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13. ABSTRACT *(Maximum 200 words)*

During Phase I, IA established the feasibility of developing a biosensor for rapidly detecting early-stage serum infection markers by demonstrating the performance of fiber sensors which are able to detect levels of IL-6 and IL-12 in buffered fetal calf serum in approximately 10 minutes with range and specificities comparable with those achieved using ELISA methods (0.25-10pg/ml). After calibration, acceptable sensor to sensor reproducibility was demonstrated and steps necessary for further improving reproducibility during Phase II have been identified. IA also developed and demonstrated a single fiber biosensor which can sequentially measure levels of both IL-6 and IL-12 in a single undiluted human serum sample. Finally, during Phase I, IA has developed a design for a hand-held instrument for use by minimally trained personnel.

The instrument which will be further developed during Phase II work will provide a means for early detection of infection in battlefield casualties as well as in civilian hospital or clinic setting. This will reduce the frequency with which infection results in sepsis by facilitating early treatment of infections.

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Judith L. Erb 6-30-97

PI - Signature Date

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Introduction

Project Summary

During Phase I, IA established the feasibility of developing a biosensor for rapidly detecting early-stage serum infection markers by developing and demonstrating fiber biosensors which are able to detect levels of IL-6 and IL-12 in buffered fetal calf serum in approximately 10 minutes with sensitivities and specificities comparable with those achieved using ELISA assay techniques. IL-6 could be detected at 0.25 pg/ml and IL-12 at 0.5 pg/ml, a sensitivity which will recognize the changes associated with early onset of infection. For these cytokines, normal levels are typically below 2 pg/ml. After calibration, acceptable sensor to sensor reproducibility was demonstrated and steps necessary for further improving biosensor reproducibility have been identified. IA also developed and demonstrated a single fiber biosensor for sequentially measuring both IL-6 and IL-12 levels in a single sample. A reasonable response of the combined IL-6 and IL-12 sensor to undiluted human serum spiked with amounts of IL-6 and IL-12 between 0.5-10 pg/ml was also demonstrated. Finally, IA designed a hand-held sensor measurement instrument and determined how to extend this technology to additional serum markers.

Demonstration of Feasibility of the Technology

IA's fiber optic biosensors have the potential for being used as early predictors of patient infection because they are capable of performing rapid point-of-care IL-6 and IL-12 assays (10 minutes or less) with measurement level sensitivities and ranges which clinical studies have shown necessary for distinguishing healthy control subjects from persons in early stages of infection.

Cytokines are small immunomodulatory proteins which serve as the soluable messengers of the immune system. Two of the immediate early response cytokines are IL-6 and IL-12.

The cytokine IL-6 orchestrates of the production of acute phase proteins during the early stages of infection as well as signaling for the proliferation of B-cells[1]. IL-12 mobilizes undifferentiated helper T-cells to differentiate and provide the necessary help for B-cell function and macrophage activation. In healthy individuals the levels in serum of these cytokines are extremely low, IL-6 is generally not greater than 2pg/ml and IL-12 levels rarely exceed 1-2pg/ml. One study even found that the levels of IL-12 were undetectable in 50% of the healthy subjects tested.[2] Figure 1a shows that IA's IL-6 biosensor produces a useful standard curve between 0.25 and 10 pg/ml of IL-6, the range which must be distinguished. Figure 1b shows the rapid response of four IL-12 biosensors (normalized to a common calibration standard) to IL-12 concentrations of 1-500 pg/ml, the IL-12 concentration range needed for identifying persons predisposed towards infection. Although the Figure 1a and 1b sensor data was measured using different sensor measure instruments and somewhat different experimental protocols, in both cases, IA's IL-6 and IL-12 biosensors exhibited close to a step level response which stabilized within seconds of sample and labeled antibody injection.

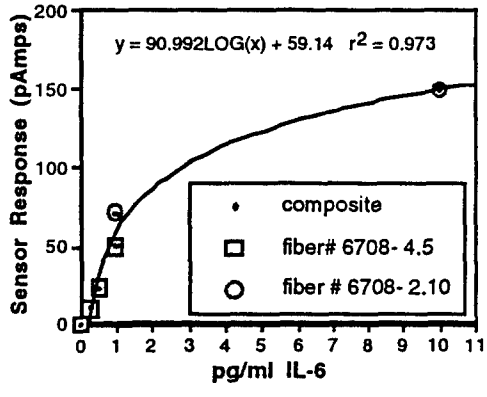


Figure 1a: IL-6 response of two sensors.

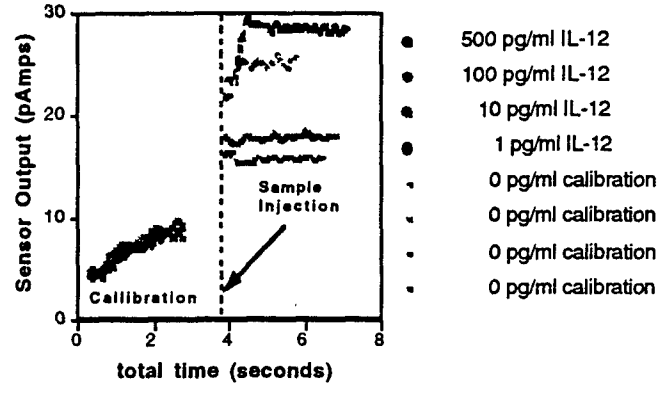


Figure 1b: IL-12 response of 4 sensors.

The Nature of the Biosensor Technology

The proposed hand-held Infection Sensor System is based on fiber optic biosensor technology

developed by IA, Inc.

Disposable fiber biosensors cartridges (0.25" OD x 4.5" long, see Figure 2a) were manufactured and chemically sensitized for detecting serum concentrations of IL-6, and IL-12. The response of the sensor cartridges to IL-6 and IL-12 was measured using fiber optic biosensor measuring instruments such as shown in Figure 2b and 2c. Additional technical details of these instruments are presented later in the subsection titled "Technical Basis for Development".

