) the average survival of CPC treated zebrafish was 83.2% versus 60.1% for untreated IAV infected zebrafish. The survival rate increased 23.1% with a 6 hpi CPC treatment compared to the untreated plates. (B) Survival curve between 0.1 µM CPC treated and untreated IAV infected zebrafish with CPC treatment administered at 24 hpi. By 5 dpf the average survival of CPC treated zebrafish was 87.1% versus 60.1% for untreated IAV infected zebrafish. The survival rate increased 26.9% with 24 hpi CPC treatment compared to the untreated plates. (C) Survival curve between 0.1 µM CPC treated and untreated IAV infected zebrafish with CPC treatment administered at 48 hpi. By 5 dpf the average survival of CPC treated zebrafish was 87.7% for treated versus 60.1% untreated IAV infected zebrafish. The survival rate increased 27.5% with 48 hpi CPC treatment compared to the untreated plates. Values presented are for a total of ~150 zebrafish, selected at random in three independent experiments. Statistically significant results are represented by ***p < 0.0001 determined by the Mantel-Cox test, the Logrank test, and the Gehan-Breslow-Wilcoxon test.
3.5. Discussion

PIP2 is a minor component of the plasma membrane responsible for a number of cellular functions (McLaughlin et al., 2002; Catimel et al., 2008; Balla, 2013) and is known to cluster at the plasma membrane (Van Den Bogaart et al., 2011; Wang and Richards, 2012; Curthoys et al., 2019). Previous studies have shown that PM targeting motifs have polybasic domains that provide PM specificity and were observed to cluster with negatively charged PIP2 and PIP3 through charge-charge and hydrophobic interactions (Won et al., 2006)

3.5.1. CPC Modulates the Cellular Distribution of PIP2-Binding Proteins.

Originally, because of its prevalence in a number of consumer products, we chose to examine the effect of the quaternary ammonium compound cetylpyridinium chloride (CPC) on cell function. CPC is positively charged and has previously been shown to have antibacterial (Ioannou, Hanlon and Denyer, 2007; Hwang et al., 2013) and antiviral properties (Popkin et al., 2017), but its effect on normal cell function is not well characterized. We found that CPC treatment caused MARCKS, a well-known PIP2 binding protein (McLaughlin et al., 2002), to fall off the plasma membrane (Figure 3.3.1). Because of a recently identified relationship between HA and PIP2 in which PIP2 modulates HA clustering and HA modulates PIP2 clustering (Curthoys et al., 2019), we became interested in whether CPC could also affect the distribution of HA. Based on our previously published results (Curthoys et al., 2019), we hypothesize that HA interacts with PIP2 through charge-charge interactions between the PIP2 head and the HA cytoplasmic tail (CTD), which has two basic residues and an estimated net charge of +6 per trimer and also has several conserved, acylated cysteines within the HA CTD which could interact with lipids within the inner leaflet of the plasma membrane (Veit, Serebryakova and Kordyukova, 2013). This led us to hypothesize that blocking this interaction would affect HA clustering, which is crucial for infectivity (Ellens et al., 1990) and the viral life cycle (Takeda et al., 2003). We used super-resolution microscopy (FPALM) to image HA-Dendra2 and the PIP2-binding domain PH(PLCδ) tagged with PAmKate (Flesch et al., 2005;
Localized molecular coordinates of HA-Dendra2 and PH-PAmKate were then quantified using single-linkage cluster analysis (SLCA) (Sneath, 1957; Curthoys et al., 2019; Sangroula et al., 2020) to determine the properties of clusters formed by the HA and PH domain. Quantification of the PH-PAmKate (PIP2) clusters showed significant disruption by CPC (Figure 3.3), thus further confirming the effect of CPC on PIP2 binding proteins. These results are important because PIP2 binding proteins have been previously shown to have polybasic domain and clusters to negatively charged PIP2 and PIP3 through charge-charge interactions (Won et al., 2006) and CPC disrupts these clustering possibly disrupting the charge-charge interactions.

3.5.2. PH-Domain Cluster Properties Compared to Published Values.

We report the PH-domain (PIP2) cluster (control) diameter of $0.365 \pm 0.003 \, \mu\text{m}$ (from the mean cluster area considering a circular shape) while previous studies have reported an average PIP2 cluster diameter of $0.073 \pm 0.042 \, \mu\text{m}$ in PC12 membrane sheets (Van Den Bogaart et al., 2011), an average diameter of $0.064 \pm 0.027 \, \mu\text{m}$ in PC12 cells (Wang and Richards, 2012). While it would be unwise to pinpoint a single factor for the discrepancies, different factors like difference in a cell line, resolution of the imaging technique, labeling density in localization microscopy technique (Pennacchietti, Gould and Hess, 2017), and difference in cluster identification technique should all be considered collectively. We report a mean PIP2 cluster area of $0.105 \pm 0.009 \, \mu\text{m}^2$ for the control which is much greater than our previously reported PIP2 area $0.0145 \pm 0.0020 \, \mu\text{m}^2$ and $0.026 \pm 0.006 \, \mu\text{m}^2$ in presence of low and high dense clusters of HA (Curthoys et al., 2019). While our previous study only identified PIP2 clusters in the vicinity of low and high dense clusters of HA, PIP2 cluster area reported in this study are the clusters (global or cell-wide) identified irrespective of their distribution with respect to the HA.

3.5.3. HA Clusters are Modulated by CPC.

Clusters of HA showed a wide variation of HA density in the cells treated with the control media (Figure 3.4A), consistent with published results showing that HA clusters on many length scales (Hess et
al., 2005). Significant disruption of the clusters was observed particularly with respect to the density and the number of HA molecules forming an HA cluster in CPC treated cells (Figure 3.4B). In order to confirm the role of local PIP2 levels in the disruption of HA co-clustering, we quantified the effect of CPC on the co-clustering of HA and PIP2. The co-clustering was significantly disrupted in 10uM CPC treated cells (Figure 3.6). This result not only further suggests that the modulation of PIP2 clusters, in turn, modulates HA clusters, but that the CPC modulates the PIP2 which is co-clustered with HA, suggesting a local effect on HA clustering due to PIP2 (we do not rule out “global” or cell-wide effects as well). This result is also of importance because dense HA clusters are crucial for efficient viral entry through membrane fusion (Takeda et al., 2003) and CPC significantly disrupts HA cluster density, thus explaining a mechanism of antiviral properties of CPC at low concentrations, consistent with our findings that influenza-infected zebrafish survival improves with CPC treatment (Figure 3.7). Although CPC is believed to work on enveloped viruses in general by disrupting their membranes (Baker et al., 2020), the doses used here are orders of magnitude lower (sub-μM to 10 μM) than those required for non-specific detergent-like membrane disruption (~mM); furthermore, a drug which is capable of nonspecifically disrupting membranes at the effective concentration would be expected to cause major cytotoxicity, which we do not observe (Figure 3.2).

A plethora of studies in different viruses show phosphoinositides, and in many cases, PIP2 interact with viral proteins through multiple basic residues, and that these interactions are crucial for the life cycles of multiple viruses (Ono et al., 2004; Chukkapalli et al., 2008; Chukkapalli and Ono, 2011; Altan-Bonnet and Balla, 2012; Johnson et al., 2016; Yandrapalli et al., 2016; GC, Gerstman and Chapagain, 2017; M., K. and R.V., 2017). More recently, phosphoinositide kinase inhibitors have been shown to inhibit the Zaire Ebola virus and SARS-CoV-2 (Kang et al., 2020). Targeting interactions between viral proteins and host cell phosphoinositides could be a novel therapeutic approach; CPC is already being used in a clinical trial against the SARS-CoV-2 (UCSF COVID-19 Trial → Antiseptic Mouthwash / Pre-Procedural Rinse on SARS-
CoV-2 Load (COVID-19), no date). Our study presents the effect of CPC on PIP2 binding proteins and illuminates a mechanism for its antiviral properties at relatively non-cytotoxic concentrations, paving the path for the future study of the effect of CPC in modulating PIP2 and PIP2 binding proteins and other membrane proteins that interact with the PIP2.
4.1. Preface

This chapter is being prepared for submission by the authors Prakash Raut, Bright Obeng, Hang Waters, Joshua Zimmerberg, Julie A. Gosse, Samuel T. Hess.

4.2. Introduction

Influenza A virus (IAV) causes significant morbidity and mortality in human populations. IAV is an enveloped, negative-sense RNA virus in the orthomyxoviridae family (Lamb and Choppin, 1983). The membrane contains three viral glycoproteins, hemagglutinin (HA), neuraminidase (NA), and the proton channel (M2) (Nayak, Ka-Wai Hui and Barman, 2004). Adjacent to the inner leaflet of the viral membrane, the matrix protein (M1) forms a layer and provides support to the integrity of the virus particle through interactions with other viral components and the bilayer itself (Calder et al., 2010). Finally, the viral core consists of nucleoprotein (NP), three polymerases (PB1, PB2, PA), eight RNA segments, and three Nuclear Export Proteins (NEPs) (Lamb, Robert and Choppin, 1983). Interaction of viral proteins with host cell membranes begins with binding to the host cell, and continues through the viral life cycle until new progeny virions bud and are released from the host cell (Gudheti et al., 2013; Chlanda and Zimmerberg, 2016; Zhao, Wang and Li, 2017; Curthoys et al., 2019). It is crucial to better understand the interaction of viral components with host cell membranes in order to understand the viral life cycle and develop new antiviral therapies.

M1 can bind to lipid bilayers through electrostatic interaction (Gregoriades, 1980; Ito et al., 1991; Zhang and Lamb, 1996; Ruigrok et al., 2000; Thaa, Herrmann and Veit, 2009) and can form virus-like particles (VLPs) in the absence of other viral components (Gómez-Puertas et al., 2000; Latham and Galarza, 2001), demonstrating its ability to interact with lipids in the plasma membrane. Upon such
binding, M1 multimerizes (Hilsch et al., 2014). This formation of multimers or clusters is suspected to play a crucial role in the viral life cycle; however, the role of the viral glycoproteins in the clustering of M1 during interaction with the plasma membrane is unclear. Previous studies show that in model membranes and cell models of infection, M1 can bind to lipid bilayers containing PS (Ruigrok et al., 2000; Hilsch et al., 2014; Bobone et al., 2017). However, there is less information about M1 binding to other lipids, as M1 can bind the lipid bilayer through multiple residues (Thaa, Herrmann and Veit, 2009), and also M1 is twice more likely to be adsorbed on phospholipidic surfaces than on neutral surfaces (Shishkov et al., 2009). In light of the recent finding that HA and PIP2 can interact and modulate their clustering in the plasma membrane (Curthoys et al., 2019), we considered the possibility that HA interactions with M1 (Ali et al., 2000; Barman et al., 2001) could be mediated by phosphatidylinositol 4,5 bisphosphate (PIP2).

