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SPRi-based adenovirus detection using a surrogate antibody method

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ABSTRACT

Adenovirus infection, which is a waterborne viral disease, is one of the most prevelant causes of human morbidity in the world. Thus, methods for rapid detection of this infectious virus in the environment are urgently needed for public health protection. In this study, we developed a rapid, real-time, sensitive, and label-free SPRi-based biosensor for rapid, sensitive and highly selective detection of adenoviruses. The sensing protocol consists of mixing the sample containing adenovirus with a predetermined concentration of adenovirus antibody. The mixture was filtered to remove the free antibodies from the sample. A secondary antibody, which was specific to the adenovirus antibody, was immobilized onto the SPRi chip surface covalently and the filtrate was flowed over the sensor surface. When the free adenovirus antibodies bound to the surface-immobilized secondary antibodies, we observed this binding via changes in reflectivity. In this approach, a higher amount of adenoviruses resulted in fewer free adenovirus antibodies and thus smaller reflectivity changes. A dose–response curve was generated, and the linear detection range was determined to be from 10 PFU/mL to 5000 PFU/mL with an R^2 value greater than 0.9. The results also showed that the developed biosensing system had a high specificity towards adenovirus (less than 20% signal change when tested in a sample matrix containing rotavirus and lentivirus).

1. Introduction

The rapid identification of the presence of a virus in contaminated food or water, or in a patient sample, is a prerequisite to efficiently counteract potential epidemics and bioterrorism (Bally et al., 2011). Human adenoviruses (HAdVs) cause epidemic, endemic, and sporadic infections worldwide, and can infect and replicate in the respiratory tract, gastrointestinal tract, eyes, bladder, and liver (Uhnoo et al., 1990; Desselberger and Gray, 2003). Adenoviruses are prevalent in rivers, coastal waters, swimming pool waters, and drinking water supplies worldwide. The United States Environmental Protection Agency (EPA) lists adenovirus as one of the nine microorganisms on the Contamination Candidate List for drinking water, because its ability to survive water treatment protocols is not yet fully understood (Kundu et al., 2013). The World Health Organization (WHO) reports that 1.8 million people die each year from diarrheal diseases, of which 90% are children under the age of five. Over 88% of diarrheal diseases are caused by waterborne or water-related viruses (Pond, 2005).

The detection of viral pathogens, such as adenoviruses, by cell culture is very complex, requires 1–2 weeks, and not all groups of viruses can be isolated with regular cell lines (Li et al., 2010). Polymerase chain reaction (PCR) assays provide more rapid and sensitive diagnostic results for adenoviral conjunctivitis than cell culture (Percivalle et al., 2003). However, PCR-based assays still utilize complicated extraction, amplification procedures, and relatively long analysis times (hours to days) (Puig et al., 1994). Therefore, novel, rapid, and sensitive methods for adenovirus detection are needed (Percivalle et al., 2003).

Label-free biosensors have recently emerged as promising detection tools for virus detection (Anker et al., 2008; Lee et al., 2008). Surface Plasmon Resonance imaging (SPRi) is an interesting alternative to conventional sensing methods. SPRi is an optical technique that allows label-free and real-time analysis, saving time and reagents in comparison to other end-point techniques (Ray et al., 2010). SPRi is suitable for the detection of many types of molecules, like DNA, proteins, (Guedon et al., 2000; Shevchenko et al., 2014), and more recently, living cells (Abadian et al., 2014; Hide et al., 2002) and cellular secretions (Milgram et al., 2012). Further, SPRi sensors monitor both the association and dissociation of biomass on the sensor surface (Kodoyianni, 2011; Malic et al., 2011; Paul et al., 2009; Scarano et al., 2010; Yanase et al., 2010).