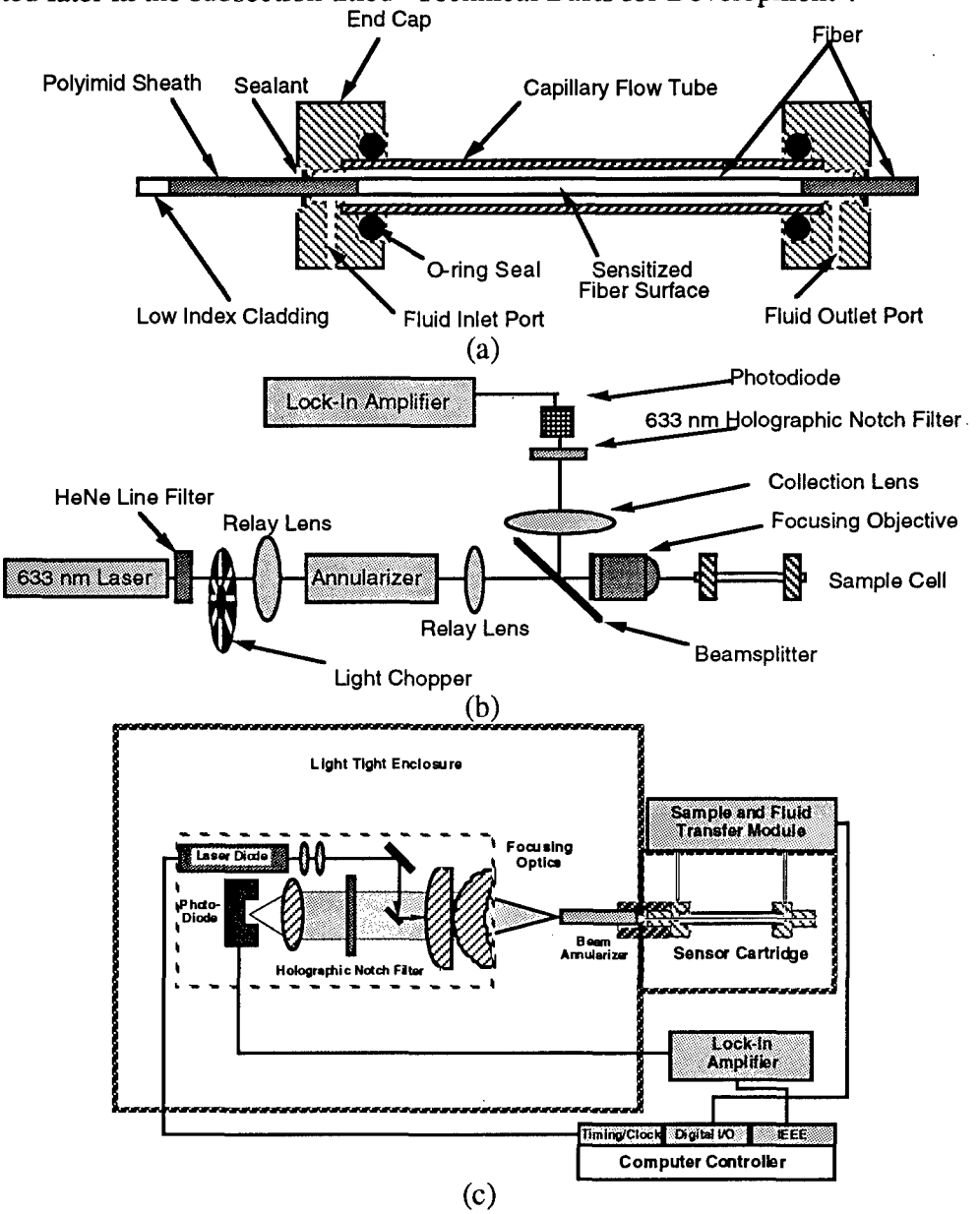


Figure 2: The response of IL-6 and IL-12 sensor cartridges (a) were measured with (b) an optical breadboard benchtop fluorometer, and (c) a compact fluorometer.

To measure an infection serum marker, a sensor cartridge is inserted into a sensor measurement instrument, such as that depicted in Figure 2c, which evanescently couples a laser radiation source (a ~635 nm laser diode) to the biosensor's chemically sensitized silica surface. After incubating a sample for a known period of time with a fluorophore label, the sample is injected into the sensor cell, where the fluorophore labeled component to be measured binds itself to the fiber surface. Because laser radiation is evanescently coupled to the sensor surface, the radiation extends only around a wavelength past the fiber surface and into the surrounding fluid. As a consequence, the fluoresence signal measured by the instrument only depends on the amount of surface-bound and labeled component adhering to the sensor surface and not that contained in the

volume of the fluid. In addition, because a laser wavelength > 600 nm is used for exciting the fluorophore, the measurement process is not compromised by biological fluoresence.

The Need for Sensor for Early Prediction of Infection Onset.

To reduce mortality and morbidity rates associated with wounded battle casualties, more effective methods are needed which allow field medical unit staff to identify infection susceptible patients and detect early stage infections. A method to detect an infection before it reaches the acute phase, would allow early medical intervention to eliminate potentially life threatening infections before they become established.

Historically, determining whether a patient is suffering from an infection has been accomplished by monitoring the patient's temperature and periodically measuring their white blood cell count (WBC). Unfortunately, by the time an elevated temperature or WBC is observed, a low level infection may have progressed into an acute phase. To address this problem, recent research, based on the past decade's rapid growth in knowledge of immune response mechanisms, suggests a variety of serum proteins may be useful as prognostic and diagnostic indicators for infections, septicemia, transplant rejections, myocardial infarctions, cancer, and response to surgical procedures.

The body's immune response is a complicated process involving many different interacting chemical messengers.[3-6] When tissue is damaged by trauma or infection, interleukin 6 (IL-6) and various chemotactic factors that attract macrophages and other lymphocytes to the site of the damage are released. IL-6 functions to tell the body that tissue damage has occurred. When an infection begins to grow in a wound, the levels of IL-6 increase dramatically (10-100 fold).[7 8] IL-6 acts on the liver, often in concert with TNF-α and IL-1a, to stimulate the production of the acute phase proteins such as c-reactive protein (CRP).[9] Besides assisting in the production of acute phase proteins, TNF-α has been directly correlated with the onset of sepsis. CRP serves to bind to bacterial cells in the presence of Ca^{2+} where it serves as an opsonin, enhancing the phagocytic properties of macrophages. Macrophages that are present at the site of infection will "signal" the other lymphocytes that an infection is present and needs to be dealt with. This signal will depend upon the nature of the pathogen. Viral and bacterial pathogens are dealt with by the T_H1 arm of the immune system.

It is important to remember that progress in understanding the human immune system's response to an infection is currently evolving rapidly. Significant new insights are being made practically daily. While no single blood serum factor has been shown to uniquely predict infection or infection susceptibility under all conditions, a system capable of providing serum level measurements of multiple blood factors will provide a promising diagnostic and prognostic tool.

CRP is a well characterized acute phase protein and is thus often looked for as an indicator of an acute phase response and has been studied as a possible serum indicator for diagnosing acute infections.[10, 11] Studies show that CRP appears to be a better prognostic indicator for monitoring the efficacy of treatment of an infection during its acute phase than conventional WBC measurements.[12] As a low level infection progresses into an acute phase, the serum level of CRP rapidly rises over a period of 24-36 hours and stays elevated until the infection starts to subside.[3] While CRP measurement can provide an improved diagnostic as to the severity of an infection and treatment efficacy, CRP measurements alone do not distinguish between tissue damage caused by trauma and damage caused by infection. Moreover, CRP measurements can not be used to predict infection susceptibility or to identify the onset of an infection at an early stage.

Recently interleukin 6 (IL-6) has been identified as being produced during early stages of an infection.[1, 13-16] Because elevated serum levels of IL-6 can precede elevated levels of CRP during an infection by several days or more,[17] IL-6 measurements may be used to identify infection onset at an earlier point in time. As a major inducer of acute phase response, elevated IL-6 levels have also been demonstrated to be a good prognostic indicator of the onset and outcome of sepsis,[15, 18] and have been demonstrated to be a good predictor of bacteremia, infection severity, and risk of death.[7, 19] IL-6 has also been shown to be the best predictor of

sub-clinical infections of the amniotic cavity which may affect pre-term delivery.[20, 21]

In addition to IL-6, IL-12 plays a pivotal role during the early stages of the immune response both by assisting in detecting that an infection is present and in determining how well the body is capable of dealing with the infection.[4, 22] The majority of viral and bacterial infections are dealt with by the T_H1 arm of the immune system and available evidence points to IL-12 as being the chemical messenger that is released by the macrophages to initiate the observed T_H1 response.[4] In addition, studies in mice have demonstrated that the existence of IL-12 determines how susceptible a mouse is to infection.[23] It therefore seems possible that IL-12 may in fact be the earliest measurable infection-specific signal to the immune system, and that IL-12, when used in conjunction with other early cytokines such as IL-6 and TNF-α would serve not only as an additional indicator of the presence of an infection but also as a measure of a given individual's ability to deal with the infection.

Cytokines such as IL-6, IL-12, and TNF-α may be measured in serum using an enzyme-linked immunosorbant assay (ELISA). Unfortunately, ELISA assays are time consuming and require an experienced technician to run them. Test results take at least 4-5 hours to obtain, possibly longer, depending on the clinical laboratory's schedule. Thus, by the time results are obtained using current diagnostic tools, an infection may have progressed substantially in severity. However, these tests do provide a better prognostic indicator of the onset of an infection than can be obtained using WBC or temperature measurements alone; and with prompt treatment, an infection may be stopped before it takes hold. Unfortunately; performing ELISA assays in a field medical unit is impractical. Field medical units do not have staff resources and clinical laboratory facilities needed for performing periodic IL-6 ELISA assays for each casualty. Moreover, the components of ELISA assays are too unstable to be used outside of a controlled clinical laboratory environment.

