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Rapid detection and identification of biological and chemical agents by immunoassay, gene probe assay and enzyme inhibition using a silicon-based biosensor

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Abstract

A rapid biosensor assay procedure that utilizes biotin–streptavidin mediated filtration capture onto nitrocellulose membrane, in conjunction with a silicon-based light-addressable potentiometric sensor (LAPS) was developed for detection and identification of biological and chemical threat agents. Sandwich immunoassays, nucleic acid hybridization assays and enzyme inhibition assays are described. For immunoassays, the lower limits of detection (LOD) per 100-μl sample were approximately 5 pg/ml for protein (Staphylococcal enterotoxin B), 2 ng/ml for virus (Newcastle disease virus), and 20 ng/ml for vegetative bacteria *(Brucella melitensis)*. In a dual gene probe assay format, the LOD was 0.30 fmol (1.8×10^8 copies per 60-μl) of single stranded target DNA. Enzyme inhibition assays on the LAPS using acetylcholinesterase were able to detect soman and sarin in aqueous samples at 2 and 8 pg (100 and 600 pM), respectively The assays were easy to perform and required a total time equal to the reaction period plus about 15 min for filtering, washing and sensing. The assay format is suitable for detection of a wide range of infectious and toxic substances. New assays can be developed and optimized readily, often within 1 or 2 days. © 2000 Elsevier Science S A. All rights reserved.

Keywords: Immunoassay, Antibodies; Nucleic acid hybridization, Gene probes, Biosensors, Urease, Enzyme inhibition, Biotin, Streptavidin, Organophosphates, Acetylcholinesterase

1. Introduction

Biosensor technologies are important tools for detection of chemical and biological warfare agents. The rapid analysis time, relatively compact size and ease of use allow biosensors to be used on the laboratory bench, the field, or in containment facilities.

The biorecognition processes employed in this work include antibody–antigen interactions, enzyme assays and nucleic acid hybridizations. Antibodies are widely used in immunoassays and have largely revolutionized the process of detection of large molecules (Tijssen, 1985). The binding domains can have high affinities for specific antigens; the binding constants are as high as 10^{10} M^{-1}. Currently, antibodies are employed in a wide variety of sensor devices, that include fiber optic, acoustic energy, semiconductor, and membrane. Gene probes are discrete sequences of nucleic acids (often labeled) that are complementary to specific areas of target DNA or RNA. The process that underlies gene probe assays is the formation of a hybridized complex consisting of a single-stranded nucleic acid target molecule and its complementary probe sequence. Enzyme assays have been used to quantitate both substrate and inhibitory compounds. Examples of substrate assays include, glucose oxidase or hexokinase for glucose (Boutelle et al., 1986; Zhang et al., 1998) and urease for urea (Watson et al., 1987, Ho et al., 1999). Inhibitory assays include the use of the enzyme acetylcholinesterase for organophosphorus detection (Rogers et al., 1991). Organophosphorus nerve agents and anticholinesterase insecticides inhibit the enzyme, acetylcholinesterase. The toxicity arises from the inability of the affected neurons to remove acetylcholine (a neurotransmitter)

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from the synaptic junctions, thus leading to over stimulation and loss of function.

In this report we describe a commercially available versatile assay system employing a silicon-based light-addressable potentiometric sensor (LAPS) that can be used for the detection of infectious agents (virus, bacteria) and toxic substances (protein, chemical) in liquid samples by immunoassay, gene probe assay, or enzyme inhibition. For immunoassays, the reagent antibodies and the antigen were incubated together in a single step as illustrated in Fig. 1. The resulting sandwich immune complexes were filtered through a biotin-embedded nitrocellulose membrane and immobilized on the membrane by means of biotin–streptavidin interactions (Green, 1975; Wilchek and Bayer, 1988). The presence of antigen on the membrane was detected by monitoring the enzyme activity associated with urease-conjugated second antibodies in the immune complex. Monitoring was carried out by wetting the membrane with a solution of substrate (urea) and pressing the membrane against the surface of a pH-sensitive light-addressable potentiometric sensor (LAPS) (Hafeman et al. 1988; Bousse et al., 1990). Using selected antibodies we have developed assays against *Bacillus subtilis var. niger* (a spore forming bacteria), *Brucella melitensis* (a vegetative bacteria), Newcastle disease virus (NDV), and proteinaceous toxins staphylococcal enterotoxin B and ricin.

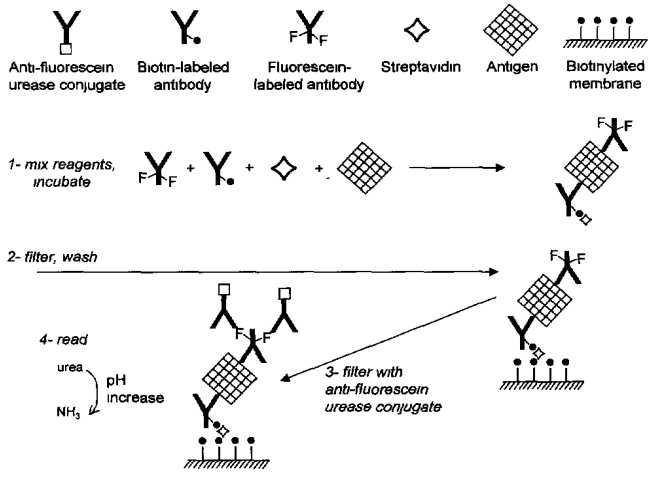

Fig 1 The standard format assay scheme for the LAPS.

