Detection of *Neisseria meningitidis* and *Yersinia pestis* with a Novel Silicon-Based Sensor

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A light-addressable potentiometric (silicon) sensor was used in an immunofiltration procedure for the detection of pathogenic bacteria. *Yersinia pestis* was detected by filtering the cells onto nitrocellulose membranes and then filtering anti-*Y. pestis* mouse monoclonal antibody and anti-mouse immunoglobulin G-horseradish peroxidase conjugate. For *Neisseria meningitidis* detection, mouse monoclonal antibody to the major outer membrane protein of this bacterium was coupled directly to horseradish peroxidase. *N. meningitidis* cell suspensions were filtered onto polycarbonate membranes, and the enzyme conjugate was allowed to react with the filtered bacteria. The presence of both enzyme conjugates was determined potentiometrically with the silicon sensor. The sensitivity of this technique relative to that of an enzyme-linked immunosorbent assay for *N. meningitidis* was determined. Fewer than 1,000 bacterial cells could be detected with the silicon sensor in a 20-min assay, whereas a 2.5-h enzyme-linked immunosorbent assay with the same antigen and antibody preparations was significantly less sensitive.

A light-addressable potentiometric sensor able to detect changes in pH or redox potential has recently been described (Fig. 1) (3). The detecting component of this sensor is insulated silicon which contains no on-chip circuitry and is impervious to biological fluids. Electrical signals are generated by directing beams of light onto the surface of the silicon chip. The photoresponse of the silicon chip is "spatially selectable" in that light beams directed to different locations on the chip surface may be used to assay a number of biochemical events occurring simultaneously. In order to detect redox changes, such as those due to enzymatic activity in the solution in contact with the silicon insulator, thin (500-nm) gold "spots" on the insulator surface are positioned under the solution in which the redox change is occurring. The electrical potential of the gold is determined by the redox potential of the solution with which it is in contact. An inorganic redox pair, such as ferricyanide and ferrocyanide ions, sets the potential of the solution and mediates the transfer of electrons from an enzyme substrate to the gold. Changes in the gold layer potential in turn are monitored by their influence on field effects in the silicon (3).

This semiconductor technology may be used as the detection component of an immunofiltration test. When a membrane filter which has captured a bacterial cell or product and then a specific enzyme-antibody conjugate is placed against the chip, enzymatic activity modulates the potentiometric signal, indicating the presence of the specific antigen. Thus the silicon sensor has the potential to detect a wide range of cellular targets, limited only by the ability to capture the target with the membrane and the availability of appropriate antibody-enzyme conjugates for signal generation.

In order to ascertain the sensitivity and precision of a silicon sensor-based immunofiltration method for bacterial antigen detection, killed-cell preparations of two well-characterized pathogens, *Yersinia pestis* and *Neisseria meningitidis*, were obtained. Rapid detection methods for these bacteria, including filtration (7), immunofiltration (1, 9), and commercially available assays for *Neisseria* species (2, 4, 6), have been previously described (8, 10–12). The immunofiltration assays described in this report were not developed for the purpose of introducing new *Yersinia* or *Neisseria* assay products to the market but rather to determine the feasibility of using a silicon sensor-based instrument for bacterial antigen detection in general. Direct comparisons of these assay methods with commercially available tests were therefore not conducted. Tests for other microbial pathogens which will use silicon sensor technology are being developed.

MATERIALS AND METHODS

Antibodies and bacterial antigens. *Escherichia coli* ATCC 25922 obtained from the American Type Culture Collection (Rockville, Md.) was cultured in Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.). *E. coli* cells used for negative controls were washed in 0.01 M sodium phosphate–0.9% NaCl (pH 7.0; PBS) and suspended in PBS to 5×10^8 cells per ml. *N. meningitidis* ATCC 13090 (serogroup B) and *Neisseria gonorrhoeae* CDC 98 Bactrol disks were obtained from Difco Laboratories. *Y. pestis* M0319 vaccine and *N. meningitidis* 7851 (serogroup A) vaccine containing Formalin-killed cells suspended in saline were obtained from the Army Medical Research and Development Command. Two antibody preparations were obtained from the latter source, including an anti-*Y. pestis* mouse monoclonal antibody prepared against F1 envelope antigen and an anti-*N. meningitidis* monoclonal antibody developed against the major outer membrane protein of strain 7851.

