Rapid Specific and Label-Free Detection of Porcine Rotavirus Using Photonic Crystal Biosensors

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Abstract—A rapid, label-free assay using photonic crystal biosensors for the direct detection of intact viable rotaviruses is demonstrated. This paper describes the design and operation of the biosensor, surface functionalization with anti-rotavirus antibody, characterization of the detection sensitivity, and demonstration of specificity with respect to non-target virus particles. A 30-min assay of a partially processed water sample yielded a detection sensitivity of 36 virus focus forming units without the use of any external reagents. This sensitivity is comparable to commercial enzyme-linked immunosorbent assays. These results suggest photonic crystal biosensors may be useful for real-time monitoring of virus contamination of environmental water resources.

Index Terms—Ground water monitoring, label-free detection, photonic crystal biosensor, rotavirus.

I. INTRODUCTION

RAPID DETECTION of pathogen contamination of a water resource, particularly virus pathogens, in a manner that allows pathogen-specific detection, would be of great benefit in determining and managing the risks of waterborne disease transmission. While numerous sensitive and pathogen-specific molecular detection methods, such as polymerase chain reaction (PCR) and enzyme linked immunosorbent assay (ELISA), have been developed and applied to environmental samples including water, they require multiple processing steps and considerable time to perform. ELISA protocols require secondary detection antibodies (Ab) conjugated with an enzyme label that reacts with a chromogenic/fluorogenic substrate to produce a colorimetric/fluorescent readout. Though ELISA assays provide high sensitivity, the numerous steps required for label application are not only labor intensive but also have the potential, especially with fluorometric assays, to create

quenching interactions among the multiple reagents [1], as well as the potential inability to distinguish intact virions from soluble virus antigens. In this paper, we explore the use of label-free optical detection using a photonic crystal biosensor technology that is rapid, pathogen-specific, and does not require chemical modification of the test sample.

Of the label-free methods that may be used to directly detect pathogens, optical biosensors provide a useful combination of high sensitivity, low cost, high throughput, and ease of use. In general, optical biosensors are designed to produce a measurable change in some characteristic of light that is coupled to the sensor surface. Rather than detecting mass directly, all optical biosensors rely on the dielectric permittivity of detected substances to produce a measurable signal [2]. The advantage of this approach is that a direct physical connection between the excitation source, the detection instrument, and the transducer surface itself is not required, thus circumventing the need for electrical connections to the transducer that must be kept separated from liquid sample media. Publications in recent years have described the application of optical biosensor methods like Surface Plasmon Resonance (SPR) and acoustic label-free technologies such as Quartz Crystal Microbalance (QCM) for detection of intact virus particles. The functionality of label-free virus detection has been demonstrated with SPR and QCM for herpes, dengue, and influenza [3]–[6]. Though both technologies offer the advantages of label-free biosensing and have high sensitivity, they are limited by a combination of factors that include sensor cost, instrumentation complexity, low assay multiplexing throughput, and lack of incorporation of multiple positive/negative controls to reduce the rate of false diagnostics. In clinical and laboratory settings, ELISA, PCR, and culture methods are still widely used, with ELISA and PCR being primary choices. Yet aside from ELISA, the use of other methods are discouraged by the World Health Organization for surveillance of pathogens like rotavirus due to their labor and resource-intensive nature, which require more highly trained personnel[7].

