OMB No. 0925-0195
Expiration Date 2/28/98

Department of Health and Human Services
Public Health Service

Small Business Innovation Research Program

Phase I Grant Application

Follow instructions carefully.

Leave blank — for PHS use only.		
Type	Activity	Number
Review Group		Formerly
Council Board *(Month, year)*		Date Received

1. TITLE OF APPLICATION *(Do not exceed 56 typewriter spaces)*
CCD-Based Analyzer for Multi-Channel Biosensor

2. SOLICITATION NO. PHS 97-2

3. PRINCIPAL INVESTIGATOR ☐ New Investigator

3a. NAME *(Last, first, middle)*
Van Wagenen, Richard A.

3b. DEGREE(S)
Ph.D | B.S. |

3c. SOCIAL SECURITY NO. *Provide on Personal Data Page*

3d. POSITION TITLE
Vice President for Research and Development

3e. MAILING ADDRESS *(Street, city, state, zip code)*
Protein Solutions, Inc.
P.O. Box 58093
Salt Lake city, UT 84158-0093

3f. TELEPHONE AND FAX *(Area code, number, and extension)*
TEL: 801-583-9301
FAX: 801-583-4463

BITNET/INTERNET Address:

4. HUMAN SUBJECTS ■ NO ☐ YES
4a. If "yes," Exemption no. or
IRB approval date
☐ Full IRB or ☐ Expedited Review
4b. Assurance of compliance no.

5. VERTEBRATE ANIMALS ■ NO ☐ YES
5a. If "Yes," IACUC approval date
5b. Animal welfare assurance no.

6. DATES OF PROJECT PERIOD
From: 10/1/97 Through: 3/31/98

7. COSTS REQUESTED
7a. Direct Costs $ 69,290
7b. Total Costs $ 99,164

8. PERFORMANCE SITES *(Organizations and addresses)*
Protein Solutions, Inc.
391 Chipeta Way, Suite 320
University Research Park
Salt Lake City, UT 84108

9. APPLICANT ORGANIZATION *(Name and address of applicant small business concern)*
Protein Solutions, Inc.
P.O. Box 58093
Salt Lake City, UT 84158-0093

10. ENTITY IDENTIFICATION NUMBER: Fed. Tax # 87-045-1813 | Congressional District: 2

11. SMALL BUSINESS CERTIFICATION
■ Small Business Concern ☐ Women-owned
☐ Socially and Economically Disadvantaged

12. NOTICE OF PROPRIETARY INFORMATION: The information identified by asterisks(*) on pages _____ of this application constitutes trade secrets or information that is commercial or financial and confidential or privileged. It is furnished to the Government in confidence with the understanding that such information shall be used or disclosed only for evaluation of this application, provided that, if a grant is awarded as a result of or in connection with the submission of this application, the Government shall have the right to use or disclose the information herein to the extent provided by law. This restriction does not limit the Government's right to use the information if it is obtained without restriction from another source.

13. DISCLOSURE PERMISSION STATEMENT: If this application does not result in an award, is the Government permitted to disclose the title only of your proposed project, and the name, address, and telephone number of the official signing for the applicant organization, to organizations that may be interested in contacting you for further information or possible investment? ■ YES ☐ NO

14. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name: J.D. Andrade
Title: President and CEO
Address: Protein Solutions, Inc.
P.O. Box 58093
Salt Lake City, UT 84158-0093

Telephone: 801-583-9301
FAX: 801-583-4463
BITNET/INTERNET Address:

15. PRINCIPAL INVESTIGATOR ASSURANCE: I certify that the statements herein are true, complete, and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

SIGNATURE OF PERSON NAMED IN 3a *(In ink. "Per" signature not acceptable.)*
Richard A. Van Wagenen
DATE: 4-11-97

16. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete, and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Service terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF PERSON NAMED IN 14 *(In ink. "Per" signature not acceptable.)*
DATE: 4-11-97

Abstract of Research Plan

NAME, ADDRESS, AND TELEPHONE NUMBER OF APPLICANT ORGANIZATION

Protein Solutions, Inc.
P.O. Box 58093
Salt Lake City, UT 84158-0093
Phone: 801-583-9301

YEAR FIRM FOUNDED: 1987
NO. OF EMPLOYEES *(include all affiliates)*: 5

TITLE OF APPLICATION
CCD-Based Analyzer for Multi-Channel Biosensor

KEY PERSONNEL ENGAGED ON PROJECT

NAME	ORGANIZATION	ROLE ON PROJECT
R. Van Wagenen, Ph.D.	Protein Solutions, Inc.	Principal Investigator
C.-Y. Wang, Ph.D.	" " "	Research Scientist
R. Scheer, Ph.D.	" " "	Research Scientist
J. D. Andrade, Ph.D.	" " "	Advisor

ABSTRACT OF RESEARCH PLAN: State the application's broad, long-term objectives and specific aims, making reference to the health-relatedness of the project. Describe concisely the research design and methods for achieving these goals and discuss the potential of the research for technological innovation. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. ***Therefore, do not include proprietary or confidential information.*** DO NOT EXCEED 200 WORDS.

The firefly luciferase reaction is the basis for the quantification of ATP via the emission of light which can be directly related to the amount of ATP present. ATP is a ubiquitous biochemical related to many metabolic biochemicals, i.e., sugars, amino acids, hormones, vitamins, enzymes, etc. The objective of this study is to assess the feasibility of developing a small, relatively inexpensive CCD-based analyzer for the direct detection and quantification of ATP and more importantly for the quantification of many of the biochemicals directly related to ATP utilization via phosphorylation and de-phosphorylation reactions catalyzed by a wide variety of enzymes. The instrument would be superior to a PMT based luminometer in that it could analyze an inexpensive, disposable multi-channel biosensor card simultaneously for a wide variety of clinically related analytes necessary for a correct medical diagnosis, i.e., biosensor cards for a family of tumor markers, cardiac damage markers, etc. A bench top prototype will be constructed and evaluated relative to well quantified luminescence standards to confirm the efficacy of a signal modeling equation. Evaluation will then be extended to a multi-channel biosensor card for ATP and in Phase II to a multi-channel, multi-parameter biosensor card.

