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Remote Sensing Using an Airborne Biosensor

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There is no current method for remote identification of aerosolized bacteria. In particular, such a capability is required to warn of a biological warfare attack prior to human exposure. A fiber optic biosensor, capable of running four simultaneous immunoassays, was integrated with an automated fluidics unit, a cyclone-type air sampler, a radio transceiver, and batteries on a small, remotely piloted airplane capable of carrying a 4.5-kg payload. The biosensor system was able to collect aerosolized bacteria in flight, identify them, and transmit the data to the operator on the ground. The results demonstrate the feasibility of integrating a biosensor into a portable, remotely operated system for environmental analysis.

Introduction

Biosensors, defined as analytical devices combining the molecular recognition capabilities of biomolecules with electronics for signal measurement, continue to capture the imagination of an ever-increasing number of scientists and engineers. Many of the currently described biosensors have relatively small analytical sensing elements, but the electronic hardware of the device is more likely to be the size of a laser printer (~40 × 35 × 18 cm) or a personal computer (~40 × 40 × 50 cm). Furthermore, with few exceptions (i.e., process control), they are designed with a research or clinical laboratory in mind. Biosensors will fulfill their true potential as practical analytical devices only when they are configured to be rugged, simple-to-use, portable, and sufficiently inexpensive for a wide variety of applications.

Basic research discoveries related to configuring an immunoassay in the evanescent wave region of an optical fiber (*1*), maintaining function of immobilized proteins (*2*), and increasing the excitation and recovery of a fluorescence signal at the surface of an optical fiber (*3*) led to the construction of the fiber optic biosensor (*4*–*6*). The fiber optic biosensor proved its ability to analyze clinical samples for pathogens (*7*), food samples for toxins (*8*), groundwater samples for pollutants (*9*), and environmental samples for biological warfare agents (*4*). In all of these applications, however, samples and reagents were manually introduced into the device. While the data confirm the sensitivity, resistance from interference, and flexibility of the biosensor as well as its ability to be operated outside a laboratory, technical training was required of the operator and sample analysis was performed as an operation distinct from sample collection.

The experiments presented here break new ground for integrating biosensors into remotely operated airborne platforms. The specific challenge as described herein was to build a system that could identify specific bacteria in an aerosol and transmit the data to an operator on the ground. Such an identification system could warn troops prior to exposure to disseminated biological warfare agents or ascertain that the destruction of a hostile production facility has not released bioagents into the environment. At present, particle detectors are available, but they offer no identification capability or discrimination of bioagents from normal background particles. To achieve this goal, the Naval Research Laboratory (NRL) designed and built an air sampler and automated fluidics system that could be integrated with an antibody-based fiber optic biosensor, a radio transceiver, and a small, remotely piloted unmanned air vehicle (UAV) (12ft wingspan). The constraints included a total payload weight, including batteries, of less than 4.5 kg, space availability of 1640 cm^3, and repetitive sampling and analysis times of 5 min. Field testing of the integrated system was performed at Dugway Proving Ground, UT, due to its EPA approval for the aerial release of the harmless bacteria *Bacillus subtilis* var. *niger* (Bsn, also commonly called *Bacillus globigii* or Bg).

Several specific tasks had to be accomplished in order to achieve the goal: First, the sensitivity of the biosensor for *Bsn* had to be increased in order to identify the low numbers of cells expected in aerosol releases, and the assay time had to be decreased from 10 to 5 min for earlier warning. Second, an automated fluidics unit had to be fabricated and integrated with the fiber optic biosensor since no manual operations were possible in the air. Third, a radio transceiver, batteries, and accompanying electronics had to be integrated with the fluidics and fiber optic biosensor for remote operation. Fourth, a lightweight air sampler had to be developed that would collect relatively high volumes of particulate-containing air into small volumes of buffer. And finally, the entire sensing system had to be integrated with the UAV and operated in the field under both warm-and-cold and day-and-night conditions, performing repetitive assays without sacrificing sensitivity.

Experimental Section

Preparation of Antibody-Coated Sensing Probes. The general principle of using tapered fiber probes to enhance the signal in immunoasssays employing the fiber optic biosensor has been described in detail previously (*3*–*5*). Briefly, multimode fused silica fibers, 600 μm in diameter and fitted with SMA connectors (Research International), were stripped of cladding over approximately 7 cm at the distal end and tapered using hydrofluoric acid and a computer-controlled dipper. The proximal 1 cm of the

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unclad region was tapered rapidly to a radius such that the number of modes carried by the clad and unclad regions were identical. The remaining 6 cm was tapered gradually to a final radius of 100−130 μm.

