Virus pH-Dependent Interactions with Cationically Modified Cellulose and Their Application in Water Filtration

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Norovirus and Rotavirus are among the pathogens causing a large number of disease outbreaks due to contaminated water. These viruses are nanoscale particles that are difficult to remove by common filtration approaches which are based on physical size exclusion, and require adsorption-based filtration methods. This study reports the pH-responsive interactions of viruses with cationic-modified nanocellulose and demonstrates a filter material that adsorbs nanoscale viruses and can be regenerated by changing the solution’s pH. The bacteria viruses Qbeta and MS2, with diameters below 30 nm but different surface properties, are used to evaluate the pH-dependency of the interactions and the filtration process. Small angle X-ray scattering, cryogenic transmission electron microscopy, and z-potential measurements are used to study the interactions and analyze changes in their nanostructure and surface properties of the virus upon adsorption. The virus removal capacity of the cationic cellulose-based aerogel filter is 99.9% for MS2 and 93.6% for Qbeta, at pH = 7.0; and desorption of mostly intact viruses occurs at pH = 3.0. The results contribute to the fundamental understanding of pH-triggered virus-nanocellulose self-assembly and can guide the design of sustainable and environmentally friendly adsorption-based virus filter materials as well as phage and virus-based materials.

1. Introduction

Contaminated drinking water is estimated to cause 485 000 diarrheal deaths each year.[1] Viral waterborne pathogens are responsible for numerous deaths and epidemics in high-income as well as emerging countries.[2] Water treatment reduces the impact of such pathogens on populations[2a] but efficient low-cost solutions for virus removal are not straightforward due to this contaminant’s nanometer scale size. Filtration of viruses by conventional size-exclusion requires pore sizes of 20 nm or smaller, resulting in the need of a high overhead pressure and an increased running cost.[3] Virus removal from drinking water by membrane filtration has been recently reviewed.[4] Moreover, membrane filtration separates contaminants only on the top surface of the filter leading to fouling problems and a need for back-flushing and chemical cleaning,[5] hence increased running costs.

As opposed to size-exclusion, depth filtration relies on the adsorption of contaminants to the filter material.[6] The main pathogens of interest, Rotavirus and Norovirus, are negatively charged within the pH range of mostly intact viruses occurs at pH = 3.0.

Tailoring the filter material to interact with the contaminants is a key parameter to achieve efficient removal in depth filtration. Related to this, membranes have been surface-modified with cationic polymers, promoting the adsorption of negatively charged viruses.[6] However, the drawback of this approach is the relatively small membrane surface at which the separation occurs. Ceramic depth filters modified with metal nanoparticles have allowed the removal of nanosize contaminants including viruses.[7] However, the potential leaching of toxic metal from the filter may be a major risk.[8] This study demonstrates the development of a sustainable adsorption-based filter material from the abundant biopolymer cellulose.

Cellulose is a promising raw material for filter design: it is widely available, cheap, biodegradable, renewable, nontoxic and its chemical modification is straightforward.[9] These characteristics make it a well-used material for membranes in water treatment processes as well as biotechnology processes.[10] The vast majority of these studies focus, for particle separation, on cellulose in size-exclusion membranes. For example, removal of swine influenza virus (SIV), a 130 nm spherical lipid enveloped virus,[11] has been achieved by size-exclusion using nanofibrillated cellulose (NFC) membrane.[12] However, the problematic water-borne pathogenic Norovirus and Rotavirus are smaller than
SIV, 40\(^{[15]}\) and 80 nm\(^{[16]}\) in diameter, respectively, and non-enveloped. Thus, SIV is not an ideal representative of these relevant waterborne pathogens due to its lipid bilayer envelope and larger size for filtration studies. Moreover, cationically modified nanocellulose and cellulose–polyethylenimine composites have a well-established capacity in removing heavy metal ions, organic contaminants, and decrease water turbidity by adsorption of contaminants.\(^{[17c]}\) Inorganic nanoparticles of dimensions down to 2 nm have been successfully adsorbed from water to positively charged nanocellulose aerogels.\(^{[18]}\) Aerogels are highly porous, foam-like structures, with large internal surface area where adsorption can occur.\(^{[19]}\) A cationic nanofibrillated cellulose (CNFC) aerogel, combining the internal porous structure of freeze-dried aerogels and the positive charge of covalently linked quaternary amine groups,\(^{[17c]}\) is of great interest to develop an eco-friendly paper-like depth filter to adsorb viruses from contaminated water and will be investigated in this study.

Water chemistry is known to influence the interactions between positive material and viruses. For example, ionic strength influences the adsorption of viruses on polyelectrolyte surface by modifying the electrostatic interactions.\(^{[20]}\) Nonetheless, to the best of our knowledge, the water’s pH and whether it has a pivotal role in the electrostatic interactions between viruses and positively charged material has not yet been comprehensively investigated. Furthermore, the impact of such interactions on the virus adsorption, the potential for filter regeneration by pH-triggered desorption as well as the nanostructural integrity of the virus cage and its biological activity are mostly unknown. This understanding is of vital importance for the comprehensive design of pH-regenerable charged virus filter materials and for virus–biopolymer composite biomaterials, and will be addressed in this work.

