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# An Array Immunosensor for Simultaneous Detection of Clinical Analytes

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**A fluorescence-based immunosensor has been developed for simultaneous analysis of multiple samples. A patterned array of recognition elements immobilized on the surface of a planar waveguide is used to "capture" analyte present in samples; bound analyte is then quantified by means of fluorescent detector molecules. Upon excitation of the fluorescent label by a small diode laser, a CCD camera detects the pattern of fluorescent antigen:antibody complexes on the sensor surface. Image analysis software correlates the position of fluorescent signals with the identity of the analyte. This immunosensor was used to detect physiologically relevant concentrations of staphylococcal enterotoxin B (SEB), F1 antigen from *Yersinia pestis*, and D-dimer, a marker of sepsis and thrombotic disorders, in spiked clinical samples.**

Immunoassay devices have become indispensable in clinical diagnostics due to the specificity and sensitivity of antibodies. The need for rapid, reliable diagnostic analysis of clinical samples provides a good niche for immunosensors, which are small, portable instruments for analysis of analytes in complex fluids. Immunosensors are designed for ease of use by untrained personnel, rapid assay times, and sensitivity comparable to ELISA methods. Antibody−antigen interactions have been utilized for detection and measurement of clinically relevant analytes using surface plasmon resonance,[1] resonant mirror,[2] continuous-flow immunosensors,[3] fiber-optic waveguides,[4−10] and planar waveguides.[11−13] However, to date, only fiber-optic immunosensors using evanescent wave measurements have detected analytes in clinical fluids as complex as whole blood.[8,10]

As immunosensors demonstrate the capability for rapid, quantitative analysis in clinical fluids, an additional feature of immunosensor performance becomes more important: the immunosensor should be able to discriminate between multiple analytes in a single sample. Reports of immunosensors capable of simultaneous detection of multiple analytes are beginning to appear in the literature,[11,12,14−18] but no data on analysis in clinical fluids are provided. In this paper, we demonstrate a planar waveguide immunosensor that can provide rapid quantitative analysis in a variety of clinical fluids without sample preprocessing and adapt it for simultaneous measurements of multiple analytes.

Three different types of analytes were spiked into buffer and clinical samples for the assays described here: staphylococcal enterotoxin B (SEB), plague F1 antigen, and D-dimer. In each case, the levels found in clinical fluids have already been defined, and the need for rapid diagnosis has been validated. The described array immunosensor was used to measure each of the three analytes in less than 35 min without prior treatment of the sample and with sensitivities similar to those previously described for other immunoassays. The use of both flow channels and avidin-coated waveguides facilitated patterning of biotin-labeled capture molecules into discrete areas. Utilization of a thermoelectrically cooled CCD camera reduced both background noise fluctuations, generally associated with room temperature CCDs, and size, associated with nitrogen-cooled CCDs, resulting in a portable, highly sensitive array immunosensor.

## EXPERIMENTAL SECTION

**Antigens and Antibodies.** The F1 antigen and monoclonal antibody YPF-6H3-1-1-IgG (6H3) were kindly provided by Dr. John

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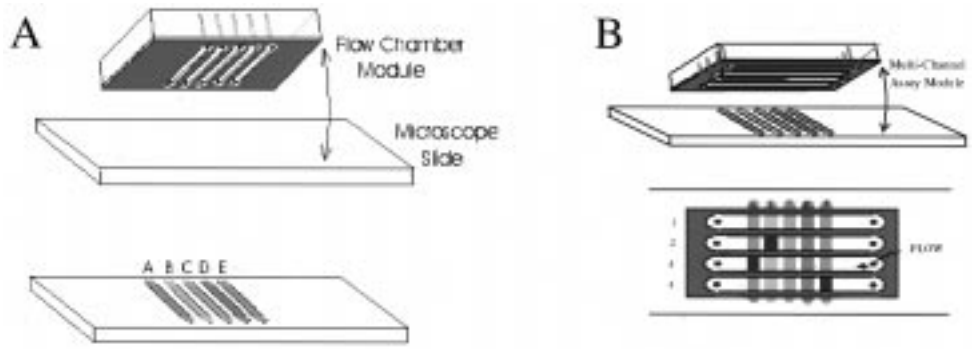


Figure 1. Patterning of capture antibodies and sample assay. (A) Physically isolated patterning. Biotin-labeled antibodies are loaded into vertically oriented channels in the flow chamber module. Upon incubation of the capture antibodies with the sensor substrate, they are effectively immobilized via an avidin−biotin bridge in vertically oriented stripes. (B) Application of sample. Samples are flowed through horizontally oriented channels in the sample flow chamber module. After rinsing, an appropriate detector molecule is flowed through the channels, and the substrate is again rinsed. If analyte is present in a sample, the corresponding spot fluoresces.

