Characterization of recombinant norovirus virus-like particles and evaluation of their applicability to the investigation of norovirus removal performance in membrane filtration processes

N. Shirasaki, T. Matsushita, Y. Matsui and K. Ohno

ABSTRACT

Noroviruses (NVs) are one of the leading causes of epidemic gastroenteritis around the world. Water treatment technologies using membrane filtration for virus removal are becoming increasingly important. However, experiments to test removal of NVs from water have been hampered because NVs do not grow in cell culture or in small-animal models and therefore cannot be easily artificially propagated. Expression of the NV genome in a baculovirus-silkworm expression system has produced recombinant NV virus-like particles (rNV-VLPs) that are morphologically and antigenically similar to native NV. Here, we characterized these rNV-VLPs and evaluated their potential use in assessing NV removal. Electron microscopic analysis and peptide mass fingerprinting showed that the rNV-VLPs were morphologically identical to native NV. In addition, surface charge and particle size distribution, which are important factors for explaining virus particle behavior during membrane filtration, were successfully evaluated by using rNV-VLPs. The rNV-VLPs were easy to quantify with a commercially available enzyme-linked immunosorbent assay kit, they remained stable for several days at 4°C after dilution in river water, and they were easy to concentrate with the ultrafiltration entrapment method used. Thus, rNV-VLPs can be used to facilitate our understanding of the behavior of NVs during membrane filtration processes.

Key words | norovirus, peptide mass fingerprinting, sample storage method, virus concentration method, virus-like particles

INTRODUCTION

Noroviruses (NVs), which belong to the genus Norovirus of the family Caliciviridae, are the dominant cause of epidemic nonbacterial gastroenteritis around the world in all age groups. Owing to their low infectious dose (Teunis et al. 2008) and environmental stability (Duizer et al. 2004; Bae & Schwab 2008), NVs are easily transmitted in a wide variety of environments, which has led to numerous outbreaks.

The main source of NV in the aquatic environment is fecal waste from infected persons, because patients excrete stools containing a high content of NV (approximately $10^{10}$ copies cDNA/g (Lee et al. 2007)). High concentrations of NV have often been detected in untreated sewage, treated sewage, and wastewater (Lodder & Husman 2005; da Silva et al. 2007). Therefore, environmental water, including groundwater and river water, are sometimes contaminated with NV via sewage discharge or inflow of treated sewage and wastewater (Powell et al. 2003; Lodder & Husman 2005). This could pose a human health risk when these waters are used as drinking water sources. Numerous outbreaks of NV gastroenteritis through consumption of contaminated drinking water have been reported (Nygard et al. 2003; Maunula et al. 2005). Accordingly, understanding NV behavior during drinking water treatment processes is important for protecting human health.

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Among water treatment processes, membrane filtration processes are becoming one of the most important technologies due to their ability to efficiently produce high quality water and their small footprint (Huang et al. 2009). Membrane filtration processes, including microfiltration (MF), ultrafiltration (UF) and nanofiltration (NF), vary in their efficiency of virus removal (Otaki et al. 1998; Langlet et al. 2009). However, because there is no cell-culture system or small-animal model for NV cultivation (Bae & Schwab 2008), studies of drinking water treatment processes for NVs, including separation and disinfection processes, have been hampered compared with those for cultivable viruses. We therefore propose that an appropriate surrogate for NVs is required to estimate NV behavior during water treatment processes.

Expression of the NV genome in a baculovirus expression system produces extremely high numbers of recombinant NV virus-like particles (rNV-VLPs) (Jiang et al. 1992) that are morphologically and antigenically similar to the native NV, but are non-infectious due to their lack of viral RNA; this negates the need for the establishment of a cell-culture system or small-animal model for NV cultivation. Therefore, rNV-VLPs have been used as a NV surrogate to develop virus detection methods (Kobayashi et al. 2000; Colquhoun et al. 2006) and to evaluate virus purification and concentration methods (Huhti et al. 2010). Moreover, adsorption and aggregation properties of NVs in various solution chemistries have been evaluated by using rNV-VLPs (da Silva et al. 2011). We therefore hypothesized that rNV-VLPs are suitable for use as a NV surrogate to estimate NV removal performance during membrane filtration processes.

