# Quantification of the Removal and Inactivation of Virus Particles



## ABSTRACT

Researchers from the Netherlands have developed and demonstrated a new method of quantifying and characterizing antiviral properties of polymer-functionalized surfaces for virus filtration and inactivation. Specifically, a polyethylenimine (PEI)-coated poly(ether sulfone) (PES) micro-filtration membrane was used to adsorb, inactivate, as well as disassemble virus capsids. Using fluorescence microscopy, spectroscopy, and single particle counting, only a small fraction (1%) of intact viruses can pass through the membrane, and a large fraction of viruses became inactivated and disassembled. Not only does the virus adsorb onto the PEI coating, it also interacts with PEI to disassemble the virus capsid. This new method provides a simpler and faster quantification and characterization technique for virus filtration and inactivation in the medical and biological world.

## VIRUS INACTIVATION

In many biological fluids and water, there is a need for viruses to be removed or inactivated to ensure the liquid is not contaminated. The simple solution is to remove the viruses completely, but in situations where this cannot be achieved, viral inactivation can stop the viruses by causing them to be non-infectious. Rather than removing all the virus particles, many viruses can be inactivated by chemical alteration and can remain in the fluid after inactivation. Some processes actually disassemble the virus particles and further the inactivation procedure, rendering them harmless to the final product. Techniques for removal or inactivation are used in health and environmental research as well as the food industry. This research is crucial for water purification as well as blood tests and treatments.

Virus removal and inactivation is nothing new to the biological and medical industry, but there is minimal understanding and documentation on how some infectious particles are reduced, removed, or inactivated by functionalized surfaces. Functionalized surfaces are chemically or biologically modified surfaces which can interact with specific molecular species. Knowing how to develop polymer-functionalized surface materials and systems for virus retention is necessary for research, sample tests, and experiments; but knowing how to quantify the reduction of viruses and understanding how different functionalized materials and methods reduce or inactivate infectious particles is even more critical. Monitoring the virus-reduction or inactivation processes of these systems and filter membranes can be laborious and time consuming. Characterization and quantification of virus retention and inactivation surfaces are needed to design more advanced and effective micro-filtration membranes for critical applications in food and drug industries, vaccines, air and water purification, and viral sample extraction.

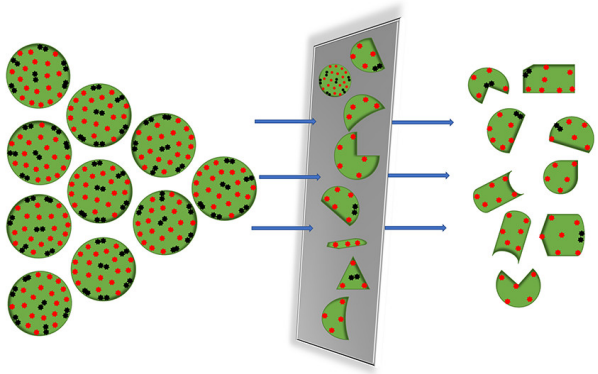

**Figure 1. Polymer-functionalized surface for removing and inactivating viruses.**[1]

## PROBLEMS AND GOALS

The most straightforward and simple solution to stopping viruses is to remove them using ultra-filtration membranes based on the size of the viruses. This can be effective, as seen in water filtration systems, but only to a certain degree of success. Large pore sizes in micro-filtration membranes enable many virus particles to move through without reduction or any inactivation. A more advanced approach is needed to improve upon this standard micro-filtration system.

A different technique uses a functionalized surface that continuously interacts with virus particles passing through. Some viruses are adsorbed on the surface while others are inactivated through interaction with the functionalized membrane. A polymer-functionalized membrane has been used for gravity or low pressure driven water filtration systems and can be used to effectively remove infective virus particles.[1]

Even with an effective functionalized membrane to counteract viruses, a simple and detailed quantification method is desired. The mechanisms behind the reduction of infectious particles from the polymer-functionalized membrane are not fully understood, and obtaining information on the quantity of viruses reduced, adsorbed, or made inactive is often complex and time-consuming.

