university of groningen

Single-particle visualization of viral fusion and its inhibition by an antiviral peptide

Chaline M.M. van Aartrijk

June 27th, 2014

Submitted in fulfillment of the requirements
for the degree of Bachelor of Science in physics
at the Rijksuniversiteit Groningen.

First advisor:
Prof. Dr. Antoine M. van Oijen
Second advisor:
Dr. Thorben M. Cordes

Single-Molecule Biophysics
Zernike Institute for Advanced Materials
University of Groningen

Abstract

The common flu is caused by the influenza virus. The influenza virus is divided in several types: influenza A, influenza B, and influenza C. Each type is divided in different subtypes due to differences in their surface proteins. Here the X31 and the Puerto Rico (PR) viruses are used, both viruses are subtypes of the influenza A type.

For any virus to cause illness, it has to deposit its viral genome into the host cell by merging its membrane with the membrane of the host cell and making a pore in the membranes. The virus achieves the merging of the membranes with its surface protein, hemagglutinin. Under the right conditions, hemagglutinin can bind to the membrane of the host cell and pull them together. If the membranes are close enough they can fuse. After fusion a fusion pore will be created so that the viral genome can enter the cell, be replicated, support propagation of the virus, and cause illness.

Virus fusion can be visualized by fluorescence microscopy. To achieve this, the viruses will be labeled with fluorescent molecules. After excitation by laser light, they emit light of a red-shifted wavelength which will be imaged with a CCD camera using a so-called Total-Internal-Reflection Microscopy (TIRF) microscope. With the TIRF microscope is a part of a flow channel observed. In this flow channel is a lipid bilayer membrane formed. Viruses will bind to the lipid bilayer and when the pH in the flow channel is lowered the viruses can fuse with the lipid bilayer. When the viruses fuse the intensity of the emitted light will increase. This way virus fusion is visualized.

By inhibiting virus fusion, the virus cannot cause illness anymore. The Baker lab at the University of Washington, Seattle (U.S.A.) designed an antiviral peptide to inhibit fusion of the Puerto Rico (PR) virus. To see whether the antiviral peptide indeed inhibits fusion of the PR virus, it will be added to the virus and visualized the same way as without the peptide. If the peptide indeed inhibit fusion, multiple questions will arise. For example: how many of these antiviral peptides are necessary to inhibit fusion? What is the dependence of the number of antiviral peptides to the fusion percentage? To be able to answer these questions the antiviral peptide will be labeled with a fluorescent molecule. This fluorescent molecule is excited with a different laser and emits a different color light than the one used for the virus. From the intensity of one fluorescent molecule on an antiviral peptide and the intensity that arises from the fluorescent molecule(s) of the antiviral peptide(s) that are attached to a virus particle, the number of antiviral peptides on the virus can be determined. With the number of peptides on the virus and the knowledge of whether the virus fused or not, conclusions of the number of peptides necessary for fusion inhibition can be drawn.

Contents

Introduction

Influenza A virus

Who did not get ill because of the flu? Having a fever, coughing all day, having a runny nose. Every year people get ill because of the flu, but the major concern is not only illness. In 2009 the influenza A (flu) virus is estimated to have approximately caused: 60.8 million cases of illness, around 275 thousand hospitalizations, but also over 12 thousand deaths in the United States[1] alone. During the pandemic in 1918 at least 50 million people died because of the flu, which is more than the number of people that were killed in the First World War[2]. Influenza A causes deaths every year, not only in humans but the virus is found in ducks, chickens, pigs, whales, horses and seals[3].

X31 (A/Aichi/68-X31) and Puerto Rico (PR, A/Puerto Rico/8/34) are two subtypes of the influenza A virus. The difference can be found in their surface proteins. The influenza A virus has two surface proteins: hemagglutinin (H) and neuraminidase (N)[3]. X31 is a H1N1 virus and PR is a H3N2 virus. Because of their differences in their surface proteins the viruses will bind to different receptor proteins in the membrane.

Viral membrane fusion

To cause illness, virus particles have to infect cells with their viral genome. Figure 1 shows a virus particle that binds to a receptor protein on the membrane of the host cell. After binding the cell will 'eat' the virus particle by endocytosis. The virus is now inside the cell but still surrounded by the cell membrane. The cell lowers its pH to pH 5 inside the endosome to digest the endosomal content. However, the low pH triggers the virus to fuse with the endosome membrane, after fusion the viral genome will enter the cell[4].

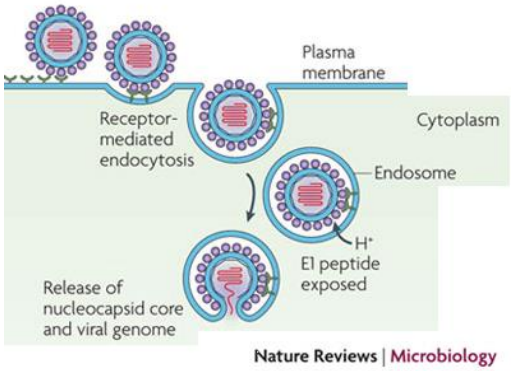


Figure 1. the alphavirus infection pathway, fusion of the virus with the endosome. Figure adapted from reference 4.

The influenza virus enters cells by clathrin-mediated endocytic pathways and will be in an endosomes[5], like the alphavirus infection pathway in figure 1. After lowering of the pH in the endosome, the virus membrane will fuse with the endosome membrane. This fusion is expected to follow the proposed mechanism for viral fusion proteins, as shown in figure 2. Hemagglutinin plays the key role in this proposed

mechanism for viral fusion. Hemagglutinin itself is a homotrimer, but as discussed in Floyd, et al.[5] the virus needs probably 3 or 4 hemagglutinin homotrimers to establish viral fusion. Figure 2 shows the hypothesized steps of viral fusion of two homotrimers. Hemagglutinin has an inactive and active state. The inactive state, denoted as HA0, is formed to prevent fusogenic properties from being present and the active state, denoted as HA1 and HA2, is formed to establish viral fusion.

Figure 2A visualizes the virus membrane and cell membrane before lowering the pH, this is called the prefusion state. When the pH is lowered protons will bind to the hemagglutinin which induces its conformational change. The hydrophobic N-terminus will bind to the cell membrane when the hemagglutinin is stretched out. This is the extended intermediate state (figure 2B). Then the hemagglutinin will collapse or "fold-back". The "fold-back" forces the two lipid bilayers to come together (figure 2C) and facilitates the proximal leaflets to fuse into a hemifusion stalk state (figure 2D). As the distal leaflets merge by subsequent rearrangements of the HA2 transmembrane domain[6], a fusion pore will be created (figure 2E). The fusion pore allows the viral genome to enter the cell.

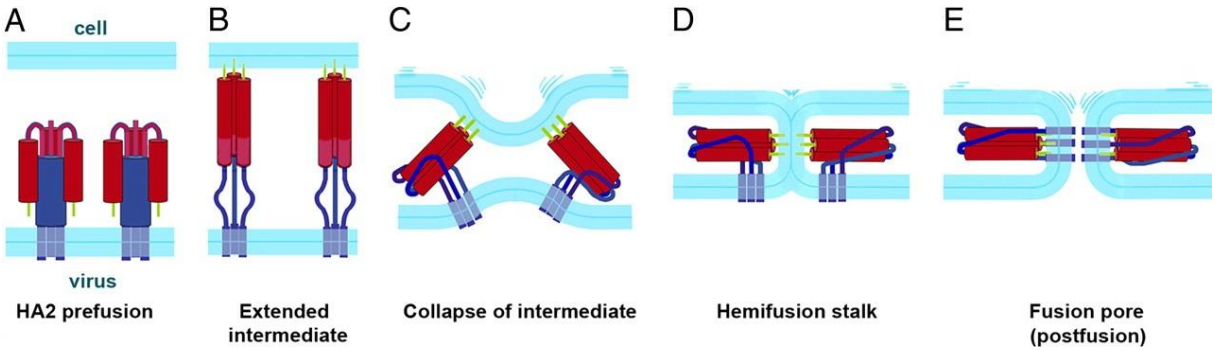


Figure 2. Proposed mechanism for viral fusion proteins. (A) In the prefusion state, the protein, anchored in the viral membrane by a C-terminal transmembrane segment, folds so that the fusion peptide (green) is sequestered. (B) A ligand-dependent trigger (e.g., proton binding, for H and many other viral fusion proteins) induces a conformational change in which the fusion peptide projects toward the target membrane, forming an extended intermediate that bridges the two membranes. (C) The intermediate collapses and brings the two bilayers together. (D) The collapse leads to formation of a hemifusion stalk where the proximal leaflets are fused. (E) By fusion of the distal leaflets a fusion pore opens up. Figure adapted from reference 5.

