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Research paper

Antibody immobilization using pneumatic spray: Comparison with the avidin–biotin bridge immobilization method

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ABSTRACT

The formation of a thin antibody film on a glass surface using pneumatic spray was investigated as a potential immobilization technique for capturing pathogenic targets. Goat-*Escherichia coli* O157:H7 IgG films were made by pneumatic spray and compared against the avidin–biotin bridge immobilized films by assaying with green fluorescent protein (GFP) transformed *E. coli* O157:H7 cells and fluorescent reporter antibodies. Functionality, stability, and immobilization of the films were tested. The pneumatic spray films had lower fluorescence intensity values than the avidin–biotin bridge films but resulted in similar detection for *E. coli* O157:H7 at 10^5–10^7 cells/ml sample concentrations with no detection of non-*E. coli* O157:H7 strains. Both methods also resulted in similar percent capture efficiencies. The results demonstrated that immobilization of antibody via pneumatic spray did not render the antibody non-functional and produced stable antibody films. The amount of time necessary for immobilization of the antibody was reduced significantly from 24 h for the avidin–biotin bridge to 7 min using the pneumatic spray technique, with additional benefits of greatly reduced use of materials and chemicals. The pneumatic spray technique promises to be an alternative for the immobilization of antibodies on glass slides for capturing pathogenic targets and use in biosensor type devices.

1. Introduction

The use of biosensors for the detection of pathogens in food and for monitoring bioterrorism pathogens has been a priority in recent years stimulating a growing interest to develop sensor devices to protect customers, military personnel and first responders (Lim et al., 2005; Arora et al., 2011). One of the most researched and developed field is the evanescent wave fluorescence biosensor (Taitt et al., 2005; Leung et al., 2007). Various devices or versions implementing the evanescent wave technology exist but share the common characteristic of immobilization of a recognition biomolecule onto an optical fiber, capillary, or planar waveguide (Taitt et al., 2005). The recognition biomolecule has affinity to a specific target, and when it comes in contact with the target, will bind to it or capture it. A detector or reporter biomolecule then binds to the captured target. The fluorophore on the detector biomolecule is excited by the evanescent wave and emits a fluorescent signal which is registered and processed by the biosensor device.

Most of the immunoassays developed for evanescent wave fluorescence biosensors use antibodies as the capture biomolecule. The antibodies are immobilized by a biotin–avidin intermediate layer covalently attached to the surface (Demarco and Lim., 2001; Golden et al., 2005; Zhu et al., 2005) indirect physical adsorption where avidin is directly adsorbed to the surface followed by a biotin conjugated antibody (Lim et al., 2005), or direct adsorption (Taitt et al., 2005). The physical adsorption method offers the advantage of simplicity, where fewer steps are performed and less

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reagents or chemicals are used. Often stated unwanted characteristics of the physical adsorption method are non-specific adsorption, denaturation on the surface at distinct pH, the probability that adsorbed proteins can leach or wash off from the surface if the coated substrate is exposed to a high liquid flow, and ionic strength of the buffers (Ulf et al., 1985a, 1985b; Lin et al., 1988). Some studies also have suggested that the physical adsorption of proteins can lead to denaturation of the biomolecule through surface–protein interactions resulting in non-specific binding of the antibodies to the target molecule (Moulin et al., 1999; Peluso et al., 2003; Karlsson et al., 2005). Most of these potential issues appear not to be a problem with the biotin–avidin covalent bridge method.

Optimization studies to the capture surface of various biosensors have been performed with the goal of improving sensitivity and reproducibility of the antibody capture surface but not all have been successful (Anderson et al., 1997; Plowman et al., 1999; Sapsford et al., 2001; Vijayendran and Leckband., 2001; Vermette et al., 2003; Cui et al., 2003; Gehring et al., 2006; Ngundi and Anderson, 2007; Batalla et al., 2008; Ikeda et al., 2009; Feng et al., 2011). In addition, requirement of trained personnel, extra protocol steps and reagents increase preparation time and material usage. This makes it harder to design and implement mass fabrication assay techniques. Alternative methods with potential for mass fabrication of waveguides incorporate technologies such as nanolithography and ink and laser printing (Borini et al., 2007; Lisboa el al., 2010; Sharma et al., 2010).

In this study, the possibility of using spray deposition of antibodies as an alternative to waveguide fabrication was investigated. The method presented here was demonstrated using goat anti-*Escherichia coli* O157:H7 antibody for the deposition onto glass waveguides through direct physical adsorption using a low flow concentric pneumatic nebulizer. The characteristics of the spray process offer advantages that include the fast deposition of waveguide films, an almost chemical free process, consistent coverage of the sprayed surface, and easy set up for manufacturing purposes. Additional potential benefits for a manufacturing environment are low cost and maintenance of the equipment. This makes the pneumatic spray an inexpensive and efficient immobilization technique.

2. Materials and methods

2.1. Materials

Plain microscopy glass slides were purchased from Globe Scientific Inc. (Paramus, NJ). The primary antibodies used for the immobilization processes were unlabeled goat anti-*E. coli* O157:H7 IgG (pneumatic spray process) and biotinylated labeled goat anti-*E. coli* O157:H7 IgG (avidin–biotin bridge process). Both antibodies were purchased from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD). The reporter antibody for the bacterial immunoassays, goat anti-*E. coli* O157:H7, was labeled with AlexaFluor 647 (AF647) using the AF647 protein labeling kit from Invitrogen (Eugene, OR) and following the manufacturer's instructions. AF647 labeled donkey anti-goat IgG (reporter secondary antibody) was purchased from Invitrogen (Eugene, OR) and Rhodamine Red conjugated AffiniPure donkey anti-goat IgG (reporter secondary antibody) was purchased from Jackson ImmunoResearch (West Grove, PA). The antibodies were rehydrated and stored according to the manufacturer's instructions. Luria–Bertani and Tryptic Soy media (Becton Dickinson Company, Sparks, MD) were used for the growth of the bacteria.