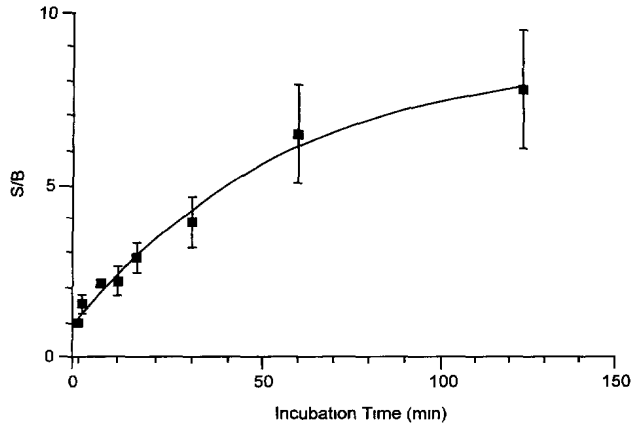

Fig 2 The signal-to-background ratio versus incubation time for a LAPS assay of NDV The signal component of the ratio was the sensor output for a 500 ng/ml sample of NDV in buffer (100 µl volume); the background component was output for reagents alone, no NDV Each point represents the quotient of the mean signal ($n = 4$) and the mean background ($n = 4$) The bars represent ± the sum of 1 s.d of signal and 1 s.d of background

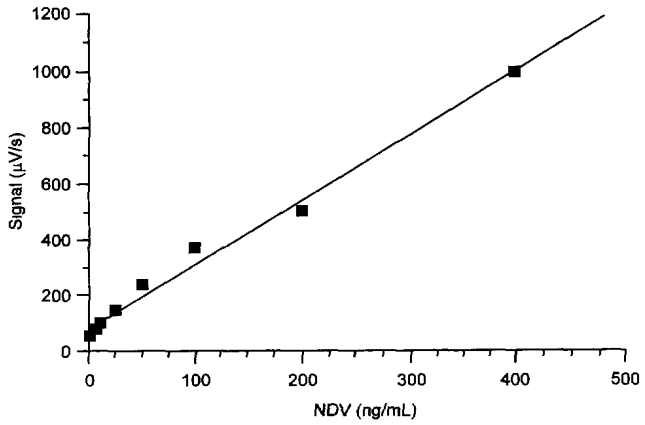

Fig. 3 Standard curve of Newcastle disease virus (60 min incubation) carried out with monoclonal antibodies 55R3 (biotin-labeled) and 25R5 (fluorescein-labeled) The designations 55R3 and 25R5 refer to specific monoclones

The specific target of the gene probe assay on the LAPS was a PCR-amplified cDNA fragment of the nucleocapsid gene of NDV, 391-bp in length. The assay employed two DNA probes of the same sense, 20 and 23 nucleotides in length, end-labeled with fluorescein and biotin, respectively. In the assay the probes and the target were mixed together in a single step, hybridized at elevated temperature and immobilized on the biotin-embedded membrane via biotin–streptavidin capture As in the immunoassays, anti-fluorescein urease conjugate was added to the immobilized hybridization product and the presence of hybridization product was detected by monitoring urease activity.

Enzyme inhibition assays on the LAPS were carried out by reacting inhibitory organophosphorus (OP) compounds with biotin-labeled acetylcholinesterase and then adding streptavidin. After the enzyme–OP reaction, the enzyme was captured on the biotin-embedded nitrocellulose membrane by filtration. Monitoring was carried out in a similar fashion as above by immersing the membrane in substrate (in this case acetylcholine) solution and pressing it against the sensor surface. The presence of organophosphates was determined by the decrease in esterase activity.

Table 1
Limits of detection for NDV as a function of antibody configuration

Sample[a]	Upper Ab[b]	Lower Ab[b]	LOD[c] (60 min)[d] ng/ml	LOD (5 min) ng/ml	LOD (1 min) ng/ml
Buffer	25R5-f[a]	55R3-b	2 0	43	82
Buffer	55R3-f	25R5-b	2.0	–	-
Buffer	25R5-f	25R5-b	4 2	–	–
Buffer	55R3-f	55R3-b	4 3	–	–
Buffer	pc-f	pc-b	0.85	–	–
Serum	25R5-f	25R5-b	400	–	–

[a] Samples were contained in 100 μl volumes of buffer or 10 μl of volumes serum as indicated
[b] f and b indicate antibody labeled with fluorescein and biotin, respectively, 55R3 and 25R5 indicate different antibody clones, pc indicates polyclonal antibody
[c] Limit of detection (S/B > 2)
[d] The incubation times are given in brackets

The model analytes used in this work were selected to represent a range of potentially infectious and toxic agents. Newcastle disease virus is an avian paramyxovirus. The B1 vaccine strain of NDV, a commercially available poultry vaccine, was employed. As a model, this strain of NDV has a number of advantages. It has been approved by Agriculture Canada for release into the air and has been used as a BW viral simulant in field trials. It has been characterized by a number of immunoassay studies (Fulton et al., 1988; Wong et al., 1991, 1992) and served as a virus analyte in the development of biosensor technologies (Lee et al., 1993; Lee and Thompson, 1996). *B. melitensis* is a gram-negative bacterium responsible for brucellosis. Although it is largely a disease of livestock and wildlife, the zoonotic nature of the bacterium makes it a threat to human health. The spore model used in this work was *B. subtilis var. niger*, often referred to as *B. globigii* (BG), a benign Gram-positive soil bacterium. BG is a model for *B. anthracis* and is used in open-air field trials and aerosol collection studies. Staphylococcal enterotoxin B (SEB) is an exotoxin protein (28.3 kDa) of the bacterium *Staphylococcus aureus*. It causes food poisoning and anaphylactic shock. Ricin (64 kDa) is a highly toxic protein extracted from castor beans (*Ricinus communis*).