Ezzell at U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) and were prepared as described.[8] Purified D-dimer, high-molecular-weight D-dimer polymer (XL-FDP), and monoclonals DD-3B6/22 and DD-4D2/182 were a kind gift from AGEN Biomedical Ltd. (Brisbane, Queensland, Australia) and have previously been described.[19−21] Affinity-purified rabbit anti-SEB IgG, affinity-purified sheep anti-SEB IgG, and purified SEB were purchased from Toxin Technology, Inc. (Sarasota, FL).

Capture antibodies were labeled with biotin for immobilization onto the sensor surface. Biotin was introduced onto the hinge sulfhydryl group of DD-3B6/22 Fab′ fragments[20] according to Savage et al.[22] Anti-SEB IgG and 6H3 were labeled with biotin-LC-*N*-hydroxysuccinimidyl ester (Pierce, Rockford, IL) at pH 9 at a 5:1 molar ratio (biotin:antibody);[22] unincorporated biotin was removed from labeled proteins by dialysis or gel filtration. Detection antibodies were fluorescently labeled using Cy5 Bis-functional Reactive Dye ($\lambda_{ex} = 649$ nm, $\lambda_{em} = 670$ nm; Amersham Life Science, Pittsburgh, PA), according to the manufacturer's instructions. Dye-to-protein ratios ranged from 1.2 to 4.

**Physiological Samples.** Blood samples were obtained from informed, healthy volunteers and from patients admitted to an intensive care unit (ICU) with clinically suspected sepsis. Septic samples were a generous gift from Dr. Paul Eisenberg at Washington University, St. Louis, MO. Platelet-poor plasma was prepared from heparinized whole blood[23] and was fast frozen and stored at −20 °C. Immediately prior to use, aliquots of plasma were quick-thawed at 37 °C and centrifuged at 3000*g* to remove cryoprecipitate. Serum was obtained from clotted whole blood by centrifugation for 10 min at 2000*g*. Whole blood samples were diluted 1:1 with buffer before spiking and subsequent assay. Normal human urine was purchased from Utak Laboratories, Inc. (Canyon Country, CA). Nasal swabs and saliva samples were taken from healthy, informed volunteers. Nasal swabs were collected by swiping the interior of the nasal cavity, using 2 cotton-tipped swabs per nostril. The cotton swabs were then placed into 4 mL of PBS/0.05% Triton X-100 and incubated for 15 min. Upon removal of the cotton-tipped swabs, the samples were slightly turbid.

**Preparation of NeutrAvidin-Coated Slides.** The procedure for immobilizing avidin on planar waveguides is based on the chemistry of Bhatia et al.[24] All steps were performed at room temperature unless otherwise indicated. Microscope slides (Daiggerbrand, Wheeling, IL) were cleaned in 1:1 mixture of methanol: HCl for 20 min and then rinsed exhaustively with deionized water. After drying, slides were treated for 2 h (under nitrogen) with a 2% solution of (3-mercaptopropyl)trimethoxy silane (Fluka, Ronkonkoma, NY) in toluene. Silanized slides were rinsed thrice in fresh toluene and briefly air-dried. The slides were then incubated for 30 min in 2.1 mM *N*-($\gamma$-maleimidobutyryloxy)-succinimide ester (GMBS; Fluka) in anhydrous ethanol. After being rinsed three times in deionized water, slides were incubated overnight (4 °C) in 33 mM ImmunoPure NeutrAvidin (Pierce) in phosphate-buffered saline (PBS). NeutrAvidin-coated slides were rinsed with PBS and stored in PBS/0.01% $NaN_3$ at 4 °C.