The fiber optic probes were then coated with avidin utilizing a thiol-terminal silane in conjunction with a heterobifunctional cross-linker (*2, 10*). An avidin−biotin linking system was utilized to minimize the denaturing effects of direct immobilization onto the glass fiber surface on the capture antibodies. For immobilization of avidin, fibers subsequently were treated in a series with (1) boiling deionized water, (2) a solution of 2% (v/v) 3-mercatopropyltrimethoxy silane (Fluka) in toluene, and (3) a 2 nM solution of the cross-linker, *N*-succinimidyl 4-maleimidobutyrate (Fluka) in absolute ethanol. After extensive washing in deionized water, fibers were immersed in a solution of avidin (Pierce) at 50 μg/mL in phosphate-buffered saline (PBS) at pH 7.4. Fibers were rinsed and stored in PBS containing 0.01% sodium azide at 4 °C.

Polyclonal rabbit serum specific for *Bsn* was generously supplied by Dr. William Lee (Defense Research Establishment, Suffield, Canada) and purified using Protein G affinity chromatography. Biotinylation was performed using *N*-hydroxysuccinimide-biotin (CalBiochem). Immediately before use, the biotinylation reagent was dissolved in dimethyl sulfoxide at 5 mg/mL. Antibody was diluted 1:1 in 0.05 M sodium borate buffer, pH 9.6, containing 0.04 M sodium chloride. The biotin solution was added at 3:1 molar excess to antibody and allowed to react at room temperature for 45 min, before purification of antibody over a Bio-Gel P-10 column (Bio-Rad) equilibrated with PBS at a flow rate of 0.5 mL/min. Purified protein peaks were identified by monitoring of fractions at 280 nm, and concentrations were estimated by adsorbance at 280 nm. Fractions were stored at 4 °C until needed.

Avidin-coated fibers were inserted into 100-μL capillary tubes shortened to cover the exposed length of the probe. Plastic T-connectors (Value Plastics) were inserted into silicone tubing endcaps, and fluid paths were established off the right angle of each T-connector (*4*). Each connector end was sealed with hot glue. Avidin-coated fibers were reacted with the purified biotinylated-goat *anti-Bsn* antibody at a concentration of 10 μg/mL in PBS for 1 h. For long-term storage, the fibers were incubated with 15 mM sodium phosphate containing 10 mg/mL BSA, 100 mM trehalose, and 0.01% sodium azide for 30 min and then dried under a stream of nitrogen. Four fibers were attached in series by connecting with silicone tubing the distal or proximal fiber T-connector of one fiber with the adjacent fiber T-connector (Figure 3). The dried fibers were stored at room temperature until use (up to 3 months).

Assay Protocol. Goat IgG antibody specific for *Bsn* was labeled with fluorescent Cy5 reagent following the manufacturers recommendations to a molar ratio of between 3:1 and 4:1 dye:protein (*5*). Briefly, protein is mixed with Cy5 labeling reagent in 0.05 M sodium borate, pH 9.6, for 1 h and separated from unreacted dye on a 10-cm P-10 (Bio-Rad) column equilibrated with PBS at a flow rate of 0.5 mL/min. Labeled antibody was identified visually as the first eluted dye fraction. Dye/protein ratios were determined by absorbance at 650 and 280 nm, respectively.

The assay was performed simultaneously on four fiber optic probes using the Analyte 2000 (Research International) and the automated fluidics system. Approximately 1 mL of sample collected in 15 mM sodium phosphate buffer, pH 7.4, was pumped from the air sampler over the fiber probes. The 1 mL represented approximately 10% of the fluid in the air sampler so that the sampler continued to concentrate all bacteria removed from the air over the entire period it was collecting the aerosol. The assay routine was repeated every 5 min.

Briefly, with the laser fluorimeter on constantly, the sample is pumped from the air sampler over the fibers and into waste for 140 s followed by a 20-s wash. Air is pumped for 15 s to prevent dilution of the Cy5-labeled antibody, which is subsequently pumped over the fibers for 80 s. The fluorescent signal is recorded every second. After incubation with the labeled antibody, the fibers are rinsed with wash buffer for 30 s to reestablish baseline. The labeled antibody is returned to a reservoir for reuse in the subsequent assay.