PIP2 is a minor component of the plasma membrane in the mammalian cells (~1% of total lipid) but is the most abundant phosphoinositide (Xu, Watras and Loew, 2003; Hammond and Balla, 2015). Despite being a minority fraction of lipids, phosphoinositides have a giant role in cell functions. PIP2 is known to play an important role in endocytosis/exocytosis (Cremona et al., 1999; Martin, 2001), actin cytoskeleton regulation (Sun et al., 1999), and cytoskeleton plasma membrane adhesion (Raucher et al., 2000), among many others (Balla, 2013). It has been observed that PIP2 clusters at the plasma membrane (Van Den Bogaart et al., 2011; Wang and Richards, 2012; Curthoys et al., 2019) and is known to interact with various cytoskeletal proteins such as actin and actin-binding proteins (Logan and Mandato, 2006; Chierico et al., 2014; Wu et al., 2014). Proteins that have been purified from the influenza virus are also known to interact with PIP2 (Shaw et al., 2008), and it has been previously shown that the influenza virus exploits several PIP2-dependent pathways (Hale et al., 2006; Fujioka et al., 2013). All this evidence point toward the role of PIP2 in the influenza virus life cycle. HIV Gag protein and Ebola matrix protein VP40 have also been shown to interact with PIP2 (Ono et al., 2005; Stahelin, 2014; GC, Gerstman and Chapagain, 2017; M., K. and R.V., 2017).
We recently showed that the quaternary ammonium compound cetylpyridinium chloride (CPC) at non-cytotoxic concentrations can be used to modulate the membrane association and clustering of PIP2-binding proteins such as MARCKS and the PH-domain from PLC-δ (see Chapter 3). CPC contains a positively charged head group, and a hydrophobic tail, allowing it to efficiently associate with membrane bilayers and micelles (Marcotte et al., 2005). The detergent action of CPC is observed when the concentration of CPC is above critical micelle concentrations (CMC) ~900 µM in water (Mukerjee et al., 1971). Studies by various methods in different buffers and temperatures have identified the CMC of CPC to be in the range ~600-900 µM (Mandal and Nair, 2002; Varade et al., 2005; L et al., 2010; Shi, Luo and Li, 2011). Although CPC has been previously shown to possess antibacterial and antiviral properties through micelle formation at millimolar concentrations (Haps et al., 2008; Hwang et al., 2013; Popkin et al., 2017); however, there is much less information on the effects of CPC on cell function at low micromolar concentrations. Our recent CPC study also showed that HA and PH (PIP2) co-clustering were modulated by CPC, and suggested a mechanism for antiviral properties of CPC at non-cytotoxic concentrations. In this study, we build on these findings and test the hypothesis that CPC modulates the assembly of HA and M1. Using super-resolution microscopy (FPALM) (Hess, Girirajan and Mason, 2006) with TIRF illumination, we also shed light on the effect of M1 on the plasma membrane distribution of PIP2 and the effect of HA in the clustering of M1 beneath the plasma membrane.

4.3. Methods

4.3.1. Cell Culture, Transfection, and Fixation:

NIH3T3 mouse fibroblast cells (ATCC, CRL-1658) were cultured in growth media (DMEM with Glucose and L-Glutamine, Lonza, 12-604F) with 10% Calf Bovine Serum (30-2030, ATCC) and antibiotics (Penicillin Streptomycin, 100µg/ml) at 37°C and 5% CO₂ until they were 70-90% confluent. Cells were passed using 0.05% trypsin, were seeded at the concentration of 35000 cells/mL in the petri dish (MatTek, P35G-1.5-20-C), and were grown in media (DMEM with Glucose without phenol red, Lonza, 12-917F) for
20-24 hours in an incubator maintained at 37°C and 5% CO₂. After this time, cells were transfected using Lipofectamine 3000 (L3000008, Invitrogen) with 2 µg of total DNA (1µg of either species in the case of two-color imaging). Cells were left in the same (previous) growth media overnight in the incubator and maintained at the above-mentioned conditions. After 20-24 hours, cells were treated with Control (0 µm CPC + Tyrodes- BSA vehicle), 5µM CPC, or 10 µM CPC for 1 hour in the incubator maintained at 37°C and 5% CO₂. After an hour, petri dishes were washed twice with phosphate-buffered saline (PBS) (Sigma-Aldrich, D8537) and fixed with 4% paraformaldehyde (PFA) (Alfa Aesar, J61899AK) for approximately 15 minutes at room temperature and again washed twice with the PBS.

4.3.2. CPC solution preparation

CPC (VWR) solution was made by dissolving the CPC in Tyrodes buffer. CPC was weighed, added to a pre-warmed Tyrodes buffer, and vortexed. The CPC-Tyrodes buffer was then sonicated in a warm Milli-Q water bath for ~20 minutes. After sonication, CPC-Tyrodes buffer is diluted with sonicated Tyrodes buffer and stirred for ~5 minutes. The absorbance was read at 260 nm to determine the concentration of the CPC solution.

4.3.3. Two-color FPALM imaging

Two lasers of wavelengths 405 nm (Crystal Laser, 5mW) and 558 nm (CrystaLaser, 100 mW) were aligned and focused into the back aperture plane of an oil immersion objective (Olympus 60X 1.45NA) by a lens (Thorlabs) of focal length 350mm. The 405 nm laser is used for the activation of fluorophores and the 558nm laser is used for readout. The laser power entering the objective was recorded as ~75 µW and ~13 mW for the activation and read-out laser, respectively, using a power meter (Thor labs). Both the lasers were roughly circularly polarized before entering the objective for better activation and readout of the sample, first by passing the activation laser through a half-wave plate (Newport, 10RP42-1) and a linear polarizer (Newport, 5511), and then by passing both lasers through a quarter-wave plate (Newport, 10RP54-1B). The quad-band dichroic (Di01 R405/488/561/635-25x36) inside the microscope reflects the
laser into the objective, resulting in widefield illumination at the sample. The incoming beam was then translated relative to the optical axis before entering the objective to result in an increased angle of incidence (measured relative to the optical axis) for the illumination; for sufficiently high angles of incidence, total internal reflection occurred at the glass-sample interface, allowing total internal reflection fluorescence (TIRF) imaging. Fluorescence from the sample was collected by the objective, then passed through the same dichroic, through a notch filter (Semrock: NF03-561E-25), and then into the tube lens within the microscope. After (below) the tube lens, fluorescence was reflected by a glass beamsplitter cube inside the microscope, resulting in a horizontal path outside of the microscope. Fluorescence then passed through an aperture and two achromatic lenses of focal lengths +20mm and +40mm, respectively, which serve as a telescope with 2X magnification. Fluorescence emerging from the telescope reaches a second dichroic (Semrock, FF580-FDi02-t3) within the multicolor module, which was aligned at 45° to reflect fluorescence of wavelength(λ) < 595 nm and transmit fluorescence with λ > 595nm, thus creating two spectral channels for multi-color FPALM imaging (please cite Gunewardene et al. Biophysical Journal 2011). Transmitted and Reflected fluorescence pass-through (for the “red” channel): an ET630/92 filter (Semrock, FF01-630/69-25) or (for the “green” channel): a 585/40 filter (Semrock, FF01-585/40-25) respectively before reaching the camera (iXon+ DU897DCS-BV, Andor Scientific, Dublin, Ireland). At least ten thousand frames were recorded at ~32 Hz and EM gain of 200. To avoid saturation (i.e. too much signal) in the camera, for the first five thousand frames, fluorescence from the M1-PAmKate molecules was recorded, and the fluorescence from the PH-Dendra2 molecules was recorded only after five thousand frames.

4.3.4. Data Analysis

All the raw images that recorded the point spread function were analyzed using custom-built Matlab code. Firstly the raw images were background subtracted (Sternberg, 1983) after that pixel values above the set threshold were sorted, and then area “grab” of 7X7 pixels was selected centered at the
highest pixel value of the selected area. Each grab is localized by fitting into the 2D Gaussian function (Hess, Girirajan and Mason, 2006). The ratio of the total background-subtracted fluorescence in each grab in the red (F_R) and green (F_G) channels were used to determine the value alpha: \( \alpha = \frac{F_R}{F_R + F_G} \) to allow different colors of molecular species to be separated (Gunewardene et al., 2011). After this, each localization was tolerated to ensure no background makes through. Now the tolerated data were drift corrected and bleed through corrected (Kim et al., 2013).

4.3.5. Manders’ Colocalization Coefficient

Manders’ Colocalization Coefficient (MCC) for the green and the red channel was computed following as previously published (Manders, Verbeek and Aten, 1993). First, the data was separated into two colors according to their \( \alpha \) values. After the separation of the color, data was binned separately in boxes of dimension 80nmX80nm. A mask was drawn around each cell and all the bins inside the mask were included for the calculation. Next, all the pixels for one species (channel) were identified that had some (non-zero) pixel value of the second species (or that colocalized with the second species). Then the pixel values (intensities) of the identified pixels were summed over, and this sum was divided by the sum of all the pixel values of the same species (channel). The resulting value was the MCC for one channel and was averaged over all the cells; similarly, MCC was computed for the second channel as well.

4.3.6. Cluster Identification

For cluster identification, we used the single linkage cluster analysis (SLCA) algorithm which has been extensively used previously (Sneath, 1957; Greenfield et al., 2009; Gudheti et al., 2013; Curthoys et al., 2019; Sangroula et al., 2020). In this method, each localization was assigned to either of the two channels according to their \( \alpha \) values (Gunewardene et al., 2011; Curthoys et al., 2019). After this, the algorithm analyzes all localizations of the same channel using a cutoff distance \( d_{max} \). All the localizations within \( d_{max} \) of that any other localization will be considered as in the same cluster. To threshold a minimum cluster size, the total localizations within a cluster was required to equals or exceeds a certain value.
“min_pts”. For our cluster identification, we set our $d_{max}=50$nm and a minimum threshold of “min_pts” = 25 localizations within a cluster.

4.3.7. Co-Clustering Analysis

Co-Clustering analysis was performed by binning the localizations separately according to their $\alpha$ values within a grid with pixels of dimension 80nmX80nm. Next, a mask was drawn around the cell, and only the bins inside the mask were considered for further analysis. Bins that had five or more localizations of both species were considered for the co-clustering analysis. Co-clustering was quantified by calculating the mean molecule number per grid pixel for HA within M1 clusters ($N_{HA-M1}$) and M1 within HA clusters ($N_{M1-HA}$). Bins that had five or more localizations were considered as a cluster. Now only those bins which had a cluster of the first and the second species at the same position, and thus co-clustering, were included in the sum. The sum was then averaged over all the cells. A similar process was repeated for the second species.