To investigate these parameters and relate them to the filtration efficiency of the cellulose-based filter material, two bacteriophages, a subclass of viruses that infect bacteria, Qbeta and MS2 are used as surrogate. Qbeta and MS2 were chosen due to their small size and similar isoelectric point to Norovirus (pH = 5),\(^{[20]}\) and Rotavirus (pH = 4), respectively.\(^{[22]}\) Qbeta and MS2 are relatively small spherical viruses with diameters of around 29 nm.\(^{[21]}\) MS2 has a relatively low isoelectric point (3.9),\(^{[22]}\) a high surface polarity,\(^{[23]}\) and a highly pH-dependent colloidal stability.\(^{[24]}\) In contrast, Qbeta has a relatively high isoelectric point (4.3) and low surface polarity.\(^{[23]}\) Qbeta is thought as a highly representative model for virus adsorption onto a hydrophilic cationic material such as CNFC and is selected to study these interactions in depth.

This study demonstrates CNFC as pH-responsive adsorbent for MS2 and Qbeta viruses, with potential for pH-triggered regeneration of the material. The main focus is to unravel the effect of pH on the structure of Qbeta, on the interactions between CNFC and Qbeta as well as the impact of CNFC on the virus’s self-assembled nanostructure in water using small-angle X-ray scattering (SAXS) in combination with cryogenic transmission electron microscopy (cryo-TEM). Based on this, a CNFC aerogel filter was designed, characterized with confocal Raman microscopy, and used as a filter to remove Qbeta and MS2 bacteriophages from water.

### 2. Results and Discussion

#### 2.1. Virus–CNFC Interaction in Water

The interactions between Qbeta and CNFC as well as their morphological changes were systematically investigated in suspension. Both systems were first studied individually. The SAXS curves of Qbeta in 3 \times 10^{-3} \text{ M} Tris-Bis and 10^{-2} \text{ M} NaCl, recorded at pH = 3.0, 5.0, 7.0, 9.0 are shown in Figure 1A. Within experimental accuracy, the curves are comparable in the q region above 0.5 nm\(^{-1}\), indicating no major change in the size and shape of the virus in the 6–2 nm size region upon pH variation. However, at q < 0.1 nm\(^{-1}\), the SAXS curves for Qbeta at pH = 3.0 and pH 5.0 show an upturn, indicating the presence of virus-aggregates that are larger than the maximum dimension that can accurately be resolved with the SAXS setup in this study.\(^{[25]}\) This is also reflected in the turbid macroscopic appearance of the sample at pH = 3.0, as shown in the Supporting Information as Figure S1, due to the multiple scattering effects between particles in the micrometer-range.

The low-q upturn in the SAXS curves is not present at pH = 70 and pH = 9.0, indicating individual nanometer-sized virus particles in these samples. This result agrees with the dispersed particles imaged by cryo-TEM shown in Figure 1D, as well as with the increase in electrostatic repulsion indicated by the decrease in \(\zeta\)-potential, shown in Figure 2.

When comparing the SAXS curves for Qbeta at pH = 70 and 9.0, the noticeable difference is the shallower minima around \(q = 0.3\) nm\(^{-1}\) and the less steep slope to this first minima at pH = 9.0 suggesting a potential modification in particle shape. The particle’s structure was further investigated by analyzing the SAXS data with the indirect Fourier transformation method.\(^{[24]}\) The \(p(r)\) curves for Qbeta at pH = 70 and 9.0 in Figure 1B show that the phage’s morphology was approximately spherical with a diameter of 29 nm, obtained from \(D_{\text{max}}\) at \(p(r) = 0\). This particle size is in good agreement with the value of 29.4 nm obtained by X-ray diffraction reconstruction,\(^{[21a]}\) and 29.0 nm asymmetrically reconstructed from cryo-TEM images.\(^{[25]}\) The \(p(r)\) function at pH = 9.0 at 15.1 nm is shifted to 15.7 nm at pH = 70. A slightly more pronounced shoulder around 5 nm appears at pH = 9.0, and the radius of gyration \(R_g\) calculated with Equation (3), increases from 10.9 nm at pH = 70 to 11.1 nm at pH = 9.0. The variation in maxima and \(R_g\) indicate the presence of larger structures and in combination with the constant \(D_{\text{max}}\) suggest a higher contribution of emptier core–shell structures. The shoulder indicates that some smaller structure (in the size range of 5 nm) with a higher excess electron density, such as the capsid’s thickness, contributes to the overall scattering. In sum, this suggests a more pronounced core–shell structure of the virus at pH = 9.0 compared to pH = 70. It is noteworthy that at pH = 9.0, an increased adsorption of counter ions to the protein shell may have an effect on the excess electron density distribution of the structures measured when compared to pH = 70 but it is thought to be minimal due to the porosity of the particles at the molecular scale. To further elaborate on the potential change in the virus morphology, the excess electron density was calculated from the \(p(r)\) by its deconvolution using a convolution square-root operation.\(^{[26]}\) The radial excess electron density is shown in Figure 1C.
loss of electron density in the core upon increase of pH from 7.0 to 9.0 is \(\approx 22\%\), indicating a potential loss of RNA or change in structure. This variation in structure between pH 7.0 and 9.0 was not observed in the MS2 bacteriophage.\[21c\]