Inhibition with the antiviral HB36.5 peptide

When the viral genome enters the cell it will be replicated, supporting the propagation of the virus, and causing illness. Since the influenza A causes a lot of illness and even deaths, it is desired to prevent infection of this virus. Nowadays the way to prevent for infection is to vaccinate people. But there is a problem with vaccinations, only antibodies for the vaccinated viruses are made. When a mutated form of the virus enters the body, it will not be recognized. New antibodies will be made, but in the meantime the virus will infect cells and cause illness. Since virus mutation is not rare, a better prevention is desired.

Antibodies will prevent infection by inhibiting virus fusion. This way, the virus is not be able to let its viral genome enter the host cell and cannot cause illness. But antibodies have a major disadvantage. Antibodies will bind to the head region of the hemagglutinin (HA). Which, as mentioned before, is highly variable and therefore only inhibits viruses with the same head region. To overcome this problem antiviral stem-binding peptides are designed in the Baker lab at the University of Washington, Seattle (U.S.A.). The stem of the HA, because of the crucial role that it plays in the

conformational change, is not so variable, in time and maybe also between strains. In Fleishman, et al.[7] is discussed how they computationally designed different inhibition peptides and how they designed several of the peptides. Figure 3 shows the computational design of the binding of the HB36 peptide to the HA2.The designed peptides have been tested in an ensemble-averaging bulk experiment and one of the peptides that showed inhibition of fusion is the HB36.5 peptide. This peptide is studied here with the single particle technique.

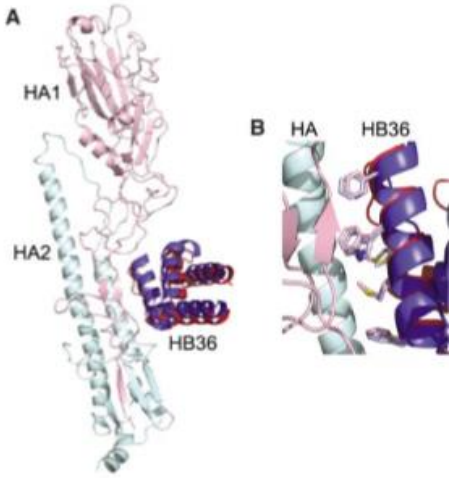


Figure 3. Computational design of HA2 – HB36 binding. (A) Shows where the HB36 peptide will bind to the hemagglutinin. (B) Zoomed in on the binding of HB36 and hemagglutinin. Figure adapted from reference 7.

Why looking at single virus particles?

From the ensemble-averaging, bulk experiments it is known that the HB36.5 peptide inhibits fusion, but besides that, the bulk experiment will not tell much more about what is going on and how it works. When looking at single particles it reveals a lot more details about the inhibition. For example, whether a virus particle is or is not bound with peptide, with approximately how many peptides it is bound, and the relation of the amount of peptide to the amount of fusion. Furthermore it can give more insight in how many hemagglutinin homotrimers the virus needs to establish fusion. Especially the last example is something that a lot of people are trying to figure out. So looking at inhibition of single virus particles may resolve some big questions about virus fusion.

Materials and methods

TIRF microscope

The visualization of viruses is done with the Total Internal Reflection Fluorescence (TIRF) microscope shown in figure 4. The TIRF microscope makes use of the fact that when light is totally reflected, a very thin layer, about 100 nm, is illuminated because of quantum tunneling[8]. This means that about 100 nm above the glass coverslip is illuminated. Therefore only the surface region of the glass coverslip will be visualized which ensures a low background.

The viruses are visualized using a 60x oil immersion objective on an Olympus IX-71 inverted microscope. The TIRF microscope is equipped with 488 nm (blue light) and 561 nm (green light) (*Sapphire models, Coherent Inc.*) lasers. The lasers are aligned in objective-based Total Internal Reflection (TIR) mode. The alignment of the laser beams is such that the bundles overlap when they undergo TIR. With the light that quantum tunnels through the glass coverslip, fluorescent molecules are excited. With the laser that radiates blue light the fluorescent molecules fluorescein and AF488 are excited. The AF488 molecules are attached to the peptide so that the peptide can be visualized. Both fluorescent molecules will emit green light after excitation. The other laser, that radiates green light, will excite the fluorescent R18 molecules. The R18 molecules are in the virus membrane. These molecules will emit red light after excitation. With a TIRF microscope and fluorescent molecules, particles and molecules that are too small to be seen with a “normal” microscope can be seen. Although the spots seen with the TIRF microscope are still diffraction-limited.

Figure 4. The Total Internal Reflection Fluorescent (TIRF) microscope setup as used during the experiments.

Visualization

To visualize the fluorescence coming out of the TIRF microscope an electron multiplier - charge coupled device (EM-CCD) camera (*Hamamatsu Photonics K.K.*) is used. Figure 5 shows schematically the setup. The blue and green light coming out of the lasers is overlapped. Then they enter the microscope objective and after excitation green and red light is emitted. The emissions are separated and simultaneously visualized on either half of an EM-CCD camera. The camera can use either a home-built or commercial dual-view (*Photometrics, Tucson, AZ, USA*) system. The left half of the camera is called the green channel, because the fluorescent molecules that emit green light are shown on this half and the right half is the red channel, because the fluorescent molecules that emit red light are shown on this half.

During visualization the EM-CCD camera shows a view of 512pixels x 512pixels in MetaVue (*MetaVue Research Imaging Software Inc.*). Since the EM-CCD camera shows a dual view, the observed field of view is 256pixels x 512pixels. The size of one pixel is 16µm x 16µm which gives a real width of $2.7*10^{-7}$ m (pixel size divided by objective magnification). The settings of the MetaVue software used are: 200 ms exposure time, and 255 EM gain. Movies of 800 frames are recorded, unless indicated differently, which corresponds to a movie of 160 seconds. The duration of the movie is determined by the number of frames times the exposure time of one frame.

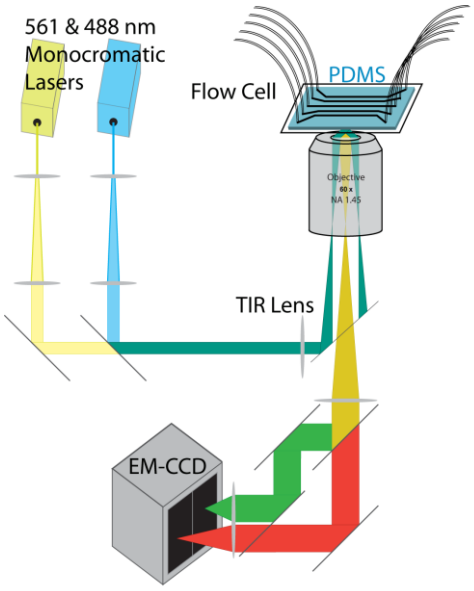


Figure 5. Setup as used to excite and collect the emission of the fluorescent molecules in the flow cell. The 488 nm and 561 nm excitation lasers are combined and excite the sample by the evanescent wave from TIRF. The resulting fluorescence is split into two color channels, each projected on a half of the EM-CCD camera to allow for simultaneous imaging. Figure adapted from J. Otterstrom.

PDMS microfluidic flow channel with tubing

The field of view of the camera will show a part of a flow channel molded in a piece of polydimethylsiloxane (PDMS). The PDMS lays on the glass coverslip above the objective (figure 5). In a piece of PDMS are five flow channels molded. The size of a PDMS piece is 24mm x 24mm x 10mm and each channel has a rectangular form of 0.2mm x 0.5mm and being roughly 1 cm in length. The channels will come out of the PDMS, which is denoted as the top side. The bottom side of the PDMS flow channel will be placed on the glass coverslip with the same size as the PDMS piece. The PDMS flow channel with coverslip will be placed into a holding clamp and placed in the holder above the microscope objective.

One end of the flow channels will be connected to PE-20 tubes and the other end to PE-60 tubes as shown in figure 6. The 20 and 60 refer to the area of the tubes, with the PE-60 having an tube area that is three times higher than the tube area of the PE-20. The other ends of the PE-20 tubes will be placed into a Eppendorf tube with the solution that is desired to flow through the channels. The other ends of the PE-60 tubes are attached to a valve manifold so that one channel at a time can be used. The valve manifold is connected to a pump, with the pump the solution in the Eppendorf tube is pulled into the flow channels.

