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A portable automated multianalyte biosensor

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Abstract

The array biosensor employs an array of capture molecules on a planar optical waveguide to interrogate multiple samples simultaneously for multiple targets. In assay development and demonstration studies published previously, we have quantified this biosensor's capability for rapid identification of a wide variety of targets in complex sample media. This paper describes the miniaturization and automation of the array biosensor for portability and on-site use. The fluidics have been redesigned and constructed with reliability and commercial production of disposable components in mind. To demonstrate the automated operation, simultaneous assays were automatically conducted on samples containing both ovalbumin and staphylococcal enterotoxin B. Results demonstrate the capability of the biosensor for detection and quantification.
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Keywords: Array biosensor; Multianalyte; Fluorescence; Portable biosensor

1. Introduction

A wide variety of sensors have been developed using optical waveguides, as is demonstrated in accompanying papers in this issue of *Talanta*. This paper focuses on an optical biosensor system designed especially for simultaneous detection of multiple targets in multiple samples. For that reason, the biosensor is based on a planar waveguide with sufficient surface area to accommodate many small (μm^2 to mm^2) sensing regions. The waveguide, a modified microscope slide, is illuminated using a 635 nm diode laser and a line generator, with the light launched into the proximal end. The first two-thirds of the slide serves as a mode-mixing region so that the light is relatively uniform in the 2.4 cm^2 sensing region near the distal end [1]. Under the conditions used here, total internal reflection is achieved and an evanescent field is produced in the sensing region. The evanescent light excites fluorophores bound in the sensing region, and the emitted fluorescence is measured at 90° using a Peltier-cooled CCD camera [2,3]. The location of the fluorescence within the array on the waveguide surface reveals the identity of the target detected.

In order to capture the target from the samples, antibodies or other molecules capable of binding to the target are immobilized on the waveguide surface in arrays of spots [4,5]. Both positive and negative controls can be included in the arrays to prevent false-positive or false-negative responses [6]. Furthermore, the use of multiple channels in combination with the arrays of sensor spots enables the analysis of multiple samples simultaneously. Assays can be formatted to detect either large molecules and microorganisms (sandwich assays) or small molecules (competitive assays, displacement assays) [7]. The use of near-infrared fluorescence prevents interference from sample components, which may autofluoresce at shorter wavelengths, making separation of the target from complex samples unnecessary prior to analysis [4,6,8,9]. In contrast to mass-sensitive sensors, such as the surface plasmon resonance (SPR), resonant mirror, or interferometric systems [10–13], the fluorescence-based array biosensor requires a fluorophore-labeled molecule for signal generation. This makes the assay relatively immune to interference from nonspecific adsorption by sample components [4,6,8,9].

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Most optical biosensors have been developed as laboratory systems. Commercially available systems that have been partially automated include the Biacore SPR system (Biacore International, Upsala, Sweden), the ORIGEN electrochemiluminescence system (IGEN, Gaithersburg, MD), the IAsys resonant mirror system (Fisons Affinity Sensors, Cambridge, UK), and the IMPACT displacement flow immunosensor (Lifepoint, Rancho Cucamonga, CA). Each of these is unique in its fluidics system design. The Biacore flows the sample over the sensor surface in a single pass through a flow channel; measurements are made continuously, usually until there is no further signal change. The ORIGEN processes discrete samples individually by capturing the target from the solution on magnetic beads, collecting them on a magnet, adding the chemiluminescent substrate, and measuring the light output. The IAsys stirs the sample in a flow-through cuvette located over the resonant mirror and measures the signal as binding occurs. The IMPACT automatically collects saliva and passes it through minicolumns containing antibodies specific for drugs of abuse. In the presence of the drug, a fluorescent analog of the drug is displaced from the immobilized antibody and measured downstream. These systems can only discriminate multiple targets by sending the sample over parallel sensing surfaces; the Biacore SPR system has four parallel channels in the standard unit, the ORIGEN system can have 8, the IAsys comes with 2 cuvettes and the IMPACT accommodates 10 flow columns.

