Effective removal of enteric viruses by *Moringa oleifera* seed extract functionalized cotton filter

Authors

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Abstract

Accessible and low-cost point-of-use technologies have significant potential to mitigate risk to public health, particularly in areas with limited resources and in disaster scenarios. Natural cotton fibers functionalized with water-soluble proteins from *Moringa oleifera* seeds (*MO*-cotton filter) are a promising technology at lab-scale with demonstrated feasibility for pathogen removal from water. Here, we showed the performance of *MO*-cotton filters under practically relevant conditions to remove mammalian virus spiked in groundwater. Specifically, *MO*-cotton filters achieved > 3.2-$\log_{10}$ reduction at a superficial velocity of 0.7 m/h of two mammalian viruses Tulane virus (TV, *Caliciviridae*, non-enveloped virus) and Transmissible gastroenteritis virus (TGEV, *Coronaviridae*, enveloped virus), which are representative of a significant portion of waterborne illnesses. We further evaluated the risk of virus particles detached due to shear forces by testing their infectivity and found that the viruses accumulated on the *MO*-cotton filters pose a minimal risk of contaminating the drinking water source.

Introduction

Enteric viruses are a significant cause of waterborne diseases and acute diarrhea, resulting in approximately 1.7 billion infections and 525,000 deaths in children under the age of 5 years annually [1]. Unfortunately, enteric viruses disproportionately affect the health of individuals belonging to low socioeconomic status [2,3]. For instance, approximately 90% of diarrhea related deaths occur in sub-Saharan Africa and South Asia, where there is lack of access to appropriate water, sanitation, and hygiene (WASH) [4]. As a result, point-of-use (POU) water treatment technologies have been studied as a cost-effective decentralized solution [5]. However, many POU water treatment technologies have not achieved sustained use at the household level because they require operational knowledge, such as chemical dose for chlorination, or rely on less accessible materials. For instance, only 1.5% of households in low- and middle-income countries, that received training to operate coagulant-chlorine disinfection systems, appropriately treat their drinking water after 6 months [5].

Due to the low cost, simple maintenance, high treatment capacity, and high performance against a wide variety of contaminants, filtration has been identified as a promising point-of-use (POU) water treatment technology. POU filters have the potential to be widely adopted sustainably in low- and middle-income countries, to improve the household water quality and reduce the risk of waterborne diseases and deaths [6–8]. However, a primary challenge associated with conventional filtration technologies, such as sand and ceramic filters, is their inability to effectively filter viruses [7,9]. For instance, rapid sand filtration achieved only a 1.26-$\log_{10}$ reduction for pepper mild mottle virus and a 0.49-$\log_{10}$ reduction for JC polyomavirus [10]. Similarly, ceramic filters were unable to achieve even a 1-$\log_{10}$ reduction of MS2 virus [11,12]. Given that viral pathogens are one of the primary causes of waterborne diseases [13], there is an urgent need for POU water filtration technologies that can effectively reduce the risk of water-borne viral infections.

Recent studies have focused on enhancing the virus removal effectiveness of filtration technology by functionalizing the surface of a filter media with easily accessible natural materials. *Moringa oleifera (MO)*, a fast-growing deciduous tree found in tropical regions, has garnered attention due to its ability to thrive in areas facing significant challenges in obtaining safe drinking water[14]. A water extract containing cationic proteins, derived from *MO* seeds can functionalize filter media and adsorb viruses that are typically negatively charged in the environment [14]. In our previous research, we successfully functionalized model sand particles with the moringa proteins and demonstrated a virus removal efficacy of up to a 7-$\log_{10}$ using MS2 bacteriophage as a model virus [15]. However, the challenges associated with low filter loading rate and the availability of appropriately sized natural sand grains (<130 μm) have necessitated the exploration of alternative filter media. Subsequently, we have shown that natural cotton can be successfully functionalized with *MO* seed extract (*MO*-cotton filters), creating an affinity-based filter for the removal of contaminants from water which shows exceptional virus, bacteria, and inorganic nanoparticle removal [16,17]. Despite the promising performance at lab-scale, research on *MO*-cotton filters to date involved the use of surrogate virus, MS2 bacteriophage, deionized water with additives like NaCl as artificial water matrix and has been conducted over a relatively short duration. These experimental conditions lack representation of the real-world application scenario for *MO*-cotton filters where consistent virus removal against mammalian viruses over long periods of operation is required limiting the wide application of *MO*-cotton filters in the field.

This study aims to evaluate the effectiveness of *MO*-cotton filters in removing enteric viruses under real-world conditions. Specifically, we focused on two mammalian viruses: the Tulane virus (TV, rhesus monkey virus) and transmissible gastroenteritis virus (TGEV, porcine virus) as representative viral species belonging to the *Caliciviridae* and *Coronaviridae*, respectively. These viruses are responsible for a significant portion of waterborne illnesses

worldwide, including diarrhea [18,19]. In addition, viruses are categorized into enveloped or non-enveloped viruses depending on the presence or absence of an encapsulating lipid bilayer membrane. Therefore, TGEV (an enveloped virus) and TV (a non-enveloped virus) have been selected intentionally for this study to represent the behavior of a variety of viral pathogens in the environment. Furthermore, the virus removal efficacy of the *MO*-cotton filters was determined by experiments using actual groundwater spiked with these mammalian viruses, creating conditions that closely resemble real-world scenarios. Through column experiments and systematic analyses, we investigated the kinetics of virus removal and determined the fate of viruses during the filtration processes. The risk of detached viruses from *MO*-cotton filters was also studied here as this is a critical factor for ensuring safety of public health. Virus detachment experiments were conducted by operating the *MO*-cotton filters at high superficial velocity and simulating high shear conditions in batch experiments. Our results showed that even when operated at a superficial flow velocity (2.1 m/h) that is three times higher than the filtration rate, no detectable viruses were released from the filter (i.e., < 2.1 gene copies/μL). Viruses detached from the filter by a strong shear force in batch experiments were also confirmed to be inactivated by the moringa protein-induced aggregation. These results demonstrate that *MO*-cotton filters can effectively remove viruses from groundwater, thereby contributing to the mitigation of waterborne diseases through their use in POU filters.

