

# Chapter 8

## Protein Microarrays for the Detection of Biothreats

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**Abstract** Although protein microarrays have proven to be an important tool in proteomics research, the technology is emerging as useful for public health and defense applications. Recent progress in the measurement and characterization of biothreat agents is reviewed in this chapter. Details concerning validation of various protein microarray formats, from contact-printed sandwich assays to supported lipid bilayers, are presented. The reviewed technologies have important implications for in vitro characterization of toxin–ligand interactions, serotyping of bacteria, screening of potential biothreat inhibitors, and as core components of biosensors, among others, research and engineering applications.

### 8.1 Introduction

Both public health and defense concerns necessitate the development and availability of analytical technologies capable of ready detection and reliable identification of biothreats. For the purposes of this discussion, biothreats consist of pathogens, viruses, and toxins. Examples of naturally occurring biothreats include emerging infectious diseases, infections that often appear unexpectedly and have the potential to spread through a population or increase in severity of each incidence. Recent outbreaks include a highly contagious African viral hemorrhagic fever (Ebola) in 1974, hantavirus pulmonary syndrome in the southwestern United States in 1993, and severe acute respiratory syndrome (SARS) in 2003 [1]. Biothreats with relevance to defense concerns include ricin, anthrax, and botulinum.

Table 8.1 summarizes biothreats of interest to the U.S. National Institutes of Allergy and Infectious Diseases, an institute of the National Institutes of Health. Historically, identification of microbes and viruses has relied upon bacterial culture and viral replication in a host, respectively, whereas identification of toxins employed

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**Table 8.1** U.S. National Institutes of Allergy and Infectious Diseases (NIAID) Priority Pathogens**Category A***Bacillus anthracis* (anthrax)*Clostridium botulinum**Yersinia pestis**Variola major* (smallpox) and other pox viruses*Francisella tularensis* (tularemia)

Viral hemorrhagic fevers

Arenaviruses (LCM; Junin, Machupo, Guanarito viruses; Lassa fever); Bunyaviruses (Hantaviruses, Rift Valley fever)

Flaviruses (Dengue)

Filoviruses (Ebola, Marburg)

**Category B***Burkholderia pseudomallei**Coxiella burnetii* (Q fever)*Brucella* species (brucellosis)*Burkholderia mallei* (glanders)Ricin toxin (from *Ricinus communis*)Epsilon toxin of *Clostridium perfringens**Staphylococcus* enterotoxin BTyphus fever (*Rickettsia prowazekii*)

Food and waterborne pathogens (bacteria, viruses, protozoa)

**Category C**

Tickborne hemorrhagic fever viruses (Crimean-Congo hemorrhagic fever)

Tickborne encephalitis viruses

Yellow fever

Multidrug-resistant TB

Influenza

Other rickettsias

Rabies

Severe acute respiratory syndrome-associated coronavirus (SARS-CoV)

Antimicrobial resistance

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Source: Accessed on September 18, 2006 at: [http://www3.niaid.nih.gov/Biodefense/bandc\\_priority.htm](http://www3.niaid.nih.gov/Biodefense/bandc_priority.htm).

biological assays [2, 3]. Consequently, these conventional means for identification of biothreats are not rapid enough to facilitate quick responses from public health agencies in a large-scale event associated with a biothreat.

Ideal biothreat surveillance necessitates the measurement of multiple analytes quickly in a single sample with little to no sample preprocessing. In reality, multiplexed quantitative measurement of analytes is difficult and samples are typically complex, being of biological or environmental origin. That said, recent engineering advancements in the development of high-throughput systems, as well as improvements regarding the affinity, specificity, and availability of molecular recognition components underpin the development and probable success of next-generation analytical methods [4]. Incorporation of advanced sensing and measurement techniques in deployable instruments would significantly improve the odds of reduced casualties

associated with either a pandemic or biodefense situation. Usage scenarios ranging from clinical diagnostics to food safety, and from industrial applications to molecular medicine and biodefense would benefit from the advantages afforded by early detection of such an event and efficient triage of victims.

Nucleic acids-based methods such as real-time polymerase chain reaction or PCR [5] and DNA microarrays [6, 7] have been instrumental in identifying pathogens based on genetic signatures, as well as allowing characterization of postexposure biological response to toxins [8]. To augment DNA-based techniques, researchers are developing multiplexed immunoaffinity-based methods for biothreat screening relevant to public health laboratories and field deployment. Immunoaffinity-based methods provide substantial advantages for multiplexed identification of biothreats and are, arguably, one of the most important technologies aside from PCR-based methods and DNA microarrays [9]. Immunoaffinity-based methods may be even more important than genomic methods when developing systems for field use. This chapter details the role protein microarray methods play in development of increasingly sophisticated analytical platforms for detection, measurement, or identification of biothreats.

## 8.2 Molecular Recognition

As is true in any immunoassay method, protein microarrays typically rely on antigen binding to antibodies as a means to detect. Protein microarrays are a plausible assay solution if (1) a high affinity exists between the antigen in question and the capture or detection antibodies and (2) capture or detection antibodies for a particular antigen are available. The former point determining the effectiveness of the microarray and the latter point establishing the degree of multiplexing available for a particular assay.

A review by Iqbal and colleagues describes advances made in molecular recognition technologies for detection of biothreats [4]. A 2003 article by Andreotti and co-workers presents a detailed review of available means for molecular recognition of antigens using immunoassay methods [1]. As mentioned by these authors, binding of recognition molecules to protein targets requires that the detection probe recognize and have sufficient affinity for specific domains on the protein target.

Polyclonal and monoclonal antibodies are often used as both capture and detection probes in protein microarrays. Polyclonal antibodies may provide sufficient assay sensitivity, but may not enable the specificity necessary to measure the presence of specific antigens. Use of polyclonal and monoclonal antibodies in combination or use of monoclonal antibodies alone can surmount the specificity requirements, sometimes at the expense of sensitivity. In certain cases, neither monoclonal nor polyclonal antibodies with the required biological characteristics are available.