2.2. Bacteria

E. coli O157:H7 ATCC 35130 transformed to express green fluorescent protein (GFP) has been previously described (Simpson-Stroot et al., 2008) and was used for this study. GFP-*E. coli* O157:H7 was grown on Luria–Bertani media containing 100 μg/ml ampicillin and 5 mg/ml arabinose (LBAA) at 37 °C for 18–24 h. *E. coli* K12 ATCC 23590, *E. coli* O124:H7 CDC 3836-65, *Salmonella enterica* Typhimurium ATCC 19585, *Shigella flexneri* ATCC 12022, and *Staphylococcus aureus* ATCC 25923 were grown on Tryptic Soy Agar (TSA) at 37 °C for 18–24 h. Cell suspensions were prepared in 10 mM sodium phosphate/10 mM sodium chloride (NaPCl) buffer and diluted ten-fold. Direct counts were performed with Cellometers (Nexcelom Bioscience, Lawrence, MA) to approximate cell concentrations followed by spread plating on LBAA agar plates (for GFP-*E. coli* O157:H7) or TSA (all other bacteria) in triplicate to determine viable cell concentrations.

2.3. Immobilization of antibody via pneumatic spray

The pneumatic spray deposition process utilized a commercially-available concentric nebulizer with a low flow rate (3–10 μl/min) (Nebulizer Model DS-5, CETAC, Omaha, NE), which was placed into an in-house constructed apparatus to hold the nebulizer and a slide (Fig. S-1). A pressure regulated N_2 gas line was connected at the top of the nebulizer. A syringe pump (Pump 11 PicoPlus Harvard Apparatus, Holliston, MA) with a 1 ml syringe was connected to the back of the nebulizer. The glass slides were immersed in a solution of 10% KOH in methanol and incubated for 30 min followed by extensive rinsing with deionized water and dried in a flow of N_2 prior to spraying. A slide was placed on the slide assembly and positioned at a certain distance away from the nebulizer. The syringe was then filled with unlabeled goat anti-*E. coli* O157:H7 IgG solution. The parameters that were explored were the antibody concentration (100 or 200 μg/ml), the outflow N_2 pressure (20–60 PSI), the spraying time (2–32 min), the distance of the slide to the nebulizer (30–70 mm), and rotational rate of the glass slide (7.5–17 RPM). The only parameter that did not change was the syringe pump speed, which was set to 4 μl/min. After varying these parameters in a set of tests, a well-defined antibody film pattern was established using a 25×75 mm metal mask with fifteen 1×9 mm rectangular openings (rows). All pneumatic spray slides for experiments were prepared with the following parameters: 200 μg/ml antibody, 30 PSI outflow N_2 pressure, 7 min spraying time, 30 mm distance of the slide to the nebulizer, and 12 RPM. These parameters yielded at least 6 rows of immobilized antibody on the glass slides. The prepared slides were stored at 4 °C until further use.

2.4. Immobilization of antibody via an avidin–biotin bridge

The procedure used to covalently immobilize antibody via an avidin–biotin bridge onto glass slides has previously been described (Rowe et al., 1999). Briefly, the glass slides were immersed in a solution of 10% KOH in methanol for 30 min, rinsed with deionized water and dried with nitrogen. The slides were treated for 1 h (under nitrogen) with a 2% solution of (3-mercaptopropyl) triethoxysilane in toluene followed by a 30 min incubation in 2.1 mM, 4-maleimidobutyric acid N-hydroxysuccinimide ester in 200 proof ethanol. The slides were rinsed with deionized water and incubated in 33 mM NeutrAvidin in NaPCl buffer at 27 °C for 2 h. The slides were rinsed with NaPCl buffer and placed in patterning templates, each consisting of a plexiglass holder and a polydimethylsiloxane (PDMS) flow module. A solution of 10 μg/ml biotinylated goat anti-*E. coli* O157:H7 IgG in NaPCl buffer was injected into 6 rows of the flow chamber. The slides were incubated for 18–22 h at 4 °C and rinsed using NaPCl with 0.5% Tween 20 (NaPClT) buffer. Slides were dried with nitrogen and used for assays or stored at 4 °C until further use.

2.5. Slide assays

Slides to be assayed with bacteria were tempered to 21 °C and placed inside plexi-glass holders with silicon gaskets. The gaskets had a 17.4×16.8 mm open area (292.32 mm^2) for sample application. One hundred microliters of the sample solution was added to the 292.32 mm^2 area and incubated for 30 min at 21 °C on a Belly Dancer Shaker (Stovall Life Science, Greensboro, NC). The slides were then rinsed 3× with 0.5 ml NaPClT buffer. Half a milliliter of 10 μg/ml AF647 anti-*E. coli* O157:H7 reporter solution was added and incubated for 15 min at 21 °C on the shaker. Each slide was subsequently rinsed 3× with 0.5 ml NaPClT buffer, removed from the plexi-glass holder and air dried.

2.6. Visualization of films and GFP bacteria

Slides prepared by pneumatic spray and avidin–biotin bridge were processed for visualization of antibody films in the following manner: 1 ml of a 5 μg/ml Rhodamine Red anti-goat IgG solution was added to each slide surface and incubated for 15 min at 21 °C. The slides were then rinsed 3× with NaPClT buffer and dried. Slides were examined on an Olympus BX60 Epifluorescent microscope (Olympus America Inc., Center Valley, PA) with UPlanFl 10× and UIS2 LUCPlan FLN 40× objectives and a Cy3 filter (Excitation 535–550, Emission 610–675, Beam splitter 565; Olympus America Inc., Center Valley, PA). Digital images were captured with an attached SPOT Flex color CCD camera (Diagnostic Instruments Inc., Sterling Heights, MI) and measurements made with the SPOT Advanced version 4.6 software.

Assayed slides with detector antibody were read using a biosensor array reader apparatus as described by (Golden et al., 2005). Each slide was illuminated using a 635 nm laser and emitted light from the AF647 fluorophores was captured as an image with a Peltier-cooled CCD camera.

Slides assayed with GFP bacteria were subjected to observation and imaging with epifluorescence microscopy using a U-MWIBA filter (Excitation 475–490, Emission 515–550, Dichroic 500; Olympus America Inc., Center Valley, PA) to determine GFP cell counts and calculate percent capture efficiencies.

2.7. Reproducibility and functionality of pneumatic spray antibody films

After establishing optimized pneumatic spray parameters, ten prepared slides were treated with reporter secondary antibody to check for pattern reproducibility of the immobilized antibody films. The slides were placed horizontally on a slide holder. One milliliter of 5 μg/ml AF647 donkey anti-goat IgG solution was added to the surface of each slide and incubated for 15 min at 27 °C. The slides were rinsed 3× with NaPClT buffer and air dried. Slides were read as described above.