In previous work, we have described a label-free optical biosensor system based on an approach that utilizes a photonic crystal manufactured from continuous sheets of plastic film and incorporated onto standard 96-, 384-, and 1536-well microplates [8]. The photonic crystal surface is comprised of a 1-D periodic grating surface structure formed by nanoreplica molding in a low refractive index polymer that is coated with a high refractive index film of TiO_2. The device is produced upon a flexible plastic substrate and bonded with adhesive to plastic microplates, so that the photonic crystal surface comprises the bottom surface of each well. The fabrication is inexpensive and the device may be disposed after a single
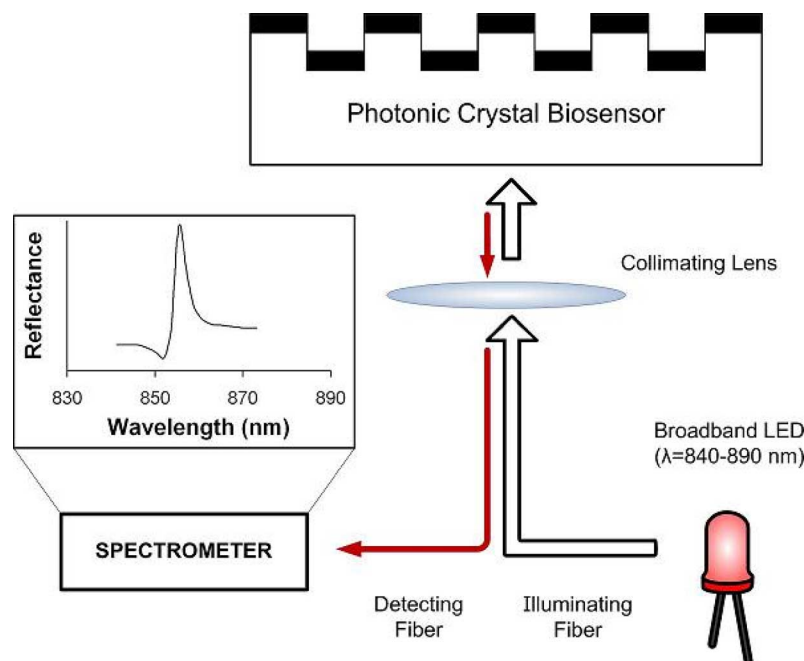
Fig. 1.   Schematic diagram of photonic crystal biosensor structure and operating principal of detection instrument.

use. We also have demonstrated rugged, compact detection instrumentation for the photonic crystal biosensor using either a miniature spectrometer or a tunable Vertical Cavity Surface Emitting Laser (VCSEL) as the source of sensor illumination [9], [10]. Light incident the photonic crystal from a $90°$ angle is strongly reflected at a single wavelength–the Peak Wavelength Value (PWV)–at which an optical resonant reflection occurs. The PWV of the resonant coupling condition is strongly modulated by the dielectric permittivity of any material that is in direct contact with the photonic crystal surface. As a result, the density of adsorbed biolayers, such as proteins, DNA, small molecules, cells or virus particles, can be quantified by measuring positive shifts in PWV.

Group A rotaviruses are the major cause of virus diarrhea in the young of most mammalian and avian species [11]–[15]. In humans, rotavirus kills over $600\,000$ children worldwide every year, and infects nearly all children before the age of 5 [16]. In agriculture, a widespread attempt to curb mortality of livestock through various vaccination programs has not been successful [12]. The predominant route of transmission for rotaviruses is fecal-oral, and fecally polluted water has been implicated as a possible source of rotavirus disease, as well as other incidences of virus gastroenteritis (1, 6, 24). Enteric viruses are excreted in large number in the feces of infected hosts, and may be dispersed in environmental waters including private wells and municipal water supplies (6, 24). The stability of rotaviruses in environmental water and their resistance to water treatment may facilitate transmission; outbreaks of gastroenteritis caused by waterborne rotaviruses have been reported, and the presence of rotaviruses have been found in various sources of water such as sewage, ground water, irrigation water, and even drinking water [17]–[25]. It is possible that early detection of water systems contamination could prevent further spread of the virus

and/or alert for proper treatment of infected individuals. In this work, we demonstrate the viability of the use of photonic crystal biosensors for the detection of whole virus particles with detection limits comparable to commercially available ELISA.

II. MATERIALS AND METHODS

A. Biosensors and Instrumentation

As previously described [10], the photonic crystal biosensor surface is comprised of a linear grating surface structure that is replica molded from a polymer material with a period of ~ 550 nm and a grating depth of ~ 120 nm. The polymer grating structure is coated with ~ 80 nm of TiO_2 high refractive index dielectric coating by a sputtering process. Both the grating molding and the TiO_2 coating are performed upon continuous rolls of flexible polyester film. Individual sensor coupons ($\sim 3 \times 5$ in^2) are cut from the film and attached with adhesive to bottomless microplate frames. With the biosensor surface exposed to water, a resonant reflection peak is produced at a wavelength of ~ 855 nm, and a peak width (half maximum) of ~ 2.5 nm.

