A NOVEL MEMBRANE-BASED ELECTRO-SEPARATION (MBES) METHOD FOR SAMPLE CLEAN-UP AND NOROVIRUS CONCENTRATION

by

WEI KANG

(Under the Direction of Jennifer L. Cannon)

ABSTRACT

Norovirus elution and concentration from complex food matrices is difficult, often resulting in inefficient recovery and inhibitor introduction in downstream molecular detection. Membrane-based Electro-separation (MBES) is a technique to exchange charged particles through a size-specific dialysis membrane from one solution to another using electric current as the driving force. Norovirus has a net negative surface charge in a neutrally buffered environment, so when placed in an electric field, it can move towards the anode (positive electrode) by electrostatic force, which can be separated from the cathodic compartment where sample is placed, and then collected in the anodic compartment for downstream detection. A MBES device was designed, developed and evaluated to concentrate and recover murine norovirus (MNV-1) from phosphate buffer. As high as 30.8% of MNV-1 migrated from a 3.5 ml sample chamber to a 1.5 ml collection chamber across 1 μm separation membrane when 20 V was applied for 30 min using sodium phosphate with 0.01% SDS (pH 7.5) as the electrolyte.

INDEX WORDS: Norovirus, murine norovirus, membrane-based electro-separation, membrane, electric field, separation and concentration, electrolyte

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CHAPTER 1

LITERATURE REVIEW

Norovirus introduction

Norovirus prevalence

Foodborne illnesses, caused by the consumption of contaminated foods, pose a big concern in public health. Two reports from the Centers for Disease Prevention and Control (CDC) estimated that 48 million people, 128,000 hospitalization cases, and 3,000 deaths are due to foodborne diseases in the United States annually (1, 2). Foodborne disease is a global issue, especially in developing countries associated with poor sanitation and few strategies to prevent, detect, and treat foodborne illnesses. Norovirus is the number one cause of foodborne illnesses in the US, responsible for 58% of foodborne gastroenteritis illnesses and 95% of nonbacterial gastroenteritis illnesses each year (3). The economic impact from foodborne and waterborne outbreaks of norovirus illnesses is estimated to be $5.8 billion annually in US (4).

Norovirus (NoV) can cause severe acute gastroenteritis (AGE) if individuals are infected, with symptoms of diarrhea, vomiting, nausea, stomach pain, fever, headache, and body aches. Children, elderly, and immune-compromised individuals are more susceptible to more severe illness due to norovirus. Since the symptoms caused by norovirus in otherwise healthy adults are often mild and self-limiting, many norovirus illnesses are not reported to public health agencies; thus, reported outbreaks and economic impacts tend to be underestimated.

Transmission

Norovirus outbreaks typically peak during the winter with heightened prevalence from November to April (5). Norovirus is transmitted by the fecal-oral route. Norovirus particles are shed by infected patients in stool or vomit, which can spread to people through direct contact with contaminated hands of the infected person or through aerosolized vomit (6). Indirect transmission can also occur through consumption of fecal contaminated foods or water, or by contact with contaminated environmental surfaces (7). Norovirus contamination of food can happen anywhere from farm to fork, but the most frequent contamination pathways can be summarized to: 1) inappropriate agriculture irrigation using contaminated water on fruits and vegetables that are consumed raw; 2) fecally contaminated shellfish grown and harvested from waters, resulting in the bio-accumulation of viruses in the shellfish ; 3) ready-to-eat deli meats, leafy-green salad mixes and other prepared foods contaminated at the point of retail or food service (8). Prepared foods are particularly susceptible to norovirus contamination due to the high level of handling often associated with their preparation.

Human norovirus has a low infectious dose, as between 18 to 1,015 viral particles can cause infection (9, 10). Normally, an infected person can shed up to 10^5 to 10^{11} viral copies per gram of feces 1 to 3 days after infection (10); while infected immunocompromised patients can continuously shed viral particles 20 to 40 days after infection (11). Without proper hygienic practices, human norovirus particles shed from infected patients can stay in the environment for days to up to months. Hospitals, hotels, cruise chips, and day-care centers can thus increase the probability of norovirus transmission if environmental surfaces are not disinfected adequately during a norovirus outbreak (6, 12).

Prevention and Control

The most effective way for an individual to prevent foodborne norovirus transmission is by proper hand hygiene, especially for food handlers. The possibility of food contamination by food handlers can happen at any stage from farm-to-fork, such as harvesting, distribution, in retail, or even at home. Washing hands carefully after using the restroom can prevent the transmission of norovirus from contaminated feces to the hands of persons, environmental surfaces (such as cooking utensils) or food. Since hand hygiene is the most effective and direct way to prevent norovirus transmission, Codex Alimentarius Commission has established hand hygiene guidelines specific for foodborne viruses (13). The United States CDC highlights the importance of food handlers' hand hygiene practices to prevent norovirus infection and recommends washing hands with plain soap and running water for at least 20 s (14). Washing fruits and vegetables thoroughly with clean water or sanitizers and avoiding eating contaminated raw shellfish are also important food safety practices to prevent norovirus infection, especially for susceptible individuals.

Since norovirus is contagious and stable in the environment for a prolonged duration, cleaning and disinfecting surfaces to prevent norovirus transmission are crucial strategies to prevent infection. According to Codex Alimentarius International Food Standards (13), for regular surface cleaning, application of a fresh solution of $\geq$ 1000 ppm free chlorine or 200 ppm concentration chlorine dioxide for 5 to 10 min is recommended. Alternative disinfection methods for some industries (i.e, healthcare) can use vaporized hydrogen peroxide treatment (VHP) with a concentration $\geq$ 100 ppm for 1 hr or use UV irradiation at 40 Ws/cm^2. Most alcohol-based chemical disinfectants are not efficient in inactivating enteric viruses, including norovirus, by more than a 3 $\log_{10}$ reduction in infectious virus titers based on their labeled recommended

concentrations and applied times. During norovirus outbreaks, it is recommended to use 1,000-5,000 ppm chlorine or an EPA-approved surface disinfectant for surfaces, cleaning twice per day or up to 3 times/day, depending on the severity of the outbreaks for complete decontamination (3, 14).

Norovirus biological and pathological properties

Human noroviruses, previously called Norwalk-like viruses, are members of the norovirus genus, belonging to *Caliciviridae* family. Other *Caliciviridae* family genera include Sapovirus, Lagovirus, Vesivirus, and Nebovirus (15). Noroviruses are non-enveloped viruses with single-strand, positive-sense RNA genomes inside a protein capsid shell (16).

Norovirus was first visualized by immune electron microscopy (IEM), in which a stool sample was obtained from an outbreak in Norwalk, Ohio in 1972 by the Kapikian research group (17). During his study, healthy volunteers were administrated oral with filtrates from rectal swabs from Norwalk gastroenteritis outbreak patients. The volunteers developed gastroenteritis symptoms and small round viruses were visualized by IEM from their stool samples.

Norovirus size ranges from 27 to 38 nm in diameter. It is grouped into 6 genogroups (GI to GVI) (18) based on the complete amino acid sequence of the major capsid protein (VP1) (18, 19). A new proposed genogoup GVII virus was recently discovered from canine specimens in Hong Kong (20). Each genogroup can be further divided into different genotypes, comprising a total of 32 genotypes or 29 genetic clusters (6). Among them, GI, GII, and GIV cause infections in humans, referred to as human noroviruses (HuNoVs). Besides humans, GII can also infect swine species and GIV can infect canine species. GIII and GVII cause infection in bovine species and canine species respectively. GV virus causes infection in murine species and is also called murine norovirus (MNV) (19). Norovirus GI and GII, especially GII.4 (GII.4 represents

genogroup II, genotype 4), are responsible for the most number of acute gastroenteritis outbreaks in humans globally (21).

The genetic analysis of the norovirus genome by DNA sequencing techniques was a major breakthrough to understanding the genome structure and function of RNA encoded viral norovirus proteins (22). The norovirus positive-sense, single-strand RNA genome is about 7.5 kb in length and consists of 3 open reading frames (ORFs). A VPg protein is linked at the 5' end of the RNA genome, functioning as the initiation of protein translation, while the 3' tail of the RNA genome is polyadenylated (23). ORF 1 encodes a single polymer of nonstructural proteins, which is cleaved by an internal viral 3C-like protease into 6 proteins: p48, NTPase, p22, VPg, 3CL protease, and RdRp (RNA dependent RNA polymerase for viral transcription). ORF2 encodes the major structural protein VP1, while ORF3 encodes the minor structural protein VP2. VP1 protein consists of 2 domains, the S (shell) and P (protruding) domains. The P domain is further divided into P1 and P2 regions; the P2 region is the most hypervariable region of the genome and is responsible for viral-cellular interaction and antigenicity (23, 24).

Treatment of Norovirus Illnesses

Vaccination is the best way to prevent viral infection, especially for high-risk populations (6). However, there is no FDA-approved vaccine available for human norovirus yet. Norovirus can self-assemble into viral-like-particles (VLPs), which are protein capsids without RNA genome inserted. VLPs are antigenically similar to norovirus and therefore capable of causing immunogenic responses in hosts. Norovirus vaccination studies thus focus on using vector systems such as yeast, *E. coli*, the Baculovirus insect cell expression system, or the Venezuelan equine encephalitis virus (VEE) mammalian cell expression systems to assemble VLPs, which are then inoculated into human hosts to trigger the host immune response (3). Several potential

vaccine candidates are under development and being evaluated. A VLP-based vaccine produced by the Baculovirus expression system was tested in gnotobiotic pigs. The vaccinated pigs developed norovirus specific antibodies which protected them from developing gastroenteritis symptoms after inoculation with infectious norovirus (25). Vaccine clinical trials were conducted as far back as 1999 by Ball and his colleagues to see if human VLPs based vaccine could cause human NoV specific immunogenic response and to evaluate the safety of VLP-based vaccines for human use (26). More recently, Atmar and his colleagues used a VLP vaccine containing adjuvants chitosan and mono phosphoryl lipid A followed by human challenge with infectious virus (27). The results showed that 70% of the vaccinated volunteers developed a NoV-specific antibody response and reduced gastroenteritis symptoms after being challenged with Norwalk virus.

A drawback of using VLP-based vaccines is that they might not be able to cause cross-protection from different genotypes of norovirus because norovirus genotypes are heterogeneous and diverse (3) due to mutation and homologous recombination (28). The binding sites of human norovirus to human cells are histo-blood group antigens (HBGAs) expressed on gut epithelial cells (29). Different genogroups (such as GI and GII) of norovirus have different binding patterns to HGBAs due to the viral major capsid protein residue diversity. Even HBGA-NoV interactions of strains within a single genotype (such as GII.4) can be variable because of antigenic drift in the major capsid protein's amino acid sequence, which is also the binding site for HBGAs (28, 30). The fast evolution of GII.4 strains thus have caused at least four global epidemics and enlarged the susceptible population (28).

Norovirus pathogenesis and host response to infection

Human norovirus cannot be cultivated in routine cell culture systems and no suitable small animal model is available to replicate and evaluate the infectivity of human norovirus yet, thus making the study of basic virology and pathogenic mechanism of norovirus slow (31). However, recently, Jones et al. has developed a B cell line, that is able to replicate human norovirus *in vitro* (32), which is a major breakthrough in human norovirus infectivity research. The system depends on co-inoculation with bacterial flora of stool specimens which express HBGA-like molecules. Validation of the method and duplication of the work in other laboratories is needed before this promising method can be used routinely in laboratory settings. Alternatively, cultivable norovirus surrogates are widely used to study the pathogenicity of human norovirus.

Viral surrogates are viruses with genetic material, size and structural similarity to the uncultivable virus being studied. Other properties that may be similar include receptor binding properties and the generated host-response mechanism after infection. Surrogates for human norovirus include murine NoV, feline calicivirus (FCV), Tulane virus, and porcine sapovirus. Murine norovirus (MNV) infects mice, causing fecal shedding in otherwise healthy mice and systemic infection in severely immunocompromised mice; feline calicivirus infects cats, causing a respiratory tract infection; and porcine sapovirus causes gastroenteritis in pigs (33, 34). Tulane virus (TV), belongs to the Recovirus genus, but can bind to HBGAs with sequence structures as those that bind to GII human norovirus (35). Murine norovirus and feline calicivirus are used mostly as surrogates to study human norovirus because of their similarities in environmental survival and genome structure (33, 34). Wobus et al. promotes the use of MNV to study human norovirus by emphasizing the similarity in molecular properties and pathogenesis and the ease of

mouse model manipulation. Although FCV and PEC (porcine enteric calicivirus) models are promising to reveal calicivirus replication and pathogenesis mechanisms, manipulation of bovine, porcine, and feline species as animal models is difficult (34).

Besides using surrogates to study the potential similarities with human noroviruses, in recent decades, human norovirus infectivity has been evaluated using human challenge studies. In Chamock's Norwalk virus infectious study back in 1972 in Maryland, volunteers were infected with Norwalk virus to study symptom development, route of transmission, and population susceptibility (36). The observation that only a subgroup of volunteers infected with norovirus during human challenging studies developed symptoms, helped scientists to reveal the relationship of HBGAs in population susceptibility (37-39). Human norovirus binds to HBGAs receptors on cells of the gut mucosa via P2 domain of the VP1 capsid protein of the virus (40, 41). HBGA expression is genetically determined. "Secretors" are people in the population expressing HBGAs on their gastrointestinal and oral mucosa, who are more susceptible to some infection by some human norovirus genotypes than "nonsecretors", who do not express HBGAs on their gastric mucosa (42).