In addition to the above described instruments for laboratory use, two optical biosensor systems, the FAST 6000 and the RAPTOR, have been commercialized for field operation by Research International (Monroe, WA). The FAST 6000 is a small (3.7 kg) displacement flow immunosensor for explosives and operates on the same principles as the IMPACT [14,15]. It is approximately the size of a laptop computer and automatically analyzes a manually added sample for up to six different targets with total assay times under 2 min. The RAPTOR fiber optic biosensor is portable (4.6 kg) and can automatically process samples added manually or pumped from a computer-controlled air sampler. The RAPTOR uses four optical fiber probes coated with antibodies to extract target from samples and generates a signal when a fluorescent tracer antibody binds to target captured by the antibody-coated probes. The entire operation, including data analysis and display, is automated [16]. Because it uses a sandwich fluoroimmunoassay, it has proven to be highly resistant to interference from complex sample components [17–19], and the current version of the automated device is proving to be highly reliable [16]. The main limitation of the RAPTOR is that it analyzes one sample at a time and is generally limited to the detection of only four targets, although the detection of eight targets has been recently reported [20].

In comparison to the sensors described above, an array format offers a number of advantages, such as the potential to analyze a sample for a large number of targets simultaneously. Furthermore, inclusion of positive and negative controls on each sensing surface is more reliable than such controls located on parallel but separate sensing surfaces. DNA array technology has led this effort in terms of laboratory devices; two notable systems employing optical waveguides include the systems marketed by Zeptosens (Witterswil, Switzerland) [21,22] and Illumina (San Diego, CA) [23]. These systems accommodate thousands of capture molecules and are highly sensitive. However, they are designed for use by highly trained laboratory personnel and have not been automated or adapted for on-site applications.

The focus of this paper is to describe the miniaturization and automation of the array biosensor. The array biosensor has progressed through a series of prototypes to obtain a reliable, small system to which a user can add six samples, with minimal, if any, sample preparation, and test for a variable number of targets [24–26]. The current system weighs less than 6 kg and is operated using a laptop computer. The user places two reservoir modules in the system, one containing up to six samples and the other containing up to six cocktails of tracer antibodies, and starts the assay. The fluidic system automatically runs the samples over the waveguide, exposes the waveguide to tracer, and washes out excess tracer. Image acquisition and data analysis are still performed offline, but the program to determine the boundaries of the spots and background controls and calculate mean increase in fluorescence over the background is semi-automated. The results of this study suggest that the array biosensor will be an effective, multi-analyte sensor for on-site testing for homeland security, food safety, agricultural monitoring, and clinical diagnostics.

2. Experimental methods

2.1. Instrumentation

The array biosensor detection system was designed to be portable and easy to use for untrained personnel (Fig. 1). The system has been mounted within a portable carrying case (Zero Enclosures, Salt Lake, UT) with a 50 W switching power supply (Sunpower, Taipei, Taiwan) where a cooled CCD camera (Retiga 1300, Q-Imaging, Burnaby, BC, Canada), peristaltic pumps (P625/66.143, Instec, Germany) and valves (Lee Company, CT) are controlled using a serial (RS-232) interface. An interface board (ADR-2000, Ontrak Control Systems, Sudbury, Ontario, Canada) converts the RS-232 signals into the required digital and analog control voltages for the pumps and valves. The image is acquired through a Firewire (IEEE 1394) connection, eliminating the need for a bulky frame grabber board and allowing a laptop to control all aspects of sensor function. A control program written with LabWindows (National Instruments, Austin, TX) provides a simple user interface to program the timing and flow of assay reagents.