4.3.8. Statistical Analyses

Tests of statistical significance of differences between groups of data were performed in GraphPad Prism 8.3.1. Data were copied to Graph Pad Prism directly from the Matlab files. Statistical significance was typically determined by one-way ANOVA followed by a Dunnett’s post-test for data comparing three or more groups or using a Mann-Whitney test for data comparing only two groups.

4.4. Results

4.4.1. CPC disrupts PIP2 and M1 colocalization.

As it has been previously shown that M1 binds to membranes primarily due to electrostatic interaction (Ruigrok et al., 2000; Thaa et al., 2009), we were curious to test whether M1 colocalized with PIP2 and whether CPC could affect the colocalization of M1 and PIP2. For this two-color super-resolution microscopy, TIRF illumination was used to image the plasma membrane of fixed NIH3T3 cells expressing Dendra2-PH (PLC-δ) and M1-PAmKate. The colocalization was quantified by using Manders’ Colocalization
Coefficient (MCC) (see Methods). An MCC of 0.79±0.02 was obtained for Dendra2-PH (PLC-δ) with M1-PAmKate, and an MCC of 0.85±0.02 was obtained for M1-PAmKate with Dendra2-PH. The MCC value for Dendra2-PH (PLC-δ) decreased significantly (p<0.0001, using ordinary one-way ANOVA followed by Dunnett’s multiple comparison test against the control) to 0.62±0.04 and 0.58±0.04 upon CPC treatment with concentrations of 5 µM and 10µM, respectively. Similarly, the MCC value for M1-PAmKate also decreased significantly (p<0.01, using ordinary one-way ANOVA followed Dunnett’s multiple comparison test against the control) to 0.71±0.05 and 0.68±0.04 with 5µM and 10µM CPC treatment, respectively.

Figure 4.1: Super-resolution image showing the colocalization of PIP2 and M1. NIH3T3 cells expressing PH-Dendra2 (PLC-δ) and M1-PAmKate were exposed to control media (0µM CPC+ Tyrodes-BSA vehicle), 5µM CPC and 10µM CPC and were incubated at 37 °C for an hour. Cells were chemically fixed after an hour using 4% PFA. Two-color FPALM imaging of the plasma membrane of the cells was carried out in TIRF illumination. Cells treated with (A) control show a higher degree of colocalization (white) relative to the (B) 5µM CPC and (C) 10µM CPC treatment. Also the areas of co-clustering (white) decrease with the CPC treatment. Examples of colocalization (white) are shown by the red triangles with the yellow outline.
Figure 4.2: CPC reduces the Manders’ Colocalization Coefficient (MCC) of PIP2 and M1 in fixed NIH3T3 cells. NIH3T3 cells expressing PH-Dendra2 and M1-PAmKate were exposed to control media (0µM CPC+ Tyrodes-BSA vehicle), 5µM CPC, and 10µM CPC and incubated at 37 °C for an hour. After an hour, cells were fixed using 4% PFA. Two-color FPALM imaging of PH-Dendra2 and M1-PAmKate was carried out with TIRF illumination. Localizations were assigned to one of the two channels according to their alpha values and were binned in pixels of dimensions 80nmX80nm. The MCC was quantified and plotted as a function of CPC treatment. Both the colocalization coefficients (A) MCC of PH-Dendra2 and (B) MCC of M1-PAmKate reduced significantly with the CPC treatment. Values presented are mean ± SEM for a total of 33 cells for control, 31 cells for 5µM, and 32 cells for the 10µM CPC treatment from three independent experiments. Statistically significant results are represented by **** p<0.0001 and by **p<0.01 determined by ordinary one-way ANOVA.
4.4.2. CPC disrupts PIP2 clusters in NIH3T3 cells expressing M1

The Pleckstrin Homology (PLC-δ) domain is an excellent marker of PIP2 that binds to PIP2 by specific electrostatic interactions and hydrogen bonds (Gambhir et al., 2004; Curthoys et al., 2019). We previously showed CPC disrupts the clustering of PIP2-binding proteins, and in some cases, their binding to the plasma membrane. Because of the interactions between HA and PIP2, we became interested in whether other viral components might also do the same. Thus, we examined the effect of CPC on clusters of PH domain (Van Den Bogaart et al., 2011; Wang and Richards, 2012) in the presence and absence of influenza A matrix protein expression. For this, two-color super-resolution microscopy was used to image the plasma membrane of fixed NIH3T3 cells expressing PH domain protein tagged with Dendra2 and influenza A M1 tagged with PAmKate. Cluster analysis was performed using SLCA (see methods). Upon quantification of cluster properties, the following results were observed. The mean area of a PH domain cluster for the control was found to be 0.12±0.02 µm² which decreased to 0.075±0.006 µm² and 0.077±0.005 µm² with the CPC treatment of the concentration 5µM and 10µM, respectively. This decrement was significant (p<0.05) by one-way ordinary ANOVA. When followed by the Dunnett’s multiple comparison test, only the 5µM CPC treatment was significantly different from the control, while the 10µM CPC treatment was barely insignificant (p= 0.056). Similarly, the mean perimeter of a PH domain cluster also decreased to 1.73±0.10 and 1.78±0.09 when compared to the perimeter of the control 2.25±0.16. The mean perimeter result was significant (p<0.01) when tested by ordinary one-way ANOVA followed Dunnett’s multiple comparison test against the control. The mean density value of the PH domain cluster decreased slightly from 1051±67/µm² (control) to 990±43/µm² and 1004±57/µm² for 5µM and 10µM CPC treated cells, respectively. However, differences in the mean density of the PH domain cluster were insignificant by ordinary one-way ANOVA followed by Dunnett’s multiple comparison test against the control. The mean number of PH molecules forming a PH domain cluster also decreased for 5µM and 10µM CPC treatment to 74±7 and 82±7 respectively when compared to the control where
125±26 PH molecules were found forming a PH domain cluster, on an average. Although the decrease was approximately by 41% and 34% for the 5μM and 10μM CPC treatment respectively, no significant difference was observed by ordinary one-way ANOVA followed by Dunnett’s multiple comparison test against the control.
Figure 4.3: CPC modulates PIP2 clustering in fixed NIH3T3 cells. NIH3T3 cells expressing PH-Dendra2 and M1-PAmKate were exposed to control media (0µM CPC+ Tyrodes-BSA vehicle), 5µM CPC, and 10µM CPC and were incubated at 37°C for an hour. Cells were chemically fixed using 4% PFA after the incubation. Two-color FPALM imaging in TIRF illumination was used to image the plasma membrane of the fixed NIH3T3 cells. Cluster properties (A) Mean area of a PH-domain cluster (B) Mean perimeter of a PH-domain cluster (C) Mean density of a PH-domain cluster and (D) Mean number of PH-domain molecules forming a cluster were quantified using SLCA and plotted as a function of CPC treatment. Values presented are mean ± SEM for a total of 33 cells for control, 31 cells for 5µM and 32 cells for the 10µM CPC treatment, pooled from three independent experiments. Statistically significant results are represented by * p<0.05 and **p<0.01 determined by ordinary one-way ANOVA.
4.4.3. CPC disrupts M1 clusters in NIH3T3 cells.

Next, we studied the effect of CPC on the distribution of IAV Matrix Protein (M1) found with PH clusters. For this, two-color FPALM was used to image the plasma membrane of NIH3T3 cells expressing M1-PAmKate and Dendra2-PH (PLC-δ) using TIRF illumination. Clusters were identified and the cluster properties were quantified by SLCA (see methods). The mean cluster area of M1 clusters for the control was found to be 0.113±0.014µm² which reduced to 0.064±0.005 µm² and 0.061± 0.004µm² for 5 µM and 10µM CPC treated cells. The mean area of M1 cluster significantly decreased (p<0.001, ordinary one-way ANOVA followed by Dunnett’s test against the control) with CPC treatment. Similarly, the mean perimeter of an M1 cluster for the control was observed to 2.24±0.15µm, which decreased significantly to 1.55±0.09µm (p<0.0001, ordinary one-way ANOVA followed by Dunnett’s test against the control) and 1.49±0.07µm on treatment with CPC of concentration 5µM and 10µM, respectively. The mean density of the M1 clusters for the control was observed to be 930±30 /µm² which slightly increased to 1020±60/µm² and 1070±60/µm² with the 5µM and 10µM CPC treatment. The mean value of the density of M1 clusters seemed to increase slightly with the CPC treatment, but no significant difference was observed by ordinary one-way ANOVA followed by Dunnett’s multiple comparison test against the control. On an average, about 109±15 molecules were found in a M1 cluster for the control while only 61±6 and 68±9 M1 molecules were found in a M1 cluster in the cells treated with 5µM and 10µM CPC respectively. This decrease was significant (p<0.01) by ordinary one-way ANOVA followed by Dunnett’s multiple comparison test against the control.
Figure 4.4: CPC modulates M1 clustering in fixed NIH3T3 cells. NIH3T3 cells expressing PH-Dendra2 and M1-PAmKate were exposed to control media (0µM CPC+ Tyrodes-BSA vehicle), 5µM CPC or 10µM CPC, and were incubated at 37 °C for an hour and fixed at room temperature using 4% PFA. Plasma membranes of fixed cells expressing PH-Dendra2 and M1-PAmKate were imaged using FPALM in TIRF illumination. Cluster Analysis was performed using SLCA. Cluster properties (A) Mean Area of a M1 cluster (B) Mean perimeter of a M1 cluster (C) Mean density of a M1 cluster and (D) Mean number of M1 molecules forming a M1 cluster were quantified and plotted as a function of CPC treatment. Values presented are mean ± SEM for a total of 33 cells for control, 31 cells for 5µM and 32 cells for the 10µM CPC treated cells, pooled from three independent experiments. Statistically significant results are represented by **** p<0.0001, ***p<0.001 and **p<0.01 determined by ordinary one-way ANOVA.
4.4.4. CPC disrupts the co-clustering of PIP2 and M1 in NIH3T3 cells.