2. Experimental

2.1. Apparatus

The detection apparatus was a commercially available LAPS marketed under the name Threshold Unit. It was purchased from the manufacturer, Molecular Devices Corp. (MDC) (Sunnyvale, CA). The instrument was controlled by a computer and custom software, supplied by MDC. The assay system was capable of processing (filtration, potentiometric sensing) eight samples simultaneously on a single membrane stick. The DNA hybridizations were carried out using a programmable DNA thermal cycler (Perkin Elmer, Mississauga, Ont.).

2.2. Reagents

Bovine serum albumin (BSA), sodium dihydrogen phosphate, Tween 20, Triton X-100, urea, electric eel acetylcholinesterase, acetylcholine chloride, ethylene diamine tetraacetic acid and phosphate buffered saline (PBS) were obtained from Sigma Chemical Co. (St. Louis, MO) and used without any further purification. Streptavidin was obtained from Scripps Laboratories (San Diego, CA). It was reconstituted in distilled water to yield a stock concentration of 10 mg/ml. Antibody labeling reagents, *N*-hydroxysuccinimide esters of carboxyfluorescein and dinitrophenylbiotin, were obtained from MDC and were used according to procedures described in the product literature. Nitrocellulose membrane filters (0.45 μm pore size) coated with biotinylated BSA and anti-fluorescein urease conjugate were

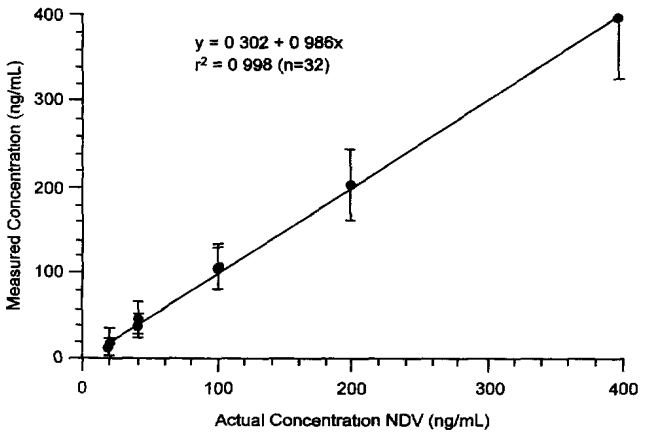

Fig 4 Comparison of measured values of NDV by LAPS assay with the actual concentrations of the test samples Each point represents the mean ($n = 4$) of an independent determination The error bars represent ± 1 s.d.

Table 2
Limits of detection for protein toxins

Assay	Antibodies[a] (upper/lower)	LOD[b] (60 min)		LOD (5 min)		LOD (1 min)	
		pg/ml	pM	pg/ml	pM	pg/ml	pM
SEB	R-f/R-b	3	0 11	30	1 1	310	11
SEB	Sh-f/Sh-b	3	0.11	20	0.7	80	2 9
SEB	Sh-f/R-b	6	0 22	20	0 7	–	–
SEB	R-f/Sh-b	7	0 25	20	0 7	–	–
Ricin	R-f/Sh-b	50	1 5	–	–	–	–

[a] R and Sh indicate rabbit and sheep antibody to either SEB or ricin, f and b indicate fluorescein and biotin-labeled antibody
[b] Limit of detection (S/B > 2), samples were contained in 100 μl volumes, incubation times are indicated in brackets

Table 3
Limits of detection of *B. melitensis*

Assay configuration	LOD (60 min)[b] ng/ml	LOD (5 min) ng/ml	LOD (1 min) ng/ml
R-f/R-b (biotin-mediated capture)[a]	20	50	150
Filtration-capture w/anti-f-U[c]	5 0	20	
Filtration-capture w/anti-R-U[d]	24	50	150

[a] R-f and R-b indicate sandwich assay using fluorescein-labeled rabbit anti-*Brucella* and biotin-labeled rabbit anti-*Brucella*, respectively, followed by anti-fluorescein urease.
[b] Limit of detection (S/B > 2), samples were contained in 100 μl volumes, incubation times are indicated in brackets
[c] Filtration-capture assay incubation of *Brucella* with fluorescein-labeled anti-*Brucella*, capture of immune complex on nitrocellulose membrane, followed by anti-fluorescein urease.
[d] Filtration-capture assay incubation of *Brucella* with unlabeled rabbit anti-*Brucella*, capture of immune complex on nitrocellulose membrane, followed by anti-rabbit urease conjugate

purchased from MDC. Soman (pinacolyl methylphosphonofluoridate) and sarin (isopropyl methylphosphonofluoridate) were obtained from the organic chemistry laboratory at Defence Research Establishment Suffield (DRES). Trichlorfon ((2,2,2-trichloro-1-hydroxyethyl)-phosphonic acid dimethyl ester) and malathion ([(dimethoxyphosphinothioyl)thiol]butanedioic acid diethylester) were purchased from Polyscience (Niles, IL). Dried sand/clay/loam- # 3 was provided by James Hancock at DRES. Naive mouse serum was provided by Dr John Cherwonogrodzky at DRES.