Air Sampler Testing and Development. Various air sampler designs based on the use of ram air to introduce the sample into aqueous media were tested for their ability to collect ovalbumin released into a wind tunnel. The ovalbumin was aerosolized and introduced into the center of an air stream flowing at 40−50 mph. Amounts of ovalbumin collected in the various air samplers after 5 min were measured using a standard ELISA. A standard all glass impinger (AGI-30) equipped with a pump drawing in 12 L of air/min was used as a reference and run simultaneously with all of the experimental air samplers. Due to variations in the total amount of ovalbumin released, values were expressed as ratios of ovalbumin collected by the ram air-driven samplers as compared to that collected by the all glass impinger.

On the basis of these data, we determined that the NRL-built cyclone functioned better than all but one other air sampler tested and that the height of the collector could be reduced approximately 3-fold without affecting the collection efficiency. The final sampler consisted of a plastic funnel as the base with an outlet at the bottom to remove fluid, a plexiglass collection tube rising from the funnel (4 cm in height and 5 cm in diameter), an air intake tube 2 cm in diameter positioned at an angle into the side of the collection tube so as to generate the cyclone, and an air exit tube 2 cm in diameter extending out the top of the collection tube. Water was injected into the air intake tube using a syringe needle bent to spray liquid into the air stream just as it entered the collection tube.

Results

Improving Immunoassay Sensitivity. The Analyte 2000 fiber optic biosensor (Research International, Woodinville, WA) was selected for this project because it weighs 1.4 kg, is capable of performing four immunoassays simultaneously, and has an evanescent wave sensing modality that is relatively immune to interference from sample components (*5*). We have developed and previously described reusable sensing probes enclosed in capillary tubes for simplified introduction of fluids (*6*). In the past, 10-min, manual sandwich immunoassays were performed on the probes by immobilizing *anti-Bsn* antibodies directly on the fiber through a silane film and heterobifunctional cross-linker (*2, 10*). Detection limits of only 10^6 cfu/mL were obtained using this standard methodology and were not deemed of sufficient sensitivity for the expected aerosol field tests.

To address the limits in detection levels, focus was placed upon moderating the detrimental effects of chemical cross-linking on the antibody molecule. Detection limits observed when utilizing the same *anti-Bsn* antibody reagent in solution by enzyme-linked immunoadsorbent assays (ELISA) indicated that much lower sensitivities were possible (data not shown). Suspecting that the antibody directly cross-linked to the glass surface was sterically hindered by excess cross-links to the surface or by concomitant adsorption, the antibody was immobilized through a biotin−avidin bridge (Figure 1). Employing an avidin−biotin cross-linking method not only limited the number of sites on the antibody used to attach it to the surface but also had the added benefit of

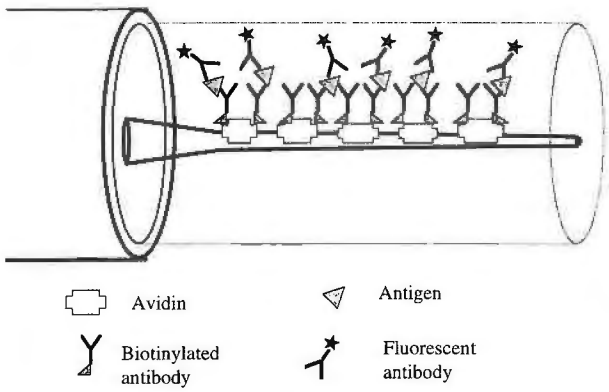

FIGURE 1. Schematic of immunoassay on the fiber probe. Avidin is covalently immobilized on a tapered fiber probe (*1*), and biotinylated antibody is attached to the avidin. Antigen (3.5 min) and fluorescent antibody (1.5 min) added in sequence form a fluorescent complex that is excited by light in the evanescent region at the surface of the optical fiber. Fluorescent light is collected by the fiber and transmitted to the photodiode.