SPRi utilizes a high refractive index glass prism coated with a

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50 nm layer of gold. The entire sensor surface is illuminated with a single collimated light source. The detection is based on excitation of surface plasmons polaritons at a resonance condition. Incident light enters the prism at the resonance angle, and it is converted to surface plasmons on the gold by exciting the electrons in the metal. The plasmons propogate as an evanescent wave, or polariton, perpendicular to the surface and extend up to 200 nm above the sensor surface. When the refractive index of the material above the surface changes, for example when a biomolecule attaches to the surface, the surface plasmon polariton cannot form and the incident light is reflect back from the sensor surface. The intensity of the reflected light is monitored pixel-by-pixel with a CCD camera. The intensity of the reflected light is proportional to the amount of attached biomolecules. This variation in the intensity of the reflected light is measured continuously and simultaneously for each spot in real-time with SPRi sensors. This provides the capability of detecting multiple biological interactions at the same time (Abdulhalim et al., 2008; Knoll, 1998; Kodoyianni, 2011). The output is a grey scale image called a difference image. This image shows pixel-by-pixel reflectivity changes of the light compared against the initial state of the surface. To obtain information about specific binding events, the gold surface can be functionalized before the start of the experiment. Biomass flowed over the sensing surface will selectively bind to the recognition elements and the remainder is washed away (Berger et al., 1998; Brockman et al., 2000; Fischer et al., 1993; Huang et al., 2012; Linman et al., 2010).

In this work, we report a biosensor utilizing SPRi for indirect detection of adenovirus. Antibodies specific for adenovirus are mixed with a sample. Unbound antibodies are collected and flowed through the SPRi, where they bind to secondary antibodies that are used as a probe molecule. Using this approach, we developed a rapid, real-time, sensitive, and label-free detection system for adenovirus infection. While the relatively high sensitivity of the assay (10 PFU) is well-suited to detect and identify viruses present in clinical and environmental samples, virus detection in food matrices may also be possible without sample preparation steps that require the virus to be removed from the raw sample or pre-concentrated prior to detection (Amano and Cheng, 2005). The EPA has not yet determined a clear quantitative limit for adenovirus concentrations; instead, they have indicated that there must be no infectious adenovirus in environmental water. Research suggests that environmental water contains between 13 and 7500 PFU/mL of adenovirus (Jiang et al., 2001; Wong et al., 2010; Prescott and Barkely, 2008).

2. Materials

Bovine serum albumin (BSA) was purchased from Sigma-Aldrich (9048-46-8). Adenovirus Hexon Mouse anti-Virus Monoclonal (3G0) Antibody (LS-C55826) was purchased from LifeSpan Biosciences Inc. Goat Anti-Mouse secondary IgG (H+L) was purchased from Life Technologies. Cross-linker dithiobis(succinimidyl propionate) (DSP, Product no. 22585) and dimethylsulfoxide (DMSO, Product no. 20684) were purchased from Thermo Fisher Scientific Inc. A buffer solution of 20 mM PBS (pH 7.4) was used for all experiments, which contained 200 mM NaCl, 25 mM KCl, 10 mM $MgCl_2$. Both adenovirus antibody and secondary antibody were dissolved in 20 mM PBS and kept frozen at −20 °C during storage. Adenovirus serotype 5 (rAd5) and Rotavirus Wa were purchased from SinoGenoMax Co., Ltd. (Beijing, China). Lentivirus (LV-CMV-vector control) was purchased from KeraFAST, Inc. (Boston, MA). All solutions were prepared with Millipore Milli-Q system ultrapure water (18 MΩ cm). A 33-mm-diameter sterile syringe filter (Durapore® PVDF membrane) with a pore size of 0.1 μm were purchased from EMD Millipore (Billerica, MA).

2.1. Adenoviruses, host cells, and plaque assays

Recombinant Adenovirus serotype 5 (rAd5), in which the E1A gene is replaced by the GFP gene (SinoGenoMax Co., Ltd.), was used as a surrogate of adenovirus during sensor development. Human lung carcinoma cell line A549 was cultured using a published protocol (Jiang et al., 2009). A549 cells were grown in Ham's F12 medium containing 5% Fetal bovine serum, 2 mM L-glutamine, 100 U ml21 penicillin, and 100 mg/mL streptomycin. Cells were subcultured at 4- to 5-day intervals with a trypsin–EDTA solution. This cell line is suitable for expressing the recombinant Adenovirus serotype 5 (rAd5) (Graham, 1987).

An adenovirus plaque assay using A549 cells was developed and described previously (Jiang et al., 2009). Briefly, serially diluted rAd5 viruses were inoculated onto confluent A549 cells in 6-well plates and incubated for 1.5 h, with gentle rocking every 20 min for viral adsorption. Then, inoculated A549 cells covered with 1.25% agarose, containing nutrients and antibiotics. A second nutrient covering was applied 5 days post infection. Inoculated cell cultures were examined microscopically every day for 2 weeks after infection, and virus plaques were counted at day 10. All the viruses used are recombinant viruses. The viruses were not infectious; however, still we used a biological safety level 2 laboratory for all the experiments.

