Chikungunya virus (CHIKV) is a rapidly spreading, enveloped alphavirus causing fever, rash and debilitating polyarthritis. No specific treatment or vaccines are available to treat or prevent infection. For the rational design of vaccines and antiviral drugs, it is imperative to understand the molecular mechanisms involved in CHIKV infection. A critical step in the life cycle of CHIKV is fusion of the viral membrane with a host cell membrane. Here, we elucidate this process using ensemble-averaging liposome-virus fusion studies, in which the fusion behavior of a large virus population is measured, and a newly developed microscopy-based single-particle assay, in which the fusion kinetics of an individual particle can be visualized. The combination of these approaches allowed us to obtain detailed insight in the kinetics, lipid dependency, and pH dependency of hemifusion. We found that CHIKV fusion is strictly dependent on low pH, with a threshold of pH 6.2 and optimal fusion efficiency below pH 5.6. At this pH, CHIKV fuses rapidly with target membranes, with typically half of the fusion occurring within less than two seconds after acidification. Cholesterol and sphingomyelin in the target membrane were found to strongly enhance the fusion process. By analyzing our single-particle data using kinetic models, we were able to derive that the number of rate-limiting steps occurring before hemifusion equals about three. To explain these data, we propose a mechanistic model in which multiple E1 fusion trimers are involved in initiating the fusion process.


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4.1 Introduction

Chikungunya virus (CHIKV) is a rapidly emerging pathogen that belongs to the alphavirus genus, which also includes Semliki Forest virus (SFV), Sindbis virus (SINV) and O’nyong O’nyong virus (ONNV). After re-emerging in 2004, CHIKV has caused large epidemics in Africa and Asia and a number of cases in Europe. Recently, CHIKV crossed the Atlantic and as of January 2015, more than 26 000 confirmed and 1 094 000 suspected CHIKV cases were reported in the Americas.

CHIKV is transmitted by Aedes mosquitoes, with A. aegypti and A. albopictus as the most important vectors. The majority of people infected with CHIKV develop chikungunya fever, which is characterized by high fever, rash, myalgia, joint pain, and headache. A common long-term implication of CHIKV fever is severe joint pain, which can persist for months to years. There is no vaccine or specific treatment for CHIKV available. For the rational design of a vaccine or antiviral drug it is imperative to acquire detailed knowledge on the molecular mechanisms involved in CHIKV infection.

Alphaviruses are enveloped viruses that infect the cell via receptor-mediated endocytosis and subsequent membrane fusion from within acidic endosomes, although direct fusion at the plasma membrane has also been reported. Viral attachment and fusion are facilitated by the envelope glycoproteins E1 and E2, which are arranged as 80 spikes at the viral surface. One spike consists of three E1/E2 heterodimers. The E2 protein contains the receptor-binding site and shields the fusion loop on E1. Upon virus uptake and delivery to endosomes, the acidic pH of the endosomal lumen causes a dramatic rearrangement within the E1/E2 heterodimers, which drives fusion of the viral membrane with the endosomal membrane. The first step in this process involves dissociation of the E1/E2 heterodimer. As a consequence, the fusion loop is exposed and inserted into the target host membrane. A core trimer of E1 proteins is formed and the E1 subunits re-fold, which causes the opposing proximal membrane leaflets to merge, a step known as hemifusion. Subsequently, a fusion pore is formed and the viral nucleocapsid is released into the cytosol. Generally, multiple copies of a viral protein trimer are thought to act in concert to catalyze hemifusion.

The characteristics of the membrane fusion reaction have been studied in detail for the alphaviruses SFV and SINV (reviewed in Kielian). For both viruses, membrane fusion is strictly dependent on low pH and the presence of sphingomyelin and cholesterol in the host cell membrane. In liposomal bulk fusion assays, the threshold of fusion has been found to be pH 6.2 for SFV and 6.0 for SINV wild-type strains. However, this threshold varies with strain and assessment method, as also lower pH thresholds for both viruses were described. For CHIKV, cell-based assays revealed that fusion is dependent on low pH and cholesterol as well.
pH threshold of fusion is around pH 5.9-6.1, depending on the strain used.\textsuperscript{254,255} However, no details are known on the membrane fusion kinetics or sphingomyelin dependence of fusion.

In this study, we characterized the kinetics of CHIKV (strain S27) fusion using both a liposomal bulk fusion assay and a single-particle fusion assay based on total-internal-reflection fluorescence microscopy (TIRF-M). We found that CHIKV fusion is strictly dependent on low pH, with a pH threshold of 6.2 and optimal fusion at a pH range of 4.5–5.6. For this CHIKV strain, we observed a sharp pH dependency of the extent of fusion, with only 0.3 pH units between conditions of near-maximal and only residual fusion activity. Both cholesterol and sphingomyelin in the host-cell membrane strongly supported CHIKV fusion activity. The single-particle assay indicated that multiple, parallel rate-limiting steps precede hemifusion, a phenomenon described earlier for other membrane-fusing viruses.\textsuperscript{181,188,189,256} We propose that these steps are arising from the parallel action of several fusion trimers.

