6 Scientific summary and perspectives
6.1 Summary
Membrane fusion is a key step in the entry pathway of enveloped viruses, as it enables mixing of the viral content with that of the target cell and introduction of the viral genome into the interior of the cell. Subsequent translation of this viral genome will give rise to new viral proteins, thereby hijacking the cell to produce new virus offspring. To overcome the high kinetic barriers associated with the fusion of two membranes, enveloped viruses bring coat proteins that catalyze the membrane fusion process. These fusion proteins enable genome delivery at the right place in the host cell for virus reproduction.

In this thesis, I have studied membrane fusion mediated by proteins from two different classes: the class I hemagglutinin protein of influenza virus and the class II E1 protein of chikungunya virus. Even though the pathways of protein-mediated fusion of influenza and chikungunya viruses differ on key aspects as identified in Chapter 1, the overall mechanism is strikingly similar, and this commonality in fusion mechanisms seems to hold universally for all enveloped viruses studied thus far.

The influenza hemagglutinin (HA) is arguably the best studied of the viral fusion proteins and we started out reviewing current knowledge of influenza membrane fusion in Chapter 2. First, we identify the kinetic barriers involved in fusion through an overview of literature-reported values. We then describe the structure of the influenza hemagglutinin and the large conformational changes that make the HA act as a fusion catalyst. The structure and pre-to-post-fusion conformational changes of HA have largely been mapped onto different steps in the fusion pathway. For example, a region in the pre-fusion structure termed the B loop extends and forms an alpha helix upon acidification, thereby projecting the fusion peptide towards the target membrane. There is however evidence that another region, termed the globular bottom, may influence the success rate of fusion peptide insertion. If the globular bottom zippers up too quickly, the HA might refold without succeeding to bridge the viral and target membranes. The existence of such nonproductive pathways has been predicted in models of single-particle data, which we review last. Single-particle assays were developed over the last decade and were extended in this thesis. They enable the study of the fusion of individual virus particles to a target membrane. Bottom-up and controlled design are key aspects of such a single-particle approach. By observing the effect of for instance fusion inhibitors on the behavior of fusing influenza particles, the cooperativity of HA-mediated fusion and the possible existence of nonproductive pathways have become clear. Influenza fusion, and viral fusion in general, is now seen as arising from the collective action of multiple, stochastically activated fusion proteins that together overcome the membrane fusion barrier.

In Chapter 3 we studied the effect of single amino-acid substitutions in the globular bottom of HA on membrane fusion. All-atom molecular dynamics simulations previously had identified residues that form a hydrogen-bonding network in the globular bottom of HA. Mutating
these residues in silico to prevent hydrogen bonding resulted in reduced unfolding times under a pulling force, indicating destabilization. I describe the use of single-particle fluorescence microscopy to observe fusion of a panel of mutant-HA influenza viruses. We found that the single-particle yields of fusion correlated with the extent of destabilization in silico. Then, we employed fusion-inhibiting antibodyFab fragments that had previously been identified by pharmacists. By titrating the concentration of these antibodies to bind a varying fraction of the available HA epitopes we controllably disabled a fraction of the HAs. By counting the number of inhibitors and correlating this number with the yield of fusion, we observed that the more destabilized mutants also were more susceptible to inhibitor neutralization. We determined that the destabilized virus mutants incorporated less HA per virion, which we modeled as resulting in reduced numbers of HA in contact with the target membrane. Using the molecular model reviewed in Chapter 2, we were able to semi-quantitatively explain the observed inhibitor sensitivity. The major effect of the mutations in the globular bottom appeared to be a change in HA incorporation, which manifested as reduced viral fusogenicity.

In the subsequent two Chapters I focus on the mechanism of chikungunya virus (CHIKV) fusion. This virus has recently globally expanded its range, while no vaccine or specific treatment of infection is available. In Chapter 4 we set out to study the pH and lipid dependency of fusion in both bulk liposomal and single-particle fusion assays. We found a very sharp fusion threshold, around which a difference of 0.1 pH units resulted in a doubling of the yield of fusion. The combined data on the extent and rate of fusion suggested that there is a window of opportunity for the acid-activated protein rearrangements beyond which inactivation occurs. Inactivation of the CHIKV particles in the absence of target membranes was rapid but partly reversible at neutral pH. We then investigated the effect of two lipids in the target membrane, cholesterol and sphingomyelin. Both cholesterol and sphingomyelin were required and resulted in larger fusion yields at higher concentrations, but for sphingomyelin this phenomenon was subject to a threshold. However, both lipids did not consistently influence the rate of fusion. Analysis of the single-particle fusion time distributions showed that at least two rate-determining steps are involved in the CHIKV fusion process.