Recently, recognition molecules such as recombinant antibodies produced via phage display, random peptides, and aptamers have become available and grown in importance. Molecules such as these may play a significant role in the future of

protein microarray, and biosensor, development [10]. Regardless of the recognition component identity, improvements in the molecular recognition functions (i.e., affinity, specificity) and the availability of the molecule will determine the success or failure of the particular detection technology.

### 8.3 Contact-Printed Protein Microarrays

#### 8.3.1 *Direct and Competitive Immunoassays for Toxin Measurement*

In work from our own laboratory, Rucker et al. report on the development of antibody-based microarray techniques for the multiplexed detection of protein toxins in solution [11]. Details regarding assay development and fabrication of a six-element monoclonal antibody microarray for cholera toxin  $\beta$ -subunit, diphtheria toxin, anthrax lethal factor and protective antigen, *Staphylococcus aureus* enterotoxin B, and tetanus toxin C fragment were presented. Samples included both a model buffer system and bovine serum samples. We reported on assessment of two detection schemes. Namely, a direct assay (fluorescently labeled toxins were detected directly by immobilized capture antibodies) and a competition assay (unlabeled toxins employed as reporters for the quantification of native toxin in solution).

In the direct assays, six unique fluorescently labeled toxins were exposed to arrayed antibodies as a means to determine the strength of the antibody–toxin interaction in both buffer and diluted bovine serum samples. Fluorescence measured at each array element was correlated with known labeled toxin concentration to yield binding information (e.g., Langmuir isotherms, affinity constants). Both dissociation constants and limits of detection for the antibody microarray were determined. Limits of detection for the direct binding assays ranged from 14 to 704 ng/mL, depending on the analyte. The dilute bovine serum sample matrix allowed investigation of cross-reactivity between the arrayed antibodies or spiked toxin and background sample constituents.

We also reported on experiments directed toward the ultimate goal of detecting and identifying unlabeled toxins at low concentration. To this end, a competition assay was designed for the detection and characterization of unlabeled toxins in solution. Competition assays have been reported previously for the detection of toxins using immobilized gangliosides (membrane-embedded receptors that are negatively charged ceramide-based glycolipids with one or more sialic residues recognized by several bacterial toxins when infecting a host cell) and G protein-coupled receptors, whereas others have used a competition assay for serum-profiling experiments 2000a.

A significant advantage of the competition assay over reported profiling assays was the minimal sample preparation required. The competition assay obviated the need to fluorescently label native proteins in the sample of interest. In our study,

the 50% inhibition constants for the competition between fluorescently labeled reporter toxin and unlabeled toxin were characterized for all six analytes in buffer and diluted bovine serum. Both the calculated inhibition constants for the binding of the unlabeled toxin to the immobilized antibodies and the calculated detection limits using this competition assay for native toxin detection were reported.

Dose–response curves and detection limits were established for both assay formats. Although the sensitivity of the direct assay was superior to that of the competition assay (limits ranging from 24 to 5300 ng/mL), detection limits for unmodified toxins in the competition assay were comparable to values reported previously for sandwich-format immunoassays of antibodies arrayed on planar substrates. As a demonstration of the potential of the competition assay for unlabeled toxin detection, a straightforward multiplexed assay was demonstrated for the differentiation and identification of both native *S. aureus* enterotoxin B and tetanus toxin C fragment in spiked dilute serum samples.

### 8.3.2 Sandwich Immunoassays for Measurement of Pathogenic Bacteria

Although analytical methods such as plate culture, enzyme-linked immunosorbent assays (ELISA), and PCR have been used to detect bacteria, multiplexed methods appropriate for screening of bacterial pathogens in biological samples are needed. Gehring and coauthors [15] have recently reported on a microarray-based method for the detection of *Escherichia coli* O157:H7. Both biotinylated and antibodies bound to biotinylated protein G were used as capture moieties. Biotinylated capture antibodies for *E. coli* O157:H7 were contact printed onto streptavidin-coated microarray slides. Printed slides were blocked by static incubation with 100  $\mu$ L of PBS plus 1% BSA (*w/v*) for 1 h at room temperature.

After washing and drying steps, 100  $\mu$ L of bacterial solution was added to each array and incubated (1 h, room temperature) to allow bacterial capture. After a second set of washing and drying steps, 100  $\mu$ L of solution consisting of fluorescently labeled reporter antibodies in PBS with 0.5% BSA (*w/v*) was added to each slide, incubated for 1 h at room temperature, washed, dried, and then scanned for fluorescence. Fluorescence detection yielded a linear detection range from  $3.0 \times 10^6$  to  $9.0 \times 10^7$  cells/mL, with an apparent limit of detection at  $3.0 \times 10^6$  cells/mL. Below  $10^6$  cells/mL, protein G-bound antibody did not produce a measurable signal.

### 8.3.3 Serotyping

Cai and coworkers report on the development of an antibody microarray that makes use of simultaneous analysis of multiple antigens inherent in protein microarray methods to perform *Salmonella* serotyping [16]. The somatic (O) and flagellar (H) antigens present on *Salmonella* bacteria result in over 2500 serovars (strains). The work

reported by Cai et al. makes important improvements to current serotyping methods; namely, the described microarray allows concurrent detection of multiple antibody–antigen interactions, requires small reagent volumes, and requires significantly less time than current three-day serotyping durations. The *Salmonella* serotyping array was designed to detect and identify the 20 most clinically relevant serovars, strains that represent more than 80% of *Salmonella* isolates collected in Canada.