Norovirus detection in foods and water

Mechanism of virus adhesion to food matric

The mechanism of virus adsorption to solid surfaces, including food matrices, can be explained by the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory of colloid stability. Viruses adsorb to surfaces, such as food surfaces, by repulsive double-layer interactions (electrostatic forces) and attractive van der Waals forces (43). The strength of the electrostatic force is affected by sorbent properties (pH, structure, and composition), the properties of the solution (pH, ionic strength, and components), and characteristic of the viruses (isoelectric

point). The isoelectric point of norovirus is around pH 5.5 to 6.5 (44). The high acidic property of some fresh produce such as berries thus favors the adherence of virus particles to food matrices by strong electrostatic interaction. High pH elution buffer, pH higher than 9, is usually used to alter the sorbent pH and change the viral particle's net charge in order to detach virus from the solid matrices (45). The ionic strength of the elution buffer can also affect virus extraction by changing the potency of repulsive electrostatic forces. Lowering the ionic strength of elution buffer favors virus detachment and avoids virus aggregation by increasing the electrostatic repulsive force (46, 47). Adding salt to elution buffer, on the other hand, can out-compete the unspecific binding of virus to matrices, thus favoring virus elution.

Besides DLVO theory, hydrophobic interactions between viruses and food surfaces can also alter virus adhesion. Hydrophobic protein residues of the virus capsid are repulsive to aqueous solvents by Van der Waals forces and short-range (SR) hydrogen bond interactions, which favors virus attachment to low energy-surface matrices (48, 49). Vega et al. have illustrated that for FCV, echovirus, and bacteriophages, the mechanism for major virus attachment to butterhead lettuce is by hydrophobic interactions, instead of DLVO interactions (50). DLVO was initially used to describe hard colloid particles. Different from hard colloid particles, the dynamic nature of virus particles and the present of its RNA genome might also affect virus attachment (47, 51).

Norovirus detection in food and water samples

The primary obstacle in developing effective human norovirus detection methods is that traditional enrichment or routine cultivation methods are not available for human norovirus. The first international standard for norovirus and HAV detection in food and water (CEN/TC275/WG6/TAG 4) uses quantitative real-time RT-PCR (RT-qPCR) for virus detection

in a variety of food matrices (52). Viral particles are first eluted and concentrated from food surfaces (upstream sample preparation), then the viral RNA is extracted for molecular detection (downstream RNA genome detection). The minimum amount of virus commonly present in complex sample matrices makes the sample preparation step challenging, requiring efficient virus elution procedures as well as avoiding inhibitor introduction which can interfere with downstream detection (53).

Downstream detection methods are well studied, yielding promising methods for RNA extraction and detection. RNA extraction methods are typically based on virus capsid disruption by chaotropic reagents (guanidine thiocyanate) and RNA absorption to silica particles (54). Such methods are very mature and typically yield highly sensitive and accurate results with high consistency. Commercialized RNA extraction assay kits based on the modification of Boom's method are available for rapid RNA extraction (55-57). The overlapping region between ORF1 and ORF 2 is the most conserved region within norovirus strains, thus this region is targeted for norovirus detection (58). To further discriminate between genotypes of norovirus, a larger genomic region with less conservation is used, such as the RdRp gene located in ORF1 and the gene encoding the major capsid protein (VP1) in ORF 2 (59). Many molecular detection assays have been developed for reliable norovirus detection from different samples from clinical, food, water, and environmental samples, which are summarized in Stals et al. review (58).

Upstream sample preparation

For most food items associated with norovirus outbreaks, sample preparation can be categorized into three groups based on the characteristic of the sample: carbohydrate- and water-based fruits and vegetables such as berries, lettuce, and green onions; fat- and protein-based ready-to-eat foods such as hams, salami, and whipped cream; oysters and other shellfish because

of their unique filter-feeding ability to filter water while accumulating virus particles in their digestive systems (53, 60).

Most virus extraction methods for food matrices can be summarized into two main approaches (D. Rodriguez-Lazaro 2013). First, elution-concentration methods wash virus particles from food matric into the elution buffer which are then concentrated. Purification steps can also be added to remove food debris and inhibitors. The purified viral particles are ready for RNA genome extraction. Alternately, direct RNA extraction usually coupled with guanidinium isothiocyanate (GITC)/phenol to extract viral RNA from the food matric followed by RNA purification and molecular detection. This method is being implemented in different kinds of food matrices, such as protein-based foods and oysters and shows reliable results (60-62). However, due to the complexity of different food matrices, direct RNA extraction is less sensitive than elution-concentration methods (61). Furthermore, direct RNA extraction method cannot be used to recover intact virus particles to further estimate virus infectivity because all virus particles are disrupted for RNA extraction.

Elution-concentration methods

Elution-concentration methods are the most commonly used norovirus sample preparation methods for carbohydrate/water-based foods and protein-/fat-based foods. These methods involve two basic steps: elution of viral particles in an appropriate elution buffer, followed by concentration of the extracted viral particles. Alkaline and neutral buffers are widely used to elute viral particles from food matrices. A recent review paper (53) describes the use of alkaline buffers at pH 9 to 10.5 to facilitate the detachment of negatively charged noroviruses from food matrices. Neutral pH buffers are used when anion exchange methods or ultracentrifugation methods are used as a subsequent concentration step. Many carbohydrate- and

water-based fruits and vegetables, such as berries, are high in acidity which can impair virus elution by favoring virus absorption to food surfaces, resulting in a subsequent reduction in detection sensitivity. This problem can be overcome by using high buffering capacity elution buffers such as alkaline tris-buffer (45), phosphate buffer (63), and bicarbonate buffer (64).

Beef extract (1%-3%) and/or glycine is frequently added to elution buffers to facilitate virus detachment from food matrices and later enhancement of viral particle flocculation during polyethylene glycol (PEG) concentration (45, 65). Proteins and amino acids, such as beef extract and glycine, are strongly negatively charged in alkaline environments, which, when added in abundance, can out-compete the nonspecific absorption of negatively-charged viruses to food surfaces which are fewer in number (45, 66). Pectinase is added to elution buffers when extracting virus from high pectin containing fruits and vegetables, such as raspberries, to break-down the pectin bonds in fruits and vegetables and to prevent the formation of a jelly-like substance caused by the residual food matric which can hinder virus extraction (45). The pH of alkaline elution buffers needs to be readjusted to neutral after extraction for subsequent analysis because high pH is not favorable for preserving infectious viral particles. Virus elution is usually followed by virus concentration to minimize the volume. This is often done by PEG precipitation, ultracentrifugation, or ultrafiltration (67).

An efficient separation method requires the elution of an adequate amount of viral particles from sample matrices and an extract that is free from any potential inhibitors of molecular methods. Many studies report low levels of norovirus recovery (10 to 50%) from food matrices due to inefficiency in virus elution and concentration from the foods (53). The complexity of food components, the presence of inhibitors in food sources, the uneven distribution of viral particles in foods, the possibility of co-eluting and co-concentrating

inhibitors with virus all pose great challenges to develop efficient virus recovery methods (67). Virus elution requires gentle handling to avoid damage of food surfaces because inhibitors, such as acidic compounds, enzymatic substances, some organic/inorganic compounds, and cytotoxic compounds, if released from food, might lower elution efficacy and interfere downstream molecular detection (45). Beef extract, an important ingredient in elution buffer to facilitate virus detachment, has shown to be a potential inhibitor when used at high concentration, interfering with downstream molecular detection (68, 69). pH, structure, and composition of foods can also affect the efficacy of eluting virus from food matric. Fresh produce can release acidic juice to the elution buffer thus causing virus absorption to food surfaces and lower viral recovery (45). Fruits, such as blueberries, might contain antiviral compounds, causing virus inactivation if released to the elution buffer (64). Low viral titers present in complex food samples pose another difficulty in recovering viruses from foods. Viral particles can be embedded inside the food matric, making them hard to elute. A literature review of elution and concentration methods for norovirus recovery and detection in different food categories was summarized in Table 1.

Norovirus detection in water

Drinking, recreational, environmental, and irrigation water contamination by norovirus can pose a significant public health concern. Unlike high norovirus concentrations often found in human feces (more than 10^5 viruses per gram of stool), the virus concentration in water samples is often much more diluted, causing difficulty in detecting such small amounts of virus particles in relatively large water samples (70). Pre-concentration of viral particles from water samples is crucial. However, when concentrating viruses to smaller volumes, eliminating the co-concentration of inhibitors is necessary. Usually, one thousand- to several thousand-fold concentrations are performed to reduce the water sample volume to less than 10 ml for further

virus analysis. The elimination of co-concentrated inhibitors is crucial in water sample preparation process to avoid their interference with molecular detection methods (71). Adsorption/concentration (also called VIRADEL) methods utilize absorption matrices such as electro-charged filters to absorb viruses from water samples and concentrate them on the filter. Next, viruses are eluted from the filters using elution buffers, usually containing 1.5% to 3.0% beef extract solution with a pH of 9.0 to 9.5 to obtain sample volume of 100-1000 ml (72-74). Secondary concentration of the eluate might also be needed to further reduce the sample volume to 5-20 ml by PEG precipitation (75, 76) or organic flocculation (77, 78). Alternatively, ultrafiltration (Hill, 2007) and ultracentrifugation (Fumian 2010) are simpler and quicker methods to concentrate viral particles from large water samples. However, these methods do not purify samples and may co-concentrate inhibitors or concentrate organic debris which will clog filters so they can't be used for high turbidity samples.

Sample preparation by electric field application

Rapid sample separation and concentration improvements by inducing an electric field has become a popular trend in analytical chemistry. Analyte extraction from complex samples is a critical step for chemical extractions to ensure a high degree of purity and concentration for downstream detection methods. Downstream methods include electrophoresis, chromatography, mass spectrometry, and capillary electrophoresis. The assistance of an electric field for sample clean-up and concentration poses a new possibility for simple, low-cost, selective, and automated method for separation technology.

Electrodialysis, or membrane liquid-phase extraction (MLPE), utilizes an electric field across a membrane to enhance analyte migration, based on either the selectivity of the membrane charge or the molecular weight cut-off or pore size (79). The applied electric field creates an

electric force to induce charged particle migration from one liquid phase (donor phase) through a charge- or size-specific membrane to another liquid phase (acceptor phase) (80). Concentration gradients and electric potential differences are the two major driving forces in electrodialysis techniques. Morales-Cid et al, explained that these driving forces create molecular and electric fluxes across the membrane barrier to facilitate charge particle movement (79).
Geroenewegen, et al, used the Nernst-Planck equation to illustrate the migration of particles in an electric field by concentration gradient and electric potential gradient:

$$J = J\ diff + J\ migr$$

The flux of analyte is driven by the flux of diffusion caused by a concentration gradient and the flux of electromigration is caused by electrical potential differences, thus charged analytes can migrate and be concentrated in an acceptor phase (81). With the assistance of electric force, the mobility of analyte is more favorable when compared with traditional separation methods, in which only the diffusion kinetic is involved (79).

Separation membranes between the donor phase and the acceptor phase used for electrodialysis can be either ion-exchange or neutral. Ion-exchange membrane electrodialysis is widely used in waste-water treatment and sea water desalination. The selectivity of ion-exchange membranes is based on ion charge; however, charged particles larger than 500 Da are unable to migrate across ion-exchange membranes (79). For higher molecular weight or larger size particles, neutral porous membranes are more suitable for separation. To overcome the pH change due to water electrolysis near the cathode (pH decrease) and the anode (pH increase) which might affect a pH-sensitive analyte and reduce the possibility of oxidoreduction of analyte to electrodes (cathode is reduction and anode is oxidation), ion-exchange membranes are usually

used as a physical boundary to separate electrodes from analyte, creating electrode compartments sandwiching the donor phase and acceptor phase (82).

The acceptor phase can be either stagnant or flowing coupled with stagnant donor, creating either static or dynamic electrodialysis (83). For example, static mode electrodialysis was used to separate and enrich negatively charged inositol phosphate using a 30 k MWCO membrane coupled with online capillary electrophoresis for automatic analysis (84). When a flowing donor phase (continuously feeding of sample) was used to enrich positively charged ephedrine, enrichment increased to 10-fold, when compared to stagnant donor phase (85). The mobility of analytes migrating through pore-selective membranes in an electric field can also be affected by molecular orientation in MLPE (79). Different molecular orientations introduce different friction forces while crossing the physical barrier (membrane), thus affecting the degree of target compound mobility.

Applications for using electrodialysis to separate charged compounds derived from foods or plant materials have been tested by different research groups. Polyphenols were isolated from tobacco extract based on the charge and size of the polyphenol compounds as they migrated through a special membrane driven by an electric field (86). Catechins and caffeine were also extracted from green tea solution by eletrodialysis (87). Furthermore, a modified eletrodialysis technique using stacking ultrafiltration membranes was tested to fractionate peptide compounds based on differences in molecular weight (88).

Based on the concept of electrodialysis and electrophoresis, utilizing electric force to mobilize charged particles to achieve separation and concentration, a series of electroialysis devices were designed and patented under Gradiflow technology (Gradipore Limited, Australia). The first and simplest generation of Gradiflow was a static mode of electrodialysis consisting of

a separation membrane to separate donor phase and acceptor phase, patented by Olge, et al (89, 90). Electrodes were fixed in the electrode chambers sandwiching the donor and acceptor phases, as well as avoiding convection by a respective membrane. The device was recommended for use in separating small volumes of sample, from 0.02 ml to 5 ml, using any type of buffer as electrolyte. Most target solutes with a charge can be separated depending on the selection of the separation membrane. In an advanced version of the Gradiflow system, a multi-port separation device consisting of multiple separation membranes was designed to handle complex samples (91). However, this static electrodialysis system without buffer circulation is not suitable for large volumes. Buffer recirculation can help to cool down the electrolyte during processing as well as mitigate the pH change due to water electrolysis. Continuous feeding or recirculation of the sample functions to increase the input volume and avoid sample fouling of membrane (92).

Rylatt and Leong (2012) adapted the Gradiflow technology to selectively separate live cells in small volumes for the first time using electric force. In his patent, erythrocytes were separated and purified from blood samples containing erythrocytes (~7 µm diameter) and leukocytes (8-20 µm diameter) by applying a 50V/cm voltage for 3-9 mins using a 10 µm polycarbonate separation membrane and 10-20 kDa restriction membranes (separating the electrodes and samples). The electric strength determined by the distance of electrodes and applied voltage, the membrane to volume ratio, and the buffer conductivity all affected cell mobility across the membrane barrier and thus the efficacy of separation (89, 93).