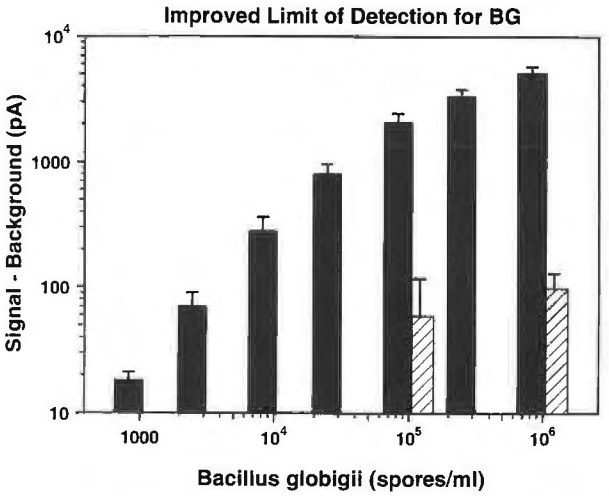

FIGURE 2. Standard curve for *Bsn* (*Bg*). Detection of various concentrations of *Bsn* was performed using a sandwich immunoassay at the surface of tapered fiber probes (*7–9*) and the Analyte 2000. Data represent the mean ± SD of three determinations. Solid bars represent data using the *anti-Bsn* immobilized on the silane-coated fiber via a biotin–avidin bridge whereas open bars represent data obtained using *anti-Bsn* immobilized directly to the silane-coated fiber.

further passivating the glass surface against nonspecific interactions with the antibody. The same silane chemistry as previously described for antibody immobilization (*10*) was used to attach avidin to the fiber probe. Although biotinylation ratios up to 20:1 are feasible for many antibodies, the optimum molar labeling ratio for the *anti-Bsn* antibody was found to be less than 4 biotin molecules per antibody. When using the *anti-Bsn* antibody bound to the probe through an avidin–biotin bridge (*11*), the detection limit was reduced to 3000 cfu/mL, nearly a 1000-fold improvement over the standard direct cross-linking method (Figure 2). Fiber probes coated with antibodies in this manner could be dried and rehydrated after several months without significant loss of activity (data not shown).

System Automation. The automated fluidics prototype and controlling software code were built upon embedded processor cards designed in house and utilized commercially available microfluidic components. Several iterations resulted in a system utilizing three-way solenoid microvalves (Lee Co., Westbrook, NJ) with a combination of peristaltic (Alitea AB, Stockholm, Sweden) and diaphragm minipumps (Lee Co., Westbrook, NJ). Design and assembly considerations focused upon development of a reliable and robust system that could withstand the environmental extremes expected at Dugway along with the mechanical stresses inherent in UAV operations. Three of the three-way valves are cascaded to allow for four inputs using a minimal amount of tubing, and a fourth valve is used to either recycle the fluorescently labeled antibody or to direct sample or wash buffer to waste (Figures 3 and 4). The peristaltic pump is dedicated to the immunoassay reagent fluidics, and the solenoid diaphragm pump serves to refill the cyclone sampler after repetitive sample removal. Except for the peristaltic pump, the entire system uses less than 1 W of power, weighs less than 100 g, and fits on a 3 × 3 in. printed circuit board. (Originally, two solenoid pumps were used to conserve weight, but the peristaltic pump proved necessary for reliable operation, especially in handling relatively dirty cyclone samples from field collections over extended time periods.)

The Analyte 2000 was controlled by manufacturer-supplied PC software (version 4.27) and is capable of recording a signal from each of the four fiber inputs at a rate up to 1 reading/s. To relay data to and from the ground, wireless modem transceivers (Freewave Technologies, Boulder, CO) were connected to the dedicated laptop computer and the Analyte 2000 through the RS-232 ports and transmitted between the two at 9600 Bd. Recorded signal data were downloaded into Excel for Windows for reduction and analysis. The airborne system was powered by two NiCad battery packs (SR Batteries, Bellport, NY). These batteries could run the integrated system for 2 h.

Air Sampling. The air sampling requirement presented a unique challenge for the project. Several types of air samplers have been developed for collecting particles from air into aqueous reservoirs (*12*). Each had serious limitations with respect to weight, power, and overall efficiency (as measured by total particles removed in a given aerosol cloud). The AGI, the reference standard for collecting 1–10 μm particles from air into water, is highly efficient, but samples air at a flow rate of only 12 L/min. The cyclone samplers previously described intake volumes of 100–1000 L/min but have traditionally required heavy vacuums or fans. The electrostatic impaction systems are efficient but require power beyond current portable batteries. To avoid these limitations, NRL built a small plastic cyclone, generally based on the design of Griffiths (*13*), but engineered to take advantage of the ram air flow from the movement of the airplane. The cyclone operates by creating a rapidly circulating aqueous film on the inner walls of the sampler as air enters at a speed between 40 and 50 mph. The cyclonic action serves to transfer micron-sized particles into the liquid from the aerosol cloud. At ram air speeds common for the UAV platform, sampling volumes were estimated at over 100 L air/min. Total particle collection of the NRL-designed ram air cyclone was determined to be 10–15-fold that of a 12 L/min AGI-30 by testing in the wind tunnel at Aberdeen Proving Ground (*14*).