4.2 Results

4.2.1 pH-dependent fusion of CHIKV with liposomes

For the bulk fusion assay, CHIKV was biosynthetically labelled with 1-pyrenehexadecanoic acid (pyrene).\textsuperscript{257} The pyrene labelling did not influence viral infectivity (shown in Table A4.1). The virus was incubated with liposomes and fusion was triggered by adding a pre-titrated volume of low-pH buffer to the reaction mixture. Fusion was followed in real time using a fluorimeter at 37 °C. The lipid concentration corresponding to optimal fusion efficiency and signal-to-noise ratio was determined to be 400 µM, and therefore used for all experiments (Figure A4.1).

Figure 4.1A shows the time traces of the extent of CHIKV fusion at different pH values as measured by the bulk assay (solid lines). The total extent of fusion as a function of pH is shown in Figure 4.1B. The highest pH showing detectable fusion was pH 6.2, with a residual fusion activity of 4.3±0.6%, compared to the pH 7.4 control (0±2%). For the CHIKV S27 strain used, we observed a sharp pH dependence: a change of 0.2 units from pH 5.9 to 6.1 resulted in an eight-fold reduction of the extent of fusion, suggesting a fusion mechanism that involves a form of cooperativity between neighboring fusion proteins. Below pH 5.6, the extent of fusion reaches a plateau value. The fusion rate, which we calculated as the inverse of the time point at which half of the extent of fusion is reached, is plotted in Figure 4.1C. In the bulk assay, the fusion rate was observed to increase with lower pH from a minimal, detection-limited value at pH 6.0–6.2, to saturating rate at pH 5.6. In the plateau region, CHIKV fusion happened promptly, with typically half of the fusion occurring within less than two seconds after acidification. The mid points of fusion extent and fusion rate were found to be 0.2 pH units apart.
Earlier work on SFV and SINV showed that the time to induce fusion is very limited, as the E1 protein rapidly rearranges into a fusion-inactive state if acidification occurs in the absence of target membranes.\textsuperscript{248,258} To assess whether this also applies to CHIKV, virions were exposed to low pH in the absence of liposomes. At the indicated time points in Figure 4.2A, pre-acidified liposomes were added to the acidic virus-containing reaction mixture. A tenfold reduction in fusion extent was observed in 9±2 s. This inactivation curve is faster than for SFV (\textasciitilde 50 s for a tenfold reduction) and for SINV (\textasciitilde 75 s). The inactivation was not described well by a single inactivation rate, but instead a sum of two exponentials was required to describe the data (Figure 4.2A; dotted curve). The fast-decaying fraction represented 76±5\% of the total population, decaying at a time scale of \(\tau = 4.2\pm0.4\) s. The remaining 24±5\% inactivated with a time...
constant of $\tau = 38\pm5$ s. To test whether inactivation was reversible, virus was acidified in the absence of liposomes for 90 s at pH 5.0, back-neutralized to pH 8, and a standard fusion measurement was performed. Inactivation was found to be partially reversible (Figure 4.2B). Approximately 55% of membrane fusion capacity was restored, when compared to the untreated control that was acidified after the same time interval (Figure 4.2C). This is slightly more than the one found for SFV (45%).

Figure 4.2 Reversible inactivation of CHIKV due to low pH exposure in the absence of liposomes. Data points show mean±SEM for at least three independent experiments. (A) CHIKV virions were incubated at pH 5.0 prior to the addition of pre-acidified liposomes for the indicated time period. The extent of fusion is compared to an untreated control. Dashed line shows a double-exponential fit, indicating the presence of two populations. The first population comprises a fraction of 0.76±0.05 with time scale $\tau = 4.2\pm0.4$ s$^{-1}$, the second, a fraction of 0.24±0.05 and $\tau = 38\pm5$ s$^{-1}$. (B) Reversibility of low pH inactivation. Representative fusion curves of each condition are shown. The exact experimental protocol is described in the Material & Methods section. (C) Comparison of the fusion extents relative to the untreated control of the experiments of 90-seconds-inactivation (5±3%) and back-neutralization (55±4%).
4.2.2 Low-pH dependent fusion of single CHIKV particles at 37 °C

The time traces of fusion obtained in the bulk assay represent an averaged readout of an ensemble of virions in different stages of the fusion process. Due to the stochastic nature of the underlying molecular transition, the population becomes increasingly asynchronized as time elapses after triggering fusion. As a result, subpopulations and short-lived intermediate states cannot be discriminated. To overcome this population averaging and obtain more kinetic detail, we designed a single-particle assay based on earlier single-particle work by our group and others (Figure 4.3).
**Figure 4.3 Experimental design of the single-particle assay.** (A) Virions labeled with a membrane dye (R18) bind to a glass-supported lipid bilayer. Hemifusion is induced by lowering the pH and observed with total internal reflection (TIRF) microscopy as the dequenching of R18 caused by its dissipation into the target membrane. Fluorescein molecules bound to the supported bilayer provide a readout of local pH conditions. (B) The microfluidic flow cell and microscope objective are kept at 37 °C. Low pH buffer is incubated proximally to the channel of observation (left half of chip) in a serpentine-shaped flow channel (right half of chip) to provide constant-temperature and short acidification times. Emitted fluorescence is separated by color and collected onto different halves of an EM-CCD camera. (C) Pre- and post-acidification movie frames (top) and an example of the fluorescence time trajectories (bottom). The fluorescein signal defines the $t = 0$ of the experiment and individual virus hemifusion lag times are identified.