We also tested if the CPC affected the co-clustering of PH and M1, or if the effect of CPC was primarily through cell-wide changes in clustering on average. The co-clustering of PH with M1 was reduced by approximately 44% and 84% with 5µM and 10µM CPC treatment respectively (see Figure 4.5). This decrement in the pixel sum with CPC treatment was significant ($p<0.01$) by ordinary one-way ANOVA. When followed by Dunnett’s multiple comparison test against the control, only the co-clustering result of 10µM CPC treatment was significant ($p<0.01$). Similarly, the co-clustering of M1 with PH was also reduced by approximately 56% and 85% with 5µM and 10µM CPC treatment, respectively, and was significant ($p<0.01$) by ordinary one-way ANOVA. When followed by Dunnett’s multiple comparison test against the control, both the 5µM and 10µM CPC treatment was significantly different from control, with the $p<0.05$ and $p<0.01$, respectively.
**Figure 4.5:** CPC reduces the co-clustering of PIP2 and M1. NIH3T3 cells expressing PH-Dendra2 and M1-PAmKate were exposed to control media (0µM CPC + Tyrodes-BSA vehicle), 5µM CPC, and 10µM CPC and were incubated at 37 °C for an hour. Cells were chemically fixed using 4%PFA at room temperature. Two-color FPALM microscopy was carried out with TIRF illumination to image the plasma membranes (lower surface) of the cells. After the separation of their color according to the α value, localizations were binned in pixels of dimensions 80nmX80nm. Pixels containing clusters (at least 5 localizations of each species) of both the species were identified. After this, pixels identified to contain PH domain clusters and M1 clusters were included in a sum of the number of localizations of M1 and PH. The mean sum of localizations in these pixels was defined as the Mean Pixel Sum and was used as a measure of co-clustering of PH-domain clusters with the M1 clusters (see methods). A similar analysis was performed on the pixel sum of M1 localizations co-clustering with PH-domain clusters. (A) Mean Pixel sum of PH domain co-clustering with M1 and (B) Mean Pixel sum of M1 co-clustering with PH domain was plotted as a function of CPC treatment. Values presented are mean ± SEM for a total of 33 cells for control, 31 cells for 5µM and 32 cells for the 10µM CPC treated cells, pooled from three independent experiments. Statistically significant results are represented by ** p<0.01 determined by ordinary one-way ANOVA.
4.4.5. M1 enhances PIP2 clustering

In order to understand the effect of M1 on PIP2, NIH3T3 cells expressing Dendra2-PH (PLC-δ) only and Dendra2-PH (PLC-δ) with IAV M1-PAmKate were fixed using PFA (4%) at room temperature. Two-color FPALM microscopy was used to image the plasma membrane of fixed cells with TIRF illumination. Clusters of PH-domain with and without of M1 were quantified using SLCA. The mean cluster area of PH-domain clusters significantly (p<0.05 using a two-tailed Mann-Whitney test) increased from 0.080±0.004 µm² in the absence of M1, compared to 0.105±0.008 µm² when co-expressed with M1. Similarly, the perimeter of the PH domain clusters also significantly increased (p<0.05 using a two-tailed Mann-Whitney test). The perimeter of PH domain clusters without the expression of M1 was 1.87±0.06 µm and the mean perimeter of a PH domain cluster with the expression of M1 was found to be 2.18±0.08 µm. The mean density of a PH domain cluster without the expression of M1 was observed to be 886±17 /µm² and with the expression of M1 was 870±21 / µm². The number of molecules found in a PH domain cluster without the expression of M1 was 74±4, which increased to 102±9 with co-expression of M1. While no significant difference was observed, the mean density of a PH domain cluster with and without of M1, and the number of molecules in a cluster increased significantly (p<0.05 by two-tailed Mann Whitney test).
Figure 4.6: M1 enhances PIP2 clustering. Cells expressing PH-Dendra2 with and without M1-PAmKate were fixed using 4% PFA at room temperature. Two-color FPALM imaging of the plasma membrane of the cells in TIRF illumination was carried out. Cluster properties (A) Mean of a PH-domain cluster (B) Mean Perimeter of a PH-domain cluster (C) Mean Density of a PH-domain cluster and (D) Mean number of molecules forming a PH-domain cluster were quantified using SLCA and compared with and without of M1. Values presented are mean ± SEM for a total of 29 cells expressing PH domain only (without M1), 36 cells expressing PH domain and M1, pooled from three independent experiments. Statistically significant results are represented by * when p<0.05, as determined by an ordinary Mann-Whitney test.
4.4.6. CPC disrupts the colocalization of HA and M1 expressed together in NIH3T3 cells

We wanted to study the effect of CPC in HA and M1 when expressed together, as it has been previously suggested about the possible interaction of HA and M1 (Enami and Enami, 1996; Ali et al., 2000; Barman et al., 2001). For this, two-color super-resolution microscopy was used to study the co-expression of HA-Dendra2 and M1-PAmKate at the plasma membrane of fixed NIH3T3 cells using TIRF illumination. To test the effect of CPC on HA and M1, we quantified the MCC as a function of CPC treatment. On quantification, a relatively high colocalization of HA with M1 was observed, compared to that of M1 with HA: a colocalization coefficient of 0.79±0.02 was observed for HA with M1, while a correlation value of only 0.44±0.03 was observed for M1 with HA. A similar trend was seen with the CPC treatment, but the coefficient values decreased significantly (p<0.0001) by ordinary one-way ANOVA followed by Dunnett’s multiple comparison test against the control. The HA colocalization coefficient with M1 decreased approximately by 15% and 20% to 0.67±0.03 and 0.63±0.03 on CPC treatment with 5µM and 10µM, respectively. Similarly, the M1 colocalization coefficient values decreased approximately by 34% and 39% to 0.29±0.02 and 0.27±0.02 on treatment by CPC of concentrations of 5µM and 10µM, respectively.
Figure 4.7: Super-resolution image showing the colocalization of HA and M1. NIH3T3 cells transfected with influenza A HA and M1 were treated with the control (0µM CPC + Tyrodes-BSA vehicle), 5µM CPC, or 10µM CPC and incubated at 37°C for an hour, then chemically fixed using PFA (4%) at room temperature. The lower plasma membrane of the cells was imaged with FPALM using TIRF illumination. HA-Dendra2 is rendered in green and M1-PAmKate in magenta. Red triangles with the yellow outline point to the regions of colocalizations (white). Cells treated with the (A) control show a higher degree of colocalization compared to (B) 5µM CPC and (C) 10µM CPC treated cells.
Figure 4.8: CPC reduces the Manders’ Colocalization Coefficient (MCC) of HA and M1 in NIH3T3 cells. NIH3T3 cells transfected with influenza A HA-Dendra2 and M1-PAmKate were treated with the control (0µM CPC+ Tyrodes-BSA vehicle), 5µM CPC, and 10µM CPC and incubated at 37°C for an hour and chemically fixed by PFA (4%). Two-color FPALM microscopy using TIRF illumination was carried out to image the lower plasma membrane of the cells (adjacent to the coverslip). Localizations were separated into two color channels according to their α values. After this, separated coordinates were binned in a grid of pixels of dimension 80nmX80nm, overlaid with the imaged region. The MCC was quantified and plotted as a function of CPC treatment. Both the colocalization coefficients (A) MCC of HA-Dendra2 and (B) MCC of M1-PAmKate reduced significantly with CPC treatment. Values presented are mean ± SEM for a total of 33 cells for control, 29 cells for 5µM, and 32 cells for the 10µM CPC treated cells, pooled from three independent experiments. Statistically significant results are represented by ****p<0.0001 determined by ordinary one-way ANOVA.
4.4.7. CPC disrupts HA clusters in NIH3T3 cells expressed together with M1

We have previously shown that CPC disrupts HA clustering and explained a mechanism for the disruption. Here we wanted to study the effect of CPC on HA and M1 when expressed together and if CPC affected the assembly of HA and M1. In order to study the effect of CPC on the co-expression of HA and M1, we used FPALM with TIRF excitation to image HA-Dendra2 and M1-PAmKate in the plasma membrane of fixed NIH3T3 mouse fibroblast cells. We quantified the HA clusters using SLCA (see methods). Quantification of HA clusters showed the mean cluster area (under normal conditions) was found to be 0.040±0.005 µm² but was lowered to 0.031±0.003 µm² for 5µM CPC treated cells and 0.028±0.003 µm² for 10µM CPC treated cells. Although this decrement was approximately by 25% and 30 % for the cells treated with the CPC of concentrations 5µM and 10 µM CPC, respectively, no significant difference was observed with an ordinary one-way ANOVA and when followed by Dunnett’s multiple comparison test against the control. The mean perimeter of an HA cluster decreased from 1.04±0.10 µm for the control to 0.87±0.07 µm and 0.81±0.07 µm for 5µM and 10µM CPC treated cells, respectively, and this decrement was significant (p<0.05) by ordinary one-way ANOVA but when followed by Dunnett’s multiple comparison test against the control only 5µM CPC treatment was significant (p<0.05). Mean HA cluster density also decreased from 4700±1100/µm² for the control to 2400±400/µm² and 1950±180/µm² for 5 µM and 10 µM CPC treated cells. This decrement in HA cluster density is significant (p<0.05) by ordinary one-way ANOVA but when followed by the Dunnett’s multiple comparison test against the control only 10µM CPC treatment was significant (p<0.05). A similar result was obtained for the number of molecules forming an HA cluster. On average, about 94±16 HA molecules formed an HA cluster for the control; only 53±7 and 42±3 HA molecules were found in a HA cluster in cells treated with 5µM and 10µM CPC, respectively. This reduced number of HA molecules in a HA cluster on CPC treatment was significant(p<0.01) by ordinary one-way ANOVA followed by Dunnett’s multiple comparison test against the control.
Figure 4.9: CPC modulates HA clustering in NIH3T3 cells. NIH3T3 cells transfected with influenza A HA and M1 were treated with the control (0µM CPC+ Tyrodes-BSA vehicle), 5µM CPC, and 10µM CPC and incubated at 37°C for an hour. After an hour cells were chemically fixed by PFA (4%) at room temperature. FPALM images of the plasma membrane of the NIH3T3 cells expressing HA-Dendra2 and M1-PAmKate were taken with TIRF illumination. After the separation of the colors, localizations were processed through SLCA for cluster identification. Cluster properties (A) Mean Area of a HA cluster (B) Mean Perimeter of a HA cluster (C) Mean Density of a HA cluster and (D) Mean number of molecules forming a cluster were quantified and plotted as a function of CPC treatment. Values presented are mean ± SEM for a total of 34 cells for control, 29 cells for 5µM, and 27 cells for the 10µM CPC treated cells, combined from three independent experiments. Statistically significant results are represented by * p<0.05 and **p<0.01 determined by ordinary one-way ANOVA.
4.4.8. CPC disrupts M1 clusters in NIH3T3 cells expressed together with HA