To provide an early infection detection system suitable for field medical units which allows for a more timely detection of the onset of an infection and which requires only minimally trained operators to use, IA is proposing to develop a field-portable, fiber-optic sensor capable of measuring a panel of prognostic and diagnostic indicators for infection including IL-6, IL-12, CRP, and TNF-α. The fiber optic sensor will be able to determine serum concentrations of IL-6, IL-12, TNF-α and CRP in approximately 10 minutes or less. As new knowledge develops regarding additional serum blood factors which may be utilized as prognostic and diagnostic indicators, such measurements may easily be incorporated into the system by adding additional sensor cartridges.

The Technical Basis for the Development

The essential feature of a fiber optic evanescent biosensor, is the confinement of the fluoresence sensing region to the immediate surface of an optical fiber by taking advantage of the evanescent (short range decaying) electromagnetic field produced by total internal reflection of light propagating within the fiber as described by Hirshfield.[24] The manner in which this functions is as follows:

Consider light incident at angle θ on the boundary between two optical media with indexes of refraction N and n. When the light is incident on the boundary at angles greater than or equal to the critical angle, θ_{crit} where $\sin(\theta_{crit}) = N/n$, the light will be totally reflected from the surface. Although, no light is transmitted past the boundary and into the media with the lower index of refraction, electromagnetic theory shows that an evanescent electromagnetic field exists at the boundary which decays exponentially with perpendicular distance from the boundary. The characteristic 1/e depth for this decay for light of wavelength λ incident at angle θ is given by the equation:[25]

$$(\lambda/4\pi)(N^2 \sin^2\theta - n^2)^{-1/2}.$$

This distance is large compared with the dimensions of antibodies. Thus, the light with wavelength λ_1 will interact with antibodies bearing fluorescent molecules which are attached near the probe's surface to generate fluorescence at wavelength λ_2. Because the fiber is very large

compared with the size of the antibody, a large fraction of the emitted fluorescence light at wavelength λ_2 will intersect the fiber optic sensor, be trapped inside it by total internal reflection, and be carried back to a solid state light detector in the control unit.

The basic concepts of evanescent sensing have been applied in the laboratory to specific assays by several investigators using various designs for launch and collection optics, fiber geometries, and surface chemistries. However, none of these assays is yet commercially available. The requirements for such sensors to be practical for real world applications are described below.

First, the sensing fiber must be held in a cartridge whose purpose is to contain the biological fluids being analyzed. Light must be launched into the fiber and subsequent fluorescence received and detected upon exiting the fiber without incurring excessive signal losses at the points at which the fiber sensor contacts the sensor cartridge's support structure.

Three basic approaches to minimizing loss at contact points have thus far been reported. The original design by Hirshfield[24] held the fiber with a very small ring positioned at a point relative to the focusing objective prior to the point at which the first light rays would contact the side of the fiber. The difficulty of making the area small enough and located precisely enough to prevent losses of a magnitude which negatively impacts sensitivity at levels of interest has led to other approaches. Slovacek's group at CIBA Corning addressed this by using a fiber which is large enough to stand up when supported only at the distal end.[26] It actually could be regarded more as a rod than a fiber. The disadvantage of this approach is that it looses the greater sensitivity which is associated with smaller fibers by virtue of the larger number of bounces which occur in smaller fibers as the light travels down the fiber.[27]

The third approach to this problem has been developed at the Naval Research Laboratory (NRL) under the direction of Francis Ligler.[28] NRL has employed a tapered fiber which alters the V-number of the fiber and channels the higher order return modes into lower order modes which are less likely to be lost when the signal passes through the point of contact. Various types of tapers have been utilized and a combined taper has been found to work most effectively. This approach has been effective in providing remote sensing for two BW agents, *Clostridium botulinum* Toxin A[29], and ricin[30] with a sensitivity of the order of ng/ml. The latter was used to measure river water and urine samples. However, the complicated manufacturing process (computer controlled etching of foot long small diameter fibers with hydrofluoric acid) used in this design, adds considerable expense to each fiber sensor. The manufacture of low-cost, disposable sensor cartridges using this approach is not feasible.

To address this problem, IA developed a fiber optic fluorometer (see Figure 2b, c) and sensor cell (Figure 2a) in which the critical fiber points of contact are treated with a low index lossless surface coating which constrains the light within the fiber without reducing the fiber numerical
* aperture (NA), thereby eliminating losses of signal. By incorporating the surface coating into our
* sensor cells, the signal to background ratio improved 10 fold.
*
* To further increase sensitivity, IA's fiber optic fluorometers reshape the excitation light beam
* entering the sensor using a "beam annularizer" to maximize evanescent coupling to the fluorophore. At angles ≤ to the critical angle of the excitation or input light, λ_1, the effectiveness of fluorescence detection varies as a function of the eighth power of the sine of the angle of incidence of the light incident at the glass fiber/water boundary. It is therefore essential that the light propagating within the fiber sensor be concentrated at angles near to (but not greater than) the angle for total internal reflection. If the sensor illumination system simply matches the numerical objective of its focusing objective to that of the glass-water interface at the sensor's surface, the majority of light traveling within the fiber will propagate at angles far from the optimum for exciting fluorescence. IA has developed a proprietary illumination system which redistributes the light entering the sensor so that nearly all of the laser energy propagates at any specific angle less than or equal to the critical angle.

To further improve the fiber optic fluorometer's sensitivity, a holographic notch rejection filter is used to block the laser radiation by a factor of $>10^6$. This technique allows the entire broad fluorescence peak of the fluorophore to be collected and improves the sensitivity of our system

by a factor of 10-100 over systems using laser line filters and monochromaters to isolate the fluoresence signal.

A fiber optic cable (Figure 2c) connects the optic package to the sensor cartridge holder. Disposable sensor cartridges "plug in" to the holder's optical connector, and fluid couplings clamp to the sensor cartridge's input and output fluid ports. A notebook computer is used to control the laser and fluid handling, and to acquire data from the lock in amplifier.

IA's optical improvements have steadily increased the sensitivity of our measurment systems, particularly as we convert optical breadboard systems to brasssboard prototypes. For example, the second generation measurement instrument (Figure 2c) is at least 13x more sensitive than the current optical breadboard system (Figure 2b). IA has recently started testing our third generation instrument, an optically similar but more compact brassboard of the instrument shown in Figure 2c. This instrument's optical package is contained within a 3.5"x13"x9" space.

Sensor Manufacture

In order for fiber optic biosensors to be efficacious for medical testing, it must be possible to manufacture the chemically treated fiber sensors' reproducibly in quantity and the fiber sensors must be provided in cartridges which can be placed in the measurement instrument. IA has developed technology which addresses both these issues. First, IA has developed processes which allow a large quantity of optical fibers to be cleaned and chemically treated at a single time. Fibers are cleaved using an electronic cleaver, placed in a batch processor, cleaned, passivated, and finally chemically sensitized. The nature of sensitization depends on the type of sensor being developed. For the lead sensor being developed by IA, a chromophor/ionophor membrane is applied to the fiber surface. For the hormone sensors being developed by IA, the silanized silica surface is covalently linked to an antibody. Processing fibers in large batches improves fiber to fiber reproducibility. Following chemical sensitization, each fiber is inserted into a sensor cartridge. The end caps of each cartridge provide fluid inlet/outlet ports. These inlet/outlet ports mate with the fluid couplings in the sensor cartridge holder. One end of each sensor cartridge incorporates a centering ferrule so that the fiber sensor may be reproducibly mated with the optical connector in the sensor cartridge holder.

Nonspecific Binding

Another problem which has made it difficult for biosensors to maintain their sensitivity when used in real biological fluids such as plasma or urine is the problem of nonspecific binding of endogenous fluorescent materials to the sensor probe. Most reported sensors have utilized fluorescein or rhodamines as the fluorophore because they can be excited by the 488 and 514 nm lines of an argon laser. Because these wavelengths also stimulate biological fluorescence as well as intrinsic fluorescence of optical components, the detection sensitivity for fluorophore above the biological background is drastically lowered. This problem is not significant for IA's fiber optic sensors because they use an inexpensive diode (~635 nm) laser along with a fluorophore, Cy5, which absorbs near this laser frequency and fluoresces at 670 nm.