Also enclosed within the carrying case is a 635 nm diode laser (LAS-635-15, Lasermax, Rochester, NY) equipped with a line generator to spread the laser beam into a fan pattern. The light launched into the edge of the microscope slide

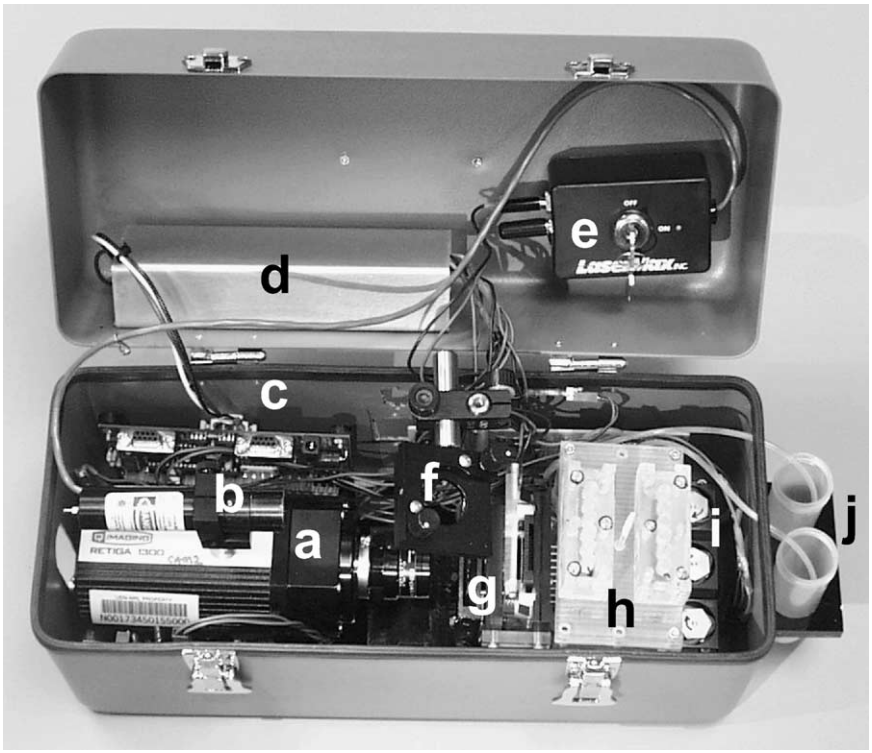

Fig. 1. Portable array biosensor components: (a) CCD camera, (b) diode laser, (c) RS232 interface board, (d) power supply, (e) laser power switch, (f) mirror, (g) slide mount, (h) removable reservoir modules, (i) peristaltic pumps, (j) waste and buffer reservoirs. Electronic control boards for pumps and valves are not visible. The valves are underneath the reservoir modules.

thus provides even fluorescence excitation across the viewing area. The CCD camera images the fluorescent array in the sensing region through a 700 ± 35 nm bandpass filter (P70-700-S, Corion, Franklin, MA) and a 665 nm longpass filter (RG665, Schott Glass, Duryea, PA) using a 17 mm C-mount lens (Schneider Optics, Hauppauge, NY).

The system uses two removable six-chamber reservoir modules (Fig. 1), each with a rubber septum on the bottom. Upon insertion of the reservoirs into the instrument, blunt needles mounted on the module platform penetrate holes pre-punched in the rubber septa, connecting the reservoirs to the rest of the fluidics system. Insertion of the prepared waveguide (described below) into the system is accomplished using a vertical mount equipped with a spring loaded plate. This plate presses the waveguide against a channel gasket molded in poly-(dimethylsiloxane) (PDMS, NuSil Silicone Technology, Carpinteria, CA), forming the six assay flow channels. Each of the six channels has an inlet located at the bottom of the chamber and an outlet at the top. Each outlet is connected to a pump that draws flow from the top of the vertical assay flow channels. The inlets at the lower end of the channels are connected to a bank of six 2-way valves. This bank is used to switch the flow through the flow channels from the two reservoir modules (Fig. 2), allowing either samples or tracer