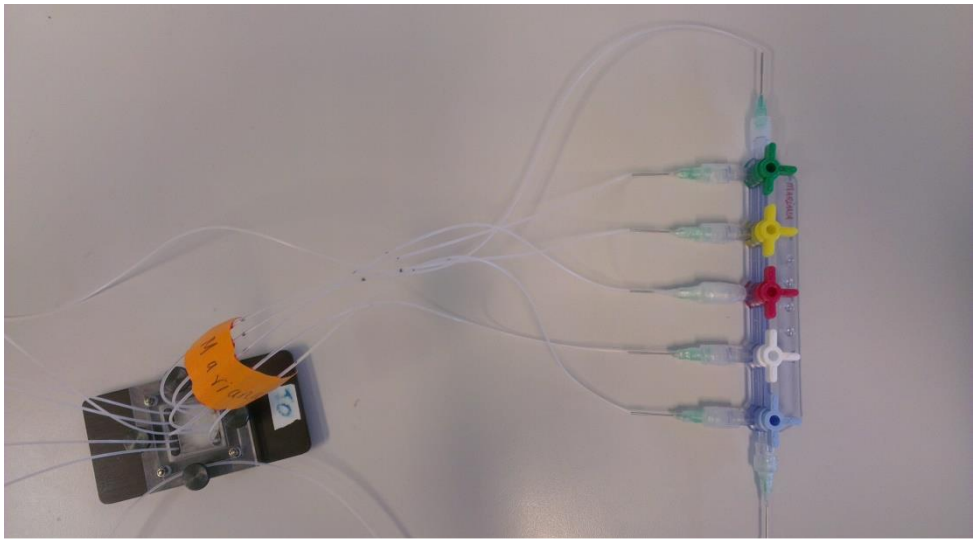


Figure 6. The piece of PDMS with five flow channels on a glass coverslip in its holding clamp. On the left the PE-20 tubes coming in the PDMS and on the right the PE-60 tubes attached to the valve manifold.

The syringe pump

The solutions in the Eppendorf tubes are pulled into the flow channel by a syringe pump (*New Era Pump Systems, Inc.*), figure 7. The solutions in the Eppendorf tubes are the liposomes, fluorescein, viruses (with or without peptide), and HNE which is mostly water. HNE is used to wash residual liposomes, fluorescein and virus out of the flow channel. To pump the solutions out of the Eppendorf tubes into the flow channel the syringe pump is withdrawing. The syringe pump can also pump, then the solution is pushed back into the Eppendorf tube. This is only used when there is an air bubble in the PE tube. An air bubble might destroy the lipid bilayer in the flow channel, so when an air bubble went through the flow channel, the flow channel cannot be used anymore.

The pump is used at a withdrawing speed of 100 µl/min. The pump is only withdrawing at a speed of 300 µl/min when the solution with pH 4.5 has to go into the flow channel. This solution with pH 4.5 is used to make the viruses fuse, and the pH drop is observed by the disappearance of the fluorescein signal. The withdrawing speed is high to prevent for a gradual pH drop. By withdrawing too fast the lipid bilayer may be destroyed. To complete the experimental setup, the liposomes, fluorescein, and virus with or without peptide has to be pulled into the flow channel and during recording the movie the pH 4.5 solution will be pulled into the flow channel to cause fusion.

Figure 7. The syringe pump attached to the PE-60 tube which is leads to the taps.

Liposomes

In order for the virus to stay in the flow channels there has to be a lipid bilayer membrane. This lipid bilayer membrane will be formed out of liposomes. The liposomes are made following protocol 1 (see Appendix A). Different lipids are dissolved in chlorophorm and, after the evaporation of chloroform, they are resuspended in HNE. Since HNE is water mostly and the nature of lipids amphiphilic, with their head hydrophilic and tails hydrophobic, they will spontaneously form micelles and bilayered liposomes. The X31 virus is able to bind to an incorporated ganglioside receptor membrane, but the PR virus requires an specific receptor, the sialoglycoprotein glycophorin A. This receptor protein will be added to the liposomes following protocol 2 (see Appendix A). After liposome preparation they will be incubated in the flow channel. During the incubation the liposomes will be absorbed on the glass surface and after that they will form a planar lipid bilayer membrane through spontaneous rupture.

Labelling of the virus and HB36.5 peptide

To be able to observe the virus and the HB36.5 peptide with a TIRF microscope, they have to be labeled with fluorescent molecules. The virus will be labeled with the octadecyl rhodamine b (R18) fluorescent molecule, this molecule will reside in the membrane of the virus with its hydrophobic tail. The HB36.5 peptide will be labeled with Alexa Fluor 488 (AF488). The labelling of the virus is done with protocol 3 (see Appendix A) and the labelling of the HB36.5 peptide with protocol 4 (see Appendix A).

Analyzing with MATLAB

When the movies are recorded they have to be analyzed. The amount of viruses that fuse and their associated fusion time will be analyzed. This is done with a home built MATLAB file (which can be found in reference 9). It determines the intensity within a circle that is fitted around a virus particle and displays the intensity of the circle in the time. When a virus particle fuses the intensity of the emitted fluorescence will increase, as can be seen in figure 9. The time at which the particle fuses is analyzed by hand and is denoted as hemifusion time.

When the number of fusing particles and all hemifusion times of similar experiments are known, they can be combined to visualize them. The visualization will be in a bar graph. The data is binned and the number of bins is determined by the square root of the number of fusing particles. The number of hemifusion events in each bin is determined and denoted as counts. And at last the number of counts divided by the total number of counts divided by the bin width is determined, this gives the frequency.

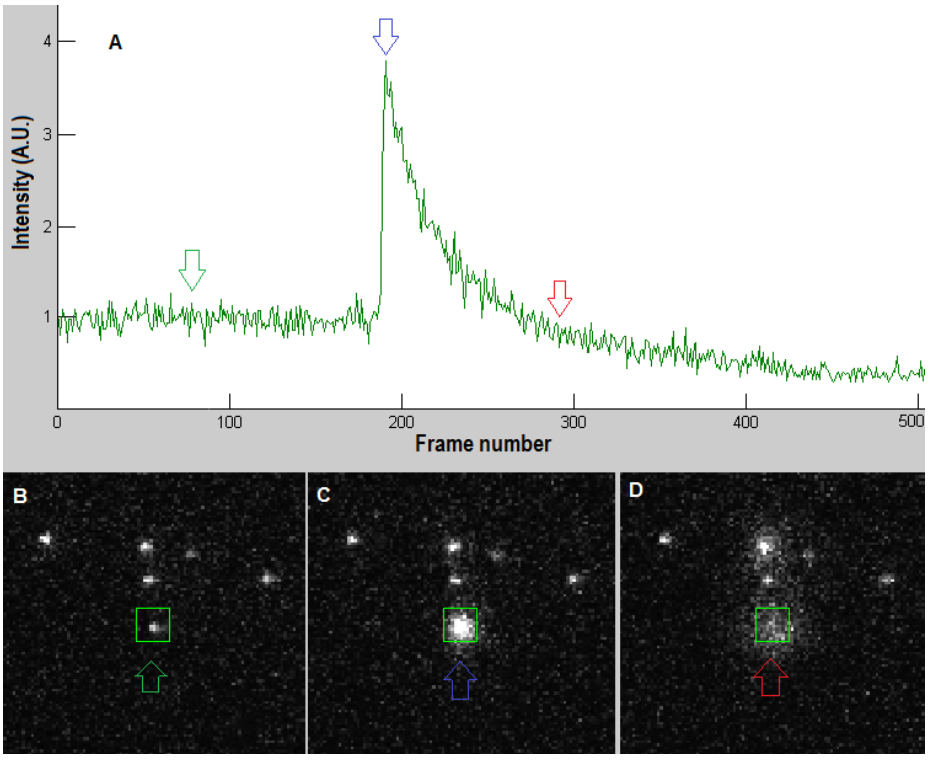
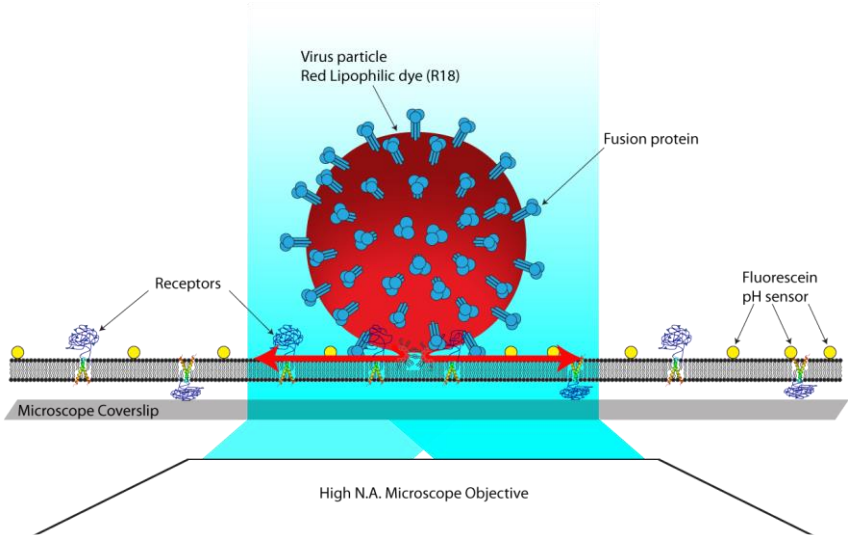


Figure 8. A fusing PR virus. (A) The intensity spectrum of the tracked particle. The tracked particle in (B) before hemifusion, in (C) during hemifusion (dequenching) and in (D) after hemifusion. The arrows in (A) corresponds with the same colored arrow in (B), (C), and (D).