The remotely piloted airplanes employed for the majority of the tests are from a family of model airplane kits called Telemasters, originally designed and used in Germany to string telegraph lines across valleys. Two 12 ft Telemasters were custom built for NRL by BAI Aerosystems, Easton, MD. The 12 ft Telemaster is a high wing utility aircraft with a wingspan of 3.6 m, an empty weight of 19 kg, and a payload volume of 0.032 m^3. The fuselage was slightly widened to allow easier access to the payload. The onboard systems include an Airtronics radio control system, a BTA wing leveling autopilot, and a Zenoah G-62 gasoline engine for propulsion. This aircraft was chosen because it can carry a 4.5-kg payload and is easy for the pilot to fly, an important

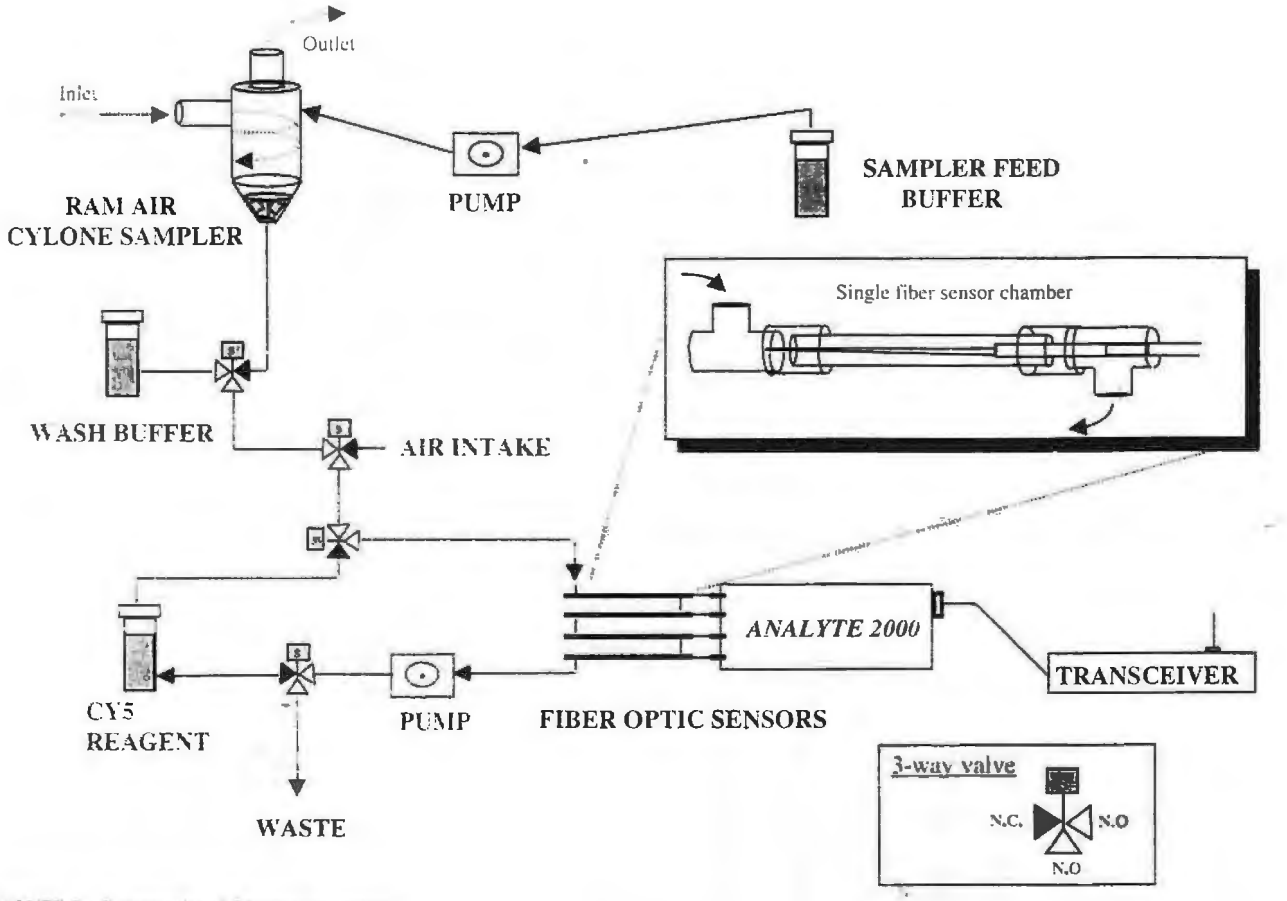

FIGURE 3. Schematic of biosensor system.