Provide key words (8 maximum) to identify the research or technology.

ATP, biosensor, luminescence, CCD, luciferase, luciferin, homocysteine

Provide a brief summary of the potential commercial applications of the research.

A need exists for a small, low cost CCD camera based monitoring device which can quantify a variety of analytes on a single, disposable multi-channel biosensor platform. Each biosensor platform would contain a panel of analytes which were clinically related to a proper diagnosis, i.e., tumor marker panel, cardiac damage marker panel, essential amino acid panel, errors of metabolism marker panel are examples of data profiles that could improve diagnoses.

Budget Justification

Using continuation pages if necessary, describe the specific functions of the personnel and consultants. Read the instructions and justify costs accordingly.

Dr. R. Van Wagenen, Principal Investigator, will devote 60 percent of his effort to this six month project. He will manage and coordinate all of the activities of the project as well as contribute technically in the area of optics, prototype device design, CCD selection and evaluation and sensor design.
Dr. C.-Y. Wang, Research Scientist is budgeted at 10 percent effort. He is a Research Scientist with extensive experience with the firefly luciferase reaction for ATP analysis. He will play a key role in the design of the miniaturized ATP biosensor and in the day to day experiments involving the sensors.
Dr. Rob Scheer, Research Scientist is budgeted at 30 percent effort on this project. He will assist with the experimental design and conduct of the experimentation and evaluation primarily as this relates to the evaluation of the luminescence standards.
A laboratory technician will be appointed on a 50 percent basis to conduct many of the daily experiments and data analyses.
Dr. J.D. Andrade, founder and President, although not budgeted in the proposal for any funding, will be available to provide support, assistance, advice and technical consulting on an as needed basis.

Total Direct personnel costs are $38,300 and fringe benefits are based on a 30 percent of direct personnel salaries basis. The companies current indirect cost rate is 60 percent of the total of salaries and fringe benefits. This indirect cost rate was established with the National Science Foundation on a Phase II STTR grant awarded in the fall of 1996.

No consultants, subcontracts, patient care costs or fees are proposed for this work. $5,000 is requested for general lab supplies and electronic and optical components. Funds are requested to purchase a high performance CCD camera from either Santa Barbara Instruments Group or Meade Instruments. This device is essential for showing feasibility of the prototype. Funds are also requested for a lap top computer which can be interfaced to the CCD in the prototype on a full time, instrument dedicated basis. $2,500 is requested for travel expenses to two meetings (clinical chemistry and electro-optics). $3,000 is requested in the Other Expenses category to maintain the companies membership in the Center for Biopolymers at Interfaces, a state-university-industry consortium. Refer to the Resources section below which details the benefits the company realizes via a membership in this organization.

Resources

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate their capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Include laboratory, clinical, animal, computer, and office facilities at the applicant small business concern and any other performance site listed on the FACE PAGE. Identify support services such as secretarial, machine shop, electronics shop, and the extent to which they will be available to the project. Use continuation page(s) if necessary.

Protein Solutions, Inc. (PSI) occupies 1,200 square feet of research, laboratory, and office space located in the Research Park adjacent to the University of Utah (391 G Chipeta Way). This space includes a chemistry lab, a biology lab, an optics lab, and two offices. The space and equipment are adequate for the work proposed with the addition of the equipment funding requested in this proposal. Office equipment is standard and is adequate to address the administrative aspects of the project, i.e., computer, laser printer, copy machine, FAX machine, etc. The company has a Technology Transfer Agreement with the University of Utah which allows for the transfer of jointly developed technologies to PSI. The company is also a member of The Center for Biopolymers at Interfaces (CBI) a state - university - industry consortium which is one of the Centers for Excellence in the Utah State System of higher education. CBI membership provides a number of key benefits including: (1) fee for service access to many analytical services at the University of Utah at a very low rate, e.g. the SEM/TEM facility and the surface analytical facility (XPS, SIMS, etc.), (2) access to specialized lab equipment in the departments of Bioengineering (optics lab) and Chemistry (analytical biochemistry labs), and (3) access to faculty members who can provide expertise on a consulting basis. Finally, the close proximity to the University of Utah makes it easy to utilize machine shop and electronic shop capabilities at rates that are competitive for the Wasatch front of northern Utah.

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

Basic laboratory equipment at PSI includes analytical balances, stirrers, hot plates, fume hood, oven, pH meter, water bath, HEPA filtered air hoods, steam autoclave, reverse osmosis water, luminometers, light microscopes, cameras, general optical components, etc. A charge coupled device (CCD) array camera and associated computer and controller are also available for the recording and quantification of luminescence experiments. A high resolution laser printer makes it possible to print out and display luminescence patterns from the CCD.

BIOGRAPHICAL SKETCH AND BIBLIOGRAPHY

Richard A. Van Wagenen, Ph.D.
Vice President for Resarch and Development

Current Employment

Protein Solutions, Inc. 391 G Chipeta Way, Suite 320, Salt Lake City, Utah 84108

Birth Date: January 11, 1948 **SSN:** 528-64-5667

Education

Ph.D., Materials Science and Engineering, University of Utah, 1976.
B.S., Mechanical Engineering, University of Utah, 1971
Associate in Science, Engineering Major, College of Eastern Utah, 1968.

Professional Experience

Vice President of Research and Development, Protein Solutions, Inc., Salt Lake City, UT 1996 - present. Current employment.

RVW Consulting, Salt Lake City, UT 1995-1996.

Director of Research and Development, OHMEDA Medical Systems, Salt Lake City, UT 1990-1994.

Vice President Research and Development, Albion Instruments, Salt Lake City, UT 1987-1990.

Director of Instrumentation, Biomaterials International, Inc., Salt Lake City, UT 1981-1987.

Research Assistant Professor, Department of Bioengineering, University of UT, Salt Lake City, UT 1978-1985.