**Patterning of Capture Antibodies (Physically Isolated Patterning, PIP).** Flow chamber modules for patterning of capture antibodies and sample analysis were made from poly-(dimethylsiloxane) (PDMS; Liquid Silicone Elastomer, NuSil Silicone Technology, Carpenteria, CA). PDMS cells were molded in a two-step process from a poly(methyl methacrylate) (PMMA) master template. The PMMA template, milled as a positive relief of the desired cell, contained six channels of dimensions 1.5 mm × 22 mm, separated by 2-mm gaps. The PMMA template was used to cast an inverse template in PDMS, and the final flow cell was molded from the inverse template. Polymerization of the PDMS templates was performed at 120 °C under vacuum to activate cross-linking and remove bubbles formed during curing.

Formation of a patterned array of capture antibodies on the sensor surface was accomplished as follows. The PDMS flow chamber module was placed onto the NeutrAvidin-coated waveguide such that the channels were vertically oriented (Figure 1A). Biotin-

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labeled capture antibody was flowed into each chamber (0.1 mL, 10 $\mu$g/mL in PBS) and was incubated for 3 h at room temperature, with shaking. Each channel was then washed three times with 1 mL of PBS/0.05% Triton X-100 (PBST). This procedure effectively produced six vertically oriented stripes of capture antibodies immobilized on the surface of the waveguide by an avidin−biotin bridge. Patterned slides were then used immediately or were treated with 15 mM sodium phosphate pH 7.4/0.1 M trehalose/10 mg/mL BSA (PTB buffer) for 15 min and dried under nitrogen. Dried, PTB-treated slides could be stored at 4 °C for at least 3 weeks.

**Analysis of Samples.** Samples were analyzed using a sandwich immunoassay format. Following patterning of capture antibodies on the surface of the waveguide, another PDMS assay template was placed onto the patterned slide such that the six channels were horizontally oriented (Figure 1B). Each channel was rinsed with 1 mL of PBST and blocked for 10 min with a solution of PBST containing 1 mg/mL BSA. After rinsing, sample was introduced to each channel and incubated, with flow (0.3 mL/min), for 15 min. Sample was then removed, and chambers were rinsed twice with PBST. Fluorescently labeled detector antibody (0.75 mL/channel, 5 $\mu$g/mL in PBST) was loaded into each lane and incubated, recirculating (0.3 mL/min), for 15 min. Following two rinses with PBST, the flow chamber module was removed, and the slide was dried under nitrogen before imaging with the CCD-based optical readout device.

**CCD-Based Optical Readout Device, Data Analysis.** The optical detection system is similar to that previously described;[11,15,25] a more detailed description of the optics, fluidics, system, data acquisition, and data analysis will be presented elsewhere (Feldstein, M. J., manuscript in preparation). Fluorescent complexes on the surface of the waveguide were excited using a 635-nm, 12-mW diode laser (Lasermax, Rochester, NY). A line generator was attached to the front of the laser to allow more even distribution of illumination throughout the waveguide. Light was launched through a 1-cm-focal length lens into the end of the waveguide at an appropriate angle, resulting in evanescent excitation of surface-bound molecules. A two-dimensional graded index of refraction (GRIN) lens array (Nippon Sheetglass, Summerset, NJ) was used to image the fluorescent pattern onto a peltier-cooled CCD camera. Long-pass (Schott 0G-0665, Schott Glass, Duryea, PA) and band-pass filters (Corion S40−670-S) were mounted onto the device scaffolding to eliminate excitation and scattered light prior to CCD imaging.

Data acquisition software allowed extraction and manipulation of fluorescence intensities from the CCD image. A virtual mask of the same size and dimensions of the array was aligned with the captured image and defined the array elements for data analysis. Mean pixel intensities, backgrounds, and signal-to-noise ratios were calculated and were available to the user in tabular format. The mean fluorescent signal for each square was determined by subtracting the background pixel intensities on each side of the square from the mean pixel intensity within the square.

***Safety Considerations.*** All clinical fluids and solutions containing SEB were handled by personnel wearing gloves and appropriate personal protective gear (lab coat, goggles). All equipment, benchtops, etc., exposed to physiological fluids and solutions containing SEB were disinfected with a 30% bleach solution and were rinsed with distilled water. Silicone tubing attached to peristaltic pumps (through which samples were pumped) and PDMS flow cell modules were rinsed with 30% bleach and distilled water after each experiment. Solutions containing SEB or clinical fluids were also treated with bleach (final concentration of 30%) and were rinsed down the sink with excess water. Contaminated disposables (test tubes, pipet tips, used sensor substrates) were placed in biohazard bags and later incinerated.

