Anal Bioanal Chem (2012) 402:315–323
DOI 10.1007/s00216-011-5381-9

ORIGINAL PAPER

Micro-total analysis system for virus detection: microfluidic pre-concentration coupled to liposome-based detection

John T. Connelly · Sowmya Kondapalli · Marc Skoupi · John S. L. Parker · Brian J. Kirby · Antje J. Baeumner

Received: 29 June 2011 / Revised: 15 August 2011 / Accepted: 30 August 2011 / Published online: 10 September 2011

Abstract An integrated microfluidic biosensor is presented that combines sample pre-concentration and liposome-based signal amplification for the detection of enteric viruses present in environmental water samples. This microfluidic approach overcomes the challenges of long assay times of cell culture-based methods and the need to extensively process water samples to eliminate inhibitors for PCR-based methods. Here, viruses are detected using an immunoassay sandwich approach with the reporting antibodies tagged to liposomes. Described is the development of the integrated device for the detection of environmentally relevant viruses using feline calicivirus (FCV) as a model organism for human norovirus. In situ fabricated nanoporous membranes in glass microchannels were used in conjunction with electric fields to achieve pre-concentration of virus–liposome complexes and therefore enhance the antibody–virus binding efficiency. The concentrated complexes were eluted to a detection region downstream where captured liposomes were lysed to release fluorescent dye molecules that were then quantified using image processing. This system was compared to an optimized electrochemical liposome-based microfluidic biosensor without pre-concentration. The limit of detection of FCV of the integrated device was at 1.6×10^5 PFU/mL, an order of magnitude lower than that obtained using the microfluidic biosensor without pre-concentration. This significant improvement is a key step toward the goal of using this integrated device as an early screening system for viruses in environmental water samples.

Keywords Biosensor · Microfluidic · Pre-concentration · FCV · Liposomes

Published in the *10th Anniversary Issue*.

J. T. Connelly · M. Skoupi · A. J. Baeumner (✉)
Department of Biological and Environmental Engineering,
Cornell University,
Ithaca, NY 14853, USA
e-mail: ajb23@cornell.edu

S. Kondapalli · B. J. Kirby
Sibley School of Mechanical and Aerospace Engineering,
Cornell University,
Ithaca, NY 14853, USA

J. S. L. Parker
Baker Institute of Animal Health, College of Veterinary Medicine,
Cornell University,
Ithaca, NY 14853, USA

Abbreviations

CPE	Cytopathogenic effects
DMEM	Dulbecco's modified Eagle's media
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HSS	HEPES–saline–sucrose
IMS	Immunomagnetic separation
LIA	Liposome immunoassay
mAb	Monocolonal antibody
μTAS	Micro-total analysis system
OG	Octyl-β-D-glucopyranoside
pAb	Polyclonal antibody
PBS	Phosphate-buffered saline
PBSS	Phosphate-buffered saline sucrose
PCR	Polymerase chain reaction
PFU	Plaque forming unit
RT-PCR	Reverse transcriptase-PCR
SERS	Surface-enhanced Raman spectroscopy
SNR	Signal-to-noise ratio

SRB Sulforhodamine B
US EPA United States Environmental Protection Agency

Introduction

Enteric viruses are any one of over 100 species that infect humans or animals via the fecal–oral route and primarily infect and replicate in the gastrointestinal tract. Although these viruses are commonly associated with gastroenteritis, they can cause a range of diseases, including respiratory infections, hepatitis, conjunctivitis, and meningitis [1]. They have even been linked to chronic diseases like insulin-dependent diabetes [2].

Once infected, humans or host animals shed virus particles in feces. Enteric viruses are then introduced into water systems mostly through leaking sewage and septic systems, urban and agricultural runoff, and directly from untreated or under-treated wastewater. Outbreaks have been linked not only to contaminated drinking water, but also contaminated recreational and irrigation water as well as shellfish harvested from contaminated waters [3]. These pathogenic viruses are highly resistant to changes in pH and temperature, as well as to common methods of wastewater treatment. It has been shown that these viruses can remain infective for up to 130 days in seawater, 120 days in freshwater and sewage, and 100 days in soil [1]. Depending on the source of contamination and water supply in question, virus particles can be present in low concentrations, complicating both detection and sterilization methods.