Post Doctoral Fellow, University of British Columbia, Vancouver, B.C. 1976-1977.

Honors

Member of Pi Tau Sigma, National Honorary Mechanical Engineering fraternity.
Winner of the NASA Innovation Award, 1981.
Recipient of the R & D Award for Innovative Design and Development of the RASCAL II; a laser based respiratory/anesthetic gas monitor utilizing Raman light scattering, 1993.

Representative Patents and Publications:

J. D. Andrade and **R.A. Van Wagenen**, "Process for Conducting Fluorescence Immunoassays Without Added Labels and Employing Attenuated Internal Reflection", January 11, 1983, United States Patent 4,368,047.

RESEARCH PLAN

A. Specific Aims.

We propose to develop a CCD camera-based analyzer for a multi-channel biosensor which employs luminescence for the simultaneous quantification of multiple analytes. The target analyte addressed in this preliminary feasibility study is ATP. ATP reacts with luciferin in the presence of luciferase enzyme and other co-factors to produce light which can then be detected via the CCD. The ATP analyzer platform can be linked to other analytes related to many metabolic reactions of clinical and biochemical interest, i.e., carbohydrates, amino acids and some hormones and vitamins.

The primary goals of this feasibility study are to:

1. Construct a functional, bench top prototype analyzer from commercially available components, i.e., a charge coupled device (CCD) imaging camera, a CCD controller, an imaging lens, biosensor plate holder, and a lap top computer for data acquisition, analysis, and display.

2. Evaluate the prototype analyzer using commercially available luminescence standards in terms of absolute sensitivity threshold and signal linearity. Validate the current signal modelling equation.

3. Convert our existing single channel ATP biosensor design to a smaller design with multiple ATP channels and evaluate its performance with the functional prototype. Parameters to be evaluated are: threshold sensitivity for analyte ATP, luminescence signal intensity as a function of time, sample and signal reproducibility, accuracy and precision. Three analysis modalities will be compared and contrasted, i.e., (a) using the CCD as a direct analytical measure of analyte concentration via measured signal intensity, (b) CCD imaging of the luminescence spatial distribution on the biosensor which can, in turn, be directly related to the analyte concentration via a pre-established spatial calibration scale, and (c) a combination of both analysis modalities for a more definitive determination of analyte concentration.

Success in Phase I will pave the way for construction of a fully functional, stand alone prototype in Phase II. Both hardware and software development and refinement will be emphasized along with the development of a specific multi-channel biosensor design based on the ATP platform now in development.

B. Identification and Significance of the Problem

1. Technical Background

There are two very special molecules that play unique and central roles in biology. They are adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NADH) and its phosphate form (NADPH). NADH is a ubiquitous electron donor and ATP is generally recognized as one of the key energy currencies in biology. The two molecules act in a cyclic manner and can be regenerated or recharged. ATP and NADH are the basic coupling agents of cellular metabolism. A very large number of biochemical enzyme processes involve one of these two molecules.

There is a large body of literature on the development of biosensors for ATP and ATP-dependent processes and for NADH and NADPH-dependent processes, using the firefly and bacterial luciferase enzymes, respectively (1). Luciferases have been extensively used as labels for a wide range of clinical diagnostic chemical tests (2). Since the firefly luciferase reaction is dependent on an ATP co-factor, it has been extensively used in the development of biosensors for the measurement of ATP. Until very recently such applications were frustrated by the instability of luciferases and the difficulties encountered in attempts to incorporate them into commercial devices exhibiting the necessary reproducibility, reliability, accuracy, and shelf life. We have overcome these problems by incorporating all of our reagents including luciferase into agarose gels (3). Many ATP based biosensors have employed fiber-optic or other wave guided means for delivering the luminescence to a sensing device such as a photomultiplier tube (PMT) that can accurately measure light intensities (4). Although one of the most portable and most sensitive photon detectors available is the human eye it is notoriously difficult to calibrate for accurate measurements of even relative light intensity. The human eye can, however, reliably and accurately measure changes in the spatial position of light given sufficient contrast. This concept is the basis for the work done thus far to develop a small, disposable biosensor for ATP which does not require instrumentation for the quantification of analyte ATP.

Our present ATP biosensor concept (5-7) is based on the fact that, for a given luciferase and luciferin concentration, a minimum concentration of ATP is required to produce a detectable light output. Above that concentration, light is visible; below that ATP concentration, no light is detected visually. By filtering or consuming some of the analyte ATP with an ATP "consumase" such as apyrase before it reacts with the luciferin and luciferase the intensity of the light at a well defined spatial position on the biosensor can be controlled. The turnover rate of apyrase is two orders of magnitude higher than that of luciferase, so the apyrase quickly moderates the ATP concentration. Refer to Figure 1 which summarizes the concept for a single discrete spatial position and one apyrase concentration. The amount of ATP that reacts with the luciferase and luciferin to produce light depends on both the initial ATP concentration in the sample and the concentration of apyrase incorporated into the biosensor. With high initial concentrations of ATP, a large concentration of apyrase is required to eliminate emission of a detectable light signal. With low initial concentrations of ATP, only small concentrations of apyrase are required to prevent emission of light at a detectable

FIGURE 1 ATP in a sample is partially consumed by a "consumase" enzyme such as apyrase. The remaining ATP then diffuses into a region containing luciferin, luciferase, and other reagents where bioluminescence photons are produced with high quantum efficiency.

signal. The goal of modulating the light signal with apyrase is to generate a specific light cut off point on the sensor which is indicative of a specific concentration of ATP in the analyte solution.