Materials and Methods

Moringa protein functionalized cotton filter preparation

Moringa oleifera seeds were obtained from Echo Global Farms (USA). One gram of seed was weighed and crushed to a fine powder using a grinder for about 10 seconds. The ground seed was then mixed with 50 mL of deionized (DI) water at 100 rpm for 15 minutes to extract proteins. The well-mixed moringa seed extract was filtered through a 0.22 μm filter to remove

the seed debris. Next, 1.75 g of cotton was weighed and soaked in DI water for approximately 10 minutes to prevent air entrapment. The dampened cotton was then packed into a clean column with an inner diameter of 1.5 cm and a height of 10 cm (7374151, Bio-Rad, USA). The column was secured with an adaptor (7380016, Bio-Rad, USA). The cotton filled 4 cm of the column, corresponding to a volume of 7.1 cm^3. Deionized (DI) water was first pumped to flow through the column filter at a rate of 2 mL/min for 30 minutes to saturate the cotton filter with water. Then, 50 mL of the filtered moringa extract was flowed through each column at 2 mL/min for 25 minutes to coat the cotton fiber with moringa proteins. The total protein concentrations of influent and effluent were measured using Quick Start™ Bradford Protein Assay Kit 2 (Bio-Rad, USA) following the manufacturer's protocol to determine the protein amount coated on the cotton fiber. **Fig. S1** shows that the total protein concentration of the effluents was saturated to that of the initial solution, indicating the cotton filter was functionalized by the moringa proteins within 25 minutes. Note that this in situ coating method was found to be more efficient compared to the batch process [15].

Propagation of testing viruses

TV and TGEV were provided by the Cincinnati Children's Hospital Medical Center [20] and the Veterinary Diagnostic Laboratory at the University of Illinois Urbana-Champaign, respectively (Oh et al., 2022a). We sequenced 6 kilo-bases (kb) of the TV genome and 10 kb of the TGEV genome, which are similar to the sequences of TV (99% identity with NC_043512.1) and TGEV (99% identity to KX900394.1) in the GenBank (Fuzawa et al., 2019 and Supplementary file 1). This finding confirmed the identity of the virus stocks used in this study. We propagated the TV and TGEV in MA 104 (CRL-2378.1, ATCC, USA) and ST cell lines (CRL-1746, ATCC), respectively, supplemented by a complete culture medium. The complete culture medium consisted of 1X minimum essential medium (MEM; Thermo Fisher Scientific, USA),

2% fetal bovine serum (FBS; Thermo Fisher Scientific, USA), 1X antibiotic-antimycotic (Thermo Fisher Scientific, USA), 17 mM $NaHCO_3$, 10 mM HEPES, and 1 mM sodium pyruvate (Thermo Fisher Scientific, USA). The cells were incubated at 37℃ with 5% CO_2 for 2 days. Following incubation, the viruses were released from their host cells through three cycles of freeze and thaw. Centrifugation at 2000 rpm (556 g) for 10 minutes using a Sorvall Legend RT Plus centrifuge (Thermo Fisher Scientific, MA, USA) was employed to separate the viruses from the cell debris. The resulting supernatant was filtered using a 0.22 μm filter (Millipore Sigma, MA, USA) and stored in 1 mL aliquots as stock solutions at -80°C until further use.

Viral RNA quantification

Viral RNA was extracted from samples using the QIAamp Viral RNA mini kit (Qiagen, Germany), following the manufacturer's instructions. The extracted viral RNA was stored at -30℃ and analyzed using reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) within three days. The RT-qPCR mixture included 3 μL of RNA sample, 0.3 μL of 10 μM forward primer, and 0.3 μL of 10 μM reverse primer, 1.275 μL of molecular biology grade water (Corning, NY, USA), 5 μL of 2×iTaq universal SYBR green reaction mix, and 0.125 μL of iScript reverse transcriptase from the iTaq™ Universal SYBR® Green One-Step Kit (1725151, Bio-Rad Laboratories, USA). The RT-qPCR mixture was dispensed in 96-well plates (4306737, Applied Biosystems, USA) and analyzed by an RT-qPCR system (QuantStudio 3, Thermo Fisher Scientific, USA). The RT-qPCR mixture was incubated with a thermocycle of 50℃ for 10 minutes, 95℃ for 1 minute, and then 40 cycles of denaturation at 95℃ for 10 seconds, annealing and extension at 60℃ for 30 seconds. Melting curves were analyzed while the temperature increased from 60°C to 95°C at the end of each RT-qPCR analysis. No primer-dimers were detected in any of the RT-qPCR analyses. The SYBR signal

was normalized to the ROX reference dye. The cycles of quantification (Cq) were determined by QuantStudio Design & Analysis Software (v1.5.1). Each RT-qPCR mixture, including synthetic DNA for a standard curve (and a positive control), molecular biology grade water as a negative control, and the viral RNA samples, was analyzed in at least three technical replicates. The linear dynamic range for the serial dilutions of synthetic DNA ranged from 10^0 to 10^5 gene copies (gc)/μL. The PCR efficiencies for the RT-qPCR assays were above 85% (R^2>0.99). The details of the RT-qPCR assays are summarized in **Table S1 and S2**, adhering to the MIQE guidelines [22]. An inhibition test was conducted by adding 1 μL of 10^3 gc/μL bovine coronavirus RNA (BCoV; Merck Animal Health, USA), which is not expected to exist in our groundwater samples, to 10 μL of RNA extract and 10 μL of molecular biology grade water. We found that the differences in Cq values for BCoV RNA between the RNA extract and the water were smaller than 1, meaning that the impact of any possible inhibitors was negligible (**Fig. S2**) (Oh et al., 2022b). The limit of detection (LOD) for the RT-qPCR assays for TV and TGEV was determined using 20 replicates of serial dilutions of synthetic DNA controls (Oh et al., 2022b). We found that the LODs for TV and TGEV were 2.14×10^3 and 8.91×10^2 gc/mL, equivalent to 2.1 and 0.9 gc/μL, respectively (**Fig. S3**).