Currently, there are multiple methods to quantify the reduction of viruses in a sample or system. End-point dilution and plaque assays are commonly used together for counting the number of infectious particles, and more simple methods include flow virometry and scanning electron microscopy for counting intact viruses but not infectivity after filtration. These methods show the total number of virus particles per volume.[1]

Other methods that measure the genetic material of viruses to determine the number of viruses, such as qPCR, do not necessarily reflect the number of infectious particles that are present as virus inactivation may result in a release of genetic material. The measured genetic materials or virus proteins, that are released as a result of inactivation from the functionalized membrane, are not infectious after the functionalized micro-filtration. Thus, these materials that are not infectious are inaccurately counted toward the virus total. Methods to accurately and easily monitor the breakdown of bio(macro)molecules such as virus capsid, or the protein shell of a virus, are challenging to find and to develop. All of these methods are experimentally complex and time-consuming and hinder the development and optimization of antiviral surfaces.[1]

## METHOD

Researchers from the Netherlands have designed a single particle counting (SPC) approach to investigate virus removal and inactivation in fluids with fluorescence microscopy and spectroscopy for observation and quantification.[1] With this design, the interactions between the functionalized membrane and the viruses are studied, specifically interactions resulting in disassembly or inactivation of the viruses. Virus removal and inactivation is demonstrated and monitored through fluorescently labeled viruses and can be tracked through changes in emission spectra and fluorescence lifetime.

For the functionalized membrane, branched polyethylenimine (PEI) was adsorbed onto poly(ether sulfone) (PES) micro-filtration membranes with pore size of 0.45µm. This membrane combination was coated in a solution of 0.52 mM PEI for functionalized modification.[1] This membrane has been shown to effectively reduce virus particles in samples, but the details are not well known or documented. The virus interaction with the PEI coating on the membrane causes the inactivation of the virus particles.

For this experiment and design, Cowpea chlorotic mottle virus (CCMV) was used with the capsid proteins of CCMV bonding with fluorophores to allow for fluorescent labeling. To count the number of viruses in the solution, the average number of attached fluorophore molecules per virus was first determined from the absorbance at 260 and 646 nm. As the CCMV does not absorb at 646 nm, the fluorophore absorption at 260 nm was taken into consideration when calculating the degree of labeling and found to be 61 fluorophores / virus.[1] With this value, the quantification of virus concentration can be discovered with SPC. An example of the SPC in both a traditional PES micro-filtration membrane and a PEI-coated PES membrane is shown in **Figure 2**. This plot shows the effectiveness of viral retention of the PEI-coated PES membrane compared to the normal PES membrane.

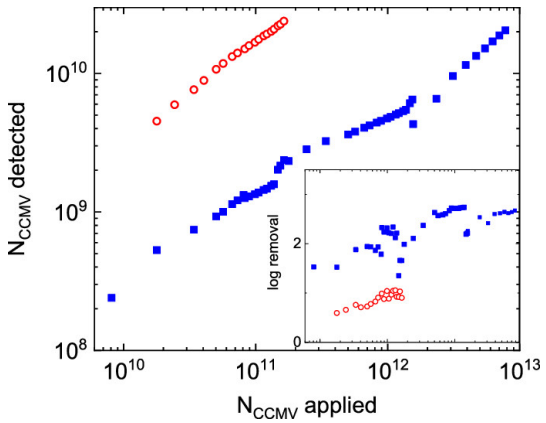


**Figure 2. CCMV removal by microfiltration membranes, quantified in single particle counting experiments. The data show the cumulative amount of CCMV particles detected in the filtrate as a function of the cumulative amount of CCMV particles that was applied to the membrane via the feed. The cumulative CCMV removal by a PES membrane with a nominal pore size of 0.45 µm is shown in red, and the removal of a PEI-coated PES membrane is shown in blue. The inset shows the log removal as a function of the cumulative number of particles applied to the membrane via the feed.[1]**

In the SPC, the fluorescence is excited using a 638 nm, 2.1 mW diode laser driven by Wavelength Electronics' LD5CHA laser driver. Diffraction limited spots from the filtered light imaged onto a camera are located and counted to determine the number of fluorescently labeled viruses. Variables such as noise and out of focus signals could create false positives, so an intensity threshold was used to counteract potential issues. The size of the virus particles is also tracked and recorded. The CCMV particles are characterized through spectroscopy using measurements of fluorescence lifetime, correlation spectroscopy, and 3D fluorescence lifetime imaging.