A low concentration of ATP will produce light only at the lowest consumase concentrations because at higher consumase concentrations all of the ATP is consumed before it reacts with the luciferase. A high concentration of ATP will still produce light even at the higher consumase concentrations because enough ATP remains after consumption to produce a measurable light output. This is illustrated in Figure 2 for three identical ATP biosensors exposed to three different concentrations of ATP. Note that there are six discrete "consumase" (apyrase) channels spatially arranged in a step gradient format. It should be emphasized that a continuous gradient may also be employed. ATP is consumed to varying degrees in each of the channels. The residual ATP not consumed then diffuses into a region of constant luciferase and luciferin concentration where the reaction occcurs which produces photons of wavelength 563 nm (for firefly luciferase). The resulting photon flux for each sensor and each channel gives the illumination pattern appearance of a "glowing thermometer" in the dark. The actual ATP analyte concentration would then be determined by observing where the luminescent glow ended and comparing this point with a pre-established scale (not shown in Figure 2). Note that it is not the light intensity that matters, but rather, the position of the termination point that indicates concentration much like the position of mercury in a thermometer indicates the temperature.

The experimental results shown in Figure 3 illustrate this concept of the dependence of spatial light output as a function of signal ATP and discrete step apyrase concentrations. There are eight identical biosensors (arranged horizontally) each having the same one dimensional step gradient of apyrase concentration (0 units/ml to 2 units/ml). The apyrase, luciferin, luciferase, and other reagents are deposited into discrete 8 x 8 test wells of a 96 well microtiter plate. Note that the apyrase gradient is a composite of linear and geometrical gradients. Eight different ATP concentrations ($2x10^{-4}$ to $1x10^{-7}$ mol/l) are applied, each to one of the eight identical biosensors and the resultant luminescent patter after five minutes was noted visually in the dark by an observer and then photographed with both Polaroid film (ASA 3000) and a two stage, thermolelectric cooled, CCD camera. The actual quantitative determination of ATP is made by looking at the spatial illumination pattern of each of the eight sensors either visually in the dark or via the CCD image or film image and noting the position of the last visible luminous circle, e.g. positions A, B, C, D, and E in Figure 3. These points correspond to ATP analyte concentrations of 0.2mM, 0.1mM, 10 µM, 1 µM, and 0.2 µM ATP, respectively. Appropriate design of the apyrase gradient can narrow the analysis range from three orders of magnitude to one with an accuracy of +/- 20 percent of full scale reading.

In 1996 Protein Solutions, Inc. was awarded a STTR Phase II grant from NSF entitled, "Direct Reading Quantitative Biosensors for ATP". This grant focuses on enhancing the sensitivity of our vision and film-based ATP-based luminescent biosensor by several orders of magnitude so that it is competitive with existing instrument based approaches. The use of the CCD camera was envisioned to be

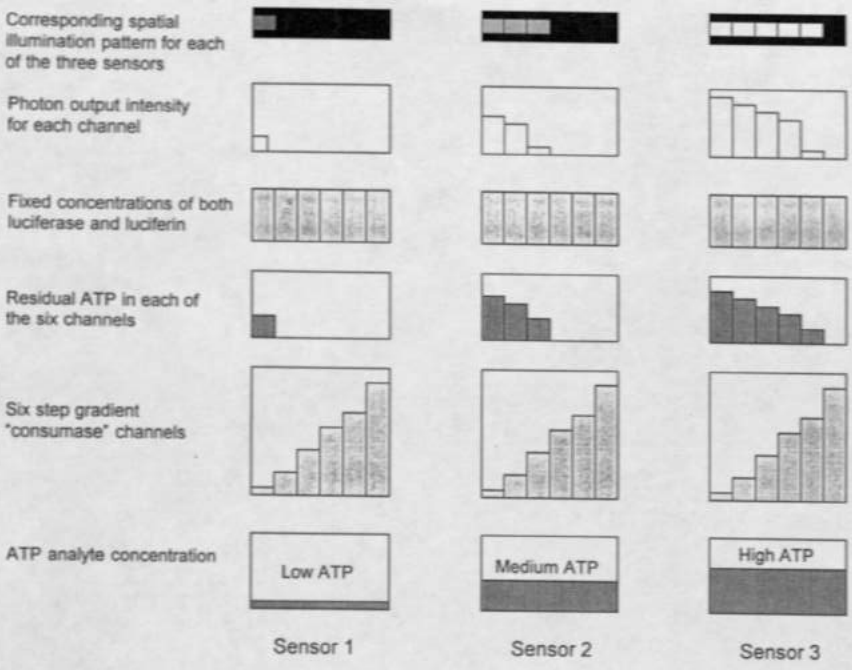

FIGURE 2 Three identical ATP biosensors each exposed to different analyte concentrations. The resultant spatial illumination pattern (not the intensity of the luminescence) gives the ATP concentration via the visual reading of a pre-established calibration scale (not shown).