The functionality of the pneumatic spray antibody films was then tested and compared against avidin–biotin bridge slides in various experiments. Slides were assayed with GFP-*E. coli* O157:H7 and non-target bacteria to determine the sensitivity and specificity, respectively, of the immobilized antibody. Five assay replicate experiments were performed in which each experiment consisted of three slides of each deposition method (overall total of 30 slides). For each experiment, one slide of each deposition method was assayed with GFP-*E. coli* O157:H7 at 1.2–4.6×10^5, 1.2–4.6×10^6, or 1.2–4.6×10^7 cells/ml. Slides from 3 different experiments (3 sets) were then subjected to the procedure for visualization of the antibody films with Rhodamine Red reporter secondary antibody. A different set of slides was assayed with non-target bacteria following the same assay procedure: *E. coli* K12, *E. coli* O124:H7, *S. enterica* Typhimurium, *S. flexneri*, and *S. aureus* suspensions at 10^7 cells/ml were used per slide.

In further testing of antibody functionally, 12 slides were prepared via the pneumatic spray process on the same day (day 0) and stored at 4 °C. On day 1 (24 h after slide prep) one slide was assayed with a GFP-*E. coli* O157:H7 sample at 10^7 cells/ml. One slide was then assayed at the same concentration each week for 12 weeks.

2.8. Data analysis

The images captured with the CCD camera were analyzed with the HLAB 5000 analysis software (Hanson Technologies, Inc., Carlisle, PA). Relative fluorescence intensities (RFU) were generated for 6 antibody rows per slide, termed regions of interest (ROI; 4.37 mm^2) and for the non-target areas to the top and bottom of the ROI (area between the rows), designated as the top and bottom backgrounds (1.09 mm^2 each) using a 1×6 array grid. Mean and standard deviation RFU values for each slide were calculated using the 6 replicates. Signal to noise ratios (SNR) were determined by subtracting the mean background fluorescence intensity from the mean intensity of the ROI and then dividing the results by the background standard deviation. Antibody rows with SNR≥3 were considered positive detection in the bacterial assays.

Percent capture efficiencies for each slide was determined by placing each GFP-*E. coli* O157:H7 assayed slide on the

epifluorescence microscope and generating a digital image of the ROI for each row (total of 6 values per slide). The area imaged was 0.09486 mm^2. GFP-*E. coli* O157:H7 cells in the imaged area were counted using DAIME 1.31 software (Daims et al., 2006). The percent capture efficiency was calculated by dividing the number of GFP cells counted per image by the theoretical number of GFP cells per image and multiplying by 100. The theoretical number was calculated by dividing the number of cells applied by the antibody film area in contact with each cell sample. Each antibody film row for the pneumatic spray slides was estimated (based on mask dimensions) to be 16.80 mm^2 and 14.94 mm^2 for the avidin–biotin bridge slides.

Differences in pneumatic spray and avidin–biotin bridge fluorescence intensity values, SNR, and percent capture efficiencies were analyzed with the unpaired *t*-test or the Mann–Whitney test for sample groups with non-Gaussian distributions (GraphPad InStat v3.0, GraphPad Software, Inc., La Jolla, CA). Differences were considered statistically significant for *P* values less than or equal to 0.05 (95% confidence level). Error bars on the graphics represent the standard deviations of replicates.

2.9. Thin film analysis

The thickness of the pneumatic spray and avidin–biotin bridge antibody films were analyzed by ellipsometry (Null point Ellipsometer, Rudolph AutoEL III) with a single wavelength of 632.8 nm and a resolution of 3–10 Å at a fixed angle of 70°. Substrates for the process were prepared in the following manner: 2.5×4 cm silicon wafers were immersed in a solution of 10% KOH in methanol and incubated for 30 min followed by extensive rinsing with deionized water and subsequently dried with N_2. Ten silicon wafers were pneumatically sprayed with goat anti-*E. coli* O157:H7 as described in the pneumatic spray process. Each wafer was sprayed in duplicate using different deposition times of 2, 4, 7, 10 and 14 min. Two silicon wafers were used to immobilize anti-*E. coli* O157:H7 IgG via the avidin–biotin bridge process. A clean silicon wafer without immobilized antibodies was retained as a reference for each of the two immobilization techniques. The measurements were performed on silicon wafer due to its highly reflective characteristics and an index of refraction far from the region of biological transparent films allowing an accurate measurement of the films.

3. Results and discussion

3.1. Characteristics of antibody films

Figs. 1 and 2 display representative images of the immobilized antibody films visualized with a 635 nm laser/CCD camera apparatus and epifluorescence microscopy, respectively, for both pneumatic spray and avidin–biotin bridge prepared slides. Initial testing did not include addition of cells but rather direct visualization to determine pattern reproducibility of the pneumatic sprayed films (Fig. 1, A1). This was accomplished as all ten slides assayed in the reproducibility experiment had defined immobilized films with at least 6 defined rows for. Two slides had high AF647 background values as indicated by the higher residual fluorescence between the rows as seen in Fig. 1, A1. Some of the antibody films were slightly off-centered but adjusted in subsequent tests and some rows were slightly thicker than other rows. With this data and further observations, it was determined that the positioning, tightening, and slight deformity of the aluminum mask caused an uneven seal between the mask and slides allowing for thicker rows and sprayed antibody to enter underneath the mask and immobilized between the rows. This caused a low-density antibody coating in these areas with resultant antigen capture capability during the functionality tests (Figs. 1, B1–B2 and 2, E2). Overall, however, all slides had immobilized multi-row films that were suitable for bacterial assay testing.