3. Experimental

3.1. Chip preparation

High-refractive index glass slides, coated with 50 nm of gold, were purchased (SPRi-Slide, Bare, J47200004, HORIBA Scientific, Inc. Edison, NJ). First, the gold surface was cleaned with ethanol (Fig. 1a). DSP was dissolved in DMSO to a concentration of 4 mg/ml. This solution was applied to the gold surface of the SPRi slide for 30 minutes at room temperature (~23 °C). The gold surface was rinsed with DMSO and then with water. The purpose of this step was to activate the gold surface with N-hydroxysuccinimide (NHS) groups (Fig. 1b). Immediately after surface activation, two sets of secondary antibodies, one specific to the adenovirus antibodies (Goat Anti-Mouse secondary antibody IgG (H+L), Life Technologies, Grand Island, NY) and one non-specific both at a 0.2 μg/mL concentration, were spotted (0.2 μL) on the gold surface and incubated for 2 h at room temperature. In this step, both specific and non-specific secondary antibodies became immobilized onto the gold surface via binding between the cross-linker agent (DSP) and the NH_2 groups of the proteins (Fig. 1c). The gold surface was rinsed with buffer to remove cross-linker by-products (NHS leaving groups) and unconjugated protein. The detailed chemistry of this reaction is shown in the Supplementary material Fig. S1. BSA (pink circles) at a 1 mg/ml concentration was used for blocking the bare gold surface from non-specific binding interactions (Fig. 1d).

3.2. SPRi measurements

The functionalized chip was placed on the high refractive index glass prism (etched prism 25 × 12.5 mm^2, 47200062, Horiba Scientific, Edison, NJ) with a drop of oil (High Index Liquid, Series B, J47200063, HORIBA Scientific, Edison, NJ) to eliminate any air between the chip and the prism, and to maintain a single refractive index across the assembly (Fig. 1e). The functionalized chip had the same length and width as the prism. Next, the functionalized chip on the prims was placed inside of a commercial SPRi instrument (SPRI-Lab+, HORIBA Scientific, Edison, NJ)