The array reported by Cai et al. was composed of 35 antibodies (11 O factor antisera, 9 H phase antisera, 13 H factor antisera, 2 O multigroup antisera) in an 8 × 15 array. Antibodies from polyclonal rabbit antisera were contact printed on commercially available epoxide-functionalized glass slides. Optimal antibody concentrations ranged from 1 to 5 mg/mL.

After printing, the authors blocked unreacted epoxide groups and applied fluorescently labeled *Salmonella* cells to the array. The cells were labeled with Eosin Y and Cy3 by incubating the cells with the dyes. For operator safety, cells were inactivated by a brief heating step. Simultaneous detection of O serogroups and H phase 1 and phase 2 antigens was accomplished using the assay. Evaluation of the array consisted of screening 117 *Salmonella* strains, covering the 20 target serovars, and 73 strains, covering 38 nontarget serovars. Correct identification of 74% of the target strains was made using the serotyping microarray.

## 8.4 Membrane Microarrays

Contact-printed supported membranes exhibiting characteristics of a physiologically fluid environment provide a microarray format especially relevant for studying cellular processes. Especially pertinent to the study of signal transduction pathways, physiological fluidity of the supported membrane allows development of high-throughput biomimetic assays, thus allowing the study of protein–receptor interactions on time scales relevant to in vivo questions. Membrane fluidity is largely dependent upon the preparation of the lipid bilayer and the characteristics of solid substrate supporting the membrane. The fluidity of molecules inserted in planar lipid bilayers facilitates dimerization and other higher-order interactions necessary for biological signaling events. Membranes also allow the study of toxin interactions with gangliosides. Gangliosides are components of the cell membrane and act to modulate cell signal transduction events, including those relevant to toxin signaling pathways.

### 8.4.1 *Gangliosides Immobilized on Optical Waveguides*

A nonantibody-based flow assay utilizing ganglioside GM1 immobilized on the surface of optical waveguides was reported for rapid detection of cholera toxin [12, 13]. Direct and sandwich assays for cholera using a flow-patterning method

(described in more detail in [Section 8.6](#)) to immobilize GM1 at discrete locations on a planar optical waveguide were described. As the authors note, interest in development of an identification and detection system for cholera stems from the difficulty associated with distinguishing cholera from other causes of acute diarrhea. In the case of cholera, mortality approaches 50% if left untreated, yet, when properly treated, cholera mortality rates can be as low as 1%. Conventional culture-based diagnosis can take 8 to 24 hours and may not be effective, as only a single serotype of *Vibrio cholerae* (O1) causes disease (WHO, 1994).

Patterning of gangliosides relied upon the hydrophobic interaction between octadecyltrichlorosilane (OTS)-silanized surfaces and ganglioside reagents. Several buffer and solvent systems were investigated in an effort to optimize ganglioside immobilization. Slides were treated with a long-chain silane to allow hydrophobic interactions between the surface and the ganglioside capture molecule. OTS was chosen due to the chain length of the sphingosine (C18). Ganglioside-based ELISAs for cholera toxin and other toxins have been reported [17–20].

Direct assays involved measurement of binding of fluorescently labeled cholera toxin to immobilized GM1. In a manner similar to ELISAs, sandwich assays using fluorescently labeled monoclonal and polyclonal detection antibodies were also developed. The sandwich assays enabled limits of detection as low as 40 ng/mL in the case of polyclonal detection antibodies and 5  $\mu$ g/mL with monoclonal detection antibodies. The detection sensitivity of the reported system for GM1 binding of cholera toxin (40 ng/mL) compared well with other reported ganglioside-based assays [21–23].

To take advantage of the multiplexing ability of the presented sensor platform, the authors explored binding of cholera toxin to a number of different glycolipids (GD1b, Gb3, Gb4, and GT1b). As was the case with GM1, each glycolipid was flow-patterned onto a silanized slide. Negligible binding to GT1b was observed, however, the authors did observe dose-dependent binding of cholera toxin to GD1b, Gb3, and Gb4. As the authors discuss, the ligand–receptor binding results from the array sensor are in disagreement with published reports. Factors such as different experimental conditions, shear fluid forces, toxin concentration ranges, orientation, and density of receptor molecules patterned on the planar surface are all discussed as possible sources of deviation in the measured ligand to receptor binding.

#### **8.4.2 *Ganglioside Microarrays for Toxin Detection***

In the early 2000s, researchers at Corning reported on microarrays of lipids containing gangliosides for detection of bacterial toxins and screening of potential inhibitors, two important applications of membrane microarrays [24–26]. A typical bacterial toxin is composed of two domains: the *A* or *activating* domain is involved

in intracellular enzymatic activity and the *B* or *binding* domain is involved in binding to the cell membrane. The surface of host cells displays several types of molecules that act as toxin-binding sites [27]. The authors provide a brief overview of surface receptors important as potential bacterial toxin inhibitors (cholesterol, carbohydrate derivatized lipids (sphingoglycolipids), and gangliosides, in particular).

At Corning, Fang and colleagues [24–26] described development of membrane microarrays as a tool to study the interaction of toxins (cholera and tetanus) with carbohydrates in near-native environments. The work also investigated screening of possible inhibitory compounds using the developed membrane microarray. Supported lipids are of interest as these features allow both immobilization and confinement of the probe ligand and the associated lipids, while maintaining lateral movement of individual molecules within the arrayed lipid microspot. Recent studies have shown that ideal supported membrane surfaces used in microarrays should (i) resist physical desorption and (ii) exhibit long-range lateral fluidity.

As background information, in 1999 Boxer and coworkers reported that supported lipids patterned on bare-glass desorbed as the glass substrate was drawn through an air–water interface [28]. In 2000, Mrksich and others used self-assembled monolayers to provide carbohydrate ligands covalently attached to the surface making studies of biomolecular recognition feasible [29]. The lack of lateral fluidity in the system precluded the biomimetic display of carbohydrate ligands, as is necessary in cases where ligand clustering is important [30]. Through use of supported lipids, Fang and coworkers have combined advantages inherent to microarray analysis (throughput, efficiency) with near-native environments to carbohydrate-mediated recognition.