We also studied the effect of CPC on M1 clusters when together with HA, expressing HA-Dendra2 and M1-PAmKate together in NIH3T3 mouse fibroblast cells and treating with control (0µM CPC), 5µM and 10µM CPC for an hour at 37°C, followed by PFA fixation at room temperature. We used FPALM with TIRF illumination to image the plasma membrane of the fixed cells. M1 clusters and HA were quantified using SLCA. On quantification we observed that the M1 clusters had a mean area of 0.128±0.011µm² for the control, 0.083±0.007µm² and 0.069±0.005µm² for 5µM and 10µM CPC treated cells, respectively. The decrement observed was approximately by 35 % and 46 % with 5µM and 10µM CPC treatment, respectively, and was significant by ordinary one-way ANOVA followed by Dunnett’s multiple comparison test against the control. A similar result was obtained for the perimeter of an M1 cluster. The mean perimeter of an M1 cluster was found to be significantly (p<0.0001) decreased from 2.53±0.14µm (control) by about 25% to 1.89±0.10µm for 5µM and by about 35% to 1.67±0.08µm for 10µM CPC treated cells when tested by ordinary one-way ANOVA followed by Dunnett’s multiple comparison test against the control. The mean density of the M1 cluster was observed to increase slightly with CPC treatment, but no significant difference was observed by ordinary one-way ANOVA followed by Dunnett’s multiple comparison test against the control. The mean density of M1 clusters for the control, 5µM CPC, and 10µM CPC, were observed to be 873±15 /µm², 888±15/µm² for and 917±23/µm², respectively. While no significant difference was observed in the density, the mean number of M1 molecules forming a cluster was found to decrease significantly (p<0.0001) by ordinary one-way ANOVA followed by Dunnett’s multiple comparison test against the control. On average, about 127±14 molecules were found in the cells treated with the control media, which decreased by about 40% and 51% to 75±7 M1 molecules and 62±5 M1 molecules with the CPC treatment of concentrations 5µM and 10µM, respectively.
Figure 4.10: CPC modulates M1 clustering in NIH3T3 cells. NIH3T3 cells transfected with influenza A HA and M1 were treated with the control (0µM CPC+ Tyrodes-BSA vehicle), 5µM CPC, or 10µM CPC and incubated at 37°C for an hour and chemically fixed by PFA (4%) at room temperature. Two-color FPALM imaging of the plasma membrane of the fixed NIH3T3 cells expressing HA-Dendra2 and M1-PAmKate was carried out under TIRF illumination. Localizations were assigned to one of the two-color channels according to their α values, then further processed through SLCA for cluster identification. Cluster properties (A) Mean Area of a HA cluster (B) Mean Perimeter of a HA cluster (C) Mean Density of a HA cluster and (D) Mean number of molecules forming a cluster were quantified and plotted as a function of CPC treatment. Values presented are mean ± SEM for a total of 34 cells for control, 29 cells for 5µM, and 32 cells for the 10µM CPC treated cells, combined from three independent experiments. Statistically significant results are represented by **** p<0.0001 determined by ordinary one-way ANOVA.
4.4.9. CPC disrupts the co-clustering of HA and M1 in NIH3T3 cells

We identified the co-clusters of HA and M1 (see method) and quantified the co-clustering of HA and M1 as a function of CPC treatment. The co-clustering of HA-Dendra2 and M1-PAmKate with each other was observed to be significantly disrupted. The mean pixel sum of the co-clustering of clusters of HA clusters within the M1 cluster was reduced by approximately 80% and 91% with 5µM and 10µM CPC treatment, respectively, when compared to the control. This result was significant by ordinary one-way ANOVA. With the post-Dunnett’s test when compared against the control, 5µM CPC treatment was not significant (p=0.66) but with 10µM CPC treatment was significant (p<0.05). The mean pixel sum of M1 clusters together with HA clusters was also observed to be reduced by about 75% and 89% on treatment with 5µM and 10µM CPC, respectively, compared to control. This decrease in the co-clustering of M1 with HA was significant (p<0.01) by ordinary one-way ANOVA followed by post-Dunnett’s multiple comparison test against the control.
Figure 4.11: CPC reduces the co-clustering of HA and M1 in NIH3T3 cells. NIH3T3 cells transfected with influenza A HA and M1 were treated with the control (0µM CPC + Tyrodes-BSA vehicle), 5µM CPC, and 10µM CPC and incubated at 37°C for an hour and chemically fixed by PFA (4%) at room temperature. Localizations from the two-color FPALM imaging of the plasma membrane of the fixed cells expressing HA-Dendra2 and M1-PAmKate were assigned to either channel according to their alpha value. Assigned localizations were binned in pixels of dimensions 80nm×80nm. Pixels were identified that had at least 5 localizations of both the species hence the co-clustering (see methods). All the pixels corresponding to the HA channel that co-clustered with M1 were summed over and averaged over all the cells. Similarly, pixels corresponding to the M1 channel that co-clustered with HA were summed over and averaged over all the cells. (A) Mean pixel sum of HA channel that contained clusters of HA and M1 and (B) Mean pixel sum of M1 channel that contained clusters of M1 and HA were quantified and plotted as a function of CPC treatment. Values presented are mean ± SEM for a total of 33 cells for control, 28 cells for 5µM, and 30 cells for the 10µM CPC treated cells from three independent experiments. Statistically significant results are represented by *p<0.05 and **p<0.01 determined by ordinary one-way ANOVA.
4.4.10. HA does not modulate M1 association with the membrane

It is generally thought that the nature of the interaction between M1 and the lipid membrane is electrostatic. However, studies show conflicting results about the role of viral glycoproteins in the membrane association of M1. In one study, M1 association was affected by the viral glycoproteins HA and NA (Ali et al., 2000) while other studies showed co-expression with the viral glycoproteins did not alter the membrane association of M1 significantly (Kretzschmar, Bui and Rose, 1996; Zhang and Lamb, 1996). In order to explore this controversy, we compared the number of localized M1 molecules using super-resolution FPALM with TIRF illumination. The number of M1 localizations per cell without HA (but expressed with PH domain) was compared with the number of M1 localizations which was expressed together with HA. Although the mean number of localizations was slightly greater for the M1 expressed without HA, no significant difference was observed by either one- or two-tailed Mann-Whitney test.

![Figure 4.12](image)

**Figure 4.12:** HA does not modulate M1 association with the membrane. The mean number of M1 localizations in fixed NIH3T3 cells expressing M1 with and without HA was calculated and compared. Values presented are mean ± SD for a total of 34 cells without HA, and 33 cells with HA, from three independent experiments. No statistical significance was observed by the one-tailed Mann-Whitney test.
4.4.11. HA enhances M1 clustering at the plasma membrane of NIH3T3 cells

M1 multimerizes after binding to the lipid bilayer (Hilsch et al., 2014). This multimerization may have important implications in the life cycle of the influenza A virus as M1 can induce lipid deformation after multimerization (Dahmani, Ludwig and Chiantia, 2019) and can also form VLPs on its own by causing outward membrane protrusions (Gómez-Puertas et al., 2000). The role of viral glycoprotein(s) in M1 clustering at the plasma membrane has not been fully explored. We compared the M1 cluster properties when expressed without HA (but expressed along with PH domain) and with HA. When expressed together with HA, the mean M1 cluster area, perimeter, and the number of M1 molecules forming a cluster increased significantly (one-tailed Mann-Whitney test) when compared to the respective M1 cluster properties expressed without HA. The mean density of the M1 cluster expressed without HA was slightly greater compared to the mean density of the M1 cluster expressed with HA but was not observed to be significantly different by the one-tailed Mann-Whitney test. These results show a role for HA in enhancing the M1 clustering at the plasma membrane.
**Figure 4.13:** HA enhances the M1 clustering in NIH3T3 cells. Cluster properties (A) Mean Area of a M1 cluster (B) Mean Perimeter of a M1 cluster (C) Mean Density of a M1 cluster and (D) Mean number of a M1 molecules forming a cluster was compared with and without of HA. Values presented are mean ± SEM for a total of 33 cells without HA, 33 cells with HA from three independent experiments. Statistically significant results are represented by * p<0.05 determined by the one-tailed Mann-Whitney test.
4.5. Discussion

Influenza A Matrix Protein (M1) is the most abundant viral protein and has been previously shown to have lipid-binding domains (Gregoriades, 1980; Gregoriades and Frangione, 1981; Ye et al., 1987). M1 in the cytoplasm binds to the plasma membrane primarily due to electrostatic interactions (Ruigrok et al., 2000; Shilova et al., 2017) suggesting it can interact with various negatively-charged (anionic) lipids (Thaa, Herrmann and Veit, 2009) and also in the absence of any other viral protein. Moreover, M1 is twice more likely to be adsorbed onto surfaces containing negatively charged phospholipids than onto surfaces with a neutral charge (Shishkov et al., 2009). PIP2, which is the most abundant phosphoinositide (Xu, Watras and Loew, 2003; Hammond and Balla, 2015), has a net charge of -3 at neutral pH (McLaughlin et al., 2002).

Our earlier studies (see Chapter 3) showed that CPC at non-cytotoxic concentrations disrupted the membrane organization of PIP2-binding proteins, causing MARCKS and PH domain proteins to fall off the plasma membrane, presumably through disruption of charge-charge interactions. Thus, we wanted to test if Influenza A matrix protein is associated with PIP2 and if CPC could disrupt this association. After this, we also tested if CPC disrupted the assembly of HA and M1 using super-resolution FPALM microscopy with TIRF illumination.

4.5.1. CPC disrupts M1 and PIP2 colocalization, clustering, and co-clustering.

In order to test the association of Influenza A Matrix Protein M1 and PIP2, we labeled PIP2 with Dendra2-PH (PLC-δ), which is an excellent marker of PIP2 (Kavran et al., 1998; Flesch et al., 2005; Szentpetery et al., 2009; Ji et al., 2015) and co-transfected M1-PAmKate. The PH domain of PLC-δ binds to PIP2 with relatively high specificity, and therefore can be used as a marker of the spatial distribution of PIP2; the PH domain from PLC-δ has been extensively used in previous studies from other groups (Yagisawa et al., 1994; Lemmon et al., 1995; Kavran et al., 1998). Dendra2-PH has been used in our previous study to label PIP2 (Curthoys et al., 2019). PAmKate is a known super-resolution probe derived from mKate (Shcherbo et al., 2007) and is spectrally well-separated with the Dendra2 which makes it
compatible with two-color super-resolution microscopy with acceptably low bleed through between the probes (Gunewardene et al., 2011; Curthoys et al., 2019). PAmKate was cloned to be expressed at the C-terminal of the M1. M1 has been shown to interact with the lipid head groups electrostatically through the positive charges in the N-terminal fragment (Arzt et al., 2001; Ruigrok et al., 2001). Some of the functions of M1 were impaired when a fluorescent protein was cloned for expression at the N-terminal of the M1 (T et al., 2018), suggesting that the C-terminal location of M1 we used could avoid some of those problems. Fluorophores like YFP and mCardinal, whose sizes are similar to PAmKate, were successfully cloned with M1 in previous studies (Hilsch et al., 2014; Bobone et al., 2017). Our results show the regions of colocalization (Figure 1) between M1 and PH domain and upon quantitative analysis, and we found that CPC treatment reduced the MCC significantly (Figure 2). This result indicates that CPC disrupts the association of PIP2 and M1, possibly by interfering with the electrostatic interactions with which M1 binds to PIP2.

