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RAPTOR: A PORTABLE, AUTOMATED BIOSENSOR

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The RAPTOR is a portable automated fiber optic biosensor for detection of biological threat agents. It performs rapid (3 to 10 minute), fluorescent sandwich immunoassays on the surface of short polystyrene optical probes for up to four target analytes simultaneously. The optical probes can be reused up to forty times, or until a positive result is obtained, reducing the logistical burden for field operations. Numerous assays for toxins, such as SEB and ricin, and bacteria, such as *Bacillus anthracis* and *Francisella tularensis*, have been developed. Research International has commercialized the RAPTOR, and development of a second-generation instrument, sponsored by the US Marine Corps, is now in progress.

INTRODUCTION

The fiber optic biosensor has developed over the last decade from a single channel laboratory breadboard into a portable, automated 4-channel sensor.[1-7] While the optical, electronic, and mechanical workings have evolved significantly, the assay methodology has remained essentially the same, consisting of a sandwich fluoroimmunoassay. Capture antibodies are immobilized onto the surface of an optical fiber. When sample flows over the fiber probes, immobilized antibody captures any analyte present. The amount of analyte bound is determined by a later step where the binding of a fluorescent tracer antibody to the bound analyte forms a fluorescent complex or "sandwich". The biosensor monitors this complex formation by evanescently exciting surface-bound fluorophores with a diode laser. The optical probe captures a portion of the emitted fluorescence, which returns back up the fiber to the photodiode detector. Since the excitation intensity and efficiency of fluorescence recovery falls exponentially with distance from the fiber probe surface, the system is highly discriminatory for the surface bound fluorophores.[8]

Figure 1. RAPTOR Fiber Optic Biosensor

The RAPTOR (Figure 1) is the only fiber optic biosensor commercially available (Research International, Woodinville, WA) that is designed to withstand the rigors of

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field-testing. The system employs a separate 635 nm diode laser to excite each of the four fiber optic probes. To match this excitation wavelength, the fluorescent dye Cy5 is used to tag the tracer antibody. Since the sensor performs a two-step sandwich assay, fluorescent tracer reagent is not contaminated by sample and negligible signals are obtained from optical probes when negative samples are analyzed. Hence, both the fiber probes and fluorescent reagent can be reused until a positive is obtained. This makes the RAPTOR ideal for continuous monitoring applications. If a positive sample is detected, the user need only replace the coupon which holds the four capture antibody-coated optical probes. The optical probes are injection-molded polystyrene, which permits inexpensive mass production. These probes are easily coated with antibody by passive adsorption, over-coated with a stabilizing solution, and dried while being glued into the coupon, thereby providing an extended shelf life. Each coupon is marked with a bar code that tells the sensor the appropriate assay protocol and the identity of the antibody on each of the probes. To use, the coupon is inserted into the holder on the RAPTOR, and sample and reagents are introduced through blunt tipped needles, which connect to the coupon upon closure of the top door. From its initial design, the RAPTOR was made to be field hardened and very simple to use, even for an operator with no technical background. It can be battery operated and accepts samples introduced either manually or automatically from an attached air particle collector.

Data analysis is also completely automated, which removes any subjective bias of the user. After the data are collected and analyzed using preloaded software, with user modifiable settings, the results are displayed on the LCD screen of the instrument in a simple display specifying the assays performed and the instrument's assessment of the results. While the results are displayed in a qualitative, easy-to-read format (negative, suspect, positive, high positive), quantitative data can be downloaded through a serial port to a computer for further analysis.

The RAPTOR and the fiber optic biosensors from which it evolved have been used to detect and quantify a variety of hazardous substances in numerous sample matrices; this work includes detection of explosives,[9-11] toxins,[6,7,12-14] bacteria,[6,7,13,15,16] and viruses,[6,7] in ground water,[10] soil extracts,[11] air extracts,[16] meat homogenates,[12,13] and clinical fluids.[15,17] A summary of the limit of detections for several different analytes is shown in Table 1.