As described in Fang et al. [24–26] the supported lipid system reported utilized surfaces derivatized with  $\gamma$ -aminopropylsilane (GAPS) to provide a substrate resistant to desorption and yet supportive of lateral fluidity (with a mobile fraction of  $\sim 0.5$ ). The fabrication protocol reported relies upon quill-printing of DLPC (1 mg/mL) in the absence and presence of 4 mol% ganglioside (GM1 or GT1b) in 20 mM phosphate buffer (pH 7.4) on the GAPS-coated slide. Quill pins were dipped into individual wells of a 384 microtiter plate to pick up each lipid solution followed by a 1 h incubation period in a humidity chamber. Repeated dipping of slides printed with fluorescently labeled lipid revealed robust performance of the described system. The lateral fluidity of supported lipids was tested by traditional fluorescence recovery after photobleaching experiments. The authors report that microarrays of G protein-coupled receptors exhibiting ligand binding affinities and specificities consistent with the literature [24–26].

To demonstrate detection of toxins in a competitive assay, arrays were incubated with 20  $\mu$ L of a solution consisting of fluorescently labeled toxin (0.031–2 nM) and varying concentrations (0–100 nM) of unlabeled toxin. Fluorescence signal was measured at the completion of the assay using a microarray scanner. The authors report detection limits of 10 nM for the unlabeled cholera toxin and 50 nM for the unlabeled tetanus toxin. For labeled toxins, the detection limits were measured at 0.03 nM for FITC-labeled cholera toxin and 0.06 nM for FITC-labeled tetanus toxins.



### ***8.4.3 Polymer Lift-Off Technique for Lipid Patterning***

Moran-Mirabal and colleagues present a characterization of bacterial toxins (cholera toxin B-subunit and tetanus toxin C-fragment) binding to micron-sized lipid domains patterned onto planar substrates and within microfluidic channels [31]. The authors detail an innovative polymer lift-off technique used to fabricate ganglioside-populated (GM1, GT1B) supported lipid bilayers.

In brief, the patterning procedure used to deposit supported lipid bilayers on planar substrates consisted of an initial deposition of a polymer film on a silicon substrate by vapor deposition. Photoresist is then spun on the coated silicon substrate and exposed with ultraviolet (UV) light. The photoresist is developed and removed from UV exposed areas. The resulting exposed parylene is etched via oxygen plasma, revealing well-defined micron-sized features of exposed silicon. The substrates are oxidized and incubated with lipid vesicles, resulting in vesicle fusion and the formation of lipid bilayers.

Finally, the polymer is peeled off and ganglioside-populated lipid bilayer patterns remain. The bacterial toxin assays involve subsequent incubation of the patterned surface with aqueous samples of interest. An adaptation of the procedure is described for the fabrication of multiple lipid bilayer elements in a microfluidic channel, as is especially relevant to biosensor development. The authors point out that the lift-off technique eliminates the need for etched barriers to contain the bilayers, as the polymer defines the bilayer structures with micron-scale resolution.

Total internal reflection fluorescence microscopy allowed extraction of apparent binding constants and suggested that protein binding to the ganglioside receptors was influenced by the microenvironment lipid bilayer and the underlying substrate. Characterization of the binding did enable estimates of the limits of detection at down to 100 pM for cholera toxin B-subunit and 10 nM for tetanus toxin C-fragment. Arrays of lipid domains having different compositions were demonstrated on a single microfluidic device and enabled segregation and selective binding from a composite mixture of the two toxins, as determined by epifluorescence microscopy.

### ***8.4.4 Lipid Bilayers on Nonglass Substrates***

Although not in a multiplexed format, Phillips and Cheng [32] report on the development of heterogeneous assays for cholera toxin using supported lipid bilayers in poly (dimethylsiloxane) (PDMS) microfluidic channels. The heterogeneous immunoassay developed could be readily adapted to a microarray format, possibly using contact printing. The technique revealed the ability to quickly and specifically detect cholera toxin using the cell surface receptor GM1 integrated into a supported lipid bilayer.

The assay provided a dynamic range spanning three orders of magnitude and a detection sensitivity of 8 fmol of cholera toxin when performed under flow conditions. In addition, the supported lipid bilayer had good fluidity, as measured by fluorescence recovery after photobleaching, exhibited minimal nonspecific protein adsorption, and was robust under flow conditions. The work highlighted advantages associated with using a microfluidic fluid delivery approach. Furthermore, the work demonstrates the potential for use of alternate materials for planar substrates, in this case PDMS.

Building on the study described above, Phillips and coworkers recently detailed development of a lipid bilayer-based sensor for detection of cholera toxin in environmental water samples [33]. The work addresses the design challenge of providing sufficient mechanical stability in the lipid bilayer while retaining lateral fluidity. In the previous work, the authors show that oxidized PDMS exhibits hydrophobic recovery over short periods of time, whereas surfaces covered with phosphatidylcholine membranes maintain hydrophilic properties.

As it is important to robust sensor development, the authors report in this recent work that PDMS surfaces treated with vesicles from cationic lipids (i.e., ethylphosphocholine, DOPC+) exhibit exceptionally strong resistance to air-dry damage. Fluorescence recovery after photobleaching measurements and protein adsorption studies conducted by the authors reveal that the mobile fraction of PC membranes decreased by nearly 10% after drying/rehydration cycles. Membrane fluidity was reduced as well, with the lateral diffusion coefficient decreasing by close to 30% after drying/rehydration of the PC membranes. The DOPC+ membranes developed in the study reveal little to no reduction in either mobile fraction or diffusivity.