2.3. Antigens

Ricin was obtained from Sigma Chemical Co., SEB from Toxin Technologies Inc. (Sarasota FL). Newcastle disease virus was obtained from the American Type Culture Collection (Rockville, MD), cultivated in hen eggs, purified by sucrose gradient centrifugation and suspended in phosphate buffered saline pH 7.0. NDV used in this work was provided by R.E. Fulton, DRES. Protein concentrations were determined spectrophotometrically with a BCA protein assay kit (Pierce Chemical Co., Rockford, IL). Formalin-killed *B. melitensis* was provided by Dr John Cherwonogrodzky. *B. globigii* (BG) was obtained as a lyophylized powder from the Baker Laboratory, Dugway Proving Ground, UT. The BG preparation was reconstituted in distilled water for use in the immunoassays. Further, a portion of the reconstituted BG preparation was centrifuged The supernatant was retained and the pellet was given two additional washes by centrifugation. This preparation (the pellet) was referred to as washed BG. A measured portion of the retained supernatant was filtered through 0.22 μm nitrocellulose membrane to remove any particulate material and evaporated at 75°C to remove the water. The residue from the filtrate was then weighed and diluted quantitatively.

Table 4
Limits of detection of *B. globigu* preparations

BG Analyte	Antibodies[a]	LOD[b] (60 min)	LOD (10 min)
		ng/ml	ng/ml
Reconstituted	SciLab	3 9	
Washed	SciLab	170	
Filtrate	SciLab	0 10	
Reconstituted	Baker		7 1

[a] IgG from rabbit serum purified on protein G column source SciLab or Baker Laboratory as indicated
[b] Limit of detection (S/B > 2), samples were contained in 100 μl volumes, incubation times are indicated in brackets

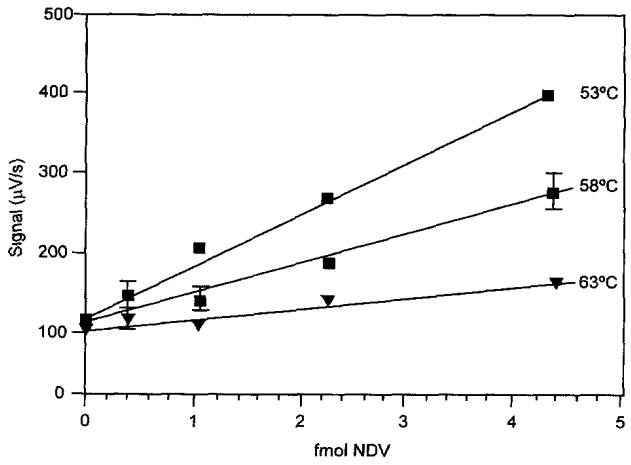

Fig 5 The effect of temperature on hybridization of probes and target The error bars, where not masked by the point markers, represent ± 1 s d

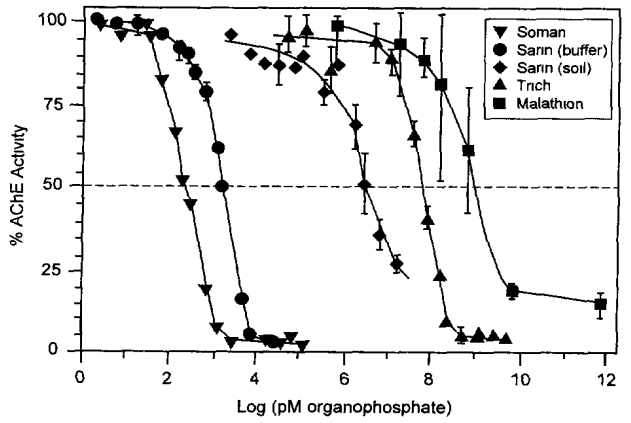

Fig 6 Comparative enzyme inhibition of organophosphorus compounds soman, sarin, trichlorfon and malathion in buffer, and soil-spiked sarin The IC_{50} points for sarin spiked into buffer and soil-spiked sarin on the inhibition curves are separated by three log units concentration

2.4. Antibodies

Anti-ricin IgG was obtained from mouse serum using protein A purification. Sheep and rabbit anti-SEB were purchased from Toxin Technologies Inc. Monoclonal antibodies against NDV (provided by R.E. Fulton) were purified from ascites fluid using high performance liquid chromatography as described previously (Wong et al., 1992). Polyclonal anti-NDV was purified from guinea pig serum using ammonium sulfate precipitation, followed by ion exchange chromatography. Rabbit anti-*Brucella* IgG was obtained from Dr John Cherwonogrodzky. Protein G-purified rabbit polyclonal anti-BG was obtained from the Baker Laboratory (DPG) and from SciLab (Medicine Hat, Alberta.). The Baker antibodies were raised against the reconstituted spore sample. The SciLab antibodies were raised against washed spores (six cycles of centrifugation and resuspension in distilled water). Anti-rabbit IgG urease conjugate and anti-mouse IgG urease conjugate were purchased from Sigma.