Accordingly, the objectives of the present study were to characterize rNV-VLPs and to evaluate the applicability of rNV-VLPs as a potential alternative to the native NV in the estimation of NV removal performance during membrane filtration processes.

**MATERIALS AND METHODS**

**Preparation of rNV-VLPs**

Subgenomic cDNA fragments derived from Chiba virus (AB042808, GL4, Chiba407/1987/JP, a Japanese strain in genogroup I of genus Norovirus, family Caliciviridae) were artificially synthesized. The synthesized cDNA fragment containing the entire second and third open reading frames and the 3’ untranslated region of the NV genome was inserted into the vector (pDONR221, Invitrogen Japan K. K., Tokyo, Japan) by using the Gateway BP reaction. After EcoRI and PstI digestion of the plasmid, the digested cDNA fragment was ligated into the baculovirus transfer vector (pM0NHT04, Sysmex Corp., Kobe, Japan, former Katakura Industries Co. Ltd, Saitama, Japan). The transfer vector was co-transfected with the linearized genomic DNA of baculovirus (Bombyx mori (silkworm) nucleopolyhedrovirus; CPd strain (Suzuki et al. 1997)) into the B. mori-derived cell line, BmN (Maeda 1989), to generate a recombinant baculovirus, which was then injected into silkworm pupae to express the rNV-VLPs. Six days after inoculation, the expressed rNV-VLPs were separated from the pupal homogenate by centrifugation and dialysis to prepare the rNV-VLP stock solution, which was stored at −83 °C in the dark until use.

**Electron microscopy**

Negative-stain electron microscopy was used to analyze the presence, integrity, and morphology of the rNV-VLPs. Ten microlitres of rNV-VLP stock solution was deposited on a 400-mesh copper grid with a collodion membrane (Nissin EM Corp., Tokyo, Japan) and adsorbed to the grid for 1 min. Excess solution was drained from the side of the grid with filter paper, and rNV-VLPs were negatively stained with 10 μL of 2% phosphotungstic acid (pH 5.5) for 45 s. After the excess stain was drained off, the grid was examined with a transmission electron microscope (HD-2000, Hitachi High-Technologies Corp., Tokyo, Japan).

**Peptide mass fingerprinting**

The target protein selected for peptide mass fingerprinting was a capsid protein of Chiba virus (NCBI accession number gi|11275373). The rNV-VLP stock solution was denatured at 95 °C for 20 min in a thermal cycler (Thermal Cycler Dice Model TP600, Takara Bio Inc., Otsu, Japan). Five microlitres of denatured rNV-VLPs were then digested with 0.5 μg trypsin (sequencing grade modified trypsin,
Promega, Madison, WI, USA) in 10 μL of buffer containing 50 mmol/L Tris-HCl and 1 mmol/L CaCl₂ at 37 °C for 20 h and then 4 °C for 30 min to produce rNV-VLP capsid peptides. The peptides were suspended in a matrix solution containing 3.0 mg/mL of α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich Co. LLC., St Louis, MO, USA), 50% (v/v) acetonitrile (HPLC grade, Wako Pure Chemical Industries, Osaka, Japan), and 0.1% (v/v) trifluoroacetic acid (Sigma-Aldrich Co. LLC.), and then deposited onto a 100-well stainless steel plate (Applied Biosystems Japan, Tokyo, Japan). The peptides were analyzed with a Matrix-Associated Laser Desorption Ionization Time-Of-Flight Mass Spectrometer (MALDI-TOF-MS, Voyager-DE STR-H, Applied Biosystems Japan) in the low-mass range (m/z 800 to 4,000 in reflector mode). The observed peptide mass spectrum was used to identify proteins by conducting a Mascot (http://www.matrixscience.com/) peptide mass fingerprint search.