The cellulose pulp was modified with quaternary amine groups as described in the Material and Methods Section in the Supporting Information and depicted in Figure 3. The content of cationic groups, quantified by conductometric titration, was 0.108 mmol of quaternary amine groups per gram of cellulose giving a degree of substitution of 0.0175 mol trimethylamine per mol glucose (details can be found in the Materials and Methods and the titration curve as Figure S3 in the Supporting Information). The degree of substitution is similar to that found in literature.\[17c\] The SAXS curves for CNFC show a \(q^{-2}\) decay in the \(q\) region below 0.6 nm\(^{-1}\), indicating Gaussian chain characteristics (see Figure 4).\[27\] This scattering is most likely not only due to the chain conformation but may also result from the interconnections between individual CNFC as well as a wide distribution of the fibril’s cross-section area. Hence, the deconvolution between the local stiffness and the chain conformation is impossible. At \(q > 1\) nm\(^{-1}\), a \(q^{-2}\) decay of the \(I(q)\) is observed which most likely results from the scattering of the relatively smooth surface of the fibrils. The \(\zeta\)-potential measurements for CNFC show a positive charge at all pH values, as shown in Figure 2. Its value increases from 15.3 \(\pm\) 0.3 mV at pH = 3.0 to 22.4 \(\pm\) 1.4 mV at pH = 9.0. This increase may be due to an increase in negative charges in the Stern layer at pH = 9.0 compared to pH = 3.0.

The SAXS data of the CNFC and Qbeta mixtures (1:1 weight ratio) are shown in Figure 5. The shape of the scattering curves with similar characteristic minima and maxima as the pure Qbeta scattering curves at the corresponding pH values indicates that the spherical virus cages are present in the mixture and no major structural change occurred to the cage. The interactions between the viruses and CNFC were investigated by modeling the scattering curves of the mixture of Qbeta and CNFC as a linear combination of the virus scattering curve at the corresponding pH, shown in Figure 1, and the CNFC scattering curves at the corresponding pH, shown in Figure 4. The fitting parameters are shown in the Supporting Information in Table S1. A good fit, with a small reduced \(\chi^2\), indicates that the mixture’s scattering curve is an addition of the scattering curves of the two separate components indicating the absence of significant interactions between them. On the contrary, a bad
fit, with a large reduced $\chi^2$ indicates interactions among the CNFCs and the viruses. The deviation from the linear combination of the two individual components in this case can result, for instance, from a change in the shape of the virus, modification in the CNFC chain characteristics, and scattering cross-terms between the viruses and the CNFC upon its adsorption that contribute to the overall signal.

At pH = 3.0, the best possible fit of the linear combination of the scattering from the two components results a reduced $\chi^2 = 5.42$. The good fit indicates no significant interactions between Qbeta aggregates and CNFC. The absence of interaction is in good agreement with the $\zeta$-potential shown in Figure 2, since at pH = 3.0 both Qbeta and the CNFC are positively charged, repelling each other.

Contrary, at pH = 5.0, the linear combination of the scattering curves from the two species does not provide a reasonable fit to the experimental data, the goodness of fit decreases to a reduced $\chi^2 = 56.57$ (see Figure S4, Supporting Information). The $q$-range of the deviation may help indicate the variations in shape. At $q < 0.1$ nm$^{-1}$, the fit underestimates the experimental data indicating that larger objects are present. At pH = 5.0, the virus particles are aggregated and the presence of CNFC may further promote the aggregation process. The minima and maxima of the scattering tend to be overestimated by the linear combination of the individual virus and CNFC scattering, indicating a change in overall shape and addition of cross-terms. The interactions agree with the opposite $\zeta$-potential values for the Qbeta particles and the CNFC at this pH value, shown in Figure 2.

At pH = 7.0, the linear combination of the two species deviates from the experimental data with a reduced $\chi^2 = 66.48$, the fit residuals are shown as Figure S4 in the Supporting Information. At $q < 0.1$ nm$^{-1}$, the deviation of the fit from the experimental $I(q)$ is less pronounced than that at pH = 5.0. At pH = 7.0, the virus particles are dispersed, hence they may adsorb as individual particles to the CNFC. The best possible fit to the experimental scattering curve deviates at the minima and maxima in a similar fashion that at pH = 5.0, indicating interactions

Figure 2. $\zeta$-Potential measurements. The $\zeta$-potential measurements of CNFC dispersions in red, Qbeta in black, and MS2 in blue at pH values between 3.0 and 9.0. All measurements were done in $3 \times 10^{-3}$ M Tris-Bis, $10 \times 10^{-3}$ M NaCl. Each $\zeta$-potential value corresponds to the average of three independent experiments.