TABLE 1. Limits of detection for toxins and pathogens using a 10-minute assay performed with RAPTOR

Hazard	Type	Limit of Detection
Staphylococcal enterotoxin B (SEB)	Toxin	1 ng/ml
Ricin	Toxin	10 ng/ml
Cholera toxin	Toxin	1 ng/ml
Yersinia pestis F1	Bacterial surface protein	10 ng/ml
Bacillus anthracis	Gram positive bacterium (vegetative form)	50 cfu/ml
Bacillus globigii	Gram positive bacterium (spore)	5×10^4 spores/ml
Brucella abortus	Gram negative bacterium	7×10^4 cfu/ml
Francisella tularensis	Gram negative bacterium	5×10^4 cfu/ml
Giardia lamblia	Protozoan cysts	3×10^4 cysts/ml

EXPERIMENTAL

Buffers and Reagents

Anti-*Giardia* antibody (Mab 7D2 ascites) and antigen were provided by Dr. Ted Nash (NIH, Bethesda, MD).[19] Mab 7D2 IgG was purified using MEP-HyperCel (Life Technologies). Additional anti-*Giardia* antibody (Mab AG1 and Cy5-labeled AG1) was purchased from Waterborne, Inc. (New Orleans, LA). The anti-cholera toxin IgG was purchased from Biogenesis (Brentwood, NH); cholera toxin antigen was purchased from Calbiochem (La Jolla, CA). Ricin, ovalbumin, *B. anthracis* Sterne strain, *F. tularensis* LVS and all antibodies directed against these antigens were provided by Naval Medical Research Command, Silver Spring, MD. Affinity-purified sheep anti-SEB IgG and SEB antigen were purchased from Toxin Technology (Sarasota, FL). RAPTOR wash buffer consisted of phosphate buffer (Sigma, 8.3 mM, pH 7.3) containing 0.05% (v/v) Triton X-100 (TX-100) and 0.01% (w/v) sodium azide (PBT). Casein and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO).

Probe and Coupon Preparation

Capture antibodies were immobilized onto fiber optic probes by passive adsorption. Injection-molded, polystyrene fiber optic probes (Research International) were first blackened at their distal ends to prevent reflection of excitation light. Probes were then placed into capillary tubes (100 µl, cut to 4 cm length) pre-filled with 36 µl of the appropriate antibody solution, in general, 100 µg/ml IgG in 0.1 M sodium carbonate buffer (pH 9.6). After overnight incubation at 4°C, unbound IgG was rinsed off the probes by brief immersion in dH_2O, and the probes were incubated for 15-30 minutes in immunoassay stabilizer solution (ABI, Columbia, MD). The probes were then mounted and glued into the disposable coupons (Figure 2), as described in detail previously.[14]

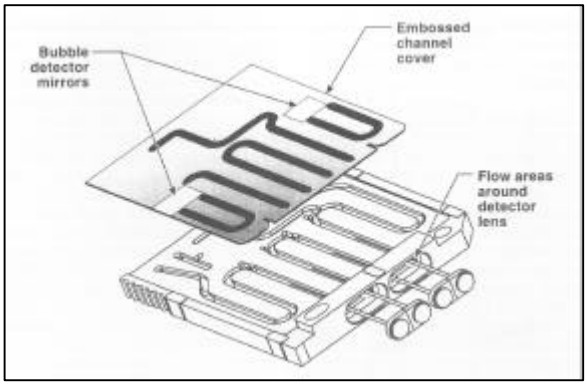

Figure 2. Schematic of assay coupon showing four fiber optic probes, adhesive channel cover and bubble detector mirrors.

Preparation of Cy5-labeled Antibodies

Cy5-conjugated antibodies were prepared by reacting 3 mg of protein (1 mg/ml) in 50 mM sodium tetraborate, 40 mM NaCl, pH 9.0 with one vial bisfunctional Cy5-reactive dye (Amersham Life Science Products, Arlington Heights, IL) for 30 minutes at room temperature in the dark. Subsequently, labeled protein was separated from unincorporated dye by size-exclusion chromatography. Dye to protein ratios ranged from 2 to 4 Cy5 molecules per IgG.

RAPTOR Design and Operation

Insertion of the assay coupon into the RAPTOR coupon compartment aligns all necessary optical paths and engages all fluidic connections required for sample analysis. A pneumatic pump moves buffer, air, fluorescent reagent (from on-board reservoirs), or sample within the system. Serpentine channels in the coupon provide a common path across the probe surfaces. Bubble detectors, which monitor liquid-to-air interfaces, control introduction of sample and fluorescent reagent (Figure 2).