A closely related, but more general problem, is that of fouling of the sensor by substances present in samples. This problem has prevented all previous fiber sensors from working reliably with high sensitivity in untreated biological fluids, particularly blood. Common methods for addressing this problem include pacification of the glass fiber with serum albumin or casein and adjustments in solution pH. IA, Inc. has addressed this problem in the context of Phase II work on a project aimed toward development of a fiber optic sensor for measuring hormones associated with the fertile period. During the fiber sensor manufacturing process, a novel proprietary treatment is used to alter the fiber's surface. This treatment nearly eliminates sensor response changes caused by the adhesion of proteins to the fiber surface as is shown in Figure 3. In an experiment to test the efficacy of this treatment (see Figure 3), an untreated fiber and one which received the proprietary treatment were both incubated with BSA and then fluorophore-labeled antibody without antigen was flowed into the sensor cell. Since no antigen was present, observed fluorescence represents nonspecific binding to the antibody to the fiber. The reading on the treated fiber changed very little with time, whereas the fiber which received only the conventional BSA treatment rapidly increased fluorescent response as antibody non-specifically

gathered on its surface.

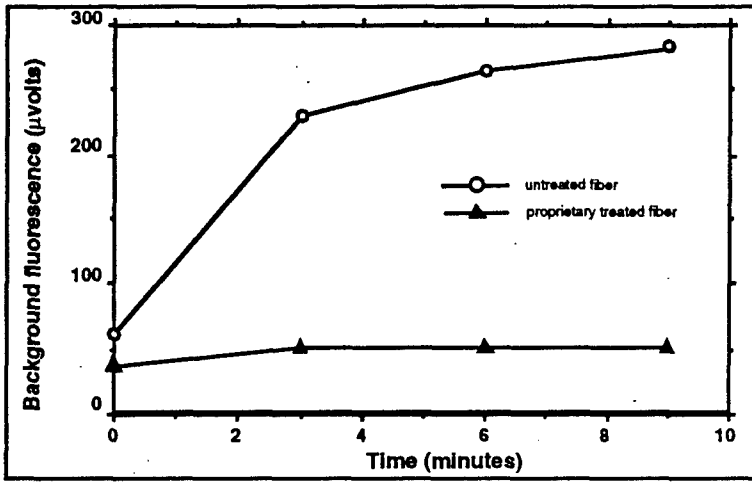


Figure 3 The fiber receiving proprietary treatment shows a greatly reduced nonspecific binding.

Body of Report

Task I: Prepare the assay components for IL-6 and IL-12 sensitive fiber optic sensor.

Anti-IL-6 and anti-IL-12 antibodies were obtained from R&D Systems. They were coupled to fibers either directly through a heterobifunctional crosslinker or through neutravidin and biotin. Other aliquots of antibodies were fluorophore labeled with Cy5.

Materials and Methods

Producing anti-IL-6 and anti-IL-12 fibers linked directly through γ-maleimidobutyric acid-N-hydroxysuccinamide ester:

400μm multi-mode fused silica fiber was cleaved to 10 cm lengths using a York sonic cleaver. Ends were inspected for flatness and the segments were cleaned, the cladding removed from the fiber's middle section and a proprietary treatment applied which protects against non-specific binding of antibodies to the fiber. The fiber cladding at the end sections of the fibers were protected by short pieces of black polyimide tubing. Fibers were placed in a batch processor and the processor was placed in a dry box. A 2% solution of 3-(mercaptopropyl)-trimethoxysilane in dry toluene was injected into the processor and left at room temperature for 2 hours. The silane was removed and the fibers rinsed by injection and removal of toluene followed by methanol. A 2 mM solution of γ-maleimidobutyric acid-N-hydroxysuccinamide ester (GMBS) in dry ethanol was injected and left at room temperature for 1 hr. The batch processor was transferred out of the dry box, the GMBS was removed and the fibers were rinsed with methanol and then water. A solution of antibody in phosphate buffer, pH 7.5 or carbonate buffer, pH 9.3, having concentration between 0.022 mg/ml and 0.1mg/ml, was injected and left at room temperature for 4 hr. at room temperature or overnight at 4°C. Following removal of the antibody, a solution of 1% casein in phosphate buffer was injected in order to block any remaining reactive sites on the fibers. This was removed and fibers were rinsed with water, allowed to air dry in a clean hood and stored at 4°C until mounted in sensor cells. Fibers were mounted in flow through sample cells of the type shown in Figure 4. To prevent fluorophore from leaking onto the distal or proximal fiber faces and generating spurious fluorescence, fiber ends were sealed to the sample cell using 5 minute epoxy. Cells were stored at 4°C until used.

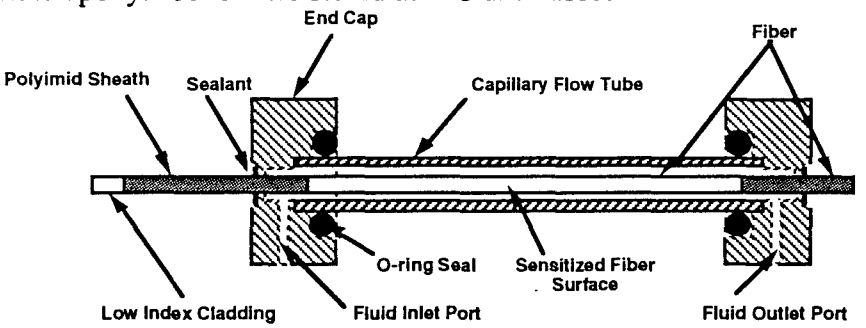


Figure 4: Sensor cartridges are designed to be easily manufactured and inserted into the measurement apparatus.

* Avidin-biotin coupled anti-IL-6 and anti-IL-12 fibers:

* Fibers were treated as described above through the reaction with GMBS. At that point, a
* solution containing 0.05mg/ml neutravidin (Pierce Chemical, Rockford, IL) was injected into the
* batch processor and left at room temperature for 4 hours. 1 mg of antibody was dissolved in
* PBS, pH 7.6 and concentrated on a Centricon 30 to remove lyophilized tris buffer. It was then
* recovered from the Centricon in 0.9 ml of 0.05M carbonate buffer, pH 8.5. To this was added
* 0.0375 mg of sulfosuccinimidyl-6-(biotinamido)hexanoate (Pierce Chemical). After a 40 minute

incubation at room temperature, the sample was concentrated to a volume of 200 µl and chromatographed on Superdex 200. The initial peak emerging from the column was diluted with PBS to the volume necessary to fill the batch fiber processor and left overnight at 4°C.

Antibody labeling with Cy5:

The fluorophore, Cy5 reactive succinimide ester was obtained from Amersham Life Science in packets sufficient for labeling 1 mg of protein. Aliquots containing 1 mg of anti-IL-6 or anti-IL-12 were placed in Centricon 30 concentrators and the original phosphate buffer was replaced with 1 ml of carbonate buffer, pH 9.3. This is the preferred pH for reaction between a succinimide ester and the lysyl amines of proteins. It is possible to perform the reaction at lower pH, however, it takes longer and the labeling density is often reduced. The antibody solution was incubated with the Cy5 reactive succinimide ester for 30-60 minutes. The solution was again placed in a Centricon 30 concentrator and the carbonate buffer exchanged with pH 7.2 phosphate buffer. This also removed the free Cy5 from the solution. Antibody was further separated from unreacted Cy5 by chromatography on Superdex 200 (Pharmacia). After fractions were collected, absorbance was measured at 280 and 650 nm and the antibody concentration and labeling density were calculated from the formulae:

$$\text{Antibody concentration in moles/liter} = A_{280} - 0.05 * A_{650} / 170000$$

$$\text{Cy5 / antibody} = (A_{650} / 200000) / \text{antibody concentration}$$

Anti-IL-12 goat polyclonal #AB-219-NA, lot #EW06 was coupled to Cy5 as previously described to yield a labeled antibody preparation having 6×10^{-7}M.

