

Applications of Array Biosensor for Detection of Food Allergens

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Although food is a necessity, compounds within food products can be dangerous and life-threatening for people with food allergies. These allergy-causing compounds, such as proteins from eggs and milk, must be identified on the labels of commercial products. Unintentional contamination of food with these compounds occurs as a result of storage, manufacturing procedures, or cleaning procedures. A sensitive, specific, and rapid method to identify foods containing allergens is required by the food industry. The array biosensor, a rapid detection system, may provide a solution to this need. The array biosensor performs fluorescent immunoassays on the surface of a planar waveguide by first running samples, then fluorescently labeled antibodies, over a surface patterned with capture antibodies. An optical image is captured by a charged-coupled device camera and converted into fluorescence values. Signal intensity and spot location provide information on the compound and its concentration. The array biosensor has been successfully demonstrated for toxin, bacteria, and virus detection at low levels in under 20 min in food, clinical samples, and environmental matrixes. An assay for detection of ovalbumin as an indicator of egg contamination has been developed with limits of detection of 25 pg/mL in buffer and 1.3 ng/mL (13 ng/g) in non-egg pasta extract (buffer:pasta 10:1, v/w).

Although the number of people with food allergies is relatively low (approximately 1–2% of adults and approximately 5% of children), allergen contamination is a priority issue with the U.S. Food and Drug Administration (FDA) and the food industry because of possible lethality (1, 2). The primary foods of concern are peanut, egg, milk, fish, wheat, tree nuts, soybean, and crustaceans (2–4). Proteins or glycoproteins from these foods are the actual trigger of the

allergic response (5, 6). Trace amounts of these food allergens can be life-threatening to sensitive individuals.

Contamination with these proteins can occur anywhere along the food processing chain from the food source to the home table. One major concern is the potential for cross-contamination during food processing. In the food industry, a processing plant is usually used to manufacture multiple products, some of which contain food allergens. Foods that may contain these allergens are required to be labeled appropriately (2, 7). Product labeling allows persons who are sensitive to certain ingredients to avoid ingesting the food product, thereby avoiding the potential adverse effects (4). The problem is that accidental contamination occurs (1, 4). Food processing procedures such as heating and/or proteolysis do not affect most food allergens (6, 8, 9). This accidental contamination has detrimental effects on food processors as a result of recalls, loss of consumer confidence, and possible liability. Procedures must be followed to clean a line prior to processing of food that does not contain allergens; however, these cleaning procedures cannot guarantee that trace amounts of the allergen are eliminated. Methods to test surfaces and products in real time and at detection limits below thresholds for an allergic response are not readily available.

Antibodies have played a major role in most allergen detection methods. Immunoelectrophoresis and immunoblot techniques have been used to detect nuts, milk, egg, and other allergens with detection limits around 5 µg/mL and 500 ng/mL, respectively (3, 10). The majority of allergen detection methods use the enzyme-linked immunosorbent assay format (ELISA; 3, 11). ELISAs have been developed for milk, almond, hazelnut, egg, peanut, soybean, and wheat (3, 8). Detections for these assays range from 0.1 to 200 ppm in food samples. There are several commercial ELISA test kits, including Veratox and Alert by Neogen, BioKits by Tepnel Biosystems Ltd., ELISA-TEK by ELISA Technologies, and RIDASCREEN by R-Biopharm, that test for milk, egg, peanut, and wheat among other assays (3). The detection for these commercial test kits ranges from 0.5 to 500 ppm. Disadvantages of ELISAs are the length of time to perform assays and the number of steps required. Polymerase chain reaction (PCR) assays are being developed, but concerns regarding processing effects on DNA and the fact that the assay does not detect the protein (the allergenic compound) need to be addressed (3). Biosensors for allergen detection have also been investigated. A peanut assay has been developed using SPREETA (Texas Instruments, Inc.), a

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biosensor that uses antibodies as the recognition molecule and surface plasmon resonance for detection with limits of detection (LOD) down to 700 ng/mL in peanut extract, corresponding to 7 ppm in the original sample (11). Another biosensor, a fiber optic biosensor, was used to detect ovalbumin as low as 20 ng/mL in spiked buffer (12). Again, antibodies were used as the recognition compound but fluorescence and evanescent wave sensing provided the detection format.