When the antibody films were treated with Rhodamine Red detector IgG and viewed with fluorescence microscopy, images revealed a difference in surface morphology between the pneumatic spray and avidin–biotin bridge prepared slides (Fig. 2). The pneumatic sprayed films had a grainy, less homogeneous surface morphology than the avidin–biotin bridge films. The differences observed are explained by the way both immobilization procedures work. During the pneumatic spray process, the nebulizer head creates an aerosol containing small droplets of antibody solution. These droplets decrease in volume size with the distance traveled from the nebulizer to the surface of the glass as the water evaporates, resulting in an increase in antibody concentration within the droplet forcing the IgG molecules to get closer to each other. Furthermore, the pH of the antibody solution influences aggregation. The isoelectric point of IgG is between 6.1 and 6.5 (Cheng et al., 2011) but the pH of the solution before spraying was 4.8 ± 0.1. Hence, the antibody had a net positive charge in the solution. Majhi et al. (2006) suggested that the protein aggregation is driven mainly by electrostatic attractive forces and that the net charges produced under non denaturing conditions are not relevant. Moreover, it was found that maximum aggregation actually occurs at a pH level where the net charge is positive. It can be hypothesized that two main factors for aggregate formation on the pneumatic spray films are an increase in antibody concentration within the droplet and enhanced electrostatic attraction due to the high antibody density. The antibody droplets are immobilized randomly and overlap each other on the surface; this creates the non-uniform, grainy film seen on the Rhodamine Red detector images. The covalently attached film produced a much more homogeneous result with some circular spots on the surface; an explanation to these circular spots is unknown.

The data obtained from the ellipsometer showed a linear behavior between the thickness of the antibody film made by pneumatic spray and the deposition time with a linear regression of 0.9874. The average thickness calculated for the avidin–biotin bridge technique was 183.16 ± 8.54 Å, whereas the standard deposition time of 7 min for the pneumatic spray process showed an average thickness of 155.25 ± 11.78 Å. The thickness values of both films were similar. However, the thickness of the sprayed slides corresponds entirely to the sprayed antibody, whereas in the case of the avidin–biotin bridge films, the thickness is a result of the covalent linked bridge (silane, cross-linker, avidin, and biotin conjugated antibody). This suggests that

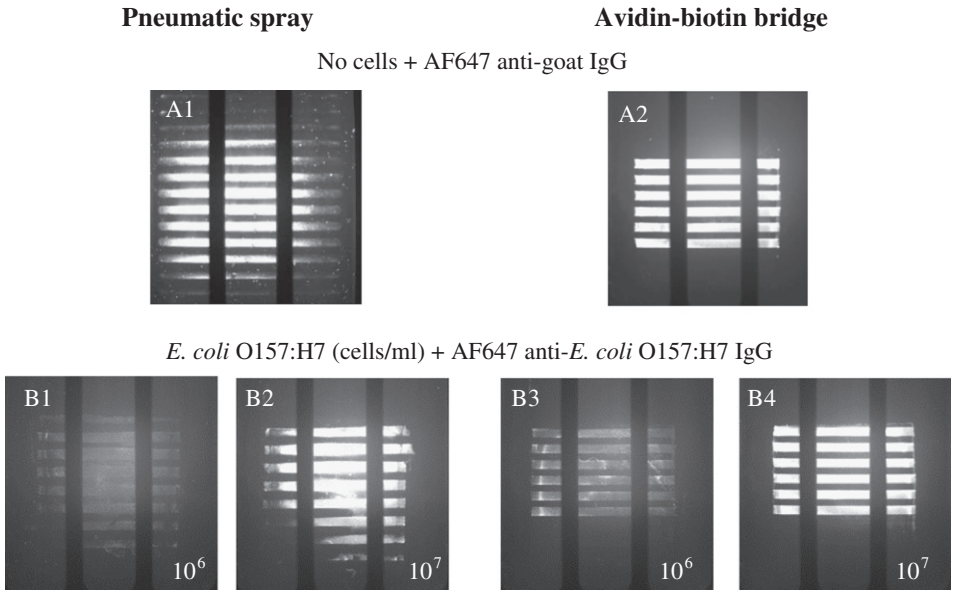

Fig. 1. Visualization of immobilized antibody film patterns using AlexaFluor 647 (AF647) labeled IgG as the reporter antibody, a 635 nm laser, and Peltier-cooled CCD camera. Fluorescence obtained in the images was due to the emitted light from AF647 excited by the 635 nm laser. Images displayed (approximately 90 mm^2 of slide area) are representative of slides prepared with pneumatic spray (A1, B1, B2) and avidin–biotin bridge (A2, B3, B4) antibody immobilization. Slides represented in images A1 and A2 show the fluorescence response after being subjected to AF647 labeled anti-goat IgG (secondary reporter antibody) which bound to the immobilized goat anti-*E. coli* O157:H7 IgG. Slides represented in images B1–B4 show the fluorescence response after addition of GFP-*E. coli* O157:H7 cells at 10^6 (B1, B3) and 10^7 cells/ml (B2, B4) followed by AF647 labeled goat anti-*E. coli* O157:H7 IgG (primary reporter antibody).

the sprayed films have a higher antibody density than the avidin–biotin bridge attached films. The corresponding mass of the deposited antibody was calculated to be around 31.69 ng/mm^2 for the pneumatic spray films, while the deposited mass for the avidin–biotin bridge has been reported to be between 2.2 ng/mm^2 (Moulin et al., 1999; Batalla et al., 2008) and 4.74 ng/mm^2 (Anderson et al., 1997; Gehring et al. 2006). This is not surprising since the spray process is a non-equilibrium technique, whereas the covalent attachment in solution is equilibrium controlled. In other words, the spray technique can load the surface with antibodies at any desired density, while the chemical attachment in solution has a maximum density that is governed by the rate constants of the participating chemical reactions.

3.2. Sensitivity and specificity of pneumatic spray assayed slides

Fig. 3A and B graphs the mean fluorescence intensities for the ROI and background areas in relative fluorescence units (RFU) as measured for *E. coli* O157:H7 samples assayed on the pneumatic spray and avidin–biotin bridge prepared slides. The mean ROI RFU at 10^5 cells/ml were similar for both slide immobilization processes with no significant difference ($P=0.1581$), but were significantly different at 6 ($P=0.0072$) and 10^7 cells/ml ($P=0.0104$) in which the avidin–biotin bridge slides had higher values. Mean SNR values (Fig. 3C) yielded no detection at 10^5 cells/ml for pneumatic spray and avidin–biotin bridge slides, 0% and 40% detection at 10^6 cells/ml and 60% and 100% detection at 10^7 cells/ml for pneumatic spray and avidin–biotin bridge, respectively. There were no significant differences ($P=0.1175$) between the 10^5 cells/ml SNR values, but there were significant differences ($P<0.0001$) at the 10^6 and 10^7 cells/ml concentrations, where avidin–biotin bridge slides had higher values. This difference was a result of the slightly lower ROI RFU (Fig. 3A) in combination with higher background values (Fig. 3B) that caused lower SNR values for the pneumatic spray slides. The lower SNR values were not an indication of decreased antibody functionality within the sprayed films but a direct consequence of the spraying process. As mentioned earlier, the uneven seal between the mask and slides allowed for a low-density antibody coating between the rows, where the background readings were taken (any difference in seal tightness on a particular slide can allow more or less antibody leak under the mask and create the large standard deviation for the samples as shown in Fig. 3C). This in turn resulted in antigen capture during these tests (Figs. 1, B1–B2 and 2, E2) and caused increased background values.