Current detection methods for enteric viruses can be divided into two main categories: cell culture assays and molecular methods. The cell culture technique was the most popular method for detection of enteric viruses prior to the development of the polymerase chain reaction (PCR) and remains the method of choice to isolate and determine infectivity of viruses. The cell culture technique requires the inoculation of a cell line, which was chosen based on the virus of interest, and the incubation for days to weeks as it is evaluated for the cytopathogenic effects (CPE) of a viral infection [4]. This long incubation time is an obvious drawback of the cell culture assay, though it is not the only one; some viruses do not grow on established cell lines, grow too slowly, or just do not show any visible CPE.

The molecular methods most commonly used for the detection of enteric viruses are variations of conventional PCR [5] or reverse transcriptase-PCR (RT-PCR) [6], including real-time PCR [7] and multiplex PCR [8], as well as nucleic acid sequence-based amplification [9]. These methods allow for the rapid, sensitive, and specific detection of enteric viruses of interest. The primary drawback to these molecular methods is the inability to limit detection to only infective viruses. However, this can be remedied by the use of integrated cell culture RT-PCR. This method involves inoculating a cell line with the sample and incubating for a short time, usually far before CPE are evident. Nucleic acids can then be extracted from the culture and processed through RT-PCR, testing for viral mRNA that would be produced only if the sample contained infective viruses. This process can, however, decrease the efficiency of detection [10].

As some enteric viruses are not cultivable and molecular techniques sacrifice efficiency of detection for an ability to identify infective viruses, many countries, including the USA, rely on indicators of fecal contamination—enterococci and coliform bacteria—rather than direct testing. Reliance on these indicators is flawed, as viruses are more resistant to disinfection processes and natural environmental conditions [11, 12].

Feline calicivirus (FCV) is a member of the *Caliciviridae* family that causes respiratory and potentially severe systemic disease in cats. FCV is used as a model for human pathogenic noroviruses, as it is a member of the same family as these viruses but is non-pathogenic to humans [13]. Enzyme-linked immunosorbent assays (ELISAs) for the detection of FCV have been previously described, which use either two antibodies [14] or one antibody and one transmembrane glycoprotein [15]. Detection limits were not reported, as the developed ELISAs were used to screen antibodies [14] or to determine the binding domain of the glycoprotein [15]. However, methods have been reported employing atomic force microscopy and surface-enhanced Raman spectroscopy (SERS) for detection of FCV with limits of detection of three million and one million virions per milliliter, respectively [16].

Biosensors are an attractive detection method for molecules and small particles, such as virions, as they can produce rapid, sensitive, and specific signals [17–22]. Both microfluidic and lateral flow assays using liposome nanovesicles as a visual or electrochemical signal generation and amplification system have been well-established using nucleic acids [17–22] and antibodies [23–25] as capture molecules, depending on the target being detected. Additionally, novel biological recognition elements have been employed in similar assays, such as using ganglioside-incorporating liposomes for the detection of cholera toxin subunit B [26].

The often-low concentration of virions in water samples can be a challenge [11]. Addressing this, herein described is the use of a microfluidic device combining pre-concentration and fluorescent detection, previously described [27], to detect FCV. As shown in Fig. 1.a, pre-concentration of the virus particles can be achieved by first allowing liposomes tagged with specific anti-FCV antibodies to bind, and then actuating the complexes toward a nanoporous membrane via electro-