Column experiments

A schematic illustration for column experiments conducted is depicted in **Fig. 1A**. The virus stock solution of either TV or TGEV was diluted 1000-fold in autoclaved groundwater, obtained from the Newmark building at the University of Illinois Urbana-Champaign. The TV-containing groundwater included TV RNA concentrations of $10^{6.6}$ gc/mL, and the TGEV-containing groundwater had TGEV RNA concentrations of $10^{5.2}$ gc/mL. The virus-containing groundwater was continuously pumped into the *MO*-cotton filter. The flow rate of the virus-containing groundwater was set to 2 mL/min and adjusted for the constant flow rate throughout

the virus removal experiments. The influent and effluent were sampled over time to evaluate the virus removal efficacy of the *MO*-cotton filters. RNA concentrations of the influent were not significantly reduced throughout the virus removal experiments ($p>0.05$ from a linear regression analysis, **Fig. S4**), meaning the natural inactivation was negligible. Log virus reduction value was determined by RNA concentrations of influent divided by that of effluent on a log scale (i.e., $Log_{10}\ C_{influent}/C_{effluent}$). Virus removal efficacy was evaluated by presenting the log virus reduction values with a normalized time scale, dimensionless column volumes (CV) (**Eq. 1**).

$$CV = \frac{elapsed\ time\ (min) \times flow\ rate\ (mL/min)}{column\ volume\ occupied\ with\ cotton\ (mL)} \quad \text{(Eq. 1)}$$

Because the flow rate and column volume were set to 2 mL/min and 7.1 mL, respectively, 3.5 minutes of elapsed time was equal to 1 CV. Virus removal kinetics was determined by two non-linear regression models, Thomas (**Eq. 2**) and Logarithm models (**Eq. 3**).

$$Log_{10}(\frac{gc_{influent}}{gc_{effluent}}) = Log_{10}(1 + exp(a + bCV) \quad \text{(Eq. 2)}$$

$$Log_{10}(\frac{gc_{influent}}{gc_{effluent}}) = cln(CV) + d \quad \text{(Eq. 3)}$$

where, $gc_{influent}$ and $gc_{effluent}$ indicate RNA concentrations of influent and effluent, CV is column volumes, normalized time term (**Eq. 1**), and a, b, c, d are constants. The Thomas model is one of the most widely used to describe breakthrough curves of the fixed-bed column [23,24] and the logarithmic model has been used to describe contaminant removal efficacy in filtration systems [25–27].

Quantification of risk from detached virus particles

In addition to evaluating the virus removal efficiency of *MO*-cotton filters, we also conducted virus detachment experiments, designed to investigate the potential detachment of viruses accumulated on the *MO*-cotton filters. Detachment of viruses was induced by two methods in this study. After the TV removal experiments described above were completed, we flowed sterilized groundwater (i.e., no viruses were spiked) at a flow rate of 6 mL/min for about 30 minutes to induce virus detachment at high superficial velocity. Furthermore, one-third of the cotton on the top was taken from the column and transferred to a sterilized 50 mL tube. We added 10 mL of DI water and vortexed the mixture for 2 minutes, incubated at room temperature for 10 min, and then vortexed for 2 minutes again to simulate high shear conditions and detach viruses from the cotton fiber. After detachment was induced, the infectious TV titer and TV RNA concentrations of the effluent solution were quantified as described below to evaluate the risk of detached viruses.

Infectious virus titer measurement

Plaque assays were conducted to determine infectious virus titers of TV and TGEV, following the protocol described by Oh et al. (2022a). A monolayer of MA104 and ST cells was prepared in the complete culture medium. The plaque assay was performed when the cell confluency reached approximately 100% on 6-well plates (USA Scientific, USA). For the plaque assay, each virus sample was serially diluted 10-fold in the complete culture medium without 10% FBS. Subsequently, 800 μL of each serial dilution was added to each well of 6-well plates and incubated for 1 hour at 37℃ with 5% CO_2. After 1 hour, the supernatants were aspirated, and a 2 mL overlay solution consisting of 1X MEM, 1% agarose, 7.5% sodium bicarbonate, 15 mM HEPES, and 1X antibiotic-antimycotic was added to the cellular monolayers. The overlay

solution was solidified by incubating at 4℃ for 20 minutes. After a 2-day incubation at 37℃ with 5% CO_2, the 2 mL of a 10% formaldehyde solution was added to each well and incubated at room temperature for approximately 1 hour. Finally, the overlay and formaldehyde solution were removed, and the plaques were visualized by adding 0.5 mL of 0.05% crystal violet dye solution. Plaque-forming units (PFU) were counted over a lightbox. One PFU on a well with an 800 μL 10-fold dilution of the original sample represented the lowest infectious virus titer. As a result, the limit of detection (LOD) for the plaque assays was $10^{1.1}$ PFU/mL in this study.