Electrophoretic mobility

The electrophoretic mobility of rNV-VLPs was measured in prepared Milli-Q water. To bring the alkalinity to 20 mg CaCO₃/L, 0.4 mmol/L NaHCO₃ was added to the Milli-Q water (Milli-Q Advantage, Millipore Corp., Billerica, MA, USA), and the pH was adjusted to approximately 2–10 with HCl or NaOH. The Milli-Q water samples were kept for 1 day at 20 °C to stabilize the pH. Just before the measurement of the electrophoretic mobility, an aliquot of rNV-VLP stock solution was diluted to approximately 10⁸ VLPs/mL in the prepared Milli-Q water, and then the suspension was filtered through a membrane filter (as described in the previous section). The particle size distribution of the rNV-VLPs was measured 100 times at 25 °C and at a 165° measurement angle by using a dynamic light-scattering particle size analyzer (ELSZ-2 plus, Otsuka Electronics Co., Ltd).

rNV-VLP assay

rNV-VLPs were quantified by using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (NV-AD (II), Denka Seiken Co., Ltd, Tokyo, Japan). The assay was performed according to the manufacturer's instructions. Optical densities at wavelengths of 450 and 630 nm were measured with a microplate reader (MTP-300, Corona Electric Co., Ltd, Ibaraki, Japan).

rNV-VLP storage method

The effect of sample storage method on rNV-VLP quantification was evaluated in water sampled from the Toyohira River (Sapporo, Japan, water quality shown in Table 1 [River water 1]), which is a water source for a drinking water treatment plant. rNV-VLPs were added to river water samples at a concentration of approximately 10⁸ to 10¹⁰ VLPs/mL. One set of rNV-VLP–spiked samples was stored at 4 °C in the dark for 3 days. At days 1 to 3 during the storage period, aliquots of the samples were returned to 20 °C and rNV-VLP concentration was quantified. The other set of rNV-VLP–spiked samples was subjected to three freeze–thaw cycles (−83 °C and 20 °C). After each thaw, aliquots of the samples were collected for rNV-VLP quantification.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Water quality of the Toyohira River</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>River water 1</td>
</tr>
<tr>
<td>Sampling date</td>
<td>12-Oct-07</td>
</tr>
<tr>
<td>pH</td>
<td>7.5</td>
</tr>
<tr>
<td>Turbidity (NTU)</td>
<td>0.50</td>
</tr>
<tr>
<td>DOC (mg/L)</td>
<td>0.90</td>
</tr>
<tr>
<td>UV260 (cm⁻¹)</td>
<td>0.027</td>
</tr>
<tr>
<td>Alkalinity (mg-CaCO₃/L)</td>
<td>19.1</td>
</tr>
</tbody>
</table>

DOC: dissolved organic carbon.
rNV-VLP concentration method

Water sampled from the Toyohira River (water quality shown in Table 1 [River water 2]) was filtered through a ceramic flat membrane filter (pore size 0.1 μm, Metawater Co., Ltd, Tokyo, Japan) to exclude large particles. Then rNV-VLPs were suspended at approximately $10^7$ to $10^8$ VLPs/mL in the filtered river water. Twelve-millilitre samples of rNV-VLP-spiked river water were concentrated using a centrifugal filter device (molecular weight cut-off (MWCO) 30,000, 50,000, or 100,000, regenerated cellulose; Amicon Ultra-15, Millipore Corp.) according to the manufacturer’s instructions in order to obtain concentrated samples of a final volume of 130 μL (theoretical 92.3-fold concentration). Samples were collected before and after the concentration procedure, and the recovery efficiency of rNV-VLPs was calculated from the ratio of total number of rNV-VLPs in the rNV-VLP-spiked river water to that in the concentrated sample.

RESULTS AND DISCUSSION

Characteristics of the rNV-VLPs

An electron micrograph of the rNV-VLPs produced by the baculovirus–silkworm expression system (Figure 1) confirmed the presence and expected icosahedral structure of the rNV-VLPs, indicating that the rNV capsid proteins successfully self-formed into VLPs in the expression system. The rNV-VLP diameters were approximately 40 nm (Figure 1), which corresponds to the reported particle diameter of native NV (Someya et al. 2000).

To identify whether the target rNV capsid protein was successfully produced by the baculovirus–silkworm expression system, peptide mass fingerprinting was conducted with MALDI-TOF-MS. Based on ExPASy PeptideMass analysis (http://web.expasy.org/peptide_mass/) with an amino acid sequence of the target rNV capsid protein, theoretical digestion of the protein with trypsin generated 14 distinct peptides in the m/z range 800 to 4,000 (Table 2), assuming no missed cleavages in the trypsin digestion. Among these 14 distinct peptides, seven were successfully observed in the peptide mass spectrum produced by MALDI-TOF-MS (Table 2, bold type). Protein identification was performed by conducting a Mascot peptide mass fingerprint search using the observed peptide masses. When the entire NCBInr database was searched with a peptide mass tolerance of ±0.1 Da, Mascot provided significant scores of $>99$ ($P < 0.05$); the match to the capsid protein of Chiba virus yielded a score of 154, indicating that the protein produced by the baculovirus–silkworm expression system was a NV capsid protein.