Figure 3. Schematic representation of the CNFC preparation process from cellulose pulp. Details can be found in the Experimental Section.
among viruses and CNFC at pH = 7.0 and pH = 5.0. The representative cryo-TEM image of the sample at pH = 7.0 showed spherical 28 nm particles adsorbing onto elongated fibril structures. This confirms the adsorption of Qbeta on the CNFC fiber which leads to the formation of large virus–CNFC aggregates together with the lack of major structural modification of Qbeta upon interaction with CNFC, see Figure 5B. A lower magnification cryo-TEM image showing the micrometer range size of the aggregates is shown in the Supporting Information in Figure S5. The aggregation between the two particles agrees with their opposite charges, their ζ-potentials are −18.2 ± 0.8 and 16.8 ± 1.3 mV for Qbeta and CNFC, respectively.

At pH = 9.0, the linear combination of virus and CNFC scattering misestimates the scattering data of the combined system with a reduced $\chi^2 = 58.46$. In the range $q < 0.1$ nm$^{-1}$ larger deviations are observed than at pH = 70, potentially indicating a higher proportion of particles adsorbing at pH = 9.0 than 70 (see Figure S4 for fit residuals in the Supporting Information). The deviation in the range $q = 0.2–0.3$ nm$^{-1}$ as well as the larger overestimation at the $q = 0.4$ nm$^{-1}$ maximum indicate an increased contribution from the cross-terms or a modification in particle morphology, both would result from a larger amount of Qbeta particles adsorbing to the CNFC. The increased interactions agree with the lower ζ-potential, $-27.6 \pm 2.9$ mV at pH = 9.0 instead of $-18.2 \pm 0.9$ mV at pH = 70, that may increase the probability of particles interacting with the CNFC. A cryo-TEM image of the micrometer-sized aggregates composed of Qbeta and CNFC is shown in the Supporting Information in Figure S5.

The interactions between CNFC and virus particles demonstrated here can guide the design of advanced virus filter materials and the optimization of virus separation processes. The lack of structural modifications in Qbeta particles upon interaction with CNFC from SAXS and cryo-TEM results indicates that the nanocages are rather stable and their biological activity may still be present. The pH-dependence of the virus–CNFC interaction that was found attractive at pH = 5.0, 7.0, and 9.0 and

**Figure 4.** CNFC’s colloidal structure. A) Experimental SAXS data (open squares) CNFC at pH = 3.0, 5.0, 7.0, and 9.0. A simulated $q^{-2}$ behavior is given as eye-guide (dashed line). B) Representative cryo-TEM image of CNFC at pH = 7.0.

**Figure 5.** Colloidal interactions between Qbeta and CNFC. A) Experimental SAXS data (open squares) for the 1:1 weight ratio mixtures of Qbeta and CNFC at pH = 3.0, 5.0, 7.0, and 9.0. The corresponding fits (red curves) calculated with a linear combination of the experimental scattering curves from the pure species at the corresponding pH value with Equation (4) are also shown. Fit residuals are shown in Figure S3 in the Supporting Information. B) cryo-TEM image of the mixture of Qbeta and CNFC at pH = 7.0. Scale bar, 100 nm. Examples of CNFC are highlighted with red arrows and Qbeta particles adsorbed on the CNFC with black arrows. A large aggregate of CNFC and Qbeta is highlighted with a black ellipse.
repulsive at pH = 3.0 is particularly interesting for the regeneration of filter materials. They may also be an efficient bacteriophage delivery material, for instance in biomedical applications such as phage-loaded antibacterial wound dressings. The next section of this study explores the design of a dynamic CNFC–virus composite that allows adsorption of viruses at pH values common for surface water and regeneration by flushing with low pH buffer, and further evaluates the biological activity of the virus upon desorption from the CNFC.