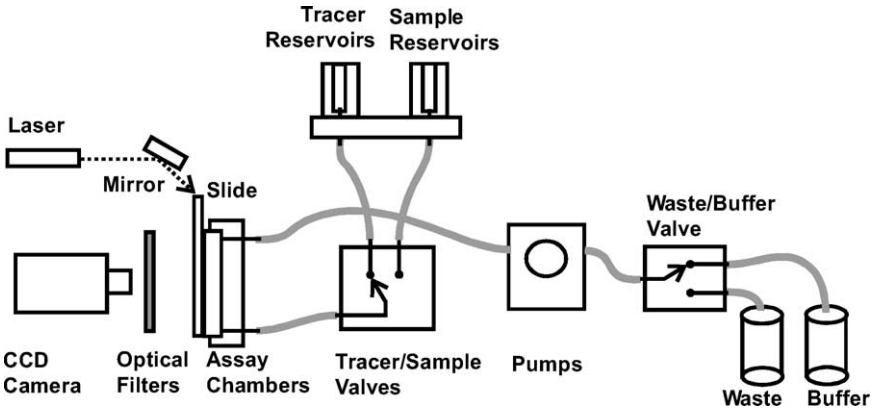

Fig. 2. Schematic illustration of array biosensor fluidics and optics systems.

reagents to be incubated with the waveguide. Valves at the pump outlets control flow to either waste (forward flow) or from a buffer reservoir to perform a final wash (reverse flow). The entire fluidics system was intentionally designed to keep the fluid components separate from the optics and electronics so that fluid leaks would not damage any optical or electronic components.

2.2. Reagents

Antibodies and antigens were purchased from the following sources: staphylococcal enterotoxin B (SEB), affinity-purified rabbit anti-SEB IgG, and affinity-purified sheep anti-SEB IgG from Toxin Technology, Inc. (Sarasota, FL); biotinylated rabbit anti-chicken IgY and Cy5-labeled chicken IgY from Jackson ImmunoResearch (West Grove, PA); rabbit anti-ovalbumin from US Biological (Swampscott, MA); and purified ovalbumin from Sigma-Aldrich (St. Louis, MO). Rabbit anti-SEB and rabbit anti-ovalbumin antibodies used as capture antibodies were biotinylated by incubation for 30 min with a five-fold molar excess of EZ-Link biotin-LC-NHS ester (Pierce, Rockland, IL) in 0.1 M borate, pH 8.5; unincorporated biotin was separated from labeled IgGs by gel filtration using Bio-Gel P-10 (Bio-Rad, Hercules, CA). Sheep anti-SEB and rabbit anti-ovalbumin antibodies used as tracers were labeled with either AlexaFluor 647 (Molecular Probes, Eugene, OR) or Cy5 dye (Amersham Biosciences, Arlington Heights, IL) as previously described [4,27,28]

The optical waveguides used in this study were standard soda lime microscope slides purchased from Daigger (Wheeling, IL). These slides were used as supplied (unclad) or were coated with a pattern of silver-based optical cladding (Opticoat Associates, Protected Silver; Chelmsford, MA). This cladding covered the area where the six-channel PDMS flow channel gasket made contact with the slide and has been shown to prevent stripping of the excitation light into the gasket material [1,29].

2.3. Preparation of substrates for assays

NeutrAvidin biotin-binding protein (Pierce) was covalently immobilized on the clad and unclad slides essentially as originally described [1]; after sequential treatment with 3-mercaptopropyl trimethoxysilane and *N*-(γ-maleimidobutyryloxy)-succinimidyl ester, the slides were then incubated overnight with 33 μg/ml NeutrAvidin in 10 mM phosphate buffer pH 7.4 containing 10 mM NaCl. Modified slides were then thrice washed and stored in the same buffer.

NeutrAvidin-coated slides were patterned with stripes of biotinylated capture antibodies using a 12-channel template molded from PDMS. Approximately 60 μl of biotinylated capture antibody (10 μg/ml, diluted in 10 mM phosphate buffer pH 7.4 containing 10 mM NaCl) was loaded into each channel (dimensions: 21 mm × 1 mm × 2.5 mm) and incubated overnight at 4 °C [1,9,27,33]. Fig. 3 shows the location