As an example application, the authors packaged the DOPC+ membrane (GM1/DOPC+ membranes) in a PDMS flow-based immunoassay. A detection limit of 250 amol for cholera toxin was obtained from on-chip calibration curves. Cholera toxin spiked into river water samples revealed similar response and sensitivity. The group has recently reported on use of a similar system for immunosensing of SEB in milk [34].

#### ***8.4.5 Microfabrication as a Means to Corral Lipid Bilayers***

Yamazaki and co-workers report a technology for fabricating multiplexed, high information-content cell membrane microarrays as a tool for high throughput biological assays [35]. The authors use the membrane microarrays to validate the approach in the study of ligand/receptor binding and interactions with live intact cells. The authors report on three classes of interactions. Specifically, the group assesses the interaction between the cholera toxin B-subunit and the membrane ganglioside GM1. The interaction of the pentameric cholera toxin B-subunit with membrane gangliosides is hypothesized to be multivalent and involve up to five GM1 receptors [36] under membrane conditions with sufficient fluidity to allow assembly of the ligand/receptor complex.

A second system of interest is the display of membrane components that are important drug targets for treating diseases. The authors present results on displaying LPS (lipopolysaccharide) from gram-negative bacteria and the mammalian proteins ICAM-1 and I-Ek to show relevance to conditions ranging from septic shock to autoimmune dysfunction.

Finally, a third system reported by Yamazaki [35] and coworkers investigates T-cell adhesion and activation on membrane microarrays displaying proteins of immunological importance. Two proteins important in mediating the adhesion and activation of T cells on antigen-presenting cells, ICAM-1 and I-Ek, were investigated using the reported methodology.

A method that combines lipid biochemistry with microfabrication methods common to the semiconductor industry and robotic handling was developed to generate high-density arrays of membranes contained within discrete “corrals” on planar substrates. Glass wafers with chrome barriers were, in some parts of the study, used to define the membrane microarray elements. To fabricate the planar supported bilayers, small unilamellar vesicles were fused onto the exposed glass regions using a microarrayer capable of programmable aspiration and dispensing.

The cholera toxin B-subunit/GM1 study substantiated the validity of the reported fabrication approach through generation of dose–response curves based on varying concentrations of membrane-incorporated GM1 and aqueous cholera toxin B-subunit. FRAP measurements were used to assess the membrane fluidity. After incorporation of lipid A, the principal endotoxic moiety of LPS, in lipid bilayers, the authors measured an approximately fourfold increase in immunoreactivity as compared with membranes not containing lipid A.

The study of T-cell adhesion revealed that monoclonal antibodies specific for the membrane-arrayed surface receptors (ICAM-1, I-Ek) nearly eliminated T-cell adhesion. The authors incorporated the ICAM-1 or I-Ek ectodomains in supported lipid bilayers (through glycosylphosphatidylinositol tethering), and examined these membrane-incorporated proteins for their ability to capture murine T cells. Results from the study suggested that each target protein, alone or in combination, mediated specific adhesion of T cells. Furthermore, the authors measured spatial clustering of membrane-incorporated I-Ek molecules as an indicator of T-cell activation. The authors have shown that incorporation of transmembrane proteins into the arrayed membranes holds promise for biomimetic studies of ligand/surface receptor interactions in both industrial and research settings.

## 8.5 Hydrogel Microarrays

Detection of biothreats using arrayed proteins and antibodies is an important potential application of microarrays, as discussed later in this chapter, however, protein microarrays have been demonstrated for characterization of biothreats. Two recent examples of the use of microarrays for measuring ligand binding properties and serotyping biological strains are presented in this section. Both studies make use

of hydrogel spots as supports for immobilization and as nanoliter reaction volumes for reactions between samples of interest and immobilized compounds. The studies presented in this section, as well as those reported elsewhere for nonbiothreat applications, highlight several advantages afforded by hydrogel-based microarrays [37–42]. Briefly, these advantages include: an increased immobilization capacity as compared to planar glass surfaces, a stable substrate amenable to covalent attachment of proteins, and minimal background fluorescence [43]. A further advantage is of particular importance to cell surface receptor–protein interactions, in that the three-dimensional (3D) gel is thought to mimic a solution-phase environment.

As an example of hydrogel-based protein microarrays, take the synthetic 3D immobilization strategy developed by Charles and co-workers which is based on a thin film crosslinked with bis (sulfosuccinimidyl) suberate under acidic conditions [44]. The work makes use of studies conducted by several groups exploring the use of hydrogels or polyacrylamide gel pads as a means to create 3D spots on planar surfaces.

In the methods developed by Charles and colleagues, pendant NHS-ester reacts with amide moieties within a hydrogel film and a secondary NHS-ester group reacts with the primary amine on SEB under neutral conditions. The protocol produces NHS as a side product for the formation of a stable bond between the protein and the 3D hydrogel. Binding measurements made using the 3D hydrogel microarray to study immobilized SEB binding with Cy5-labeled anti-SEB revealed significant differences in background fluorescence between the 3D and the 2D substrates. Fluorescence signal from the 3D hydrogels was threefold higher than that of planar glass surfaces when immobilized SEB was at concentrations greater than 10  $\mu\text{g/mL}$ .

The authors conclude that binding epitopes on hydrogel-immobilized SEB were more accessible for antibody binding than epitopes of SEB printed on the planar glass surface. The hydrogel-based SEB microarray exhibited a linear detection range from 0.1 to 30  $\mu\text{g/mL}$  and demonstrated low background signal. Citing previous work on hydrogel-based platforms, the authors conclude that femtomolar (pg/mL) detection sensitivities should be possible.

