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C. Technical Content

1. Identification and Significance of the Opportunity.

There are two very special molecules that play unique and central roles in biology; adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NAD) and its phosphate form (NADPH). NADPH 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. "They are the basic coupling agents of cellular metabolism." (Harold). A very large number of biochemical enzyme processes involve one of these two molecules.

It is very fortuitous that biology has evolved two bioluminescent processes dependent on these two molecules: the firefly luciferase reaction, which acts on firefly luciferin in the presence of ATP to produce an oxidized product which chemiluminesces; and the bacterial luciferase reaction, which in the presence of alkyl aldehydes, such as decanal, together with NADPH, also produces an excited chemiluminescent product. Both reactions produce photons with high efficiencies in the presence of oxygen. However, both the luciferases and luciferins involved are chemically very different (*Naval Research Reviews*, Lee, Hastings).

There is a large body of literature on the development of biosensors for ATP and ATP-dependent processes and for NADPH and NADPH-dependent processes, using the firefly and bacterial luciferase enzymes, respectively. Such biosensors generally employ fiberoptic or other wave guided means of delivering the luminescence to a device which can accurately measure light intensities (Roda, Blum, Coulet, Worsfold, Qollenberger, Griffiths, Nabi, Gautier, Ugarova). Although one of the most portable and most sensitive photon detectors available to the scientist or physician is his or her own eye, the eye is notoriously difficult to calibrate for accurate measurements of even relative light intensity. The human two dimensional photon detection system, however, can reliably and accurately measure changes in spatial position.

We propose to exploit the human eye's spatial detection capabilities as the readout system for quantitative, inexpensive, disposable, analytical devices for the analysis of carbohydrates using ATP-dependent, kinase-based phosphorylation reactions. Although the work proposed with this application is focused on the several carbohydrates of most immediate interest and importance to food and dairy industries, it is important to note that there are literally hundreds of phosphorylation-dephosphorylation enzymes whose substrates and whose activity are of growing importance in food quality, nutrition, and related areas (Werner).

Thus, **by proving the applicability of this technology to the assay of monosaccharides, we demonstrate the feasibility of applying our sensors to the assay of numerous substrates that are acted upon by kinases in phosphorylation reactions**, providing the assay has sufficient sensitivity to provide accurate results in the range of interest.

2. Background & Rationale

Protein Solutions, Inc. (PSI) was founded in early 1988 to develop and produce innovative science educational products and personal sensors. We now manufacture a bioluminescence-based science kit designed for students and their teachers, parents, and other genuinely inquisitive human beings.

Bioluminescence is an enzyme-dependent chemical oxidation process which results in photon emission (*Naval Res. Reviews*, Lee). The photoproteins involved in these processes, the luciferase series of oxidative enzymes, are now readily available in inexpensive form, produced by recombinant means (Wood). PSI, itself, has developed, along with the University of Utah, a recombinant process that has allowed us to produce luciferase at 1/100 of commercial cost.

For the last 5 years, PSI has been involved in numerous scientific investigations and development projects covering a wide range of bioluminescent phenomena, including several studies dealing with applications of bioluminescent reactions for sensing applications. We funded a study at the Center for Biopolymers at Interfaces at the University of Utah to characterize the interfacial behavior of firefly luciferase, the photo protein/enzyme responsible for catalyzing bioluminescence in the North American firefly, in which its adsorption at solid/liquid interfaces,

UNITED STATES DEPARTMENT OF AGRICULTURE

SMALL BUSINESS INNOVATION RESEARCH
PHASE I AND PHASE II
PROJECT SUMMARY*

9.2
OMB Approved 0524-0025
Expires 5/98

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Program Office	Solicitation No.	Proposal No.	Topic No.

TO BE COMPLETED BY PROPOSER

Name and Address of Firm	Name and Title of Principal Investigator(s)
Protein Solutions, Inc. 6009 Highland Drive Salt Lake City, UT 84121	Philip M. Triolo, Ph.D. President

Title of Project (80-character maximum)

Rapid, Direct-Reading Devices for Carbohydrate Assay

Technical Abstract (200-word limit)

PSI recently proved feasibility of spatially distributing the light emitted by the ATP-sensitive firefly luciferase bioluminescent reaction so that the position of the light is indicative of ATP concentration. Here we propose to employ a kinase as a front end enzyme to modulate ATP concentration by catalyzing ATP's reaction with an unknown quantity of monosaccharide. ATP will be depleted in direct proportion to the monosaccharide consumed in the general reaction:

$$\text{monosaccharide} + \text{ATP} \xrightarrow{\text{kinase}} \text{phosphorylated monosaccharide} + \text{ADP}$$

We will employ our recently developed spatial luminescent detection system to detect the remaining ATP concentration, thereby providing an indirect measure of monosaccharide concentration.

By using the position of the emitted light to indicate analyte concentration we enable the human eye, which is very good at discriminating the position of a light source but poor at quantifying light intensity, to serve as the readout device. Alternatively, high speed film can serve as detector and readout vehicle.

In this SBIR Phase I Project we propose to stabilize the required enzymes, incorporate all reactants into a suitable prototype configuration, and evaluate the performance of the prototype.