characteristic for testing at night. From the point of view of mimicking a real-life scenario, it was considered important to be able to operate at night as well as in the daytime due to the steadier wind conditions at night and the potential for sunlight to degrade some biological agents. To operate at night, the pilots wore night vision goggles, and the aircraft were equipped with night vision goggle-compatible lighting for the pilots to distinguish aircraft attitude.

At Dugway Proving Grounds, tests were conducted from a small airstrip located approximately 1.6 km from the aerosol release area. The ground monitoring station was run inside a mobile vehicle operating approximately 100 ft from the airstrip. The *Bsn* was released in varying amounts using either a truck-based or airplane-based crop sprayer. The UAV was flown in an oval racetrack pattern, 300−500 m in size and 10−30 m above the ground, during nine releases. Each flight averaged 12−15 min duration and was conducted at night. The *Bsn* aerosol was not visible in the darkness. UAV flights were conducted approximately 1.6−2.0 km downwind from release area.

On the first night of aerosol release testing, no *Bsn* was detected in samples collected during flight. However, the *Bsn* was detected using an Analyte 2000 on the ground to analyze eluants from wipes of the wings, proving that the UAV had indeed passed through the aerosolized *Bsn*. With the intention of increasing the collection efficiency of the cyclone, the air intake was extended from near the fuselage to a position forward of the wing strut.

During the next four flights, positive identification of *Bsn* in samples from the ram air cyclone by the fiber optic biosensor was relayed to the ground (Figure 5). The assays, run in quadruplicate, showed good agreement, although there was some variation in signal magnitude. The last flight test indicated a positive signal although the cyclone was operational for approximately 20 min before a positive sample was generated (in comparison to 5 min for two tests and 10 min for one test). It was later determined that only a half pound of *Bsn* had been released during this test. One flight was conducted when there was no *Bsn* release (negative control) and no signal was observed by the UAV.

Temperatures during the flights ranged from 6 to 24 °C (Table 1). While low temperature combined with high humidity (7 °C, 96% humidity) caused icing of the wings on one flight, the operation of the sampler and sensor demonstrated no impairment. In one test flight, interference with the radio control frequency forced the airplane into an autopilot-controlled crash on the sagebrush-covered test range. As an indication of the ruggedness of the integrated design, although the airplane was damaged beyond repair, the biosensor payload was found completely intact and still operating in the payload bay of the fuselage. The entire package was removed, certified as operational, and flown successfully the next evening in a second UAV.

Discussion

The demonstration of a fully autonomous, remotely operated biosensor opens the way for numerous real-world applications. The integration of an air sampler with the biosensor demonstrates both that the requirement of biosensor for aqueous sample does not prevent its use for detection of airborne analytes and that the biosensor can, in theory, be melded to a variety of automated samplers for remote operation. Replacement of manually operated fluid manipulations (such as pipetting by hand) with automated fluidics systems resulted in reduced variation within and between assays (data not shown). Also, the likelihood for operator-induced error is eliminated. As such, the operational parameters defined by this integrated system are