During the two-step sandwich immunoassay, sample is flowed over the four fiber optic probes mounted in the assay coupon. Antigen present in the sample binds to the fiber optic probe coated with capture antibody specific for that antigen; unbound material is washed away by a brief rinse with PBT. Fluorescently labeled antibody is next introduced and binds to the antibody-antigen complexes on the probe surface, completing the

sandwich assay. This tracer reagent is maintained at a suitable temperature in an onboard thermal storage module and recovered after each assay cycle, allowing multiple sequential analyses to be performed. Excitation light from four 5 mW Sanyo laser diodes (635 nm) within the RAPTOR is focused into the fiber optic waveguides. An evanescent wave is created along each probe, exciting the fluorescent emission of specifically bound Cy5-labeled antibodies. The portion of the fluorescence captured by the optical probe is collimated by the probe's molded lens and focused onto a photodiode using a ball lens, chosen for its light-gathering power and short focal length (Figure 3). A long-pass dichroic filter (665 nm) rejects reflected laser light. If each fiber optic probe has been coated with capture antibodies with differing specificities, one sample is interrogated for four different analytes simultaneously.

Assay Procedure

Each assay consists of multiple steps, which are performed automatically by the RAPTOR. Immediately after loading the coupon into the instrument, prior to analyte challenge, the RAPTOR automatically initiates a five-minute baseline protocol. The probes are briefly rinsed with PBT (for hydration), and are then incubated for 90 seconds with fluorescent tracer antibody. After tracer reagent is returned to its compartment, the probes are rinsed with PBT and an initial background wash value is determined. This background rate represents the rate of nonspecific binding of the fluorescent antibody to the probe surface and must be determined before samples are analyzed.

To analyze a sample, it is first loaded into the sample port using a 1 ml syringe equipped with a blunt-tipped needle or a transfer pipette. After the coupon is rinsed briefly with PBT, the sample is flowed into the coupon and is incubated with the probes for a seven minutes. A subsequent wash with PBT eliminates unbound material; the RAPTOR automatically flushes the sample port with PBT at the same time as the PBT wash, thus preventing sample carryover. Next, the coupon is cleared with air to prevent dilution of the incoming tracer reagent. Cy5-antibody reagent is flowed into the coupon and incubated for 90 s to interrogate the amount of antigen bound to the probes. The tracer reagent is returned to its reservoir for reuse and a final PBT wash completed. The entire standard assay cycle, including all wash steps, is completed in ten minutes.

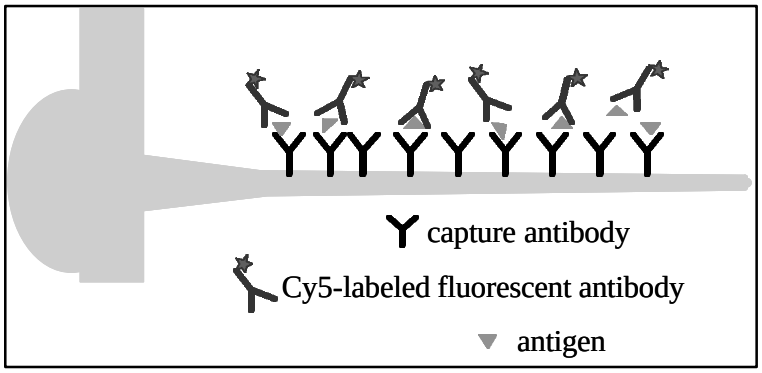

Figure 3. Schematic of optical probe and immunoassay

The rate of fluorescence increase during incubation with the Cy5-antibody is calculated ("assay rate"), and a final reading is taken to determine the increase in fluorescence due to Cy5-antibody bound during the entire assay cycle ("wash delta"). While one parameter is sufficient for strongly positive samples, use of both factors greatly reduces the number of false positives obtained.[6,7] Assay data are stored by the RAPTOR and can be downloaded through a serial port for quantitative analysis. Samples are considered positive if the both values are greater than the means of the background values plus three standard deviations.