We modified our earlier experimental design\(^{188}\) to enable the observation of fusion both at elevated temperatures and with short acidification times (also see Methods). Purified CHIKV particles were labelled with octadecyl rhodamine B (R18) as described before.\(^{263}\) R18 labelling did not influence the specific infectivity of the virus (Table A4.1). After introduction of the labelled virus to the surface-supported lipid bilayer in the flow cell, we observed that the particles bind to the membrane in a nonspecific manner, likely mediated by electrostatic interactions (Figure 4.3C; red channel in left panel). Fusion was triggered by a rapid injection of low-pH buffer from a proximal storage channel in the microfluidic flow cell. Hemifusion of individual virions was visualized using TIRF microscopy (Figure 4.3A). The flow cell was kept constant at 37±1 °C and acidification of the channel was achieved within 0.9 s. On a particle-by-particle basis, R18 dequenching traces were extracted from the fluorescence movies (Figure 4.3C) and the elapsed time between acidification and hemifusion was determined.

Representative curves showing the percentage of particles in the field of view that fused over time are shown in Figure 4.1A (dashed lines), revealing similar population-level kinetics as in the bulk assay. Mean extents of fusion at $t=60$ s are plotted in Figure 4.1B (open squares). The population-level fusion rate, calculated here as the inverse of the median fusion time, is plotted in Figure 4.1C (open squares). As depicted in the graph, the main features of CHIKV pH-dependent fusion found in the bulk assay were reproduced in the single-particle assay.

**4.2.3 Efficient CHIKV fusion is dependent on cholesterol and sphingomyelin in the target membrane**

Following previous observations that SINV and SFV fusion is dependent on cholesterol and sphingomyelin in the target membrane,\(^{246-249}\) we investigated the fusion characteristics of CHIKV with membranes consisting of varying concentrations of these lipids. As expected, cholesterol in the target membrane strongly supported CHIKV fusion (Figure 4.4A; top and middle panels). The total fusion extent followed a sigmoidal curve, with higher amounts of cholesterol in the target membrane leading to higher extents of fusion. Maximal fusion was found at 38–42 mol-% of cholesterol in the target membrane. The fusion rate did not differ considerably between the different cholesterol concentrations (Figure 4.4A; bottom panel).
Furthermore, membrane fusion was strongly enhanced by sphingomyelin in the target membrane (Figure 4.4B top and middle panels). In contrast to the sigmoidal cholesterol dependency, relatively low amounts of sphingomyelin were sufficient to achieve optimal fusion. The total extent of fusion using membranes containing 22.2 mol-%, 11.1 mol-% and 6.6 mol-% was equal to the maximum observed. Even if the sphingomyelin concentration was reduced 10-fold compared to the standard liposome composition (from 22% to 2.2%), still about 27% of the particles fused with liposomes. Also here, the fusion rate did not vary significantly with target membrane sphingomyelin content (Figure 4.4B; bottom panel).

4.2.4 Hemifusion of CHIKV is a process with multiple rate-limiting steps
The data described above demonstrate that the bulk and single-particle fusion assays are mutually consistent in the quantitative information they provide on the extent of fusion and its
kinetics at the population level. The strength of the single-particle approach lies in the fact that a particle-by-particle analysis of the kinetics provides additional information which is not accessible by the bulk approach. To evaluate the kinetic determinants for CHIKV fusion, the time elapsed between pH drop and hemifusion was obtained for a large number of particles for two pH points close to the threshold of fusion (pH 6.2 and 6.0) and one pH point within the optimum pH of fusion (pH 4.7).

The distributions of virion lag times from the time of acidification to the hemifusion event are shown in **Figure 4.5**. At all three pH points, the frequency distributions show a rise and decay. We analyzed these distributions by fitting them to gamma functions that provide a fitting parameter $N$ describing the number of rate-limiting steps occurring before hemifusion. We showed previously$^{193}$ that this is a powerful tool to determine the number of kinetic intermediates in a process. A single rate-limiting step results in a single-exponential distribution and multiple rate-limiting steps introduce the rise-and-decay in the histogram. Performing the fits with gamma functions resulted in $N = 2.1 \pm 0.4$ for pH 6.2 and $N = 3.2 \pm 0.4$ for pH 6.0. At pH 4.7, the typical timescale of hemifusion and the time to drop the pH become comparable in magnitude. To make sure the observed rise-and-decay cannot be explained solely from the pH drop, we did a correction in the fit (see **Figure A4.2**). Taking the effect of the finite width of the pH drop into account, we obtained a value of $N = 3$ for pH 4.7 (**Figure 4.5** and **Figure A4.3**).

