Membrane fusion of Influenza and chikungunya viruses

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1 Introduction
1.1 Enveloped viruses and disease

Many of the viruses causing disease in humans are enveloped with a lipid bilayer that helps to protect the viral genome and avoid immunogenic detection.\(^1\) Well-known diseases caused by enveloped viruses are measles, mumps and rubella, for which vaccination programs are in place in a large number of countries.\(^2\) For another enveloped virus, HIV-1, the causative agent of AIDS, no vaccine exists, but the availability of antiviral treatment makes it a chronic disease in the developed world.\(^3\) Recent years have seen outbreaks of other enveloped viruses, such as SARS-coronavirus, causing severe acute respiratory syndrome, in 2003, and Ebola starting in 2014.\(^4,5\)

Many developing countries are under continuous burden of disease caused by enveloped viruses. Two such examples are dengue virus and the recently globally spread chikungunya virus,\(^6\) which is one of the two subjects of this thesis. Developing antivirals to treat these infections and developing vaccines that counter all variants of these viruses is therefore paramount to improve health conditions in the developing world and prevent global pandemics. The successful eradication of smallpox and rinderpest viruses by large-scale vaccination efforts represents a hopeful signal in this respect.\(^7\) Finally, one of the best-studied viruses is influenza virus, giving rise to the flu. This virus is the cause of yearly epidemics and has the potential to cause a new pandemic.

1.2 Influenza virus

Influenza is a virus that infects humans but also other animals such as birds, pigs and bats which can act as reservoirs for new viruses to infect humans (Figure 1.1).\(^8,9\) It spreads through air or contaminated surfaces.\(^10\) Recent history has seen multiple outbreaks of influenza pandemics, the best known and most deadly in 1918 known as the Spanish flu, killing over 50 million people, about 5% of the world population at that time.\(^11\) There are several subtypes of influenza virus, designated by H and N combined with a number. An example is H1N1, where H stands for the hemagglutinin protein, and N for the neuraminidase protein. Multiple subtypes have caused pandemics in the past as overviewed in Figure 1.2. The H1N1 subtype was responsible for the 1918 pandemic. Furthermore, it led to a novel subtype that caused a pandemic in 2009.\(^9\) Two other strains that originated from birds, H5N1 and H7N9, have become highly pathogenic and have already caused many hospitalizations. There are fears that these will become pandemic due to their continued mutation in birds and ability to adapt to humans.\(^9\) In this thesis, we study an H1N1 and an H3N2 strain.
Figure 1.1 Mechanisms for the emergence of pandemic influenza virus strains. The virus keeps circulating among own species and sometimes jump the species barrier to generate a novel strain of pandemic potential. Reproduced with permission, copyright Springer Nature (2018).9

Figure 1.2 A time line of major influenza pandemics and the responsible influenza strains. Adapted with permission, copyright Springer Nature (2018).9

Subtypes form by antigenic drift, where random mutations lead to a change in antigenicity, and antigenic shift, where infection of a host by multiple influenza subtypes leads to a new subtype, a recombination of the two.12 This latter mechanism makes it possible for human-circulating strains and animal-circulating strains to combine if they happen to infect the same host, leading to a new subtype to which humans are immunogenically completely naive. The formation of such new, recombined strains is the major reason for influenza’s potential of causing a new pandemic.13

Antivirals targeting one of the proteins of influenza virus, the M1 proton channel, have been used as treatment for infection, but resistance quickly developed rendering them ineffective.14 Current antiviral treatments target the neuraminidase protein. Thereby, they counter the production of new virions, but also here resistance is emerging.14 However, to remove the threat
of a new pandemic, there remains the need for a universal flu vaccine that both is able to neu-
tralize all subtypes of influenza virus and targets sites in the virion that are not subject to much genetic variation. In this thesis we employ an antibody that targets a conserved region on an influenza protein; the protein that is responsible for a crucial step in the influenza infection pathway: fusion of the viral and host cellular membranes.

1.3 Chikungunya virus
Chikungunya virus causes high fever and potentially long-lasting symptoms like joint pain. It has recently greatly expanded its geographic range to encompass most of the subtropical regions of the globe (Figure 1.3). The virus is spread by the yellow fever mosquito (Aedes aegypti). A recent mutation has allowed the virus to use the tiger mosquito (Aedes albopictus) as host as well, which is an invasive species. This, together with climate change expanding the Ae. albopictus range, makes further expansion of chikungunya virus likely.

There is no vaccination or specific antiviral treatment available against chikungunya infection. Together with other viruses from the flaviviridae family, such as dengue and zika viruses, it constitutes a significant burden on healthcare in developing countries.

![Current or previous local transmission of chikungunya virus](image)

Figure 1.3 Countries and territories where chikungunya cases have been reported as of April 22, 2016. Source: Centers for Disease Control and Prevention; public domain.