2.2. Design of a CNFC-Based Depth Filtration Process

An aerogel filter was produced from the CNFC by freeze-drying to investigate the pH-dependence of interaction with the bacteriophages. The quaternary amine content in the aerogel is 0.107 mmol per gram of CNFC (see the Material and Methods Section in the Supporting Information for details), and the pore diameter is between 250 and 10 000 nm with a wide pore-size distribution.[18] The porous structure of the filter in air was studied using confocal Raman microscopy, a representative image is shown in Figure 6A. The fingerprint region from 700 to 1600 cm\(^{-1}\) and the CH stretch region from 2700 to 3100 cm\(^{-1}\) of a spectrum corresponding to the imaged structure as well as a reference spectrum of the nonmodified cellulose are shown in Figure 6B with the full spectra in Figure S6 in the Supporting Information. The spectrum reflects that of cellulose, with bands corresponding to the HCC and HCO bending at the C-6 ring around 900 cm\(^{-1}\), the bands corresponding to the COC of the glucose ring at 1096 cm\(^{-1}\), the C–OH stretching at 1122 cm\(^{-1}\), the shoulder at 1155 cm\(^{-1}\) corresponding to the C–OH anti-symmetric stretching, the CH deformations band at shifts of 1261, 1340, 1379, and 1470 cm\(^{-1}\), and the CH stretching at 2898 cm\(^{-1}\).[28] Additional bands are found at 761, 971, 2963, and 3030 cm\(^{-1}\). The 761 and 971 cm\(^{-1}\) bands correspond to the antisymmetric stretching and the symmetric stretching of the quaternary amine group, respectively.[28b] The 761 cm\(^{-1}\) band is not visible in the spectra from nonquaternized microfibril cellulose (MFC). Whereas, the 971 cm\(^{-1}\) band is covered up by the shoulder of the strong 1069 cm\(^{-1}\) band, making it hard to distinguish in both samples. The 2963 and 3030 cm\(^{-1}\) bands correspond to the antisymmetric and symmetric stretching vibration of the CH\(_3\) present in the quaternary amine groups.[28c] The broadening of the shoulder at 2963 cm\(^{-1}\) when comparing the MFC and the CNFC spectra indicates a contribution from an additional band in this spectral range. The band at 3030 cm\(^{-1}\) is only visible in the modified cellulose spectra. These bands confirm the chemical modification of the cellulose and agree with the positive charge of the fibers throughout the pH range tested, shown in Figure 2, as well as with the content of quaternary groups of 0.107 mmol g\(^{-1}\).

2.2.1. Filtration Studies

The filtration experiments were done by filtering 50 mL of \(10^6\) plaque forming units per milliliter (PFU mL\(^{-1}\)) through the filter. A schematic of the set-up is shown in Figure 6C, the

![Figure 6. Filter characterization and filtration efficiency. A) A confocal Raman image of the filter of 30 \(\mu\)m by 30 \(\mu\)m by 30 \(\mu\)m (imaging details in the Experimental Section). B) The fingerprint region (700–1600 cm\(^{-1}\)) and the CH stretching region (2700–3100 cm\(^{-1}\)) of on top a representative Raman spectrum of the CNFC aerogel image and, under a Raman spectrum of an aerogel of the nonmodified microfibril cellulose for comparison. C) The filtration set up used to test virus removal with a photograph of the CNFC aerogel used as filter. D) The removal efficiency of MS2 and Qbeta by filtration with the CNFC filter at pH = 7.0 and MS2 at pH = 3.0. The log removal was calculated with Equation (7). The values are an average of three experiments.](image-url)
cellulose filter is placed on a wide meshed glass filter (see the Experimental Section for details) ensuring mechanical support and no filter material release. The viruses were quantified by a double layer agar assay as described in the Material and Methods Section in the Supporting Information. The filtration rate was adjusted with a vacuum pump in order to have comparable contact times. It was set to ≈0.82 mL h⁻¹ cm⁻².

At pH = 7.0, the virus removal was 99.9% for MS2 and 93.6% for Qbeta, as shown in Figure 6D. The higher removal of MS2 may be due to its lower ζ-potential of –30.1 ± 1.0 mV for MS2 against –18.3 ± 0.8 mV for Qbeta, leading to stronger interactions between the filter surface and viruses. Although, the performance of the CNFC filter is comparable to cationic polyethyleneimine coated membrane filters that also had an MS2 removal efficiency of 99.9%,⁸⁻ the CNFC filter has some major advantages; it is made from a sustainable material, the modification to incorporate positively charged groups is straightforward and relatively cheap compared to sophisticated polymer brushes.

On the other side, at pH = 3.0, the removal of MS2 was only 17.7%. The removal of Qbeta was not measured due to rapid inactivation of Qbeta at pH values under 4.⁴⁻ The difference in removal between the negatively charged MS2 at pH = 7.0 and positively charged MS2, at pH = 3.0, as shown in Figure 2, indicates a removal mechanism that is highly depending on electrostatic interactions and adsorption of the virus on the cationic filter material. The importance of the electrostatic interaction and its pH-dependence is further highlighted by washing the filter with 50 mL of Tris-Bis buffer at pH = 3.0, 8.2% of the MS2 particles that were adsorbed to the filter at pH = 7.0 were recovered and found to be still infectious, corresponding also to a 2 log increase in PFU mL⁻¹ compared to the filtrate at pH = 7.0. This is a high number considering the relative small volume used to wash the filter and the potentially increased importance of additional short range interactions, such as van der Waals forces and hydrogen bonding, once the virus particles are in contact with the CNFC. This result is in good agreement with the findings on structural integrity from SAXS and cryo-TEM reported above. The release of MS2 particles at pH = 3.0 indicates the possibility of cleaning the filter in case of fouling by simple acid baths with no need for expensive and/or toxic chemicals, hence increasing the sustainability of the filtration process. Furthermore, the result that the released particles are still biologically active suggests that the material is virus-static rather than antiviral and indicates that a CNFC filter would also allow to concentrate viruses from large water volume to the smaller washing volume, hence decreasing the limit of detection for commonly used virus detection methods.