![Figure 4.5 Fusion lag time distributions of CHIKV at pH 6.2, 6.0 and 4.7](image)

Figure 4.5 Fusion lag time distributions of CHIKV at pH 6.2, 6.0 and 4.7. Elapsed time of individual virions between acidification and hemifusion for pH 6.2, 6.0 and 4.7 ($n = 148, 605, 977$ fusing virions respectively). The rise and decay indicates that several intermediate states precede the hemifusion event. For pH 6.2 and 6.0, fitted Gamma distributions (solid line; see text) are shown, with $N = 2.1 \pm 0.4, 3.2 \pm 0.4$ and $k = 0.34 \pm 0.08$ s$^{-1}, 0.9 \pm 0.2$ s$^{-1}$ respectively. For pH 4.7, the fits were modified to include the finite length of the pH drop (solid line; see text) and such fits with fixed $N = 1$ (dashed line; fitted $k = 0.53 \pm 0.09$ s$^{-1}$) and $N = 3$ (dotted line; $k = 1.22 \pm 0.06$ s$^{-1}$) are shown.

### 4.3 Discussion

Fusion of CHIKV with endosomal membranes is a crucial process in the viral life cycle that has not yet been investigated in great detail. In our study, we established two assays to measure
CHIKV fusion in vitro at 37 °C with remarkable agreement between these approaches. We observed that CHIKV fusion is receptor-independent, triggered by low pH, and enhanced by cholesterol and sphingomyelin in the target membrane. With this approach, we were able to obtain detailed kinetic information on the fusion process up to and including hemifusion.

We observed fusion of the majority of the viral particles within seconds after acidification. This observation is in line with earlier results on the other alphaviruses SINV and SFV. A slightly higher rate is observed in the single-particle assay (Figure 4.1C). This may be explained by the fact that in the single-particle experiments all observed particles are already docked to the membrane before lowering of the pH, while in the bulk assay a subpopulation of virions may still have to associate with a liposome after acidification.

We found that the fusion threshold for CHIKV is pH 6.2. Optimal fusion occurs within the pH range of 4.5–5.6. Remarkably, the pH dependence of fusion for the S27 strain is very sharp: there is an eightfold reduction of fusion extent over 0.2 pH units. This pattern suggests that there is a high degree of cooperativity involved in the steps leading to hemifusion. A similarly sharp pH dependence was observed for the fusion rate, although with its half-point shifted 0.2 pH units towards lower pH. This is most visible at pH 6 and might be due to bound particle pre-selection. The steepness of the pH dependence seems to be related at least partially to the amino acid at the E1 226 position. We and others observed that CHIKV strains with an alanine at this position (like S27) exhibit a sharper pH dependence than strains with a valine at E1 226. Together with the altered cholesterol dependence observed in strains with an A226V mutation, this change might have an influence on the location of viral fusion within the endosomal pathway and subsequently alter viral fitness.

Pre-exposing CHIKV to low pH for different time intervals showed a reduction in extent of fusion of tenfold over 9±2 s, corresponding to an inactivation rate of $k_{\text{inact}} = 0.24±0.04 \text{s}^{-1}$, which is of similar magnitude as the overall fusion rate observed in both assays. We speculate that there is a competition between activation and inactivation of fusogenic trimers at the viral surface under low pH conditions. Within a limited time window, a minimal number of trimers need to act simultaneously to mediate fusion before inactivation occurs. Residual fusion activity remained at high time intervals, suggesting heterogeneity in this CHIKV strain. We modelled this with a double-exponential model and found a fast-fusing and quickly inactivating population (~76%) and a second population having longer fusion times and slowly inactivating (~24%).

CHIKV fusion is strongly enhanced by the presence of both cholesterol and sphingomyelin in the target membrane. The cholesterol dependence of fusion extent followed a sigmoidal curve, flattening at around 40 mol-%. This observation is consistent with earlier studies showing that cell infection of CHIKV is dependent on cholesterol. Furthermore, our findings are in concordance with results obtained for SFV and SINV. Cholesterol is known to influence the physical properties of membranes such as curvature, stability and fluidity and was found to promote insertion of the E1 fusion protein into the target membranes.
observation that cholesterol does not influence the kinetics of fusion suggests that it indeed functions as a binding cofactor rather than exerting its function by altering the physical properties of the target membrane, with no role in the rate-limiting steps leading to fusion.

No data on sphingomyelin dependency was available so far for CHIKV. We found that CHIKV fusion is strongly dependent on sphingomyelin in the target membrane. Relatively small amounts (6.6 mol-%) are sufficient for near-optimal fusion efficiency. In the absence of sphingomyelin, we observed residual fusion activity in the bulk assay (7±1%), and to a lesser extent (1±1%) also in the single-particle assay. Residual fusion activity at 37 °C in the absence of sphingomyelin has been described for SFV,269,270 but not for SINV.248 It has been found that sphingolipids support cholesterol-mediated virus binding and stimulate the conformational changes required for membrane fusion.247,271-273 In line with these findings, we observed that the fusion rate of CHIKV is not dependent on sphingomyelin concentrations, and therefore not dependent on the physical properties of the membrane that would vary with changing sphingomyelin concentrations.