The photonic crystal biosensor detection instrument employs paired bundled optical fibers that illuminate and collect light at normal incidence from the bottom of the sensor's surface. Light reflected from the ~ 2 mm diameter region of the each microplate well bottom is measured by a spectrometer, and shifts in the reflected PWV are determined by software (Fig. 1). An automated stage allows for parallel collection from eight sensor wells at timed intervals; a 384-well microplate may be measured in less than 2 min. A complete description of the detection instrument has been previously published by Cunningham *et al.* [8], [10].
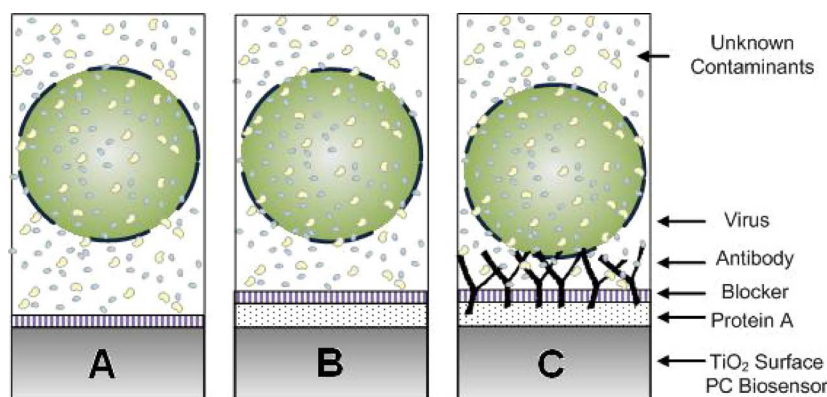
Fig. 2. Illustration of virus detection on the PC biosensor. (A-B) Reference wells measure NSB of virus and other compounds in solution. (C) Active well determines affinity of analyte to detection Ab.

B. Porcine Rotavirus Preparation

Group A Porcine rotavirus OSU strain (P9 [7], G5) obtained from the American Type Culture Collection catalog # VR-892 was passaged two additional times in Ma104 cells; an infectious rotavirus stock was harvested from the culture fluid of Ma104 cells and infectious units were determined in a focus forming assay (FFU), as described in [12]. Three colostrum deprived newborn piglets were inoculated with 1 mL (3×10^6 FFU), each of the cell culture rotavirus stock and the rotavirus infected feces were pooled and collected over a two day period. All subsequent operations were done at 4 °C. The combined fecal material was homogenized at 4 °C with a Dounce homogenizer with an equal volume of Vertrel XF cleaning agent (Miller–Stephenson) to facilitate rotavirus separation from fecal debris. After centrifugation to separate layers, the aqueous top layer was centrifuged at $13\,000 \times$g to pellet debris. The supernatant was centrifuged at $180\,000 \times$g pelleting the rotavirus. The pellet containing the rotavirus was suspended in TNC buffer and diluted to 1.154×10^5 FFUml^{-1}.

C. Feline Calici- and Parvoviruses

Feline calicivirus tissue culture stock (2×10^7 TCID$_{50}$ per ml, approximately equal to 2×10^{10} particles ml^{-1}) and feline parvovirus tissue culture stock (256–512 HA units ml^{-1} estimated to be 0.6–1.2×10^{10} particles ml^{-1}) were gifts from Dr. Gail Scherba from the Department of Pathobiology College of Veterinary Medicine, University of Illinois at Urbana–Champaign.

D. Preparation of Polyclonal Anti-Rotavirus Antibody

Pure triple layered virus particles (TLP's) were obtained by further purification of the 48 kDa rotavirus pellet by cesium chloride density centrifugation [26]. The purified TLP's (416 μg of virus protein, 4×10^8 FFU) were pressure treated in a high-pressure apparatus, as described in Pontes [27]. Immunization of a New Zealand white rabbit with 30 μg pressure treated TLP (without adjuvant) was performed. Twenty days later, a second immunization was done followed by collection of rabbit serum 28 days later. The antiserum was treated with dextran sulfate and calcium chloride to precipitate serum lipoproteins [28]. The soluble gamma-globulin fractions were precipitated with 50% saturated ammonium sulfate at 4 °C. After centrifugation and dissolution of the precipitate in 0.1 M potassium phosphate buffer (PBS) pH 8.0, the soluble fraction was precipitated in 40% saturated ammonium sulfate and the final precipitate was dissolved in phosphate buffer and applied to an 80×2 cm Ultragel AcA 34 molecular sieve column (Sigma Chemical Company) equilibrated in pH 8.0 phosphate buffer. The column had been previously calibrated with purified rabbit IgM, IgG and serum albumin as molecular weight markers. The IgG fraction was collected; the protein determined to be 2.1 mg ml^{-1} by absorbance at 278 nm, and anti-rotavirus activity was determined by ELISA to have a titer of 0.078 μg ml^{-1}, as described in [29].