All of the methods described above, including the commercial ELISA kits (13–16), are designed to detect a single compound. A method to detect multiple compounds simultaneously with similar detection limits and shorter turnaround time would be extremely useful. The array biosensor was developed to detect multiple types of antigens in multiple samples simultaneously. In this paper, we will examine the potential use of the array biosensor to detect food allergens. Ovalbumin, one of several proteins thought to be involved in egg allergic reactions, will be used as the model for egg allergens. It is found in both the egg white and the yolk. In the egg white, ovalbumin is 54% of the protein. The other major allergenic egg proteins in decreasing percentages are ovomucoid, ovotransferrin, and lysozyme (17).

Array Biosensor

Principle of Operation

The array biosensor is a small portable device that simultaneously analyzes multiple samples for multiple analytes and is appropriate for on-site analysis. The system consists of 3 parts: a planar optical waveguide patterned with an array of molecular recognition elements, an image capture and data processing system, and a fluidics unit. The patterned array of recognition elements, usually antibodies, is immobilized onto one side of a microscope slide (planar waveguide) to capture any target analyte present in samples. After incubation of the patterned slide with sample, bound analyte is then quantified by means of fluorescent detector molecules (tracers). Upon excitation of the fluorescent label using a small diode laser to generate an evanescent wave, a charged-coupled device (CCD) camera detects the pattern of fluorescent antibody:antigen complexes on the sensor surface. The evanescent wave, an electromagnetic field, is produced when light is guided down a waveguide placed into a medium of lower refractive index under conditions of total internal reflection. The evanescent wave extends from the waveguide surface into the medium of lower refractive index and decays exponentially with distance. It is exquisitely selective for surface-bound fluorophores and allows detection of analytes in turbid or nonhomogeneous samples with little contribution or interference from other components in the bulk solution. The limited penetration depth of the evanescent wave also enables real-time measurement of binding events on the surface of the waveguide (18). This and other evanescent wave techniques in biosensing have been well established (12, 19–22). Image analysis software quantitates the fluorescent signals that can be associated with the identity

and concentration of the analyte based on the locus of the fluorescent spot. A semi-automated data analysis program (18) is used to calculate the mean fluorescence signal (MFS) values for each square in the matrix. Values that are 3 standard deviations above the fluorescent values for blanks are considered positive.

The array biosensor has been used to identify and quantify a variety of different targets in less than 20 min with little, if any, sample pretreatment or concentration (23–26). Samples with large particulates require filtering or centrifugation to remove particles that may block the fluidic system. Toxins (e.g., cholera toxin, botulinum toxoid, and ricin) and physiological markers (e.g., D-dimer, F1 from *Yersinia pestis*) have been detected in the low ng/mL (ppb) range; bacteria (e.g., *Erwinia herbicola*, *Francisella tularensis*, and *Bacillus globigii*, *B. anthracis*, and *Brucella abortus*) have been detected in the 10^3 – 10^5 colony-forming units (CFU)/mL range. Detection of these various agents has been performed in environmental (soil and sand suspensions, clay, pollen, and smoke extracts) and clinical matrixes (blood, sputum, and urine). Staphylococcal enterotoxin B has been detected down to 500 pg/mL (or pptr) in 6 different food matrixes (27). Assays for *Salmonella*, *E. coli* O157:H7, *Campylobacter*, and *Shigella* have been developed and tested in a variety of food matrixes with little, if any, loss of detection limits (mid 10^4 – 10^5 CFU/mL; 28–30, unpublished). Multi-analyte assays also have been demonstrated with several of the proteins and bacteria mentioned above (19, 23–25, 30, 31).

METHODS

Materials

Rabbit anti-ovalbumin antibodies were obtained from U.S. Biologicals (USB, Swampscott, MA) and provided by Robert Bull of Naval Medical Research Command (NMRC, Silver Spring, MD). EZ-Link Biotin-LC-NHS and NeutrAvidin were obtained from Pierce (Rockford, IL). Ovalbumin, phosphate buffer (PB), pH 7.4; phosphate-buffered saline (PBS), pH 7.4; bovine serum albumin (BSA); and Tween 20 were purchased from Sigma Chemical Co. (St. Louis, MO). The assay buffer is PBS containing 0.05% Tween 20 and 1 mg/mL BSA (PBSTB). 3-Mercaptopropyltrimethoxy silane (MTS) and N-(γ -maleimidobutyryloxy) succinimide ester (GMBS) were purchased from Fluka Chemical Co. (St. Louis, MO). Alexa Fluor 647 succinimidyl ester was purchased from Molecular Probes (Eugene, OR). Bio-Gel P10 was purchased from Bio-Rad (Hercules, CA).