PIP2 is a minor lipid component and yet is responsible for a large number of cellular functions (McLaughlin et al., 2002; Catimel et al., 2008; Balla, 2013). PIP2 has been previously shown to cluster at the plasma membrane (Van Den Bogaart et al., 2011; Wang and Richards, 2012; Curthoys et al., 2019). PIP2 binding domains in proteins typically interact with PIP2 through electrostatic interactions and hydrogen bonds and can bind to negatively charged PIP2 and PIP3 (Won et al., 2006). Our results show CPC significantly disrupts the clustering of the PH domain in the plasma membrane (Figure 3), consistent with our previous result confirming the effect of CPC on PIP2-binding proteins. Our reported PH domain cluster area (control) is in good agreement with our previously reported PH domain cluster area of the control-treated with CPC (Chapter 3). The density of the PH cluster and the number of molecules forming a PH domain cluster of the control is less than the previously reported (Chapter 3). This could be because M1 binds to the anionic lipids including PIP2 which decreases the amount of free PIP2 for PH domain to bind. Also, in our previous study we didn’t observe CPC significantly decreasing the area and the perimeter
of the PH cluster which we observed in this study. In our previous study PH proteins were expressed along with HA due to which there is more free PIP2 available for PH proteins to bind so less of an effect of CPC was observed as compared to this study.

Influenza A Matrix Protein (M1) multimerizes on binding to a lipid bilayer (Hilsch et al., 2014). This higher-order clustering of M1 may have an important role in the viral life cycle, as M1 plays a critical role in virion assembly, and this clustering is thought to induce membrane curvature (Dahmani, Ludwig and Chiantia, 2019). Also, it has been previously shown that M1 can produce VLPs on its own (Gómez-Puertas et al., 2000) and there is very little quantitative knowledge about the clustering of M1. Hence we studied and quantified the cluster properties of M1 at the plasma membrane and the effect of CPC on M1 clustering. While three of the cluster properties (area, perimeter, and the number of molecules forming a cluster) were disrupted by CPC, the density remained unaffected (Figure 4). Since the CPC is expected to inhibit the binding of M1 to anionic lipids, it is understandable that M1 clusters are getting smaller. However, not all M1 molecules are being disrupted from binding to the membrane. This could be due to the ability for M1 to self-assemble into clusters once it binds to other lipid components of the membrane (Harris et al., 2001; Hilsch et al., 2014), or due to some remaining anionic lipids which are not neutralized by the CPC. Note the distance threshold \(d_{\text{max}}\) puts a lower limit on the density \(D\) of a cluster, i.e. \(D_{\text{min}} = 1/(d_{\text{max}}^2)\). In general, it is difficult to achieve \(D < D_{\text{min}}\), because at such lower densities, the molecules will be farther apart than \(d_{\text{max}}\). So if this happens, the area of the cluster may decrease to include only those regions with a density larger than the minimum. If the cluster has regions with different densities, the higher-density regions may remain detected by the algorithm, but the others will be lost. However, if the cluster is a uniform density, then it will either be detected if its density is above the minimum detectable density, or it will be missed altogether if its density is lower than the minimum.

In addition to cell-wide “global” cluster quantification, we were also interested in whether CPC disrupted the co-clustering of PIP2 and M1 locally. In order to quantify the local effect of CPC on PIP2 and
M1, we quantified co-clustering of PIP2 and M1 using the Mean Pixel Sum, observing that PIP2 clusters co-clustering (measured by PH domain clustering) with M1 (Figure 4.5A) and M1 clusters co-clustering with PIP2 (measured by PH domain clustering) decreased significantly with the CPC treatment (Figure 4.5B). These results suggest that CPC disrupts PIP2 clustering and in disrupting PIP2 clusters, M1 clusters in turn, get disrupted thus further suggesting possible interaction of PIP2 and M1.

4.5.2. Influenza A M1 enhances the PIP2 clustering

Our previous results of CPC disrupting colocalization and co-clustering of M1 and PIP2 in this study suggested a possible interaction between PIP2 and M1. We decided to further explore this possibility by quantifying cluster properties of PIP2 expressed with and without of M1. We observed that in the presence of M1, PIP2 cluster area and perimeter significantly increased and as more PIP2 molecules were incorporated into the PIP2 cluster, while no change in the density was observed (Figure 4.6). This enhancement of the PIP2 clusters by M1 further suggests a possible interaction between PIP2 and M1.

4.5.3. CPC disrupts the assembly of HA and M1

It has been previously suggested that Influenza A Matrix Protein M1 interacts with the CTD of HA which might be crucial for the viral life cycle (Enami and Enami, 1996; Ali et al., 2000; Barman et al., 2001). The quantitative study of the distributions of the viral proteins NA with HA and M2 with HA have been previously studied but lacks the information on M1 and the effect of HA in M1 clustering (Leser and Lamb, 2005). Our previous studies have shown that Influenza A Hemagglutinin (HA) clustering is modulated by PIP2 clustering (Curthoys et al., 2019) and the co-clustering of HA and PIP2 is reduced by CPC (Figure 4.11). With these in mind, we decided to study the effect of CPC in the HA and M1 assembly mediated by PIP2. Super-resolution images showed the reduced regions of colocalization HA and M1 with CPC treatment (Figure 4.7). After this, we quantified the colocalization of HA and M1 using MCC and plotted as a function of CPC treatment. We observed CPC significantly reduced the MCC (Figure 4.8).
We also investigated the effect of CPC on the clustering of HA. Our results show significant disruption of perimeter, density, and number of HA molecules forming a cluster by CPC, consistent with our previous results (Chapter 3), while the decrease in the area of an HA cluster was not observed to be significant (Figure 4.9). The reported HA cluster density range is well within the range of previously published work (Gudheti et al., 2013).

In addition to the HA clusters, we also quantified the M1 cluster properties as a function of a CPC treatment (Figure 4.10). While the cluster properties like area (Figure 4.10A), perimeter (Figure 4.10B) and the number of M1 molecules forming a cluster (Figure 4.10D) were significantly disrupted by CPC, the density of M1 clusters (Figure 4.10C) remained fairly similar and no significant difference was observed again. We did not see any significant difference in the number of localizations of M1 expressed with and without HA (Figure 4.12) at the plasma membrane of the fixed cells, which rules out the possible role of HA in the membrane association of M1. However, a significant difference was observed in the M1 cluster properties when M1 was expressed with and without HA: in the presence of HA, M1 cluster area, perimeter and the number of M1 molecules forming a cluster all increased significantly, while no significant difference was observed with the density of an M1 cluster (Figure 4.13). These results indicate a possible role of HA in the incorporation of M1 in a budding virion by incorporation of more M1 into plasma membrane clusters.

To observe the local effect of CPC in the assembly of HA and M1, we quantified the co-clustering of HA and M1. We observed the mean pixel sum of HA clusters co-clustering with the M1 clusters (Figure 4.11A) and the mean pixel sum of M1 clusters co-clustering with clusters of HA was significantly disrupted with the CPC treatment (Figure 4.11B). This confirms the modulation of HA leads to the modulation of M1 and vice-versa.

CPC consists of a positively charged head group with a long hydrophobic tail. It destroys the membrane integrity to gain its antibacterial and antiviral properties at concentrations in the range of
millimolar (Haps et al., 2008; Hwang et al., 2013; Popkin et al., 2017). We previously showed that CPC is non-cytotoxic at concentrations in the range of micromolar and displaces some PIP2-binding proteins from the plasma membrane, presumably by disrupting electrostatic interactions. In our previous study, we also showed CPC disrupts PH clustering, HA clustering, and their co-clustering. Building on these studies, we investigated more into the mechanism of the antiviral properties of CPC at non-cytotoxic concentrations. In this study, we extend our hypothesis and establish a mechanism for the anti-influenza properties of CPC by disruption of the assembly of the viral proteins and their membrane clustering. We also show an effect of HA on clusters of M1 in the plasma membrane and rule out an effect of HA on M1 membrane association.

4.6. Summary

M1 is the most abundant viral protein present underneath the viral lipid bilayer. M1 can interact with the lipid bilayer and can form VLPs even in the absence of other viral proteins (Gómez-Puertas et al., 2000). M1 interaction with the lipid bilayer is considered to be primarily electrostatic interaction (Gregoriades, 1980; Ito et al., 1991; Zhang and Lamb, 1996; Ruigrok et al., 2000) and can M1 can bind lipid bilayer by multiple residues (Thaa, Herrmann and Veit, 2009). M1 is known to interact with PS in the cell model and in the cells (Bobone et al., 2017) however M1 interaction with other lipid is not well studied. If the M1 interaction with the lipid bilayer is primarily electrostatic and can interact with the lipid bilayer by multiple residues, M1 interaction with the other lipids that are more negatively charged than PS is very viable. We considered testing this hypothesis and we saw M1 colocalized with the PH domain (PLC-δ) at the nanoscale. PH domain (PLC-δ) is an excellent marker of free PIP2. After this, we tested if this interaction could be disrupted by CPC. CPC is a positively charged quaternary ammonium compound that can disrupt the PIP2 binding proteins (Chapter 3). We observed CPC significantly disrupted the colocalization (Figure 4.2), clustering (Figure 4.3 and 4.4), and the co-clustering of M1 and PIP2 (Figure 4.5). In order to further investigate the effect of M1 in PIP2, we analyzed the cluster properties of PIP2
with and without of M1. We observed that the PIP2 clustering was significantly enhanced in presence of M1 (Figure 4.6). This result further supports the notion that M1 interacts with the PIP2 at the plasma membrane.