Using the single-particle assay, we found that multiple rate-limiting steps precede CHIKV hemifusion. At pH 6.2, 6.0 and 4.7, we obtained hemifusion kinetic data for a large number of individual particles to be able to resolve the rise-and-decay behavior in the distribution of hemifusion times. This is a characteristic of a process having multiple, equally fast rate-limiting steps. These steps could be sequential or parallel. For the range of proton concentrations (thirtyfold difference between pH 6.2 and 4.7) investigated, the number of steps was found to be in between 2 and 3. In the case of mechanistically distinct sequential steps that happen to have the same rate of progression, the one being proton-dependent would become the slowest, rate-limiting step at high pH, reducing the $N$ to a value close to 1. In line with a similar reasoning previously used to rationalize single-particle fusion kinetics of influenza virus,188 it seems more plausible from our data that there are several parallel steps required. In our opinion, it is likely that this feature reflects the requirement to have several copies of the fusion trimer to mediate fusion. Indeed, low-temperature electron micrograph experiments have shown the assembly of rings of fusion protein trimers on the outside of the virion.274 The concerted action of assembled trimers then could give rise to the observed rise-and-decay hemifusion distributions. Alternatively, the formation of the fusogenic trimer from the individual E1 monomers could be rate limiting. We are currently working on obtaining an even more detailed molecular insight in the fusion process to test these hypotheses.

We report here the application of a bulk and single-particle fusion assay to study CHIKV hemifusion and show good consistency in results between these two approaches. The main advantage of the bulk assay lies in the fact that it possesses a high throughput and can therefore be used for a broad and detailed characterization of fusion. The single-particle approach enables kinetic information to be obtained at higher time resolution and devoid of dephasing effects as present in bulk assays. On the other hand, because of the technically challenging nature
of the single-particle experiments, the single-particle experiments have a lower throughput and require more labor-intensive data analysis compared to the bulk assay. By using the combination of the two assays we arrive at an improved kinetic picture of CHIKV fusion, proving it a promising route for further research into the mechanistically guided search of fusion inhibitors. Further study of CHIKV fusion involving mutant virions and fusion-inhibiting antibodies will be needed to further elucidate the molecular mechanisms involved in fusion.

### 4.4 Methods

**Production, labelling and inactivation of viruses.** CHIKV strain S27 (kindly provided by S. Günther, Bernhard-Nocht-Institute for Tropical Medicine), which was isolated in Tanzania in 1953, was propagated in Vero-WHO cells to obtain seed stocks. The cells were maintained in DMEM (PAA laboratories) supplemented with 5% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/mL streptomycin at 37 °C and with 5% CO₂. For virus production, a confluent monolayer of Vero-WHO cells was infected at a multiplicity of infection (MOI) of 0.01. At 48 hours post infection (hpi), the cell supernatant was harvested and cleared from cell debris by low-speed centrifugation, frozen in liquid nitrogen, and stored at -80 °C.

Virus for the bulk fusion assay was labelled biosynthetically with pyrene, essentially as described before for SFV and SINV. Briefly, baby hamster kidney cells (BHK)-21 were cultured in the presence of 15 µg/ml of 1-pyrenehexadecanoic acid (Invitrogen) 48 h prior to infection in RPMI (Life Technologies) supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37 °C and with 5% CO₂. BHK-21 cells were infected at MOI 4 and at 24 hpi, the cell supernatant was harvested and cleared from cell debris by low-speed centrifugation. Subsequently, the pyrene-labelled CHIKV particles were pelleted by ultracentrifugation in a Beckmann type 19 rotor at 54 000×g for 2.5 h. The virus was purified on a continuous sucrose (20/55% w/v) gradient by ultracentrifugation in a Beckmann SW 41 rotor overnight at 50 000×g.

The virus preparations used for the single-particle fusion assay were generated and purified in the same fashion, except that the virus was propagated in the absence of pyrene. The purified CHIKV particles were subsequently labelled with the octadecyl rhodamine B chloride (R18; Invitrogen) fluorophore. For this purpose, 1×10¹¹ to 2.2×10¹¹ particles of purified and inactivated (see section *single-particle assay*) CHIKV were diluted in HNE (5 mM Hepes, 145 mM NaCl, 0.2 mM EDTA) and R18 dissolved in DMSO was added to a final concentration of 1µM. Subsequently, the virus solution was rotated at room temperature for 1 h. A gel-filtration column (PD-10 desalting column; GE Healthcare) was used to separate the virus from unincorporated dye. The most concentrated fractions were combined and used undiluted in the experiment. To test whether labelling influences viral infectivity, active virus was labelled using the same methods for use in infectivity assays. At the timescale of our experiments, no R18 flip-flop occurred, which would be visible as a loss of virus particle intensity before the pH drop.