2.5. Immunoassays

For immunoassays, the wash solution consisted of 150 mM NaCl, 10 mM phosphate buffer pH 6.5, plus 0.2% Tween-20 detergent. The dilution buffer was the wash solution titrated to pH 7 0, containing 0.1% BSA and 0.25% Triton X-100. The substrate solution was prepared by adding 100 mM urea to the wash solution The reagent solution consisted of 20 μl of biotin-labeled and fluorescein-labeled antibody preparations (typically 0.5 mg/ml) and 5 μl streptavidin solution in 10 ml of dilution buffer. Aliquots of reagent solution (100 μl) were added to 100 μl portions of antigen (the test analyte), suspended in dilution buffer, mixed, incubated for the required time, filtered under partial vacuum through the biotin-embedded nitrocellulose membrane and rinsed with wash solution (500 μl) (see Fig. 1). After the rinsing step, an aliquot (200 μl) of anti-fluorescein urease conjugate (stock solution diluted 1/10 in dilution buffer) was slow filtered over 10 min through the membrane that contained the trapped immune complex. Following filtration-capture, the membrane sticks were inserted into the reader compartment that contained the pH-sensitive sensor and substrate solution. The urease enzyme reaction produced ammonium hydroxide and thus an increase in pH. A plunger in the reader compartment pressed the membrane against the surface of the sensor. The instrument was designed so that the eight spots on the surface of the membrane aligned with the eight pH-sensitive sites on the surface of the silicon sensor The data was recorded and stored on the computer. The rate of change of pH with respect to time at the surface of the silicon sensor was monitored by the rate of change with respect to time of the surface potential as μV/s. For immunoassays, the 100 μl-aliquot of reagent cocktail typically contained 50–100 ng, each, of biotin-labeled and fluorescein-labeled antibody and 0.5–1.0 μg streptavidin. Starting with these amount of reagents, the assays were optimized by adjusting the concentration of each labeled antibody in the cocktail to obtain maximal signal to background readings.

2.6. Gene probe assays

The final concentration of the hybridization buffer was 30 mM sodium phosphate (pH 7.4), 3 mM EDTA, 450 mM NaCl, and 0.25% Triton X-100. The reverse transcription PCR primers, the 20-mer fluorescein probe (5′ fluorescein-GCTCCTCGCGGCT-CAGACTC-OH 3′) and 23-mer biotin probe (5′

biotin-TAGCGAGGATGCCAACAAACCAC-OH 3′) were prepared by the Regional DNA Synthesis Laboratory (University of Calgary, Calgary, Alberta). The probes were designed based upon sequence information from Ishida et al., 1986. The wash solution consisted of 10 mM sodium phosphate buffer (pH 6.5), 1 mM EDTA, 150 mM NaCl and 0.05% Tween-20 detergent. Assay buffer consisted of the wash solution titrated with HCl to pH 7.0, plus 0.1% BSA. The wash solution, hybridization buffer and assay buffer were each filter-sterilized through 0.22 μm nitrocellulose before use.

The target analyte was a 391 bp cDNA fragment of the nucleocapsid protein gene that was prepared by reverse transcription polymerase chain reaction (RT-PCR) of purified genomic RNA from NDV B1 strain (American Type Culture Collection) as described elsewhere (Bader and Lewis, 1995). In the assay, the target cDNA was combined with 300 fmol of each probe in 60 μl assay buffer, denatured at 100°C for 15 min and cooled to 10°C for 5 min. Hybridization was carried out by heating the mixture to the required temperature for the prescribed period of time (e.g. 58°C and 30 min) then cooling to 4°C. The probes were of the same sense, such that the hybridization product contained both biotin and fluorescein components. After hybridization, a 50-μl aliquot of reaction mixture was added to 100 μl of assay buffer containing 1 μg streptavidin and thoroughly mixed. An aliquot of this mixture (135 μl) was filtered under partial vacuum and captured on the biotin-embedded nitrocellulose membrane (same membrane used in immunoassays). After filtration-capture the membrane was washed with 1 ml wash buffer. The vacuum was reduced and 100-μl aliquot of anti-fluorescein urease conjugate was slow filtered through the membrane over 10 min. The membrane was washed with 2 ml of wash buffer to remove excess anti-fluorescein urease conjugate. The membrane stick was removed from the filter apparatus and inserted into the reader compartment that contained the pH sensor and substrate solution. As in the immunoassays the presence of target analyte (in this case, dual-probe-cDNA hybridization product attached to anti-fluorescein urease) was monitored as urease activity at the membrane surface. The output signal from the sensor was recorded by the computer as μV/s.

2.7. Enzyme inhibition assays

The procedure for biotinylation of acetylcholinesterase was described previously in detail (Lee and Hall, 1992). For the assays, aliquots of biotinylated acetylcholinesterase (AChE-b), containing 50 ng in 100 μl were mixed with 10 μl of organophosphorus inhibitor (OP) and streptavidin (0.5 μg in 50 μl), incubated for 10 min, filtered under partial vacuum through the biotin-embedded nitrocellulose and rinsed with 0.5 ml PBS The membrane containing the immobilized AChE-b was inserted into the reader compartment of the LAPS that contained substrate solution, 25 mM acetylcholine chloride dissolved in 5 mM PBS (pH 7.4). The sensor monitored enzyme activity as a decrease in pH resulting from the generation of H^+ in the hydrolysis of acetylcholine. The presence of OP was indicated by a decrease in the enzyme activity compared to the control samples.