After this, we tested if CPC disrupted the assembly of HA and M1 mediated by disrupting the PIP2. We observed CPC significantly disrupted the colocalization (Figure 4.8), clustering (Figure 4.9 and 4.10), and co-clustering of HA and M1 (Figure 4.11). CTs of HA and NA have been suspected in the interaction of M1 with the viral surface glycol proteins although the direct evidence of interaction is yet to be proven. Detergent resistant studies show contradicting results on membrane association of M1 by the viral surface glycoproteins (Kretzschmar, Bui and Rose, 1996; Ruigrok et al., 2001). We observed although HA didn’t stimulate the membrane association of M1, but significantly enhanced the clustering of M1. The enhancement of the M1 clustering by HA might have a significant impact on the viral life cycle as M1 can induce lipid deformation via multimerization (Dahmani, Ludwig and Chiantia, 2019).
CHAPTER 5
CONCLUSIONS AND FUTURE DIRECTIONS

5.1. Conclusions

Influenza A viral protein HA is responsible for attaching the virus to the host cell receptor. After binding it catalyzes the host cell membrane barrier for the viral entry. After the HAs are translated within the ER and trafficked through the Golgi apparatus of the cell, HA depends on actin comets for its delivery to the plasma membrane where it spontaneously clusters (Gudheti et al., 2013). HA clusters are found in a wide range of sizes (Hess et al., 2007; Curthoys et al., 2019). These clusters of HA are observed to be associated with actin-rich regions (Gudheti et al., 2013) however the exact mechanism of the spontaneous clustering of HA at the plasma membrane is not known yet. The actin comets that deliver to the HA at the apical membrane of the cells are mediated by PIP2 (Rozelle et al., 2000; Guerriero et al., 2006). Also, PIP2 plays a crucial role in regulating the actin cytoskeleton (Takenawa and Itoh, 2001). More recently HA was observed to colocalize with the PIP2 by both diffraction-limited and super-resolution microscopy and also the large HA clusters were observed in the regions of high levels of PIP2 (Curthoys et al., 2019). Also, the CT (tail) of a HA consists of two positively charged arginines, and thus a cluster of HA can create a highly basic region underneath the inner leaflet of the plasma membrane which could lead to potential interaction and modulation of the PIP2 clustering by the HA cluster. Hence this evidence led us to hypothesize that PIP2 is a possible nexus between actin and HA and that modulating PIP2 clusters can modulate HA clusters. Modulation of the HA cluster is important, and specifically the modulation of HA cluster density because dense HA clusters directly correlate with infectivity (Ellens et al., 1990) of the virus and also it is suspected that dense HA clusters may mark the budding (assembly) site (Nayak, Ka-Wai Hui and Barman, 2004). Hence if the clusters are modulated in such a way that the modulation decreases the density of HA clusters on average, it not only reduces the infectivity of the virus but also disrupts the
assembly of the virus, thus resulting in reduced budding (fewer progeny viruses) or budding of defective virions.

CPC is a positively charged and quaternary ammonium compound capable of destroying the cell membrane integrity (toxic) at high concentrations. However, there is less known about the effect of CPC in normal cell functions at lower concentrations, and about whether CPC is capable of binding negatively charged lipids without destroying the membrane integrity. Diffraction-limited images showed that the CPC displaced MARCKS a well-known PIP2 binding protein from the plasma membrane to the cytoplasm in RBL cells. On quantification, the ratio of plasma membrane fluorescence to cytoplasm fluorescence decreased significantly with the CPC treatment. Quantification of the clusters of PH domain another well-known PIP2 binding protein was disrupted in CPC treated NIH3T3 cells at the nanoscale. This result further corroborates our first result that CPC disrupts the PIP2 binding protein. HA, which is hypothesized to interact with PIP2, is also significantly disrupted with the CPC treatment. HA cluster density and the number of HA molecules forming a cluster were significantly disrupted by the CPC treatment in NIH3T3 cells. In order to further strengthen our hypothesis that modulation of PIP2 clusters led to modulation of HA clusters, we quantified the co-clustering of HA and PIP2 as a function of CPC treatment. Co-clustering of HA and PIP2 were significantly decreased by the CPC treatment of concentration 10μM.

M1 interaction with certain lipids can occur through electrostatic interactions (Arzt et al., 2001; Baudin et al., 2001). M1 can interact with negatively charged lipids through multiple regions (Thaa, Herrmann and Veit, 2009) and is twice likely to be adsorbed onto a negatively charged phospholipidic surface than onto a neutral surface (Shishkov et al., 2009). It has been shown that M1 can interact with the negatively charged lipid phosphatidylinerine (PS) in membrane models and in cell models (Hilsch et al., 2014; Bobone et al., 2017) however there is less information about M1 interactions with other negatively charged phospholipids that have net charge greater than PS. Curiously, we tested if M1 colocalized with the PH domain and the effect of CPC in their distribution. We observed that M1 colocalized with the PH
domain and the CPC significantly disrupted the Manders’ colocalization coefficient. Cluster analysis showed both M1 and PH clusters were significantly disrupted by the positively charged CPC. These results further encouraged us to explore the possible interaction between M1 and PIP2. After this, we quantified the cluster properties of PIP2 with and without M1. Results showed that in presence of M1, PIP2 cluster area, perimeter, and the number of PIP2 molecules forming a cluster significantly increased, while no significant difference was observed in other parameters. These results further hint at the possible interaction between M1 and PIP2.

It is thought that the cytoplasmic tail of HA and NA may serve as a docking site for M1 (Rossman and Lamb, 2011) and that either of the glycoproteins can recruit M1 (Ali et al., 2000; Zhang et al., 2000; Barman et al., 2001) to the assembly site. So we also tested if CPC affects the assembly of M1 with HA. We observed that CPC significantly reduced the colocalization of HA and M1. The colocalization was quantified using Manders’ Colocalization Coefficient. We further observed that CPC also significantly disrupted the clustering of HA and M1 and also the co-clustering of HA and M1 together. These results tell us that CPC is able to disrupt the clustering HA and M1 and as well as HA disruption, CPC led to M1 disruption and vice-versa, disrupting the assembly of HA and M1 mediated by PIP2.

Furthermore, we tested if CPC possesses antiviral properties at non-cytotoxic concentrations. For this, we used zebrafish as our animal model. We saw that CPC significantly rescued zebrafish that were infected with IAV and then administered CPC after 6 hpi (hours post-infection), 12hpi, and 24hpi compared to the zebrafish that were not treated with CPC. This confirms the antiviral property of CPC at non-cytotoxic concentrations (in the range of µM) as well.

**Future Directions**

In the search for new influenza drug targets, we began an investigation of quaternary ammonium compounds, hypothesizing that we could attack the interactions we previously discovered between HA and the phosphoinositide PIP2. We studied the effect of CPC on HA clustering, on HA-PIP2 co-clustering,
and on the assembly of HA and M1 in cell models. We also measured the effect of CPC on IAV-infected zebrafish (as an animal model). In future experiments, it would be worthwhile to extend the studies to test different cell lines, including the A549 and WHTBF-6 human lung cell lines, as well as other cell lines such as MDCK and Vero-E6 which are frequently used for IAV and SARS-CoV2 infections. It would be very interesting to study the effect of CPC on the HA clustering and assembly of HA and M1 in zebrafish; *in vivo* super-resolution imaging of zebrafish has been successfully conducted previously in our lab (Gabor *et al.*, 2015).

Because of the dependence of HA clusters on PIP2 clusters (Curthoys *et al.*, 2019), further study of the role of PIP2 kinase inhibitors on HA cluster properties would be one possible future experiment. PI4K and PI5K phosphorylate the fourth and fifth position of PI5P and PI4P to generate PIP2, so the study of PI4K and PI5K inhibitors would be follow-up experiments to know which path is more crucial to HA clustering. Perhaps other quaternary ammonium compounds with lipidic groups could also be investigated. Phenylarsine Oxide (PAO) and Quercetin have been previously shown to inhibit PI4K and PI5K (De Souza Santos *et al.*, 2013). PIP2 can also be synthesized from PIP3 by dephosphorylating the third position of the inositol ring of PIP3 by the actions of PTEN (Ball, 2013), hence studying this pathway could also give valuable information on the role of phosphatase and PIP2 in HA clustering. In addition, studying the PI3K/Akt pathway could also be an important experiment to be conducted in the future as PI3k/Akt pathway has been associated with different types of viruses including MERS and HIV (Kindrachuk *et al.*, 2015; Mohr *et al.*, 2015; Tian *et al.*, 2015; Wang *et al.*, 2017). To study the PI3k/Akt pathway, drugs like Wortmannin and LY294002 could be used, as these have been well known PI3K inhibitors (Walker *et al.*, 2000; De Souza Santos *et al.*, 2013) and was previously used to understand the role of PI3K pathway in the infection of the West Nile virus (Wang *et al.*, 2017). Wortmannin has been previously used to study the infectivity of the influenza A virus (Ehrhardt *et al.*, 2006; Ehrhardt, Wolff and Ludwig, 2007; Zhou *et al.*, 2009) however its role on the HA clustering at the plasma membrane has not been studied yet.
Similar experiments can also be conducted on M1, as we showed M1 colocalized with PIP2 at the nanoscale and in presence of M1. The polybasic region at helix 5 (Liu and Ye, 2004; Das et al., 2012; Kerviel et al., 2016), and helix 6 of the matrix protein are exposed towards the membrane, which can electrostatically interact with the PIP2 head. We also showed the presence of M1 enhanced PIP2 clustering, hinting further towards the possible interaction between PIP2 and M1. In order to further strengthen this result, we can add a positive control and a negative control as a future experiment. As proteins are able to recruit PIP2 (Hammond, 2016), two-color super-resolution experiments on the live cells expressing M1-PamKate and PH domain would be very valuable to understand the mechanism behind the enhancement of the PIP2 clusters by M1.


Shtykova, E. V. et al. (2017) ‘Influenza virus Matrix Protein M1 preserves its conformation with pH, changing multimerization state at the priming stage due to electrostatics’, *Scientific Reports*, 7(1), pp. 1–16. doi: 10.1038/s41598-017-16986-y.


A.1. Introduction

The COVID-19 pandemic has turned the lives of people upside down and has already resulted in the deaths of over 3.8 million people worldwide as of June 2021. Covid-19 pandemic which started in late 2019 is caused by the SARS-CoV-2 virus. SARS-CoV-2 belongs to the coronavirus family known as Coronaviridae (Haque et al., 2020). Although elderly and immunocompromised people are at the greatest risk from this virus, severe symptoms have resulted in persons of all ages. The SARS-CoV-2 virus genome is single-stranded positive sense RNA and contains four structural proteins Spike(S), Membrane Protein (M), Envelope Protein (E), and Nucleocapsid Protein (N), 16 non-structural proteins (nsp1-16) (Wang et al., 2020).

Although SARS-CoV-2 viruses are assembled inside the cell, not all translated S protein gets incorporated to form progeny virions. The S proteins that are not incorporated in the daughter viruses travel to the plasma membrane (Fehr and Perlman, 2015). The nanoscale distribution of the SARS-CoV-2 S protein at the plasma membrane has not been well studied, and such information could be crucial to reducing the infectivity of the virus because the virus can also infect neighboring healthy cells through fusion mediated by S protein (Tang et al., 2020; Hörnich et al., 2021). Moreover, S protein interactions with the lipids at the plasma membrane have hardly been studied. The cytoplasmic tail (CT) of S protein consists of several cysteines which are acylated and basic residues. These features in the CT of S proteins make the interaction between S protein and lipids highly possible.