## RESULTS AND DISCUSSION

**Detection of *Staphyloccus aureus* Enterotoxin B (SEB).** Staphylococcal enterotoxin B (SEB) is a common cause of food poisoning, causing nausea, vomiting, and diarrhea when injested;[26] exposure by an aerosolized route is far more serious and may lead to irreversible hypotension, respiratory distress, shock, and death.[27,28] Animal studies have demonstrated that serum SEB levels can reach 500 ng/mL, and SEB can be detected in the urine of intoxicated animals at concentrations of 1−10 ng/mL for up to 24 h after exposure.[29]

Samples were assayed for SEB using NeutrAvidin-coated slides previously patterned with six columns of biotin-labeled rabbit anti-SEB IgG. Cy5-labeled sheep anti-SEB IgG was used as detector antibody. Figure 2A shows the pattern of fluorescent signals generated when SEB was spiked into samples at different concentrations. The 36-square array showed higher fluorescence intensity in those rows where samples containing higher concentrations of SEB were applied. To account for variability in patterning of capture molecules, as well as in excitation efficiency along the length of the slide, a sample of known concentration (in PBST buffer) was applied to each slide (row 5). The mean fluorescent signal for each square was then normalized to the corresponding square from the standard row. This method of normalizing data resulted in lower coefficients of variation when compared to nonnormalized signals. Coefficients of variation in sample intensities were approximately 17% (6−21%). Less than half of this variability was due to uneven illumination (4−8% coefficient of variation).

Figure 2B shows normalized signals obtained when SEB was spiked into buffer (open bars) or nasal swabs (closed bars) at final concentrations of 1, 5, 25, and 125 ng/mL. Unspiked controls are also included. A standard of 25 ng/mL SEB (in buffer) was run on all slides for normalization of signals and to allow comparisons between slides. Limits of detection (value greater than the blank $\pm$ 3 standard deviations) of both spiked buffer and spiked nasal samples were 1 ng/mL under the described assay conditions. A limit of detection of 0.02 ng/mL has recently been observed under different conditions using the same array immunosensor (S. Scruggs, unpublished results).

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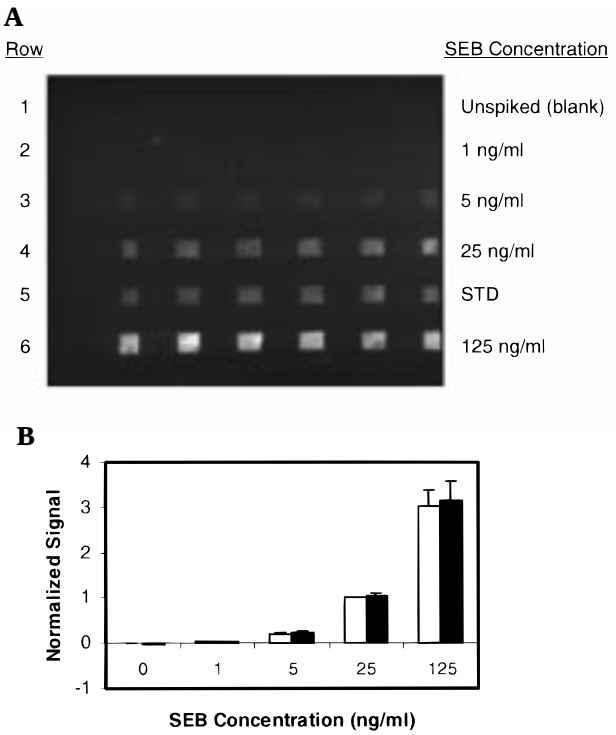


Figure 2. Detection of SEB. (A) SEB spiked into nasal swabs—pattern of fluorescence. SEB was spiked into nasal swabs at concentrations of 0, 1, 5, 25, and 125 ng/mL (indicated) and assayed on a slide patterned with six vertical stripes of biotin-labeled rabbit anti-SEB capture antibodies. After exposure of the array to Cy5-labeled sheep detector molecules, the slide was rinsed and imaged using the cooled CCD optical readout device. A standard of 25 ng/mL SEB in buffer was also assayed (channel 5) for calculation of normalized signals. (B) SEB spiked into buffer and nasal swabs—normalized signals. Buffer and nasal swabs were spiked with SEB at concentrations of 0, 1, 5, 25, and 125 ng/mL and assayed using the array biosensor as described. A standard of 25 ng/mL SEB in buffer was included on each slide and was used for calculation of normalized signals. Shown are mean normalized signals of six replicate assays of spiked buffer samples (open bars) and nasal swabs (closed bars) ± SEM.