These results suggest that rNV-VLPs were successfully produced by the baculovirus–silkworm expression system, and were morphologically identical to native NV.

Electrophoretic mobility and particle size distribution of the rNV-VLPs

The electrophoretic mobility (i.e. surface charge) of virus particles is an important factor in the control of virus removal performance in physicochemical water treatment processes. The electrophoretic mobilities of rNV-VLPs in prepared Milli-Q water at pH values less than 4 were positive, whereas those at pH values larger than 6 were negative (Figure 2). The pH value at which the electrophoretic mobility of rNV-VLPs was equal to zero (i.e. isoelectric point) was approximately 5, which is roughly in accordance with reported values for rNV-VLPs (Redman et al. 1997; da Silva et al. 2011). This result implies that rNV-VLPs have a negative surface charge over the environmentally relevant pH range (i.e. near-neutral), although the electrophoretic mobility of virus particles is influenced by the ionic strength or presence of multivalent cations such as calcium and magnesium ions in solution (da Silva et al. 2011).
The particle sizes of rNV-VLPs in prepared Milli-Q water at approximately pH 7 were distributed over the range 30‒50 nm (Figure 3). The median value, 38.1 nm, corresponded with the particle diameter of rNV-VLPs observed by electron microscopic analysis (Figure 1). The negative surface charge and the particle size distribution of rNV-VLPs indicate that no virus‒virus aggregate was generated and that rNV-VLPs were stably monodispersed under near-neutral pH conditions as a result of electrostatic repulsive interactions between the VLPs.

The mechanism of virus removal during membrane filtration depends on the morphology of the viruses (particle size, surface charge, hydrophobicity), the characteristics of the membranes (pore size, surface charge, hydrophobicity), and the characteristics of the solution (pH, ionic strength, ion composition) (van Voorthuizen et al. 2001). Because the rNV-VLPs produced here were morphologically identical to native NV, as described above, and the surface charge characteristics and particle

### Table 2

| Theoretically generated and experimentally observed peptide masses, and the corresponding amino acid sequences |
|---|---|---|
| Theoretical mass [M + H⁺] | Positions | Amino acid sequences |
| 1147.6 | 111–122 | VLAGNAFTAGK |
| 1418.7 | 123–135 | VIICVPQGR |
| 1952.1 | 136–155 | TLSIAQATLPHVIADVR |
| 1481.8 | 154–166 | TLDPVEVPLEDVR |
| 1702.8 | 167–180 | NVLYHNDTQPTMR |
| 1222.7 | 181–190 | LLCMLYTPLR |
| 1438.7 | 191–206 | TGGASGTDTSFVAGR |
| 2461.3 | 207–220 | VLTCPGPDFNLFLVPTVEQK |
| 1382.8 | 229–240 | TRPFTVPNIPLK |
| 2670.3 | 247–270 | IPNPQEGMSLSDPQTQNVQFQNGR |
| 2047.0 | 271–290 | CDIDQGQLGTTPVSXMLCK |
| 3934.8 | 301–337 | VLNLELDSPFMADAPAPAGFPDLGSCDWHIEMSK |
| 3742.9 | 489–523 | LYPGGLTVPNSSTGPQLPLDVFDVFASWVSR |
| 1504.8 | 524–537 | FYQLKPVTAGPAR |

Peptide masses that were both theoretically generated and experimentally observed are highlighted in bold and underlined.

![Figure 2](image2.png)

**Figure 2** | Electrophoretic mobility of rNV-VLPs in the prepared Milli-Q water. Each value represents the mean ± standard deviation of 25 measurements. The rNV-VLP concentration in each sample was approximately 10⁸ VLPs/mL.

![Figure 3](image3.png)

**Figure 3** | Particle size distribution of rNV-VLPs in the prepared Milli-Q water. Values represent the means of 100 measurements. The rNV-VLP concentration in each sample was approximately 10¹¹ VLPs/mL.
size distribution of rNV-VLPs were successfully evaluated in the present study, rNV-VLPs can be used to estimate the efficiency of NV removal during membrane filtration processes, and to explore the mechanism of NV removal during membrane filtration processes, such as size exclusion and electrostatic interactions between NVs and membrane surfaces.