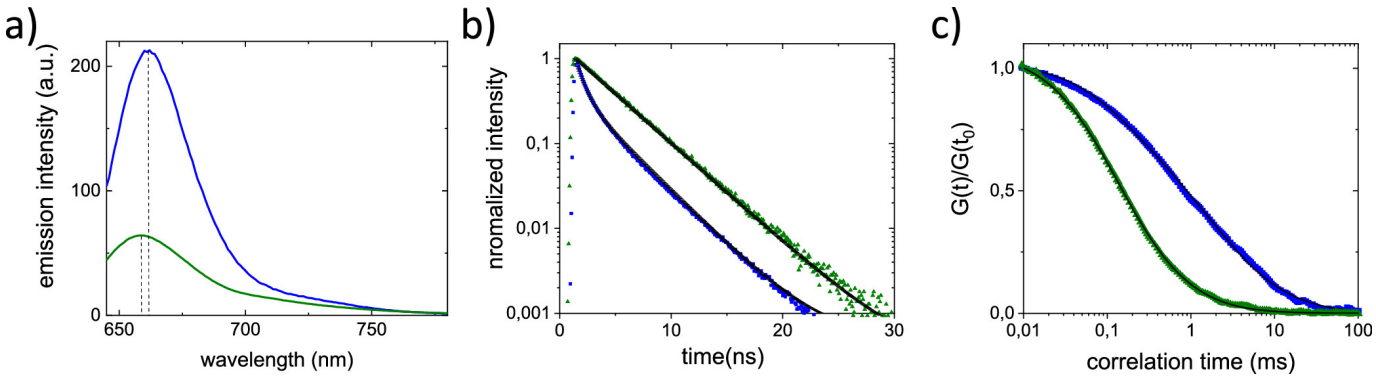


**Figure 3. Disassembly of CCMV capsids upon filtration over a PEI-coated PES membrane. (a) Emission spectra of Atto647N-labeled CCMV of the feed (blue) and the filtrate (green). The dashed vertical lines indicate the position of the emission peak maxima. (b) Peak normalized fluorescence decays of the CCMV particles in the feed (blue) and in the filtrate (green). The lines through the data points represent a double- and single-exponential fit, respectively. (c) FCS autocorrelation curves of the Atto647N-labeled viruses in the feed (blue) and in the filtrate (green). The fit to the data is shown as a line. The data were normalized to G(t) at 0.01 ms.[1]**

## RESULTS

The non-enveloped virus, CCMV, with fluorescently labeled virus capsids, was fed through the micro-filtration system in a diluted solution. Both the PES and PEI coated PES micro-filtration membrane were investigated as shown in **Figure 2**. There is a difference in fluorescence for intact virus capsids compared to disassembled capsids. This is used to characterize any inactivated and disassembled viruses after filtration. **Figure 3** shows the bulk spectroscopy experiments on labeled viruses before and after filtration and interaction with the PEI-coated membrane. Previous experiments showed the presence of PEI in the virus solution doubled the fluorescence as well as blue-shifted the peak fluorescence position by ~3 nm.[1] This ensures that the decrease in infective particles is not only an effect of virus adhering to the membrane.

Because the addition of PEI results in disassembly and inactivation of the CCMV, **Figure 3** displays the retention of the virus as well as effects on the virus integrity from the PEI-coated PES membrane. Although the peak emission intensity of the filtrate only dropped by a factor of 4 (compared to 99% of intact viruses removed by filtration during SPC experiments), the blue-shift of the peak wavelength indicates the presence of disassembled virus capsids. This is confirmed in **Figure 3**b,c with the fluorescence lifetime and correlation experiments. With the single particle tracking spectra and bulk spectroscopy spectra, both intact and disassembled virus capsids are demonstrated to be present in the solution after membrane filtration. Because the filtrate contains only 1% of intact viruses, the fluorescence can be attributed to disassembled virus capsids. If the virus capsids are either fully intact or disassembled, it is calculated that 12.5% of the viruses that were present before the micro-filtration membrane are present after the PEI-coated PES membrane in the disassembled state.[1] Because only a trivial amount of viruses are infectious in the filtrate, this method has the advantage over traditional methods and accurately reflects the number of disassembled, inactivated viruses.

Only ~13.5% of the virus capsids are found in the filtrate, correlating to a large fraction left remaining on the membrane filter. This is confirmed through fluorescence microscopy shown in **Figure 4**. **Figure 4**a,b,c show a typical image of the fluorescence intensity at a large virus load ($10^{12}$), fluorescence lifetime of that membrane in (a), and fluorescence lifetime image of a membrane with a lower virus load ($10^{11}$). As seen in **Figure 4**d, an increase in virus load correlates to an increase in fluorescence intensity, implying no saturation of the membrane with the virus.