The separation of live cells using the electro-separation technique with small volumes is an innovative methodology for upstream sample clean-up for microbe detection, especially since there is a possibility of integrating automatic online downstream detection, similar to membrane-based chemical separations coupled with online analysis by chromatography or capillary

electrophoresis (85, 94, 95). The combination of size- and charge-specific selection technologies and the application of electricity as the driving force might be able to help to simplify the sample clean-up process and norovirus concentration from complex food matrices. Noroviruses, with a net negative surface charge in a neutral pH environment, might be able to mobilize in an electric field and become separated from food matrices across a separation membrane which will restrict the movement of large food debris. Small inhibitors will also move along with norovirus across the separation membrane, but might be able to be further filtered out if an additional membrane is added to the device. With further design of a membrane-based electro-separation device, large volume separation and automatic downstream detection might be feasible for laboratory and industry applications.

Table 1. A summary of elution and concentration methods for norovirus recovery and detection in foods

Food categories	Food sample	Virus	Extraction	Concentration	Recovery	Detection limit	Reference
Carbohydrate-based	Frozen forest fruit mix (10 g) Fresh raspberry (10 g) Fresh strawberry puree (10 g)	NoV GI & GII (10^4 RNA copies)	Washed with 30 ml 0.1 M tris-HCl, 3% beef extract, 0.05 M glycine, with 150 µl pectinase 1XL (pH 9.5) for 20 min. Then pH adjusted to 7.2-7.4 after centrifugation	PEG 6000 precipitation overnight and chloroform/butanol treatment to remove inhibitors	Frozen mix: 7.42-20.68% Raspberry: 21.5-35.2% Strawberry puree: 25.26-61.06%	GI: $3.99X10^4$ RNA copies GII: $9.63X10^4$ RNA copies	(96)
	RTE (mixed food salad, mixed lettuce, raspberry), 50 g	GII-4:10^4 RT-PCRU	Washed 20min with three different elution buffers: (1) 30 ml 0.05 M glycine-0.3 M NaCl, pH 9.5 (2) 150 ml 0.1 M tris-HCl-0.05 M glycine, 3% beef extract, pH 9.5 with pectinase 1XL (3) 150 ml 0.05 M glycine-0.15 M NaCl, pH9	PEG 6000 precipitation and Chloroform/butanol treatment followed by proteinase K treatment	Positive detection for all three methods	10^3 RT-PCRU for raspberry	(60)
	Fresh whole strawberry (5 g) and lettuce (25 g)	NoV: 4,800-0.48 RT-PCRU	Wash with elution buffer 0.05 M glycine-100 mM tris (pH 9.5) for 30 min	Two different methods (1) adjust pH to 7.0-7.2 following centrifugation to remove debris, then	2.9% in lettuce (PEG precipitation) 2.9% in lettuce (ultrafiltration)		(97)

				PEG 8000 precipitation (2) ultrafiltration through negatively charged membrane, the filter was then washed with 1.5% beef extract-0.05 M glycine solution, pH 9.5	50% in strawberry (PEG precipitation) 40% in strawberry (ultrafiltration)		
	Green onion (25 g)	GII4: 10^4-10^1 RT-PCRU	Washed with 20 ml 2.9% tryptose phosphate with 6% glycine, pH 9.5	PEG 8000 precipitation		1 RT-PCRU	(98)
	Tomato sauce (10 g)	10^7 PFU HAV	Acid-adsorption, elution and concentration (AEC): eluents (1) 0.5M threonine, pH 7.2 (2) 3% beef extract and 2M $NaNO_3$, pH 7.2		51% when threonine used 28% when beef extract used		(66)
Protein-based	Mix of 10 g iceberg lettuce, frozen raspberries, cooked ham	$2X10^6$ RT-PCRU norovirus	Elution method: rinse with 100 mM tris-HCl, 50 mM glycine, 1% beef extract (pH 9.5) for 10 min followed by 180 units of pectinase addition. pH was adjusted to	Elution method: PEG 8000 precipitation for 2 hours Ultrafiltration method: the filtered supernatant was concentrated by ultrafiltration using	Elution: 23% for lettuce, 7% for raspberry, 24% for ham. Ultrafiltration: 9% for lettuce, 3% for raspberry, and	Elution: 20 RT-PCRU on ham. Ultrafiltration: 200 RT-PCRU for ham.	(99)

			7.2 after centrifugation Ultrafiltration method: rinse with PBS for 10 min, followed by centrifugation for 10 min.	Vivaspin 50,000 MWCO	7% for ham.		
	Delicatessen meat (roast pork chop, salami, gammon), 20 g	FCV: $1.3X10^4$ (TCIU50)	Washed by 80ml 0.5 M glycine with 1% bovine albumin (250 ul CatFloc TL was added to facilitate removal of food debris) for 30 min, followed by 3 min centrifugation	Ultracentrifugation for 2 hr at 23,500 X g	12.5% for roast pork, 3.4% for salami, and 5.9% for gammon.		(100)
	Deli turkey 25 g, chocolate cake 25 g	HAV: 10^3-10^5 PFU	Washed by 0.05 M glycine-saline, (pH 9.0) for 2 min, then pH was adjusted to 7.0.	Cationic particles processing and magnetic separation		Turkey: $2X10^2$ RT-PCRU. Chocolate cake: $2X10^6$ RT-PCRU.	(101)
Shellfish	25 g oyster and clam	HAV: 15-0.15 PFU Norovirus: $2.24X10^5$-22.4 RT-PCR	Shellfish was blended with 175 ml glycine buffer, pH 9.0 (0.1 M glycine, 0.3 M NaCl)	16% PEG precipitation for 1 hr		0.015 PFU RT-PCRU for HAV 22.4 RT-PCRU for Norwalk virus	(102)

	Mussel	GII. 4	2 g inoculated mussel sample was homogenized with 6 ml 0.05 mol/l glycine-0.15 m/l NaCl (pH 9) for 20 min. pH was adjusted to 7.2-7.4 after centrifugation.	PEG 6000 precipitation overnight		20-100 RT-PCRU	(103)
	oyster 25g	HAV: $8.3X10^4$ to 0.85 TCID50	shellfish tissue was homogenized with glycine buffer (0.25N, pH 10) followed by chloroform purification Supernatant after centrifugation was adjusted to pH 7.	ultracentrifugation to concentrate the virus		TCID50/g	(104)

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CHAPTER 2

A NOVEL MEMBRANE-BASED ELECTRO -SEPARATION METHOD (MBES) FOR SAMPLE CLEAN-UP AND NOROVIRUS CONCENTRATION [1]

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Abstract

The most widely used extraction methods for norovirus detection on food matrices are based on elution and concentration methods. Such methods are complex, cumbersome, and labor intensive, with the potential of introducing inhibitors to downstream molecular detection and inefficient recovery from complex food matrices. The membrane-based electro-separation (MBES) method is a technique to exchange charged particles through a size-specific dialysis membrane from one solution to another using electric current as the driving force. Norovirus has a net negative surface charge in a neutrally buffered environment, so when placed in an electric field, it can move towards the anode by electrostatic force, which can be separated from the cathodic compartment where sample is placed and then collected in the anodic compartment for downstream detection. A MBES system was designed, developed and evaluated to concentrate and recover murine norovirus (MNV-1) from phosphate buffer in a proof-of-concept study. As high as 30.8% MNV-1 migrated from the 3.5 ml sample chamber to the 1.5 ml collection chamber across a 1 μm separation membrane when 20 V was applied for 30 min using sodium

phosphate with 0.01% SDS (pH 7.5) as the electrolyte. In optimization of the method, weak applied voltage (20 V), moderate duration (30 min), and low ionic strength electrolytes with SDS addition were needed to increase virus movement efficacy. The electric field strength of the system was the key factor to enhance virus movement, which could only be improved by shortening the electrodes distance, instead of increasing system applied voltage because of virus stability.

This study successfully demonstrated the norovirus mobility in an electric field and migration across a size-specific membrane barrier in sodium phosphate electrolyte. With further modification of the MBES system and optimization of applied parameters, a novel, quick, easy,

and cost-effective sample clean-up technique might be developed to separate norovirus particles from food matrices by electric force.

Introduction

Norovirus (NoV) is recognized as the major cause of foodborne illnesses in the U.S. each year, responsible for 58% foodborne gastroenteritis illnesses and 95% nonbacterial gastroenteritis illnesses (1, 2). The economic impact of norovirus related outbreaks is estimated to be $5.8 billion annually in the U.S. (3). Norovirus is highly contagious through person-to-person contact and can affect humans of all ages (4). Indirect norovirus transmission occurs by consumption of fecally contaminated foods or water, or contact with contaminated environmental surfaces (5). Fresh fruits and vegetables, ready-to-eat deli meats, leafy-green salad mixes, and shellfish are susceptible to norovirus contamination (6). Oysters can bio-concentrate virus in their tissue when grown in contaminated water. High-level post-handling processing of ready-to-eat deli meats and salad mixes is the primary contamination route of food handlers. Fresh fruits, vegetables, and raw oysters have higher chances of causing norovirus infection because they are often consumed without cooking. National Outbreak Reporting System (NORS) reported 1,008 norovirus foodborne outbreaks in U.S. from 2008-2012, wherein 30% were caused by consumption of norovirus contaminated leafy vegetables, 21% by fruits, and 19% by shellfish (7).

To protect public safety, sensitive, fast, and reliable methods to detect norovirus in a variety of foods and drinking water is needed. Such detection systems must also be cost-effective for industry and laboratory uses. Norovirus detection from foods can be divided into two processes, upstream sample preparation and downstream molecular detection. Upstream sample preparation involves virus elution from food matrices and further concentration and

purification if needed. Downstream molecular detection involves viral RNA extraction and RT-qPCR detection. Reliable sample preparation requires efficient elution of virus from different kinds of complex food matrices without the co-elution of possible inhibitors for RT-qPCR assays (8). Elution-concentration is the most common sample clean-up and norovirus concentration method used for a wide range of foods such as carbohydrate-based fresh produce, protein-based read-to-eat foods, and shellfish (9). However, since this procedure is cumbersome and labor-intensive, inefficient in terms of virus recovery, and potentially introduces inhibitors, there is a need to develop better norovirus detection methods from foods (10).

Membrane liquid-phase extraction (MLPE) (11) methods utilize either size- or charge-specific electro-separation membranes and apply an electric field across the membrane to selectively mobilize charged compounds for separation and concentration. MLPE, also known as electrodialysis is widely used in sample pretreatment in analytical chemistry to extract analytes from complex samples (12-15). By coupling electro-separation with chromatography or capillary electrophoresis online or offline, an automated detection system can be designed to combine sample pretreatment and analysis for time and cost reduction (16-18). Utilization of an electric field to separate live cells was first patented using a modification of Gradiflow Technology (Gradipore, Australia), which was a membrane-based electrophoresis method. In that study, negatively charged erythrocytes (7 µm diameter) were mobilized in an electric field across a size-selective membrane (10 µm) and were separated from leukocytes (8-20 µm diameter) in small volume (450 µl) blood samples (19). Norovirus capsids are 27 nm to 38 nm in diameter and have isoelectric points (pI) of pH 5.5 to 6.5 (20), which means they have a negative surface charge in a neutral or basic pH environment. Similar to red blood cells, they should be able to move in an electric field towards a positive electrode (anode). Therefore, selective separation of

norovirus from food samples, with simultaneous separation of inhibitors may be accomplished with a method using the integration of a pore-size specific membrane barrier and application of electric field.

The objective of this study was to evaluate the feasibility of using a membrane-based electro-separation (MBES) method to evaluate norovirus mobility using an electric field as the sole driving force across a pore-size specific selective separation membrane. This is a proof-of-concept study where an ElectroPrep electrodialysis device (Harvard Apparatus, MA) was adapted for our uses and murine norovirus (MNV-1) was used as the model virus to study this objective. The study provides a foundation for designing a detection device to be used for separation and concentration of norovirus from food samples.

Materials and methods

MNV-1 stock preparation

Murine norovirus (MNV-1) was a gift from Dr. Herbert Virgin at Washington University. RAW 264.7 cells for MNV-1 infection were purchased from ATCC® (TIB-71; Manassas, VA). To prepare high titer virus stocks, confluent RAW 264.7 cells in T175 flasks were inoculated with MNV-1 and incubated at 37°C with 5% CO_2 for 48 hrs. The infected flasks were frozen (-70°C) and thawed (room temperature) for 3 cycles, followed by centrifugation at 2,000 X g for 15 min and vacuum filtration (0.2 µm PES membrane filter) to remove large cell debris and clarify viruses. The filtrate was further concentrated by ultracentrifugation in a Beckman Coulter Ultracentrifuge using a Type 35 rotor at 100,000 X g for 1 hr. The pellet was dissolved in sterile PBS overnight at 4°C to prepare virus stock. Sucrose purification was performed to further concentrate the virus stock. Virus stock was loaded on top of a 30% sucrose solution for

centrifugation using a NVT 90 rotor at 100,000 X g for 1 hr at 4°C and the pellet was dissolved in PBS.

MNV-1 quantification by plaque assay

MNV-1 virus stock titer was estimated by plaque assay. RAW 264.7 cells were infected with 100 µl of virus stock and incubated for 1 hr with gentle rocking manually at 15 min intervals. The surface medium was then aspirated and an agar containing 50:50 of 1% Seakem LE agarose (Lonza, Rockland ME) and 2X minimum essential medium (MEM) was poured over the infected cells. 2X MEM consisted of Cellgro MEM powder (VWR, Radnor PA), 10% Hyclone fetal bovine serum (VWR), 3% HEPES (VWR), 2% penicillin/streptomycin (VWR), 2% sodium pyruvate (VWR), 2% L-Gutamine (VWR), 2% non-essential amino acid (VWR), 3% sodium bicarbonate (VWR). The plates were incubated at 37°C with 5% CO_2 for 48 hrs. PBS containing 3.7% formaldehyde (Acros Organics, Geel, Belgium) was then added to fix the cells on the plates for 2 hrs. The agar layer was removed and the plates were stained with 1% crystal violet (Alfa Aesar, Ward Hill, MA). Visual plaques were counted to calculate the particles forming units per ml (pfu/ml).