The ability to detect SEB in spiked nasal swabs is potentially a very important result, since nasal swabs are the preferred diagnostic sample for exposure to aerosolized infectious or intoxicating agents.[30] Spiked nasal swabs generally gave higher normalized signals than those from identically spiked buffer samples. Although these differences are not statistically significant, this slight enhancement was reproducible. This effect may presumably be due to the presence of *S. aureus* in the nasal samples assayed. A large percentage of the population (20−65%) carry nasal enterotoxigenic *S. aureus* asymptomatically.[31−33] However, signals from unspiked nasal swabs were the same as or less than those from buffer controls. No attempt was made to culture *S. aureus* from the nasal samples used in this study.

The array immunosensor was unable to detect physiologically relevant SEB concentrations (≤125 ng/mL) in spiked urine, saliva, and blood products; no fluorescence above background levels was observed. These negative results were obtained with samples taken from five healthy volunteers who showed no symptoms of *S. aureus* infection or SEB intoxication. It is uncertain whether the negative results were due to interfering substances in these samples or to endogenous antibodies directed against SEB. Although SEB has been detected in spiked serum and urine samples at appropriate concentrations using a fiber-optic immunosensor,[9] there was a 30% decrease in signal in the presence of serum and a 50% decrease in spiked urine samples. Moreover, a considerable number of samples were screened to obtain samples which would give positive results in the fiber-optic assays (G. Anderson, Naval Research Laboratory, personal communication). Several studies have shown that a significant percentage of healthy individuals have detectable titers of antibodies directed against staphylococcal enterotoxins, in some cases up to 90% of the population.[34] The effect of these endogenous antibodies could be to prevent binding of added SEB to the immobilized capture molecules or to block binding of labeled detector antibodies in the sandwich assay.

**Detection of *Yersinia pestis* F1 Antigen.** Plague may be responsible for the deaths of 200 million people throughout recorded history and continues to persist in a number of countries.[35] While DNA hybridization, PCR, and ELISA techniques provide more timely on-site diagnosis of plague than standard bacteriologic and serologic tests,[36−38] these methods are not rapid diagnostic tools and are generally used in a confirmatory manner.

The glycoprotein, F1, is a major component of the outer membrane of *Y. pestis*, the etiologic agent of plague. F1 serves as a protective antigen and is secreted by *Y. pestis* only upon invasion of a mammalian host. Direct detection of F1 by ELISA and RIA is now becoming a more routine diagnostic test for plague.[39,40] F1 is detected in clots and serum samples in the high nanogram per milliliter to the low microgram per milliliter range 2−3 days after exposure.[37,39,41]

F1 samples were assayed using the same sandwich immunoassay format as used for SEB. Due to the polymeric nature of F1, monoclonal antibody 6H3 was used for both capture and detection of antigen. Figure 3 indicates normalized signals for buffer and clinical fluids spiked with 25, 125, and 625 ng/mL F1 (stippled, solid, and striped bars, respectively); unspiked controls are indicated with open bars. A standard of 125 ng/mL F1 in buffer was included on all slides for calculation of normalized signals and comparison of results between slides.

F1 was detectable at 25 ng/mL in buffer and in all spiked body fluids; concentrations below this level are not generally encountered in clinical samples[37,39,41] and were not analyzed using this

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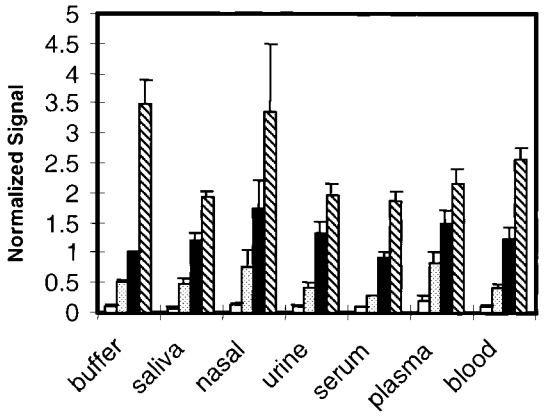