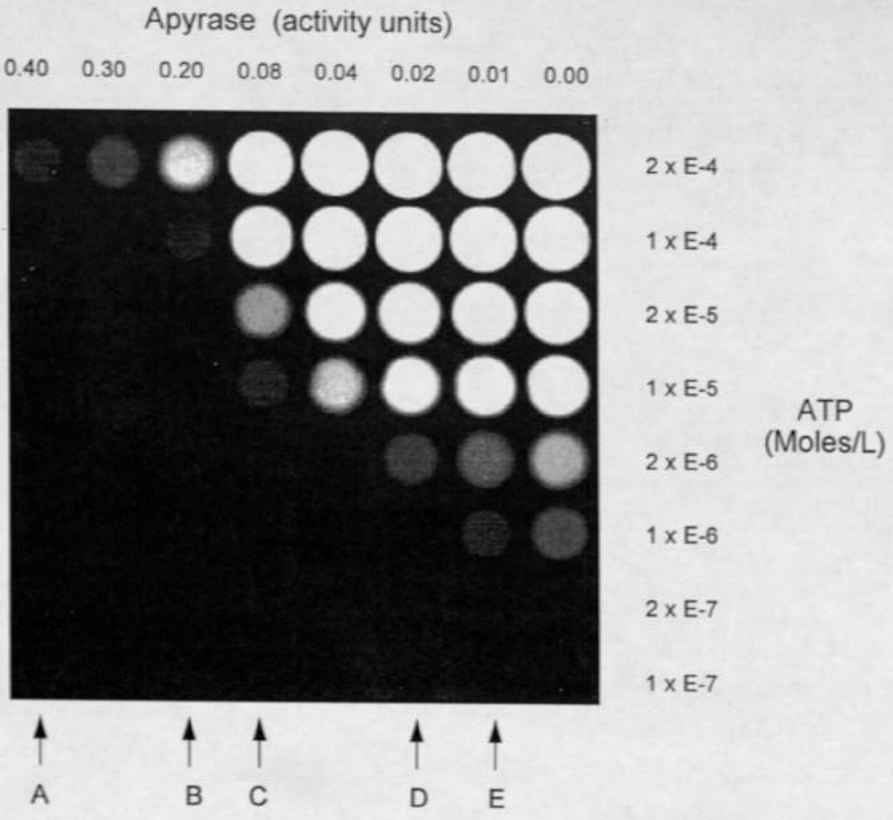

FIGURE 3 Luminescence patterm from an 8 X 8 multi-well plate experiment. See text for details.

utilized merely as an approache to preserving the data. Other areas of the research and development effort address optimizing the "consumase" gradients, enhancing the long term storage of the sensor device and associated enzymes, reagents and luciferase by incorporating them into agarose gels, enhnancing the luminescence with the addition of proprietary additives and evaluating the applicability of the visual detection and film-based detection modes. We, therefore, have the commitment and resources to fully develop the direct reading, quantitative ATP sensor platform We refer to this work as development of the ATP monitoring platform.

The effort to date has resulted in the development of the single channel, prototype ATP sensor card shown in Figure 4 which has a detection capability that ranges from 1 x 10^{-4} to 1 x10^{-7} molar ATP. Since our sample volume is 10 microliters the overall detection threshold is about 10^{-12} moles or 5 x 10^{-10} grams of ATP based on visual detection with the eye given a 5 minute dark adaptation. Refer to TABLE I. Film and CCD based detection thresholds with short integration times are about one order of magnitude better, i.e., 10^{-13} moles of ATP. Current ATP detection and quantification instrumentation (based on PMT detectors and photon counting electronics) for hygiene testing is now in the 10^{-15} to 10^{-16} mole range. This is a factor of 100 to 1000 times more sensitive than our current design based on visual observation. However, it should be stressed that the detection of such an extremely low amount of ATP (sub femtogram) is the most extreme example of the required sensitivity threshold. There are many more applications of monitoring biochemical reactions related to ATP or NADH which are in the range of 10^{-10} to 10^{-14} moles of ATP.

2. The Problem and the Solution

Given our experience to date, we have concluded that subjective testing based on visual perception of luminescence position in the dark is not a realistic monitoring modality for most applications, especially for any clinical monitoring opportunities. The inconvenience of having to observe the biosensor with the dark adapted eye is unrealistic and there is no permanent, quantitative record of the result. Finally, as previously noted, the detection thresholds with the eye, with film contact exposures of several minutes or imaging with our CCD camera (currently limited to about 10 seconds integration time due to high dark current performance) are simply not sufficient to detect many clinically significant analytes in the submicromolar range.

The solution is to develop a CCD camera based analyzer for a multi-channel luminescence biosensor which can quantify the luminescence from multiple sensor sites on a single platform. In this way an entire panel of significant and clinically related analytes can be analyzed based on one sample applied to the biosensor.

3. Commercial Opportunities

Multi-channel biosensors capable of quantifying analytes comprising groups or panels of clinically important diagnostic markers should find wide acceptance since numerous analytical tests can be accomplished simultaneously with one sample of blood or urine. Possible application areas would be for the identification and

041197D.PPT

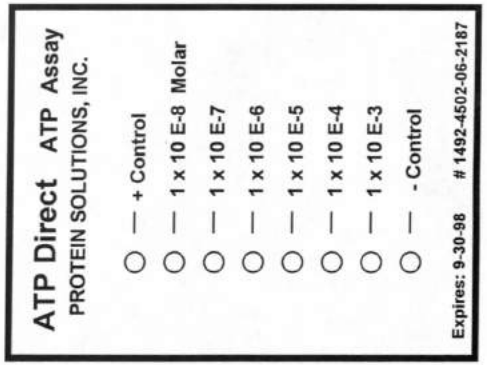

FIGURE 4 ATP biosensor card prototype measuring 75 mm x 100 mm. All biochemical reagents and sample are applied to the individual circles. Luminescence in the dark is correlated with the numerical scale printed in phosphorescent ink.

TABLE I Correspondence between molar concentration of sample ATP, moles of ATP in 10 microliter sample volume, grams of ATP in sample volume and anticipated total photon signal obtainable with CCD detection at 100 seconds integration time. The ▬ bar indicates the current level of performance for our visual detection ATP sensor. The ▭ bar indicates the current level of performance for many commercial ATP analyzers. The ▒ bar indicates the analysis range for many biochemical molecules and proteins of relevance to clinical monitoring needs.

Molar ATP	Moles ATP	grams ATP	Anticipated Total Signal, Nc
10^{-4}	10^{-9}	5×10^{-7}	10^{12}
10^{-5}	10^{-10}	5×10^{-8}	10^{11}
10^{-6}	10^{-11}	5×10^{-9}	10^{10}
10^{-7}	10^{-12}	5×10^{-10}	10^{9}
10^{-8}	10^{-13}	5×10^{-11}	10^{8}
10^{-9}	10^{-14}	5×10^{-12}	10^{7}
10^{-10}	10^{-15}	5×10^{-13}	10^{6}
10^{-11}	10^{-16}	5×10^{-14}	10^{5}

TABLE II Performance Characteristics of the CCD Cameras Discussed in This Proposal