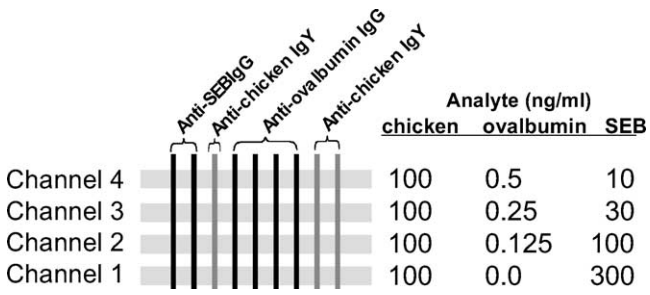

Fig. 3. Diagram of detection assay pattern. Recognition antibodies were immobilized in vertical columns with the above pattern, as described in Section 2.3. To perform the assay, each sample was flowed through channels (rows) along the length of the slide, and had the concentrations of ovalbumin and SEB noted at the right. After the sample step, the tracer solution containing 10 μg/ml Cy5-rabbit anti-SEB and 10 μg/ml Alexafluor 647-labeled rabbit anti-ovalbumin to form the sandwich assay, and 100 ng/ml Cy5-chicken IgY as a direct-assay positive control was flowed through the channels.

of each column of capture antibody patterned on the slides. After removal of the patterning solution, each channel was rinsed with 0.6 ml of phosphate buffered saline pH 7.4 containing 0.05% Tween 20 and 1 mg/ml bovine serum albumin (PBSTB). The patterning template was then removed and the slides were incubated for 30 min with 10 mg/ml bovine serum albumin in 10 mM phosphate buffer pH 7.4 (blocking/drying solution). The slides were then dried under a stream of nitrogen without further rinsing and stored at 4 °C until use; patterned slides treated in this manner have been stored up to 17 weeks with no significant loss in activity [9].

2.4. Assays

Dried, patterned slides were inserted into the instrument's vertical slide mount such that the columns of capture antibodies were perpendicular to the six-lane flow channel gasket. After insertion, a spring-loaded plate pressed the slide into the PDMS gasket, forming a fluid- and air-tight seal between the gasket and the surface of the patterned slide. Samples (Fig. 3) and tracer cocktail were pipetted into their appropriate reservoirs (approximately 0.6 ml in each reservoir) and the reservoir modules attached to the system. The tracer cocktail contained 10 μg/ml Cy5-rabbit anti-SEB, 10 ug/ml Alexafluor 647-labeled rabbit anti-ovalbumin, and 100 ng/ml Cy5-chicken IgY; thus, while multiplexed sandwich format assays were performed for SEB and ovalbumin, a direct binding assay was simultaneously performed for the positive control, Cy5-labeled chicken IgY.

3. Results and discussion

3.1. Instrumentation

3.1.1. Imaging

The components in the array biosensor were designed and selected for making a lightweight and durable portable sens-

ing system. A CCD imager has been shown to be better than other imaging alternatives, including a CMOS camera [30]. The array biosensor imaging system reported previously was a large format (1 in.^{2}, 1024 × 1024) CCD with a GRIN lens array used to provide 1:1 imaging of the slide surface onto the CCD [2,31]. Although this format provided a wide field of view in a compact space, the camera required a frame grabber card, consuming precious space and power. The present CCD camera is one-third the cost, requires half the space, has a digital shutter instead of a mechanical one, and needs only a firewire connection to acquire the images. Since the current CCD is significantly smaller (0.09 in.^{2}, 1280 × 1024, commercial 2/3 in. format) than the previously reported CCD, the GRIN lens (1:1 imaging) is not required. The standard C-mount lens used here provides macro imaging, but unlike the GRIN lens array, it exhibits slight distortion at the edges.

3.1.2. Reflective cladding

Since this system relies on total internal reflection for production of an evanescent field, any material that touches the surface of the waveguide can act as a cladding, or can scatter the excitation light out of the waveguide, reducing excitation energy. The slides used in these assays have a silver-based reflective coating (cladding) that is patterned on the surface wherever the PDMS material forming the flow channels touches the slide, leaving a clear portion in contact with the flowing sample that can be imaged at a 90° angle. Some slides were prepared and tested without the reflective cladding. These waveguides showed a high degree of scattering due to the PDMS material (Fig. 4a). The light scatter is so severe, it overwhelms the rest of the image, making the fluorescent spots undetectable. Fig. 4b shows an identical assay performed on a duplicate slide that had the reflective cladding. Both images were taken with identical exposure times. As is evident here, the reflective cladding not only prevents stripping of the excitation light as previously demonstrated [1], but also prevents light scatter from the interface between the PDMS and the sensor surface. Thus the reflective cladding is essential for obtaining reliable data.