In contrast, the slightly lower ROI RFU values for the pneumatic sprayed slides were likely a result of the detachment of some of the antibody from the surface during sample incubation. The representative images D1 and E1 in Fig. 2 demonstrate that the *E. coli* O157:H7 cells applied and captured on the sprayed slide surface were fluorescent after treatment with Rhodamine Red detector IgG whereas the cells on the avidin–biotin bridge slides were not. This suggests that cells immobilized on sprayed slides were coated with a certain amount of capture antibody during assay sample incubation. Once the Rhodamine Red anti-goat IgG was added, it bound to the goat anti-*E. coli* O157:H7 IgG on the cells' surfaces. This coating most likely results in blockage of some of the epitopes on the cell surface thereby

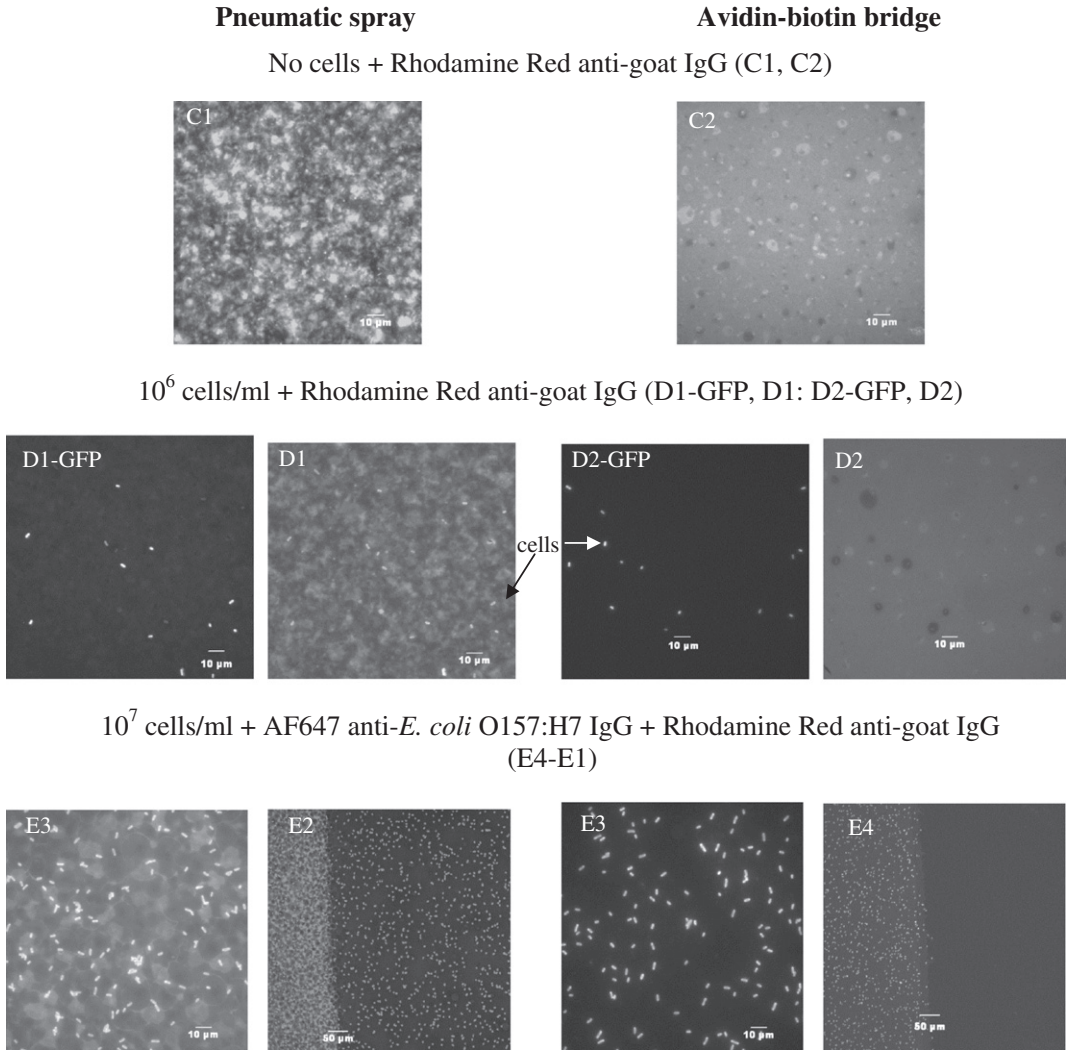

Fig. 2. Visualization of GFP-*E. coli* O157:H7 cells and immobilized antibody films by epifluorescence microscopy. A Cy3 filter (excitation 535–550, emission 610–675, beam splitter 565) and SPOT Flex CCD camera was used to obtain all images except for D1-GFP and D2-GFP where a U-MWIBA filter (excitation 475–490, emission 515–550, dichroic 500) was used to view only the GFP-*E. coli* O157:H7 cells. Images displayed are representative of slides prepared with pneumatic spray (C1, D1-GFP, D1, E1, E2) and avidin–biotin bridge (C2, D2-GFP, D2, E3, E4) antibody immobilization. Slides represented in images C1 and C2 show the fluorescence response after incubation with secondary reporter antibody, Rhodamine Red (RR) donkey anti-goat IgG (which bound to the immobilized goat anti-*E. coli* O157:H7 IgG). Slides represented in images D1-GFP (same area as D1), D1, D2-GFP (same area as D2), and D2 show the fluorescence response after incubation with GFP-*E. coli* O157:H7 cells at 10^6 cells/ml followed by RR anti-goat IgG. Slides represented in images E1–E4 show the fluorescence response after incubation with GFP-*E. coli* O157:H7 cells at 10^7 cells/ml followed by AF647 labeled goat anti-*E. coli* O157:H7 IgG (primary reporter antibody) and then RR anti-goat IgG. Color images were transformed to grayscale where white is fluorescence and black is no fluorescence but do not necessarily represent quantified fluorescence intensities.

reducing the amount of attachment of reporter antibody. As a consequence the measured fluorescence intensity is lower. This phenomenon appears absent on assays on the covalently attached films. This is a qualitative observation and further studies will need to be performed to confirm and quantify this process.