Effect of water quality on rNV-VLP quantification

The sensitivity of a commercially available ELISA kit was determined by using 1-log-fold serial dilutions of the rNV-VLP stock solution with Milli-Q water or Tōyohira river water (Table 1 [River water 1]). In the ELISA, an excellent linear correlation between the rNV-VLP concentration and absorbance was observed in the range from $10^8$ to $10^{10}$ VLPs/mL; the rNV-VLP quantification limit was approximately $10^8$ VLPs/mL, for both Milli-Q water and river water (Figure 4). These results indicate that the rNV-VLPs produced here could be quantified using a commercially available ELISA kit, and that the effect of water quality (Milli-Q water vs. river water) on rNV-VLP quantification was negligible. Our findings demonstrate that we can quantitatively estimate the efficiency of removal of NVs, as particles, during membrane filtration processes by using rNV-VLPs and ELISA even when the rNV-VLPs are injected into environmental water such as source water of a drinking water treatment plant.

Effect of sample storage method on rNV-VLP degradation

In general, it is desirable to quantify the virus concentration in samples immediately after sample collection, because some researchers have reported that the stability of viruses decreases with time in water, especially at room temperature (Long & Sobsey 2004; Bae & Schwab 2008). However, virus-containing samples sometimes need to be stored for several days because of limited laboratory availability (Haramoto et al. 2008). Therefore, we investigated the effect of different storage conditions on rNV-VLP degradation by examining the stability of rNV-VLPs in river water samples stored at 4°C or subjected to freeze–thaw cycles. The rNV-VLP concentrations quantified by ELISA were almost constant for 3 days at 4°C (Figure 5(a)). Long & Sobsey (2004) investigated the survival of F-specific DNA and RNA bacteriophages in environmental water, and reported that the time taken to reduce the infectivity of the bacteriophages by 99% was longer at 4°C than that at 20°C. Similar trends were observed for the infectivity of feline calicivirus and poliovirus (Bae & Schwab 2008). In the present study, storage of rNV-VLP-containing samples at 4°C seemed to be better than that at 20°C. In contrast, the rNV-VLP concentrations were markedly decreased by one or more freeze–thaw cycles (Figure 5(b)), indicating that part of the target rNV capsid protein was probably degraded by the freeze–thaw process.

Taken together, the results indicate that sample storage method strongly influences rNV-VLP quantification, and rNV-VLP-containing samples need to be stored at 4°C after dilution in river water, since freeze–thaw cycles probably degrade the rNV-VLP structure.

Concentration of rNV-VLPs by UF entrapment method

Because the quantification limit of rNV-VLPs with the ELISA was approximately $10^8$ VLPs/mL (Figure 4), further concentration of VLPs is required for the quantification of samples with a low rNV-VLP concentration (i.e. <$10^8$ VLPs/mL). A wide variety of virus concentration methods, such as adsorption–elution methods with glass wool or electropositive/electronegative membranes, ultracentrifugation, and entrapment with UF membranes have been used for
detecting human enteric viruses in low virus concentration samples (Albinana-Gimenez et al. 2013). Among these methods, entrapment with UF membranes has been used to concentrate rNV-VLPs (Huhti et al. 2013). Here, we investigated the recovery efficiency of rNV-VLPs concentrated by the entrapment method using centrifugal UF devices with different MWCOs (Table 3). When a 100,000-Da MWCO membrane was used and the centrifugation was conducted at 5,000 × g for 30 min, the recovery efficiency of rNV-VLPs from filtered river water was 8.9%. Recovery efficiency increased slightly as the MWCO decreased, with a 13.5% recovery efficiency observed for the 30,000-Da MWCO membrane. This result suggests that a 30,000-Da MWCO is superior to a 100,000-Da MWCO for concentrating rNV-VLPs. Moreover, mean recovery efficiencies of 21.7% to 34.4% were observed for 30,000-Da MWCO membranes when the centrifugation time was extended to 45 min; these recovery efficiencies provide 20.6- to 31.8-fold concentration of rNV-VLPs. These results indicate that entrapment with UF membranes with a 30,000-Da MWCO can concentrate rNV-VLPs stably from samples with a rNV-VLP concentration that is too low to be directly quantified by ELISA.