The performance of CNFC materials may be further improved by increasing the concentration of quaternary amine groups on the NFC, and optimizing flow-path and conditions that would increase the probability for interaction between the viruses and the filter surface. The virus removal efficiency of the CNFC filter from water is comparable to that of commercially available filter that use depth filtration for virus removal.⁹⁻ The fabrication of the CNFC filters requires less components, they do not use, for instance, diatom earth and are thus more sustainable.

A point worth considering is that the interaction described in this work is specific to aqueous-based mediums. Indeed, there has been a lot of development in recent time on air filters and face masks.¹⁰⁻ It is worth mentioning that the interactions described in this work are not directly relatable to those in air due to the difference in dielectric constant (εirr = 1.0 and εwater = 80.2 at 20 °C),¹¹⁻ the change of hydration state, the presence of virus containing aerosol particles of various sizes, etc.

### 2.3. Implications and Applications of Virus–CNFC Interactions

The findings on virus–CNFC interactions from this work can guide the design of biomaterials including bacteriophage-based antimicrobial materials. The strong interactions between CNFC and viruses around physiological pH, together with the integrity of the virus cage may even have implications for the design of future vaccines: The CNFC virus aggregates can provide high local concentration of the antigen for an immune response.¹²⁻ Moreover, cationic-modified cellulose nanocrystals as well as bacteriophages have shown immunomodulatory properties,¹³⁻ providing possibilities to use them in combination to present antigens efficiently to the immune system by, e.g., conjugating the antigen on the bacteriophage and self-assembling them with cationic cellulose material.

The rapid growth of antibiotic multi-resistant bacteria has led to a renewed interest in phage therapy and the design of antibacterial materials based on bacteriophage.¹⁴⁻ The conservation of the phage’s self-assembled structure along with its bioactivity upon adsorption to CNFC indicate that CNFC-based materials such as hydrogels may be an ideal material for delivery of bacteriophages to infections. At physiological pH, a stain-specific antibacterial activity would be activated upon surface contact of the specific bacteria and the hydrogel. Whereas at low pH values comparable to those of the stomach,¹⁵⁻ the phages would be released from the material allowing higher mobility and potentially high antibacterial activity.

### 3. Conclusion

CNFC material was found to be an excellent pH-dependent virus sorption material. The 29 nm diameter spherical core–shell structure of the bacteriophage Qbeta in suspension is observed by SAXS and cryo-TEM at pH = 7.0 and 9.0. The virus particles are less charge stabilized and aggregate at pH = 5.0 and 3.0. The CNFC structure in suspension is pH independent, and resembles Gaussian chain characteristics. At pH = 5.0, 7.0, and 9.0, opposite ζ-potential was measured and attractive interactions were recorded by SAXS, between Qbeta and CNFC, whereas at pH = 3.0, no attractive interactions were found, indicating a potential filter regeneration process.

Based on these results, a CNFC-based aerogel was designed as filter material. At pH = 7.0, a high filtration efficiency was measured for MS2 and Qbeta with 99.9% and 93.6% removal, respectively. At pH = 3.0, no attractive interactions were observed and a low filtration efficiency was measured with 17.7% removal of MS2. The high pH dependence of the interactions is shown both on a filter and in solution, leads to the possibility of recovering the virus particles and regenerating the filter by acidic bath. Moreover, the combination of intact adsorbed particles observed by SAXS and cryo-TEM, with the...
biologically active bacteriophages released from the filter at pH = 3.0 after being adsorbed at pH = 7.0, indicates the potential design of a pH-triggered phage delivery material.

4. Experimental Section

**Virus Propagation and Purification:** The detailed virus propagation and purification protocol were described in previous study.[25] In short, MS2 (DSMZ 13637, DSMZ, Germany) and Qbeta (DSMZ 13768, DSMZ, Germany) were replicated in *Escherichia coli* strain W1485 (DSMZ 5695, DSMZ, Germany). A virus to bacteria ratio of 0.1 was used for the infection. After 4–5 h incubation, the bacterial debris were separated by centrifugation at 2600g for 20 min (Eppendorf 5810R, Eppendorf Germany) followed by sterile filtration (MillexGP, Millipore, Ireland). The filtrate was concentrated using centrifugal filters and washed with phosphate-buffered saline (PBS) containing 0.78 g L⁻¹ NaH₂PO₄·2H₂O, ≥98.0% purity and 0.58 g L⁻¹ NaCl (≥99.5% purity) at pH = 7.0 using centrifugal filters with a 100 kDa cutoff membrane (100 kDa Amicon, Ultra-15 centrifugal filters, Millipore, Ireland). The bacteriophage solution was passed through 0.1 µm pore filters (MillexVV, Millipore, Ireland). The buffer was then changed to Tris-Bis buffer at pH 7.0 containing 3 × 10⁻³ M Tris-Bis and 10⁻² M NaCl, ≥99.5% purity. 1 ml of sample was washed with at least 100 ml of Tris-Bis buffer by multiple 13–15 ml washing steps and concentrating to 1 ml in the centrifugal filters (100 kDa Amicon, Ultra-15 centrifugal filters, Millipore, Ireland). The final concentration was adjusted to 10⁹ PFU ml⁻¹, corresponding to about 1 mg ml⁻¹. All pH adjustments were performed with NaOH (≥99% purity, Carl Roth GmbH, Karlsruhe, Germany) and HCl (ACS reagent grade, Sigma-Aldrich, Buchs, Switzerland).