Batch experiments

Interactions between moringa proteins and TV were investigated through batch experiments to understand the mechanism of inactivation for viruses on *MO*-cotton filters. Schematic illustration for the batch experiment is depicted in **Fig. 3A**. Groundwater was spiked with TV to achieve a final RNA concentration of $10^{6.1}$ gc/mL. A total of 675 μL of the TV-containing groundwater was then mixed with 75 μL of moringa proteins, with varying total protein concentrations ranging from 0 to 25.3 mg/mL. After a 5-minute reaction period, 750 μL of FBS was added to stop the reactivity of the moringa proteins. Subsequently, the final reactants were examined for infectious TV titers, total TV RNA, and TV aggregates. The TV aggregates were analyzed according to the aggregation assay described in the subsequent methods. Note that **Fig. S5** represents that FBS-quenched moringa proteins did not cause a significant impact on infectious TV titers compared to that of PBS, indicating that FBS can quench the moringa protein activity under our experimental conditions.

Aggregation assay

The diameter of a single TV particle was less than 0.1 μm (Oh et al., 2022b). Therefore, TV particles with a diameter larger than 0.1 μm were assumed to be aggregated TV particles in this

study. To quantify the TV aggregates with a diameter larger than 100 μm, we employed the aggregation assay developed by (Oh et al., 2022b). In brief, the TV samples were passed through a 0.1 μm syringe filter (Sartorius, Germany). The number of gene copies particles in the TV samples (i.e., total TV particles) and the filtrate (i.e., TV gene copies with a diameter less than 100 μm) were quantified using the RT-qPCR assays as described earlier. The aggregated virus particles were determined by subtracting the number of TV gene copies with a diameter of less than 100 μm from the total TV particles.

Statistical analysis

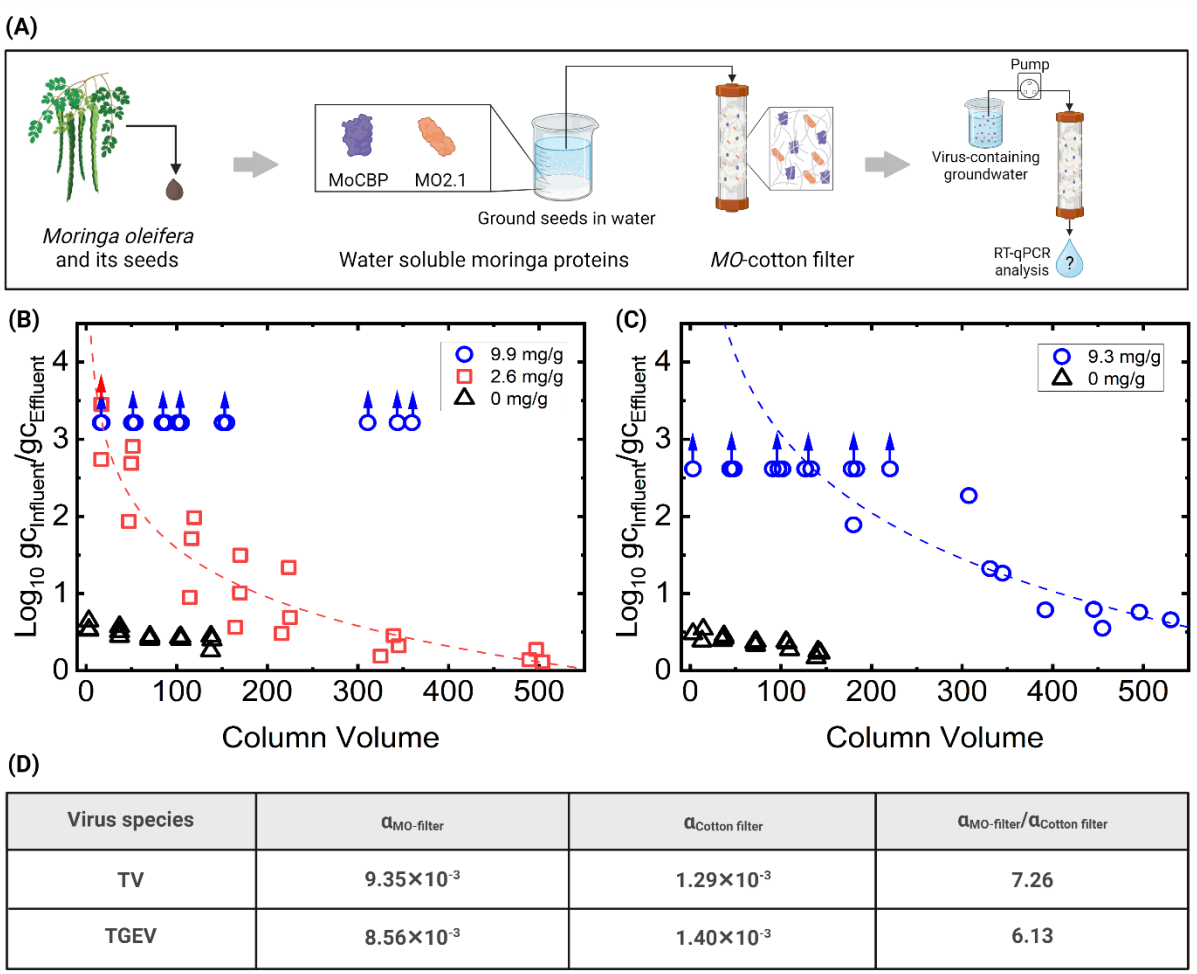
Linear regression analyses were carried out to examine whether the influent TGEV RNA concentrations decreased over the experimental time frame (**Fig. S4**), as well as to investigate the effects of moringa protein concentrations on virus aggregation and inactivation (**Fig. 3**). Two-sample t-tests were performed to assess the quenching effects of FBS on the affinity of moringa proteins to viruses (**Fig. S5**), to evaluate the impact of moringa protein functionalization on virus removal efficacy of cotton filters (**Fig. 1**), and to compare the extent of virus aggregates detached from *MO*-cotton filters (**Fig. 2**). Mean-squared errors (MSE) were calculated to compare the goodness-of-fit of two non-linear regression models (the Thomas and logarithmic models), for TV removal kinetics (**Fig. S6**). All statistical analyses were conducted using OriginPro 2023.