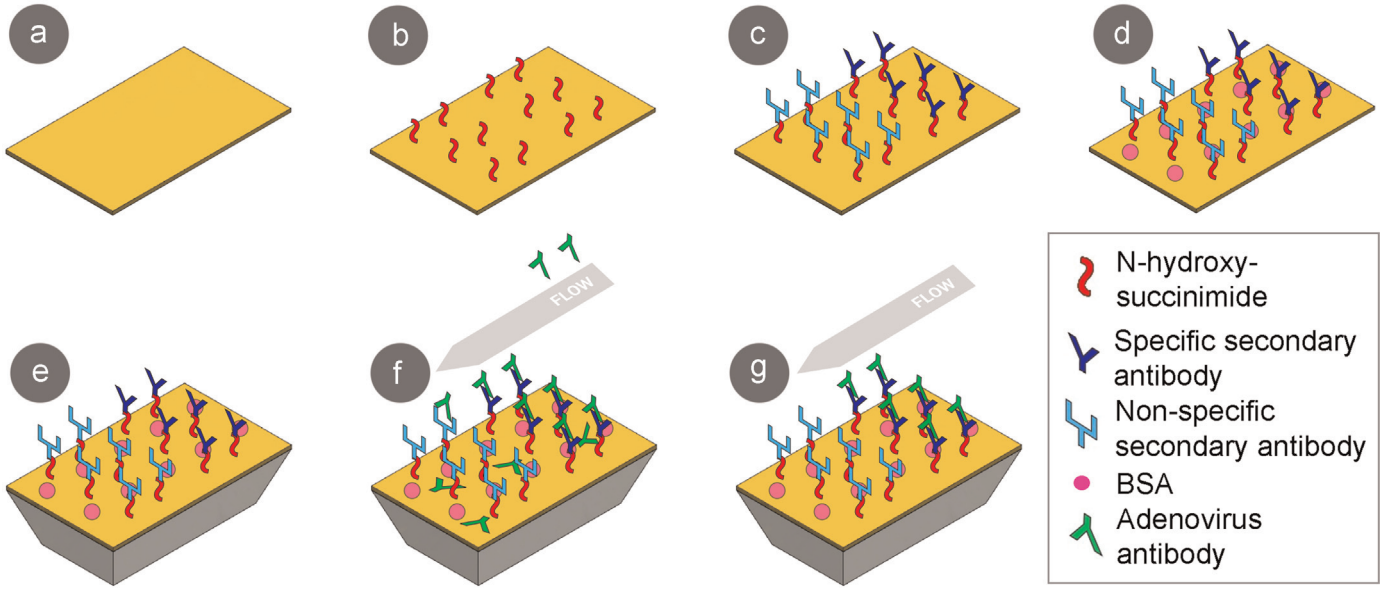

Fig. 1. Schematic representation of the experimental protocol for adenovirus antibody detection using a SPRi platform. (a) Clean gold surface, (b) gold surface activated with N-hydroxysuccinimide (NHS), (c) specific and non-specific antibodies immobilized on surface, (d) gold surface blocked with BSA, (e) chip affixed to prism using index matching liquid, (f) antibody solution flowed over surface allows binding to occur and (g) non-specific attachments removed by a buffer rinse. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.).

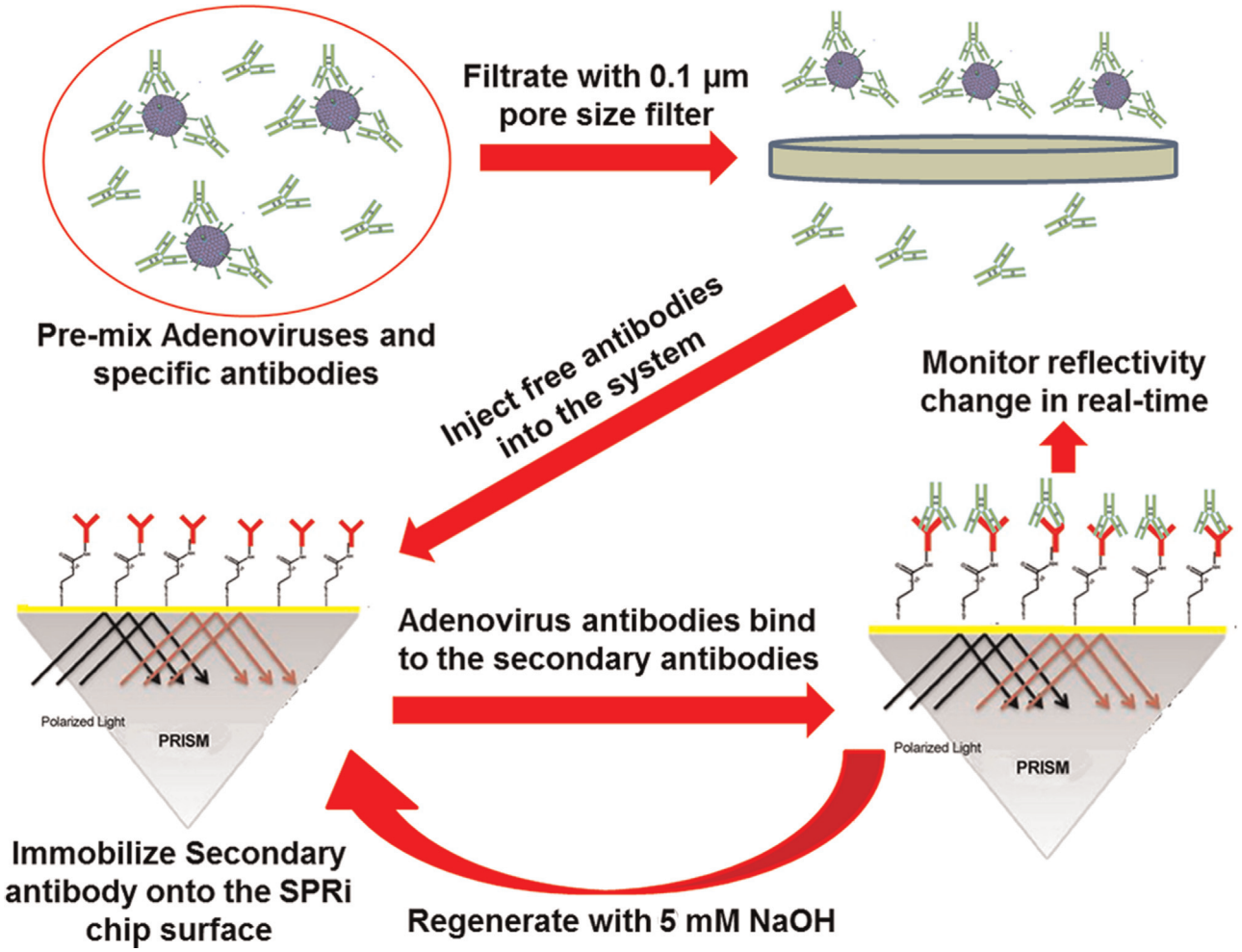

Fig. 2. Schematic representation of sensing protocol for indirect adenovirus detection using SPRi-based biosensor system. The sensing mechanism and procedure involves pre-mixing and incubation of adenovirus samples with a known concentration of adenovirus antibody, filtration, binding of residual free adenovirus antibodies with secondary antibodies immobilized onto the chip surface, and measuring the change in reflectivity. The sensor surface can be regenerated several times with 5 mM NaOH and reused.