Fusion experiment

The labeled viruses, with or without peptide, are immobilized by binding to the specific proteins in the lipid bilayer. The lipid bilayer is formed out of liposomes in the flow channel. When the bilayer is formed, fluorescein-labeled streptavidin is pulled into the flow channel. It will bind to the clean glass and is seen in the 'red channel'. After the fluorescein, the viruses, with or without peptide, are pulled into the flow channel so that they can bind to the lipid bilayer.

With the TIRF microscope is a part of the flow channel observed. Since the virus particles will not fuse at normal pH, but at a lower pH, the pH will be lowered by flowing a citric acid buffer with pH 4.5 through the flow channel. The pH drop is shown by the disappearance of the fluorescein signal. After the pH drop the virus particles might or might not fuse. When the virus does fuse the lipids of the lipid bilayer will mix with the lipids of the membrane of the virus (figure 9). The merging of the membranes will be seen as an increasing intensity (dequenching) of the membrane dye. This is because the space between the R18 dye on the membrane increases, before the lipid mixing the R18 dye extinct each other, so when the space between the R18 molecules increase the intensity of the signal will increase as well. By recording the flow channel for 160 seconds, starting just before the pH drop, the intensity of the virus particles can be tracked and analyzed (figure 8). The pH drop will be set as time t=0. Further analysis is done with MATLAB, ImageJ, OriginPro, and Microsoft EXCEL.



Figuur 9. Virus immobilized onto a glass supported lipid bilayer. Figure adapted from J. Otterstrom.

Results

Before using the single-particle approach to study the effect of the antiviral HB36.5 peptide on the influenza A virus, the approach was used to study the fusion kinetics of the influenza A virus without the antiviral peptide. First the X31 virus was studied, the kinetics of the X31 virus are studied before and reported by Floyd, et al.[5], then the PR virus without peptide was studied and as last the PR virus with the HB36.5 peptide. When the PR virus with the HB36.5 peptide was studied, two different HB36.5 peptide concentrations were used to see their effect. When the HB36.5 peptide was added, there was always at least one channel used to check if fusion would take place for the virus without peptide. The results of each single experiment can be found in table 1, 2, 3, and 4 in Appendix B.

The X31 virus

In the first experiment two channels were observed for fusion of the X31 virus. The number of frames recorded during this experiment were 600 instead of 800. In the two channels a total of 331 viruses were found of which 199 fused, this gave a fusion percentage of 60.1%. The results of the single channels can be found in table 1 in Appendix B. To visualize the fusion kinetics, when and how many viruses fuse, a bar graph was made (figure 10). Figure 10 shows on the x-axis the time after which the virus fuse (hemifusion time) and on the y-axis the frequency (discussed in Materials and Methods, analyzing with MATLAB). The bar graph was fitted with the gamma distribution. The gamma distribution, discussed in reference 10, enables the study of the not directly observable rate-limiting steps. It fits the number of steps and the rate at which each step occurs. The gamma distribution in figure 10 has a fixed number of steps, which equals 3. After that the gamma distribution determined a rate of 0.28 s^{-1}, which is in agreement with Floyd, et al.[5].

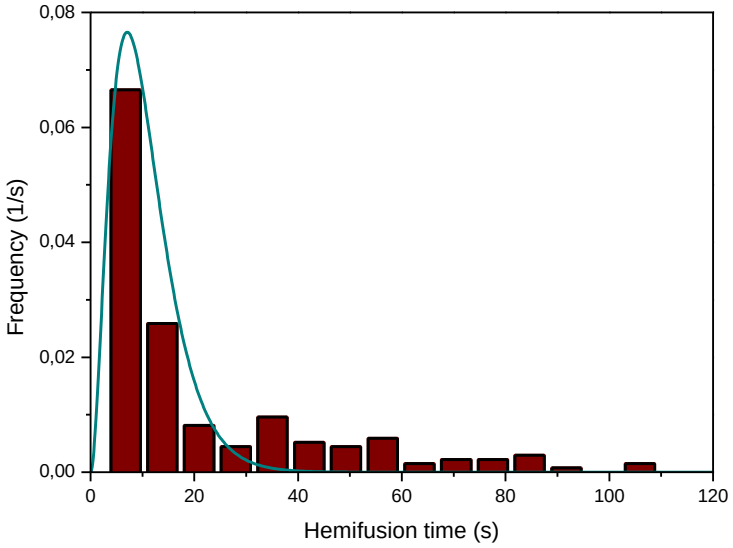


Figure 10. Distribution of 199 hemifusion times of the X31 virus. A gamma distribution is fitted in the bar graph. This fit had a fixed number of steps which equals 3 and determined a rate of 0.28 s^{-1} at which each step occurs.

The PR virus

Then the experiments with PR virus was done. A total of six flow channels were observed and analyzed. Now were 800 frames recorded. The viruses were observed for a longer period of time, because less is known about the hemifusion times of this virus. In the six flow channels a total of 1128 viruses were found of which 483 fused, this gave a fusion percentage of 42.8%. The results of the single channels can be found in table 2 in Appendix B. Figure 11 shows the distribution of the hemifusion times of the 483 fusing viruses. With this data a gamma distribution is fitted through the bar graph. The fitting resulted in a number of steps of 1.5 and a rate at which each step occurs of 0.037 s^{-1}.

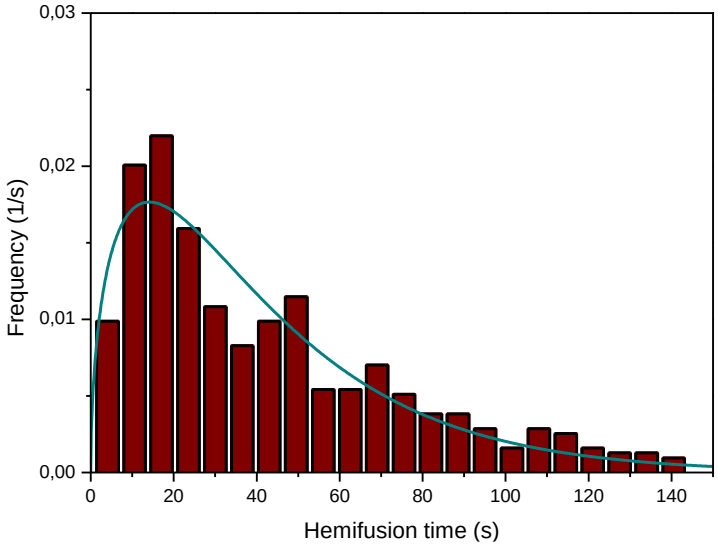


Figure 11. Distribution of 483 hemifusion times of the PR virus. A gamma distribution is fitted in the bar graph. Resulting in 1.5 as the number of rate limiting steps and 0.037 s^{-1} as the rate at which each step occurs.

The PR virus with antiviral peptide

To inhibit the fusion of the PR virus, the HB36.5 peptide was added to the virus. For the high peptide concentration the amount of peptide added to the virus was way above the dissociation constant (K_D) of the peptide. This way inhibition of fusion should easily be seen when the peptide is able to inhibit. The K_D is determined by the concentration of unbound hemagglutinin times the concentration of unbound peptide divided by the concentration that is bound in the solution (equation 1).

$$K_D = \frac{[HA]\,[HB36.5]}{[HA{+}HB36.5]} = 0.9\ nM \qquad [1]$$

The virus with peptide was studied for two different peptide concentrations, both way above the K_D. First the virus with a peptide concentration of 149 nM was studied and second the virus with a peptide concentration of 74.9 nM. The 149 nM concentration will be denoted as high peptide concentration and the 74.9 nM concentration as low peptide concentration. For the high peptide concentration there were 1.88 peptides per HA in the solution, which should lead to a binding of 99% of

all HA's with a peptide. For the low peptide concentration this were 0.94 peptides per HA, which should lead to a binding of 87% of all HA's with a HB36.5 peptide. This was determined from an estimated virus concentration of 0.2 nM, the assumption that there are 400 HA's per virus particle, and the known peptide concentrations.