Membrane-based electro-separation (MBES) device configuration and experimental variables investigated

An ElectroPrep System including an ElectroPrep tank, two dialyzer chambers (0.5 ml and 1.5 ml), and a union (3.5 ml) were obtained from Harvard Apparatus (Holliston, WA). As shown in Figure 1a and 1c, the original distance between the electrodes of the system was 18 cm. This electrode distance was used initially, but the system was later reconstructed in order to obtain a shorter distance between the electrodes. The electrodes originally fixed on the edges of the

electroprep tank were moved to the center of the tank, making the distance between two electrodes shorter to 6 cm (Figure 1b).

The union (3.5 ml) was screwed with two dialyzer chambers (0.5 ml and 1.5 ml) on each side to create a linked chamber. A pore-size selective polycarbonate membrane (either 1 µm or 10 µm; SterliTech Corp, Kent, WA), was fitted into the linked chamber and two 300 kDa MWCO cellular acetate restricting membranes (Harvard Apparatus) were flanked on both sides of the linked chambers to create two membrane-separated chambers; one for sample addition (4.0 ml sample chamber) and one for sample collection (1.5 ml collection chamber) (Figure 2). Both chambers were filled with sodium phosphate buffer solutions (pH 7.5; concentration range from 20 mM to 100 mM) with or without the addition of 0.01% SDS or other norovirus elution buffers (PBS, tris-glycine, or TGBE), all with 0.01% SDS addition.

In each experiment, 50 µl of MNV-1 (containing about 10^7 genomic copies) was added into the sample chamber. The linked chambers were then submerged into the ElectroPrep tank containing 1 L of electrolyte (the same as the buffer solution used to fill the linked chamber), with the sample chamber facing the cathode and the collection chamber facing the anode. A constant voltage potential (range from 20V to 150V) was supplied by a BioRad PowerPac 300 for a period of time (range from 5 min to 60 min) at room temperature to generate an electric field. Samples for which no constant voltage (0V) was applied to the linked chambers were immersed in electrolyte for 30 min as a control for assessing simple diffusion of virus across the selective membrane. At the end of each experimental trial, 300 µl of sample was collected from both the sample and collection chambers and frozen immediately at -70°C until RNA extraction.

For each experiment, electrolyte conductivity and pH were measured by an Orion Star A215 Meter (ThermoScientific, Waltham, MA). The corresponding current for each applied constant voltage was recorded and the system resistance was calculated using Ohm's Law:

$$current = \frac{voltage}{resistant}.$$

RNA extraction

The RNA extraction method used in all the experiments was a modification of Boom's nucleic acid purification method using an in-house made Guanidine thiocyanate (GuSCN) lysis buffer as the chaotropic agent and silica as binding agent (21). To make the GuSCN lysis buffer, 60 g guanidine thiocyanae was dissolved in 50 ml 0.5X TE (Tris-EDTA) buffer, followed by addition of 5.5 ml 5M sodium chloride, 5.5 ml sodium acetate, and 1.1 ml PolyAdenosine. Equal volumes of GuSCN lysis buffer and sample (300 µl) were vortexed thoroughly followed by room temperature incubation for 10 min. To determinate the virus input, 50 µl MNV-1 aliquot was mixed with lysis buffer. To precipitate nuclei acid out of solution, a 2X volume of 100% ethanol was added to the mixture and vortexed before transferring into a RNA spin column (Omega Bio-Tek, Norcross, GA) for centrifugation at 14,000 X g for 1 min. The supernatant was discarded and 500 µl of 75% ethanol was added into the column for washing. This was followed by another cycle of centrifugation for 1 min. Then, an additional centrifugation step for drying the column was applied for 1 min after discarding the supernatant. The RNA bound to the column was eluted with 40 µl nuclease-free water (EMD Millipore, Billerica, MA) into a 1.5 ml microcentrifuge tube by centrifugation at the same speed for 1 min.

Real-time RT-PCR

The quantification of MNV-1 RNA genomic copies was carried out by real-time reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) using a MNV-1 specific probe

G54808P (CTA CCC ACC AGA ACC CCT TTG AGA CTC) and primers pairs G54763F (TGA TCG TGC CAG CAT CGA) and G54863G (GTT GGG AGG GTC TCT GAGA CAT) (22). PCR amplification was performed using a Stratagene Mx3005P qPCR System (Aligent Technologies, Santa Clara, CA) using thermal cycle conditions: one cycle of 50°C for 30 min as reverse transcription step; one cycle of 95°C for 15 min as initial PCR activation step; 50 cycles of 95°C for 10 s, 50°C for 30 s, and 72°C for 30 s as denaturation, annealing, and extension steps, respectively. Data collection and analysis was performed using the MxPro software based on a standard curve, consisting of a 10-fold serial dilution of a MNV-1 RNA transcript (see below) ranging from 0 to 10^7 genome copies per µl. All the samples and controls were tested in duplicate and the PCR efficiency for each standard curve generated ranged from 90% to 110%.

The RNA transcripts were prepared using a MEGA shortscript high yield transcription kit (Ambion, Austin, TX) by *in vitro* transcription of MNV-1. The RNA template for *in vitro* transcription was produced by RT-PCR using MNV-1 pairs G54-T7 (TAA TAC GAC TCA CTA TAC GTC TTG ATC GTG CCA GC) and G54-linker (TAG TAC ATA GTG GAT CCA GCC ATT AGT TGG GAG GGT CTC). The PCR products were transcribed using a MEGAshortscript T7 kit following the manufacturer's instructions, incubating at 37°C for 4 to 5 hrs, followed by TURBO DNase treatment to clean the transcript. Transcript concentration was measured using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and confirmed by RT-qPCR using probe G54808P and primer pairs G54763F and G54863G (described above).

Data analysis

After each experiment, recovery percentages of MNV-1 from both the sample and collection chambers were calculated by dividing the viral genomic copy number recovered in each chamber by the initial input in the chamber and multiplying 100%.

% recovery in sample chamber =

$$\frac{genomic\ copies\ detected\ by\ RT-qPCR\ after\ electro-separation\ in\ sample\ chamber}{input\ (genomic\ copies)} \times 100\%$$

% recovery in collection chamber =

$$\frac{genomic\ copies\ detected\ by\ RT-qPCR\ after\ electro-separation\ in\ collection\ chamber}{input\ (genomic\ copies)} \times 100\%$$

Norovirus mobility in electric field without the presence of membrane

In a subset of experiments, a simple device was constructed to evaluate norovirus mobility in electric field without the effect of physical membrane barrier. Two 5 ml syringe barrels (Becton, Dicknson and Company, Franklin Lakes, NJ) were connected with a 6 cm tubing (1/2'' ID × 5/8'' OD × 1/16'' wall Tygon®S3™ E-3603 laboratory tubing; United States Plastic Corp, Lima, OH). Platinum electrode wires connected to the power supply were inserted into the open ends of the syringe barrels to create an electric circuit. In one set of experiments, the device was bent into a U-shape and fixed on a rack (Figure 3a and 3c). Sodium phosphate buffer (500 mM, pH 7.5) with 0.01% SDS was filled into the device followed by 50 µl (7 log genomic copies) of MNV-1 addition to the syringe barrel with negative electrode wire (cathode) inserted. 20V constant voltage (corresponding to 18 mA electric current) was applied to the U-shape device for 30 min. Then the middle section of the tubing was clamped by an office clamp to stop the fluidic flow. After removing the applied electricity, the total sample volumes from the cathode and anode compartments were collected for RNA extraction and RT-qPCR detection. In the second set of experiments, the U-shape device was modified and a horizontal device was

constructed. The plungers for the two syringe barrels were inserted to the 6 cm tubing (described above) to prevent liquid flow out of the tubing. Platinum wire connected to power supply was inserted into holes drilled on each plunger (self-sealed with its rubber gasket) to establish an electric circuit (Figure 3b and 3d). Holes were punched in the top of the horizontally placed tubing to allow the passage of gas generated when the electric current was applied. Sodium phosphate buffer with 0.01% SDS (60 mM, pH 7.5) was filled into the tubing along with 50 µl (7 log genomic copies) MNV-1. 40V (corresponding to 30 mA electric current) was applied to the system for 10 min and 30 min, then the middle section of the tubing was clamped and the samples processed as described above. In both sets of experiments, no voltage (0V) was applied for 30 min to serve as the control for assessing virus movement by simple diffusion in the system.

Dragon green beads (DG beads) mobility in MBES system

To evaluate the movement of a surrogate particle in MBES device, 5 µg/ml florescent carboxyl polymer fluorescent microspheres FCO2F (diameter <0.49 µm, dyed with dragon green with 480 nm excitation wavelength and 520 emission wavelength) (Bangs Laboratories, Inc) was used. In these experiments, a 0.6 µm separation membrane was used and the following variables were examined; 1) electrolyte type added to the sample chamber: 1X PB (phosphate buffer), 5X PB, 10X PB, tris-glycine, or 5X TAE; 2) voltage applied: 20V to 100V electrical potential for 30 min. In each experiment, the fluorescent intensity of the microspheres collected in the sample and collection chambers was measured using a Qubit 2.0 Fluorometer (Invitrogen, Grand Island, NY).

Statistical analysis

Statistical analysis of experimental data was performed by JMP 12 (SW) (SAS Institute Inc., Cary, NC). Significant differences in % MNV-1 genomic copies recovered from collection chambers impacted by SDS addition to the electrolyte buffer and impacted by electric field strength were analyzed by one-way ANOVA. The means of % MNV-1 genomic copies recovered from collection chambers affected by different electrolyte buffers (PBS, tris-glycine, TGBE, 20 mM sodium phosphate; all with 0.01% SDS addition), different durations, and voltages were compared using student's t-test for independent samples. Alpha (α) = 0.05 was used to determine significance differences between the means.

Results and discussion

Lessons learned from initial experiments using DG beads and MNV-1:

Prior to the evaluation of MNV-1 movement in an electric field using the membrane-based electro-separation (MBES) system, fluorescent carboxyl-functionalized polystyrene microspheres (~ 50 nM in diameter) were used to evaluate the feasibility of negatively charged particle movement using the MBES system. Since the Dragon green bead (DG bead) used had a negative surface charge due to their carboxyl-functionalized surface and their concentration could be measured by fluorescence intensity, they were thought to be good candidates for testing the system efficiency before the use of virus. In these initial experiments, where the bead input was 50 µg/ml in the sample chamber, the applied voltage was 20V for 30 min and a 0.6 µm separation membrane was used, 10% to 44% of beads could be collected from the collection chamber using the following electrolyte buffer: 1X PB, 5X PB, 10X PB, 5X TAE, and tris-glycine with electrolyte conductivity measured to be 1.5 mS/cm, 5.7 mS/cm, 12.5 mS/cm, 5.4 mS/cm, and 7.1 mS/cm, respectively, and pH to be 7.9, 7.4, 7.5, 8.2, and 7.6, respectively (data

not shown). DG beads migration to the collection chamber reached its highest (44%) when 5X TAE was used as electrolyte, followed by tris-glycine (32%) and 5X PB (30%). For nearly all electrolyte buffers tested, bead recovery in the collection chamber decreased as applied voltage increased from 20V to 100V. For example, 44% DG bead migrated to the collection chamber when 20V was applied for 30 min in 5X TAE, but migration decreased to 37.8% and 6.4% when 60V and 100V were applied, respectively.

In initial experiments, to test MNV-1 movement using the MBES system, tris-glycine (pH 7.5) was used as the electrolyte buffer with a 0.6 μm polycarbonate membrane as the separation membrane. Less than 1% virus was recovered in collection chamber with applied voltages of 20V-120V for 30 min (data not shown). Then, with the addition of 0.1% sodium dodecyl sulfate (SDS) to the electrolyte buffer (tris-glycine) and substitution with a 1 μm separation membrane, virus recovery in collection chamber increased to 14.5% with 20V applied voltage for 30 min. The addition of 0.1% SDS to tris-glycine electrolyte increased virus recovery in the collection chamber from below 1% to 14.5%. When another electrolyte (sodium phosphate, concentration ranged from 20 mM to 100 mM, pH 7.5, 0.1% SDS added) was used, the virus recovery in collection chamber ranged from 5.6% to 19.3% with 20V applied voltage for 30 min. Increased electrolyte concentration seemed to slightly increase virus recovery. Because virus inactivation can occur when too high of a concentration of SDS (0.1%) is used, SDS addition was decreased to 0.01%, which also resulted in a 14.2% virus recovery in collection chamber when 20V was applied for 30 min using a 20 mM sodium phosphate electrolyte buffer (pH 7.5). The pH of the 20 mM sodium phosphate was increased to 8.5 in an attempt to increase the negative charge of norovirus to see if this would improve virus movement

to the sample chamber, but this resulted in similar virus recovery (15.3%) in the collection chamber with the same voltage (20V) and duration (30 min) applied.

Based on the preliminary results using the MBES system to separate the negatively charged microspheres and MNV-1, up to 44% of beads and 14.2% MNV-1 moved from sample chamber to collection chamber across a separation membrane with mild voltage (20V) applied for 30 min. Although the electrolyte buffer selection seemed to have an impact on DG bead and virus recovery in the collection chamber, increasing the strength and duration of the voltage applied did not seem to increase virus or DG bead recovery in the collection chamber.