Figure 3. F1 spiked into buffer and physiological fluids. F1 antigen was spiked into buffer and undiluted saliva, nasal swabs, urine, serum, and plasma at concentrations of 0, 25, 125, and 625 ng/mL; whole blood was diluted 2× with PBST before spiking. Samples were assayed on slides patterned with biotin-labeled 6H3 IgG; Cy5-labeled 6H3 IgG served as detector molecule. A standard of 125 ng/mL F1 in buffer was assayed on all slides and was used for calculation of normalized signals. Bars for samples spiked with 25 (stippled), 125 (solid), and 625 ng/mL (striped) represent the mean normalized signals of four replicates ± SEM. Bars for unspiked samples (open bars) represent mean normalized signals of eight replicates ± SEM.

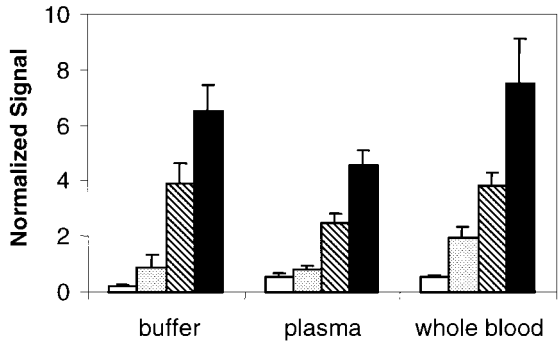


Figure 4. High-molecular-weight XL-FDP spiked into buffer, plasma, and whole blood. High-molecular-weight XL-FDP at concentrations of 50 (stippled bars), 200 (striped bars), and 1000 ng/mL (solid bars) was spiked into buffer, control plasma, and whole blood diluted 1:1 with buffer. Unspiked samples are indicated with open bars. Samples were assayed for D-dimer using biotinylated DD-3B6/22 as capture molecule and Cy5-labeled DD-4D2/182 for detection of bound analyte. Normalized signals (shown) were calculated using a sample of 100 ng/mL D-dimer in buffer as standard. Each bar represents the mean normalized signals of 12 (buffer, plasma samples) or 6 replicates (whole blood) ± SEM.

method. Negative controls (unspiked samples) indicated that these signals were due to added F1 rather than to endogenous components of the samples. There was no significant difference in signals obtained with spiked blood products, urine, and saliva and those from buffer spiked with 25 and 125 ng/mL F1. However, at 625 ng/mL F1, signals from these clinical fluids leveled off; a maximum 2.3-fold increase in normalized signal was observed for a 5-fold increase in F1 concentration (versus a 3.5-fold increase in signal for samples spiked into buffer). This effect was not due to simple protein−protein interactions; up to 30 mg/mL BSA could be added to spiked buffer samples without a decrease in fluorescent signals (data not shown).

A trend was observed with F1-spiked nasal swabs which was similar to that observed with SEB-spiked nasal samples (above). Although differences were not statistically significant, spiked nasal swabs generally resulted in normalized signals which were higher than those from identically spiked buffer controls. As with the other spiked clinical fluids, this effect was not due to background fluorescence from unspiked swabs.

**Detection of D-Dimer.** D-dimer is the most well characterized product of plasmin-mediated proteolysis of cross-linked fibrin. Although this 189-kDa protein is a normal component of the blood in healthy individuals, high concentrations of D-dimer are indicative of disseminated intravascular coagulation, pulmonary embolism, myocardial infarction, and deep venous thrombosis,[20,21,42,43] as well as sepsis and infection.[44,45]

D-Dimer concentrations were assessed in buffer, plasma, and whole blood, using the antibody pair of DD-3B6/22 and DD-4D2/182 as capture and detector antibodies, respectively. High-molecular-weight XL-FDP was spiked into whole blood (diluted 1:1 with buffer before spiking), buffer, and undiluted plasma at concentrations of 50, 200, and 1000 ng/mL; the concentrations utilized here encompass the range found in normal, healthy individuals (25−150 ng/mL) and in patients with sepsis, myocardial infarction, and thrombotic disorders (≥125 ng/mL). Lower concentrations of D-dimer (<50 ng/mL) were not assayed using the array immunosensor; quantitation of these low (normal) concentrations is not relevant to clinical diagnoses. Although D-dimer was used as a normalization standard (due to its uniform composition), high-molecular-weight XL-FDP was the cross-linked fibrin species used to spike buffer, plasma, and whole blood. This polymerized form of D-dimer is more representative of the native species found in the circulation than the 189-kDa form.[42]