Six flow channels were observed and analyzed for the high peptide concentration. In total 1303 viruses were found of which 82 fused, this gave a fusion percentage of 6.3%. The results of the single channels can be found in table 3 in Appendix B. Figure 12 shows the distribution of hemifusion times of the 82 fusing viruses. Because just 82 viruses fused and they seems not to follow the gamma distribution, no gamma distribution was fitted through the bar graph.

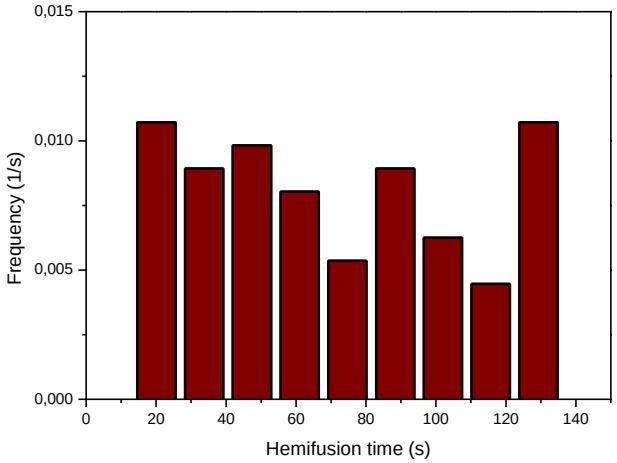


Figure 12. Distribution of 82 hemifusion times of the PR virus with HB36.5.

Then the low peptide concentration was studied. For the low peptide concentration only two flow channels were observed and analyzed. In total 447 viruses were found of which 92 fuse, this gave a fusion percentage of 20.6%. The results of the single channels can be found in table 4 in Appendix B. The fusion percentage of the low peptide concentration is more than threefold higher than for the high peptide concentration. Figure 13 shows the distribution of hemifusion times of these 92 viruses. The number of fusing viruses is again really low and again they not seems to follow the gamma distribution, so no gamma distribution is fitted through the bar graph.

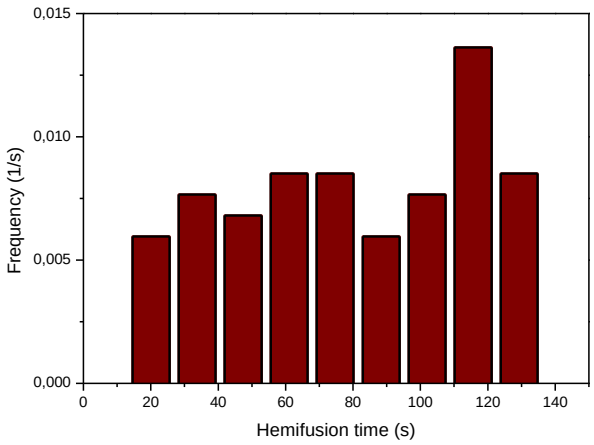


Figure 13. Distribution of 92 hemifusion times of the PR virus with HB36.5.

Besides the PR virus and the corresponding fusion percentage, there was also looked at the HB36.5 peptide which was visible on the other half of the EM-CCD camera. The MATLAB program tracked the intensity of this half of the EM-CCD camera as well. The circles of which the intensity is determined was relatively on the same place for both halves of the camera. To determine the number of peptides bound to the virus from the intensity, the intensity of one peptide has to be measured. This was done by diluting the HB36.5-AF488 solution with HNE to 1:150 000, then looking at a droplet of the dilution with the TIRF microscope and recording a time lapse movie. The time lapse made every half a second a frame for ten minutes long. In total 1200 frames were recorded and each frame had an exposure time of 200 ms.

Before the intensity of the spots were determined, the intensities of the frames of the time lapse movie were average, shown in the left picture of figure 14. This was done to reduce the background signal. After that, corrections were made for the imperfections in of the field of view and from here on the intensity of the HB36.5-AF488 could be measured. Figure 14 shows the image of the field of view before the corrections in the left picture, and after the corrections in the right picture. Figure 15 shows a zoom-in from figure 2 in Appendix B. The figure shows the deviation of the intensities of the spots in the right picture of figure 14. Multiple peaks seems to appear, but it is expected that the first peak corresponds to the intensity of a single AF488 bound to a single peptides. The line fitted through the first peak determines the average intensity of a single AF488 bound to a single peptides. The determined average intensity was 116.7 A.U.

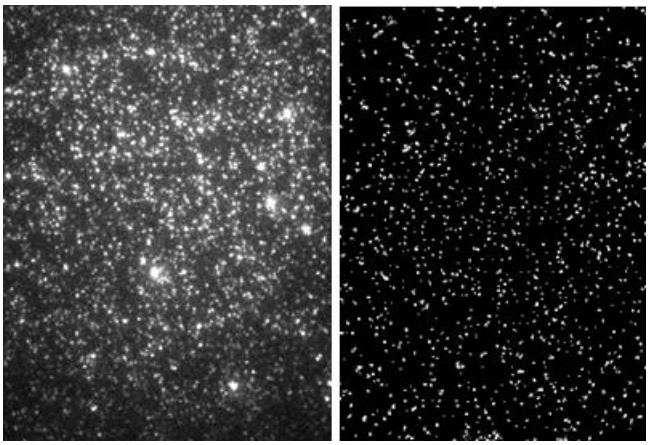


Figure 14. The measured intensities for HB36.5 bound to AF488 before (left) and after (right) corrections to reduce the background and imperfections in the field of view. The peak in figure 14 represents a single HB36.5 bound to a single AF488.

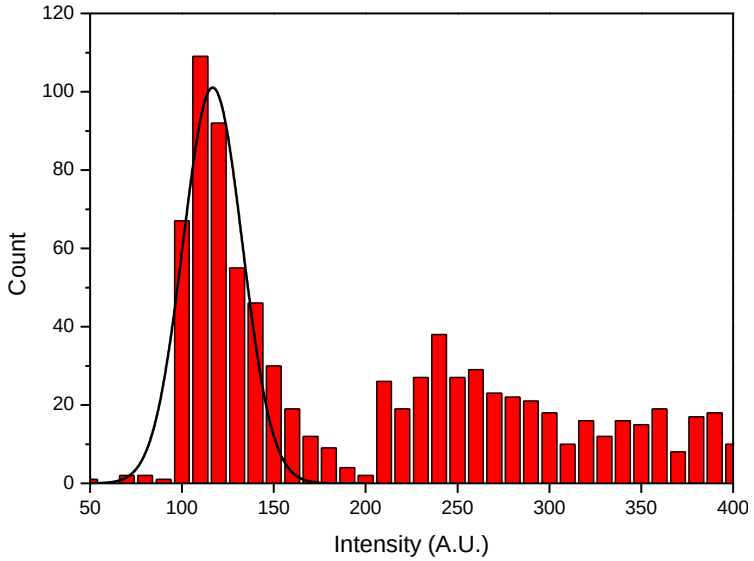


Figure 15. The intensities for (single) HB36.5 bound to AF488 after corrections for background and imperfections in the field of view. The peak lays at an intensity of 116.7 A.U. with 101 counts.

With the average intensity determined in figure 15 and the intensity of the peptide(s) bound to the virus, the number of peptides bound to the virus was measured. Figure 16 shows the average number of peptides bound to the virus plotted against the hemifusion efficiency. The most left point gives the results of the experiment without peptide, the point in the middle the results of the experiment with the low peptide concentration, and the most right point the results of the experiment with high peptide cocnentration. The outmost error bars in figure 16 were determined

by the standard deviation of the population (SE), the amount of variation from the mean. The inner error bars show the standard error of the mean (SEM).