E. Commercial ELISA

An enzyme linked immunosorbent assay specific for detection of rotavirus group A antigen was performed on the porcine fecal rotavirus preparation using a commercial kit (IDEIA, Rotavirus K6020) from DakoCytomation, according to the manufacture's instructions.

F. Surface Functionalization and Rotavirus Detection

A schematic of the surface functionalization of "active" and "reference" biosensor wells is shown in Fig. 2. A 384-well surface photonic crystal biosensor microplate (SRU Biosystems) was functionalized by the following procedure: 12 μL of 5 mg ml^{-1} Protein A (Sigma-Aldrich) in PBS pH 7.0 incubated on the TiO$_2$ sensor surface for 4 h, followed by washing of wells three times with an equal volume of PBS. "Active" wells were prepared by incubating 20 μL of target antibody (anti-rotavirus IgG) solution on the immobilized Protein A layer for 12 h at 4 °C. Four blockers [Bovine Serum Albumin (BSA), sugar, Sea Block (Pierce Biotechnology), and Starting Block] have been previously tested to determine that Starting Block yielded minimum NSB on sensor surface (data not included in this paper). All wells were washed with PBS and then blocked with 20 μL of undiluted Starting Block (Pierce Biotechnology) to prevent nonspecific binding (NSB); a set of TiO$_2$–only wells without Protein A were also blocked to serve as secondary reference. The blocker was incubated in the microplate wells for 2 h at ambient temperature and the wells were washed and filled with 20 μL of TNC. The PWV shift induced by each stage of the
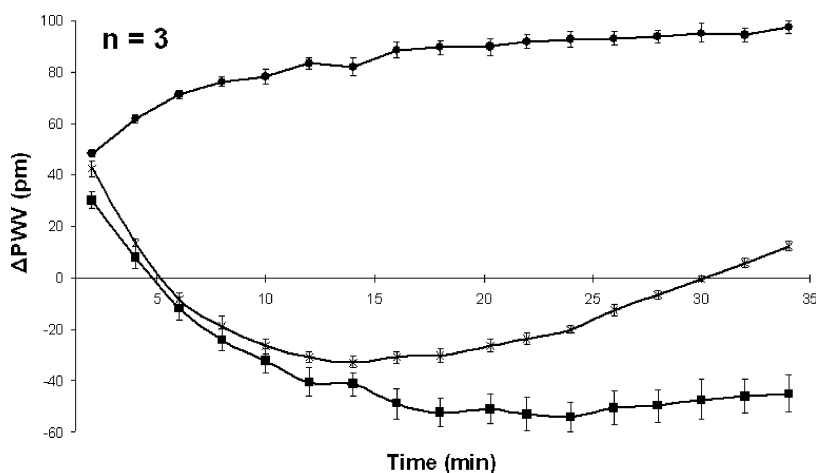
Fig. 3. Kinetic data for rotavirus detection on blocked wells with anti-rotavirus IgG ($\bullet$), Protein A ($\blacksquare$) and TiO_2 ($*$). Decrease in ΔPWV on Protein A and TiO_2 indicate detachment of starting block; no decrease is seen on Ab wells due to simultaneous binding of particles. Increase in PWV on TiO_2 wells indicates NSB of unknown contaminants to the surface; a smaller degree of NSB is observed on other wells.

biosensor preparation process was measured using the detection instrument described above.

G. Rotavirus Sensitivity Detection Assay

The porcine rotavirus was half-diluted with PBS into nine concentrations ranging from 5.77 to 0.02×10^4 FFU ml^{-1} in a clear 384-well plate (Falcon); photonic crystal biosensor wells were functionalized as described above with 0.2 mg ml^{-1} anti-rotavirus IgG. 20 μL of virus solutions were transferred to biosensor microplate. Measurements of ΔPWV due to adsorption of intact virus particles were performed on triplicate active wells, anti-rotavirus IgG, and two types of reference wells: Protein A-coated and the uncoated TiO_2 surface. This assay is to establish a relationship between PWV shift and virus concentration that can be used to create a standard curve, and to determine the limit of detection of the biosensor on the virus.