Concentrations of 50 ng/mL XL-FDP or greater could be detected in buffer, plasma, and diluted whole blood (Figure 4). Fluorescent signals from unspiked plasma and whole blood were higher than those from unspiked buffer samples (open bars). This result was probably due to the presence of endogenous D-dimer, which is normally present in the circulation at low concentrations. However, despite endogenous levels of D-dimer, normalized signals for spiked plasma samples were lower than those of spiked buffer controls; an inhibitory effect of plasma on antibody binding to D-dimer has previously been documented.[10]

However, signals from spiked whole blood were greater than those from spiked plasma and did not differ significantly from spiked buffer controls. This increment over signals obtained with spiked plasma may be due to additional D-dimer species present in the whole blood samples. As there is no commercially available method for quantifying D-dimer levels in whole blood, the results presented in Figure 4 indicate the amount of XL-FDP *added*, rather than the final concentration.

Plasma samples taken from five septic patients were assayed in the same manner as the spiked control plasma samples, above. Figure 5 shows normalized signals obtained with the high molecular weight XL-FDP-spiked plasma samples (○) and plasma

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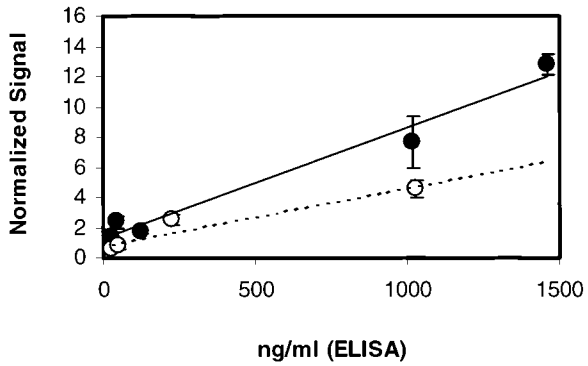


Figure 5. Normalized signals from spiked plasma and samples taken from septic patients. Spiked control plasma (○) and samples from septic patients (●) were assayed for D-dimer using ELISA and the array biosensor, as described for Figure 4. Shown on the ordinate are the mean normalized signals from 5 (septic samples) or 10 replicates (spiked plasmas) ± SEM. Values on the abscissa indicate mean concentrations determined by ELISA. The linear regressions to which the data were fitted are represented by the equations $y = 0.0038x + 0.83$ for spiked plasma samples (dashed line) and $y = 0.0058x + 0.70$ for septic samples (solid line). $R^2$ values were 0.92 and 0.98, respectively.

samples from septic patients (●) as a function of *final* D-dimer concentration. The final concentration of D-dimer in all samples was determined by ELISA (DimerTest EIA, AGEN Biomedical Ltd.); values for spiked samples also included endogenous D-dimer. Although the number of data points is limited, the data were fit to linear regressions with $R^2$ values of 0.92 and 0.98 for the spiked and septic samples, respectively. The slopes of the trendlines indicate that the septic samples tended to result in higher fluorescent signals than XL-FDP-spiked controls with comparable D-dimer concentrations. This phenomenon has been previously observed using an immunosensor assay based on the same capture:detection antibody pair.[10]

**Comparison to Other Immunoassay Methods.** In addition to ELISA and RIA, other immunoassay methods for detection and quantitation of these three analytes have been described. Rapid, bedside tests have been developed based on agglutination of latex particles or red cells. Latex agglutination assays for D-dimer in plasma and SEB in food samples are commercially available. Although easy to perform, these assays are semiquantitative at best. Latex D-dimer tests require only 3−5 min for results, but sensitivity is limited to approximately 200−250 ng/mL.[46] The latex agglutination assay for SEB, on the other hand, is sensitive to 0.5 ng/mL but requires 24 h and has demonstrated matrix effects.[47,48]

A 2-min whole blood test for D-dimer has recently been described.[49] This test, which agglutinates red cells in the presence of high D-dimer concentrations, requires no sample preparation. However, false negatives and results inconsistent with other diagnostic procedures have been documented.[49−51]