Anticipated Results/Potential Commercial Applications of Research (100-word limit)

We anticipate demonstration of the feasibility of applying PSI's bioluminescent sensing technology to determine monosaccharide concentrations. By proving the applicability of this technology for monosaccharide assays, we demonstrate the feasibility of applying our sensors to the assay of numerous other substrates that are phosphorylated by kinases.

Rapid analysis of sugar and carbohydrate content of foods is becoming increasingly important in the food, dairy, and biotechnology industries. The sensors developed will provide a means for rapid analyses of sugars in these industries, and provide the technical foundation for development of more complete panels of bioluminescent sensors in the future.

Keywords to Identify Technology/Research Thrust/Commercial Application (8-word maximum)

ATP, Luciferase, Bioluminescence, Biosensor

*The Project Summary must be suitable for publication by USDA in the event of an award. Do not include proprietary information on this page.

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air/water interfaces, and denaturability or stability in solution (Wang), were examined. Recently a similar set of studies was initiated to characterize bacterial luciferase, the enzyme responsible for bioluminescence in various species of marine bacteria. These studies, together with our commercial work on bioluminescent phytoplankton (the dinoflagellate *Pyrocystis lunula*) have provided a deep and diverse background in practical bioluminescence.

Luciferases are extensively used as labels for a wide range of clinical diagnostic chemical tests (Kricka, Baldwin, Griffiths). Since the firefly luciferase reaction is dependent on an adenosine triphosphate (ATP) co-factor, it has been extensively used in the development of biosensors for the measurement of ATP. Likewise, bacterial luciferase utilizes NADPH and has therefore been widely applied for biosensor applications that include a need for assay of NADPH. 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 required reliability, accuracy, and shelf life (Ugarova, Roda).

Several years ago PSI researched a new science education product based on another unique biological phenomenon -- the ability of certain plants and animals to survive almost total desiccation for extended periods of time and to spring back to life when rehydrated, a phenomenon called anhydrobiosis (Crowe). In developing our new science educational product, Resurrection™, we learned that many of these plants and animals depend on a unique disaccharide, trehalose, for their ability to withstand severe desiccation stress without denaturation of their proteins and enzymes or disruption of their cell membranes (Arakawa, Carpenter, Crowe, Colaco, Gaber).

Trehalose apparently serves two major functions. In high concentration it tends to 1) prevent phase separation and crystallization, and 2) substitute for the water normally hydrogen bonded to proteins and cell membranes, thereby stabilizing their structures when the last molecules of water are removed by desiccation or drought. Trehalose is now being widely applied to the stabilization of enzymes and antibodies and is being studied for the stabilization of air dried cells, tissues, and organisms (Arakawa, Carpenter, Crowe, Colaco).

Mr. C.Y. Wang, a Ph.D. student in the Department of Bioengineering at the University of Utah, has been working on the trehalose stabilization of firefly luciferase for the past year under a contract between PSI and the Center for Biopolymers at Interfaces. Mr. Wang has succeeded in stabilizing firefly luciferase in agarose gels for periods of up to six months. More importantly, the agarose/luciferase gels he has formulated can be dehydrated and desiccated, and retain their clarity and transparency when rehydrated. Full enzyme activity returns with rehydration, even after extended periods of dehydration (Wang). If such rehydrated luciferase gels are exposed to solutions containing ATP and luciferin they luminesce.

We have initiated several projects to develop this gel preservation technology for educational and commercial purposes. In one of these, Mr. Dong Min is performing studies with bacterial luciferases with the goal of producing stable gels of these bioluminescent reactants.

PSI has collaborated closely with researchers at the Center for Biopolymers at Interfaces and the Dept. of Bioengineering at the University of Utah for the past five years. PSI's existing Technology Transfer agreement with the University of Utah allows for transfer of jointly developed technologies, and is relevant to all topics covered in this SBIR application (patent reference).

Joe Andrade, CSO of PSI, has accumulated a wealth of experience germane to the proposed work. He has worked with Dr. V. Hlady and Mr. C-Y Wang at the University in investigations and development of fluorimmunosensors (Andrade), thin film and waveguide optics, protein and enzyme immobilization (Andrade, Wang), protein and enzyme stabilization, protein and enzyme purification and characterization, and, more recently, bioluminescence of both intact organisms and purified luciferases (Andrade, Wang). Additionally, Joe has relevant experience with dry reagent analytical systems. Studies addressing capillarity and spreading primarily directed to develop dry reagent systems for bioluminescent sensors, performed by Dr. Scheer of PSI, provide additional experience and the basic knowledge base to support the proposed investigation.

The extensive multi- and inter-disciplinary background of PSI personnel and its collaborators has enabled PSI to successfully develop its spatial bioluminescent sensor, and is essential to ensure the successful completion of this feasibility study and the development of a commercially viable product.