The number of physical particles was determined by a standard phosphate assay using a value of 4.6×10⁻²⁰ mol of phosphate per particle and with quantitative PCR (qPCR). The qPCR was performed as described previously for Dengue virus. Briefly, viral cDNA was synthesized by reverse transcriptase (RT) PCR using the forward primer 5′-AGCTCCCGGTTCCTTACC-3′ and the reverse primer 5′-GCCAAAATTGCTGGTCTTTCC-3′. For the qPCR, the TaqMan probe 5′-FAM-CACGTTGACCCGGAGG-TAMRA-3′ was added. DNA was amplified for 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Determination of the number of RNA copies was performed with a standard curve (correlation co-efficient > 0.995) of a quantified CHIKV plasmid containing the E1 sequences (pCHIKV-LS3 1B) constructed with standard DNA techniques. The infectivity of the virus was determined by a standard plaque assay on Vero-WHO cells. The specific infectivity was calculated by dividing the number of physical
particles or genome-containing particles (GCP) by the number of PFU. As can be seen in Table S1, there is no significant difference between the specific infectivity calculated with GCPs and the specific infectivity calculated with the physical particle concentration.

**Preparation of liposomes and supported lipid bilayers.** Liposomes (200 nm in diameter) were prepared by a freeze-thaw extrusion procedure as described before. Unless specified otherwise, liposomes consisted of phosphatidylcholine (PC) from egg yolk, phosphatidylethanolamine (PE) prepared from transphosphatidylation of egg PC, sphingomyelin (SPM) from porcine brain, and cholesterol from ovine wool in a molar ratio of 1:1:1:1.5. In experiments with lower SPM concentrations, SPM was replaced with an equal molar amount of PC to maintain the phospholipid-to-cholesterol ratio of 2:1. All lipids were purchased from Avanti Polar Lipids. Lipids and the phospholipid-to-cholesterol-ratio were chosen to approximate the lipid composition within the endosomal compartment.

For the single-particle assay, liposomes (200 nm) were also prepared by freeze-thaw extrusion. Liposomes consisted of 1:1:1:1.5:2×10⁻⁵ ratio of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), porcine brain sphingomyelin (SPM), ovine wool cholesterol and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(biotinyl) (Biotin-PE), unless otherwise specified.

**Fusion assays.** Throughout the report we refer to (hemi)fuson as fusion, as the assays used do not distinguish content mixing from lipid mixing.

**Bulk fusion assay.** Fusion of pyrene-labelled CHIKV with liposomes at 37°C was monitored in a Fluorolog 3-22 fluorometer (BFi Optilas, Alphen aan den Rijn, The Netherlands), as described before. Pyrene-labelled CHIKV (1.5 µM viral phospholipid, corresponds to 4x10¹⁰ virions) was mixed with an excess of liposomes (400 µM phospholipid, corresponding to 6x10¹⁰ liposomes) in a total of 665 µl in HNE buffer (5 mM HEPES, 150 mM NaCl, 0.1 mM EDTA) in a quartz cuvette. After 60 seconds of incubation with constant magnetic stirring, the pH was lowered by adding 35 µl of 0.1 M MES with 0.2 M acetic acid pretitrated with NaOH to achieve the desired pH. The fusion scale was calibrated such that 0% fusion corresponded to the initial excimer fluorescence value. 100% of fusion was set equal to the signal obtained by adding 35µl of 0.2 M octaethyleneglycol monododecyl ether (C₁₂E₈; Sigma-Aldrich) which caused an infinite dilution of the probe. The total fusion extent was determined by calculating the average signal between of 50 and 60 s after the pH drop. Curves were corrected for bleaching of the dye by subtracting the linearized control curve at pH 7.4.

To analyze whether CHIKV is inactivated by low pH in the absence of target membranes, the protocol was slightly adapted. In this case, HNE was mixed with 4x10¹⁰ virions, and the pH was lowered to pH 5.0 with 0.1 M MES, 0.2 M acetic acid pre-triturated with NaOH. Pre-acidified liposomes were added to the measurement at the indicated time points to measure remaining fusion activity. For the back-neutralization experiments, the virions were acidified as described above. After 90 seconds of acidification, the mixture was back-neutralized to pH 8.0 by a pre-triturated volume of NaOH. Then, liposomes in HNE (pH 7.4) were added and the mixture was re-acidified to pH 5.0 (t=0) and fusion was measured. Control experiments were performed with the same time intervals without pre-acidification and back-neutralization.

**Single-particle fusion assay.** Use of the virus in our single-particle microscope outside a BSL-3 environment necessitated inactivation, which was achieved by UV radiation using a 2×8-watt 254 nm UV lamp (VWR) until infectivity remained below 75 PFU/mL. The single-particle fusion assay showed that CHIKV is still fusogenic after treatment with UV radiation, with no significant change in fusion characteristics when compared to the bulk assay data.