Improved virus recovery by shortening the distance between electrodes:

In 2005, Ogle et al. patented an electrophoresis device (Gradiflow^{TM} Technology) and demonstrated the migration of charged macromolecular solutes through a dialysis membrane (23). In the report, they described how the distance between electrodes affected the system electric field strength by equation $E = V/d$ (E=electric field strength, V-voltage, d=distance). Therefore, a shorter distance between electrodes will increase the electric field strength of the system when the applied voltage is kept constant. Thus shortening the distance between electrodes may also enhance virus movement across the separation membrane. The electrodes originally fixed in the electroPrep tank used in this study were 18 cm apart (d = 18 cm), giving E = 1.11 V/cm when the applied voltage was 20V. After shortening the distance between electrodes to 6 cm (d = 6 cm), E increased to 3.33V/cm with the same constant voltage (20V) applied.

As shown in Figure 4, the recovery of MNV-1 in the collection chamber was less than 1% when no voltage (0V) was applied to the system due to passive diffusion of virus across the membrane. Using a constant voltage of 20V, MNV-1 movement from sample chamber to

collection chamber increased significantly (p=0.0255) when the electric field strength increased, resulting in recovery percentages of 14.2% and 31.7% for the electric field strengths of 1.11 V/cm and 3.33V/cm, respectively.

The addition of SDS to the electrolyte buffer improves norovirus recovery:

In our preliminary experiments, including SDS in the electrolyte buffer was found to be important for virus recovery from the collection chamber (data not shown). Therefore it was important to demonstrate this phenomenon using the newly designed system having a 6 cm distance between the electrodes. As shown in Figure 5, the addition of 0.01% SDS to the electrolyte buffer improved virus recovery in the collection chamber. With SDS, 31.7% of MNV-1 was recovered from the collection chamber when 20V were applied for 30 min, while 53.3% was recovered in sample chamber. Comparing these results to the MNV-1 recovery results using the electrolyte buffer without SDS, 0% and 61.3% were recovered from collection and sample chambers, respectively. In the control experiments, where no voltage was applied for 30 min, the majority of MNV-1 was recovered in sample chamber, 90.6% and 107.8% with and without 0.01% SDS, respectively, while 0.4% and 0.2% were recovered from the collection chambers, respectively, due to passive diffusion. The addition of 0.01% SDS to the electrolyte buffer significantly (p=0.0177) increased the virus movement from sample chamber to collection chamber when 20V were applied for 30 min using the MBES system.

We found that the addition of 0.01% SDS to buffer electrolyte was necessary to enhance viral recovery assisted by an electric field. The MNV-1 genomic copies recovery increased from 0% (without 0.01% SDS) to 31.7% (with 0.01% SDS), as shown in Figure 5. There are two explanations for this phenomenon. First, the addition of detergents such as SDS (also known as sodium lauryl sulfate (SLS)) can reduce surface tension of viral particles, thereby reducing virus

absorption to the membrane (24). Second, the binding of SDS to norovirus particles may have increased the negative surface charge of the particles, thus increasing their movement in the electric field.

When determining particle size of type I poliovirus by syringe filtration in 1968, the addition of 1% SLS to the virus suspension enhanced virus movement through the filter membrane such that 100% of virus particles were recovered in the filtrate using a membrane with a pore-size at least twice as large as the virus. When comparing this to a virus suspension without the addition of SLS, 99% of the virus absorbed to filter membrane. Keesom et al. reported that polycarbonate membranes had a slight negative charge due to a negative zeta potential (-27mV) when the membranes were placed in a buffer with a pH above 6 (25). Anionic surfactants such as SDS enhance the negative charge of polycarbonate membranes by co-ion surfactant adsorption (25, 26). SDS could have been bound to the negatively charged polycarbonate membrane used in this experiment, thereby reducing the absorption of MNV-1 to the membrane and leading to an increase in MNV-1 movement across the membrane which would have improved viral recovery in collection chamber. On the other hand, SDS may have been bound to the surface of viral particles, thereby increasing the particles' electrostatic repulsion and thus decreasing virus aggregation and their adherence to the membranes. In a study recovering microorganisms from drinking water by ultrafiltration, the addition of the negatively-charged surfactant, sodium pholyphosphate (NaPP), to tap water enhanced recovery of bacteriophage MS2 (108%) and *Salmonella* (49%); when compared to the control samples without NaPP, the recoveries were 51% and 31%, respectively (27). NaPP added to the water samples was thought to bind to the microorganisms, which decreased the surface zeta potentials of the microorganisms, making their surface charge more negative (28). SDS, also a strong

negatively-charged surfactant, may have the same function as NaPP in this study. The sum of virus recovery in the sample and collection chambers was about 60% and 80% without and with SDS added to electrolyte when 20V was applied, respectively (Figure 5). Therefore, it is likely that without the binding of SDS to both membrane and viral particles, virus adherence or absorption to the membranes was increased.

Although norovirus viral particles have a negative charge under neutral or basic aquatic environments (20), the surface charge appeared to be too weak to allow particle movement in such a weak electric field (20V). Similar to the function of SDS in SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) (29), the hydrophobic binding of the anionic detergent, SDS, to the surface of viral particles likely increased the overall negative viral surface charge, thereby strengthening the viral particle movement towards the anode (positive electrode).

It is very likely that both hypotheses of the function of SDS contributed to the improvement of virus recovery in collection chamber. Based on the findings in Figure 5, 0.01% SDS was included in the electrolyte buffer for all subsequent experiments optimizing our MBES system.

Electrolyte type and concentration is important for virus movement and survival in the system:

With the modification of the Ogle et al. (2005) electrophoresis device, a fixed boundary electrophoresis method to separate living cells in a membrane-based electro-separation methodology was patented under Gradiflow™ Technology (19). They demonstrated that 10^5 to 10^{10} cells/ml of erythrocytes (7 µm in diameter) migrated through a 10 µm polycarbonate membrane in 2 min with an electric field of 50V/cm using a broad variety of electrolyte buffers such as Bis-Tris, HEPES, glucose, sucrose, and phosphate buffer salts (all pH 7.4, conductivity 4 mS/cm) in small volume (anodic and cathodic chambers were both 420 µl). They also described

how the buffer concentration and conductivity, electric field strength, and applied voltage all affected cell migration, but the selection of buffer did not seem to matter. As described in our preliminary studies, the type of buffer used in MBES system did appear to impact virus movement; therefore, we further investigated if the buffer type and concentration would affect norovirus migration across the separation membrane under the mild electric field strengths used in our system.

PBS, TGBE (30, 31), and Tris-glycine (32, 33) are common elution buffers used to extract and recover norovirus from the surfaces of different foods or other environmental matrices. Table 2 indicates the pH and buffer conductivity measurements of these elution buffers. A wide range of conductivities (0.7-13.3 mS/cm) are represented by these buffers. However, we also wanted to include a buffer that had a conductivity and pH similar to that recommended in Rylatt and Leong's patent (buffer pH 7.4 and conductivity 4 mS/cm) (19). Therefore, we selected a 20 mM sodium phosphate buffer having a conductivity of 3.5 mS/cm and pH 7.4. As showed in Figure 6, when no voltage was applied for 30 min, most of the virus was recovered in the sample chamber (range from 86.1% to 95.3%) for all electrolyte buffers used (PBS, try-glycine, and 20 mM sodium phosphate, all with 0.01% SDS), while less than 1% virus was recovered in the collection chamber. After applying 20V to the MBES system (electrodes distance 6 cm) for 30 min using each electrolyte buffer with a 7 log genomic copies/ml MNV-1 input, virus recoveries in the collection chamber differed ($p < 0.0001$) for each buffer (Figure 6). No virus recovery was recovered in both sample and collection chambers when PBS was used. Respective sample and collection chamber MNV-1 recovery rates were 65.8% and 17.7% with tris-glycine, 65.3% and 6.9% with TGBE, and 60.7% and 30.8% with 20 mM sodium phosphate. For tris-glycine and 20 mM sodium phosphate buffers, the sum of the percent

recovery values for the sample and collection chambers totaled approximately 90%. However, this was not the case for TGBE and PBS.

With TGBE, the sum of virus recoveries in the sample chamber and collection chambers was only 72.2% with a large stand deviation for the sample chamber recovery results (65.3% ± 18.8) when 20V was applied. Likely due to the complexity of TGBE, components of this buffer could have interfered with virus movement across the membrane when voltage was applied. No virus was recovered from either the sample chamber or collection chamber when PBS was used as the electrolyte buffer. After applying 20V to the system for 30 min, the pH and conductivity of each buffer remained stable, with the exception of the PBS buffer (Table 2). The pH of the PBS electrolyte in the anode compartment decreased to 3.0 and the pH in the cathode compartment increased to 11.3 after the 20V application for 30 min. This pH change was likely due to the electrolysis of sodium chloride contained in PBS. Hydrolysis caused hydroxide and hydrogen ion formation and a migration of these ions toward the cathode and anode, respectively, resulting in pH changes. Viral capsids and subsequently viral RNA in the sample chamber might have degraded due to the pH increase to 11.3 using PBS. Although a low pH of 3 should not cause MNV-1 inactivation (34), free chlorine generation due to the dissociation of sodium and chloride ions of NaCl in PBS could have caused virus inactivation. Measuring free chlorine levels in this compartment was attempted using the DPD-FEAS (ferrous ethylenediammonium sulfate) titration method (HACH company, Loveland, CO), but free chlorine production could not be detected. It is possible that the volume of electrolyte (1 L) was too diluted for accurate chlorine detection (detection range of DPD-FEAS method was 0 – 3.00 mg/L free chlorine production in a 25 ml sample volume) or the free chlorine could have been rapidly consumed by the virus before it could be measured.

As our preliminary findings suggested, that electrolyte buffer conductivity may also impact virus recovery, we examined different concentrations of phosphate buffer using the MBES system. According to the equation analogous to Ohm's Law, electric field strength (E) = current density (J) / conductivity (σ), where current density (J) = current (A) / membrane area (m^2) (the membrane area in our system was 4.9 X 10^{-4} m^2). Increasing the concentration of phosphate buffer in the system did increase the current density, but because of the larger increase of buffer conductivity, the net electric field strength decreased slightly as the electrolyte buffer concentration increased (Table 3). Interestingly, this slight decrease of the electric field strength for increasing concentrations of phosphate buffer in the system resulted in a substantial decrease of virus recovery from 31.7% to 8.2% (Figure 7). The highest virus recovery in the collection chamber was achieved when 20 mM sodium phosphate electrolyte (conductivity 3.5 mS/cm) was used, while increasing the electrolyte concentration in an attempt to increase conductivity deceased virus recovery. The optimized electrolyte conductivity (3.5 mS/cm) in this study was similar to Rylatt and Leong's patent (19), in which the optimized electrolyte conductivity was 4.0 mS/cm. Comparing the conductivity of different concentrations of sodium phosphate and other buffers tested (Table 2 and 3), a relatively low electrolyte conductivity was needed to increase overall virus movement in a weak electric field. Insufficient electric field was generated to mobilize virus particles when the conductivity was too low, while the net electric field strength started to decease if conductivity was too high.

<u>The impact of the duration of electric field application on norovirus recovery and stability:</u>

From Figure 8, the recovery of MNV-1 from the collection chamber increased from 13.0% (after 10 min) to 30.8% (after 30 min) when 20V was applied; while MNV-1 recovered from the sample chamber decreased from 83.8% (after 10 min) to 60.7% (after 30 min). These

results showed that as electro-separation duration increased from 10 min to 30 min, more MNV-1 moved from sample chamber to collection chamber, but the result was not statistically significant ($p = 0.0992$). However, when duration increased from 30 min to 60 min, MNV-1 recovery in the collection chamber remained identical ($p = 0.9910$), 30.8% (30 min) and 30.7% (60 min), but MNV-1 recovery in the sample chamber decreased from 60.7% (30 min) to 37.9% (60 min).

In a study utilizing stagnant electrodialysis to separate negatively charged inositol with a size-selective membrane (18), inositol phosphate enrichment increased when the electrodialysis time increased from 1 min to 5 min with 600V applied. A non-linear curve of time vs. target enrichment could be plotted. As time increased to 8 min and 10 min, no more enrichment was observed. The results in Buscher's study were similar to our study in that as duration increased, the recovery increased until a point where increases in virus recovery could no longer be achieved. Gas bubble formation around the electrodes was reported in Buscher's study and was also observed in the current study. Bubble formation for a long duration may alter and mitigate the electric field strength provided by the same constant voltage due to electrical resistance build-up and current deprivation (18).

Besides the possibility of electric current deprivation causing an increase of electrical resistance (18), viral particles may destabilize with prolonged electric application, causing RNA exposure and degradation by innate RNases present in the electrolyte, since RNase-free buffers were not used in this study. This was likely the reason for the sharp decrease in MNV-1 recovery from the sample chamber after the 60 min duration. The sum of MNV-1 genomic copy recovery rates in the sample chamber (37.9%) and in the collection chamber (30.7%) after 60 min of

applied voltage was 68.6%, meaning 31.4% MNV-1 genomic copies were lost due to RNA degradation (Figure 8).

Moderate applied electric field strength is important to norovirus recovery and stability:

MNV-1 recovery rates in the collection chambers were 30.8% and 31.3% when the applied voltages were 20V and 40V, respectively; while MNV-1 recovery in sample chambers were also similar, 60.7% and 65.5%, respectively. However, when the applied voltage increased to 60V, no MNV-1 was detected in the collection chamber and only 31.6% was recovered in the sample chamber. Almost 70% of MNV-1 was lost after 60V was applied for 30 min (Figure 9). Figure 9 results indicated that when a high voltage is applied for prolonged durations, this could cause viral destabilization and RNA degradation.

When high voltage was applied for short durations in the MBES system, as shown in Figure 10, 18.8% and 24.5% of MNV-1 was recovered in the collection chamber when 100V and 150V was applied for 5 min, respectively. When the applied voltage duration was increased to 10 min, no increase in the collection chamber recovery rates of MNV-1 were observed, but a significant MNV-1 recovery loss ($p = 0.0092$) in the sample chamber was observed when 150V was applied. The sum of MNV-1 recovery percentages from the sample and collection chambers was 75.4% and 59.1% when 100V was applied for 5 min and 10 min ($p = 0.2940$), respectively; while 70.8 % and 37.2% was observed when 150V was applied for 5 min and 10 min ($p = 0.0688$), respectively, These two results were not statistically significant probably because of the low number replicates ($n = 2$) being tested in the experiment.