In this study, we use super-resolution microscopy (FPALM) and TIRF illumination to study the nanoscale distribution of the S proteins at the plasma membrane of the cell. We also studied the colocalization of S protein with the PIP2. Further, we also studied the effect of CPC at the non-cytotoxic concentrations in clustering of S protein and PH domain (PLC-δ). We previously showed CPC at the non-cytotoxic concentrations displaces PIP2 binding proteins. Our results show the colocalization of PIP2 and S protein...
at the plasma membrane and CPC significantly disrupts the clustering of S-protein and PH domain (PLC-δ).

A.2. Methods

Please see the Methods Section in Chapter 3.

A.3. Results

A.3.1. SARS CoV2 Spike colocalizes with PIP2

NIH3T3 cells expressing SARS CoV2 Spike-Dendra2 and PH-PAmKate (PLC-δ) were fixed and two-color super-resolution FPALM imaging was carried out with TIRF illumination. We labeled the PH domain (PLC-δ) with PAmKate to allow visualization of the distribution of free PIP2. The PH domain from PLC-δ is an excellent way to label free PIP2 and has been used extensively (Won et al., 2006; Curthoys et al., 2019). We observed overexpressed SARS CoV2 Spike at the plasma membrane of the cell. We also observed that the SARS CoV2 Spike colocalized with PIP2 at the nanoscale at the plasma membrane.
Fig A1: SARS-CoV-2 S protein colocalizes with PIP2. Super-res images showing the colocalization of Spike-Dendra2 in green and PH-PAmKate in magenta. Yellow triangles with the red outline point towards the regions of colocalization. Scale bar=1 μm.
### A.3.2. CPC disrupts PIP2 clustering

Two-color FPALM imaging of the plasma membrane of the fixed NIH3T3 cells expressing Spike-Dendra2 and PH-PAmKate (PLC-δ) was carried out in TIRF illumination. Localizations were assigned to either channel according to their alpha value (Gunewardene et al., 2011). Identification and quantification of cluster properties were performed by using SLCA (Gudheti et al., 2013). On quantification, the mean density of the PH domain clusters and the mean number of PH domain molecules forming a PH domain cluster as a function of a CPC treatment were not significantly different from control by ordinary one-way ANOVA followed by Dunnett’s test against the control. The mean density of a PH domain cluster was 1440±70 /µm² for the control and 1400±30/µm² and 1440±30/µm² with 5µM and 10µM CPC treatment respectively. On average, 104±9 PH domain molecules were found in a PH domain cluster, which reduced to 89±5 and 85±6 molecules with 5µM and 10µM CPC treatment, respectively. Although the number of molecules forming a cluster reduced by approximately 14% and 18%, no significant difference was observed by ordinary one-way ANOVA followed by Dunnett’s multiple comparison test against the control. The mean area and the perimeter of the PH domain cluster were significantly (p<0.05) disrupted by CPC. The mean area of a PH domain cluster was observed to be 0.064±0.004 µm², which reduced to 0.056±0.002 µm² and 0.052±0.002 µm² with 5µM and 10µM CPC treatment, respectively. When followed by the post Dunnett’s test against the control only the 10µM CPC treatment was observed significant (p<0.05). The mean perimeter of the PH domain cluster was observed 1.25±0.05 µm for the control which reduced to 1.15±0.03 µm and 1.11±0.03 µm with 5µM and 10µM CPC treatment respectively. With the post Dunnett’s test against the control mean perimeter of a PH domain cluster for 10µM CPC treatment was significantly different (p<0.01).
Figure A2: CPC modulates the PH clustering in NIH3T3 cells. NIH3t3 cells expressing PH-PAmKate and Spike-Dendra2 were exposed to control media (0µM CPC+ Tyrodes-BSA vehicle), 5µM CPC and 10µM CPC and were incubated at 37°C for an hour and fixed at room temperature using 4% PFA. Plasma membrane of fixed cells expressing PH-PAmKate and Spike-Dendra2 were imaged using FPALM in TIRF illumination. Cluster Analysis was performed using SLCA. Cluster properties (A) Mean Density of a PH cluster (B) Mean number of PH molecules forming a PH cluster (C) Mean Area of a PH cluster and (D) Mean perimeter of a PH cluster were quantified and plotted as a function of CPC treatment. Values presented are mean ± SEM for a total of 33 cells for control, 31 cells for 5µM, and 30 cells for the 10µM CPC treated cells from three independent experiments. Statistically significant results are represented by *p<0.05 determined by ordinary one-way ANOVA.
A.3.3. CPC disrupts Spike Clustering

Two-color FPALM imaging of fixed NIH3T3 cells expressing Spike-Dendra2 and PH-PAmKate was carried out at the TIRF illumination. Localizations were processed for cluster identification and quantification after assigning each localization to either of the channels according to their alpha value (Gunewardene et al., 2011). On quantification, the mean density of the spike cluster was significantly (p<0.05) disrupted by CPC. Mean density of control was observed 2070±306 /µm^2 which decreased to 1367±34/µm^2 and 1561±115 /µm^2 with 5µM and 10µM CPC treatment respectively. When followed by Dunnett’s test only the 5 µM CPC treatment was significant (p<0.05). On average 98±13 spike molecules formed a spike cluster which reduced to 80±7 and 81±5 spike molecules with 5µM and 10µM CPC treatment respectively. Although the mean number of spike molecules forming a cluster decreased by approximately 18% no significant difference was observed by ordinary one-way ANOVA and post Dunnett’s multiple comparison test against the control. Both the mean area and the perimeter of the Spike cluster were not observed to be significantly different as a function of a CPC treatment. The mean area of a spike cluster for the control was observed 0.052±0.004 µm^2 which remained fairly the same as a function of CPC treatment. The mean area spike cluster for 5µM and 10µM CPC treatment was observed 0.052±0.003 µm^2 and 0.051±0.003 µm^2 respectively. Similarly, the mean perimeter of the spike cluster also remained fairly similar as a function of CPC treatment. The mean perimeter of the spike cluster for the control was observed 1.11±0.05 µm for the control, 1.11±0.04 µm for the 5µM and 1.08±0.05 µm for the 10 µM CPC treatment.
Figure A3: CPC modulates SARS-CoV-2 S protein clustering in NIH3T3 cells. NIH3t3 cells transfected with SARS CoV2 Spike-Dendra2 and PH-PAmKate were treated with the control (0µM CPC+ Tyrodes-BSA vehicle), 5µM CPC and 10µM CPC and incubated at 37°C for an hour. After an hour cells were chemically fixed by PFA (4%) at room temperature. FPALM images of the plasma membrane of the NIH3t3 cells expressing Spike-Dendra2 and PH-PAmKate were taken at the TIRF illumination. Localizations after separation of the color was processed through SLCA for cluster identification. Cluster properties (A) Mean Density of a Spike cluster (B) Mean number of Spike molecules forming a cluster (C) Mean Area of a Spike cluster and (D) Mean Perimeter of a Spike cluster were quantified and plotted as a function of CPC treatment. Values presented are mean ± SEM for a total of 33 cells for control, 31 cells for 5µM, and 30 cells for the 10µM CPC treated cells from three independent experiments. Statistically significant results are represented by * p<0.05 determined by ordinary one-way ANOVA.
A.4. Discussion

SARS CoV2 Spike is a trimeric protein responsible for attaching the virus to the host cell (Haque et al., 2020; V’kovski et al., 2021). S protein catalyzes the membrane barrier and infects the healthy cells with the SARS CoV2 virus genome (Tang et al., 2020). SARS CoV2 progeny virions are formed inside the cell and the viral proteins don’t travel to the plasma membrane for assembly like in the Influenza virus, however, S proteins that are not incorporated into the progeny travel to the plasma membrane (Haque et al., 2020) and can infect neighboring healthy cells by fusion (Hörnich et al., 2021; Wang et al., 2021). We observed some of the S protein at the plasma membrane (Figure A1); however, we also observed intracellular S protein as well (data not shown) which has been previously observed (Sadasivan, Singh and Sarma, 2017).

We also observed at the plasma membrane S protein colocalizes with the PIP2 which was labeled by labeling the PH domain (PLC-δ) (Figure A1). CT of S protein contains several cysteines and basic residues. These cysteines are acylated. Acylations have been previously reported for their role in membrane association and interaction with the lipid (Gambhir et al., 2004; McLaughlin and Murray, 2005; Won et al., 2006). So we hypothesize these acylations and basic residues in the CT of S protein are important for the membrane association and the colocalization with PIP2, and possibly other phosphoinositides.

The results of colocalization with PIP2 at the nanoscale suggested it would be interesting to test the effects of CPC on the clustering of the S protein. CPC is a positively charged quaternary ammonium compound that has been previously shown to possess antibacterial and antiviral properties (Hu et al., 2009; Popkin et al., 2017). We previously showed that CPC at non-cytotoxic concentration displaced the PIP2 binding protein MARCKS from the membrane to the cytoplasm, and altered their clustering (Fig 3.1 and 3.2), presumably by disrupting the electrostatic interaction between the PIP2-binding proteins and PIP2. Our results show that the CPC significantly disrupts PIP2 clustering (Figure A2), in line with our previous results (Figure 3.2). We also observed CPC significantly disrupted S protein clustering (Figure A3).
Phosphoinositides (PIs) have been previously shown to play role in SARS CoV2 infection (Kang et al., 2020) and PI kinase inhibitors have been shown to reduce the infection of SARS-CoV-2 and other viruses like Ebola and Zaire (Kang et al., 2020). Although the CPC is not a known PI kinase inhibitor, our results show that it can modulate PIP2 binding proteins. CPC treatments have already been in studies in relation to the SARS CoV2 infections (Green et al., 2020; Muñoz-Basagoiti et al., 2020; Seneviratne et al., 2021), in our study we show CPC at non-cytotoxic concentrations disrupts PIP2 binding protein and S protein clustering. Although our results are not sufficient to prove CPC reduces SARS-CoV-2 infection, it is certainly novel considering that S protein colocalized with PIP2 at the plasma membrane and the spike protein clustering can be modulated by CPC.
BIOGRAPHY OF THE AUTHOR

Prakash Raut was born in Biratnagar, Nepal on Dec 06, 1988. He was raised in Biratnagar, Nepal along with the two sisters. He attended St. Xavier’s college and graduated in 2009 with a Bachelor’s degree in Physics. He then attended University of Delhi for the Master’s in Physics program and graduated from the University of Delhi in 2012. After receiving his degree, Prakash returned to Nepal and started teaching in a high school. He then moved to Maine where he entered the graduate program in the Department of Physics and Astronomy at the University of Maine in the fall of 2015. Prakash is a candidate for the Doctor of Philosophy degree in Physics from the University of Maine in August 2021.