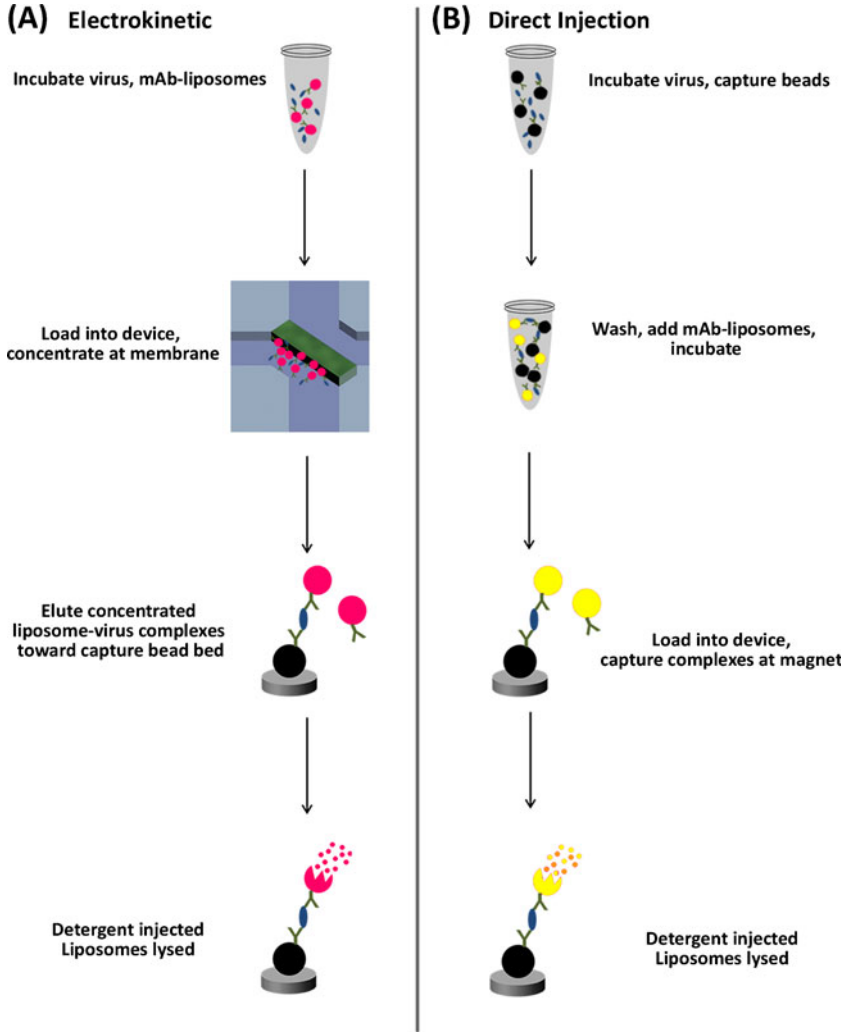

Fig. 1 Schematic of assays with and without pre-concentration. The assay employing electrokinetic pre-concentration (A) begins with loading the device with anti-FCV pAb-labeled Protein A superparamagnetic beads to create a capture bed and incubating the anti-FCV mAb-labeled fluorescent liposomes with FCV. The sample is then loaded into the inlet well, concentrated at the nanoporous membrane, and eluted toward the capture bead bed. Following washing, detergent is injected to lyse the liposomes, releasing the fluorescent dye for quantification. The assay without pre-concentration (B) begins with incubating an FCV sample with the same capture beads as before. The virus–bead complexes are washed and incubated with electrochemical liposomes. This sample is pulled into the microfluidic channel where the detection complexes are captured at a magnet and washed, and the bound liposomes are lysed with detergent. This releases the electroactive species, which undergoes redox cycling at a downstream IDUA

kinesis [27, 28]. These complexes can then be eluted from the membrane as a bolus and applied to a downstream capture and detection zone, where the non-specifically bound liposomes may be washed away prior to lysis and signal quantification. This was compared with an optimized microfluidic electrochemical detection assay, outlined in Fig. 1.b, in which all incubation steps are conducted off-chip, in suspension.

Materials and methods

FCV purification and titration

The F9 vaccine strain of FCV (ATCC; VR-782) was propagated on Crandell–Reese feline kidney cells (ATCC; CCL-94). Viral stocks were prepared from twice plaque-purified viruses. Purified FCV-F9 was prepared and titrated as previously described [29, 30], by extraction from cell lysates using trichlorotrifluoroethane followed by banding of virus on CsCl gradients (1.30–1.45 g/mL). Purified virus was dialysed into 150 mM NaCl, 10 mM Tris base, 15 mM MgCl2, pH 7.2 then stored at 4 °C prior to use.

Biotinylation of antibodies

Biotin was conjugated to antibodies using the EZ-Link® NHS-PEG_4-Biotin kit and purified using the Slide-A-Lyzer® mini-dialysis kit (Pierce Rockford, IL). Briefly, 100 μL of 1 mg/mL antibodies were added to the Slide-A-Lyzer tubes and dialyzed against 1× PBS, pH 7.0, to exchange the buffer and assure appropriate pH. Biotin was then added at more than a 20-M excess to assure good conjugation at the relatively low antibody concentration, and the samples were incubated for 30 min at room temperature. The samples were again dialyzed against 1× PBS, pH 7.0, in order to remove the excess biotin. Samples were collected out of the dialysis tubes and stored in the refrigerator.