In the inositol phosphate separation study (18), an increase in applied voltage from 150V to 600V increased inositol migration from the donor phase to receptor phase through a 30,000 MWCO membrane in 20 seconds, but voltage higher than 600V did not increase inositol

phosphate migration due to intense bubble formation near the electrodes and thus destabilization of the electric field. Issaq et al., discussed the relationship between the applied voltage and a compound's mobility (35) using dansylalanine and mesiyloxide to confirm their hypothesis. An increase in applied voltage caused heat formation near the electrodes, leading to uneven electrolyte temperature increases. Partial temperature increases can cause an increase in buffer conductivity but also a decrease in buffer density and viscosity. The combination of all these effects could result in an increase in solute mobility and thus improve recovery. In a more advanced design based on electric field separation, Balchen et al. extracted angiotensin peptides from human plasmas by electro-membrane extraction (EME) through a supported liquid membrane (SLM). (36). Angiotensin recovery increased to 30% when voltage increased from 5V to 15V, but no recovery increase was observed when the voltage increased from 15V to 20V. In this case, increasing the applied voltage initially favored angiotensin migration because of the higher electric current generated, but increasing the voltage too much caused electrolyte electrolysis near the electrodes. Unstable electrical system thus formed because of bubbles and heat generation. This phenomenon was also observed when basic drug substances were extracted from human plasma and human urine by EME (37).

In capillary electrophoresis (CE), extremely high voltages (usually measured in kilo-voltage (kV)) are used to mobilize biological particles (viruses, bacteria, and eukaryotic cells). Differences in particle sizes, particle surface amino acid residues, and charged sugars can contribute to the different particle mobility across a capillary when voltage potential is applied (38). A UV or florescence detector is connected to the end of the capillary to measure the absorbance of viral capsids or residual viral genomes and impurities pass through (39). When determining norovirus VLPs (virus-like particles) isoelectric point, a whole-column UV

absorption imaging detector was coupled with a capillary to separate VLPs by 3 kV dc voltage (20). In such a system, both intact and damaged VLPs would be detected. RT-PCR is usually not used to detect viruses separated by CE due to the likely disruption of intact viruses by high voltage potential. This is the reason why voltages as high as those used in CE technique were not implemented by this MBES study.

The impact of separation membrane pore size on norovirus mobility;

Since the norovirus particle size is around 28 nm to 36 nm in diameter, noroviruses should be able to pass through a membrane with pore-size of 1 μm singularly or as duplets. However, virus aggregation could be problematic for these membranes and larger pore-size membranes may be necessary if virus aggregates are present. As shown in Figure 11, the use of a 10 μm pore-size separation membrane generated a slightly higher passive diffusion rate for MNV-1 passing through the membrane (2.23%), compared to the 1 μm pore-size membrane (0.36%) when no voltage was applied for 30 min using the 20 mM sodium phosphate electrolyte buffer containing 0.01% SDS (pH 7.5). With a 20V application for 30 min, collection chamber recoveries were 22.6% and 11.2% for the 1 μm and 10 μm separation membranes, respectively. This experiment demonstrates that increasing the pore size does not lead to increased virus recovery from the collection chamber and therefore this does not appear to be a limiting factor for the MBES system. This experiment was conducted after significant modifications to the MBES system were made (see section below), followed by re-assembly of the MBES system to the format described for the experiments above. In this set of experiments, using the same applied voltage (20V) and electrolyte conductivity as described in the experiments above (20 mM phosphate buffer with 0.01% SDS), the virus recovery rate in the collection chamber was lower than previously observed. In this experiment, the current generated was also slightly lower

(18 mA) than those of all the other experiments (23 mA). It is unclear why this decrease in current was observed, but it is possible that it was due to unstable wire connection upon re-assembling the MBES system. This decrease in current might have been the cause of the low MNV-1 recovery in the collection chamber for this experiment.

Restriction membranes separating the electrodes from norovirus in the sample and collection chambers is necessary to avoid virus inactivation:

When measuring the system resistance with an applied voltage of 20V using the 20 mM sodium phosphate with 0.01% SDS (pH 7.5) as the electrolyte buffer, the system resistance increased respectively from 385Ω to 769Ω without and with the presence of the restriction and separation membranes in the device. The increased resistance also caused a decrease of electric current from 52 mA to 26 mA (voltage = current X resistance), which might affect virus movement causing low virus mobility, and thus a decrease in virus recovery in the collection chamber. To decrease the resistance of the system in an attempt to increase virus mobility, the system was re-designed to remove all membranes. In the first re-design, a U-shaped device was constructed (Figure 3a and 3c). However, due to the vertical nature of the fluid movement in the device and the narrow connection between the syringe barrels and tubing, electron flow was decreased, and thus no electric current was established using the 20 mM sodium phosphate (with 0.01% SDS) buffer electrolyte with 20V applied to the U-shaped device. In an attempt to compensate for this unexpected increase in system resistance, a high concentration of electrolyte buffer (500 mM sodium phosphate) was used to obtain a sufficient electric circuit. When 20V constant voltage was applied, 23 mA electric current was recorded, which was similar to the MBES system's electric current (26 mA) when using the 20mM sodium phosphate (with 0.01% SDS) electrolyte. However, when 500 mM sodium phosphate (with 0.01% SDS) was used, a

precipitate was formed after the samples underwent the normal freeze-thaw cycle prior to the RNA extraction step. No viral RNA genomic copies were detected in either the sample or collection chambers using this system, possibly due to of the presence of the precipitate that clogged the membrane of the spin column used during the RNA extraction step.

To cope with high system resistance and low electric current due to insufficient electrons flowing in the U-shaped vertical device, the system was again re-designed in a horizontal format (Figure 3b and 3d). As shown in Figure 12, when 0V electrical potential was applied, 50% and 48% MNV-1 was recovered in the cathode and anode compartments, respectively, due to passive diffusion in the tubing. When 20V was applied for either 10 min or 30 min, no RNA was detected in either the cathode or anode compartments.

Without the shielding of the electrodes with a restriction membrane barrier, it is likely that the negatively charged virus was attracted to the anode surface by electrosorption. This would have been followed by direct electron transfer caused by oxidoreduction of the electrolysis buffer, resulting in instant virus inactivation. This phenomenon is similar to the inactivation of *E. coli* by electrochemical disinfection using a Pt (platinum) anode and a chlorine-free electrolyte (40). In Rylatt and Leong's patent, physical membrane barriers (10 or 20 kDa CTA membrane) were used to prevent the convection of electrolyte between the electrode compartment and sample compartment as well as from the direct contact of the cells to the electrodes (19). A physical membrane (either pore-selective membrane or ion-exchange membrane) is thus needed to separate the virus sample from direct contact with the electrode surface, which can cause virus inactivation.

Conclusion

This study is the first to illustrate the possibility of mobilizing norovirus by electric force across a pore-size selective separation membrane in order to achieve separation, concentration and purification of noroviruses. A MBES method for recovering murine norovirus from phosphate buffer was successfully designed, developed, and evaluated in this proof-of-concept study. In a subset of experiments, as high as 30.8% of MNV-1 migrated from sample chamber to collection chamber across a 1 μm separation membrane, when 20 mM sodium phosphate buffer with 0.01% SDS was used with 20V applied for 30 min. Of all variables tested, this set of parameters yielded the highest virus recovery percentage, implying that weak voltage, moderate duration, and low ionic strength electrolytes are optimal for norovirus movement across an electric field without disrupting the stability of the virus particles. Since the negatively charged surface of norovirus is not strong enough to be mobilized in a weak electric field, SDS addition to electrolyte is essential to increase norovirus mobility and recovery. The electric field strength of the system is a key factor to enhance norovirus mobility in an electric field, but from this study it was determined that this can only be achieved by decreasing the distance between the electrodes instead of increasing applied voltage because the virus is not stable at high voltages as demonstrated by RNA degradation. From this study it was also found that a horizontal electric system design is important to ensure the smooth flow of electrons and decrease the system resistance. Last but not least, although it was found that the integration of restriction membranes in the system increases system resistance, a membrane barrier separating the electrode from the collection chambers is essential to avoid virus inactivation by oxidoreduction when in contact with positive electrode (anode).

This study suggests that in future designs, either increasing the separation membrane surface area, decreasing the distance between electrodes, or both can help to improve norovirus recovery beyond what was observed in this study. To accommodate larger sample input volumes, a continuous input feeding design could be explored with this system. MNV-1 infectivity was not evaluated using the MBES system because the virus concentration in each chamber was too low to be detected by plaque assay. Future studies should include a virus concentration step prior to analysis of virus infectivity in order to evaluate whether or not the system could be used to detect infectious virus.

In conclusion, this study was the first to explore the movement of norovirus in an electric field as a part of a sample clean-up and preparation method for norovirus detection. With further modifications of the system and optimization of its parameters, the membrane-based electro-separation method may provide a novel, quick, easy, and cost-effective method for norovirus detection.

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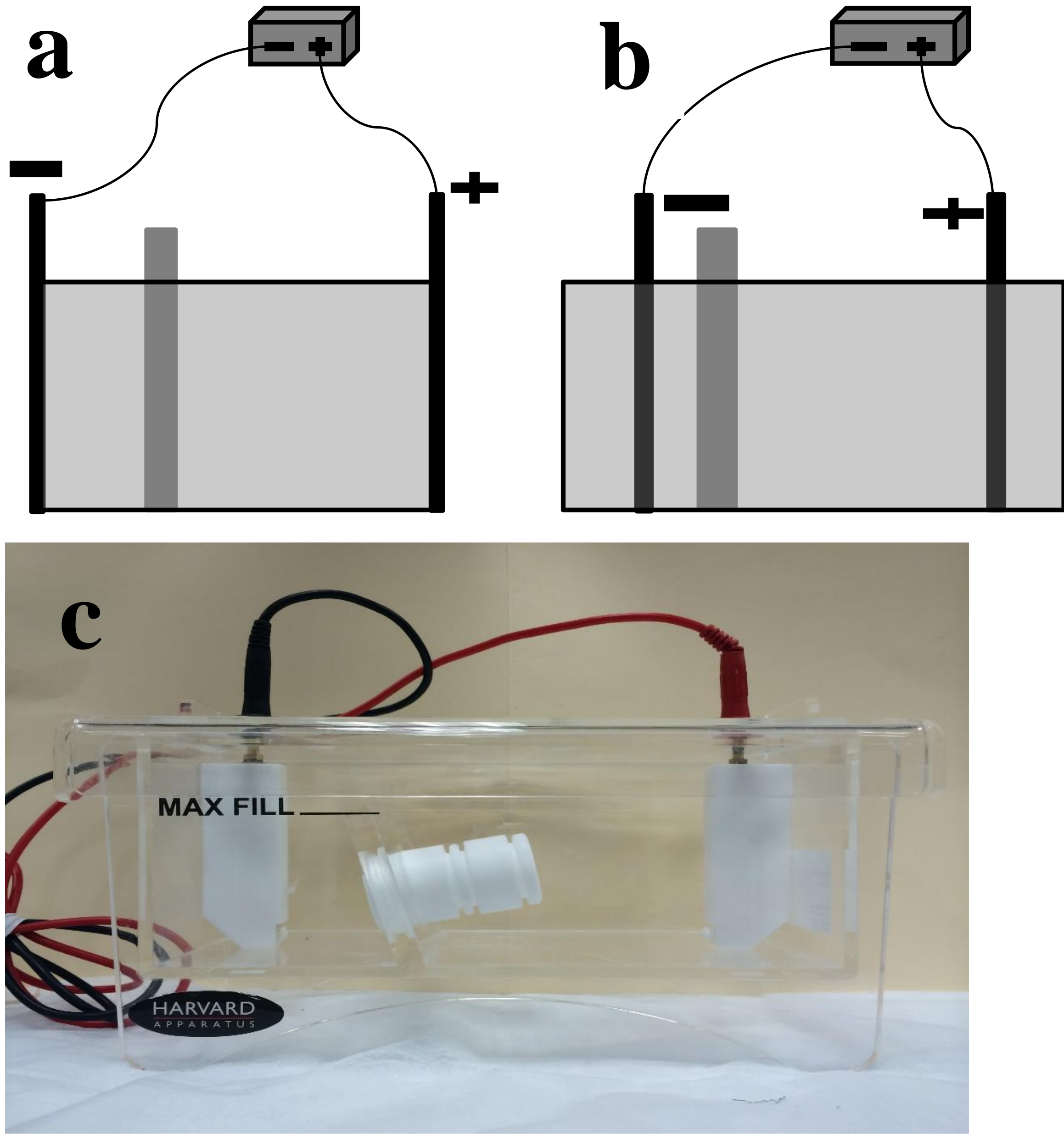


Figure 1: Eletroprep tank used for membrane-based electro-separation (MBES). a = electrodes originally fixed on the edges of the tank with distance = 18 cm. b = electrodes reconstructed to the center of the tank with distance = 6 cm. c = actual picture of the electroprep tank with distance = 18 cm.