Antibody Preparation

In a sandwich immunoassay, antibodies perform 2 functions: capture and tracer. The capture antibodies provide the initial specificity. They were immobilized onto NeutrAvidin-coated sensor surfaces with an avidin-biotin bridge. This immobilization procedure improves antibody functionality when compared with surfaces using direct covalent linkage (32, 33). Antibodies were biotinylated with a 5-fold molar excess of biotin-LC-NHS ester. After a 30 min

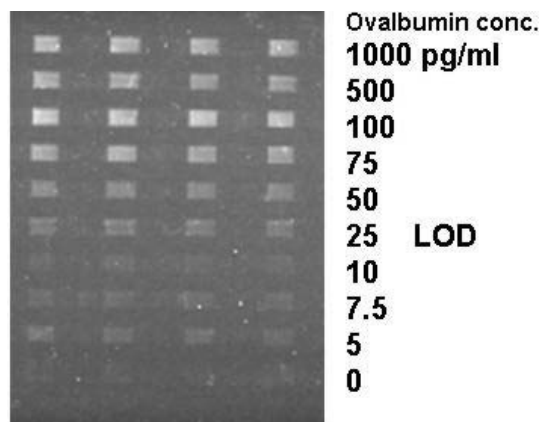


Figure 1. Image showing dose-response detection of ovalbumin with an array biosensor. Ovalbumin concentrations ranged from 1 to 1000 pg/mL.

incubation with activated biotin at pH 8.5, labeled antibody was separated from unincorporated biotin by gel filtration on Bio-Gel P10.

The second antibody (tracer) is used to identify that compound of interest that is bound to the antigen:immobilized capture antibody complex. The fluorophore Alexa Fluor 647 was coupled to the tracer antibody by incubating 1 mg antibody with 100 μ g Alexa Fluor 647 for 1 h at pH 9 (34). After incubation, the unincorporated dye was removed by gel filtration on Bio-Gel P10. The dye-to-protein ratio and the antibody concentration were determined from the absorbance at 280 and 650 nm.

Preparation of Antibody-Coated Substrates

The antibody immobilization procedure has been described in detail elsewhere (23). Borosilicate glass microscope slides (Daigger Scientific, Vernon Hills, IL) were used as waveguides. They were cleaned by immersion for 30 min in KOH (10%, w/v) in methanol, followed by copious

rinsing with deionized water and drying under a nitrogen stream. Immediately, the cleaned slides were silanized by immersion for 1 h in an anhydrous toluene solution containing 2% MTS under a nitrogen atmosphere. The slides were then washed in toluene and nitrogen-dried. The silanized slides were then immersed in a solution of 1mM GMBS in absolute ethanol for 30 min at room temperature. The waveguides were washed with water and incubated in 30 μ g/mL NeutrAvidin in PBS overnight at 4°C, and then washed in PBS and either used immediately for patterning with biotinylated antibody or stored at 4°C in phosphate buffer.

Patterning of the antibody was performed as previously described (25). Briefly a 6- or 12-channel polydimethylsiloxane (PDMS; MED-4011, Nusil Silicone Technology, Carpinteria, CA) flow cell was clamped onto the surface of a waveguide that had previously been coated with NeutrAvidin. Biotin-conjugated antibody (10 μ g/mL in 10mM sodium phosphate containing 10mM NaCl and 0.05% Tween, pH 7.2) was introduced into the channels of the flow cell and incubated overnight at 4°C. The antibody solution was removed, and the channels were rinsed with PBSTB. The slide was removed from the PDMS flow cell, rinsed in deionized water, and dried with N₂.