We set out to further study the cooperativity of CHIKV fusion in Chapter 5 by using fusion-inhibiting antibodies. Antibody CHK-152 had been determined before\textsuperscript{292,294} to bind to the E2 protein on the viral surface and to be an effective inhibitor of infection. We first showed that CHK-152 strongly shields the virus particles from interaction with membranes, both at neutral and low pH. Then, we used single-particle microscopy to study CHK-152’s mechanism of neutralization. At a sub-stoichiometric number of antibodies per virion we observed differential inhibition of fusion: at pH around 6.1, fusion was strongly reduced, whereas at lower pH this effect was reduced. The CHK-152 were observed to dissociate at low pH. Taking into account the partial dissociation of the antibodies and considering the number of unbound protein spikes,
we deduced that CHIKV fusion is cooperative, needing three to five E1 trimers in close proximity to mediate fusion.

Overall, this dissertation aimed to unravel the molecular mechanisms of viral fusion of influenza and chikungunya viruses. Collaboration between scientists enabled the observation of collaboration between proteins. We demonstrated that it is now possible to bridge the length scales between experiment and simulation: to map in-silico-derived, single amino-acid substitutions to function at the level of a virion; to titrate in inhibitors on virions in order to find the cooperativity at the level of the proteins. By identifying the molecular mechanism of virus fusion this work may help to advance the rational design of antivirals to prevent or eliminate future viral infections.

6.2 Perspectives
The field of viral fusion has seen great advancements over the last decade due to the advent of single-particle assays. In the years 2006 to 2008, seminal papers were published demonstrating that fusion for single virions can be observed. This new experimental paradigm has instigated a synergy of experiment and modeling, with the complementarity of these approaches providing new testable hypotheses. Models of influenza fusion have since then been refined to the point that there are now predictions on the conformational changes of viral proteins residing on the viral surface from the observed behavior of the whole virus particle. We further extended the bridging of length scales by combining atomistic modeling and single-particle fusion, to trace the effect of single amino-acid substitutions to the protein and virion level (Chapter 3). The findings of this thesis then also suggested several venues of further study, detailed below.

Virion-membrane interaction geometry. Designing influenza viruses with controllably varied densities of hemagglutinin protein on the viral surface will allow to test the current hypotheses on non-productive refolding pathways of hemagglutinin. A changed density of hemagglutinins leads to a change in the size of the contact patch in contact with the target membrane, a poorly defined parameter in current models. Observing the fusion properties of such hemagglutinin-density variant viruses will allow researchers to refine or refute parameters of the current model of influenza fusion, for example on the non-productive pathways. Another effect not currently modeled is that influenza virus particles are heterogeneous in size. Some preliminary explorations by the author not included in this thesis showed that such heterogeneity can, by itself, give rise to a rise-and-decay distribution of fusion times, due to the variation of the contact patch over virus particles. Having a measure of individual virion contact patch sizes is therefore important to further refine the estimates on the cooperativity and robustness of fusion by the hemagglutinin proteins. In addition to clarifying the geometry of virion-membrane interaction, separating the fusion and binding functions of viral proteins will help to elucidate both processes as discussed in Chapter 5. For example, virions could be tethered to the
target membrane artificially, so that the effect of inhibitors on membrane fusion can be studied separately from binding. Additionally, this approach would provide more control over experimental statistics, and hence, throughput, which is generally a limiting factor for single-particle experiments.

**Protein conformational dynamics.** Crucial details of the dynamic conformational changes of viral fusion proteins are typically inferred from static pictures as obtained from x-ray crystallography or other structural approaches. An important recent tool able to probe sub-nanometer distance changes is single-molecule Förster resonance energy transfer (smFRET). It has been used to probe the conformational dynamics of HIV-1 fusion proteins. In combination with all-atom or coarse-grained modeling, similar to our approach in Chapter 3, this will open a very promising direction of future studies where the dynamic protein states and the reversibility of these states can be directly probed.

**Membrane composition and crowding.** The exact role of the composition of the target membrane in viral fusion is largely unknown. We determined the influence of the composition of the target membrane in chikungunya fusion and found that the two factors required for fusion, sphingomyelin and cholesterol, affected the fusion very differently (Chapter 4). Recent work has shown that the influence of cholesterol on fusion depends on the type of receptor used, suggesting that lipid raft domains may be involved. HIV-1 has been shown to fuse at the edge of such membrane domains, at the phase between ordered and disordered regions. As the receptor to which the virus attaches appears to influence the properties of fusion, an effect that remains unclear, inhibitors targeting receptor or receptor-binding sites may prove insightful when used in single-particle assays. Moreover, the environment of a live cell is highly crowded, including crowding of membrane-anchored components. An important step in fusion may therefore be de-proteination of the target membrane before fusion can occur. Knowing more about the local ordering and structure of the membranes involved, for example by employing fluorescence or atomic force microscopy, will help to extend viral fusion models to include environmental factors as discussed above.

In the end, integrating the insights from different fields of study of membrane fusion may lead to a picture of a very universal nature, as has already been obtained for viral fusion. Freely after L.V. Chernomordik and B. Podbilewicz, this will define the rising field of ‘fusionology’.