H. Anti-Rotavirus Antibody Specificity Detection Assay

Several biosensor microplate wells were functionalized as described above with 0.2 mg ml^{-1} of five different target IgGs derived from: human, goat, sheep (Sigma-Aldrich), along with anti-rotavirus and fractions of light and heavy chains of goat anti-mouse (Cappel Laboratories). 20 μl of partially purified rotavirus (5.77×10^4 FFU ml^{-1}) were added in triplicate wells to the biosensor plate. Two nontarget viruses, feline calicivirus, and feline parvovirus, were exposed to the biosensor at high concentrations, 1×10^{10} and 0.6×10^{10} particles ml^{-1}, respectively, on blocked wells with anti-rotavirus IgG or Protein A.

I. Scanning Electron Microscopy (SEM) of Sensor Surface

Excess buffer from each well was removed and the surface was blow-dried using Nitrogen gas. Both active and reference wells sensors were cut out from the plastic surface using a 1 mm^2 hole punch. The sensors were mounted and examined using a standard SEM instrument, where both active and reference surfaces were scanned for attachment of rotaviruses.

J. Animal Experiments

All animal procedures mentioned in this paper were part of animal care and use protocols approved by the University of Illinois Animal Care and Use Committee (IACUC).

III. RESULTS AND DISCUSSION

A. Sensitivity of Rotavirus Detection

The detection limits of the assay were tested by twofold series dilution of porcine rotavirus. Kinetic data gathered immediately after exposure of the virus sample to the biosensor indicated a combination of binding of virus particles and detachment of immobilized molecules from the surface. Virus solutions were exposed simultaneously to "active" and both types of "reference" surface layers to establish which component of the surface immobilized layer was being removed from the surface, as shown by a decrease in ΔPWV in Fig. 3. The "active" sensors were functionalized with anti-rotavirus IgG on the immobilized Protein A and blocked with Starting Block. One of the "reference" sensors was immobilized only with blocker on TiO_2 surface, and the other was immobilized with Protein A and blocker. The "reference" sensors were used to compensate any drift or noise signal, which was a downward drift in this assay. These results suggest that an unknown factor in the virus solution initiated a small degree of detachment of the Starting Block because both blocked TiO_2 and Protein A wells experienced an equivalent negative drift in ΔPWV after the introduction of the virus. After approximately 30 min, a net increase in ΔPWV of the blocked TiO_2 wells is recorded. The NSB was the highest on the blocked TiO_2 surface, suggesting Protein A and anti-rotavirus IgG layers acted as blockers. Since NSB was observed only in the three highest virus concentration and small negative drifts in ΔPWV was observed in all concentrations (data not shown), we hypothesized that NSB and detachment of blocker were caused by independent sources. Wells with immobilized antibody were referenced to the mean ΔPWV measurement from Protein A
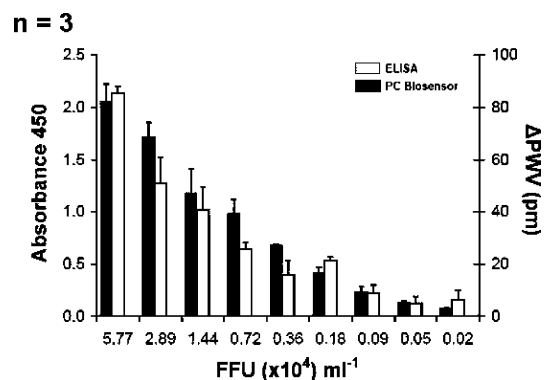
n = 3

Fig. 4. Twofold serial dilutions of group A porcine rotavirus measured on ELISA (filled bars) and PC biosensors (open bars). PC biosensor measurements were referenced to the ΔPWV of Protein A wells. Data are represented as $\mathrm{mean} \pm \mathrm{SEM}$, $n = 3$.