On the membrane itself are viruses that are disassembled, although the fluorophores remain in close proximity. This adsorption and disassembly is likely due to interactions between the positively charged PEI and the net negatively charged CCMV surface.[1]

To confirm the charge-charge interaction, the pH level is changed and the fluorescent signal from the membrane is studied. This is seen in **Figure 5** where the fluorescent signal of the membrane is recorded before any change to the pH level, after exposure to pH 3.8, and the signal of adsorption of CCMV at neutral pH after the initial exposure to pH 3.8. The observation of these fluorescent signals confirm the electrostatic nature of the attraction between the virus and the PEI coating. This could also lead to future research on functionalized membrane regeneration.[1]

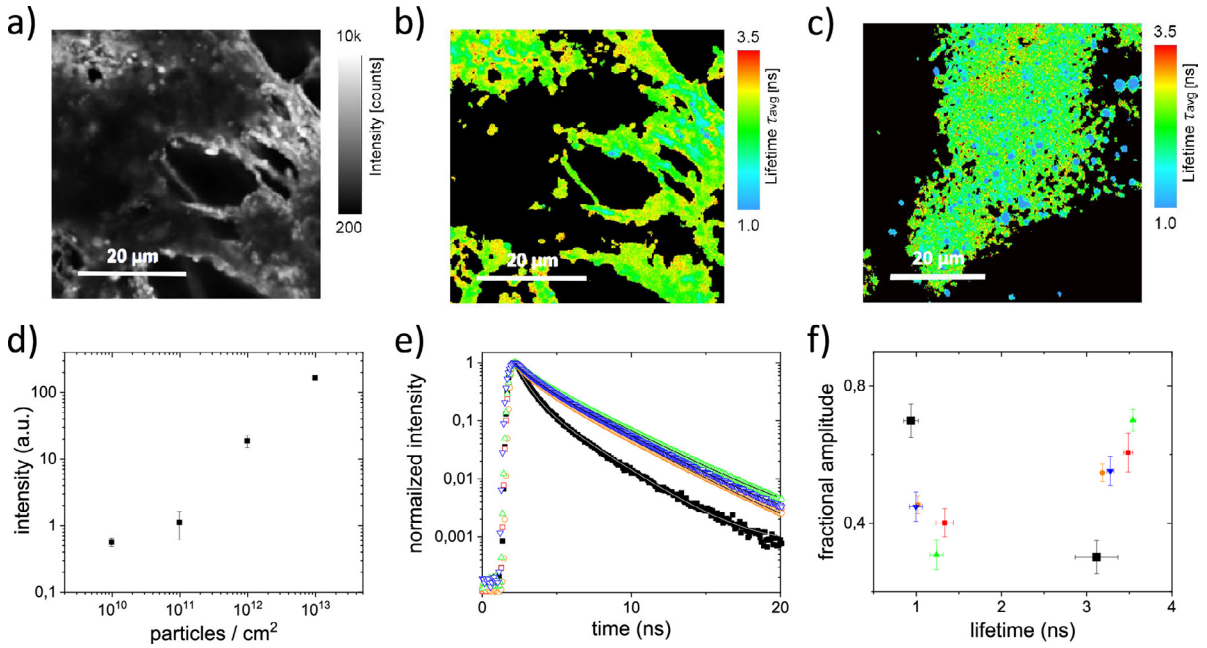


**Figure 4. Fluorescence lifetime and intensity of labeled CCMV on PEI-coated PES membranes. (a) Fluorescence intensity measured on a PEI-coated membrane after exposure to a virus load of $7.7 \times 10^{12}$ particles labeled. (b) Fluorescence lifetime image of the membrane shown in (a) obtained by fitting the measured fluorescence decays at each pixel to a single-exponential decay. (c) Fluorescence lifetime image of a PEI-coated PES membrane at a lower virus load of $7.7 \times 10^{11}$ labeled particles. The fluorescence lifetime was obtained by fitting a single exponential to the fluorescence decay curves for each pixel. (d) Average fluorescence intensity per pixel on the PEI-coated PES membrane as a function of the virus load. (e) Peak normalized fluorescence decay observed on the filter after cumulative virus loads of approximately $10^{13}$ (red), $10^{12}$ (orange), $10^{11}$ (green), and $10^{10}$ virus particles/cm$^2$ (blue) and the feed (black). The gray lines represent double-exponential fits to the data. (f) Lifetimes and fractional amplitudes obtained from fitting a double exponential to average fluorescence decays shown in (e).[1]**