Preparation of capture beads

Polyclonal anti-FCV antibodies (Baker Institute, Ithaca, NY) were purified from rabbit serum with a HiTrap Protein A HP column (GE Healthcare Uppsala, SE) as per manufacturer suggestions. Once purified, polyclonal antibodies were then conjugated to Protein A magnetic beads from Dynabeads Immunoprecipitation kit (Invitrogen, Carlsbad, CA) as per manufacturer provided instructions.

Preparation of streptavidin-conjugated liposomes

Fluorescent streptavidin-conjugated liposomes were prepared via the reverse-phase evaporation method with 150 mM sulforhodamine B (SRB), 20 mM HEPES, pH 7.5, as the encapsulant, as previously described [31] with modification. To allow for visualization of the liposomes during the concentration procedure, a fluorophore-labeled lipid (Avanti Polar Lipids Alabaster, AL), 0.33 mol% 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl), was added to the initial lipid mixture. Liposomes coupled to streptavidin were incubated for 15 min at room temperature with 1 μg anti-FCV monoclonal antibody (Abcam Cambridge, MA), biotinylated as above. The liposome–antibody conjugate was then diluted to a working phospholipid concentration of 0.7 mM.

Liposomes with the same bilayer composition and streptavidin-modification were also prepared with an encapsulant of potassium ferri/ferrohexacyanide with a combined concentration of 200 mM for experiments using amperometric detection. These liposomes were prepared in 1× HEPES–saline–sucrose (1× HSS), containing 10 mM HEPES, 200 mM NaCl, and 200 mM sucrose, pH 7.5, but the liposomes were then dialyzed against 1× PBS, 20 mM sucrose, pH 7.5, as HEPES has been shown to interfere with electrochemistry [32, 33].

Microtiter plate liposome immunoassay for antibody selection

Previously reported protocols for the use of liposomes in microtiter plates [34] were adapted and modified for virus detection. High-binding Nunc Maxisorb® polystyrene plates were prepared for a liposome immunoassay (LIA) by washing each well with 200 μL of 1× PBS. Anti-FCV antibodies were diluted with 1× PBS to 5 μg/mL and 200 μL was added to each well. The plates were then incubated overnight in the refrigerator. After incubation, wells were emptied, tapped dry, and washed with 200 μL of 1× PBS. Wells were blocked for 1 h at room temperature with 200 μL of blocking reagents containing either 0.05% Tween-20 or 0.1% Tween-20 in 1× PBS. Plates were then emptied, dried, and washed twice with 200 μL per well of 1× PBS.

Prepared plates were then loaded with 100 μL per well of varying concentrations of FCV in 1× PBS in triplicate and incubated for 2 h in the refrigerator with gentle shaking. Wells were tapped dry and washed twice with 200 μL of 1× PBS. Biotinylated anti-FCV antibodies were diluted in 1× PBS to a concentration of 1 μg/mL, and 100 μL of solution was added to each well. Plates were incubated for 1 h at room temperature with gentle shaking.

The plates were washed twice in 200 μL per well of 1× HSS. Streptavidin-conjugated liposomes diluted to 50 μM phospholipids concentration and 100 μL were added to each well. Plates were again incubated for 1 h at room temperature with gentle shaking.

Plates were emptied, dried, and washed three times with 200 μL per well 1× HSS, respectively. For measuring the fluorescence emission at 590 nm, 50 μL of 30 mM octyl-β-D-glucopyranoside (OG) was added to each well.

Concentration and detection of FCV

Concentration and detection of FCV were carried out in a device integrating electrokinetic concentration at a nanoporous membrane with downstream liposome-based fluorescent detection, fabricated as previously reported [27]. Prior to performing concentration and detection experiments, the channels of the device, shown in Fig. 2, were primed with 1× HSS. A permanent magnet was positioned