## 8.6 Sensor Technologies

Significant progress regarding detection of chemical agents has been made over the last few decades; that said, chemical agents are more readily detected than biothreats, as victims of biological agents typically display a delayed physiological response owing to incubation periods on the order of up to several days. As Ivnitcki and coauthors describe [5], the potential threat from biological agents has sharpened owing to advances in molecular biology, genetic engineering, and the engineering of efficient delivery and dispersion systems, including increased civilian air travel.

Sensors capable of environmental monitoring as a means to detect the presence of biothreat agents, as well as portable systems capable of monitoring physiological conditions to detect and diagnose illness are two important components of biothreat surveillance. An ideal surveillance network would rely upon fast analysis for detection of the presence of biothreats, acting in concert with more sophisticated analytical sensor systems that would enable identification of the biothreat present.

In a clinical setting, timely identification is also critical, but difficult to implement with generally accepted diagnostic indicators commonly relied upon by public health and hospital-based clinical labs. Clinical indicators are largely based on physiological observables related to a patient's general health state (e.g., reported flulike symptoms). Unfortunately, the initial clinical signs and symptoms of many agents are nonspecific and similar to those observed from common infections. Improvements in clinical diagnostic technologies would fill this gap. Such a diagnostic system would be desirable if it were a sensitive, specific, inexpensive, easy-to-use system that could rapidly and accurately identify toxins. Additional capability regarding the ability to detect the presence of a variety of possible biothreats in a single sample is of special interest.

Portable multiplexed tools are viewed as one means to achieve these goals. Although chemical sensing systems are more advanced than those developed for detection of biothreats, technological advances arising from miniaturization (e.g., microarrays, microfluidics, microelectromechanical systems) have accelerated the development of portable inexpensive sensing [5]. Ivnitski and co-authors present a review of DNA-based detection and identification of biothreat agents. Commercial systems, as well as those developed by national laboratories and academic groups are discussed. Further technological innovation regarding automation of sample collection and preparation are required before real-time environmental monitoring (water treatment plants, agriculture, food products) and clinical diagnosis fulfill their potential.

### ***8.6.1 Flow-Patterned Protein Array Biosensors***

Sensor development relying upon immunological recognition of proteins, bacteria, and viruses at the Naval Research Laboratory (NRL) is one nascent example of such a system, as summarized in review articles by the NRL authors [45, 46]. A summary of select applications is given in [Table 8.2](#). The platform developed over the last decade at the NRL is designed to provide field-portable instrumentation for use in military and civilian settings.

Immobilization of capture probes on planar substrates has been developed as a detection cartridge for integration into a fully automated, user-friendly instrument. Major performance requirements for such instrumentation include: rapid analysis times (assays complete in roughly ten minutes), concurrent analysis of multiple samples for the presence of multiple analytes, and a small form factor amenable to

**Table 8.2** Summary of NRL Array Platform Assays and Sensitivity Estimates

Category	Toxin	Limit of Detection
Protein toxins	SEB	0.5 ng/mL
	Cholera toxin	1.6 ng/mL
	Botulinum toxoid A	40 ng/mL
	Botulinum toxoid B	200 ng/mL
	Ricin	8 ng/mL
Protein allergens	Ovalbumin	0.025 ng/mL
Physiological markers	Y. Pestis F1	25 ng/mL
	D-dimer	25–50 ng/mL
Gram-negative bacteria	E. herbicola	10 <sup>4</sup> cfu/mL
	B. abortus (killed)	3 × 10 <sup>3</sup> cfu/mL
	F. tularensis LVS	10 <sup>5</sup> cfu/mL
	Salmonella	8 × 10 <sup>4</sup> cfu/mL 8 × 10 <sup>3</sup> cfu/g excreta
Gram-positive bacteria	B. globigii	10 <sup>5</sup> cfu/mL
	B. anthracis	10 <sup>3</sup> cfu/mL
Virus	MS2	10 <sup>7</sup> pfu/mL

Source: Adapted from information available from the *World Technology Evaluation Center (WTEC) Workshop on International R&D in Biosensing* held on May 13, 2003.

portable operation. The sensor is designed for end-use by minimally trained personnel in a field setting. The following sections detail protocols and results of select studies performed with the NRL platform.

Rowe and coworkers reported on a fluidic method for immobilization of capture probes in a small array patterned on a planar waveguide [47]. Biotin-labeled capture molecules were immobilized on NeutrAvidin-coated slides, as described in Bhatia et al. [48]. A flow-patterning process developed by the authors at NRL was used to pattern capture antibodies. A standard sandwich assay format was designed to rely on fluorescence reporting of analyte concentration in a sample. Excitation light was incident on the end of the waveguide to generate an evanescent excitation of the surface-bound fluorescent detection antibodies. Evanescent wave techniques enable rapid response time and relative insensitivity to complex biological matrices. Relying upon detection of fluorescent signal eliminates confounding signal arising from nonspecific adsorption. Furthermore, the narrow penetration depth (100–200 nm) of evanescent waves allows detection of events occurring on the surface only, with little signal from the bulk solution. Real-time measurements of turbid or inhomogeneous samples are possible. A two-dimensional graded index of the refraction lens array allowed imaging of the surface using a CCD camera.

The two-step flow process utilized two fluid modules: (1) a molded PDMS flow chamber module consisting of six channels was used to pattern six vertical lines of biotinylated capture antibody on the waveguide surface, and (2) a second PDMS assay module consisting of six flow channels to introduce sample and detection

antibody over the patterned waveguide. After patterning, a washing procedure was implemented and patterned slides were either used immediately or treated with a storage preparation buffer (15 mM sodium phosphate/0.1 M trehalose supplemented with 10 mg/mL BSA) for 15 min and then stored at 4°C for at least three weeks.