Description	ST-6A	ST-7I	416 XT
CCD Chip	TI TC241	Kodak KAF-0400	Kodak KAF-0400
Chip Dimensions	8.6 x 6.5 mm	6.9 x 4.6 mm	6.9 x 4.6 mm
Pixels	375 x 242	768 x 512	768 x 512
Pixel Size	23 x 27 microns	9 micron square	9 micron square
Full Well Capacity	400,00 electrons	80,000 electrons	80,000 electrons
Dark Current	150 e^-/pixel/5s	<1 e^-/pixel/5s	<1 e^-/pixel/5s
Readout Noise	25 e^- rms	15 e^- rms	15 e^- rms
A/D Conversion	16 bits	16 bit	16 bit
Brightness Level	65,536	65,536	65,536
Data Transfer	parallel	serial	serial
Data Transfer Time	about 20 seconds serial interface	about 1 second SCSI interface	about 1 second SCSI interface
Temperature Control	2 stage, TE to -20 C	2 stage, TE to - 20 C	2 stage, TE to - 20 C
Thermal Control	+/- 0.1 C	+/- 0.1 C	+/- 0.1 C
Shutter	Electromechanical	Electromechanical	Electromechanical
Minimum Exposure Time	0.01 second	0.01 second	0.01 second
Camera Head Dimensions	6" dia. x 3" thick	6" dia. x 3" thick	5" dia. x 2" thick
Controller Dimensions	9" x 6" x 2"	9" x 6" x 2"	9" x 7" x 1"

quantification of tumor markers, (8) heart disease susceptibility (9) markers (cholesterol, homocysteine, lipoproteins, etc.) cardiac damage markers, and inborn errors of metabolism (10) markers (various sugars and amino acids). A CCD camera based analyzer utilizing bioluminescence for low light detection should be able to attain both very low detection thresholds and wide dynamic range for a variety of clincially related analytes that must now be analyzed separately at considerable overall cost.

4. Relationship to Phase II

The focus in Phase II will shift to hardware development and software refinement. We do not propose to develop our own CCD based camera, but rather we will work with a vendor of such cameras to meet our technical specifications. There is a local company (Process Instruments) which is developing a low cost CCD camera for industrial process control monitoring via Raman spectroscopy. Many of their technical specifications are similar to ours and early communication and collaboration may yield a low cost camera and software appropriate for our needs. Our monitoring application will certainly dictate unique user interface requirements which will have to be addressed by changes to the software currently supplied by the CCD camera vendor. In Phase II we will almost certainly have to modify the software which controls the CCD camera. Another very high priority in Phase II will be the development of one clinically relevant multi-channel biosensor which targets the quantification of a series or panel of relevant clincical parameters such as cardiac tissue damage markers, tumor markers, heart disease markers, errors of metabolism markers, etc. The multi-channel sensor development activity of Phase II will build on the ATP platform sensor work which is now underway and is being funded by other sources.

C. Relevant Experience

1. Principle Investigator

Dr. R. Van Wagenen, *Ph.D.*, Vice President of R and D, is a bioengineer with considerable product research, design, and development experience in the medical device industry. Before joining PSI in 1996, he spent ten years working on laser based biomedical instrumentation as VP of R & D and Director of R & D for Albion Instruments and then Ohmeda Medical Systems, respectively. During this time, Rick and his co-workers developed a unique Raman spectroscopy respiratory/anesthesia gas monitor that initially utilized photomultiplier tubes and subsequently CCD array detectors for light signal detection. His instrumentation experience plus his background in Materials Science, and his earlier research work dealing with the characterization of surfaces for biomedical applications are also directly relevant particularly to the design and characterization of the sensors. His product development background will enable this concept to be effectively developed into commercial products. His bio-sketch is included.

2. Other Key Personnel

Dr. C.Y. Wang, Research Scientist, recently completed his Ph.D. studies under Joe Andrade's supervision at the University of Utah. Dr. Wang has worked on the Firefly luciferase system for five years. He recently received the M. DeLuca Award at the 9th International Symposium on Bioluminescence and Chemiluminescence in Woods Hole Massachusetts for the studies of recombinant luciferase. He is an expert on enzyme immobilization and stabilization. He has developed a technique of immobilizing luciferase with agarose and dehydrating the system with the protection of disaccharides. Such gels can be desiccated and successfully rehydrated with full enzymatic activity. This technique has supported the success of our ATP sensing platform. He is now working as a Research Scientist at PSI and supervising a team that is continuing to develop the bioluminescence based direct reading quantitative biosensor for ATP. His biosketch is included.

Dr. Robert Scheer, Research Scientist, received his Ph.D. in Materials Science and Engineering in 1993 and was Principal Investigator of PSI's NSF-STTR Phase I grant on the development of ATP-based biosensors using firefly luciferase. He has had considerable experience with the handling of native firefly luciferase and its stabilization in agarose gels and fiber matrices. He has worked and will continue to work closely with Dr. C.Y. Wang. Rob's background is in polymers, polymer structure and morphology, and the modeling and testing of polymeric materials. His biosketch is included.

Dr. Joseph Andrade is founder, President, and CEO of Protein Solutions, Inc. Joe has worked extensively with proteins, enzymes and antibodies for the past 25 years, focusing his efforts on elucidating their behavior at surfaces and interfaces. He is a full professor in the departments of Bioengineering, Materials Science, and Pharmaceutics at the University of Utah. Eight years ago he became involved in bioluminescence particularly in firefly and bacteria luminescence systems. Joe will be available to assist and consult in the areas of interfacial biochemistry, bioluminescence, and biosensor expertise when required.