**CNFC Preparation:** Never-dried elemental chlorine-free cellulose fibers from bleached softwood pulp (Picea abies and Pinus spp. Stendal GmbH, Berlin, Germany) were suspended in an aqueous solution of NaOH (≥99% purity, Sigma-Aldrich, Buchs, Switzerland) to a final concentration of 2.5 and 5 wt%, for the pulp and NaOH, respectively. The quaternary amine groups were linked to the cellulose by reacting glycidyltrimethylammonium chloride (1.25 ml per gram of the pulp) under stirring at 65 °C for 8 h with the fiber suspension. The product was neutralized with HCl (ACS reagent grade, Sigma-Aldrich, Buchs, Switzerland), and subsequently filtered and washed with distilled water. The dispersion’s volume was adjusted to a concentration of ~1% w/v and disintegrated into CNFC using a grinding process (Supermass Colloidor MKZ10-20), CE, Masuko, Saitama, Japan). The energy consumption of the process was recorded with a power meter (5 kWh kg⁻¹ of dried pulp). A close contact between the two grinding stones was maintained. The rotating stone was spun at 1500 rpm. The final concentration of quaternary amine is 0.107 mmol g⁻¹ (more information can be found in the Material and Methods and in Figure S3 in the Supporting Information).

**CNFC Aerogel Preparation:** The filter was prepared via freeze-drying as previously shown.[26] 15.0 ml of the CNFC dispersion at 0.75 wt% was placed in a 6 cm diameter petri dish (Greiner Bio-One Vacutte Schweiz GmbH, St Gallen, Switzerland) and solidified at ~50°C. The ice was removed by sublimation in a freeze-dryer (Alpha 3–4 LSC basic, Martin Christ GmbH, Osterode am Harz, Germany). The highly porous CNF foam obtained was hot-pressed for 10 min at 80°C and 2.2 bars (Carver Inc., USA).

**SAXS:** The scattering data were recorded at the Austrian SAXS beamline at Eletra, Trieste, Italy. X-ray of wavelength was 0.154 nm (8 keV) and sample-to-detector distance was 906.18 mm, providing a range in scattering vector magnitude, q, from 0.7 to 5.0 nm⁻¹, q = 4π/λ sin(θ/2), λ is the wavelength and θ is the scattering angle. The diffraction images were recorded using a Pilatus 1M detector (Dectris Ltd., Baden, Switzerland) with a total area of 169 × 179 mm² and pixel size of 172 × 172 µm². The resulting 2D scattering patterns were azimuthally integrated into the 1D I(q) functions. Three repeats for each measurement were done allowing to confirm the absence of beam damage. The average of the three measurements was used for further analysis. To prevent adsorption of CNFC on the negatively charged glass surface, the borosilicate glass capillaries (Hilgenberg, Malsfeld, Germany) were coated with Sigmacote (Sigma-Aldrich, St-Louis MO, USA). Each capillary was injected with 200 µl of Sigmacote, after an incubation at room temperature overnight the excess was removed and the coated capillaries were dried at 100°C for 30 min in an oven and then rinsed with excess water. All samples were measured at an estimated concentration of 1 mg ml⁻¹ virus, corresponding to Qbeta to 10¹⁴ PFU ml⁻¹ in Tris-Bis buffer, composition can be found in the Supporting Information. The scattering of buffer was subtracted as background from all scattering curves.

**SAXS Data Analysis:** The quasi-spherical virus, the chain-like fiber shape of the CNFC, and the mixture of both required different approaches to analyze the data, the Qbeta virus particles scattering curves were modeled with model independent means, the CNFC scattering curves were discussed from the decays observed, the mixture of Qbeta and CNFC was analyzed using a linear combination of the scattering of the individual components.

The Qbeta virus particles’ structure was investigated as following. The indirect Fourier transformation method was used to calculate the pair distance distribution function, p(r), from the I(q) with Equation (1).[26] The p(r) was the real space representation of the scattering data, allowing a direct determination of the size and shape of the scattering objects.[24a,26b]

\[
l(q) = 4\pi \int_0^\infty p(r) \frac{\sin(qr)}{qr} dr
\]

where

\[
p(r) = r^2 \Delta \rho^2(r)
\]

with \(\Delta \rho^2(r)\) being the convolution square of the spatially averaged excess electron density \(\Delta \rho(r)\) of the virus.