The protocol for sample analysis consisted of flowing fluid over the patterned surface using the PDMS assay module. The assay module also consisted of six flow channels. The module was placed on the patterned planar waveguide, but perpendicular to the patterned vertical stripes allowing probing of immobilized capture antibodies at locations where patterned lines intersected with assay module channels. The sample was incubated in the channels for 15 min under flowing conditions (0.3 mL/min). After PBST washing, a second assay step introduced fluorescently labeled detection antibody that was recirculated through the channels at 0.3 mL/min for a 15 min period.

As a demonstration of the instrument for detection and identification of multiple analytes, the authors report on a study focused on measurement of Staphylococcal enterotoxin B (SEB), F1 antigen from *Yersinia pestis*, and a marker of sepsis and thrombotic disorders, D-dimer, spiked into minimally pretreated biological sample matrices at clinically relevant concentrations [49]. SEB causes food poisoning, nausea, vomiting, and diarrhea when ingested [50]. Inhalation of aerosolized SEB can be life threatening and may lead to hypotension, respiratory distress, shock, and death [51].

The glycoprotein F1 antigen is a major component of the outer membrane of *Y. pestis*. F1 antigen is the etiologic agent of plague and is secreted by *Y. pestis* only upon invasion of a mammalian host. F1 antigen is routinely detected as a means to diagnose plague [52]. DNA hybridization, PCR, and ELISA are more rapid than bacteriological and serological tests, however, these assays are not rapid enough to be used as diagnostic tools and are used mainly as confirmatory tests. Although d-dimer is a normal component of blood in healthy individuals, high concentrations are indicative of disseminated intravascular coagulation, pulmonary embolism, myocardial infarction, and deep venous thrombosis [53], as well as sepsis and infection [54].

Biological samples analyzed with the immunosensor included serum, nasal swabs, and saliva. In the serum analyses, blood samples were collected from healthy volunteers and from ICU patients clinically suspected as having sepsis. Platelet-poor plasma was prepared from heparinized whole blood, centrifuged at 3000 g, with serum obtained from clotted whole blood by centrifugation at 2000 g for 10 min. Prior to spiking with analytes of interest, the serum was diluted 1:1 with buffer. Nasal fluid samples were collected from healthy volunteers by simply swiping the interior of the nasal cavity using two cotton-tipped swabs per nostril. After collection, the swabs were placed in 4 mL of a PBS/0.05% triton x-100 buffer and incubated for 15 min. Swabs were removed and the fluid was retained.

Animal studies have shown that SEB concentrations can reach 500 ng/mL in serum and 1–10 ng/mL in urine for up to 24 h postexposure. The described immunosensor

provided limits of detection at 1 ng/mL in buffer and spiked nasal fluid. The authors highlight that analysis of nasal swabs is potentially important as nasal fluid is a preferred means to diagnose exposure to aerosolized infectious agents. The immunosensor was not able to detect physiological levels of SEB in spiked serum, saliva, or urine. F1 antigen has been detected in clots and serum at the high nanogram per mL to low microgram/mL range two to three days after exposure [55].

The described NRL immunosensor platform yielded detection of F1 antigen at 25 ng/mL in buffer, spiked serum, urine, nasal swabs, and saliva. The authors assessed the sensitivity of the platform for d-dimer in buffer, plasma, and whole blood samples. Normal healthy individuals typically have between 25 ng/mL and 150 ng/mL levels of d-dimer in their blood, whereas patients with sepsis, myocardial infarction, or thrombotic disorders can have levels of d-dimer greater than 125 ng/mL. The described immunosensor detected concentrations of d-dimer greater than 50 ng/mL in buffer, plasma, and diluted whole blood. The authors did note an apparent inhibitory effect of plasma on antibody binding to D-dimer. The platform was used to assay for all three analytes individually, as well as both F1 and D-dimer in plasma.

The NRL array biosensor has also been employed to analyze 126 blind samples for the presence of bacterial, viral, and protein analytes [49]. Specifically, the authors investigated limits of detection and assay throughput regarding analysis of the nonpathogenic gram-positive sporulating soil bacteria *Bacillus globigii*, the small RNA bacteriophage MS2, and the toxin SEB. Single analyte assays were run in parallel with the analysis of a mixture of the three analytes, thus demonstrating the sensor's capability to detect multiple species in a single assay. Sensitivity limits of the 14 minute *Bacillus globigii* and MS2 assays approached those of ELISA, with limits of detection for *Bacillus globigii* and MS2 reported to be  $10^5$  cfu/mL,  $10^7$  pfu/mL, respectively. The array sensor had a 10 ng/mL limit of detection for SEB, a factor of tenfold less sensitivity than ELISA. The authors attributed the poorer SEB sensitivity to use of polyclonal, not monoclonal, antibodies. Analysis of the 126 samples yielded a 0% false negative rate. False positives were present at 0.8%, the same level reported via ELISA.

Detection of toxins, toxoids, and killed or nonpathogenic (vaccine) strains of pathogenic bacteria has also been demonstrated on the array flow sensor [13]. Again, a sandwich format was employed in conjunction with fluorescence detection. An automated version of the array flow sensor was introduced. In the reported study, improvements regarding the optical coupling of the waveguide and flow cell assembly to the detector were made by incorporating a patterned reflective silver-based cladding to optically decouple the waveguide and the flow cell. Detection of bacterial analytes (*B. anthracis* (Stern), *Francisella tularensis* LVS, *Brucella abortus*) and toxins (*Botulinum* toxoids A and B, *S. aureus* enterotoxin B (SEB), ricin, and cholera toxin) was reported. Although the sensitivity of the semiautomated system was compatible with immunosensors, the sensitivity was dependent on the type of antibody used (i.e., monoclonal vs. polyclonal). An automated version of the array biosensor suffered from fivefold less sensitivity, mainly arising from higher variation in background signal attributed by the authors to fluorescence arising from materials used in the flow cell.