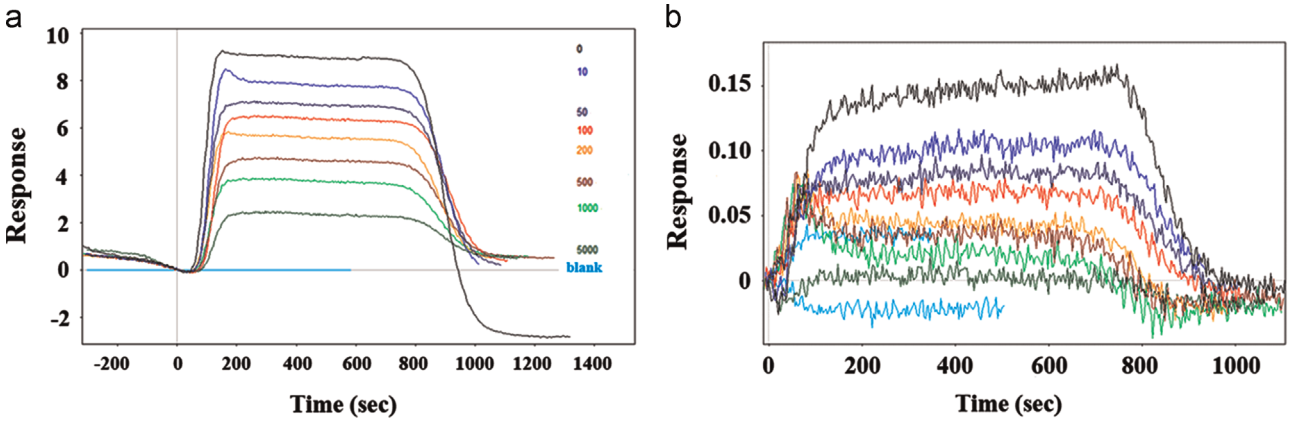

Fig. 3. SPRi data showing the change in sensor response (a.u.) upon exposure of (a) specific secondary antibodies and (b) non-specific antibodies on the sensor surface to various concentrations of adenovirus antibodies in sample eluent. The signal shown for each sample concentration is the average response from three separate patterned areas. The background from the control blank has been subtracted from each experiment. The values on the right side of the graph in (a) indicate the number of PFU/mL of adenovirus in the initial sample.

(Abadian and Goluch, 2015). The instrument uses an 810 nm light-emitting diode (LED) to excite surface plasmons along the entire imaging area simultaneously. The incident angle for the light was selected to maximize the visibility of functionalized surface areas. A peristaltic pump was used to move fluid over the sensing surface during the experiment. The solutions were degassed inline (Model 2003 Degasser, Biotech, Sweden), prior to coming in contact with the chip surface.

Before starting the experiment, the surface was regenerated with 200 μL of regeneration solution (5 mM NaOH) to remove unbound ligands from the surface. During the entire experiment, the flow rate was 15 μL/min and the running buffer was 20 mM PBS. To calibrate the system, 200 μL of buffer with a higher concentration (25 mM PBS) was injected. To stabilize the output signal, 5 blank samples, containing only running buffer solution, were injected over the sensor surface.

The developed indirect adenovirus detection protocol is presented in Fig. 2. First, adenovirus at various concentrations (200 μL sample) was mixed with 200 μL of Adenovirus Hexon Mouse anti-Virus Monoclonal (3G0) Antibody (LS-C55826, LifeSpan Biosciences, Inc., Seattle, WA) to yield a final antibody concentration of 100 ng/mL. These antibodies bind directly to the hexon proteins that are located on the outer shell of the adenovirus. The mixture was incubated for 30 min to allow the binding between adenovirus and antibodies to occur. Then the incubated media was filtered so that only the unbound antibodies could pass through the filter. 200 μL of the filtered solution, containing only the remaining free antibodies, was then injected into the system (Fig. 1f) at a fixed flow rate (15 μL/min). Some of the antibodies in the filtered solution specifically bind to the secondary antibodies (Goat Anti-Mouse secondary antibody IgG (H+L), Life Technologies, Grand Island, NY) while the rest adsorb onto the gold surface or intermingle with non-specific antibodies. Continuous flow of running buffer dislodges and removes these weakly-bound antibodies, leaving only those bound specifically to the secondary antibodies (Fig. 1g).