Assay

A sandwich immunoassay was performed to detect ovalbumin. To perform an assay, antibody-patterned slides were placed in contact with 6- or 12-channel PDMS assay flow guides; the channels in these flow guides were oriented perpendicular to the stripes of immobilized capture antibody and briefly exposed to PBSTB. Each test sample (0.8 mL/channel) was pulled through the channels at 0.08 mL/min by a peristaltic pump (Sarah model, Barnant Co., Barrington, IL). Each channel was then rinsed with PBSTB, and tracer antibody (0.4 mL/lane) was applied to each channel at a flow rate of 0.08 mL/min. Following a rinse of each channel with PBSTB, the assay flow guide was removed, and

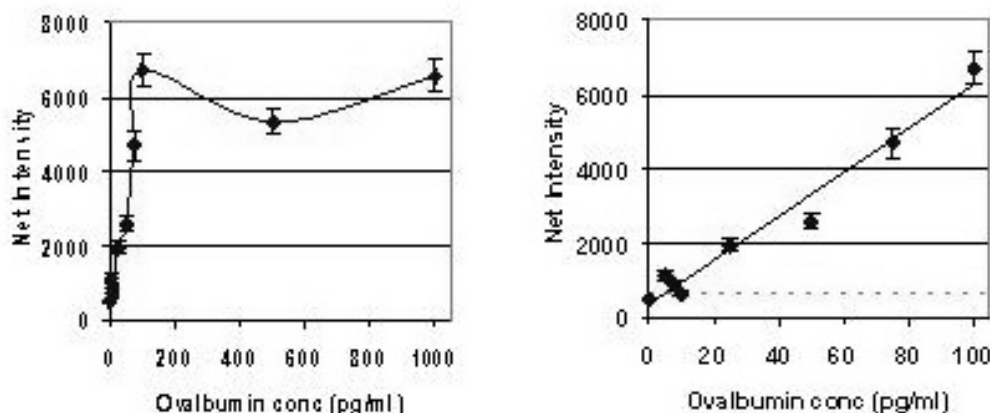


Figure 2. Ovalbumin dose-response curve. The left panel shows the full range of ovalbumin concentrations tested (0–1000 pg/mL). The right panel shows the linear range. The dotted line indicates the value for 3 standard deviations above the blank and the limit of detection. Values represent a minimum of 12 replicates $\pm$ standard error of the mean (SEM).

the slide was rinsed with deionized water, dried under N₂, and imaged.

Imaging

Optical components of the array biosensor have been described in detail elsewhere (19, 22). Excitation light from a 635 nm, 12 mW diode laser, fitted with a line generator, was launched into the end of the microscope slide opposite the end where the antibodies were patterned, and the biochemical assays were performed to generate an evanescent wave. The fluorescent spots were focused through a 2-dimensional GRIN lens array (35) onto a Peltier-cooled CCD camera. Long-pass and band-pass filters were installed to reject stray excitation light. Background intensities and fluorescent intensities within the array loci were determined with software from SpectraSource and Fortner Transform. The mean fluorescent signal for each square was calculated by subtracting the average of background intensities on each side of the square from the mean fluorescent intensity within the square. Because all fluids that pass over the antibody spots also pass over the background spots, the close proximity of the background spots to the antibody spots enables a correction both for non specific binding and for minor variations in the intensity of the excitation light. A signal was considered positive only if the mean fluorescence signal was higher than 3 standard errors of the mean above the mean signals from negative control lanes (buffer blank).

Pasta Sample Preparation

Two types of dry pasta, 100% durum wheat farina and egg pasta, were purchased at the local grocery store. The dry pasta was ground in a Waring Blender on high for 1 min. A 5 g portion of ground pasta was mixed with 50 mL PBST (1:10, w/v) and incubated at 60°C for 2 h with mixing. The mixture was clarified by centrifugation at 3500 rpm for 10 min. The supernatant was stored at 4°C until use. For the non-egg pasta, various concentrations (1 pg/mL–2.6 µg/mL) of ovalbumin were spiked into the supernatant. The egg pasta was used directly.