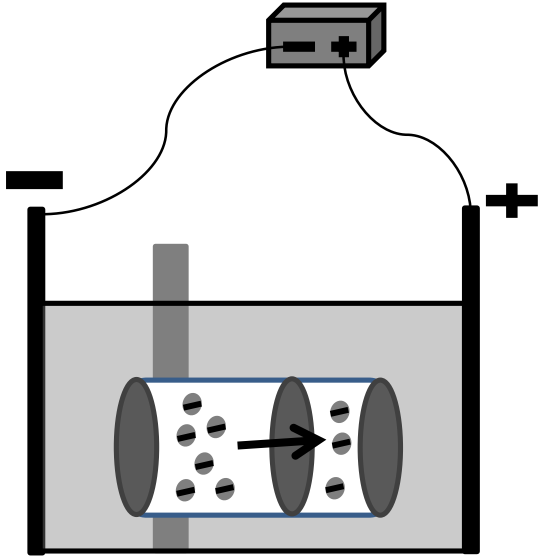
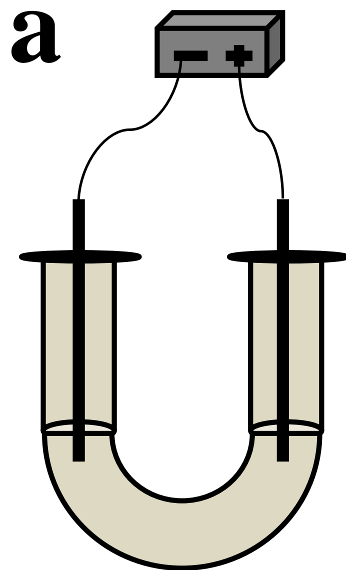
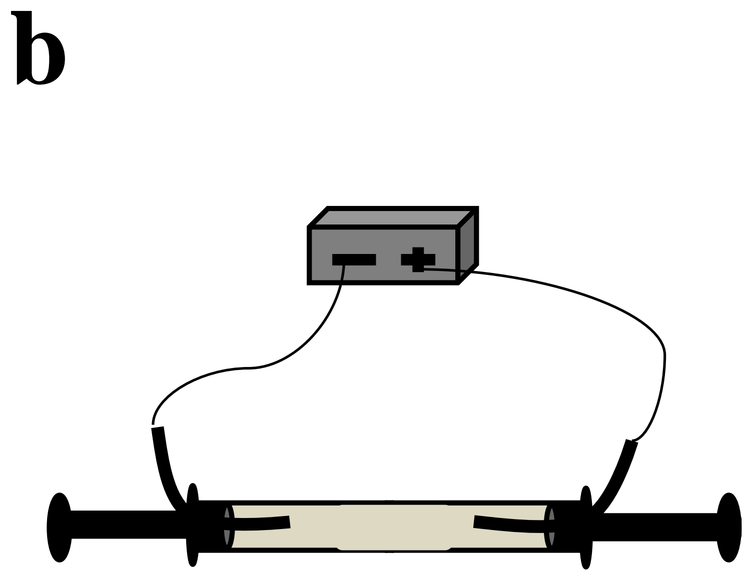
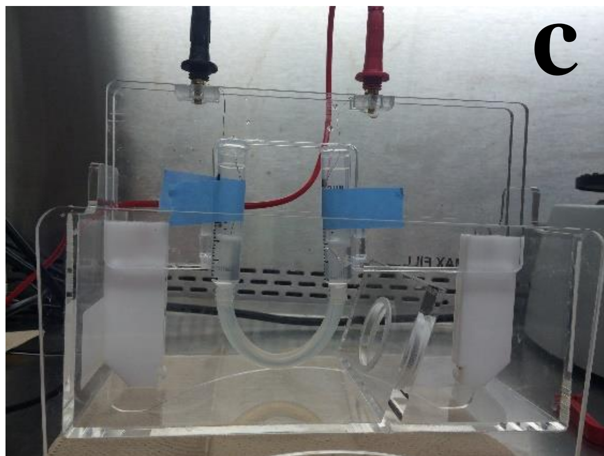


Figure 2: Illustration of viral particle movement in the MBES device.







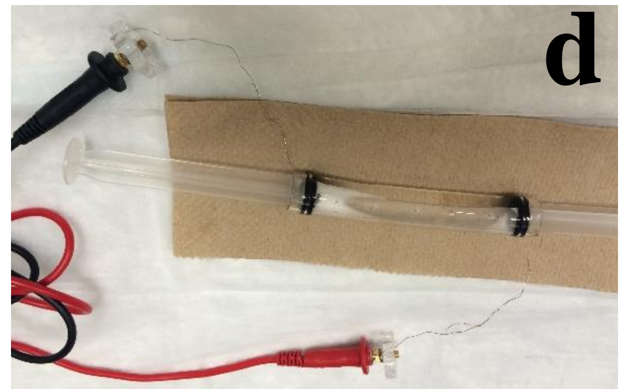


Figure 3: Devices used to investigate viral particles movement in electric field without the presence of membrane barrier. a = U-shape device. b = horizontal device. c = actual picture of the U-shape device. d = actual picture of the horizontal device.

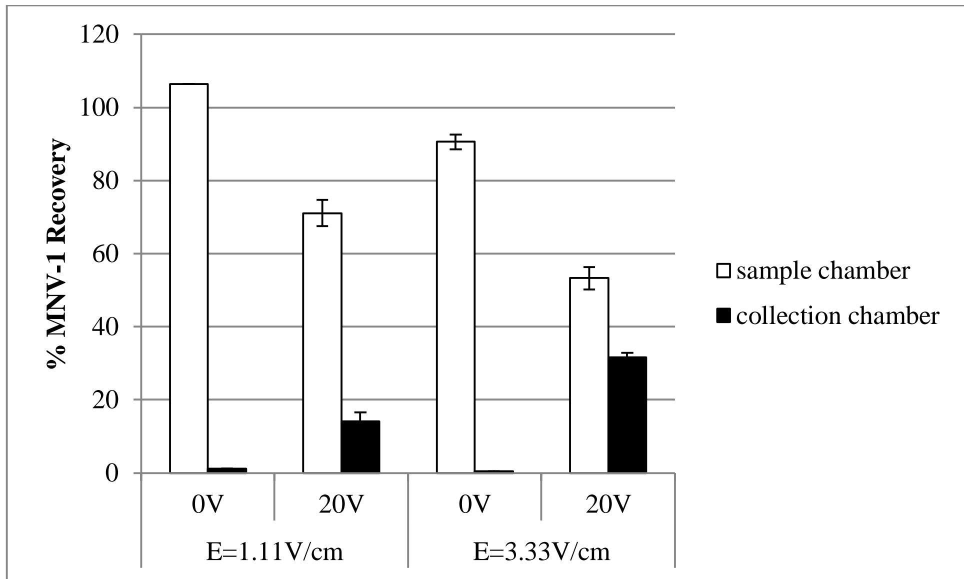


Figure 4: The impact of electric field strength on MNV-1 recovery in the MBES system. Using the system with an electrode distance of 18 cm (E=1.11V/cm) or 6 cm (E=3.33V/cm) apart, 7 log genomic copies of MNV-1 were added to the sample chamber prior to voltage application (20V) or without voltage (0V) application for 30 min in a 20 mM sodium phosphate with 0.01% SDS electrolyte buffer. Error bars represent standard deviations, n=2.

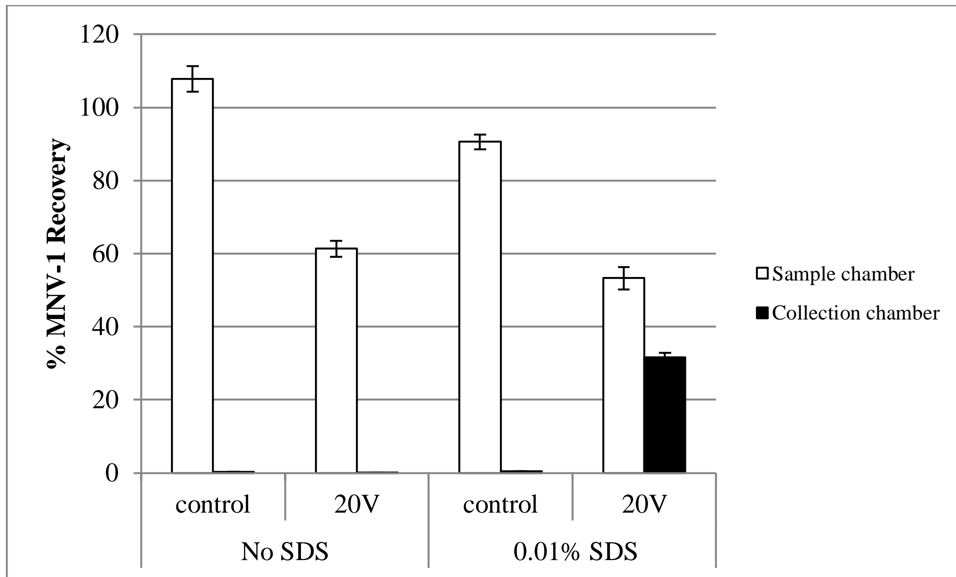


Figure 5: The impact of 0.01% SDS addition to the electrolyte buffer on MNV-1 recovery in the MBES system. Using a 20 mM sodium phosphate electrolyte buffer (pH 7.5) with or without the addition of 0.01% SDS, 7 log genomic copies of MNV-1 were added to the sample chamber prior to voltage application (20 V) or without voltage (0V) application for 30 min. Error bars represent standard deviations of the means, n=2.

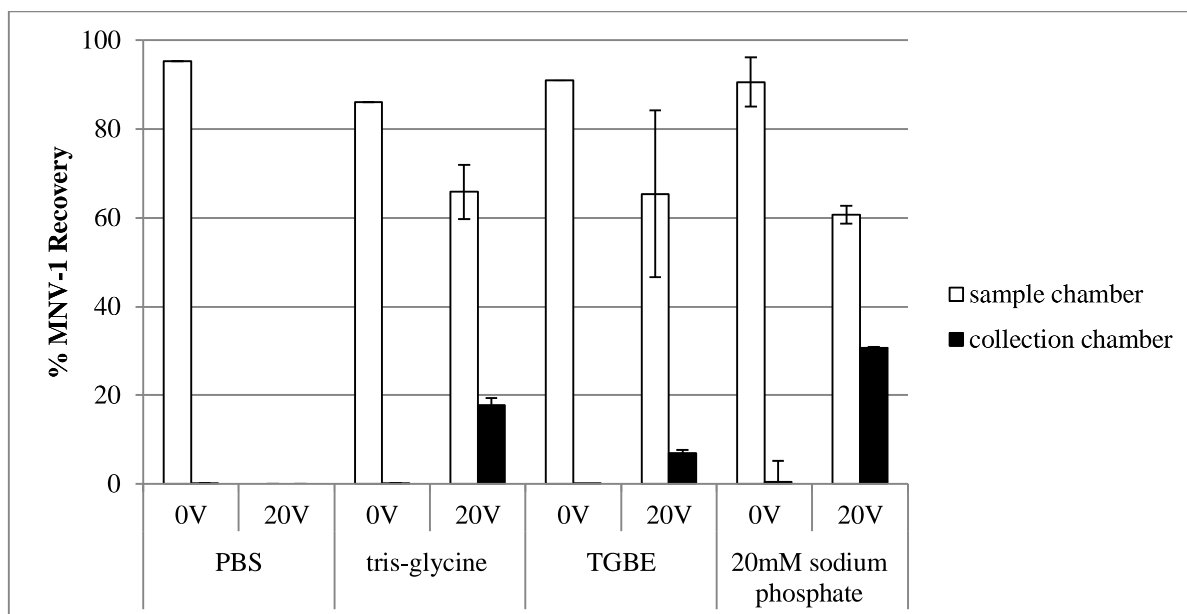


Figure 6: The impact of buffer selection on MNV-1 recovery in the MBES system. Using PBS (pH 7.4) with 0.01% SDS, tris-glycine (pH 8.3) with 0.01% SDS, TGBE (pH 8.8) with 0.01% SDS, or 20 mM sodium phosphate (pH 7.5) with 0.01% SDS as electrolyte buffers, 7 log genomic copies of MNV-1 were added to the sample chamber prior to voltage application (20V) and no voltage (0V) application for 30 min. Error bars represent standard deviations, n=2.

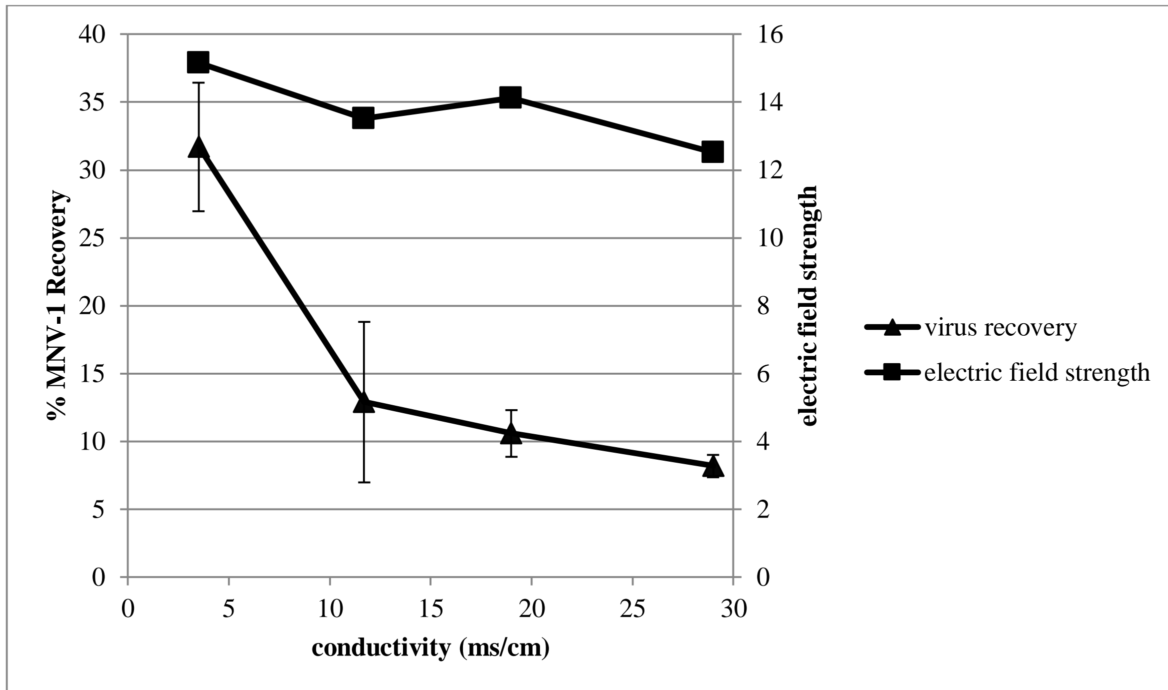


Figure 7: Relationship between phosphate buffer electrolyte conductivity and its associated electric field strength to virus recovery in the collection chamber. Using 20 mM, 100 mM, 200 mM, or 300 mM sodium phosphate (all with 0.01% SDS, pH 7.5) as the electrolyte buffer, 7 log genomic copies of MNV-1 were added to the sample chamber prior to voltage application (20V) and no voltage (0V) application for 30 min. Error bars represent standard deviations, n=2.