The ROI RFU values resultant from the shelf life study were 1600–4300 with no significant increase or decrease of fluorescence intensity over the 100 days of storage (Fig. S-2). The ROI RFU values were similar to those generated by the antibody functionality experiments (Fig. 3A) at the same concentration with no significant difference ($P=0.7155$). Mean SNR values (Fig. S-2) yielded positive detection of *E. coli* O157:H7 at 10^7 cells/ml, showing that there was no loss of detection for the pneumatic spray slides stored up to 100 days. There was a significant difference ($P<0.0001$) between the SNR values for the stored slides and the antibody functionality experiments (Fig. 3C). The SNR values for the stored slides were higher. This difference was due to the lower background RFU achieved for the stored slides in comparison to the significantly higher ($P<0.0001$) values for the antibody functionality experiment slides (Fig. 3B). The mean background RFU values were between 1400 and 2500 in the first 3 slides but then decreased to 900–1700 for the

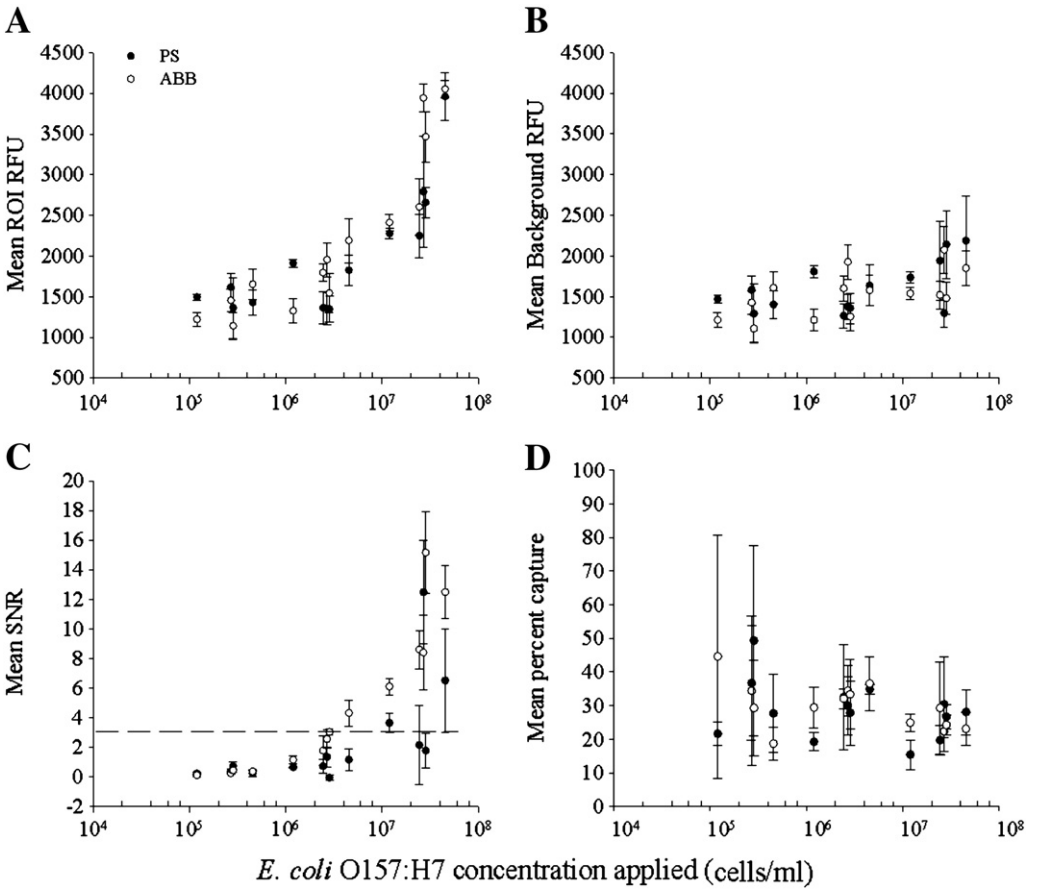

Fig. 3. *E. coli* O157:H7 assay and capture data obtained using pneumatic spray (PS) and avidin–biotin bridge (ABB) prepared slides. One sample concentration ranging from 10^5 to 10^7 cells/ml was used per slide with each slide having 6 replicate points (rows). Five slides were assayed per log concentration except for 10^5 cell/ml of which 4 were done. [A] Mean region of interest (ROI) and [B] background relative fluorescence units (RFU) for each slide assayed with *E. coli* O157:H7 and AF647 detector antibody. [C] Mean signal to noise ratios (SNR) calculated for each slide by subtracting the mean background RFU from the mean ROI RFU and then dividing by the mean background standard deviation. Sample slides with SNR≥3 (displayed via the horizontal dotted line) were considered as positive detection for *E. coli* O157:H7. [D] Mean percent capture efficiencies obtained for each slide assayed with *E. coli* O157:H7. Each circle point in each graph is 1 assayed slide at 1 cell concentration with error bars representing the standard deviation of the 6 replicates.

rest of the slides. It is possible that the cause was loss of the antibody indirectly immobilized between the film rows.

The specificity experiments yielded no detection of any of the non-*E. coli* O157:H7 strains tested. The mean SNR values of all pneumatic spray and avidin–biotin bridge assayed slides were $\leq 0.2 \pm 2$. In comparison, the *E. coli* O157:H7 positive control yielded a mean SNR of 15.2 ± 10.5 for the pneumatic spray and 14.6 ± 6.4 for the avidin–biotin bridge slides.