3.1.3. Slide mount

The microscope slides are inserted into the vertical mount through a guide slot machined to high tolerance, ensuring that the slide positioning, and thus alignment of the reflective coating with the PDMS flow channels, is highly reproducible. The user then releases a spring-mounted aluminum plate that presses the slide against the PDMS, forming the flow channels. The active area of the slide is imaged through a hole in the plate. This method for insertion of the substrate significantly improves the reproducibility of array positioning, which also simplifies array search algorithms for automated data analysis.

3.2. Reservoirs

Each reservoir module has a rubber septum on the bottom, through which the needles pierce upon mounting in the instrument. Should the septa become leaky through repeated use, a plate mounted atop the modules presses them into the module mount, using the rubber septum as a gasket and providing double security on the fluidics seal. Previously, sample and tracer reservoirs were in the same fluidics block [1,3]. The current system has separate reservoir modules for each, allowing more flexibility in reagent/sample preparation. This design also allows the user to prepare a battery of sample and tracer modules to perform a series of experiments in a short time. The reservoirs are simple in design and would be amenable to manufacture by injection molding. The tracer reagents can be lyophilized directly in the tracer reagent reservoirs for storage, distribution, and field deployment [9]. The sample reservoirs can easily be replaced with connections to continuous monitoring systems if multiple sequential analyses are desired, rather than discrete assays. This feature extends the potential uses of this biosensor to include

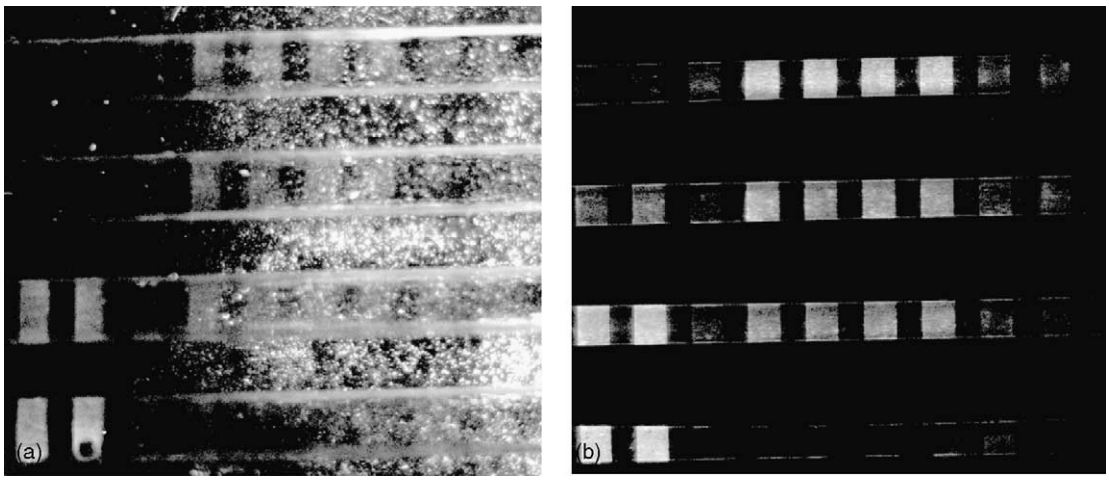

Fig. 4. (a) Image of assay results using an unclad slide; (b) image using a replicate slide with the reflective cladding. Both assays used the same samples and fluorescent reagents described in Methods and had the same image exposure time (2 s).

autonomous remote sensing in conjunction with electronically controlled air samplers such as the SASS (Research International).