1.4 Cellular entry by enveloped viruses
This thesis will discuss studies on two enveloped viruses: influenza virus and chikungunya virus. Even though the structures of these two viruses are quite distinct, the working mechanisms in cell entry are very similar. I start by discussing the structure of each virus and then proceed to explain the role of membrane fusion in enveloped viral entry.
1.4.1 Virion structure

Both influenza and chikungunya viruses have a lipid bilayer envelope that encapsulates their genome and contains proteins that mediate entry into the host cell. A schematic of the structure of an influenza virion is shown in Figure 1.4. The hemagglutinin protein (Figure 1.4, blue) mediates both attachment to a target cell and entry into that cell, the last step by catalyzing the fusion of the viral and host cellular membranes. Neuraminidase (Figure 1.4, red) enzymatically releases newly produced virions from the cell. The M2 proton channel (Figure 1.4, purple) allows protons to enter the virus to allow the M1 protein coat (Figure 1.4, maroon) to disintegrate upon entry, releasing the viral genome (Figure 1.4, green). Influenza viral particles are heterogeneous in size (average approximately 100 nm) and can be round or filamentous in shape.

![Figure 1.4 Schematic view of an influenza virus particle. Main constituents: hemagglutinin (blue); neuraminidase (red); the M2 ion channel (purple); the viral ribonucleoprotein (RNP), which contains the RNA (green); the lipid bilayer (light brown) and the M1 protein coat (maroon). Source: Centers for Disease Control and Prevention; public domain.](image)

In contrast, chikungunya virus particles all have the same size, 70 nm in diameter, and have protein heterodimers covering the surface in an icosahedral fashion with triangulation $T = 4$, giving 80 spikes, which each are a trimer of heterodimers, corresponding to a total of 240 heterodimers.

The surface layout is shown in Figure 1.5. Protein E1 (Figure 1.5, grey), responsible for cell entry by mediating virus-cell membrane fusion, is covered by its companion protein, E2 that
mediates cell attachment. The E2 proteins are colored red, blue, green and yellow. An asymmetric unit (Figure 1.5, triangle) comprises one E2 of each color together with its E1 companion and the 5-fold, 3-fold and pseudo-2-fold symmetry axes are indicated. As such, the virion surface is divided up into hexagons and pentagons of spikes.

![Figure 1.5 Chikungunya viral particle structure.](image)

**Figure 1.5 Chikungunya viral particle structure.** The cryo-EM density of Sindbis virus (a related alphavirus) showing \( T = 4 \) symmetry. The four E2 molecules in one asymmetric unit (outlined in black) are colored red, green, blue and yellow. The 5-fold, 3-fold and pseudo-2-fold symmetry axes are indicated. These give rise to one trimeric spike on each icosahedral 3-fold axis and one generally positioned spike. The E1 molecules are colored grey. Adapted with permission, copyright Springer Nature (2010).

### 1.4.2 Attachment and endocytosis

Entry of the virus into the host cell begins with attachment of the virus to host cell receptors that differ between different cell types and are specific for every virus. For influenza, the hemagglutinin protein binds to sialic-acid moieties on the cell surface. The chikungunya cell receptors are unknown, but several candidates have been identified, so there may be multiple receptors involved. After attachment, some viruses fuse at the cell membrane envelope and some are first taken up into an endosome before fusion. Since the latter mechanism is the pathway for influenza as well as chikungunya entry, we will focus here on entry through endocytosis.

The sequence of events in endosomal entry of chikungunya virus is shown in **Figure 1.6**, a process similar to that for influenza virus. The virus attaches to the host cell receptors (**Figure 1.6**, step 1), which triggers signaling pathways leading to the cell taking up the virus by clathrin-mediated endocytosis. Here, clathrin molecules stimulate the formation of a vesicle (**Figure 1.6**, step 2). The virus then resides in an endosome that is gradually acidified by proton...
pumps in order to digest the contents of the endosome (Figure 1.6, steps 3 and 4). Many viruses, including influenza and chikungunya, have evolved to use the acidification as a trigger to fuse the viral and endosomal membranes so that the viral genome is delivered into the cell cytoplasm (Figure 1.6, step 4). This fusion step is mediated by proteins, for influenza the hemagglutinin and chikungunya the E1, and these undergo large conformational rearrangements to insert into the target membrane and then catalyze the merger of the membranes.

Figure 1.6 Viral entry via the endosome. (1) Viruses attach to the cell through receptors and are internalized through different pathways depending on the virus. (2) Clathrin-mediated endocytosis is shown here as receptor-mediated uptake pathway, leaving the virus in an endosomal compartment. (3) The endosome gradually is acidified, triggering the virus to fuse to the endosome and (4) releasing the viral genome. Adapted from Richter et al.27 under license CC BY 4.0.

1.4.3 Membrane fusion
Fusion of two lipid membranes is impeded by kinetic barriers, making spontaneous membrane fusion too slow for biological timescales.28 As with many biochemical reactions that are sped...
up by enzymatic activity, viruses use catalysts to mediate membrane fusion under the right conditions.