3. Results and discussion

3.1. Immunoassays on the LAP sensor

3.1.1. Virus model

3.1.1.1. Signal-to-background ratio versus incubation time. In this work, the sandwich antibody–antigen complexes (the products of the immunoreactions) were derived from one-step incubations. Biotin-labeled antibodies were used for capture and fluorescein-labeled antibodies for detection. Fluorescein served as the hapten-target for the signal generating anti-fluorescein urease conjugate. The rate of change of pH versus time (the output signal) depended upon the number of urease-containing antibody–antigen sandwiches immobilized on the membrane during the filtration process. The efficiency of the biotin-mediated filtration capture was high, approximately 95% under present conditions, (Olson et al., 1990), thus the number of sandwiches immobilized was a measure of the number formed during the incubation period.

The effect of incubation time upon the output response for the virus model is given in Fig. 2 as signal-to-background ratio (S/B) versus time. The signal component was the output of the sensor from a test sample of 50 ng (500 ng/ml) NDV plus reagents (contained in 100 μl buffer); the background was the sensor output derived from reagents alone, no antigen. For a given incubation time, four of the test sites on the membrane contained NDV samples, the other four sites were reserved for reagents alone. The test samples were incubated at room temperature for periods of time ranging from 1 min to 2 h. There was a continuous increase in S/B up to and including the longest incubation time. The value of S/B at 125 min was 7.6 which appears close to a maximum. For incubation times of 1 and 5 min, S/B was found to be 1.5 and 2.2, respectively, or 20 and 29% of the signal obtained from an incubation of 125 min. For the protein and bacteria models, the plots of S/B versus incubation time showed similar features with maximal ratios occurring in the 60–120 min range.

3.1.1.2 Limits of detection. The lower limits of detection (LOD) for the LAPS immunoassays of NDV were determined at three incubation times (1, 5, and 60 min) using a selection of monoclonal and polyclonal antibodies. The data were represented by linear plots. Fig. 3 is typical of the sensor response. The error bars associated with the individual points were masked by the point markers. At the low concentration region of the dose–response curves, that is, near the limits of detection, the standard errors of the background were approximately equal to the standard errors of the samples. In this work, the LOD was defined as the smallest sample amounts having an output greater than background plus two standard deviations of the background. The coefficients of variation (CV: defined as the ratio of the standard deviation to the mean) were about 6%. For assays run in buffer, the LODs of the 60-min incubations were about 0.4 ng (4.0 ng/ml) or less, depending on the antibody combination. Assays of NDV for incubations of 1 and 5 min gave LODs of 4.3 ng (43 ng/ml) and 8.2 ng (82 ng/ml), respectively. The lowest result was obtained with a polyclonal preparation. Table 1 summarizes the data.

3.1.1.3. Quantitation of NDV. The ability of the LAPS to provide quantitative analysis of NDV was demonstrated. Series of NDV calibration standards and NDV test samples were prepared from NDV preparations of known concentrations and then assayed on the LAPS. The measured concentrations of the test samples were determined by reference to the calibration standards. In Fig. 4, the measured values are plotted against the actual values and show good agreement. For eight different test samples of NDV with concentrations in the range of 2–40 ng (20–400 ng/ml), the mean difference between the measured and actual values was about 9%.

3.1.1.4. Model clinical assays of NDV. The sensor system was also useful in developing model clinical assays for infectious viral agents and for immune response. Immunoassays for NDV (with NDV spiked into 10-μl aliquots of naive mouse serum) were analyzed similar to those for NDV in buffer. The measured LOD of NDV in mouse serum was 4 ng (400 ng/ml). In clinical assays, the presence of an immune response to an infectious agent is often used for diagnosis and to direct medical treatment. Model assays to determine the presence of antibody to NDV in mouse serum were developed. In these assays, the analyte was the unlabeled anti-NDV mouse monoclone (clone # 25R5), spiked into 10 μl-aliquots of naïve mouse serum The assay procedure was modified slightly to be able to detect and measure antibody rather than antigen (see Experimental Section 2). Reagents for the immune response assay were biotin-labeled NDV (the molecular recognition and capture element) and anti-mouse IgG urease conjugate (for signal generation). The reaction products were captured by biotin–streptavidin interaction. For this model, the LOD for spiked anti-NDV in mouse serum was determined to be 4 ng (400 ng/ml).

Clinical assays generally do not rely on actual quantitation of infectious agents or antibodies to them. More often, the relevant information is the degree of dilution that the agent or antibody can be detected above a control sample. Typically, an immune response will produce antibodies in the serum to the infectious agent in excess of 100 μg/ml (Harrow and Lane, 1988; McIntosh, 1990). Naive mouse serum was spiked with unlabeled anti-NDV (clone # 25R5) at 100 μg/ml and then serially diluted in buffer and assayed on the sensor. Using a cut-off value of background plus two standard deviations, the analyte was detected as low as 1/200 dilution, equivalent to a concentration of 500 ng/ml (5 ng total mass). This dilution data was consistent with the LODs determined from standard curves.