Results

Moringa protein functionalized cotton filter can achieve 4-log virus reduction, meeting regulations set by WHO and USEPA

The TV removal efficacy of the *MO*-cotton filter is presented in **Fig. 1B**. When 9.9 mg/g of moringa protein was coated on the cotton fiber, the filter showed TV removal efficacy of

greater than 3.2-$\log_{10}$ until 360 CV (blue circles in **Fig. 1B**). In contrast, cotton fibers without moringa protein coating showed a maximum removal efficacy of less than 0.6-$\log_{10}$ at the beginning of the column experiment (i.e., 3 CV) which was significantly lower than that of the cotton fiber with the 9.9 mg/g of moringa protein (two sample t-test; $p<0.001$). This finding indicates that the significant increase in virus removal efficacy is attributed to the proteins of the *MO* seed extracts.



(D)

Virus species	$\alpha_{MO\text{-}filter}$	$\alpha_{Cotton\ filter}$	$\alpha_{MO\text{-}filter}/\alpha_{Cotton\ filter}$
TV	9.35×10^{-3}	1.29×10^{-3}	7.26
TGEV	8.56×10^{-3}	1.40×10^{-3}	6.13

Fig. 1. **(A)** Schematic illustrations for column experiments. **(B)** TV and **(C)** TGEV removal kinetics were determined by column experiments with cotton fiber functionalized by water-soluble moringa proteins. The symbols and lines represent measurements by RT-qPCR and regression analysis by a logarithmic model, respectively. The legend on each figure indicates the protein amount functionalized on the cotton filter. The arrows above symbols indicate that the RNA concentrations of effluent were below the limit of detection. **(D)** Sticking coefficients by a clean bed filtration model.

However, TV removal kinetics and the maximum TV removal efficacy of the filter were not determined by the filter with 9.9 mg/g of moringa protein because the virus concentrations in the effluent were all below the LOD for the entire period of the experiment. Therefore, we conducted another experiment with a reduced amount of moringa protein of 2.6 mg/g. The column with 2.6 mg/g of moringa protein showed a decrease in removal efficacy as the virus filtration progressed to 503 CV (red rectangles in **Fig. 1B**). We fitted data of virus removal efficacy, which were quantified by RT-qPCR (i.e., RNA concentrations of effluents were above the LOD), with two nonlinear regression models (the Thomas and logarithmic models) to understand virus removal kinetics and to estimate the maximum removal efficacy. We found that the logarithmic model described the virus removal kinetics better, showing 0.59 and 0.11 of mean squared error (MSE) for TV and TGEV, respectively, compared to the Thomas model, which was 0.79 and 0.16 of MSE (**Fig. S6**). In particular, the differences in removal efficacy between the two models were more distinct at the beginning of the column experiment. For example, the TV removal efficacies at CV of 16 by the Thomas models were 1.4-$\log_{10}$ reduction, which was much lower than the experimental data, >3.2-$\log_{10}$ reduction (**Fig. S6**). Instead, we found that the logarithmic model estimated the virus removal efficacy at CV of 16 to be 3.3-$\log_{10}$ reduction (**Fig. S6**). With the logarithmic regression model, the maximum virus removal efficacy of the cotton filter with 2.6 mg/g of moringa protein was calculated to be higher than 4-$\log_{10}$ reduction as a direct evaluation of this high removal efficiency was not possible because the concentration of viruses in the effluent samples was lower than the LOD of the RT-qPCR analysis. Based on these results obtained with a *MO*-cotton filter functionalized with 2.6 mg/g of protein, it can be concluded that the filter with enough moringa protein, such as 9.9 mg/g, would show virus removal efficacy of higher than 4-$\log_{10}$ reduction.

We also evaluated TGEV removal by the *MO*-cotton filter because TGEV is an enveloped viral species that could show different behavior from non-enveloped viruses. First,

the TGEV removal efficacy of the filter with 9.3 mg/g of moringa protein was significantly higher than that of the cotton filter without the protein coating (t-test, $p<0.001$). This finding again supports that moringa protein is the key ingredient for TGEV removal. Next, the logarithmic model, excluding the data below the LOD, showed that the maximum TGEV removal efficacy was estimated to be higher than 4-$\log_{10}$ until a CV of 67. Given the column experiments with the two different viral species in groundwater, the *MO*-cotton filters tested here have promising potential to meet the US EPA and WHO virus treatment requirements for 4-$\log_{10}$ reduction or inactivation of viruses [29].

In addition to the kinetics models described above, clean bed filtration models have been widely used to calculate the sticking coefficient, a parameter evaluating the interaction between particles and filter media[30,31]. Based on the removal efficacies achieved by the *MO*-cotton filters at 350 CV (i.e., greater than 3.2-$\log_{10}$ reduction for TV and 1.2-$\log_{10}$ reduction for TGEV in **Fig. 1C**), the sticking coefficients for TV and TGEV to *MO*-cotton were calculated to be 7.3 and 6.1 times higher than those for cotton without *MO* functionalization, respectively. This result corroborates that the significant increase in virus removal efficacy is attributed to the *MO* proteins. Additionally, considering the experimental data and clean bed filtration theory, increasing the column heights to 7.5 cm and 19.6 cm (equivalent to a 24% and 227% increase from the initial 6 cm) would achieve a 4-log reduction of TV and TGEV, respectively, at a CV of 350 (**Fig. 1D**, **Supplementary File**). It is important to note that the sticking coefficients shown here along with clean bed filtration models represent a widely established tool that can be used as a basis for design and optimization of filter size and operating flowrate for field-scale applications.