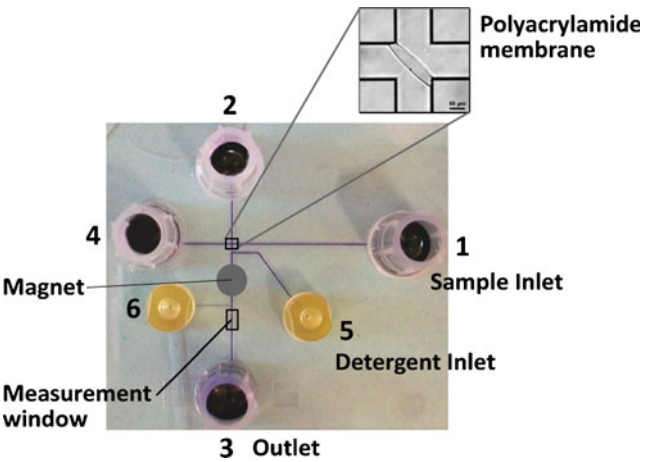

Fig. 2 Combined concentration and detection device. After the channels are filled with 1× HSS and the capture bead bed is packed at the magnet, a virus–liposome solution is introduced to port 1 and a potential is applied across the membrane (*inset*). Once concentrated, the virus–liposome bolus is eluted from the membrane by switching the potential to port 3, downstream of the magnet. Once the sample is captured, non-specifically bound liposomes are washed away by wash buffer, applied via port 5 using pressure-driven flow. Liposomes are then lysed using a detergent introduced through the same port. (Note: device filled with visible dye for illustrative purposes)

on the top surface of the device upstream of the detection region by use of adhesive putty. One microliter of polyclonal antibody-conjugated superparamagnetic beads was injected towards the magnet through port 5 using a syringe pump at a flow rate of 1 μL/min. The packed bead bed at the magnet constitutes the capture region of the device. The liposome–antibody conjugate was then mixed with FCV of the required concentration and incubated for 2 h. This virus–liposome solution was loaded into the inlet well of the device whereas all the other wells were filled with 1× HSS. The pressure-driven flow in the system was eliminated by adjusting the heights of the solutions in the wells. The virus–liposome complexes were then electrokinetically concentrated at the membrane by applying a voltage difference of 150 V across the membrane. After concentrating for 90 s, the concentrated bolus was eluted towards the bead bed by applying a voltage of 150 V to the outlet port 3 downstream of the magnet. This results in the capture of the virus–liposome complexes at the bead bed, as illustrated in Fig. 1. Wash buffer was injected at a flow rate of 20 μL/h to wash off any unbound liposomes in the device through port 5. A detergent solution of 60 mM OG was then introduced through the same port towards the bead bed at a flow rate of 40 μL/h and the emitted fluorescence from the lysis of the bound liposomes was recorded downstream of the bead bed. Video was captured during lysis, and the fluorescent intensity was integrated over time to yield the final signal.

Detection of FCV without electrokinetic concentration

To show the effect of pre-concentration on detection of FCV, the assay was also carried out in a microfluidic device outfitted with an interdigitated ultramicroelectrode array (IDUA) fabricated on polymethylmethacrylate (PMMA), shown in Fig. 3, as previously described [35]. The assay without electrokinetic concentration is similar to the procedure outlined above with several modifications required for the electrochemical transducer and pressure-driven flow. Streptavidin-conjugated liposomes encapsulating the ferri/ferrohexacyanide redox couple were substituted for those encapsulating SRB. To provide the most pertinent comparison, the procedure used was that which proved optimal for the device. Polyclonal antibody-conjugated Protein A superparamagnetic beads were prepared as described and 5 μL was mixed with 70 μL of FCV in Dulbecco's modified Eagle's media (DMEM) with 10% fetal bovine serum (FBS) and incubated, gently shaking at room temperature for 10 min. The sample was then applied to a magnet, separating the beads such that they could be washed twice with 75 μL 1× PBS, once with 75 μL 1× PBS with 0.2 M Sucrose (1× PBSS), and finally resuspended in 5 μL 1× PBSS. To this sample, 5 μL monoclonal antibody-coupled electrochemical liposomes were added and incubated, gently shaking at room temperature for 10 min. This sample was then pulled into the device, captured at the magnet, and washed with 20 μL 1× PBS at 5 μL/min to remove any unbound liposomes. Liposomes were then lysed, releasing the electroactive species to produce a signal, by the injection of 30 μM OG at 1 μL/min until the signal returned to baseline. Potential was applied and signals recorded using an Epsilon Electrochemical Analyzer (BASi, West Lafayette, IN) as previously described [26]. Though using a different signal transduction method, previous work has shown detection limits on the same order of magnitude for fluorescent and electrochemical transduction [26, 36].