3.1.2. Immunoassay of protein–SEB and ricin

Assays for toxic proteins were developed using polyclonal antibodies derived from sheep and rabbit. For SEB, several combinations of sheep and rabbit antibodies were employed for capture and detection giving LODs of 0.3–0.7 pg (3–7 pg/ml), 2–3 pg (20–30 pg/ml) and 8–31 pg (80–310 pg/ml) for 60, 5 and 1-min incubations, respectively. The various combinations of antibodies yielded approximately similar LOD values. There was no striking advantage in using antibodies from different sources for the capture and detection components of the sandwich immune complex. As in the NDV assay, good results could be obtained using a single polyclonal antibody preparation, part labeled with biotin and part with fluorescein.

The assay for ricin had an LOD of about 10 pg (100 pg/ml) comparable in sensitivity to a fiber optic-based ricin assay (Narang et al., 1997). The assay was developed quickly (within 2 days after delivery of the reagents from the supplier) in response to receiving an unknown sample reputed to contain ricin. On LAPS analysis, the unknown did contain ricin at about 3.5% by weight (later confirmed by mass spectrometry). The data for the protein immunoassays is given in Table 2.

3.1.3. Immunoassay of vegetative and spore bacteria

The vegetative bacteria model was a formalin-killed preparation of *B. melitensis* Sandwich assays were carried out in the standard format (Fig. 1) using polyclonal antibodies labeled with biotin for capture and with fluorescein for detection (via anti-fluorescein urease conjugate). The LODs were determined to be 2, 5 and 15 ng (20, 50, 150 ng/ml) for 60, 5, and 1-min

incubations, respectively (Table 3). Based on a weight of 10^{-12} g per cell these values represent 2000, 5000, and 15 000 cells.

The pore size of nitrocellulose membrane (0.45 μm) was sufficiently small to trap the bacteria in the matrix. Thus, assays could also be carried out by filtration-capture, without using the biotin-labeled antibody and streptavidin (as in Fig. 1). The other components of the assay (fluorescein-labeled antibody and anti-fluorescein urease) remained the same. LODs were comparable to the biotin-mediated capture of 0.5 and 2 ng (5 and 20 ng/ml) for 60 and 5-min incubations. Also, a variation of this filtration-capture assay that required no antibody labeling was used. Unlabeled anti-*Brucella* IgG (from rabbit) was incubated with *B. melitensis* and filtered under partial vacuum through the membrane to trap the complex. Then anti-rabbit IgG urease conjugate was slow filtered through the membrane. This latter antibody was substituted for the anti-fluorescein urease conjugate. The results of the modified filtration-capture assay were similar to assays using fluorescein-labeled antibodies and anti-fluorescein urease conjugate. The LODs are summarized in Table 3. This assay was very easy to develop compared to the standard assay of Fig. 1; the rabbit anti-Brucella IgG was obtained from the DRES freezer inventory and used 'as-is' and the anti-rabbit urease conjugate was purchased commercially

Immunoassays of *B. globigii* using polyclonal antibodies were performed according to the scheme in Fig. 1. The LOD for the lyophilized BG preparation, reconstituted in distilled water, was 0.39 ng total mass (3.9 ng/ml). It was observed that the soluble fraction of the BG preparation (i.e., the non-spore material) contained a substantial portion of the total antigenicity (Lee et al., 1998). When the spore samples were washed by several cycles of centrifugation and re-suspension to remove any residual soluble components, only BG spores remained. The sensitivity of the assay to these washed spores decreased by about 40-fold to 17 ng (170 ng/ml) even though the total number of spores used in the assay remained relatively constant. When the supernatant from the first wash (that is, the soluble fraction from the spore preparation) was dehydrated, weighed and re-suspended, it was found to give high signals in the LAPS assay, detectable to 10 pg (100 pg/ml) (see Table 4). The discovery of the relative insensitivity of the assay to the spores, themselves, and the high antigenicity of the soluble component was made during development of rapid assays for field trials. For simplicity we ran filtration-capture assays of the BG spores (similar to those described above for *Brucella*) and were surprised to see the signals drop to low values even though the spores were large enough (approximately 1 μm diameter) to be readily trapped in the filter matrix.

3.2. Gene probe assay — NDV

The LAPS provided a very reliable method for gene probe detection of specific DNA sequences. The assay employed two probes, a 20-mer labeled with biotin and a 23-mer labeled with fluorescein directed at a 391 bp cDNA fragment. Analogous to immunoassays, the signal output for the LAPS gene probe assay is a measure of the number of probe-target complexes formed during the hybridization process. The effect of temperature on the hybridization process is shown in Fig. 5, wherein lower hybridization temperatures yielded higher signals. For a hybridization temperature of 58°C (22° below the calculated melting temperature of the probes to the target), the limit of detection was about 0.30 fmol (1.8×10^8 molecules) per well or 38 pg, based on a molecular weight of 1.27×10^5 g for the single-stranded 391-nucleotide target. This corresponds to a concentration of 5.0 pM in a sample volume of 60 μl. The CVs were about 10–12%. The assay time, including hybridization, filtration capture and potentiometric sensing, was about 45–60 min. This is significantly less time than conventional hybridization assays on membranes (Bader and Fisher, 1994) or titer plates (Bader and Lewis, 1995) which require 6–8 h for completion. In addition to detection and identification applications, the LAPS provides a useful method for measuring hybridization kinetics. The small sample volumes allow for reproducible temperature control and the rapid filtration/separation step provides a sharp cut-off or endpoint for the reactions.