3.3. Cell capture efficiencies of assayed slides

The mean percent capture efficiencies (Fig. 3D) were similar for both slide immobilization processes at each concentration. There were no significant differences between the two techniques at 10^5 ($P = 0.5600$), 10^6 ($P = 0.1673$), or 10^7 cells/ml ($P = 0.9964$). Mean percent capture efficiencies against time stored ranged from 11 to 73% with the median range at 10–40% (Fig. S-2D). There was no significant difference ($P = 0.6599$) between these percent capture efficiencies and those generated by the antibody functionality experiments.

The similarity of the results suggests a compensatory process between antibody density and antibody orientation. The sprayed prepared slides have a higher antibody density with randomly immobilized antibody on the surfaces whereas the avidin–biotin bridge prepared slides have less antibody but clearly have a higher degree of orientation (Gehring et al., 2006). Studies suggest that random orientation reduces the ability of the antibody to react with the antigen due to the impact of steric hindrance generated by the arbitrary alignment of the antibodies (Lu et al., 1996; Xu and Zhao et al., 2006b). However, a reduction on antigen binding activity is not just related to randomly oriented films, because (Spitznagel et al. (1993) suggested that, despite the favorable orientation of the antibody at the surface of covalently attached films, molecular crowding can denature the fragment antigen binding (Fab) region making it necessary to find an optimal maximal coverage, which is not necessarily achieved by the avidin–biotin bridge method.

Peluso et al. (2003) found an average increase of 33% in antigen binding activity for specifically oriented antibodies when compared with randomly oriented antibodies with the same antibody density at the surface. At this point, it would seem likely that the avidin–biotin bridge method with orientated antibodies would supersede the sprayed method in terms of capturing cells but instead the sprayed prepared slides compensate this with antibody density.

As mentioned earlier, the pneumatic spray process creates a grainy surface morphology where antibody droplets are immobilized on top of each other or are overlapping. This creates a type of multi-layer antibody film where only the outer layer of randomly adsorbed antibody is responsible for the interaction with the antigen (Ngundi and Anderson, 2007) while the inner antibody layer acts as an attachment support by the antibody–antibody and antibody–surface interactions. (Kamyshny et al. (2001) suggested that the formation of antibody aggregates can favor the adsorption at the surface due to attachment of the aggregation unit to the surface, and that an increase in antigen binding activity is expected with a denser antibody layer. Xu and Williams et al. (2006a) noted that having a dense antibody surface reduces antibody structural unfolding and thereby increases the antigen binding capacity. Separate experiments (data not shown) confirmed this property by demonstrating that increases in antibody film thickness initially increased both capture efficiency and sensitivity, but ultimately reached thresholds after which no further improvement was achieved. Similarly, the avidin–biotin bridge film has a maximum threshold on antibody density at the surface. Peluso et al. (2003) using a gold coated glass surface, found that the oriented antibody immobilization achieved with the covalent attachment method usually resulted in a collateral decrease of the surface coverage (antibody density) when compared with random attachment.

The stability and functionality of an immobilized antibody film strongly depend on the type of substrate used, antibody density, and configuration of the antibody at the solid surface as well as its surrounding environment. In this study, immobilization of *E. coli* O157:H7 IgG on a glass surface using the pneumatic spray technique yielded a high density film with characteristics of antibody aggregation which in turn supported immobilization, stability, and potentially increased shelf life.

4. Conclusions

Polyclonal IgG antibody was successfully immobilized in a multi-row film onto glass waveguides using a pneumatic nebulizer. The results showed that the functionality of the high density, randomly immobilized antibody was similar to assays prepared with the often used avidin–biotin covalent attachment strategy. An investigation of the morphology of the pneumatic spray films suggests that about one to three compact layers of antibody are attached to the glass surface. The adherence of the layers is strong enough to withstand standard rinsing processes, allowing the efficient performance of assays. In summary, the reproducibility of the spraying method, the good stability of the films, capture efficiency, and the significant reduction in preparation time and chemical waste makes this new technique a promising alternative to traditional avidin–biotin covalent methods.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jim.2012.08.007.

References

Anderson, G.P., Jacoby, M.A., Ligler, F.S., King, K.D., 1997. Effectiveness of protein A for antibody immobilization for a fiber optic biosensor. Biosens. Bioelectron. 12, 329–336.
Arora, P., Sindhu, A., Dilbaghi, N., Chaudhury, A., 2011. Biosensors as innovative tools for the detection of food borne pathogens. Biosens. Bioelectron. 28, 1–12.
Batalla, P., Fuentes, M., Grazu, V., Mateo, C., Fernandez-Lafuente, R., Guisan, J.M., 2008. Oriented covalent immobilization of antibodies on physically inert and hydrophilic support surfaces through their glycosidic chains. Biomacromolecules 9, 719–723.
Borini, S., Staiano, M., Rocchia, M., Rossi, A.M., Rossi, M., D'Auria, S., 2007. Advanced nanotechnological approaches for designing protein-based "lab-on-chips" sensors on porous silicon wafer. Recent Pat DNA Gene Seq, 1, p. 1.
Cheng, M.S., Lau, S.H., Chow, V.T., Toh, C.S., 2011. Membrane-based electrochemical nanobiosensor for Escherichia coli detection and analysis of cells viability. Environ. Sci. Technol. 45, 6453–6459.
Cui, X., Pei, R., Wang, Z., Yang, F., Ma, Y., Dong, S., Yang, X., 2003. Layer-by-layer assembly of multilayer films composed of avidin and biotin-labeled antibody for immunosensing. Biosens. Bioelectron. 18, 59–67.
Daims, H., Lucker, S., Wagner, M., 2006. Daime, a novel image analysis program for microbial ecology and biofilm research. Environ. Microbiol. 8, 200.
Demarco, D.R., Lim, D.V., 2001. Direct detection of Escherichia coli 0157:H7 in unpasteurized apple juice with an evanescent wave biosensor. J. Rapid Methods Autom. Microbiol. 9, 241–257.
Feng, B., Huang, S., Luo, G.F., Jia, D., Dai, Y., 2011. 3D antibody immobilization on a planar matrix surface. Biosens. Bioelectron. 28, 91–96.
Gehring, A.G., Albin, D.M., Bhunia, A.K., Reed, S.A., Tu, S., Uknalis, J., 2006. Antibody microarray detection of Escherichia coli O157:H7: quantification, assay limitations, and capture efficiency. Anal. Chem. 78, 6601–6607.
Golden, J., Shriver-Lake, L., Sapsford, K., Ligler, F.S., 2005. A "do-it-yourself " array biosensor. Methods 37, 65–72.
Ikeda, T., Hata, Y., Ninomiya, K., Ikura, Y., Takeguchi, K., Aoyagi, S., Hirota, R., 2009. Oriented immobilization of antibodies on a silicon wafer using Si-tagged protein A. Anal. Biochem. 385, 132–137.
Kamyshny, A., Lagerge, S., Partyka, S., Relkin, P., Magdassi, S., 2001. Adsorption of native and hydrophobized human IgG onto silica: isotherms, calorimetry, and biological activity. Langmuir 17, 8242–8248.
Karlsson, M., Ekeroth, J., Elwing, H., Carlsson, U., 2005. Reduction of irreversible protein adsorption on solid surfaces by protein engineering for increased stability. J. Biol. Chem. 280, 25558–25564.
Leung, A., Shankar, M.P., Mutharasan, R., 2007. A review of fiber-optic biosensors. Sens. Actuators B. Chem. 125, 688–703.
Lim, D.V., Simpson, J.M., Kearns, E.A., Kramer, M.F., 2005. Current and developing technologies for monitoring agents of bioterrorism and biowarfare. Clin. Microbiol. Rev. 18, 583–607.
Lin, J.N., Herron, J., Andrade, J.D., Brizgys, M., 1988. Characterization of immobilized antibodies on silica surfaces. IEEE Trans. Biomed. Eng. 35, 466–471.
Lisboa, P., Valsesia, A., Colpo, P., Rossi, F., Mascini, M., 2010. Nanopatterned surfaces for bio-detection. Anal. Lett. 43, 1556–1571.
Lu, B., Smytha, R.M., O'Kennedy, R., 1996. Oriented immobilization of antibodies and its applications in immunoassays and immunosensors. Analyst 121, 29–32.
Majhi, P.R., Ganta, R.R., Vanam, R.P., Seyrek, E., Giger, K., Dubin, P.L., 2006. Electrostatically driven protein aggregation: B-lactoglobulin at low ionic strength. Langmuir 22, 9150–9159.