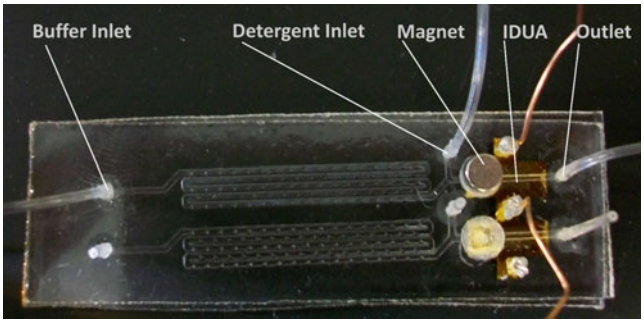

Fig. 3 Device without pre-concentration module. The device, fabricated in PMMA, has two microfluidic channels and IDUAs side-by-side for two separate samples. The sample is pulled into the device through the outlet via negative pressure on the buffer inlet, allowing for the capture of the detection complexes at the magnet. The sample is then washed by buffer flow actuated via the buffer inlet and bound liposomes are lysed with the introduction of OG through the detergent inlet. Signals are obtained by applying a potential across the IDUA and recording the current resulting from the oxidation–reduction cycling of the electroactive encapsulant

Results and discussion

For the development of the integrated device for pre-concentration and detection of viruses, a standard immunoassay using liposome amplification was initially developed using a microtiter plate format. This was subsequently transformed to capture antibodies immobilized on superparamagnetic beads and implemented with fluorescent liposomes in the integrated device. Secondly, the assay was adapted to a microfluidic electrochemical biosensor using electrochemical liposomes as these have been found to be as sensitive as fluorescent liposomes [36], or slightly more sensitive [26], and enable the development of portable and rapid microfluidic biosensors requiring little hardware [37].

Selection of antibodies and assay optimization

A series of commercially available and custom antibodies were screened via the microtiter plate LIA described and a sample of highly purified FCV in PBS. It was found that many antibody pairs would not result in effective capture

and detection of FCV. Some pairs generated highly reproducible results and representative data of two combinations are shown in Fig. 4; here, antibody pairs employing the polyclonal antibody as capture antibody generated high signals and signal-to-noise rations (SNR). Based on all combinations tried, it was determined that using a custom polyclonal rabbit-derived anti-FCV for capture was best in conjunction with the monoclonal labeled mAb1 (Abcam clone number FCV1-43) as it yielded an SNR just under 9 for a concentration of 5,000 ng/mL.

Further optimization of the assay employed FCV in lysed cell culture medium, containing DMEM with 10% FBS, and focused on blocking to reduce non-specific binding. This was done as purification of the virus, which is time consuming, and the cell debris and additional proteins provide a more challenging sample matrix than pure buffer. Further, the used of lysed cell culture allows for more direct comparison to standard methods described in the literature as it provides concentrations as PFU/mL, which is more commonly used. A dose–response curve was developed for the microtiter LIA for future comparison to microfluidic devices, as shown in Fig. 5. Here, the limit of detection is approximately 4×10^4 PFU/mL.

Comparison of FCV detection with and without electrokinetic concentration

FCV detection experiments were performed to show the improvement in detection sensitivity with the inclusion of the electrokinetic pre-concentration step. In the first set of experiments, FCV was detected by use of the integrated microfluidic device that includes the pre-concentration step. Fluorescent liposomes were used in these experiments and

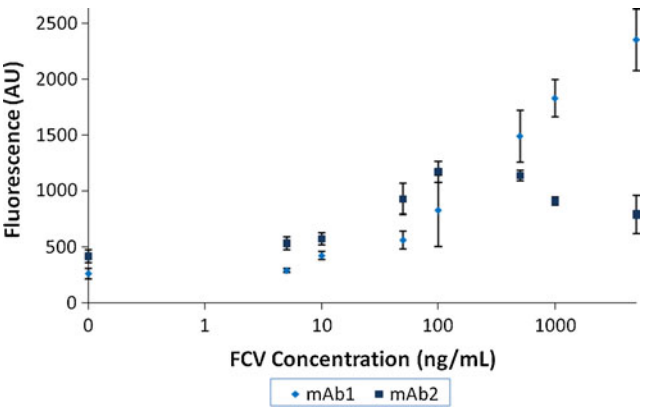

Fig. 4 Dose–response curves for polyclonal capture antibody with monoclonal reporter antibodies. The best antibody pair for the detection of FCV was determined by screening all variations in a microtiter plate liposome immunoassay (LIA). Here a custom polyclonal anti-FCV was immobilized to the plate and biotinylated anti-FCV monoclonal antibodies and streptavidin-conjugated fluorescent liposomes were used for signal generation