3.3. Assays

After installing the reservoir modules with the sample and tracer solutions, the user selects the pump speed and timing for the introduction of sample, tracer and wash buffer from the assay control program. After the user actuates the programmed protocol, the entire sandwich assay is performed automatically, with no user interaction, and is completed within 15 min. After initiation of the assay by the user, approximately 0.6 ml of the appropriate sample was flowed through each channel for 480 s. The inlet valves were then automatically changed, such that the tracer cocktail (0.6 ml/channel) was then flowed through the channels for 360 s. After completion of the tracer cycle, the control program reversed the pumps and switched to the buffer reservoir, allowing a wash cycle to remove residual fluorescent reagent. An image was then made using camera control software.

To provide a system comparable to those previously described [1,3,4,8,9,24,27], a net flow rate of 0.1 ml/min was desired for the sample and tracer steps. However, the pumps were unable to reproducibly produce flow rates of less than 0.2 ml/min. They were therefore programmed to flow forward 10 s at full speed and then backward for 10 s at 3/4 speed to yield a lower equivalent flow rate. This back-and-forth movement of the samples and tracer solutions not only allows a lower overall flow rate to be achieved, but may also increase the mass transport of analyte and tracer to the surface of the waveguide [16].

Each sample contained either SEB, ovalbumin or both. Four samples were analyzed in parallel on each slide patterned with capture antibodies directed against SEB, ovalbumin, and chicken IgY (Fig. 3). The cocktail of tracer antibodies, used in the second step of the assay contained 10 μg/ml Cy5-rabbit anti-SEB, 10 μg/ml AlexaFluor 647-labeled rabbit anti-ovalbumin, and 100 ng/ml Cy5-chicken IgY. The anti-SEB and anti-ovalbumin antibodies bound to captured antigen in the sandwich assay, while the chicken IgY was bound directly by the rabbit anti-chicken on the surface to produce the positive control.

Images were acquired using camera control software (Qcapture, Q-Imaging, BC, Canada), and then analyzed using an image analysis program written in LabWindows. The areas in the channels surrounding the active (antibody-coated) regions are monitored as control regions to account for any non-specific binding of fluorophore, and thus are considered to be background above which the signal has to be detected [6]. The average intensity of the pixels in these background areas was subtracted off the average intensity of the fluorescent regions to yield a signal above background.

Fig. 5a and b shows a limited dose–response curve for SEB and ovalbumin, respectively, for each of three slides. The standard deviation for all concentrations on all three slides ranged from 1600 to 2100 for SEB and 1900–2100 for ovalbumin, with an overall average of approximately 2000 fluorescence units per assay, regardless of the assay or sample concentration. Values measured ranged from 0 to 37,000 fluorescence units; thus an error of ±2000 units was