The protein-mediated membrane fusion pathway of chikungunya virus is shown in Figure 1.7. Under low pH conditions, a domain of the E2 protein moves to expose the E1 fusion loop (Figure 1.7b). The E1 protein then inserts its fusion loop into the target membrane and trimerizes to form the fusion-active unit (Figure 1.7c). Multiple trimers are then thought to refold together, dimpling and apposing both membranes (Figure 1.7d) so that they first merge their proximal leaflets, termed hemifusion (Figure 1.7e), and finally a full pore opens (Figure 1.7f).

The influenza fusion pathway is similar from panel c onwards, as the influenza hemagglutinin starts out as a trimer. Also, multiple hemagglutinins are thought to be involved in catalyzing fusion. The key strategy of enveloped viruses appears to be to use fusion proteins that surmount small energy barriers themselves in order to conquer the large barrier to membrane fusion.

We elaborate more on the barriers of membrane fusion, the influenza hemagglutinin structure and conformational changes, and the action of multiple hemagglutinins in Chapter 2. The experimental part of this thesis focuses on enveloped virus membrane fusion, as detailed below.
1.5 Motivation for in vitro single-particle assay

Ideally, membrane fusion of enveloped viruses is studied in their native environment, that of the live cell, so that the findings are directly known to be biologically relevant. However, the complex environment of a cell, or even only the membrane and contents of an endosome, make it difficult to ensure reproducible conditions, may provide technical challenges such as background in fluorescence microscopy, and provide limited control over biochemical parameters such as changing concentrations and using modified proteins. We therefore choose a bottom-up approach, where we reproducibly create fusion conditions using a reconstituted system. This system provides exquisite control over parameters such as the composition of the target bilayer or the target pH to trigger fusion.

Many experimental assays report on properties of an average of an ensemble. The advantages are high throughput and generally less technically demanding experimental setups. However, instead of showing an ensemble-averaged readout, single-particle assays obtain the distribution of observed events, allowing inference on the processes that lead to the event. By supplementing the single-particle data with modeling, novel insight has been obtained into the molecular mechanism of multiple viruses.30-34 In this thesis, we extend the integration of single-particle work with other assays, from bulk fusion, through biochemical assays, to all-atom molecular dynamics simulations.

1.6 Thesis outline

This thesis focuses on studying membrane fusion in influenza and chikungunya viruses, in order to determine the role of their respective fusion proteins in this process. Research questions we pursued for either of these viruses were:

• How many fusion protein trimers are necessary to overcome the membrane fusion barrier?
• How does the sequence of the fusion protein relate to its fusogenic function?
• What are the rate-determining steps in the fusion process?
• Do all proteins successfully engage in fusion, or are some of them “duds” not productively involved?
• How does the target bilayer composition modulate fusion?
• What is the mechanism of action of fusion-neutralizing antibodies?

The following two chapters concern influenza fusion. In Chapter 2 we review current knowledge of the hemagglutinin-mediated membrane fusion of influenza virus. We start by exploring the intermediates of fusion and the barriers between them as determined in experimental and computational studies. The hemagglutinin structure and conformational rearrangement under acidic conditions is explained, relating these to its function in mediating hemifusion or pore opening, and discussing hypotheses of multiple conformational pathways that may be
involved. We then review the growing body of single-particle work that intimately connects with modeling work at the level of single and multiple proteins, providing new insights on the collaborative action of hemagglutinin.

Chapter 3 describes the single-particle experimental efforts to study the effect of single amino acid substitutions discovered in silico, that destabilize a critical region of the influenza hemagglutinin. We correlate this destabilization with reduced fusogenicity and enhanced susceptibility to fusion-neutralizing antibodies. Combined with other assays, we find a mutation-induced reduction of the number of hemagglutinin incorporated per virion. Using our current molecular model of influenza fusion with a reduced amount of hemagglutinin, the resulting fusion characteristics are well explained. Although we could not find new evidence of non-productive pathways in hemagglutinin, our work does demonstrate a powerful synergy between molecular dynamics and single-particle fusion assays in bridging the length scale from single amino acids to whole virus particles.

Chikungunya fusion was studied in the next two chapters, with Chapter 4 focusing on a side-by-side comparison of both a bulk liposomal fusion assay and a newly developed single-particle assay at elevated temperature. The dependence of chikungunya fusion on the presence of two lipidic components in the target bilayer, cholesterol and sphingomyelin, was explored. These co-factors of fusion are essential and stimulating factors, respectively. We observe that multiple rate-limiting steps are involved in the fusion process.

Then, in Chapter 5 we use fusion-inhibiting chikungunya antibodies and find that these inhibit E1 trimerization. We then use single-particle fluorescence to count the number of antibodies bound to individual viruses. The magnitude of inhibition of fusion diminished with lower pH due to dissociation of the antibodies from the virions. The observed stoichiometries imply a cooperative fusion mechanism, in which multiple spikes in a surface ring need to be available for fusion. The requirement of the involvement of multiple fusion trimers therefore appears universal across enveloped viruses.

We conclude with summarizing remarks and prospects of the field of “fusionology”, and single-particle and single-molecule techniques.