3.3. Determination of sensor specificity

To determine the specificity of the adenovirus antibody, Rotavirus and Lentivirus were tested with the sensing mechanism described above. The virus strains were measured at concentrations of 10, 100, and 500 PFU/mL.

4. Results and discussion

4.1. Immobilization and optimization

In a SPR imaging setup, the entire sensor surface is illuminated with a large LED light source. The light is collimated before it enters the prism, thus providing uniform illumination of the surface. An image of the surface is generated by the light that is reflected back out from the prism. The areas where antibodies are attached to the surface are defined (Supplementary material Fig. S3) and the change in intensity at those areas is monitored during the experiment. Using this approach, any inhomogeneity in the antibody density on the surface is accounted for in the data analysis.

The incubation time for reacting the hexon adenovirus antibodies with the adenovirus particles was optimized previously, along with the amount of hexon adenovirus antibodies to add to the sample (Yildirim et al., 2013). We immobilized several concentrations of the secondary antibodies on the sensor surface and flowed a solution containing a 100 ng/mL hexon adenovirus concentration to optimize the surface antibody concentration. We selected the surface secondary antibody concentration that gave the largest signal change, as 100 ng/mL is the highest concentration of hexon adenovirus that would be flowed over the sensor surface (corresponding to 0 PFU/mL of adenovirus in the sample). Spots made with 0.2 μg/mL concentrations of secondary antibody gave the largest signal change. Concentrations of secondary antibodies above 0.2 μg/mL did not result in a greater signal, likely because of molecular overcrowding on the surface, which causes binding sites on the capture antibodies to be blocked.

BSA is a well-known blocking agent that is commonly used in many biosensor studies. We use the a previously developed protocol for blocking the sensor surface and minimizing non-specific binding (Yildirim et al., 2012). The functionalized biosensor was reused up to 20 times in 1 day by regenerating the surface using 200 μL of regeneration solution (5 mM NaOH) to remove unbound ligands from the surface. The biosensing surface was also stored in a sealed container for up to 2 weeks at 4 °C prior to use with minimal loss of function.

4.2. Non-specific binding

Fig. 3 shows the reflectivity changes on the regions coated with specific and non-specific antibodies. An increase in reflectivity was detected after injection of the target adenovirus antibodies. After non-specifically bound antibodies were dissociated and unbound

antibodies washed away, a decrease in the reflectivity was observed due to the replacement of target with running buffer. However, the reflectivity signal did not return to zero, indicating the presence of strongly bound antibodies. This final reflectivity change was used in the measurements. When the concentration of adenovirus in the initial sample was increased, the concentration of unbound antibodies in the injected filtered solution decreased. This resulted in an inverse relationship between the measured reflectivity change and the initial concentration of adenovirus (Fig. 3a).

When a sample is injected into the system, it takes almost 5 min to reach to functionalized sensor surface at a flow rate of 15 μL/min. When the sample reaches the sensor, there is a slight increase in the reflectivity in the areas of the sensing surface that have non-specific secondary antibodies (Fig. 3b), due to mass change and consequent refractive index variation. However, after the removal of non-specifically bound antibodies with buffer flow, the response signal was negligible compared to the regions of the sensor functionalized with specific antibodies (Fig. 3a). These results confirmed that minimal non-specific binding occurred during the injections.