Results and Discussion

The anti-ovalbumin antibodies were tested to determine the best antibody combination for a sandwich immunoassay to obtain low detection levels and low background values caused by nonspecific binding. Both antibodies were biotinylated and fluorescently labeled to act as capture or tracer antibodies, respectively. The dye-to-protein ratio for the Alexa Fluor 647-labeled antibodies was 10:1 for the NMRC antibody and 4.2:1 for the USB antibody. Slides were patterned with several concentrations of each biotin-labeled antibody and tested with samples containing 0, 20, or 100 ng/mL ovalbumin in PBSTB. The 2 tracer antibodies (AF647-NMRC antibody, AF647-USB antibody) were also tested at several concentrations. The slides were evaluated for signal intensity at 20 and 100 ng/mL ovalbumin concentrations, and for

nonspecific fluorescent binding at 0 ng/mL ovalbumin and in the background readings.

The fluorescent signals from specific binding were similar for all antibody combinations tested. There was some nonspecific binding of the AF 647–anti-ovalbumin from NMRC at all concentrations of tracer tested, but no significant nonspecific binding from the USB tracer. The combination giving highest specific signal intensity and lowest nonspecific binding was the NMRC antibody at 10 µg/mL as the capture antibody and the USB antibody at 10 µg/mL as the tracer antibody.

An image of ovalbumin detection using the best combination of antibodies is shown in Figure 1. An ovalbumin dose–response curve in buffer was generated by using this pair (Figure 2). The LOD was 25 pg/mL (parts per trillion) in this 15 min assay, the lowest detection level yet observed for the array biosensor; the linear range was 25–100 pg/mL. Currently, there are no detection threshold values for allergens, as it is unclear how to establish that limit (2, 36). Most commercial assays detect in the low parts per million. Because such a low detection limit in buffer was obtained with the array biosensor, possible matrix interferences from food samples can be reduced or eliminated with buffer dilution.

To determine whether this method could be used to test real-world samples, buffer was used to extract the ovalbumin from egg and non-egg pastas. Samples (10:1, v/w, PBSTB/ground non-egg pasta) were spiked with ovalbumin to achieve final concentrations of 1 pg/mL–2.6 µg/mL; these concentrations correspond to initial concentrations of 10 pg/g–26 ng/g pasta. The LOD in wheat pasta was 12 ng/g (1.2 ng/mL) with a linear detection range of 12 ng/g–3.3 µg/g (1.2–327 ng/mL). From these preliminary studies of spiked samples, it appears that there is some loss of sensitivity between the non-egg pasta and buffer. Figure 3 shows an image of non-egg pasta spiked with 4 concentrations of ovalbumin and straight extracts from egg pasta. We detected ovalbumin in the egg pasta samples but have not yet

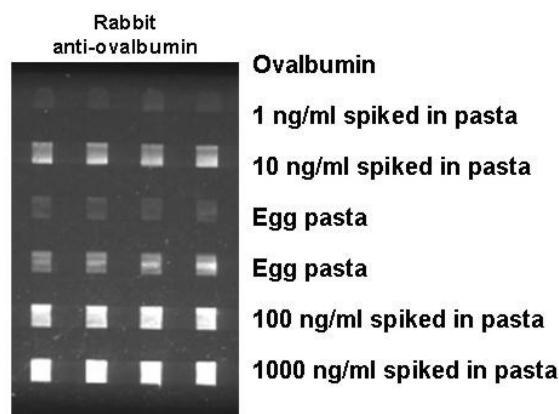


Figure 3. Image of ovalbumin detection in pasta. Non-egg pasta extract was spiked with 1, 10, 100, or 1000 pg/mL ovalbumin. Egg pasta extracts was used directly without spiking.

performed studies for the quantitation of ovalbumin in egg pasta. Currently, there is no standard validated method for allergen and, more specifically, ovalbumin detection against which these results can be compared. Further studies are being performed in collaboration with the FDA for the detection, quantification, and validation of this method for ovalbumin in a variety of food samples.

Conclusions

Low-level detection of bacteria and proteins in complex matrixes such as food, clinical, and environmental samples has been previously demonstrated with the array biosensor. In this study an assay was developed for ovalbumin as a model system for the detection of food allergens. The assay was performed in 16 min with 25 pg/mL (or parts per trillion) LOD in buffer and a detection limit of 12 ng/g in pasta extract. Future studies will include the detection of ovalbumin in a variety of foods, which, based on previous work, should not pose an insurmountable problem, assuming that the allergen is extractable by current methods.

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