The radius of gyration \(R_g\) was calculated from the \(p(r)\) with the following equation[26]

\[
R_g^2 = \frac{\int p(r)r^2 dr}{\int p(r)dr}
\]

In the case of spherical particles, the deconvolution of the \(p(r)\) gave the radial excess electron density profile \(\Delta \rho(r)\) relative to the electron density of the buffer.[26]

In order to investigate the interaction between Qbeta and CNFC, the scattering data of the mixture of CNFC and Qbeta virus particles was fitted with a linear combination of the experimental scattering curve of the individual components

\[
l_{\text{mix}}(q) = S(A_{\text{Qbeta}}(q) + B_{\text{CNFC}}(q))
\]

where \(l_{\text{mix}}(q)\) is the scattering intensity of the mixture at a specific pH value, \(l_{\text{Qbeta}}(q)\) is the experimental scattering of Qbeta at the corresponding pH value, \(l_{\text{CNFC}}(q)\) is the experimental scattering of CNFC at the corresponding pH value, A and B are the coefficient fitted to the curve. These parameters were optimized to achieve the best possible fit of \(l_{\text{mix}}(q)\) to the experimental data.

The goodness of the fit, \(\chi^2\), was used as an indicator for interactions between CNFC and the virus through change in structure, and crossover scattering terms. It is defined by

\[
\chi^2 = \frac{\sum (O_i - C_i)}{\sigma_i^2 (N_{\text{points}} - N_{\text{free}})}
\]

where \(O_i\) is the value of measure data, \(C_i\) is the value of the calculated fit, \(\sigma_i^2\) is the standard deviation of the experimental data, \(N_{\text{points}}\) is the number of data points, and \(N_{\text{free}}\) is the number of variables fitted.
Cryo-TEM: 4.0 μL of the sample at a concentration of around 1 mg mL⁻¹ was transferred onto glow-discharged Quantifoil R2/1 300 (Quantifoil, Groβlöbichau, Germany) mesh copper grids. The samples were vitrified by plunging into a liquid ethane/propane (37/63) mixture at 77 K using Vitrobot Mark IV (ThermoFisher Scientific, USA) in an environmental chamber at 100% humidity and 22 °C. The vitrified grids were imaged on a Tecnai 2 (ThermoFisher Scientific, USA) electron microscope in a Gatan 626 cryo-holder (Gatan, USA). A Falcon 2CMOS direct detector (ThermoFisher Scientific, USA) was used to integrate micrographs representing cumulative dose of ~45 electrons Å⁻². For the high magnification images (62 000x), the defocusing was set to ~3 μm and the pixel size was 1.7 Å. For the lower magnification images (about 15 035x), the defocusing was set to ~15 μm and the pixel size was 7.01 Å.

The images were processed for background subtraction in image.β So far as the phase electron microscopy (PFEM) of the Gatan Imaging Suite software. The CNF aerogel was deposited on the glass filter of a 75 mL Duran funnel filter. It was allowed to hydrate by flowing 50 mL of Tris-Bis buffer at the pH of interest for the experiment through the filter. 50 mL of Tris-Bis buffer at pH = 7.0 or 3.0 (see the Supporting Information for composition) was spiked with Qbeta or MS2 stock suspension giving a total concentration of about 10⁶ PFU mL⁻¹. The virus containing solution was filtered through the CNF filter under vacuum (MZ 2C, Vacuubrand, Wertheim, Germany). The phages were quantified by conventional double layer agar method.α The dilution series were done with PBS at pH = 7.0 (composition in the Supporting Information). The experiments were done in triplicates, the average was presented with standard deviation. The log removal was then calculated as following:

\[
\log \text{removal} = - \log \left( \frac{N}{N_0} \right)
\]

where \(N\) is the number of PFU mL⁻¹ after filtration and \(N_0\) is the number of PFU mL⁻¹ before filtration.

Confocal Raman Microscopy: The confocal Raman images and corresponding spectra were acquired with a WITec Confocal Raman Microscope (WITec alpha300 R, Ulm, Germany). A 532 nm laser with maximum power of 60 mW in combination with a Nikon 100x objective was used. The 2D image was composed of a stack of 60 2D images. Each 2D image was 60 × 60 pixels. The image volume was 30 μm × 30 μm × 30 μm and the pixel size was 500 nm. A Raman spectrum from 700 to 3675 cm⁻¹ was recorded for each pixel. The image processing was done with the WITec data processing software. The CH band (from 2780 to 3040 cm⁻¹) was used to identify the high cellulose content volumes. The processed 2D images were stacked into a 3D image using Image.β A single spectrum of an aerogel made of the cellulose pulp prior to chemical modification was taken with a low-magnification Nikon 10x objective as reference.

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**Conflict of Interest**

The authors declare no conflict of interest.

**Data Availability Statement**

Research data are not shared.

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