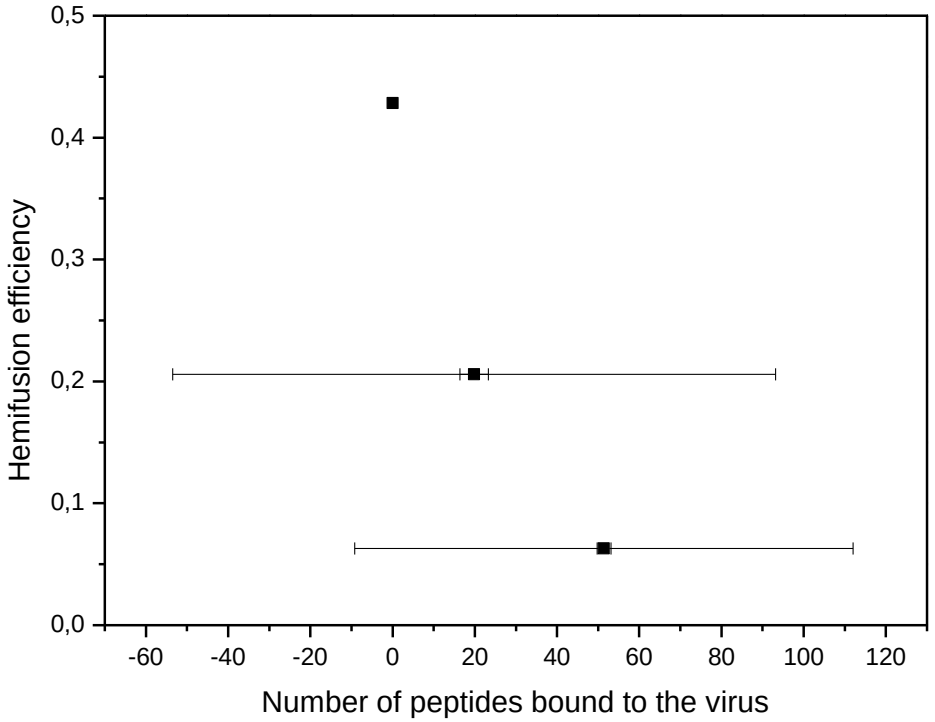


Figure 16. The number of peptides bound to the virus against the hemifusion efficiency. Left point, no peptide was added to the virus, middle point has a peptide concentration of 74.9 nM and at the right point a peptide concentration of149 nM.

To find out whether all peptides were labeled and so if all the peptides that were bound to the viruses were found or not, the percentage of labeled peptide was determined. By determining the percentage of AF488 bound to the HB36.5 peptide, a nanodrop experiment was done. With the results from the nanodrop experiment the concentration of AF488 and HB36.5 in the HB36.5-AF488 solution was determined. The nanodrop experiment measured the Optical Density (O.D.) at different wavelengths. The wavelength that gave the highest O.D. for HB36.5 and AF488 are the wavelengths that were used for determining the O.D. of the HB36.5-AF488 solution. With the measured O.D., the concentration of the samples (which must be known beforehand), and the path length, which is 1 mm, the extinction coefficient (ε) of the samples was calculated. The calculation was done with the Lambert-Beer law (eq. 2).

$$O.D. = \varepsilon * c * d \qquad [2]$$

With the calculated extinction coefficients and the measured O.D. of the HB36.5-AF488 solution, the concentration of HB36.5 and AF488 was calculated with equation 2. It turned out that the concentration of HB36.5 is about 25.2 µM and the concentration of AF488 is about 23.8 µM (see calculation 1 in Appendix B). Assuming that all the AF488 molecules that were found in the HB36.5-AF488 solution are bound to the peptide, 94.4% of the peptides are labeled with AF488.

Discussion

The X31 virus

By plotting the gamma distribution of the X31 virus without any restrictions, e.g. not fixing the number of steps,1.5 was determined as the number of steps and 0.017 s^{-1} as the rate at which each step occurs (see Figure 1 in Appendix B). Comparing this result with the results in Floyd, et al.[5], the number of steps and the intermediate transition rate is too low. But the detected number of fusing particles here, is about fivefold lower than in Floyd, et al. To be certain that there is a difference in the number of steps and the rate at which each step occurs, about the same number of particles should be analyzed. Another explanation might be that the binning of the bar graph turned out unfortunate, and therefore caused a difference.

The PR virus

Both, the X31 and the PR virus make use of the surface protein hemagglutinin, therefore it is expected that the number of steps is the same for the viruses. This is indeed the case when the number of steps for the X31 is not fixed. But since the number of steps for the X31 virus is lower than expected, there are more experiments with the PR virus necessary to find out if the number of steps will increase to 3 as is expected for the X31 virus. The rate at which each step occurs does differ between the results of the viruses. This can be explained by the fact that the surface proteins of the viruses differ and therefore the rate of the steps may be different.

The PR virus with antiviral peptide

From the results shown in table 4 (Appendix B) can be seen that the fusion percentage of the experiments on 14-05-16 is large compared to the other dates. An explanation for this might be that the virus with labeled peptide of 14-05-16 was flowed through the PD-10 and at the other dates not. This was done because when there was looked at the fluorescent signal, of the solution with labeled peptide and labeled virus, on a glass coverslip the fluorescent signal was really high. By flowing the solution through the PD-10, the unbound labeled peptide was tried to be removed, but the signal did not reduce significantly. When the peptide-virus solution was flowed into the flow channel, the fluorescent signal was too high for the camera but after washing with HNE it was fine. Therefore the virus-peptide solution did not flow through the PD-10 for the other experiments.

When looked at table 5 in Appendix B, it seems like the explanation that less peptides are bound to the virus, which would be a reasonable explanation, is not valid. But the laser intensity over the different days was inconsistent. The intensity is measured every time and it turned out to fluctuate over the days. The intensity of the lasers was higher during the experiment of 14-05-16, so this might influence the result of the determined number of bound peptides and therefore the explanation of less peptides bound to the virus is still an option and very likely.

In figure 2 in Appendix B can be seen that there are multiple peaks at different intensities. This may be caused by the aggregation of AF488 molecules or the aggregation of the peptide and since the peptide is labeled, more than one AF488 molecule is seen. By diluting the labeled peptide solution more, this problem may be overcome. Since it is expected that the first peak represents a single AF488 bound to a single peptide and what the other peaks represent is more uncertain, only through the first peak a line is fitted.

Figure 16 shows the results of the number of peptide bound to the virus plotted against the hemifusion efficiency. The outmost error bars from the standard deviation are large because of the inaccuracy of the determination. The inner error bars from the standard error of the mean are small, so the data is useful when the corrections for the background is improved. For now, the corrections that are made for the background are too rough. The rough corrections may have caused an underestimation of the intensity of some particles, that would also be an explanation for the negative running errors. That there is an underestimation is supported by the fact that expected fraction of bound HA's is 87% and 99%, which corresponds to 348 and 396 HA's bound with peptide per virus particle. But as seen in figure 16 the results gave 20 and 51 bound peptides per virus. Besides the background corrections, the inconsistency in the laser intensity (as mentioned before) and binding of unlabeled peptide to the virus, causes uncertainties in the results.

As discussed in the results part and shown in calculation 1 (Appendix B), the concentration of HB36.5 is 25.2 µM and of AF488 is 23.8 µM in the HB36.5 – AF488 solution, which is added to the virus. This would lead to 94.4% of HB36.5 to be bound with one AF488. Since the nanodrop experiment is a bulk measurement it is unknown whether all of the AF488 in the solution is bound and of the binding ratio of HB36.5 – AF488 is 1:1. It is most likely that all of the AF488 in the HB36.5 – AF488 solution is bound since the solution went three times through an Zeba spin desalting column. The Zeba spin desalting column removes unbound AF488. Furthermore the expectation is that the ratio of HB36.5 – AF488 is 1:1 since AF488 binds to N-acetylglucosaminy.

Conclusion and perspectives

The X31 virus

Carefully binning and observing more fusing X31 particles are necessary to have a better comparison with Floyd, et al.[5]. After that more conclusion can be drawn about the similarity of the results.

The PR virus

The comparison between the number of steps and the rate at which each step occurs of the X31 and the PR virus can be more precise. Then can be seen whether the number of steps stays the same and of the rate at which each step occurs stays different.

The PR virus with antiviral peptide

From the obtained results of the experiments that are done can be concluded that the HB36.5 peptide, designed in the Baker lab, does indeed inhibit fusion. It can also be concluded that when the concentration of peptide mixed with the PR virus is increased the fusion percentage is decreased. To be able to say more about the exact relation of the fusion percentage and the concentration of peptide, more experiments have to be done. With the more results can also be looked at the hemifusion times, because by adding peptide a delay in the hemifusion time of the fusing viruses may arise.

In figure 16 the number of peptides bound to a virus particle is plotted against the hemifusion efficiency. From such results, the dependence of the number of peptides bound to the virus and the hemifusion efficiency could be determined. But since the error here is so large, it is not possible with these results. From the results shown in figure 16 not much more can be concluded. But it seems like the number of peptides bound to the virus increased, and the hemifusion efficiency decreased for the high peptide concentration when compared to the low peptide concentration. A relation between the number of peptides bound to the virus and hemifusion efficiency cannot be made yet. More experiments and especially more precise signal extractions are necessary for a good estimation of the number of peptides necessary for fusion inhibition.