Affinity purified anti-IL-6 goat polyclonal #AF-206-NA was used at a concentration of 8.2×10^{-7} M.

Labeling density was between 3.5 and 5.8 Cy5 per antibody.

Task II: Determine the sensitivity and response time for IL-6 and IL-12 sensors.

Various permutations of antibodies covalently coupled fibers with Cy5-labeled antibodies were evaluated to optimize biosensor response to IL-6 and IL-12 sample concentrations. IL-6 sensors consisting of Cy5-anti-IL-6 goat polyclonal #AB-206-NA used with fibers covalently coupled to anti-IL-6 monoclonal #6708.111 discriminated between 0.25 and 10 pg/ml of IL-6. IL-12 sensors consisting of Cy5-anti-IL-12 goat polyclonal #AB-219-NA used with fibers covalently coupled to anti-IL-12 monoclonal #24910.1 exhibited a range of response between 0.5 and 500 pg/ml of IL-12.

Experimental Methods and Results

During Phase I, in order to minimize the time spent optimizing assay protocol details, both the IL-6 and IL-12 assays include a 10 minute incubation step of the sample to be assayed with an antibody-fluorophore reagent. This incubation time was picked because, based on experience with other immuno-assays, staff felt it would be long enough for a significant fraction of the labeled antibody to become bound to the assay analyte. While this assumption proved correct, the 10 minute incubation time used during Phase I, should be considered as an upper limit for the incubation time which will be utilized in the final assay protocols. These will be standardized during Phase II, since it is possible that the assay might work equally well with a reduced incubation time of 1, 3, 5, etc. minutes.

Protocol for IL-12 measurements

The antibody combination which yielded the best IL-12 sensor utilized anti-IL-12 monoclonal #24910.1 on the fibers and Cy5-anti-IL-12 goat polyclonal #AB-219-NA mixed with the sample. IL-12 measurements were carried out using the benchtop optical breadboard fiber optic

fluorometer to measure sensor response. Two tubes received 135µl each of the above anti-IL-12 solution. One tube, hereafter referred to as the calibration tube, then received 15µl of buffer. The other, hereafter referred to as the sample tube, received 15µl containing IL-12 sufficient to produce final concentrations or 1, 10, 100, or 500 pg/ml of IL-12. Both were mixed by vortex and allowed to sit at room temperature for 10 minutes. Contents of the calibration tube were first injected into a fiber sensor cartridge and data was collected every 1/100 of a second for 4 seconds. Then the contents of the sample tube were injected and data collected for an additional 4 seconds. The results of the calibration solutions were used to normalize the IL-12 data so that the responses of the different fibers could be compared. To achieve normalization, the factor which would bring data point 66 of the calibration solution to a standard value was calculated. Each data point was then multiplied by the resulting number. Results of this experiment are shown in Figure 5. It can be seen that the fiber sensors show an almost immediate binding response which is related to the concentration of IL-12 in the sample. This response is too fast to be related to equilibrium or even diffusion phenomena. It represents binding of the IL-12 in the layer which comes into contact with the fiber at the moment of injection. The exact mathematics of data processing for this type of data will be worked out in Phase II in order to produce reliable information from the sensor cartridge. For Phase I demonstration of feasibility, it represents success in that response is rapid and discrimination is seen in the range of IL-12 concentrations which will indicate early onset or predisposition toward infection.

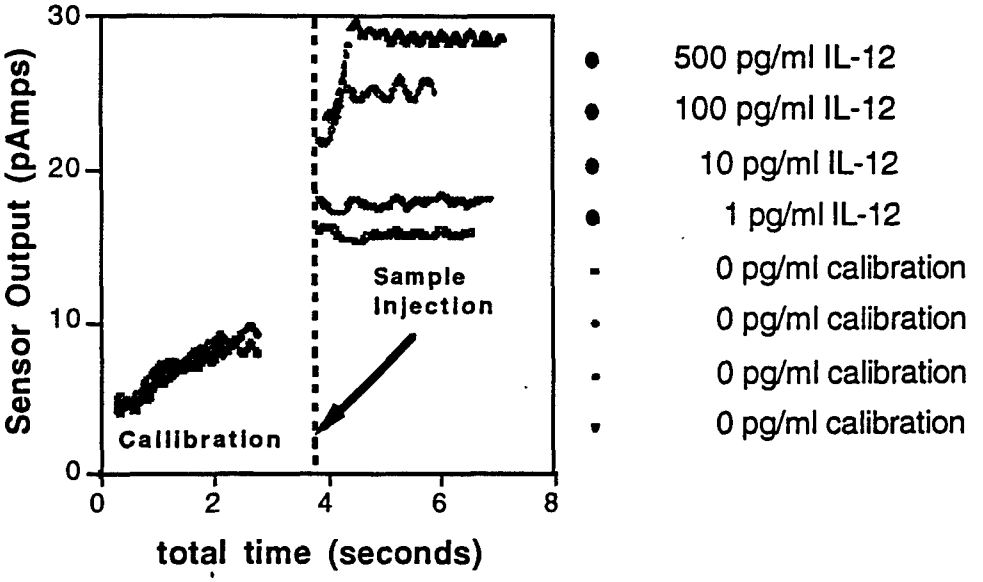


Figure 5: IL-12 fibers show a rapid response in the range of interest.

Protocol for IL-6 measurements

* Of the combinations tested, the best results for IL-6 measurement were obtained using anti-IL-6
* monoclonal #6708.111 on the fiber and Cy5-anti-IL-6 affinity purified goat polyclonal #AF-206-
* NA mixed with sample. Using these assay reagents and a similar protocol to that used for the IL-12 biosensor assay, far fewer labeled fluorophores appeared to attach themselves to the fiber sensor's surface. This difference may be because IL-6 is a smaller molecule than IL-12 (~20.3 kDa vs. ~75 kDa). Its smaller diameter would allow fewer Cy5-labeled antibodies to attach themselves around and tag each IL-6 molecule before the tagged IL-6 molecules are attached to the fiber antibody.

As a result, sufficiently sensitive IL-6 measurements could not be performed using the optical breadboard fluorometer used with the IL-12 sensor and the IL-6 assay was performed using IA's 13x more sensitive, second generation evanescent sensor instrument (currently being used for measuring lead in blood). This instrument differs from the optical breadboard in that the
* annularized laser input beam output propagating at the correct evanescent cone angle is directly
* butt coupled to the sensor cartridge. In addition, the input angle of the light is closer to the critical angle for a quartz fiber in aqueous medium than was the case for the breadboard

instrument.

As a result, this instrument has both greater sensitivity (13x) and increased background due to scatter at the sensor surface into the solution. Since all samples are mixed with similar concentrations of Cy5-antibody, the increased scatter background can be subtracted from all measurements and does not pose a problem. The increased sensitivity provided a successful demonstration of a fiber optic sensor for IL-6. IL-6 measurements shown in Figure 6 below were performed on two fibers, each fiber being used to read more than 1 sample. The first fiber
* received PBS solutions containing 8.2×10^{-7}M Cy5-anti-IL-6 #AF206-NA, 20% RD6 (an additive
* used by R&D Systems in ELISA assay kits) and either 0, 1 or 10 pg/ml IL-6, injected in that order. Between each injection the fiber sensor was rinsed with PBS. The second fiber sensor was used to see if the useful range could be extended below 1 pg/ml. It was used with similar antibody solutions containing 0.25, 0.5 or 1 pg/ml IL-6, injected in that order. Data was acquired for each solution every 2 seconds over a period of 168 seconds. Results shown in Figure 6 demonstrate the ability of the sensors to produce a reasonable standard curve for the very low levels of IL-6 which must be discriminated in identifying the early onset of infection.