D. Experimental Design and Methods

1. Constuct Functional Bench Top Prototype

The CCD imaging camera used thus far in our luminescence sensor development work is a model ST-6A manufactured by Santa Barbara Instrument Group. Performance specifications are summarized in TABLE II. This camera is typical of those produced by several companies that have designed inexpensive, medium performance imagining devices that can used by amateur astronomers with relatively low aperature telescopes. The ST-6A has met our needs in terms of imaging the luminescence patterns for our ATP biosensors, however, the CCD chip has a relatively high dark current (150 electrons/pixel/5 second @ -20 C) and signal integrations longer than about 10 seconds are not possible. Funds are requested to purchase a higher

performance CCD camera that has a much lower dark current level, i.e., less than 1 electron/pixel/5second @ -20 C. This will make it possible to increase our signal integration time by a factor of about 150 so integration times of as much as 25 minutes should be attainable. TABLE II summarizes the specifications of two higher performance CCD cameras. They are the ST-7 from Santa Barbara Instruments and the Pictor 416 XT from Meade Instruments. Both of these cameras are based on the Kodak KAF-0400 CCD chip. Both cameras have smaller pixels with higher packing densities than the ST-6A and both cameras have about half the readout noise (15 electrons rms) of the ST-6A. Finally, both the ST-7 and the Pictor 416 XT chips have SCSI parallel data transfer to the host computer which requires about one second as opposed to the serial transfer mode of the ST-6A which requires about 20 seconds for data download to the host computer. Rapid chip downloading and lower dark current will make it possible to integrate all of the photon emission from any luminescence source for the full duration of the process.

As soon as funding is assurred the camera with the shortest lead time will be ordered. Delivery times of several months are typical for these high demand cameras. During the interim, the ST-6A camera will be used to test and refine the experimental protocols and establish a baseline level of performance. Also, during this time the optical system will be evaluated. We propose to evaluate, compare and contrast the performance of both lens extension rings (12, 24, 36 mm) and a macrolens for enhanced imaging of the luminescence patterns on the biosensor. To date we have employed a 50 mm camera lens with a working length of approximately 20 inches. This makes for a rather large prototype. The use of a short focal length marco lens or lens extension rings will shorten the overall CCD-optics-sample cell to a dimension of about 6 - 7 inches and allow us to more efficiently fill the entire image frame of the CCD.

Also during the first two months our existing biosensor (75 mm x 100 mm) will be redesigned to be smaller (25 mm x 30 mm) and to have multiple ATP channels (six) rather than a single ATP channel. Thus far in our work, the prototype single channel biosensors have all been rather large microtiter plates or thin paper sheets where ATP sample was added and the sensor was transferred to the CCD imager for analysis. We propose to design a sample holder that is fixed relative to the CCD and signal collection optics so that data analysis can begin even before sample is added to the sensor. In this way, we can collect virtually all of the emmitted luminescence that can be captured given the speed of the collection lens.

Finally, we will work with the source of the luminescence standards (Biolink, Ltd. in the U.K.) to design a series of luminescence sources that have an emission range of 1 x 10^2 to 1 x 10^{11} photons/second/sterradian. The cirrcular emission area will be 5 mm in diameter. Aperatures of diameter 1, 2, 3, and 4 mm will also be machined or purchased which will allow us to vary the emission size area from the standards.

At the end of the first three months all of the prototype components and luminescence standards should be in house, all of the protocols should have been tested and refined, the optimal optics for the luminescence collection and imaging should be complete and the machined sample cell holder and miniaturized biosensor design should be completed.

2. Evaluate the Prototype and Compare Results to Modelling Equation

As noted above, the Biolink luminescence standards will allow us to test the sensitivity threshold of our CCD based device down to very low luminosity levels with much longer sample integration times. The wide range of luminescence available (nine or ten orders of magnitude) will also allow us to test the linearity of the CCD chip. Finally, the use of various diameter (1, 2, 3 and 4 mm) circular aperatures which can be placed over the luminescence standards will allow us to test the effect of image size and subsequent signal attenuation on overall sensitivity thresholds and linearity. The Biolink standards are based on the use of tritium gas in sealed chambers that emit beta particles which in turn strike a phosphor screen and emit visible light photons.

The modelling equation for the luminescence standards is as follows:

$$Nc = [F] \cdot [T] \cdot \left[\frac{Sr}{4\pi}\right] \cdot [1 + Rs] \cdot [Tcl] \cdot [QE] \cdot [Ceff] \qquad \text{EQ 1}$$

where:

Nc = Total integrated signal counts detected with the CCD.
F = Photon flux from luminescent standard (phontons/second/sterradian).
T = Signal integration time in seconds.
Sr = Imaging lens solid ange of collection in sterradians [$Sr = \pi/4\ (f\#)^2$] where Sr = .24 for an f/1.8 camera lens.
Rs = Reflectivity of the substrate reflecting photons back in the direction of the of the collection lens (typcially this is greater than 80 percent).
Tcl = Transmission of the collection lens (typically greater than 95 percent).
QE = Quantum efficiency of the CCD at 550 nm (about 45 percent).
Ceff = CCD read electronics conversion efficiency. For the Model ST-6A camera the value is 1 A/D count per 6.7 photoelectrons.

Equation 1 predicts that for a photon emission of 1×10^{11} photons per second per sterradian and a one second integration of the CCD and using a f/1.8 lens it should be possible to detect 219×10^6 total counts summed from all the pixels comprising the image of the luminescent standard. Similarly, for a photon emission of 10^4 photons per second per sterradian and a 1000 second integration time we would anticipate a total signal of 21,900 total counts summed from all the pixels comprising the image of the luminescent standard. With the existing ST-6A camera this would be impossible to see due to the high dark current of 30 electrons/pixel/second, but with the higher performance CCD it should be feasible. Note from TABLE II that the pixels comprising the CCD do not have an infinite storage capacity for photoelectrons, i.e., the full well capacity of the pixels in the Kodak KAF-0400 chip is only 1/5th of that for the TI chip in the ST-6A. Consequently, it will be necessary to configure the signal/image collection optics in such a manner that the image of the luminescence standard is spread over a

sufficient number of pixels that the photons thus collected do not completely saturate idividual pixels and compromise both the response linearity and the overall ability to accurately quantify the signal.

Prototype evaluation in terms of linearity over nine or ten orders of magnitude of photon flux and ultimate signal detection threshold (limited by dark signal) using the luminescent standards should consume most of the effort of the third month.