Finally, with the knowledge of the number of peptides necessary for fusion inhibition, more information about the number of hemagglutinin homotrimers necessary for fusion can be found. This number will give a deeper insight in how viruses establish fusion.

Acknowledgements

I thank Prof. Dr. Antoine M. van Oijen, Dr. Thorben M. Cordes and Jelle Blijleven for continuous input throughout the course of my experiments and reviewing my thesis report. Especially Jelle Blijleven for teaching me everything that I needed to know in the laboratory, the microscope room, and for guiding me. Jason Otterstrom for software support, David Baker and Aaron Chevalier from the Institute for Protein Design Harvard for designing and sending the HB36.5 peptide. Last but not least, thank you to the whole SMB group for helping me out where necessary and the great time.

References

1. S.S. Shrestha, D.L. Swerdlow, R.H, Borse, et al. Estimating the burden of 2009 pandemic influenza A (H1N1) in the United States (April 2009–April 2010). *Clin Infect Dis* **2011**; 52(suppl 1):S75–82.

2. http://www.flupandemic.gov.au/internet/panflu/publishing.nsf/content/history-1

3. http://www.cdc.gov/flu/about/viruses/transmission.htm, http://www.cdc.gov/flu/about/viruses/types.htm

4. M. Kielian and F. A. Rey. Virus membrane-fusion proteins: more than one way to make a hairpin. *Nature Reviews Microbiology* **2006**, 4(1):67–76.

5. D.L. Floyd, J.R. Ragains, J.J. Skehel, S.C. Harrison, and A.M. van Oijen. Singleparticle kinetics of influenza virus membrane fusion. *Proceedings of the National Academy of Sciences* **2008**, 105(40):15382–15387.

6. J. Otterstrom and A.M. van Oijen. Visualization of membrane fusion, one particle at a time. *Biochemistry* **2013**, 52, 1654-1668.

7. S.J. Fleishman, et al. Computational design of proteins targeting the conserved stem region of influenza hemagglutinin. *Science 332*, **2011**, 816-821.

8. http://www.olympusmicro.com/primer/techniques/fluorescence/tirf/tirfintro.html

9. J. Otterstrom, *Visualizing Influenza Virus Membrane Fusion: Inhibition and Kinetics*, PhD thesis, **2013**, Appendix 3

10. D.L. Floyd, S.C. Harrison, and A.M. van Oijen, *Analysis of kinetic intermediates in single-particle dwell-time distributions*, Biophysical Journal, **2010**, 99(2): 360-366

Appendix A

Protocol 1: Making liposomes

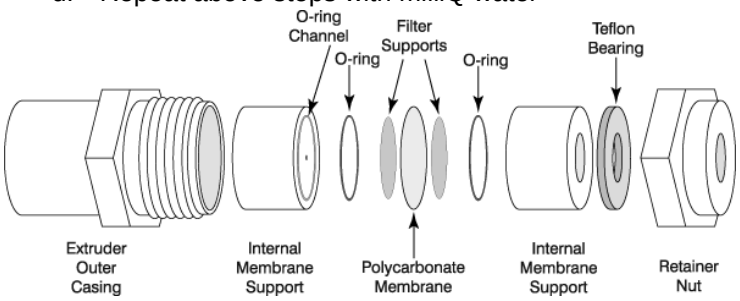
- Clean 2 test tubes ("Reagenz-Gläser") by rinsing it with chloroform 2x
- Place about 1mL chloroform in one test tube
- Get pipette in touch with chloroform from the 1st tube before pipetting the lipid. Before closing the lipid stock, let argon fall into the tube via the cap. Add all lipids this way into the 2nd tube.

Lipid	DOPC	Cholesterol	GD1a	B-PE
µl	314.4	38.6	91.8	49.0

- Evaporate all chloroform under an argon flow. Keep an eye that the lipids are not distributed all over the glass surface.
- Place the tube in vacuum for 30 minutes.
- In the meantime clean and prepare the extruder:

Cleaning the extruder:

1) Place extruder with thermometer on the heating platform: 43 ºC
2) Clean the movable parts of the extruder with ethanol and then milliQ
   a. Pull O-rings out
   b. Rinse the syringe with ethanol by sucking in and spraying out 20x
   c. Put the O-rings, plastic membrane support, Teflon bearing, outer casing in a beaker glass, fill it with ethanol, shake & spill it 2 times
   d. Repeat above steps with milliQ water



Preparing the extruder

1) Put 2 membrane supports on each plastic membrane support. Hydrate it well, dab remaining water.
2) Place larger membrane 0.2 µm in the center of plastic membrane support.
3) Place the plastic membrane support on top of each other (membrane to membrane).
4) Put the white plastic part in the metal casing.
5) Place the 2nd metal casing on top of it and screw it.
6) Place the metal casing and the syringes in the extruder, let it heat up.

- Take the lipids out of the desiccator, for opening purge with argon.
- Put 250 µl HNE on top of the lipids
- Place back into desiccator and let hydrate.
- In the meantime:
  1) Prepare liquid nitrogen in an ice barrel dim
  2) Prepare a glass of hot water
- Dissolve lipids: by pipetting it up and down.
- Purge solution with argon.
- Seal it with 6 fold folded parafilm.
- Freeze it in nitrogen (20 sec).
- Heat it in hot water (20 sec). Repeat these last two steps three times.
- To test if syringes are leaky and equilibrating with buffer: extrude with 250 µL HNE.
- Extrude the lipids by pulling the syringe back and forth 10 cycles (i.e. 20 times in total). Put the out coming substance in an Eppendorf cup.
- Extrude 250 µl HNE for 10 cycles and put the out coming by the lipids.

Protocol 2: Making proteoliposomes

- Mix lipids following protocol 1: Making liposomes, but with following modifications:
    - The lipid amounts:

Lipid	DOPC	Cholesterol	B-PE
µl	44.60	13.70	17.36

    - Place the tube for 2 hours in vacuum instead of 30 minutes.
    - Freeze thaw at least five times, instead of three times.
- Add 2.5 µl Triton-X to the liposomes.
- Incubate at 37 ºC for 15 minutes, then on ice for 15 minutes.
- Add 18.9 µl GYPA [0.5 g/l] to the lipid-detergent suspension.
- Mix on a nutator at 8 ºC for 30 minutes.
- Prepare 2 samples of Bio-beads, take 1.410 ml Bio-beads suspension and remove the solution using a fine-gauged syringe. Store on ice until used to prevent for bacterial growth.
    - Making Bio-bead solution:
    - Weigh 5 grams of Bio-beads, put it in a 50 ml falcon tube.
    - Wash 2x with methanol (~30-40 ml).
    - Wash 2x with ethanol (~30-40 ml).
    - Wash 4x with milliQ (~30-40 ml).
    - Suspend in 20 ml milliQ.
    - Keep Bio-bead solution stored at 4 ºC.
- Incubate the GYPA-Triton-lipid solution with Bio-beads for 2 hours.
- Repeat the last step.
- Store lipids in the fridge. Useful until 3 days after preparation.

Protocol 3: Labelling virus

- Take 45 µl HNE.
- Add 15 µl virus.
- Add 3 µl R18 0.2 mM in DMSO.
- Let the tube turn for 3 hours at room temperature at an angle of ±30 degrees from horizontal.
- To remove the sucrose and R18 from the virus-R18 solution.
  Start ±15 minutes before virus has finished labelling.
    - Prepare PD-10 by equilibrating with 25 ml of HNE
    - Add the 63 µl virus/HNE/R18 solution on column, let absorb completely
    - Add 2.437 ml HNE to complete one column volume
    - Prepare 8 tubes to catch fractions with
- Add 200 µl HNE on column and collect in the tube (repeat 8 times).

Protocol 4: Labelling HB36.5

- Add 30 µl of HB36.5 [169 µM] to 50 µg AF488 and dissolve the AF488 completely and mix on the nutator for 4 hours at 8 ºC.
- Equilibrate 3 Zeba spin desalting columns (0.5 ml):
    - Mark the site that goes upward in the centrifuge.
    - Remove the bottom closure, loosen the top cap and rotate for 1 minute at 1400g.
    - Place 300 µl PBS buffer on top of the Zeba spin column and rotate for 1 minute at 1400g.
    - Repeat above step 3 times.
- Place the 30 µl HB36.5-AF488 solution on the column and let absorb. Then add 15 µl PBS and rotate for 2 minutes at 1400g.
- Repeat last step 2 times with new Zeba spin columns.
- 5.5 µl of HB36.5-PBS solution is added to 250 µl virus for 1:1.88 HA - HB36.5 ratio and 2.75 µl of HB36.5-PBS solution to 250 µl virus for 1:0.94 HA - HB36.5 ratio.