Enzyme conjugate preparation. Anti-*N. meningitidis* monoclonal antibody was coupled to horseradish peroxidase (HRP; Sigma Chemical Co., St. Louis, Mo.) by the method of Ishikawa et al. (5).

Immunofiltration. The concentration of killed cells in both vaccine preparations was reported by the suppliers and confirmed in our laboratory by direct count. The *Y. pestis* vaccine preparation was diluted in PBS. Since nitrocellulose membranes retain more of the *Y. pestis* antigen than several other membranes tested (unpublished data), this type of membrane was chosen for this assay. Diluted cell suspensions (200 µl) were filtered through 0.22-µm-pore-size nitrocellulose membranes (Millipore Corp., Bedford, Mass.)
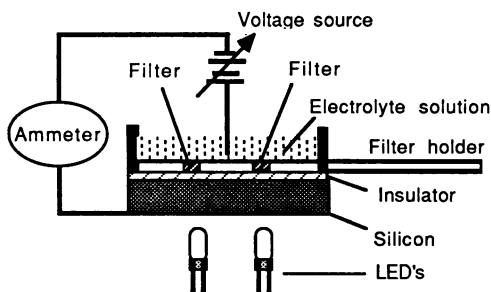
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FIG. 1. Schematic diagram of silicon sensor described by Hafeman et al. (3). The silicon is covered with an insulator which separates it from the electrolyte solution containing enzyme substrate. The electrical potential of thin gold layers on the surface of the insulator (located under the filters) is measured via a light-generated alternating current. By eliminating the liquid above the membrane with a plunger (not shown), reduced volume and thus greater sensitivity are achieved. LED, Light-emitting diode.

TABLE 1. Summary of immunofiltration assay results

Strain and no. of cells	Kinetic rate (mean ± SD μV/s)[a]	CV (%)[b]
N. meningitidis		
0	−68 ± 13	15
800	−127 ± 17	13
8,000	−227 ± 36	16
80,000	−942 ± 48	5
Y. pestis		
0	−21 ± 12	57
4,000	−44 ± 14	32
40,000	−56 ± 17	30
400,000	−155 ± 22	14

[a] Results of five determinations.
[b] CV, Coefficient of variation.

mounted on plastic dipstick holders. Filtration was facilitated by mounting each filter dipstick in a plastic filtration manifold which pressed the membrane against an absorbent pad and directed the liquid through a circular area 4 mm in diameter. For negative controls, either PBS, PBS containing 5×10^8 E. coli cells per ml (10^8 cells per membrane), or N. gonorrhoeae CDC 98 suspended in PBS (10^9 cells per membrane) was filtered through nitrocellulose filters. Monoclonal anti-Y. pestis was diluted to 1.0 μg/ml in a buffer containing 40% fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.), 0.05% Tween 20, 0.05 M Tris hydrochloride, 0.01% sodium thimersol, and 1 mM EDTA (pH 7.4) and prefiltered through a Uniflo 0.2-μm-pore-size filter unit (Schleicher & Schuell, Inc., Keene, N.H.). Of this solution, 200 μl was filtered through the membranes (approximately 5 min at room temperature). The membranes were washed with PBS containing 0.05% Tween 20. A 1/3,000 dilution of affinity purified goat anti-mouse immunoglobulin G (heavy and light chain)-HRP conjugate (Bio-Rad Laboratories, Richmond, Calif.) (200 μl) was passed through the filters. The membranes were washed a final time with 200 μl of PBS containing 0.05% Tween 20. Each filtration step took approximately 5 min and was conducted at room temperature. The total time of this filtration procedure was approximately 30 min.