wells. Endpoint measurements were taken after ~ 20 min of exposure to virus, once the slope of ΔPWV in Protein A and anti-rotavirus IgG wells reduced to approximately zero. The increase in NSB of contaminants in reference wells at approximately 30 min of binding reduces the signal-to-noise ratio in higher concentrations of virus solution, thus limiting detection time shown in Fig. 4. Nevertheless, the label-free photonic crystal biosensor has a sensitivity limit comparable to the commercially available ELISA kit. Even without the use of amplification steps or labels, the assay can detect fewer than 36 FFU (20 μl of 0.18×10^4 FFU ml^{-1} virus solution in Fig. 4) demonstrating that photonic crystal biosensors have sufficient sensitivity to detect virus particles in partially purified water samples. The function of the photonic crystal biosensor is to detect the absolute amount of virus bound to the sensor surface, which is directly proportional to the virus concentration. The PWV shift signal has been shown to be proportional to analyte concentration, thus the virus infectious units (FFU) can be determined from a standard curve relating the PWV shift signal to amount of analyte bound or concentration on the biosensor. Virus titers or concentration are measured and reported as infectious units rather than absolute mass/volume. Infectious units can be related to the absolute number of virus particles, but vastly dependent on the virus. Therefore, in order to determine virus concentration from a given PWV shift, an initial standard curve was created using known varying virus concentration (Fig. 4).

B. Specificity of Detection

Having measured the dose-response characteristic of the biosensor for porcine rotavirus detection, we sought to test specificity of detection in two ways. First, we demonstrate that the immobilized anti-rotavirus IgG will not bind nonspecific proteins by comparing the specificity of rabbit anti-rotavirus IgG against IgGs from human, sheep, goat and fraction of light and heavy chains of goat anti-mouse. Second, we compared the affinity of anti-rotavirus IgG to porcine rotavirus and two nonspecific virus samples of feline calici- and parvoviruses, exposed at a concentration $\sim 10^8 \times$ higher than the porcine rotavirus detection limit.

A biosensor microplate was functionalized with equal concentrations of antibodies as described previously. Though the
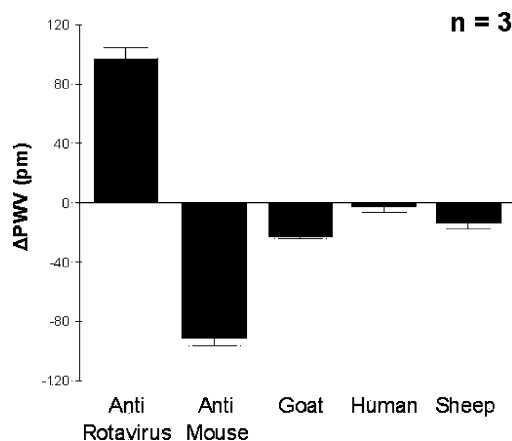
n = 3

Fig. 5. Specificity of various Ab against group A porcine rotavirus. All wells were referenced to measurements from Protein A wells. Anti-rotavirus IgG was developed specifically against porcine rotavirus in rabbit, anti-mouse IgG was developed in goat; all other IgG were nonspecific. Data are represented as $\mathrm{mean} \pm \mathrm{SEM}$, $n = 3$.
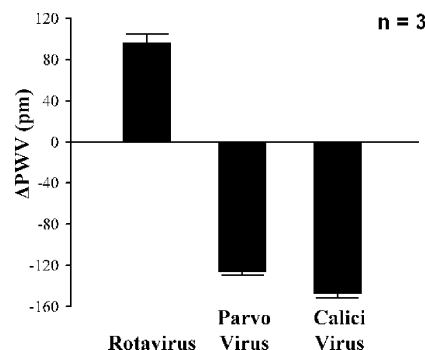

Fig. 6. Specificity of anti-rotavirus Ab against porcine group A rotavirus, feline calici and parvoviruses. All wells were referenced to measurements of respective viruses on Protein A wells. Data are represented as $\mathrm{mean} \pm \mathrm{SEM}$, $n = 3$.

affinity of IgGs to Protein A is not uniform throughout the species tested, randomized blocked ANOVA did not find a significant source of variation for virus detection. Endpoint measurements after 20 min of incubation (Fig. 5), demonstrate the specificity of the anti-rotavirus IgG. Measurements of rotavirus binding to anti-human, -sheep, and -goat IgG antibodies is comparable to the NSB on a Protein A layer; however, anti-mouse IgG coated wells showed no affinity towards the virus, as the NSB observed in Protein A control wells is higher than that measured on wells with anti-mouse IgG (resulting in a negative signal when referenced). In a similar manner, anti-rotavirus IgG failed to detect the presence of high concentration of feline calici- and parvoviruses. In both cases, the ΔPWV in Protein A reference wells was significantly greater than in wells with anti-rotavirus IgG (Fig. 6). The photonic crystal biosensor is able to detect individual target pathogen by prefunctionalizing the sensor with specific antibodies. It can be further expanded into a multiwell format, where various pathogen antibodies are immobilized into different wells in order to detect pathogens in an unknown contaminated sample.