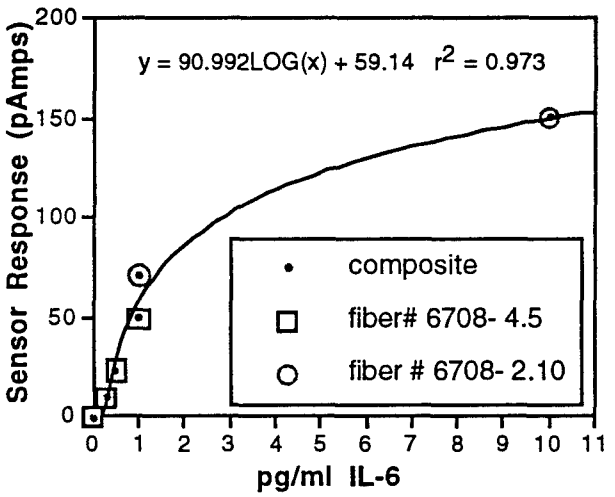


Figure 6: The IL-6 sensor produces a standard curve in desired range after normalizing for the baseline reading.

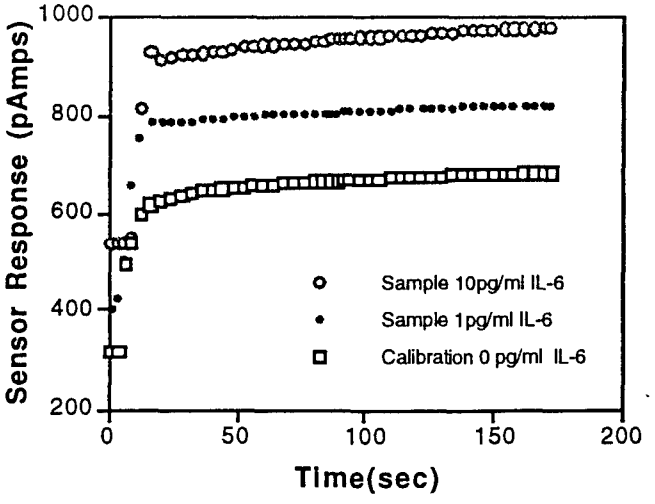


Figure 7: IL-6 sensor response has stabilized after 30 seconds.

Real time data shown in Figure 7 indicates that the initial binding between the IL-6-Cy5-anti-IL-6 complex in the sample and the antibody on the fiber has stabilized by 30 seconds.

Task III: Assess the reproducibility of sensor performance in buffer and serum.

Acceptable fiber to fiber sensor reproducibility was achieved by normalizing IL-6 and IL-12 sensors to low concentration standards. This normalization compensates for slight differences in the optical coupling of sensor to the measurement instrument and chemical sensitization of the fiber surfaces.

Reproducibility of Sensor Response

Sensor reproducibility was tested using five IL-12 fiber sensors created as described in Task 1 * using anti-IL-12 monoclonal #24910.1 on the fibers and Cy5-anti-IL-12 goat polyclonal #AB- * 219-NA mixed with the sample. The Cy5 reagent had a concentration of roughly $2x10^{-8}$M anti- * IL-12. In order to eliminate variations due to unequal incubation times of sample and antibody, * 15µl of IL-12 standard was mixed with 135µl of Cy5-anti-IL-12 reagent, mixed and left overnight at 4°C. The following day five anti-IL-12 fibers were mounted, incubated with a 1% casein solution for 10 minutes and standard curves run on each fiber according to the protocol described for IL-6 fibers under Task II. While all fibers displayed a curve which was basically linear for samples containing 0, 0.5, and 1 pg/ml and appeared to have a more logarithmic relationship between 1 and 10 pg/ml, the degree of attenuation of this basic form varied considerably between the fibers. This variation could be the result of either non-equivalent surfaces on the fibers or non-uniform coupling of the laser into the fiber sensor cartridge or both. As was indicated in the Phase I proposal the work of this task was not to achieve the requisite reproducibility for a final product, (which would be difficult within a Phase I time frame), but rather to assess data from the sensors in ways which would help to formulate the Phase II tasks which will bring these aspects of sensor production under control to provide the requisite reproducibility. Plans for this are addressed under Objective III of Phase II work.

Monitor Fiber Silanization Reproducibility

In noncompetitive immunoassays such as these, it is not necessary to effect precise immobilization of capture antibodies, but simply to have a large excess of binding sites over the number of antigen molecules available in solution. Nevertheless, for the sake of reproducibility in fiber production it is advisable to carefully control each step in the modification of the fibers. Analysis of each chemical step, when possible, will enable greater control over the entire process.

During Phase I, IA started developing QC procedures to improve batch to batch reproducibility of fiber sensor response to analytes. The first step in this process requires developing a method to monitor the reproducibility of the fiber silanization step which occurs at the start of the sensor manufacturing process.

After stripping fiber cladding and cleaning the bare silica surface, the initial chemical treatment is to couple MPTS (3-mercaptopropyl trimethoxysilane) to the optical fibers. Initial attempts to determine the number of resulting thiol groups by chemical testing (using DTNB; 5,5'-dithiobis-(2-nitrobenzoic acid); Ellman's reagent) failed, likely due to the insensitivity of the method. A second, more sensitive method[31, 32] is available in a commercially available thiol quantitation kit (kit # T-6060; Molecular Probes, Inc.). By this method, papain hydrolysis of a colorometric substrate, L-BAPNA (N-benzoyl-L-arginine-p-nitroanilide) provides a signal that is proportional to the amount of free thiol in solution. Free thiol is generated by the reaction of cystamine with thiol groups on the fiber (or protein, etc.), resulting in the simultaneous oxidation of thiol on the fiber and reduction of cystamine to cysteamine in solution. Cysteamine is then used to reduce an oxidized form of papain (papain-SS-CH_3). Since the activity of papain depends on the availability of the reduced thiol in the active site of the enzyme, enzyme activity is thereby coupled to the number of free thiol groups in the sample, e.g., on the surface of the fiber, and the latter can be calculated by hydrolysis of L-BAPNA.

As indicated in Figure 8a, the detection limit for the colorimetric method is ~200 pmol/reaction. Initial experiments indicated that the colorimetric method is still too insensitive to detect -SH on fibers. To further increase the sensitivity of this method, we have substituted a fluorogenic papain substrate, BAMC (Nα-benzoyl-L-arginine-7-amido-4-methylcoumarin). This substrate is hydrolyzed to give the fluorophore, 7-amino-4-methylcoumarin. BAMC is reported to give up to 100-fold increased sensitivity for determination of papain activity,[2] so BAMC was employed in the method. Silanized fibers were enclosed within capillary tubes, with a total included volume ~50 µL. A mixture of cystamine and papain was added to each, followed by 1 hour incubation at 25°C. Following the sulfhydryl exchange reaction, BAMC was added to each reaction mixture, and the resulting compound, 7-amino-4-methylcoumarin was quantified by fluorescence excitation at 380 nm and emission at 440 nm. The calibration curve for BAMC (Figure 8b), indicates a linear response was obtained to ~20 pmol/reaction. Initial attempts to determine -SH on fibers using the BAMC method indicated ~50 - 100 pmol free thiol per fiber, so it appears that the modified enzymatic method will provide a means to monitor batch-to-batch variations in fiber silanization.