4.3. Linear range

Different concentrations of adenovirus (5000, 1000, 500, 200, 100, 50 and 10 Plaque Forming Unit (PFU/mL)) were incubated with 200 μL of 100 ng/mL free adenovirus antibodies for 30 min to allow complete binding between the antibodies and adenovirus. Adenoviruses are approximately 90 nm in diameter, which after binding with antibodies become slightly larger than the 0.1 μm pore size membrane. It has been shown that over 240 antibodies can bind to each virus particle, or 1 antibody per hexon protein trimer on the virus coat, resulting in a particle size great than 100 nm in diameter (Varghese et al., 2004). When the media was filtered, it was expected that only the excess (unbound) antibodies (approximately 8 nm in size) passed through the filter pores. This

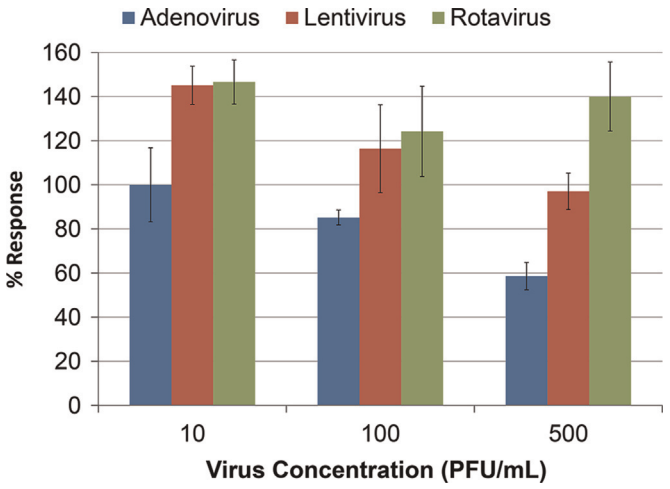

Fig. 5. Sensor specificity assessment comparing percent response of adenovirus with other pathogen strains. Virus strains compared at 10, 100 and 500 PFU/mL concentrations. At least 4 independent areas patterned with specific antibodies were measured in each experiment. The brightness of the areas was averaged and then normalized using 10 PFU/mL of adenovirus. The error bars represent one standard deviation from the normalized mean value.

filtrate, containing the unbound antibodies, was then injected into the SPRi system. This filtration step was optimized to yield reproducible and reliable results by adjusting the ratio of antibodies to potential virus particles and the sample volume.

Before measuring filtrate solutions, a control solution was injected containing 100 ng/mL (optimized concentration) adenovirus antibody. Using this control, a percent response was calculated and used for all experiments. Percent response was calculated using the formula: % Response$=(\Delta R_{sample}/\Delta R_{control})*100$. Three analysis areas were defined per spot containing antibodies. The brightness from the spots was determined using Photoshop (CS6, Adobe). Areas distorted by air bubbles (confirmed by the raw