Protocol 5: Cleaning

Coverslips

- Sonicate 15 min. at 30 ºC in 0.5% Triton-X.
- Sonicate 15 min. at 30 ºC in Acetone.
- Sonicate 15 min. at 30 ºC in ethanol.
- Sonicate 10 min. at 30 ºC in KOH.
- Rinse 3 times with milliQ.
- Blow dry with air stream.
- Dry in oven at 110 ºC for 1 hour.
- Plasma clean for 10 min.
- Store desiccated.

PDMS

- Sonicate 10 min. at 30 ºC in 0.5% Triton-X.
- Sonicate 10 min. at 30 ºC in $NaHCO_3$.
- Sonicate 10 min. at 30 ºC in 70% ethanol.
- Blow dry with air stream.
- Store desiccated.

Clean PE-60 tubes

- Empty the tubes with 5 ml syringe.
- Rinse with 70% ethanol, 5 ml per tube.
- Rinse with milliQ, 5 ml per tube.
- Empty the tubes.

Appendix B

Calculation 1.

	HB36.5	AF488	HB36.5 + AF488
O.D. at 280 nm	0.569	-	0.085
O.D. at 493 nm	-	0.329	0.169
Concentration (µM)	169	46.25	-

$$\varepsilon\,(280|HB36.5) = \frac{O.D.\,(280|HB36.5)}{c * d} = \frac{0.569}{169 * 10^{-6} * 0.10} \approx 33\,669\ M^{-1}cm^{-1}$$

$$\varepsilon\,(280|AF488) = \frac{O.D.\,(280|AF488)}{c * d} = \frac{0.329}{46.25 * 10^{-6} * 0.10} \approx 71\,135\ M^{-1}cm^{-1}$$

$$c\,(HB36.5|HB36.5 + AF488) = \frac{O.D.\,(280|HB36.5 + AF488)}{\varepsilon\,(280|HB36.5) * d} = \frac{0.085}{33\,669 * 0.10} \approx 25{,}2\ \mu M$$

$$c\,(AF488|HB36.5 + AF488) = \frac{O.D.\,(493|HB36.5 + AF488)}{\varepsilon\,(493|AF488) * d} = \frac{0.169}{71\,135 * 0.10} \approx 23.8\ \mu M$$

$$fraction\ bound = \frac{c\,(AF488|HB36.5 + AF488)}{c\,(HB36.5|HB36.5 + AF488)} * 100\% = \frac{23.8}{25.2} * 100\% \approx 94.4\%$$

Figure 1.

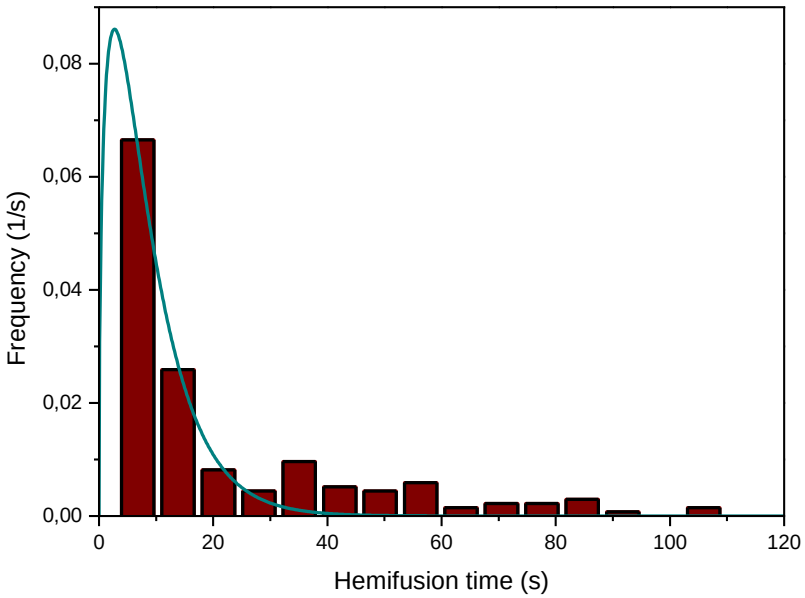


Figure 1. Distribution of 199 hemifusion times of the X31 virus. Fitted with a gamma distribution, giving 1.5 as the number of steps and 0.017 s^{-1} as the rate at which each step occurs.

Figure 2.

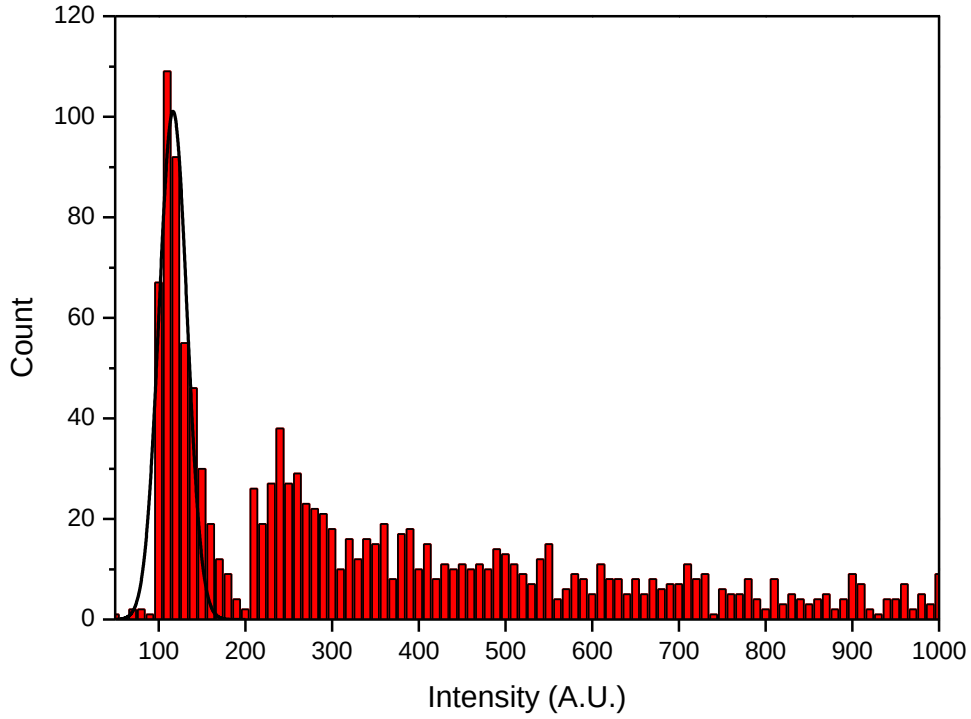


Figure 15. The intensities for (single) HB36.5 bound to AF488 after corrections for background and imperfections in the field of view. Multiple peaks can be seen, the first peak is fitted and gives an intensity average of 116.7 A.U. with 101 counts.

Table 1.

Date	14-04-30	14-04-30	Total
Number of viruses	241	90	331
Number of fusing viruses	168	31	199
Fusion percentage	69.7%	34.4%	60.1%

Table 1. Results of the X31 virus.

Table 2.

Date	14-05-16	14-05-23	14-05-23	14-05-29	14-05-29	14-05-31	Total
Number of viruses	50	305	316	281	92	84	1128
Number of fusing viruses	26	117	86	151	52	51	483
Fusion percentage	52.0%	38.4%	27.2%	53.7%	56.5%	60.7%	42.8%

Table 2. Results of the PR virus, no peptide.

Table 3.

Date	14-05-31	14-05-31	Total
Number of viruses	257	190	447
Number of fusing viruses	49	43	92
Fusion percentage	19.1%	22.6%	20.6%

Table 3. Results of the PR virus, low peptide concentration (74.9 nM).

Table 4.

Date	14-05-16	14-05-16	14-05-23	14-05-23	14-05-31	14-05-31	Total
Number of viruses	97	72	366	329	178	261	1303
Number of fusing viruses	31	20	10	7	11	3	82
Fusion percentage	32.0%	27.8%	2.7%	2.1%	6.2%	1.2%	6.3%

Table 4. Results of the PR virus, high peptide concentration (149 nM).

Table 5.

Date	14-05-16	14-05-23	14-05-31
Average number of peptide bound to the virus	67.3	48.1	50.6
Hemifusion efficiency	28.7%	2.4%	3.2%

Table 5. The average number of peptide bound to the PR virus and hemifusion efficiency per day of experiment of table 4.