Attachment of rotavirus on the sensor surface was confirmed by SEM imaging [30]. Individual and clusters of 70–90 nm diameter virus particles, typical of the morphology of triple-
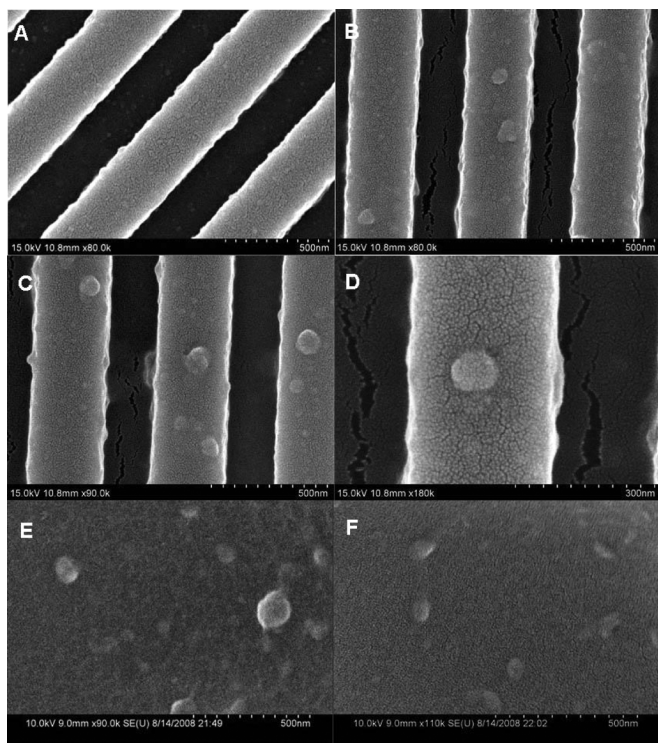
Fig. 7. SEM images of rotavirus attached to the photonic crystal sensor surface. (b) and (c) Individual and clusters of rotaviruses on multiple gratings of photonic crystal (80 and 180 k) and (a) the absence of rotavirus particles on the reference sensor surface (80 k). (d) A zoomed in image of a single rotavirus particle on sensor grating. SEM images of rotavirus attached to a plastic surface [90 and 110 k, (e) and (f), respectively].

shelled rotavirus virions [31], [32] were observed in the anti-rotavirus IgG coated wells. In contrast, attachment of rotavirus was not detected in the reference wells [Fig. 7(a)-(d)]. In order to confirm the SEM image obtained from the photonic crystal surface represented intact rotavirus, a SEM image of purified rotavirus absorbed to a plastic surface was taken as a reference image [Fig. 7(e)-(f)].

IV. CONCLUSION

We have demonstrated the use of photonic crystal biosensors for sensitive and specific detection of intact viruses in partially purified solutions; furthermore, incorporation of the biosensor onto standard size microplate wells allows for parallel quantification of contaminants, as well as screening for a range of viruses in an environmental water sample. Simplicity of the assay and technology would also permit detection in nonlaboratory settings without the use of additional label reagents, given the prior functionalization of the sensor. The current sensitivity of ~ 36 FFU is similar to that previously reported for environmental water samples (2–200 cytopathogenic units) using combined immunofluoroescence and flow cytometry [33]. We expect that detection limits may be further improved through the use of higher sensitivity versions of the photonic crystal sensor that have been published by our group [34]. With minimal sample processing, the use of photonic crystal biosensors for sensitive and specific detection of virus particles could provide the means for rapid detection of pathogens.

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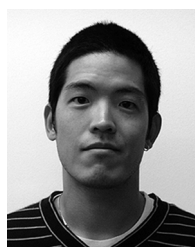
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