Moulin, A.M., O'Shea, S.J., Badley, R.A., Doyle, P., Welland, M.E., 1999. Measuring surface-induced conformational changes in proteins. Langmuir 15, 8776–8779.
Ngundi, M.M., Anderson, G.P., 2007. Failure of layer-by-layer multilayers composed of neutravidin-biotin-labeled antibody for sandwich fluoroimmunosensing. Biosens. Bioelectron. 22, 3243–3246.
Peluso, P., Wilson, D.S., Do, D., Tran, H., Venkatasubbaiah, M., Quincy, D., Heidecker, B., Poindexter, K., Tolani, N., Phelan, M., Witte, K., Jung, L.S., Wagner, P., Nock, S., 2003. Optimizing antibody immobilization strategies for the construction of protein microarrays. Anal. Biochem. 312, 113–124.
Plowman, T.E., Durstchi, J.D., Wang, H.K., Christensen, D.A., Herron, J.N., Reichert, W.M., 1999. Multiple-analyte fluoroimmunoassay using an integrated optical waveguide sensor. Anal. Chem. 71, 4344–4352.
Rowe, C.A., Tender, L.M., Feldstein, M.J., Golden, J.P., Scruggs, S.B., MacCraith, B.D., Cras, J.J., Ligler, F.S., 1999. Array biosensor for simultaneous identification of bacterial, viral, and protein analytes. Anal. Chem. 71, 3846–3852.
Sapsford, K.E., Liron, Z., Shubin, Y., S., Ligler, F.S., 2001. Kinetics of antigen binding to arrays of antibodies in different sized spots. Anal. Chem. 73, 5518–5524.
Sharma, H., Nguyen, D., Chen, A., Lew, V., Khine, M., 2010. Unconventional low-cost fabrication and patterning techniques for point of care diagnostics. Ann. Biomed. Eng. 39, 1313–1327.
Simpson-Stroot, J.M., Kearns, E.A., Stroot, P.G., Magaña, S., Lim, D.V., 2008. Monitoring biosensor capture efficiencies: development of a model using GFP-expressing Escherichia coli O157:H7. J. Microbiol. Methods 72, 29–37.
Spitznagel, T.M., Jacobs, J.W., Clark, D.S., 1993. Random and site-specific immobilization of catalytic antibodies. Enzyme Microb. Technol. 15, 916–921.
Taitt, C.R., Anderson, G.P., Ligler, F.S., 2005. Evanescent wave fluorescence biosensors. Biosen. Bioelectron. 20, 2470–2487.
Ulf, J., Magnus, M., Inger, R., 1985a. Immobilization of immunoglobulins on silica surfaces. Biochem. J. 227, 363–371.
Ulf, J., Magnus, M., Inger, R., 1985b. Immobilization of immunoglobulins on silica surfaces stability. Biochem. J. 227, 373–378.
Vermette, P., Gengenbach, T., Griesser, H.J., Divisekera, U., Kambouris, P.A., Meagher, L., 2003. Immobilization and surface characterization of NeutrAvidin biotin-binding protein on different hydrogel interlayers. J. Colloid Interface Sci. 259, 13–26.
Vijayendran, R.A., Leckband, D.E., 2001. A quantitative assessment of heterogeneity for surface-immobilized proteins. Anal. Chem. 73, 471–480.
Xu, H., Lu, J.R., Williams, D.E., 2006a. Effect of surface packing density of interfacially adsorbed monoclonal antibody on the binding of hormonal antigen human chorionic gonadotrophin. J. Phys. Chem. B. 110, 1907–1914.
Xu, H., Zhao, X., Grant, C., Lu, J.R., 2006b. Orientation of a monoclonal antibody adsorbed at the solid/solution interface: a combined study using atomic force microscopy and neutron reflectivity. Langmuir 22, 6313–6320.
Zhu, P., Shelton, D.R., Karns, J.S., Sundaram, A., Li, S., Amstutz, P., Tang, C., 2005. Detection of water-borne E. coli O157 using the integrating waveguide biosensor. Biosens. Bioelectron. 21, 678–683.