RESULTS

The RAPTOR has been used to detect a number of different toxins and pathogens. In a recent trial, we set up coupons of two different types: a pathogen coupon, which tested for *B. anthracis*, *Giardia*, and *F. tularensis*, and a toxin coupon, which tested for ricin, cholera toxin, and SEB. In addition to the agent-specific probes, each coupon also contained a positive control probe coated with an anti-ovalbumin IgG. The control probe was

utilized to check the activity of the coupon and tracer reagent prior to sample testing and again at the completion of a series of tests to confirm that both the probe and tracer reagent retained their activity.

To test that pathogen and control assays (ovalbumin, *B. anthracis*, *F. tularensis*, and *Giardia*) were compatible for use in a single assay coupon, 10 μg/ml of each Cy-5 labeled tracer antibody (40 μg/ml total) was mixed and placed in the reagent bag. This fluorescent reagent cocktail was then used in assays performed with multi-analyte coupons housing one fiber directed against each of the above analytes. To ensure that all components of the system were functional, a control containing a low concentration of ovalbumin was analyzed at the beginning of each trial. An additional ovalbumin control was included at the end of each trial as a final test to gauge coupon and reagent activity. Each of the other analytes was tested sequentially and a dose response curve for each determined (Figure 4). For each analyte, both the wash delta (shown in Figure 4) and the assay rate remained above background levels (>0) during subsequent analyses, even in the absence of additional analyte. These results indicated that the surface-bound capture antibody: antigen complexes were not saturated with tracer antibody until after several additional incubations with tracer antibody (cocktail). The magnitude of this effect may vary depending on tracer affinity and avidity, the number of binding sites on the antigen, and reaction kinetics. However, since coupons

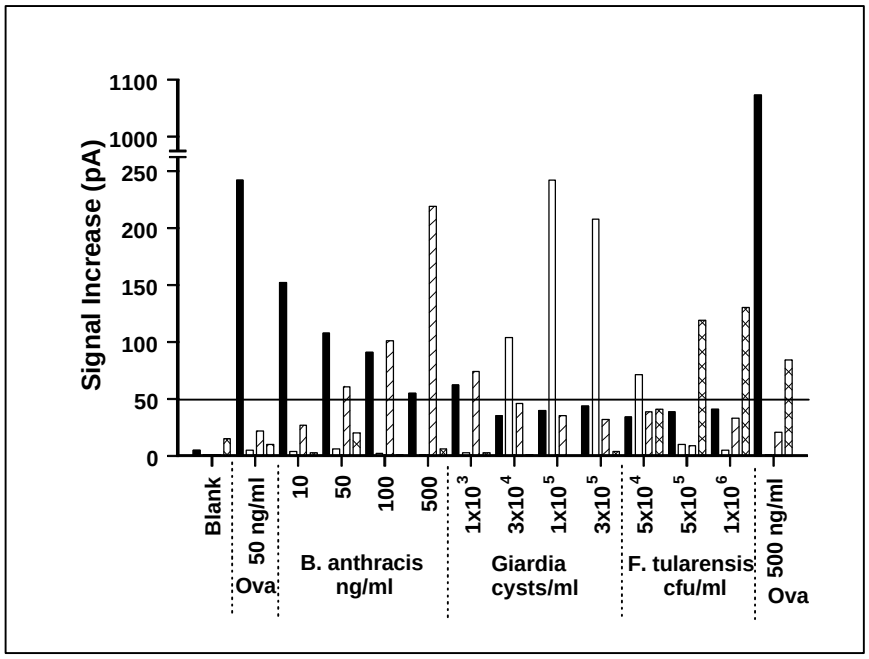

Figure 4. Dose response curve for each analyte on the pathogen coupon assay set. The signal increase obtained after being challenged with increasing amounts of each analyte is shown for each probe. Ovalbumin: solid, Giardia: open, B. anthracis: hatched, F. tularensis: cross-hatched.