3.3. Enzyme inhibition assays

Enzyme inhibition assays for organophosphorous compounds were carried out using biotin-labeled acetylcholinesterase, streptavidin and acetylcholine substrate. A comparison of the inhibitory action of four OP compounds (two classical nerve agents, two commercial pesticides) is given in Fig 6. These plots demonstrate the well-known differences in toxicity and acetylcholinesterase inhibition between nerve agents and pesticides. The limit of detection for the inhibition assays on the LAP sensor was defined as the amount of OP that reduced the mean activity (plus twice the standard deviation) to less than the control value. LODs for soman and sarin were determined to be about 2 and 8 pg (or 100 and 600 pM), respectively, for a 10 min assay.

The relative inhibitory capacity of various preparations of OP could be readily determined on the LAPS. An example is sarin in soil. For this, freshly prepared aliquots of aqueous sarin were spiked into 50 mg portions of dry soil and the water evaporated. The dried soils were then treated with water and filtered to extract the sarin. A comparison of spiked-dried-ex-

tracted sarin is shown also in Fig 6 From the IC_{50} (50% inhibition concentration) of these two curves, it can be seen that the extracted samples are about three log units less inhibitory. Analysis by these methods provides a quick and reliable way to test the toxicity of contaminated soils or surfaces and an equally quick and reliable method of monitoring decontamination. The LAPS provides a rapid and easy method for determining the presence of inhibitory compounds and for comparative toxicology Other useful applications lie in measuring aging of OP–AChE complexes and regeneration effects of oxime compounds (e.g. PAM, toxigonin, HI-6) on poisoned acetylcholinestase

4. Conclusions

This report has described a potentiometric sensor capable of detection and identification of a range of chemical and biological threat agents. Immunoassays for protein (toxins), viruses and bacteria have been developed. The limits of detection for standard volume samples (100 μl) were 0.5–5.0 pg (5–50 pg/ml) for proteins, 0.1–0.5 ng for virus (1–5 ng/ml) and 0.5–2 ng (5–20 ng/ml) for vegetative bacteria. This increase in LOD with analyte size is mostly due to the decreasing number of epitope sites per unit weight and is observed in other assays (Thompson and Lee, 1991, 1992; Lee and Thompson, 1996). A gene probe assay for PCR-amplified cDNA, derived from Newcastle disease virus, gave a limit of detection for the target of 0.30 fmol or 1.8×10^8 molecules.

The assays employed a pair of molecular recognition elements. For immunoassays, a pair of antibodies directed at the analyte, one labeled with biotin, one with fluorescein was used. For the gene probe assay, two distinct probes, one labeled with biotin and one with fluorescein, were employed. In the assays, the experimental methods were similar; the analyte and the particular reagents, consisting of the recognition elements plus streptavidin, were mixed in a single step, incubated, and then immobilized on nitrocellulose membrane by means of biotin–streptavidin interaction. The reaction complex was detected potentiometrically upon addition of anti-fluorescein urease conjugate. The assays were rapid and easy to perform; the total time required to perform the assay was the reaction period plus about 15 min for mixing, filtering, washing, and sensing. In field trial studies, reagents for several different immunoassays were pooled into a single cocktail and were found to work effectively. Thus, a single test could screen for four or five analytes. In addition, the assay-development or 'work-up' time was short and relatively easy to carry out. Starting with a pair of purified antibodies, the time required to label with biotin and fluorescein, optimize the assay, and determine the limits of detection was about 10–15 h. For assays of vegetative bacteria, good assay results could be obtained without any labeling at all by using filtration capture onto the membrane and signal generation with an anti-species urease conjugate. With this method assay development times could be as short as 2 h. The individual reagent solutions made one assay different from another and so, analyzing a variety of sample unknowns in succession does not present a technical problem. The immunoreagents were found to be stable. They have been lyophilized and stored for up to 1 year; reconstituted immunoreagent solutions are stable at 4°C for several months.

The sensor system was also useful in enzyme inhibition assays Potent organophosphate compounds such as soman and sarin could be detected below 10 pg total mass. This value is about 10^7 times less than a human incapacitating dose (Compton, 1987). Thus OP-containing samples can be analyzed at concentrations well below the hazardous level. To switch the sensor assay from a signal based on urease, to one based on acetylcholinesterase, only required a change of buffer solution in the reader compartment (approximately 2 min) or the use of two readers (30 s).

The LAPS apparatus is rugged and durable. We have operated the device for several years and experienced minimal 'down time' from instrument malfunction. The sensor unit requires a bench area of about 0.06 m^2 (3 sq. ft.) and as such, is suitable for a mobile field laboratory The work to date has primarily been with environmental samples, but the system has also been able to sample blood serum for viruses and immune responses to infectious agents. This latter work indicates utility as a diagnostic tool in a medical field laboratory. The system has a moderate logistics burden with regards to consumables and is manually operated. It requires a skilled operator, although in the hands of a qualified technician it is easy to use. The main role of the LAPS is in laboratory-based analysis for identification of unknown samples arriving from areas where military personnel would be deployed for peacekeeping or inspection duties.

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