Potential risk of infection due to virus detachment from the moringa protein functionalized cotton filter is negligible

Even though *MO*-cotton filters show significant potential for removal of mammalian viruses from realistic water matrix, it is important to consider the risk of virus detachment during filter operation to ensure public safety. Viruses accumulated on the filter could be released back into the effluent [32–34] in situations when the virus concentration on the filter is higher than that of the influent or when the shear flow of groundwater disrupts the virus particles bound to the filter, resulting in the contamination of drinking water, as evidenced by previous studies [35]. Thus, we conducted virus detachment experiments after the virus removal experiment was completed to understand the potential virus detachment risk when using it for point-of-use drinking water purification.

First, we pumped sterilized groundwater without spiking viruses (i.e., no TV included) through the columns after the TV removal experiments (**Fig. 2A**) were completed to create conditions where the concentration of viruses was higher on the filter compared to the water matrix. The flow rate was 6 mL/min, three times faster than the influent flow rate of the virus removal experiment to ensure higher shear compared to normal operation. This experiment was conducted to simulate the possible virus detachment during the filter operation. TV RNA concentrations and infectious TV titers of the effluent are summarized in **Fig. 2B**. Note that the virus removal experiment with 0 mg/g of moringa proteins also showed a noticeable level of TV and TGEV removal efficacy (t-test; $p<0.001$), which was about 0.5-$\log_{10}$ reduction (**Fig. 1**). This finding implies that cotton fibers itself (without moringa protein functionalization) contributed to trapping a portion of viruses from the groundwater. The effluent from the column with 0 mg/g of moringa protein contained infectious TV (**Fig. 2B**), which means that the binding between viruses and cotton is not strong enough to hold viruses from the shear flow of groundwater. On the other hand, the total TV RNA and infectious TV titer of the effluent from

the column with 9.9 mg/g of moringa proteins were not detected (**Fig. 2B**). Since this filter provided high removal efficacy of TV (i.e., higher than 3.2-$\log_{10}$ reduction) at the end of the removal experiment, there were available adsorption sites on moringa proteins where viruses are strongly attracted via electrostatic force over cotton fiber (blue circles in **Fig. 1B**). Thus, direct attachment to moringa proteins can prevent viruses from detachment due to the shear flow of groundwater. In the case of the column with 2.6 mg/g of moringa proteins, virus removal efficacy was reduced to values comparable to the column without moringa proteins at the end of the experiment (**Fig. 1B**). We detected infectious TV from the effluent, similar to the scenario of the column without moringa protein functionalization. The detection of infectious TV from this column can be explained by the fact that the available adsorption sites of moringa proteins were depleted in the column with 2.6 mg/g of moringa proteins, and thus a part of TV was loosely trapped on the cotton fiber.

In another set of experiments, we forced viruses to detach from the filter by vortexing the a portion of the filter substrate with 10 mL molecular biology-grade water to simulate high shear conditions. This experiment was designed to demonstrate the possible maximum shear force by unexpected physical disruptions to the filter, such as instant injection of influent to the filter or external disturbances that could occur during daily use of a point-of-use filter. Vortexing cotton filter detached TV particles from all three columns with 0, 2.6, and 9.9 mg/g of moringa proteins (**Fig. 2C**), which indicates viruses could be detached by the shear force. However, interestingly, the detached viruses were infectious only when they were from the filter without moringa protein functionalization, whereas viruses were not infectious when they were from the columns with 2.6 and 9.9 mg/g of moringa proteins. The virus inactivation may be attributed to the moringa proteins also being detached from the filter by the shear force. Specifically, we detected proteins only from the two columns with 2.6 and 9.9 mg of moringa proteins. Also, the level of virus aggregates was significantly higher when moringa protein was

present in the extract (t-test; $p<0.001$, **Fig. 2C**). Given the results from the detachment experiments, we can conclude that the availability of adsorption sites of moringa proteins is essential not only to achieve more than 4-$\log_{10}$ virus reduction but also to reduce the risk of the potential release of viruses from the filter.

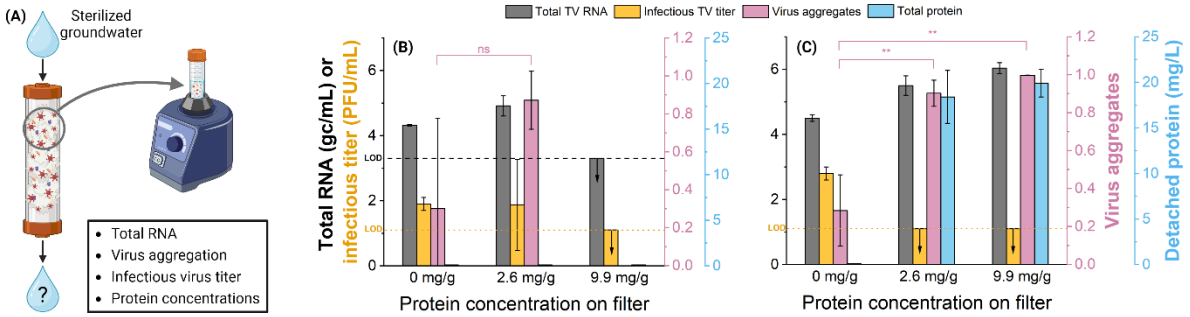


Fig. 2. **(A)** Schematic illustrations for detachment experiments. Results showing total TV RNA concentration, infectious TV titer, virus aggregates (normalized to total RNA), and total protein concentration in **(A)** the effluents of flowrate at 6 mL/min and **(B)** the extracts from cotton vortexing (n=3). Two-sample tests were conducted for virus aggregate comparison (ns: $p>0.05$, **: $p<0.001$). Dashed and dotted lines indicate the limit of detection for total TV RNA concentration and infectious TV titer, respectively. The downward arrows represent the true values could be lower than the presented values.