The N. meningitidis vaccine was diluted in PBS with 0.05% Tween 20. Diluted cell suspensions (200 μl) were filtered through 0.2-μm-pore-size polyvinylpyrrolidone-free polycarbonate membranes (Nuclepore Corp., Pleasanton, Calif.) using the filtration manifold described above for the Y. pestis assay. This membrane was chosen because it retains more of the N. meningitidis antigen than other membranes tested (unpublished data). For negative controls, PBS with 0.05% Tween 20, N. meningitidis ATCC 13090, or N. gonorrhoeae CDC 98 suspended in this buffer (10^8 cells per membrane) was used. A 200-μl volume of monoclonal anti-N. meningitidis-HRP conjugate diluted 1/400 into 40% fetal bovine serum was filtered through the membranes (approximately 5 min at room temperature). The membranes were then washed with deionized water. For the N. meningitidis sensor-based assay, the order in which the dipsticks were placed in the sensor was varied from one experiment to the next to offset the possibility of measurement bias. Each filtration step took approximately 5 min and was conducted at room temperature. The total time of this filtration procedure was 16 to 20 min.

Detection of pathogens in the silicon sensor. The membrane dipsticks used in the immunofiltration procedure were placed in the silicon sensor reading chamber for enzyme rate determination (Fig. 1). The reading chamber had been previously filled with a substrate solution of 0.01% 3,3',5,5'-tetramethylbenzidine (Sigma) in 0.1 M sodium acetate–1 mM EDTA–10% ethanol–0.2 mM potassium ferricyanide–0.2 mM potassium ferrocyanide–250 μM H_2O_2, pH 5.5. A plunger was pressed against the membrane to decrease the volume within the reading chamber. The silicon-to-plunger distance was 75 μm for nitrocellulose membranes and 25 μm for polycarbonate membranes. The determination of enzyme rate was started 10 s after immersion of the membrane to allow the solution entrapped within the membrane time to equilibrate with the substrate solution. The time required to determine the rate of change of the redox potential of the solution due to the presence of HRP was 1 min per membrane.

Enzyme-linked immunosorbent assay (ELISA). Polyvinyl chloride microdilution plates were coated with N. meningitidis 7851 (serogroup A) cells prepared as twofold dilutions in 100 μl of PBS (pH 7.0) for 1 h at 37°C. The plate was then washed with 0.9% NaCl–0.05% Tween 20. Anti-N. meningitidis monoclonal antibody-HRP conjugate was diluted 1/200 in 40% (vol/vol) fetal bovine serum. Of this conjugate solution, 100-μl was added to each of the wells of the microdilution plate. The plate was incubated for 1 h at room temperature and then washed with 0.9% NaCl–0.05% Tween 20. A 100-μl volume of 0.01% 3,3',5,5'-tetramethylbenzidine (the enzyme substrate) in 0.1 M sodium acetate–1 mM EDTA–10% ethanol–250 μM H_2O_2 (pH 5.5) was added to each well of the microdilution plate. The plate was incubated at room temperature for 20 min before the reaction was stopped with 50 μl of 1 N H_2SO_4. The A_{450} of each well was determined in a Vmax Kinetic Microplate Reader (Molecular Devices Corp., Menlo Park, Calif.).

RESULTS

Y. pestis **sensor-based assay.** In the *Y. pestis* assay, cell suspension buffers alone produced a change in the redox potential of the substrate solution of −21 μV/s (Table 1). Cell suspensions of E. coli or N. gonorrhoeae did not produce redox potential changes significantly different from those of the PBS negative controls (−26 and −22 μV/s, respectively). All suspensions containing Y. pestis cells produced changes in the redox potential of the substrate solution statistically
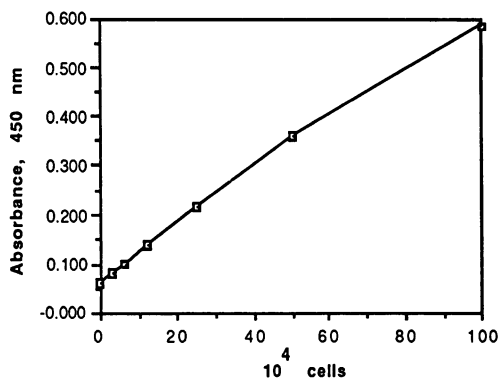
FIG. 2. Absorbance of various cell dilutions in *N. meningitidis* ELISA. The lowest number of cells determined to produce an absorbance statistically greater than that of the background was 6×10^4.

greater than that of the negative controls. These included the suspensions with fewest cells (4,000), which produced a mean kinetic rate of -44 μV/s. The mean kinetic rates of the samples containing the fewest cells and the negative controls were determined to be significantly different with a two-tailed *t* test ($P = 0.02$).