In summary, we found that rNV-VLPs are easy to quantify with a commercially available ELISA kit, they remain stable for several days at 4 °C after dilution in river water and they are easy to concentrate with the UF entrapment method. We therefore consider that rNV-VLPs can be applied to experiments, including batch and long-term membrane filtration experiments, to understand the behaviors of NVs, as particles, during membrane filtration processes such as MF, UF, and NF. In addition, we propose that the use of rNV-VLPs, which are non-infectious, can be applied to cases in which the use of native NV is hampered: e.g. native NV

Table 3 | Recovery efficiency of rNV-VLPs in river water by using centrifugal UF devices

<table>
<thead>
<tr>
<th>MWCO</th>
<th>Centrifugal condition</th>
<th>Injected VLPs</th>
<th>Recovered VLPs</th>
<th>Recovery efficiency (%)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>30,000</td>
<td>5,000 × g, 30 min</td>
<td>107.0 VLPs/mL × 12 mL = 1.3 × 108 VLPs</td>
<td>107.1 VLPs/mL × 0.13 mL = 1.7 × 107 VLPs</td>
<td>13.5</td>
<td>1</td>
</tr>
<tr>
<td>50,000</td>
<td>5,000 × g, 30 min</td>
<td>108.0 VLPs/mL × 0.13 mL = 1.3 × 107 VLPs</td>
<td>107.9 VLPs/mL × 0.13 mL = 1.1 × 107 VLPs</td>
<td>10.3</td>
<td>1</td>
</tr>
<tr>
<td>100,000</td>
<td>5,000 × g, 30 min</td>
<td>109.0 VLPs/mL × 0.13 mL = 1.3 × 106 VLPs</td>
<td>108.9 VLPs/mL × 0.13 mL = 7.9–9.5 × 106 VLPs</td>
<td>8.9</td>
<td>1</td>
</tr>
<tr>
<td>30,000</td>
<td>5,000 × g, 45 min</td>
<td>107.0 VLPs/mL × 12 mL = 1.3 × 108 VLPs</td>
<td>107.8 to 107.9 VLPs/mL × 0.13 mL = 2.1–3.4 × 107 VLPs</td>
<td>22.3 ± 5.3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>108.0 VLPs/mL × 12 mL = 4.0 × 108 VLPs</td>
<td>108.0 to 108.1 VLPs/mL × 0.13 mL = 7.9–9.5 × 108 VLPs</td>
<td>21.7 ± 2.1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>109.0 VLPs/mL × 12 mL = 1.3 × 109 VLPs</td>
<td>109.5 to 109.6 VLPs/mL × 0.13 mL = 4.0–4.6 × 109 VLPs</td>
<td>34.4 ± 2.7</td>
<td>3</td>
</tr>
</tbody>
</table>

Figure 5 | Stability of rNV-VLPs in river water samples stored at 4 °C (a) or subjected to freeze–thaw cycles (b). White, gray, and black symbols represent initial rNV-VLP concentrations of 10^7, 10^8, and 10^9 rNV-VLPs/mL, respectively.
cannot be injected into actual membrane filtration plants because of the obvious risk of infection in humans. The rNV-VLP quantification, storage, and concentration methods applied in the present study will further advance our understanding of which membrane filtration processes are effective for NV removal and have the potential to control the human health risk of NVs in drinking water.

CONCLUSIONS

1. Electron microscopic analysis and peptide mass fingerprinting revealed that the rNV-VLPs produced in the present study were morphologically identical to native NV.
2. The surface charge and particle size distribution of NV particles, which are important factors to explain the behaviors of virus particles during membrane filtration processes, were successfully evaluated by using rNV-VLPs: rNV-VLPs were negatively charged and stably monodispersed at near-neutral pH conditions.
3. The rNV-VLPs were easy to quantify with a commercially available ELISA kit in the rNV-VLP concentration range from $10^6$ to $10^{10}$ VLPs/mL, they were stable for at least 3 days at 4 °C after dilution in river water, and they were easy to concentrate 20.6–31.8-fold by UF entrapment with a 30,000-Da MWCO membrane; therefore they can be applied to experiments to understand the behaviors of NVs as particles during membrane filtration processes.

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