Electrostatic interactions drive the virus removal and subsequent inactivation if detachment occurs due to high shear

Moringa proteins have been used as a coagulant that destabilizes contaminants by manipulating charges of particles suspended in water [36]. To understand interactions between the moringa proteins and viruses, we conducted batch experiments where the TV was incubated in varying concentrations of the moringa proteins and analyzed levels of inactivated viruses and aggregated viruses. **Fig. 3B** shows that the infectious TV titer significantly reduced as protein concentration increased ($p<0.001$ from a linear regression analysis), indicating that the moringa proteins potentially lead to virus inactivation. **Fig. 3C** represents the relationship between levels of aggregated viruses and inactivated viruses after the reactions with the moringa

424 proteins. We found that the linear regression analysis shows the slope is significantly higher
425 than zero ($p<0.001$), meaning virus aggregation could be related to virus inactivation. This
426 analysis suggests that moringa proteins interact with TV, causing TV aggregation.
427 Consequently, aggregation would likely prevent the TV from entering host cells, similar to how
428 other plant-based extracts inactivate viruses (Oh et al., 2022b).
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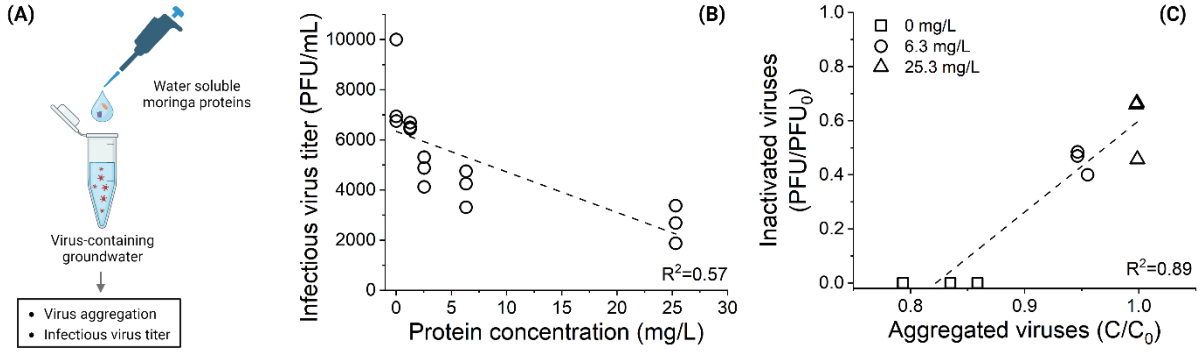

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431 **Fig. 3. (A)** Schematic illustrations for batch experiment designed to mimic the interaction
432 between water-soluble moringa proteins and TV in column effluent. **(B)** Infectious TV titer
433 changes with total protein concentrations. **(C)** A relationship between levels of aggregated
434 viruses and inactivated viruses after the reactions between TV and the moringa proteins were
435 completed.
436

437 The strong affinity of the moringa proteins to viruses has been explored for filter media
438 functionalization. Many studies appreciated natural cotton fiber as filter media because it
439 provides high water permeability and its surface is easy to be functionalized [16,37,38]. Our
440 previous study proved that cotton fiber is negatively charged (zeta potential of approximately
441 −20 mV) in the pH range of 5–8, which allows for the adsorption of cationic moringa proteins,
442 *Moringa oleifera* coagulant protein (MO2.1) and *Moringa oleifera* chitin binding protein
443 (MoCBP), on the cotton fiber [16]. We confirmed the amount of protein saturated at the elapsed
444 time of 17 minutes, showing that 6.7 mg/g of the maximum amount of the moringa proteins on
445 cotton fiber when the protein concentration of the influent was 0.62 mg/mL (**Fig. S1**). Samineni
446 et al. (2022) also found that the cationized cotton fiber attracted bacteriophage (MS2) via

electrostatic force, trapping them in the cotton filter. The moringa protein-functionalized cotton filter is also expected to remove TV and TGEV from the groundwater using similar mechanisms for the following reasons. First, the groundwater used in the current study contained 1.7 mM of calcium ions, and the pH was stable between 7.3 and 7.5 (**Table S3**, Shen et al., 2017). Second, isoelectric points (IEPs) of many enteric viruses are known to be below the pH of our groundwater. For example, IEPs of *caliciviridae*, such as norovirus and TV, were 6.0 [40,41] and 4.8 [42], respectively. In addition, SARS-CoV-2 and bovine coronavirus [43], which are in *coronaviridae*, show IEP of 5.3 and 4.6, respectively. This low value of IEPs suggests that the TV and TGEV are negatively charged in our groundwater and electrostatically bind to the moringa protein functionalized filter.