Single-particle fusion experiments were performed using an assay we developed previously to visualize influenza fusion, modified to perform all experiments at 37 °C. Glass microscope coverslips (24 × 50 mm, No. 1.5; VWR) were cleaned using 30-min sonications in isopropanol and acetone, rinsing in between with deionized water, and finally 10 min in an oxygen plasma cleaner. Coverslips were stored in desiccated
vacuum. Polydimethylsiloxane (PDMS) flow cells were prepared by pouring and hardening on a photolithography masks, essentially as before.222

A schematic overview of the setup is shown in Figure 4.3A–C. Imaging was performed on a home-built Total Internal Reflection Fluorescence (TIRF) microscope, using an inverted microscope (Olympus IX-71) and a high numerical aperture, oil-immersion objective (NA 1.45, 60x; Olympus). The flow cell was kept at elevated temperature in a heating block (Pecon GmbH), with the microscope objective thermally isolated from the microscope using a spacer ring (Bioptechs Inc.) and heated with a ring heater (Tokai Hit) to prevent local cooling of the flow cell. To provide pre-heated low pH buffer at short notice, a serpentine-shaped channel was included on the flow cell proximally to the channel of observation (see Figure 4.3B), enabling acidification times of down to 0.5 s, necessary for the fast-fusing CHIKV. Liposomes were flushed into the flow cell and a planar lipid bilayer was allowed to form over the course of 20 minutes by the vesicle-spreading method.282 Membrane fluidity was confirmed by Fast Recovery After Photobleaching (data not shown). Virions were docked non-specifically to the lipid bilayer (see Figure 4.3C). Fluorescein-labelled streptavidin (Life Technologies) was introduced into the flow cell to bind to the membrane-incorporated Biotin-PE and serve its pH-dependent fluorescence as an optical readout of the exact moment of the pH decrease. The aqueous environment was acidified by flowing in citric acid buffer (10 mM, 140 mM NaCl) of calibrated pH at 300 µL/min for 8 s. The fluorophores were excited using 488 nm and 561 nm lasers (Coherent Inc.). Viral membrane fluorescence (red) and fluorescein pH drop fluorescence (green) were projected on different halves of an EM-CCD camera (Hamamatsu). Movies were acquired at 20 frames per second.

**Analysis.** Home-written software in MATLAB was used to extract the fluorescence signals corresponding to the pH drop signal and individual virions, essentially as described before (Figure 4.3C).188 The fluorescein pH-drop signal was integrated over the entire field of view and the t=0 of the experiment defined as the point at which $Erfc[1]/2$ (~8%) intensity remained, with Erfc[] denoting the complementary error function. The lag time to hemifusion of individual particles was then determined manually, binned per time unit and plotted in a histogram with $n^{1/2}$ bins. Next, we fitted a gamma distribution to the histogram to obtain the number of steps $N$ and rate $k$ of each step, the distribution resulting from $N$ identical, rate-limiting steps.193 To take into account the finite width of the pH drop at pH 4.7, the gamma distribution was convoluted in Mathematica with the known fluorescein signal derivative (a Gaussian function, see Figure A4.2). For different values of $N$ the rate $k$ was then fitted.

### 4.5 Acknowledgements

We are grateful to S. Günther, Bernhard-Nocht-Institute for Tropical Medicine, Hamburg, Germany, for providing the chikungunya virus strain S27. We thank Jason Otterstrom, Mariana Ruiz Silva and Tabitha Hoornweg for technical assistance and helpful discussions. This work was supported by the Dutch Organization for Scientific research (NWO- Earth and Life Sciences (grant to JMS) and by the University Medical Center Groningen (grant to MKSvD-R). AMvO would like to acknowledge funding from the Dutch Organization for Scientific Research (NWO; Vici 680-47-607) and the European Research Council (ERC Starting 281098). JSB is supported by the Zernike Institute for Advanced Materials.
4.6 Appendix

Table A4.1 Effect of virus labeling on CHIKV infectivity. Viral infectivity was determined by standard plaque assay on Vero-WHO cells. The number of physical particles was measured by phosphate determination or RT-qPCR. Averages of (*) 4 different virus preparations and (#) 3 independent labeling procedures. GCP = genome containing particles. PFU = particle-forming units. Errors show SEM.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Particles/ml</th>
<th>PFU/ml</th>
<th>Particle/PFU ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified CHIKV*</td>
<td>6 ± 1 \times 10^{12}</td>
<td>1.3 ± 0.8 \times 10^{11}</td>
<td>198 ± 96</td>
</tr>
<tr>
<td>Pyrene labeled purified CHIKV*</td>
<td>9 ± 1 \times 10^{12}</td>
<td>3 ± 2 \times 10^{11}</td>
<td>125 ± 90</td>
</tr>
<tr>
<td>Purified CHIKV#</td>
<td>1.3 ± 0.7 \times 10^{13}</td>
<td>9 ± 5 \times 10^{10}</td>
<td>128 ± 69</td>
</tr>
<tr>
<td>R18 labeled purified CHIKV#</td>
<td>3 ± 1 \times 10^{11}</td>
<td>1.4 ± 0.6 \times 10^{9}</td>
<td>197 ± 66</td>
</tr>
</tbody>
</table>

Table A4.2 CHIKV fusion rate versus pH. The Table shows the data corresponding to Figure 4.1C. For the experiments in the bulk assay, the fusion half-point was found by linearly interpolating the data. For the single-particle experiments, the fusion half-point was calculated as the median fusion time point of all single events. Data are mean ± SEM of at least three independent experiments in the bulk assay, and mean ± SEM of 360-1316 studied particles per condition in the single-particle assay. Empty cells either represent values that were not detectable (bulk) or not determined (single-particle).