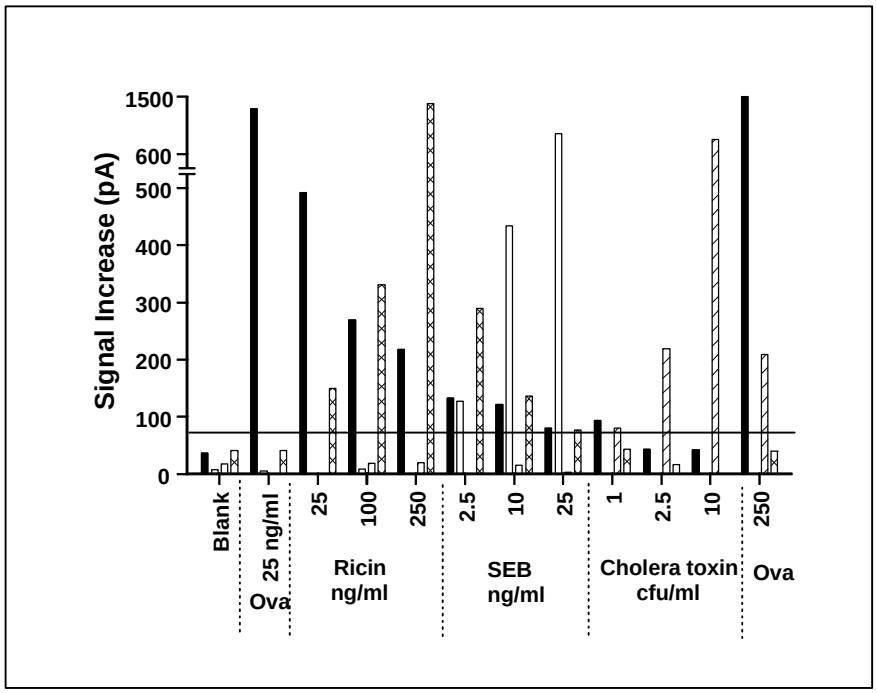

Figure 5. Dose response curve for each analyte on the toxin coupon assay set. The signal increase obtained after being challenged with increasing amounts of each analyte is shown for each probe. Ovalbumin: solid, Cholera toxin: hatched, SEB: open, Ricin: cross-hatched.

would normally be used only until the first positive is achieved, the lack of saturation does not present a problem for field-testing.

An analogous experiment was carried out using the toxin coupon, which contained probes specific for ricin, SEB, cholera toxin, and an anti-ovalbumin IgG control probe. As was seen for the pathogen coupon the control probe for ovalbumin gave strong positive responses at both the beginning and end of the trial (Figure 5). Then each target was tested at concentrations near the predetermined limits of detection (Table 1) and a dose-response curve for each determined.

For both the pathogen coupon and the toxin coupon, no significant cross reactivity was observed at the concentrations tested. As the concentration of analyte increased the response for the probe specific for that analyte increased. Had the concentration of analyte been increased further the signal would continue to rise. Signals can go to as high as 20,000 pAmps, before the instrument's range is exceeded. This is observed best with the toxin assays, as the pathogen/bacterial assays do not generally result in such large signals. This large range permits a good dynamic range for each assay, from ng/ml to μg/ml. Even if a sample were introduced at a huge excess it would not cause a problem; since the assay is a two-step process, no hook effect will occur.

At the completion of the assay validation, fifty coupons of each type and thirty vials of lyophilized reagent were prepared. These materials, along with two RAPTORs, are now in the field to provide an enhanced threat detection capability and produce data on long-term system and assay endurance.

CONCLUSIONS

The RAPTOR fiber optic biosensor is well suited for testing for pathogens and toxins and can be used as an in-line monitor or to analyze discrete samples. Only minimal sample preparation (e.g. dilution or coarse filtration) is required. Disposable coupons can be made with optimum assay combinations, allowing multiple samples to be analyzed for multiple pathogens without changing probes or reagents. Assay procedures and data analyses are both fully automated, but can be modified by the user to fit the user's specifications. Furthermore, antibody-coated probes and fluorescent reagents can be stored for over 1 year. The RAPTOR is portable (12 lb) and can be operated on batteries for use in the field. The assays are fast (3-10 min assays) and sensitive (limits of detection = 1-10 ng/ml, 50-5000 cfu/ml, depending on the antibody:analyte combinations). The second-generation device, which will be available in late 2000, will utilize custom-built peristaltic pumps instead of pneumatic pressure for liquid movement. It is expected that this change, along with other improvements, will increase both RAPTOR's sensitivity and reliability for field operation.

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