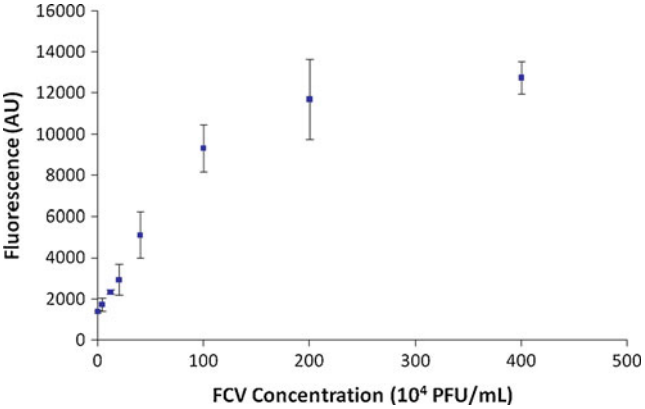

Fig. 5 Optimized assay for FCV detection in cell culture lysate. Using the previously optimized antibody pairs, FCV was detected in cell culture lysate consisting of DMEM and 10% FBS in a microtiter plate LIA

the fluorescence intensity signal from the lysis of the captured liposomes was estimated using image processing. These experiments were done for different concentrations of FCV ranging from 0 to 6.0×10^5 PFU/mL. The limit of detection for these experiments performed with the integrated device was estimated, from the data shown in Fig. 6, to be 1.6×10^5 PFU/mL.

Electrokinetic concentration requires the application of high voltage to the sample. As the buffer required for the stability of the liposomes is highly conductive, owing to the salt and sucrose concentrations, the sample is susceptible to resistive heating. To avoid this, the potential was applied across the membrane to allow the virus–liposome com-

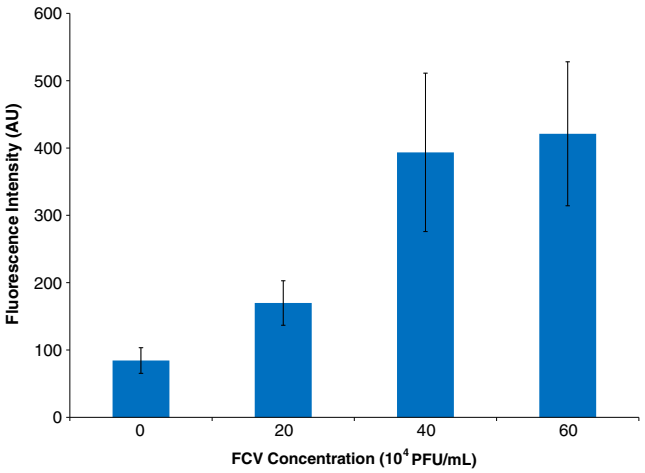

Fig. 6 FCV detection after electrokinetic concentration. FCV samples were incubated with anti-FCV-coupled liposomes for 2 h at the indicated concentrations. Samples were then concentrated for 90 s by application of a potential across a nanoporous membrane and then eluted to the capture bead bed. After washing, liposomes were lysed with detergent and the fluorescence intensity downstream was integrated over time

plexes to concentrate for 90 s. This, by no means, depletes the 60 μL sample of virus–liposome complexes, but does allow for detection.

A second set of experiments were performed excluding the pre-concentration step by directly injecting the virus–liposome–bead detection complexes towards the magnet. Electrochemical liposomes were used in these experiments and the integrated current signal from the lysis of the captured liposomes is plotted as a function of the concentration of FCV as shown in Fig. 7. The limit of detection was estimated as 3.2×10^6 PFU/mL, as it is more than 3 standard deviations above the negative control.