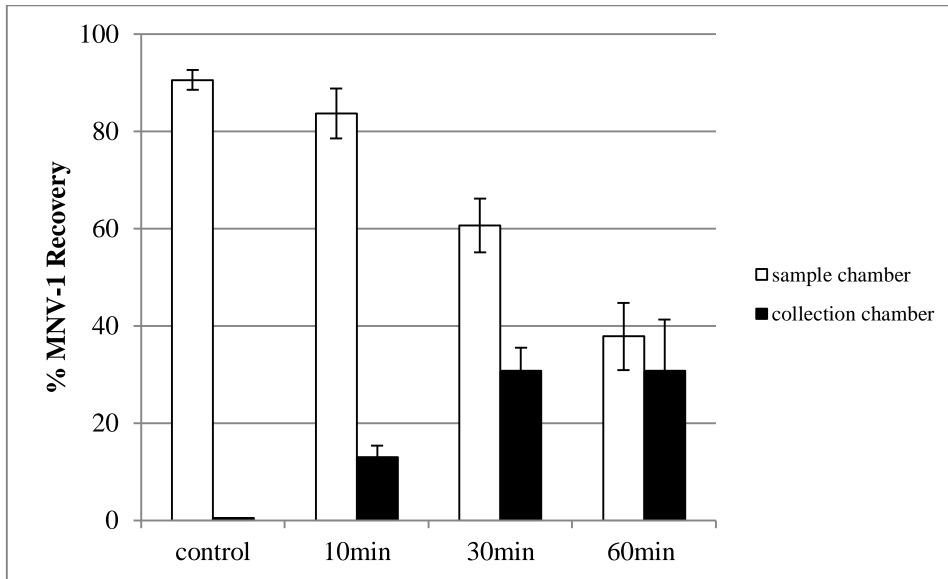


Figure 8: The impact of duration of the applied voltage on MNV-1 recovery in the MBES system. A constant voltage (20V) was applied in the MBES system for 10 min, 30 min, and 60 min or no voltage (0V) for 30 min (control) with 7 log genomic copies of MNV-1 added to the sample chamber prior to voltage application using 20 mM sodium phosphate with 0.01% SDS as the electrolyte buffer. Error bars represent standard deviations, n=2.

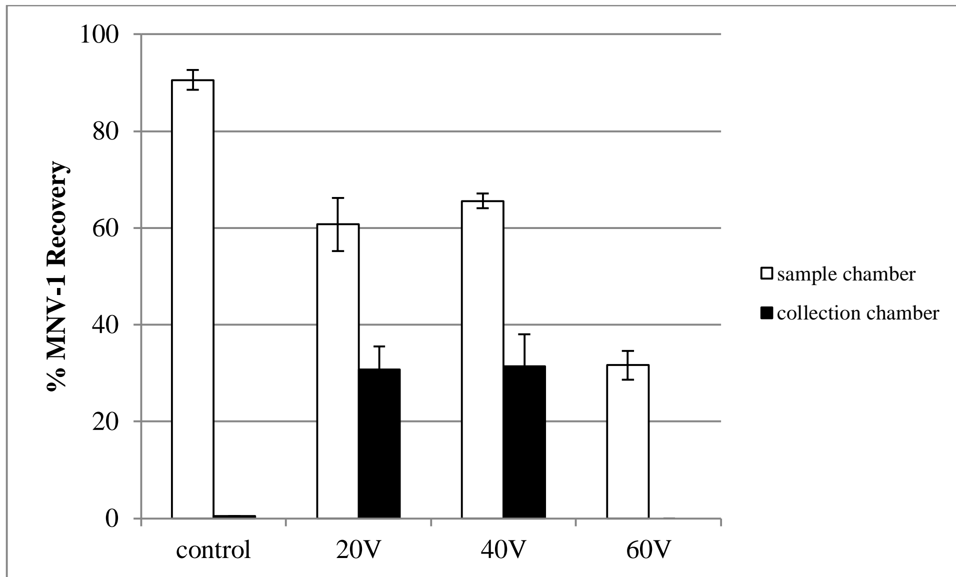


Figure 9: The impact of applied voltage strength on MNV--1 recovery using the MBES system. Applying a constant voltage of 20V, 40V, or 60V and no voltage (control) to the system for 30 min, 7 log genomic copies of MNV-1 were added to the sample chamber prior to voltage application using 20 mM sodium phosphate with 0.01% as the electrolyte buffer. Error bars represent standard deviations, n=2.

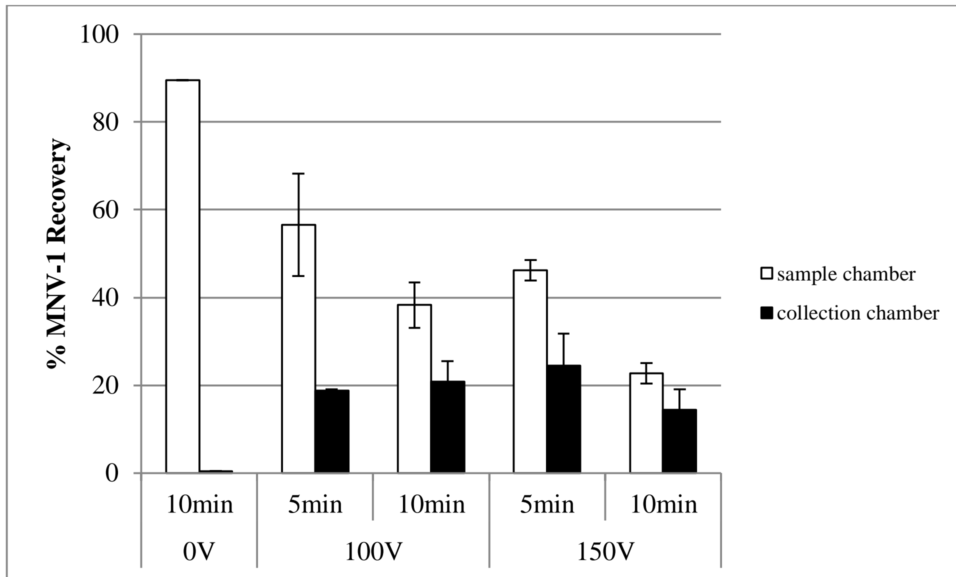


Figure 10: The impact of high applied voltage for a short duration on MNV-1 recovery using the MBES system. Constant voltages of 100V or 150V were applied to the system for 5 min or 10 min and no voltage for 10 min (control). 7 log genomic copies of MNV-1 were added to the sample chamber prior to voltage application using 20 mM sodium phosphate with 0.01% SDS as the electrolyte buffer. Error bars represent standard deviations, n=2.

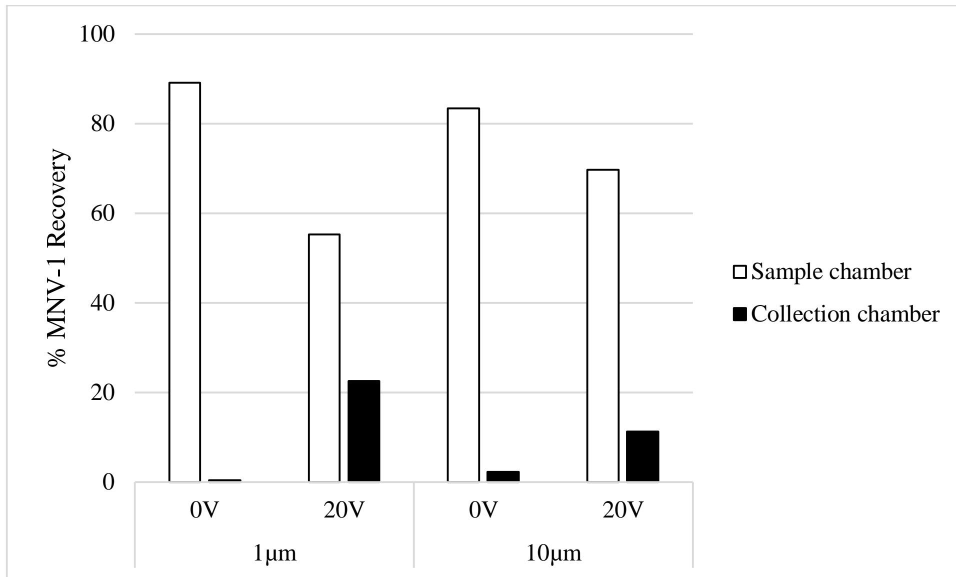


Figure 11: The impact of separation membrane pore-size on MNV-1 genomic copy recovery rate. Using the system with separation membrane pore sizes of 1 µm or 10 µm, 7 log genomic copies of MNV-1 were added to the sample chamber prior to voltage application (20V) or without voltage (0V) application for 30 min using a 20 mM sodium phosphate with 0.01% SDS electrolyte buffer, (n = 1).

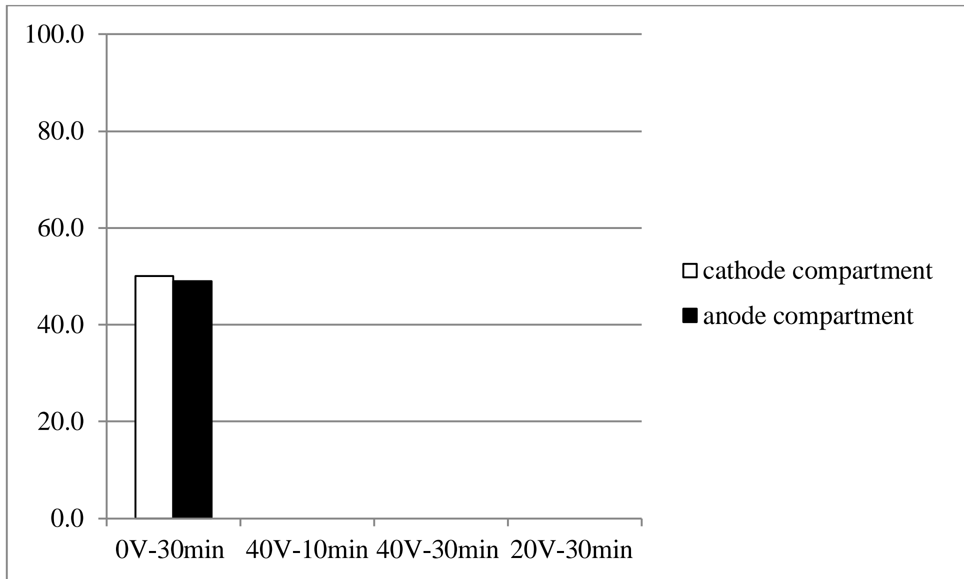


Figure 12: MNV-1 recovery using a horizontal electro-separation device without the addition of membrane barriers for restriction or separation. Using a horizontal device (6 cm length) filled with 60 mM sodium phosphate buffer (pH 7.5) with 0. 01% SDS, 7 log genomic copies of MNV-1 were added to the device prior to voltage application (40V for 10 min, 40V for 30 min, and 20V for 30 min) or no voltage (0V) application for 30 min, (n=1).

Table 2. pH, conductivity, and electrical current measurements and calculations for common virus elution buffers containing 0.01% SDS.

Electrolyte	pH		Conductivity (ms/cm)		Current (mA)	System resistance (V/mA)	Current density (A/m^2)	Electric field strength
	Initial	After	Initial	After				
20 mM sodium phosphate	7.5	7.5	3.5	3.5	26	0.8	53.1	15.2
Tris-glycine	8.3	8.3	0.7	0.7	4.0	2	8.2	11.7
TGBE	8.8	8.8	2.2	2.2	12.5	1.6	25.5	11.6
PBS	7.4	3.0 (anode compartment) 11.3 (cathode compartment)	13.3	13.3	92	0.2	187.7	14.1

*20V constant voltage was applied for 30 min in the MBES system.

*System resistance was calculated by Ohm's law R= V/I where current (I) was recorded during the voltage (20V) application. Current density was calculated by the analogous Ohm's Law equation, electric field strength (E) = current density (J) / conductivity (σ), where current density (J) = current (A) / membrane area (m^2) (the membrane area in our system was 4.9 X 10-4 m^2). The electric field strength was calculated by equation E = V/d (E=electric field strength, V-voltage, d=distance).

Table 3. pH, conductivity, and electrical current measurements and calculations for sodium phosphate buffers of different concentrations containing 0.01% SDS.

Concentration	pH		Conductivity (ms/cm)		Current (mA)	System resistance (V/mA)	Current density (A/m^2)	Electric field strength
	Initial	After	Initial	After				
20 mM	7.5	7.5	3.5	3.5	26	0.78	53.1	15.2
100 mM	7.5	7.5	11.7	11.7	77.5	0.3	158.2	13.5
200 mM	7.5	7.5	19.0	19.0	131.5	0.2	268.4	14.1
300 mM	7.5	7.5	29.0	29.0	178	0.1	363.3	12.5

*20V constant voltage was applied for 30 min in the MBES system.

* System resistance was calculated by Ohm's law R= V/I where current (I) was recorded during the voltage (20V) application. Current density was calculated by the analogous Ohm's Law equation, electric field strength (E) = current density (J) / conductivity (σ), where current density (J) = current (A) / membrane area (m^2) (the membrane area in our system was 4.9 X 10-4 m^2). The electric field strength was calculated by equation E = V/d (E=electric field strength, V-voltage, d=distance).