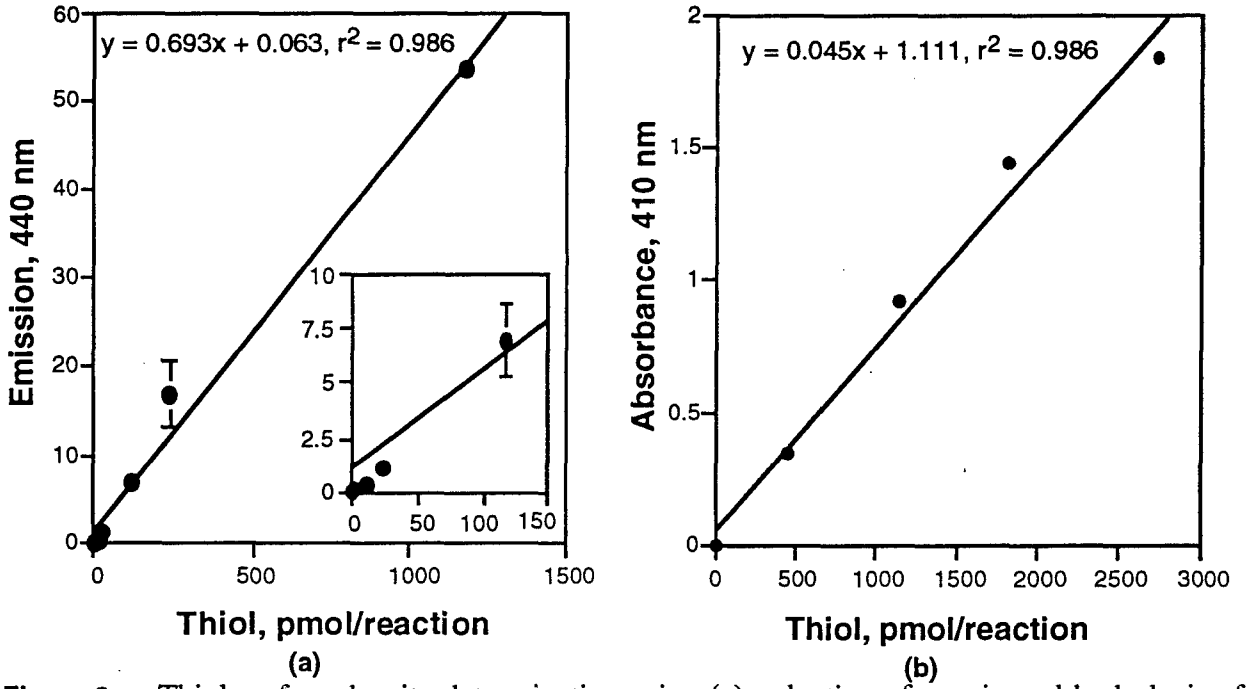


Figure 8: Thiol surface density determination using (a) reduction of papain and hydrolysis of the fluorogenic substrate, BAMC improves assay lower limit sensitivity by a factor of 10 over (b) the colorometric substrate, L-BAPNA assay.

Normalization of Sensor Response

The appearance of the data such as that shown in Figure 6 when observed for several different fibers leads to the intuitive impression that the points on the curves bear a relatively constant internal relationship to one another, but differ by an attenuation factor. If this is the case, then if the zero reading is subtracted from all values for a given fiber and the 0.5 pg/ml sample is used as a normalization factor for both optical and chemical differences between fibers, results between the fibers sensors should appear more similar. As can be seen from Table 1, this appears to be the case. What is particularly interesting about the data in this table is the observation that for four out of five fibers the difference between the results of this normalization applied to the 1 pg/ml data point and applied to the 10 pg/ml data point is very close to 1.0, which is the log of the factor relating the two concentrations to which this normalization was applied. This suggests that if manufacturing differences or optical differences cannot be overcome, the use of a low level calibration solution can potentially allow data to be reliably translated into an IL-12 concentration. This possibility will be further developed during Phase II work.

Ratio of 1 pg/ml read to 0.5 pg/ml read	Ratio of 10 pg/ml read to 0.5 pg/ml read
2.27	3.28
2.49	3.40
2.01	3.07
2.19	3.62
2.02	3.00

Table 1: Normalization of sensor response by low concentration standard.

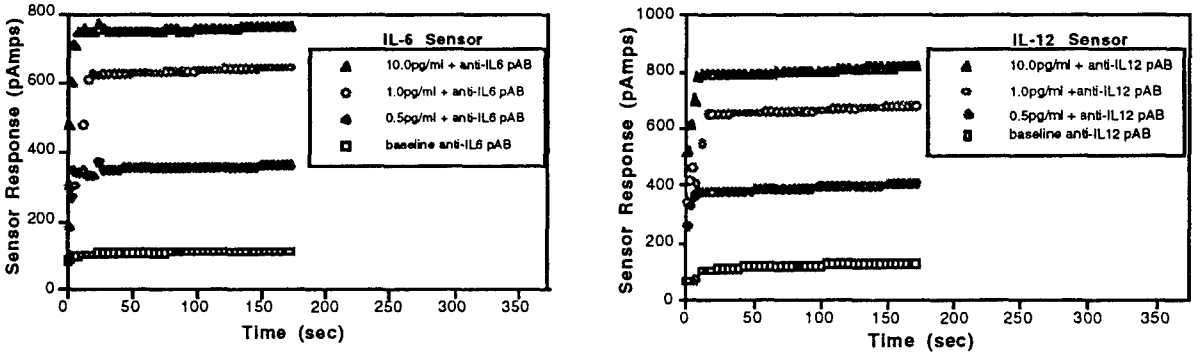
Data obtained on undiluted human serum will be reported under Task IV because it was obtained using the combined IL-6 and IL-12 fiber described in that Task.

Task IV: Evaluate performing both IL-6 and IL-12 assays on a single fiber sensor.

During Phase I, IA developed a multi-analyte sensor cartridge capable of assaying a serum sample for both IL-6 and IL-12 using a single cartridge.

IL-6 and IL-2 combined assay using avidin-biotin coupled anti-IL-6 and anti-IL-12 fibers:

In order to perform assay for both IL-6 and IL-12 on a single fiber, the following protocol was
* followed. A solution containing Cy5anti-IL-6 goat polyclonal #AB-206-NA and another
* containing Cy5-Anti-IL-12 goat polyclonal #AB-219-NA were diluted to identical
* concentrations of 2.4×10^{-8}M specific antibody. Each of these was injected separately into the
* sensor to obtain a zero reading for that antibody solution. The sensor was washed through with
* buffer. Following this a sample containing known amounts of IL-6 and IL-12 was injected into
* the sensor and allowed to sit for 10 minutes. This was removed and the sensor washed with
* buffer. The Cy5-anti-IL-6 solution was returned to the cell and data acquired as described in
* Task II. This was removed, the sensor washed with buffer and the Cy5-anti-IL-12 solution was
* injected and data acquired as before. This procedure was repeated for samples containing 0.5, 1,
* and 10 pg/ml levels of IL-6 and IL-12. Data obtained in this way is shown in Figures 9a and 9b.



(a) (b)

Figure 9: IL-6 and IL-12 assays in the range of interest can both be performed on a combined fiber.

It is quite clear that both assays yield a standard curve at concentrations between 0.5 and 10 when test by this procedure on the same fiber. This demonstrates feasibility of testing more than one cytokine on a single fiber sensor cartridge which has been exposed to the sample. During Phase II work the extent to which this concept can be extended beyond two will be examined.

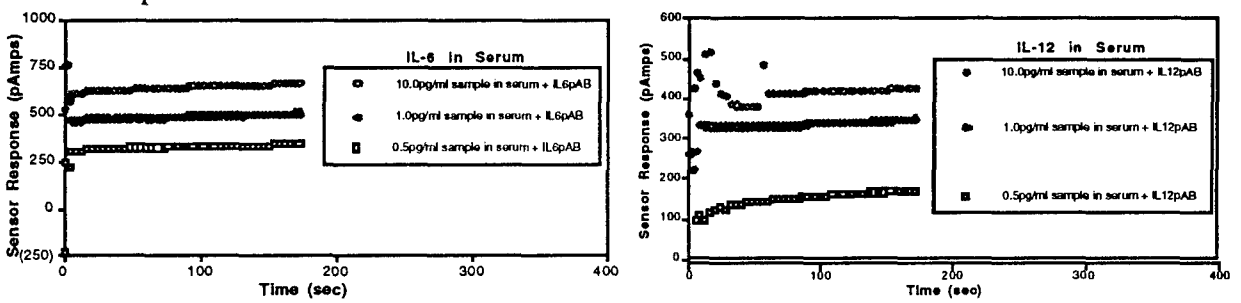
Application of the normalization method described in task 3, using the 0.5 pg/ml sample as the normalizing factor yields the results shown in Table 2.

sample	Ratio of sensor readings for 1 pg/ml and 0.5 pg/ml	Ratio of sensor readings for 1 pg/ml and 0.5 pg/ml
buffer IL-6 standard	2.13	2.63
buffer IL-12 standard	2.00	2.52

Table 2: Normalized data for IL-6 and IL-12 measured on a single fiber sensor.