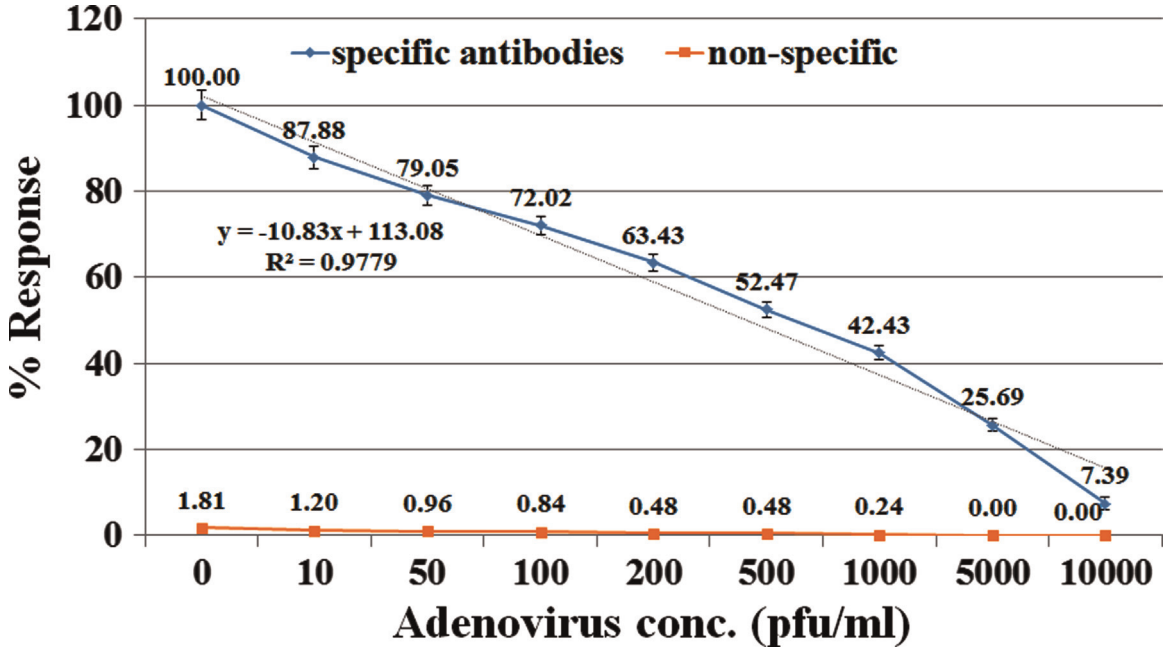

Fig. 4. Adenovirus antibody detection response curves for secondary antibodies (Blue diamonds) and non-specific antibodies (Red squares). Experiments started by mixing 200 μL of 100 ng/mL adenovirus antibody with samples containing known concentrations of adenovirus. Free adenovirus antibodies were removed from the samples and injected into the SPRi. The mean value was obtained by averaging the response from several spotted areas on the sensor chip for both the specific and non-specific patterned antibodies. Shown is the normalized mean value for each sample, which was obtained by dividing by the negative control sample containing no adenovirus. The error bars represent one standard deviation from the mean value. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

Table 1
Comparison of adenovirus detection methods.

Detection system	Recognition element	Detection limit (PFU/mL)	Detection time	Pre-processing	Re-usability	Ref.
Fluorescence-activated cell-sorting (FACS) assay	Hexon antibody	1	3 days	None	NA	Li et al. (2010)
Two step PCR-based detection	DNA	10	Several hours	DNA extraction	NA	Puig et al. (1994)
Immunosensor with conducting copolymer surface	Antibody	10^3	Up to 2 h	None	NA	Caygill et al. (2012)
Optical fiber biosensor systems	Hexon antibody	100	30 min incubation + 6 min detection	None	5 times	Yildirim et al. (2013)
SPRi-based indirect detection	Hexon antibody	10	30 min incubation + 10 min detection	None	Up to 20 times	This system

data image) were removed from the analysis. Fig. 4 shows the % Response vs. the concentration of the initial adenovirus upon binding to the specific secondary antibodies (blue curve) and to non-specific secondary antibodies (red curve). The error bars represent one standard deviation from the mean value. The percent response upon association of the adenovirus antibodies to specific secondary antibodies shows a linear decrease from 87.88 to 25.69 with increasing concentration of initial adenovirus from 10 PFU/mL to 5000 PFU/mL. This is expected as the concentration of free adenovirus antibodies in the filtered solution decreases with increasing amounts of virus in the sample.

Reflectivity changes were minimal in the regions with non-specific secondary antibodies upon interaction with each concentration of adenovirus antibodies (red curve). The reflectivity changes for non-specific secondary antibodies do not show a correlated trend with respect to concentration.

4.4. Assessment of sensor specificity

To determine the specificity of the adenovirus antibody, rotavirus and lentivirus were tested with the sensing protocol described above. The samples tested contained 10, 100, or 500 PFU/mL concentrations of either rotavirus or lentivirus. 500 PFU/mL was chosen because this represents the higher end of the range of virus concentration often found in environmental water samples.

The data has been normalized based on the percentage of respose to 10 PFU/mL of adenovirus. The results (Fig. 5) show that the developed biosensor system has high specificity towards adenovirus, although there is some cross-reactivity of the hexon adenovirus antibodies against the rotavirus and lentivirus particles. With the present sensing mechanism, we measured a 20% signal decrease with the other viruses at the highest concentration, which is likely due to non-specific adsorption of the adenovirus antibodies onto the other virus particles. However, a 20% non-specific signal for specificity experiments is acceptable for these types of antibody-based biosensor systems (Long et al., 2010).

5. Conclusion

SPRi biosensors are providing new techniques for label-free and rapid detection of biomolecular interactions. The method presented in this paper offers a simple new approach for detection of viral pathogens. In this work, SPRi sensors were used to indirectly detect various concentrations of adenovirus by monitoring the binding of adenovirus antibodies to specific secondary antibodies on the SPRi surface. The concentration of remaining free antibodies was inversely related to the concentration of virus in the media. Preparation of the biosensor surface took about 4.5 h, but the sensors can be regenerated and reused up to 20 times in one day, and they can be stored for up to 2 weeks at 4 °C with minimal loss of sensitivity. The detection limit was 10 PFU/mL with a detection time of 5 min after an initial 30 min incubation step. This is the tested experimental detection limit, not a calculated theoretical limit of detection. Table 1 compares the results of this work against methods for adenovirus detection. Further, the assay format used in this paper can be applied for the simultaneous detection of multiple antibodies.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2015.07.047.

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