There is an order of magnitude improvement in the limit of detection with the integrated microfluidic device over the direct injection system as the increased concentration of the analytes improves the antibody–antigen binding kinetics in the detection region [38]. This improvement is more remarkable as the direct injection device employed an optimized procedure allowing the magnetic beads, virus and liposomes to incubate, sequentially, off-chip in solution. This dispersion of assay components in solution decreases diffusion distances and takes full advantage of the bead surface area, providing ample antibody–antigen interaction [39]. Future planned experiments will include the addition of electrochemical detection into the integrated device in order to render it more field portable. The equipment needed for electrochemical detection is relatively inexpensive, portable and can provide quantitative signal readout making it better suited for on-site detection than the currently employed fluorescence-based detection described here [40].

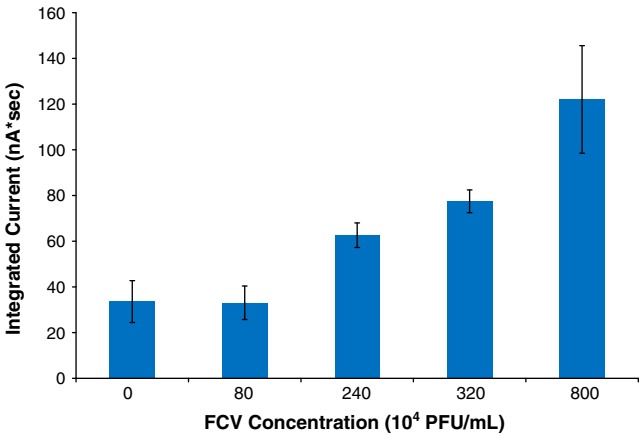

Fig. 7 FCV detection without pre-concentration. FCV samples were incubated with anti-FCV-coupled magnetic beads and anti-FCV-coupled liposomes for a total of 20 min, with wash steps, at the indicated concentrations. Samples were then injected into a microfluidic channel toward a magnet, where the detection complexes were captured and washed. Bound liposomes were lysed with detergent and the current measured across the IDUA resulting from the released electroactive species was integrated over time

Current literature reports limits of detection of FCV on the order of 10^6 particles/mL using SERS [41]. Concentration is not easily converted from virus particles/mL to PFU/mL as they require the estimation of the infectivity, which is dependent not just on the particular strain but also the cell culture media and cell line. Based on an approximate ratio of infectious to non-infectious particles for enteric viruses in general [42], this corresponds to a limit of detection on the order of 10^4 PFU/mL, which is comparable, though lower, than that reported herein. Although there have been strides in the miniaturization of SERS instruments, the equipment costs approximately $15,000 [43] and is best suited to laboratory analysis, particularly due to the approximately 20 h of incubation time required for the assay. Instead, the reported device, adapted for electrochemical detection, will be well suited to field portable assays due to its significantly shorter assay time of only 2.5 h, small size, and comparable sensitivity.

Most of the portable microfluidic biosensors for enteric virus detection reported in the literature are based on RT-PCR techniques [44–46]. PCR is highly susceptible to inhibitors, primarily humic acid, that are present in environmental water samples, which can reduce sensitivity or completely inhibit the signal [42]. Further, microfluidic PCR systems also face the challenges of adsorption of enzymes to channel walls [47], difficulty in precisely controlling temperature, sample evaporation, and formation of bubbles in the channels [48]. The advantage of our integrated microfluidic device is that it has on-chip detection times on the order of a few minutes and does not involve any temperature cycling issues.

Conclusion

Using a device integrating a liposome immunoassay with an upstream pre-concentration of the virus–liposome complexes, we have shown a limit of detection of 1.6×10^5 PFU/mL for FCV. This detection limit is an order of magnitude lower than that obtained with an optimized detection device that does not include pre-concentration. The here described system can be extended to electrochemical detection by patterning gold electrodes in the device and using electrochemical liposomes similar to those used in the described PMMA device. Electrochemical detection is inexpensive and portable with quantitative signal readout.

In addition, the current protocol described uses a concentration time of 90 s to avoid resistive heating. As this does not deplete the sample of virus–liposome complexes, in the future, we intend to avoid this problem by using several short pulses, sending several boluses of highly concentrated virus–liposome complexes to the capture bead bed. As significantly

more liposome–virus complexes would end up concentrated on the membrane, we predict that this would result in a limit of detection decreased at least by an order of magnitude.

Acknowledgments This publication was made possible by USEPA cooperative agreement #CR-83360101. Its contents are solely the responsibility of the recipient and do not necessarily represent the official views of the USEPA. Further, USEPA does not endorse the purchase of any commercial products or services mentioned in the publication.

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