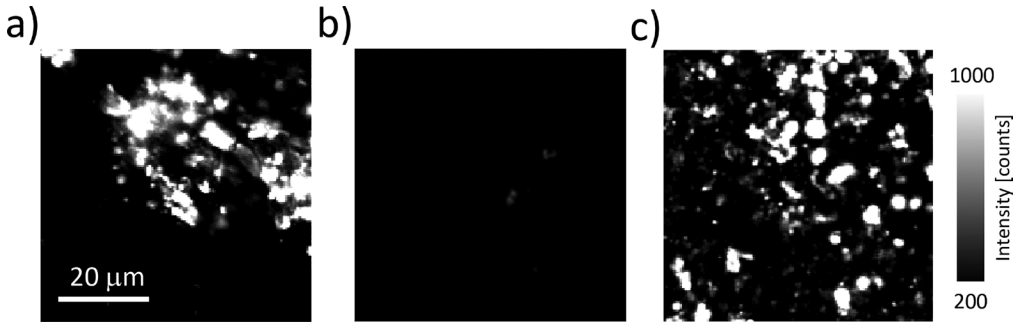


**Figure 5. Fluorescence intensity of labeled CCMV on PEI-coated PES membranes: (a) before exposure to pH 3.8 and (b) after exposure to pH 3.8. (c) Adsorption of labeled CCMV at neutral pH after exposure to pH 3.8.[1]**

The experiments of CCMV adsorption, inactivation, and disassembly on a PEI-coated PES membrane demonstrate a new method of quantifying the number of assembled virus particles in a filtrate and also characterizing the virus particles after membrane filtration. This technique also provides a new way to approach quantification of the fraction of disassembled, inactivated viruses in the filtrate, as well as a visualization of the inactivated viruses adsorbed on the membrane.

Not only did this functionalized micro-filtration membrane adsorb viruses on the surface, but the interaction between the virus and PEI resulted in disassembly of the virus capsids, inactivating the virus.[1] Further research may study different methods of membrane regeneration. This method of quantification and characterization of virus retention and inactivation with a polymer-functionalized membrane can be used for a wide range of applications in the medical and biological fields engaging in virus filtration.

## WAVELENGTH'S ROLE

In this single particle counting design with fluorescence microscopy, researchers benefited from Wavelength Electronics' laser diode driver, LD5CHA, with worry-free setup and easy integration. The LD5CHA enabled precise current control of the laser diode with minimal electronic noise from the driver. As noise could contribute to any false positive readings, lower electrical noise may lead to better spectra recordings and data analysis. The peak fluorescence wavelength red- or blue-shifted by ~3 nm for intact or disassembled virus particles, and the laser driver ensured narrow linewidth from the laser diode for precise detection of the fluorescence spectra.

The LD5CHA has RMS noise current as low as 9 µA at 100 kHz as well as a bandwidth of 1 MHz. Compliance voltage to the laser ranges from 3 V to 28 V depending on single or dual power supplies. Additional features, such as clipping current limit, slow start circuit, brownout, and over-voltage circuit protect the user and the laser from potential damage and electrical faults.

The LD5CHA laser diode driver enabled fluorescence microscopy for single particle counting of a polymer-functionalized surface designed to reduce and inactive virus particles. This makes the developed method for quantifying and characterizing the anti-viral properties of the functionalized membrane a reliable tool for biological and medical applications in virus retention and inactivation.

## REFERENCES

1. Chatterjee, S., Molenaar, R., de Vos, Wiebe, M., et al., Quantification of the Retention and Disassembly of Virus Particles by a PEI-Functionalized Microfiltration Membrane. *ACS Applied Polymer Materials*. 2022, **4** (7), 5173-5179. https://doi.org/10.1021/acsapm.2c00560

## USEFUL LINKS

- LD5CHA Product Page

## PERMISSIONS



A caption was added to Figure 1. The captions were modified for Figures 2 & 4 for length. No changes were made to the other captions or images. They are presented here in their original form.

| PRODUCTS USED |
|---|
| LD5CHA Laser Driver |

| KEYWORDS |
|---|
| Virucidal surface, fluorescence microscopy, fluorescence spectroscopy, micro-filtration, virus inactivation, virus retention, polymer-functionalized surface, single particle, LD5CHA, laser driver |

**REVISION HISTORY**

Document Number: CS-LD10

| REVISION | DATE | NOTES |
|---|---|---|
| A | October 2023 | Initial Release |