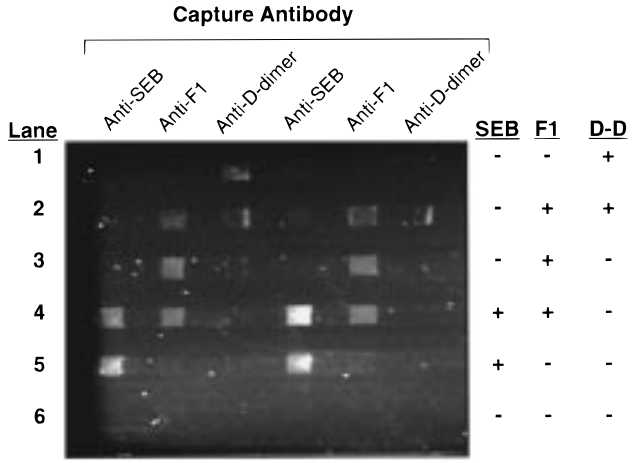


Figure 6. Simultaneous detection of multiple analytes. Samples were analyzed on a NeutrAvidin-coated slide patterned with biotinylated antibodies directed against F1, SEB, and D-dimer (indicated above the frame). Samples included PBST buffer spiked with SEB (50 ng/mL), F1 (625 ng/mL), D-dimer (500 ng/mL), or combinations thereof (indicated). After samples were washed from the channels, a cocktail containing 10 μg/mL each of fluorescent antibodies directed against SEB, F1, and D-dimer was applied to detect bound analytes.

Recently, other evanescent wave immunosensors have been described for rapid detection and measurement of F1, SEB, and D-dimer. These assays consist of sandwich fluoroimmunoassays performed on the surface of an optical fiber[8−10] or a planar waveguide.[11] The planar array system of Wadkins and co-workers[11] is a direct precursor of the immunosensor described here. Each of these systems has demonstrated the advantages of evanescent wave sensing:[5,52] analyte detection in complex, "dirty", or opaque samples; freedom from electromagnetic and radio frequency interference; lack of sample destruction; decreased optical interference from fluorescent components of the samples; rapidity of measurement; and ease of use. Not surprisingly, limits of detection and assay times (15−35 min) reported for these systems were similar to those of the current array immunosensor since antibodies were the same and surface chemistries were similar.

**Multianalyte Detection.** Although the assays described above utilized the patterned array of capture molecules as replicate tests for each sample, each channel of capture antibody could be used to test for a different analyte. Figure 6 shows the pattern of fluorescence obtained when the sensor substrate was patterned with capture antibodies directed against all three analytes; two columns of each capture molecule were patterned to provide duplicates for each sample (indicated above each lane). Samples were applied as indicated. For detection, a cocktail containing 10 μg/mL each of labeled sheep anti-SEB IgG, 6H3 IgG (anti-F1), and DD-4D2/182 (anti-D-dimer) was utilized.

Although there was an increase in nonspecific binding of labeled detector molecules, the pattern of fluorescent signals was clearly observed. Samples containing only a single analyte (rows

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1, 3, and 5) gave rise to fluorescent signals only in those locations where the appropriate capture antibody had been patterned. Similarly, multiple fluorescent signals could be observed where samples contained more than one analyte (rows 2 and 4).

In addition, a dual-analyte assay was performed to detect those analytes which could be measured in plasma samples, F1 and D-dimer (data not shown). All plasma samples tested (unspiked and F1-spiked control plasmas, septic plasmas) gave rise to fluorescence in columns where D-dimer antibody had been patterned, indicating the presence of endogenous D-dimer in the plasma samples. However, signals from septic samples were significantly greater than those from control plasmas, as expected. Likewise, only samples spiked with F1 gave rise to fluorescence in the appropriate channels.

## CONCLUSION

The array immunosensor described here takes multianalyte sensing a step further than previous demonstrations. While the fiber-optic biosensor[16] and the flow immunosensor[3] have been used for multianalyte detection, each capture antibody must be immobilized on a separate sensing surface. Although the Wadkins array biosensor[11] put multiple capture antibodies on a single surface, the detection antibodies were added sequentially. While other array immunosensors have potential for the detection of multiple analytes, supporting data have been shown for detection of only a single species at a time.[12,13,17] The immunosensor described herein, however, demonstrates analysis of multiple samples in parallel and simultaneous detection of more than one analyte per sample. Finally, the array immunosensor was effective for detection and measurement of analytes at physiologically relevant concentrations in a variety of clinical samples.

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