3. Design, Constuct ATP Biosensor and Evaluate via the CCD based Prototype

The modelling equation for a bioluminescent source comprising one element of the multi-channel ATP biosensor is as follows:

$$Nc = [C_{ATP}] \cdot [Vs] \cdot [N_A] \cdot [QE_R] \cdot \left[\frac{Sr}{4\pi}\right] \cdot [1 + Rs] \cdot [Tcl] \cdot [QE] \cdot [Ceff] \qquad \text{EQ 2}$$

where:

Nc = Total integrated signal counts detected with the CCD.
C_{ATP} = Molar concentration of ATP in sample.
Vs = Biosensor sample volume in liters in the reaction area (typically 10 micro-liters.
N_A = Avogadro's number (6×10^{23} molecules per mole)
QE_R = Quantum efficiency of the bioluminescent reaction. This is 88 percent for firefly luciferase.
Sr = Imaging lens solid ange of collection in sterradians [$Sr = \pi/4\ (f\#)^2$] where Sr = .24 for an f/1.8 camera lens.
Rs = Reflectivity of the substrate reflecting photons back in the direction of the of the collection lens (typically this is greater than 80 percent).
Tcl = Transmission of the collection lens (typically greater than 95 percent).
QE = Quantum efficiency of the CCD at 563 nm (about 45 percent).
Ceff = CCD read electronics conversion efficiency. For the Model ST-6A camera the value is 1 A/D count per 6.7 photoelectrons.

For our current sample detection threshold of 10^{-7} moles/L ATP, a 10 microliter sample volume, an f/1.8 collection lens and the ST-6A CCD camera the total number of photon counts detectable with the system should be 1.5×10^9 counts. See Table II. This is 0.3 percent of the total photons generated during the course of the complete reaction that consumes all the analyte ATP, i.e., 5.28×10^{11} photons. It should be stressed that with the current design which incorporates all of the reagents in an agarose gel support for preservation, it requires some time for the 10 microliter aqueous ATP sample to rehydrate the dessicated gel, diffuse into the reaction area and undergo the luciferin oxidation reaction which generates light. In fact, the time it takes to consume all the ATP in the reaction referenced above using our current biosensor

design is at least one hour. While essentially all of these photons (10^9) could be detected with the proposed analytical design having a high performance CCD with long integration time and rapid data downloading, the practical realities of clinical chemistry dictate a much shorter sample time. Accordingly, we are currently modifying our ATP sensor platform design to accelerate the course of the overall rehydration reaction so that most of the ATP is consumed and the photons generated within ten minutes of adding sample.

Modelling Equation 2 above indicates that the anticipated signal level for the reaction of 10^{-11} molar ATP should produce at least 10^5 counts from the CCD. See TABLE II. This should be adequate to detect as long as the overall sensor source imaged on the pixels is not so extended that the dark current from any given pixel does not overwhelm the signal on the pixel. The challenge to making a multi-channel sensor with a wide dynamic range for analyte will be to balance the photon flux on a given number of pixels so that there are not so many that the full well capacity of any one pixel is exceeded nor so few that the dark count in any given pixel exceeds the overall photon flux on that pixel. This will be accomplished by varying both the size of the discrete luminous areas comprising the sensor and the concentration of reagents which will generate the luminescence for each luminous area comprising the sensor. The overall collection optics design will have to be considered as well. In addition to this optimization the biosensor platform will be redesigned to incorporate six ATP channels in an area of about 25 x 30 mm.

Once the sensor design is finalized it can be evaluated using the prototype. Three analysis modalities will be compared and contrasted. First, we will use the CCD as a direct quantitative measure of analyte concentration via measured signal intensity - essentially the classical approach of signal intensity (predicted by EQ 2) versus concentration. Second, we will employ the more sensitive CCD in a direct image modality to image the spatial distribution of the luminescence pattern which will then be directly related to the analyte concentration via a pre-established spatial calibration scale. This is essentially our current approach but the more sensitive CCD camera will replace the human visual system. Third, we will employ a combination of the previous two analysis modalities for what will, hopefully, be a more definitive and possibly more sensitive assay of analyte concentration.

In each of the three approaches noted above the assay will be evaluated in terms of threshold sensitivity for analyte ATP, luminescence signal intensity as a function of time, sample and signal reproducibility, and overall accuracy and precision. The work in objective three should consume months 4 through 6 of the grant.

The work to address object 3 will occur during months 5 and 6 of the grant. Also during month six a final report will be completed.

E. Human Subjects

No human subjects will be used in this research.

F. Vertebrate Animals

No vertebrate animals will be used in this research.

G. Consultants and Advisors

No external consultants are proposed for this research. Our strong Scientific Advisory board will continue to serve in an advisory and consulting capacity with reimbursement from other sources. The board members are as follows:

Dr. Woody Hastings - Professor of Biology at Harvard University. He is internationally recognized for his basic research on bioluminescence.

Dr. Vladimir Hlady - Associate Professor of Bioengineering at the University of Utah. He is an expert on the study of proteins at surfaces using interfacial fluorescence spectroscopy in conjunction with CCD detectors.

Dr. Don Johnson - former New Biotechnology Product Manager for DuPont and currently and independent consultant and Chairman of the University of Utah Center for Biopolymers at Interfaces a University-Industry-State consortium Center of Excellence.

Dr. Henry Kopecek - Professor of Pharmaceutics and Bioengineering at the University of Utah. He is an internationally recognized authority on hydrogels and related polymers for drug delivery and biocompatibility.

Dr. Larry Kricka - Director of the General Chemistry Lab and Professor of Pathology and Laboratory Medicine at the University of Pennsylvania. He is an internationally recognized for his work on applying both bioluminescence and chemiluminescence to clinical chemistry.

Dr. Jerry Nelson - Microbiologist and founder and President of Nelson Labs, a nationally recognized service laboratory providing a wide range of biological testing and compliance monitoring for industry.

Dr. Russell Stewart - Assistant Professor of Bioengineering at the University of Utah. He is an expert on recombitant techniques for the synthesis and study of luciferases and motor proteins.

H. Contractual Arrangements

No subcontracts are proposed in this research.

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