One conclusion that may be drawn from comparison between data of Table 1 and that of Table 2 is that differences in antibody density on the fiber are reflected in the normalized ratios obtained at different concentrations of the measured cytokine. One would expect that there is a linear response region of the standard curve where the ratio of the calibration reading to that of the sample reading will be equivalent to the ratio of the concentration of the calibration solution and that of the sample. The 1 pg/ml samples appear to be in that region for most fibers on both tables. As a certain fraction of the available sites are used up, the ratio will show more deviation from this ideal ratio, becoming lower than the actual range of concentrations. This reasoning is consistent with the lowered ratio in the 10 pg/ml samples on the combined fiber. Since sites are shared between anti-IL-6 and anti-IL-12, a larger fraction of these sites is used up by the higher sample concentration, placing it in a more nonlinear region of the standard curve. Further work will be carried out in Phase II to more thoroughly understand and utilize information obtained in this manner.

As this proposal is being written, IA has just begun testing the IL-6 and IL-12 biosensors in blood serum. Although there has been no time to optimize sensor fabrication or assay protocols for use in blood serum, the initial results taken with a combined IL-6/IL-12 sensor show exceptional promise and suggest that the sensor is basically working as predicted. Figure 10, shows the biosensor response to IL-12 when an IL-6/IL-12 combined fiber was used in a serum sample. While the sensor's response to increasing IL-12 concentrations behaves as anticipated, the sensor is quite sensitive and apparently detected the presence of IL-12 (~0.5-0.75 pg/ml) in the serum unspiked serum sample which we had intended to use for zero calibration. Since the sensor can detect sub pg/ml IL-12 levels, we will not be able to use an "unspiked" serum sample as a zero IL-12 standard, and will need to revise our assay protocol to provide different calibration points.



(a) (b)

Figure 10: The combined IL-6/IL-12 sensor responds to (a) IL-6 and (b) IL-12 concentrations of 0.5 to 10 pg/ml in blood serum..

Conclusions

This series of experiments had as its purpose the demonstration that the two assays did not interfere with one another and that the mixed fibers still possess sufficient sites for each cytokine so that the useful range of the assay was not compromised. That purpose was achieved. These experiments demonstrate that a combination fiber can be used to perform both IL-6 and IL-12 assay. Simplified procedures for using combined fibers will be developed during Phase II work.

Task V: Design a field portable instrument.

During Phase I, the hardware, electronic, data acquisition, and software requirements for making a hand-held sized instrument were developed. The key issue which was addressed, was whether or not all component sizes could be sufficiently reduced to meet the overall requirement of

providing a sensor readout instrument which could be hand-held by a field medic. To do this a hardware drawing for a hand-held field-portable instrument concept was generated, and in conjunction with electronics consultants and in house staff, estimates made for the space which would be needed for data acquisition, analysis and instrument control electronics. The results of this effort were hand-held instrument concepts which fit within a volume less than 8" x 5" x 2.2". To verify that an instrument can be manufactured which meets these size constraints, concept drawings and interior layouts for the hand-held instrument have been developed (see Figure 11). Details of the hand-held instrument concept will be discussed in the "Phase II Technical Objectives and Approach" Section of this proposal.

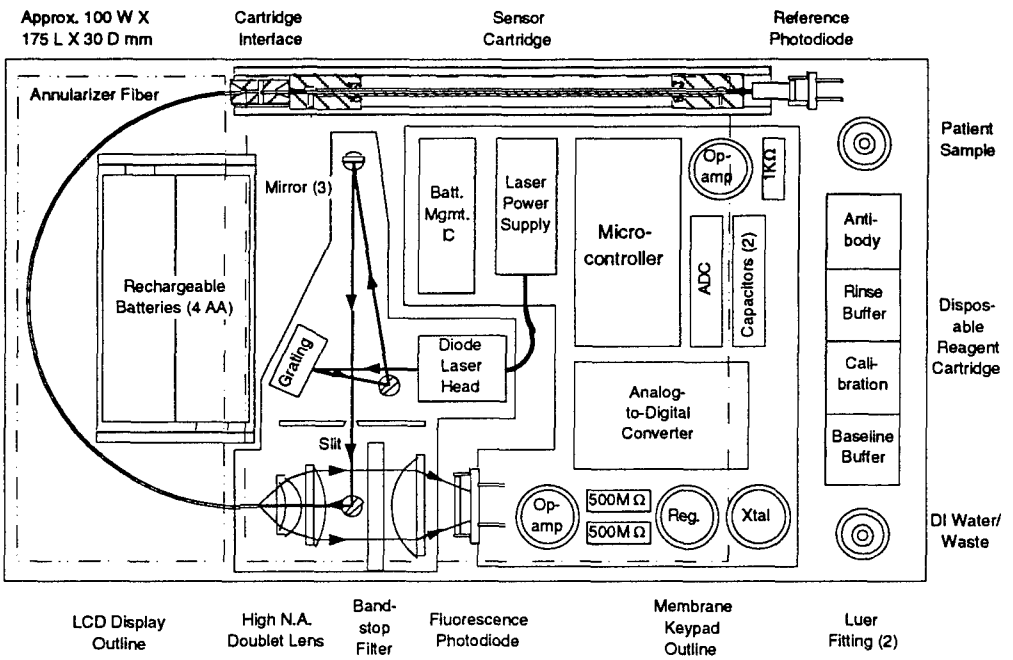


Figure 11: The infection detection instrument will be a hand-held device.

Conclusions

During Phase I, IA designed a hand-held biosensor instrument and successfully demonstrated the feasibility of using fiber optic biosensors with this instrument to rapidly detect the elevation of early stage infection markers IL-6 and IL-12. After calibrating, the biosensors developed during Phase I, are reproducibly sensitive down to sub-pg/ml concentrations of IL-6 and IL-12. After drawing and incubating a serum sample for 10 minutes with the fiber sensor, this sensor system will allow measurements of IL-6 and IL-12 levels and other early infection markers to be made in the field within 30 seconds of labeled antibody injection into the instrument. Because of the rapidity with which sepsis sets in to produce multiple organ failure and death, it is not currently feasible to detect sepsis onset by sending serum samples to clinical laboratories for analysis. Thus, the development of this biosensor instrument for early-stage infection detection has the potential for significantly improving the prognosis for critically injured patients.

This project has permitted IA, Inc. to develop a product with exciting potential. We thank the Army for providing the support which made this possible and we hope the work will be deemed worthy of Phase II support.

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Bibliography of Publications Resulting from This Contract

A poster session was presented at the 1997 meeting of the Clinical Ligand Assay Society in Chicago which included data which was obtained under this contract. The abstract was entitled:

"Fiber Optic Sensors for Real Time Observation of binding between Antibodies, Receptors and Nuclear Response Elements.

Authors: Judith L. Erb, John R. Erb-Downward, James G. Downward, Bradford Henderson, James L. Wittliff, Wolfgang Raffelsberger

List of Personnel Supported by This Contract

IA staff who performed work in support of this project included:

Judith L. Erb	Principal Investigator	- Biosensor and assay design
James G. Downward	Principal Scientist	- Data acquisition system design
Richard H. Smith	Senior Biochemist	- Assay development
Otho E. Ulrich	Senior Optical Engineer	- Optical system design
John R. Erb-Downward	Biochemist	- Assay evaluation and development
Ingrid Picazzo	Technician	- Fiber chemical sensitization
Nina Rackham	Technician	- Fiber manufacturing and quality control
Brad Henderson	Technician	- Chemistry/standards

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REPLY TO
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MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

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PHYLIS M. RINEHART
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