Comparison of *N. meningitidis* assays. The *N. meningitidis* sensor-based assay also distinguished between its negative controls (mean kinetic rate, -68 μV/s) and all suspensions containing cells (Table 1). The membranes with 800 cells adsorbed and labeled with a specific antibody-HRP conjugate produced a mean kinetic rate of -127 μV/s, which was statistically different from that of the negative control ($n = 5$, $P = 0.0002$). The membranes with other bacteria adsorbed (*N. meningitidis* ATCC 13090 and *N. gonorrhoeae* CDC 98) did not demonstrate enzymatic rates different from those of the negative controls (-65 and -68 μV/s, respectively). These results were consistent with expectations; according to the suppliers, the monoclonal antibody used in this assay was specific for *N. meningitidis* serogroup A.

In contrast to the number of cells detectable by the sensor-based assay, the lowest number of *N. meningitidis* cells detectable by ELISA was 60,000, despite the fact that this assay used the same original cell suspension and antibody conjugate solution (Fig. 2).

DISCUSSION

In the *N. meningitidis* ELISA and the filtration assay, bacterial cells are immobilized by adsorption onto a solid surface. An obvious advantage of filtration assays is that the rate of capture of bacteria on the membrane surface is limited largely by the rate of filtration, which is usually rapid. Adsorption of bacteria to a microdilution plate well is a much slower process limited by the rate of cell settling or diffusion. One important advantage of a clear microdilution plate ELISA is that a sensitive, quantitative colorimetric determination is possible. Colorimetric-based filtration assays have tended to be faster than comparable microdilution plate ELISAs, but sensitive methods for the measurement of enzymatic reactions occurring on the filter have not been available.

To overcome this shortcoming of filtration assays, two silicon sensor-based immunofiltration assays were developed for enzyme-labeled bacteria bound to microporous membrane surfaces. The sensitivity of both filtration assays

is largely achieved by eliminating the liquid above the membrane and pressing the membrane against the sensor surface. The resulting peroxidase-catalyzed redox reaction occurs within a very small volume of liquid (the volume of liquid trapped within the membrane). The feasibility of this approach was demonstrated with two types of assays: an indirect second-antibody conjugate assay and a direct antibody conjugate assay. The *Y. pestis* assay, which was the first to be developed, was not as sensitive or as precise as the *N. meningitidis* assay (Table 1). Possible reasons for these differences may include suboptimal and inconsistent capture of the antigen by this filtration system or an inferior monoclonal antibody preparation with lower affinity or stability than its counterpart used in the *N. meningitidis* assay. These issues would have to be addressed before this assay could be optimized. One likely reason for the difference in sensitivities of the two assays is that the nitrocellulose membrane used in the *Y. pestis* assay was considerably thicker than the polycarbonate membrane used in the *N. meningitidis* assay. Since the plunger-to-silicon distance in the *Y. pestis* assay (75 μm) was greater than in the *N. meningitidis* assay (25 μm), the volume in which the enzyme reaction occurred was proportionately greater and thus the sensitivity of the *Yersinia* assay was less. The more sensitive *N. meningitidis* assay detected fewer than 1,000 cells within 20 min of the start of filtration and was 2 orders of magnitude more sensitive than the 2.5-h ELISA with the same antigen and antibody preparations. The *N. meningitidis* filtration assay also proved to be more precise than the *Y. pestis* assay at all cell concentrations tested (Table 1).

The immunofiltration assay may be adapted for other cellular or noncellular antigens. The use of less specific antibodies directed against other strains or species would change the identification profile of this assay. Thus the sensor-based assay may be used to quantitate any microorganism which can be bound to a membrane support. Other membrane capture systems, including those onto which a specific capture antibody has been bound, have been used successfully in our laboratory for the detection of chlamydial antigen (H. G. Wada, J. M. Libby, L. S. Rice, R. S. Masino, K. C. Kasper, J. Moncada, and J. Schachter, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, C-122, p. 413).

The assays described in this report were conducted under somewhat ideal conditions free of body fluids, environmental contaminants, etc. However, the purpose of these studies was to demonstrate that a sensor-based microbial antigen detection system could compare favorably with an existing rapid method (e.g., an ELISA) and that the potential for practical applications exists.

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