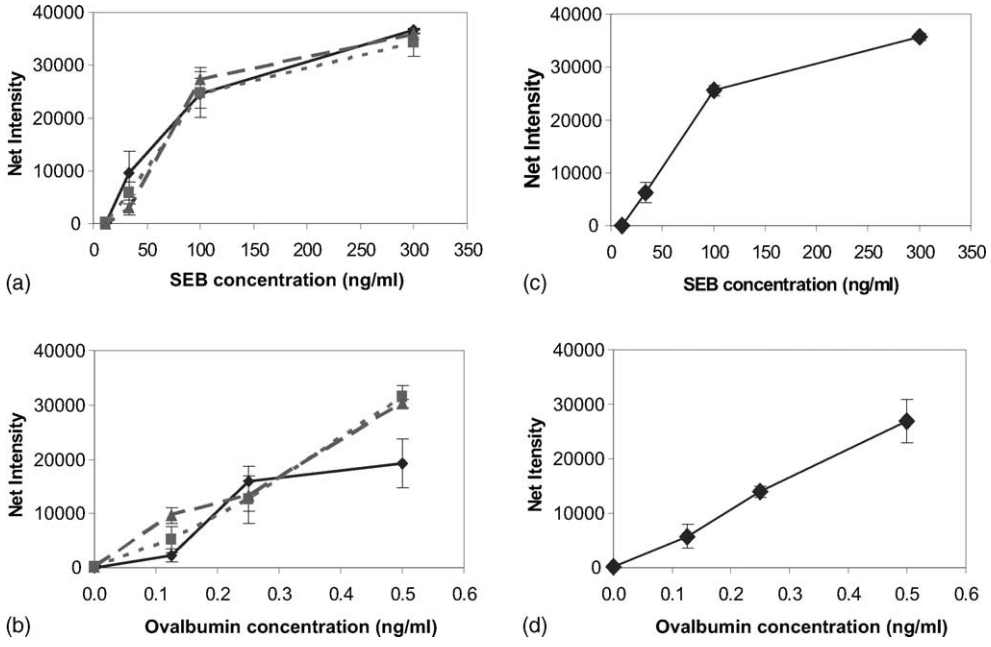

Fig. 5. Plot of assay data from three replicate slides. Fluorescence data was extracted from the images using the image analysis program. Plots in figures (a) and (b) show limited dose–response curves from a single slide for SEB and ovalbumin, respectively. Error bars show standard deviation of replicate assay spots on each slide. Plots (c) and (d) show the average of the mean signals from plots (a) and (b) with error bars showing standard error of the mean.

considered very good. The deviations appear independent of analyte concentration or assay, so they are more likely to be due to systematic variation rather than of biochemical origin. Though a full dose–response curve was not evaluated, the data indicate that the limits of detection are in the same range as determined previously using a manually operated array sensor and the identical antibodies, 10 ng/ml and 25 pg/ml for SEB and ovalbumin, respectively [24,33].

Fig. 5c and d shows the averages of the mean values for the three slides for the SEB and ovalbumin, respectively, and the standard errors of the mean. The variation *between* slides when assays were performed on different sensing surfaces averaged 900 fluorescence units for SEB and 1800 for ovalbumin. Thus the interslide variability was much lower using the automated biosensor than was previously observed using the same SEB antibodies in a nonautomated version [32], indicating that the reproducibility of the procedure for fabricating the sensing arrays, aligning them in the array biosensor, and performing the assays is greatly improved. Note that the concentrations of ovalbumin ranged from 0 to 0.5 ng/ml in contrast to those of SEB which ranged from 10 to 300 ng/ml. The higher variation in the values for ovalbumin could be partly explained by the greater difficulty in transferring such low concentrations reproducibly through the system.

4. Conclusions

Biosensors are widely touted as solutions to detection problems that can be used outside the laboratory. Yet few biosensors have actually been made portable and sufficiently automated to accomplish that goal. Nonetheless, while portable systems have been slow to reach the field, the capabilities of laboratory biosensors continue to expand. As a consequence, the expectations of potential users continually increase. The array biosensor described here combines optical waveguide technology and the capacity to test multiple samples simultaneously for multiple targets with portability and automation.

In addition to reducing the size of the biosensor, including fluidics, to that of a shoebox and demonstrating that the analyses can be run by simply loading the sample and pressing a laptop key, we have engineered a number of features important for reliability and commercialization into the system. First, the optics and fluidics components are divided into separate portions of the biosensor to prevent any unanticipated contact between the electronics and the fluids. Second, the optics are configured such that the tolerances for the addition of the replaceable components are generous; the waveguide is easily inserted without any tedious alignment and the reservoir modules have a needle/septum/clamp arrangement that is very simple for the operator and resists leakage. Third, the reservoir modules are designed to be amenable to injection molding for mass production. Finally, separation of the tracer and sample reservoir modules (1) facilitates lyophilization of the tracer reagents in the module, (2) eliminates the potential for sample and tracer mixing prior to the assay (which is important for preventing high dose hook effects), and (3) simplifies substitution of the sample module with connections to a continuous monitoring device.

Potential applications for on-site use of the array biosensor include diagnosis of infectious disease, monitoring for environmental pollutants, detection of biohazardous agents, and testing of foods for toxins and pathogens. The capacity of the array biosensor to detect relevant targets in these complex samples with little or no sample preparation has already been demonstrated using a non-automated prototype. Future studies with the automated system will determine how effectively the system actually transfers from the laboratory to the field.

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