<table>
<thead>
<tr>
<th>pH</th>
<th>Liposomal bulk assay</th>
<th>Single-particle assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fusion half-point *</td>
<td>Fusion rate †</td>
</tr>
<tr>
<td></td>
<td>(s)</td>
<td>(1/s)</td>
</tr>
<tr>
<td></td>
<td>Fusion half-point *</td>
<td>Fusion rate †</td>
</tr>
<tr>
<td></td>
<td>(s)</td>
<td>(1/s)</td>
</tr>
<tr>
<td>4.5</td>
<td>1.67 ± 0.03</td>
<td>0.601 ± 0.008</td>
</tr>
<tr>
<td>4.7</td>
<td>1.58 ± 0.08</td>
<td>0.63 ± 0.04</td>
</tr>
<tr>
<td>4.9</td>
<td>1.8 ± 0.3</td>
<td>0.55 ± 0.07</td>
</tr>
<tr>
<td>5.1</td>
<td>1.9 ± 0.2</td>
<td>0.52 ± 0.04</td>
</tr>
<tr>
<td>5.3</td>
<td>1.9 ± 0.2</td>
<td>0.52 ± 0.05</td>
</tr>
<tr>
<td>5.5</td>
<td>1.7 ± 0.3</td>
<td>0.59 ± 0.08</td>
</tr>
<tr>
<td>5.6</td>
<td>1.70 ± 0.07</td>
<td>0.59 ± 0.03</td>
</tr>
<tr>
<td>5.7</td>
<td>2.3 ± 0.3</td>
<td>0.44 ± 0.06</td>
</tr>
<tr>
<td>5.8</td>
<td>2.7 ± 0.3</td>
<td>0.38 ± 0.04</td>
</tr>
<tr>
<td>5.9</td>
<td>4.1 ± 0.8</td>
<td>0.25 ± 0.05</td>
</tr>
<tr>
<td>6.0</td>
<td>22 ± 4</td>
<td>0.046 ± 0.007</td>
</tr>
<tr>
<td>6.1</td>
<td>17 ± 6</td>
<td>0.06 ± 0.02</td>
</tr>
<tr>
<td>6.2</td>
<td>11 ± 10</td>
<td>0.09 ± 0.08</td>
</tr>
<tr>
<td>6.4</td>
<td></td>
<td>9 ± 5</td>
</tr>
</tbody>
</table>

* The fusion half-point is defined as the time point at which 50% of the fusion measured at 60 seconds is reached.
† The fusion rate is defined as the inverse of the fusion half-point.
Figure A4.1 CHIKV fusion extent versus total concentration of lipids used in liposomal bulk assay. Membrane fusion was measured at pH 5.0 in the presence of increasing concentrations of liposomes consisting of PC:PE:SPM:Chol in a molar ratio of 1:1:1:1.5. Fusion extent was normalized to the value at the concentration used in the main article (400 µM). Error bars show SEM. At least 3 independent fusion measurements were performed.

Figure A4.2 Deconvolution of the hemifusion lag-time distribution with the pH drop signal at pH 4.7. For the single-particle experiments, time t = 0 was defined as the point where 87% of the fluorescein pH sensor signal had disappeared. At pH 4.7, the mean CHIKV particle hemifusion time (~2 s) and the time needed for acidification (0.80±0.09 s) were too similar and led to particles fusing during the pH drop. To check whether the observed rise-and-decay in fusion lag time distribution is not solely caused by the comparatively slow pH drop, we modified our fitting procedure to take the kinetics of the pH drop into account. (A) An average fluorescein signal, indicating the drop in pH in the experiment, was used to define a sigmoidal curve representing the pH as a function of time. The differential of this curve with respect to time was taken to obtain a Gaussian shape representing the pH change per time unit as a function of elapsed time (B). The Gamma distributions for specific N (C) were convoluted with the distribution from (B) to obtain the curves in (D). These were then fit to the hemifusion lag time distributions using least-squares fitting to obtain the rate parameter k. Resulting goodness of fit for different N is shown in Figure A4.3. n hemifusion data points were binned to n^{1/2} bins. (C) and (D) y-axes show frequency (s^{-1}), label was omitted for clarity. Convolution and fitting was performed analytically in Mathematica (Wolfram Research).
Figure A4.3 Goodness-of-fit of the pH-drop corrected Gamma distribution versus the parameter $N$, apparent number of steps, at pH of fusion 4.7. For each fit of the convoluted Gamma distribution for different number of steps $N$, the R-squared value was calculated. It indicates goodness-of-fit and is plotted versus $N$. The R-squared values seem to indicate that multiple, rate-limiting steps are involved in CHIKV membrane hemifusion also at pH 4.7.

Movie S1. Time series of images of the single-particle assay. For a description see Figure 4.3 and main text. To see this movie, go online.