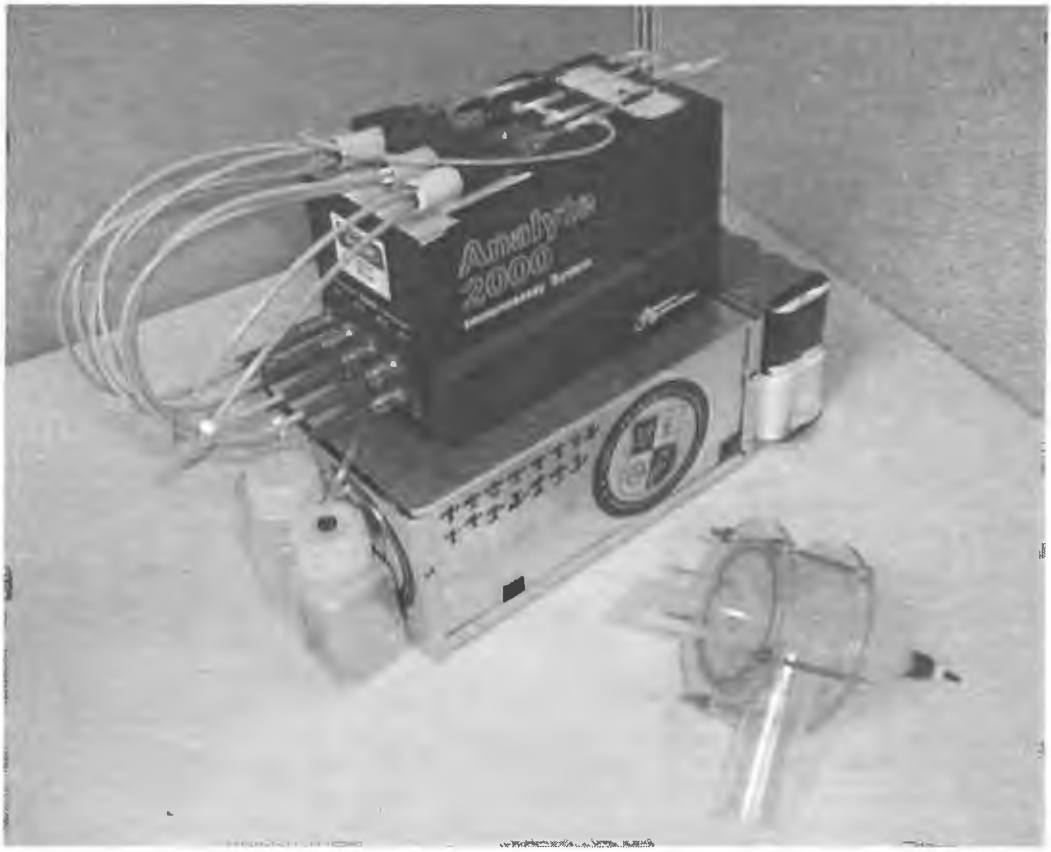

FIGURE 4. Photograph of automated fluidics unit and Analyte 2000.

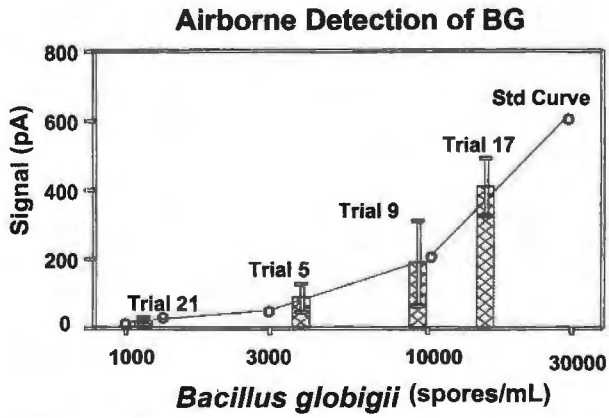

FIGURE 5. Data from test flights. The signal increase obtained from successful flights is plotted with the standard curve shown in Figure 1 for comparison. For each test, the biosensor had four probes attached, each coated with avidin and a biotinylated rabbit *anti-Bsn* antibody. The biosensor ran repetitive 5-min assays from take off until landing. Sample was withdrawn from the cyclone for 3 min and slowly pumped over the probes. The probes were then washed with buffer and then interrogated for the presence of *Bsn* by cycling a solution of Cy5-labeled rabbit *anti-Bsn* (20 μg/mL) over the surface for 2 min. The average fluorescent signal increase for the final cycle from the four probes ± SD after washing is plotted.

pertinent to air or water monitoring for any number of environmental or industrial applications. For example, to monitor environmental remediation, intermittent groundwater samples could be injected into an automated sensor with the resulting signal relayed back to a central monitoring facility. Combined with air sampling capabilities, the integrated biosensor system is uniquely positioned for any number of bioanalytical challenges where full-time operator presence is neither practical nor possible.

TABLE 1. Weather Conditions during Flights

flight no.	sample identification	temp (°C)	humidity (%)
1–4	negative	15–20	41–43
5	positive	22	36
6	positive	18	52
7	positive	7	92
8	negative	6	80
9	positive	24	57

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