Discussion

Moringa protein functionalized cotton filter is a plausible point-of-use filter to reduce the risk of virus infection in drinking water

Although various water filtration technologies have been developed, only a few can successfully be applied for gravity-driven point-of-use filters for reducing the risk of virus infection to the level of drinking water because of the following two reasons. First, the successful point-of-use filter must demonstrate a 4-$\log_{10}$ reduction or inactivation in pathogen concentrations (regulations by USEPA or WHO), over long periods of time to provide drinking water for daily use. Gutierrez et al. (2009) used a column (1.5 cm in diameter and 13.2 cm high) filled with hematite nanoparticle-coated glass fiber that removed rotavirus from actual groundwater (flowrate at 3 mL/min) by up to 4-$\log_{10}$ reduction. However, its performance soon decreased to less than 3-$\log_{10}$ reduction after 3-bed volumes (BV). Mthombeni et al. (2012) examined the use of resin beads coated with silver nanoparticles to deactivate microbes in drinking water in a column filtration system. *E. coli* containing water (2 mL/min) was flowed

through columns of 2 cm diameter and 30 cm length with silver nanoparticle-coated resin beads, and *E. coli* reduction efficacy was less than 1-$\log_{10}$ after 30 CV. Our study supports that the *MO*-cotton filter can remove TV and TGEV by more than 4-$\log_{10}$ reduction, which meets regulations by USEPA and WHO. Also, we experimentally confirmed that the filter achieved a 3-$\log_{10}$ TV reduction of up to 300 CV, which is a noticeable period of efficient virus removal. Additionally, the size of the *MO*-cotton filters can be easily optimized as the materials required for fabrication are easily accessible and the method of fabrication is robust and scalable.

Second, the filter should not pose any risk of releasing harmful materials, which could be adsorbate (i.e., viruses) or adsorbent (i.e., filter media) [32–34]. For example, Chung et al. (2015) created hydrochar-amended sand beds (2.5 cm inner diameter and 10 cm bed height) and achieved a 2.4-$\log_{10}$ removal of rotavirus and 2.4 for adenovirus up to 2 CV, but the viruses were released into the effluent at concentrations of 1% to 10% of initial concentrations when clean water was passed through it. In addition, the potential release of filter media could cause adverse impacts on human health. For example, release of nanomaterials from engineered filter medium could impact human health [47,48]. In this context, the moringa protein functionalized filter shows a minimal risk of infection by the viruses released from the filter as long as available adsorption sites of moringa proteins exist. Although we found that the moringa protein could be released from the filter by external physical force, the moringa proteins are considered safe for human health, and thus it has been used as a health supplement [49,50], and the viruses accumulated on the moringa proteins were no longer infectious (**Fig. 2**). For the above two reasons, the functionalization of cotton filters with moringa protein could be a plausible point-of-use filter to reduce the risk of virus infection in drinking water.

Moringa protein functionalized cotton filter is applicable to a wide range of drinking water sources

Considering that virus adsorption to moringa proteins is the main removal mechanism, virus concentration in the influent will affect the removal efficacy of the moringa protein functionalized cotton filter. In this study, infectious virus titers in the influent groundwater were higher than 10^3 PFU/mL, which are likely much higher than those found in actual drinking water sources. For example, rotavirus concentrations in river water, treated wastewater, and untreated wastewater are reported to be $10^{-3.0}$, $10^{-2.2}$, and $10^{-1.3}$ focus-forming unit (FFU)/mL, respectively (Lodder et al., 2005; Rutjes et al., 2009). Given the findings that the moringa protein functionalized cotton filter functions properly with orders of higher virus concentrations, this filter is expected to reduce the risk of virus infection in actual water sources for a large number (estimated to be 1000s) of bed volumes.

In addition, organic matter has been reported to compete with adsorption sites of filtering systems with target contaminants [51]. We tested the performance of a filter using actual groundwater containing 1.6 mg/L of total organic carbon (TOC) (**Table S3**), a similar level to that found in typical groundwater used as a source of drinking water [51–54]. While groundwater is the most common form of self-supply in South Asia and Southeast Asia, rainwater collection is also prevalent in the Pacific [55]. Rainwater typically contains 2 mg/L or less of TOC [56,57], indicating that the moringa protein functionalized cotton filter could also be effective in turning rainwater into safe drinking water.

Moringa protein functionalized cotton filter is a plausible point-of-use filter for those with limited resources and under disaster or emergency scenarios

The findings of this study provide valuable information for designing a point-of-use filter. The treated groundwater used in this study had a flow rate of 2 mL/min, which translates to a

superficial velocity of 0.7 m/h. If we increase the moringa protein functionalized cotton filter to 15 cm in diameter and 40 cm in height (1000 times increase in volume), the filter could provide 30 L of drinking water, a necessary amount of drinking water per day for a household [58], just in 2.4 hours (i.e., 12.4 L/h capacity). Our experiments showed that the column can achieve a 3.2-$\log_{10}$ reduction or higher in TV until at least 350 CV, and this capacity is equivalent to 2545 L or 85 days of drinking water for a household. To construct a household-scale point-of-use filter, we would require 1.75 kg of cotton and 1 kg of moringa seeds, each of which costs approximately 5 US dollars or less in places where lack of access to centralized water supply systems [59,60].

The filter fabrication and maintenance should be manageable by local people for sustainable operation. Various chemicals, such as zero-valent iron (ZVI) and magnesium oxide (MgO), have been proposed to functionalize filter media and improve virus removal efficiency [61,62]. The critical limitation of these chemicals is that they are not reusable and thus rarely applicable to the places with limited resources and disaster or emergency scenarios [63,64]. However, the moringa protein functionalized fiber filter is made of accessible natural materials (*Moringa oleifera* seeds and cotton fiber). This filter is also regenerable only with 600 mM NaCl and additional moringa proteins [16]. In addition, due to the high-water permeability of cotton filters, this filter does not require a pump to flow water through the filter.

Conclusion

We conducted systematic experiments under practically relevant conditions to determine virus removal efficacy and mechanisms of moringa protein functionalized cotton filter. Our experimental data clearly demonstrated that the moringa protein functionalized cotton filter can be used as a point-of-use filter to reduce the risk of virus infection to the level of drinking water.

In particular, this filter will contribute to providing clean drinking water to people in low- and middle-income countries.

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