### 8.6.2 Carbohydrate-Based Flow Biosensor

More recently, the NRL array biosensor provided an experimental platform to assess the binding interactions between sugars and protein toxins, as well as bacterial cells [56]. Carbohydrate derivatives were of interest to the authors, as a large number of bacterial toxins (including cholera toxin, *Escherichia coli* heat-labile enterotoxin, shigalike toxins, pertussis toxin, botulinum toxin, and tetanus toxin), viruses, and bacteria target carbohydrates on the cell surface as a means to attach and, ultimately, enter a cell. Recent carbohydrate-based sensors have employed gangliosides as receptors for protein toxins [12, 13, 22, 24–26, 57, 58], thus limiting information regarding specific carbohydrates involved in key protein toxin–carbohydrate interactions.

In order to characterize and detect specific protein–carbohydrate interactions, a model array consisting of immobilized *N*-acetyl galactosamine (GalNAc) and *N*-acetylneuraminic acid (Neu5Ac) derivatives on a planar waveguide surface was developed. GalNAc and Neu5Ac were used as receptors to assay for *Salmonella typhimurium*, *Listeria monocytogenes*, *Escherichia coli*, and staphylococcal enterotoxin B (SEB), cholera toxin, and tetanus toxin. To immobilize the model receptors on maleimide-modified glass slides, the sugars were converted to anomeric thiophenyl glycosides containing para-hydroxyl thiophenol. An acid linker was added and coupled to a thioacetate terminating linker. The monosaccharide array was constructed using a flow patterning approach similar to that described previously, albeit with a 12-channel PDMS patterning template. Fluorescently labeled bacterial cells and protein toxins were used to probe the array.

The authors observed no binding of *Salmonella typhimurium*, *Listeria monocytogenes*, *Escherichia coli*, and staphylococcal enterotoxin B to either GalNAc or Neu5Ac. Measurable binding of both cholera toxin and tetanus toxin to GalNAc and Neu5Ac was observed. The authors conclude that the results illustrate the semiselective binding behavior of the Neu5Ac and GalNAc derivatives toward cholera and tetanus toxin, as supported by previous studies. Results of dose–response studies with cholera and tetanus toxins showed a carbohydrate density dependence on the observed semiselective binding of these two toxins to the carbohydrates. Detection limits for both toxins were 100 ng/ml and the assays were complete in 65 minutes.

### 8.6.3 Microarray Cartridge System for Malaria Detection

The emergence of antimicrobial-resistant strains of pathogens such as malaria and tuberculosis, in conjunction with increased global travel and trade, has made centuries-old diseases important biothreats. According to the Centers for Disease Control and Prevention, malaria afflicts an estimated 300 to 500 million new victims each year. With funding from the U.S. Special Operations Command, Wave 80

Biosciences, Inc. has developed a field-portable, quantitative biosensor for detection of antigens associated with malaria in whole blood [59].

As described in Laser et al., the system provides detection and quantification of malarial antigens using low-density microarrays in a cartridge format. A 20 microliter whole blood sample (lysed and labeled within the cartridge) passes over a 12 spot antigen/antibody microarray in a flow chamber 120 microns deep  $\times$  3 mm wide. The malarial antigens HRP-II (associated with *Plasmodium falciparum*) and aldolase (a pan-malarial antigen relevant to *Plasmodium vivax* and other species) are detected and quantified with dynamic range greater than two orders of magnitude through variations in solid-phase probe surface concentration. With a double-sandwich assay configuration, the optical detection limit is below 100 ng per milliliter. The malaria cartridges run on a battery-powered handheld instrument with external volume (package size) less than 0.5 liters.

## 8.7 Future Directions

Protein microarrays, whether based on conventional recognition moieties such as antibodies or on newer affinity capture probes such as aptamers, are already playing a strengthening role in the identification, detection, and ultimate measurement of biothreats. Developed for basic science at the bench or robustly packaged as deployable sensors, the information available from such multiplexed systems promises to complement that available through genomic studies in the laboratory and DNA-based detection systems in the field.

Early protein microarrays were built upon concepts that borrowed from DNA microarrays and conventional immunoassays, ELISAs in particular. Recent protein microarrays for the measurement of biothreat agents have become more complex and, arguably, more adaptable. One recent example is a system based upon nanoscale glassification of gold substrates for surface plasmon resonance of supported lipid membranes, thus allowing glass surface-based assay techniques to be readily adapted for label-free SPR analysis without the need to rely on thiol-based materials [60]. Flexible detection schemes, as well as less stringent surface requirements [61], will indelibly lead to more versatile sensor platforms.

Although this chapter has focused primarily on laboratory-based protein microarrays, two commercially sponsored protein microarray systems were launched. Invitrogen, Inc. was contracted by the U.S. Department of Defense to develop Invitrogen's ProtoArray™ protein microarray technology to both detect and analyze agents such as hemorrhagic fever viruses, poxviruses, *Bacillus anthracis*, smallpox, and *Yersinia pestis*. The goal of the work is to improve the military's ability to detect dangerous bacteria and viruses in air, food, and water.

Invitrogen also demonstrated their PathAlert™ system in public venues (2006 Winter Olympics, 2006 Commonwealth Games, Technical Readiness Assessment at the U.S. Army's Dugway Proving Grounds) and reported the system as a sensitive, specific molecular approach for detecting agents such as anthrax and plague. The

ProtoArray technology is proprietary, however, Invitrogen describes the protein microarray as capable of analyzing entire pathogen and yeast proteomes or up to 5000 human proteins in a single experiment. Furthermore, the ProtoArray technology has been designed to analyze proteins, antibodies, and small molecules in a single array. Prior to 2006, Invitrogen has used the ProtoArray platform for cancer and autoimmune disease protein biomarker discovery. Protein microarray technologies developed for important areas such as biomarker discovery and proteomics form a core technology base that is beginning to prove essential to solving defense and public health biothreat concerns.

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