PSI recently submitted a STTR Phase II proposal to the NSF entitled, "Direct Reading Quantitative Biosensors for ATP-Dependent Processes" which focuses on enhancing the sensitivity of our bioluminescent spatial sensor by the three or four orders of magnitude. We established feasibility of the spatial detection sensors via a National Science Foundation STTR Phase I grant which ended on August 15, 1995. Our current device is suitable for quantitatively determining ATP concentrations as low as 10^{-7} molar (10^{-9} g). Increasing device sensitivity by three orders of magnitude will enable it to be applied as a hygiene monitor to determine the bacterial load on surfaces in sensitive food, beverage, and pharmaceutical processing areas (Fung).

The carbohydrate analyses proposed in this application do not require enhanced sensitivity of PSI's luciferase-based sensor. The sensor is more than sensitive enough to be used for carbohydrate analysis, where 0 - 25 g/l is the range of commercial interest. The goal of this scientific development project is to confer specificity for monosaccharides to PSI's spatial detection system by employing appropriate kinases as up front enzymes. Feasibility of employing the sensing system to accurately reflect monosaccharide concentrations will verify that the sensing scheme is sound, and open up opportunities to develop assays for other substrates (vitamins, complex sugars, etc.) that are phosporylated by kinases at the expense of ATP.

3. Relation to Future R & D -- Phase II

In Phase I we propose to demonstrate the feasibility of developing a series of sensors for the assay of monosaccharides based on specific kinase-catalyzed phosphorylation of the monosaccharide. Dephosporylation of ATP occurs in the reaction, resulting in a decreased ATP concentration which is detected through the ATP-specific firefly luminescence reaction using our unique spatial modulation technology. **(For a complete description of the spatial detection system, please refer to page 11 of this report.)**

In Phase II we will optimize the technology for monosaccharide analyses, including analysis of fructose (employing fructokinase). In addition, we will extend the sensing system to include assay of disaccharides. Based on our experience in Phase I, we will determine which of the other kinase-dependent analytes is most suitable for assay by our sensors. Currently, we anticipate developing a series of sensors for Vitamins B-1, (Thiamine); B-2, (riboflavin); and B-6, (pyroxidine), based on their phosphorylation by Thiamine kinase, riboflavin kinase, and pyroxidine kinase, respectively.

At the conclusion of Phase II, we expect to have demonstrated feasibility for an entire family of carbohydrate sensors. Each individual channel of the sensor will contain a series of gel layers containing appropriate concentrations of enzymes and other reactants. We will incorporate the appropriate combination of these individual channels to form multi-channel devices which will provide a complete panel for carbohydrate analysis. Similar development of a vitamin panel is anticipated.

The selection of optimal enzyme concentrations begun in phase I will be greatly aided by theoretical models and simulation which will be developed in Phase II. Models will include consideration of capillarity-based analyte delivery, and the diffusion of analyte through the gel and its interaction with enzymes. The various turnover numbers will be simulated. Existing enzyme kinetic models, diffusion models and capillarity models will be used.

With the growing interest in the food, dairy, and beverage industry for more comprehensive analysis and characterization of the carbohydrate and sugar content of their products, we fully anticipate that quantitative, rapid, easy to use, inexpensive and disposable carbohydrate and vitamin panels will have a substantial market potential.

4. *Phase I Technical Objectives*

The overall research objective is to assess the feasibility of developing a direct reading sensor for the quantification of glucose and galactose employing PSI's patented spatial luminescent ATP detectors. The sensors have been shown capable of yielding a visible light output whose position is indicative of ATP concentration. When simple sugars such as glucose and galactose are phosphorylated by their respective kinases, they consume ATP. It is anticipated that these changes in ATP concentration can be sensed by our luminescent detector, and that the decrease in ATP concentrations will be proportional to the concentrations of the sugars present.

Feasibility will be demonstrated if we can show that PSI's sensor is capable of reproducibly displaying a spatial distribution of light that can be correlated with the concentration of the simple sugars in sample solutions.

Objective 1. Prepare and Characterize Kinases.

Glucokinase (enzyme 2.7.1.2) and galactokinase (enzyme 2.7.1.6) will be obtained from Sigma Chemical Company, characterized, purified, and their respective stabilities and activities determined. This work will be performed in the laboratory of our collaborator at the University of Utah, Dr. Russell Stewart. Dr. Stewart will also assess the feasibility of producing these enzymes by recombinant means, as well as continue to provide PSI with recombinant luciferase, an essential component of our sensors.

Objective 2. In Solution, Confirm Ability Of Bioluminescent Reaction To Detect And Quantify ATP Consumption During Phosphorylation Of Simple Sugars By Kinases.

Before proceeding with studies incorporating the enzymes in gels, we will assess the ability of our sensor to detect changes in concentrations of sugars in solution. These preliminary experiments will be performed to obtain estimates of the concentrations of analytes and reactants that are required, and to identify any unanticipated interferences or incompatibilities of the system which can be addressed before proceeding to development of the gels described below.

Objective 3. Enhance Stability Of Enzymes and ATP by Incorporating Them Into Agarose Gels Using Trehalose as a Stabilizer.

Our present ATP sensing technology incorporates firefly luciferase, an ATP "consumase" (apyrase), and trehalose as a preservative and stabilizer, into agarose gels. The gels are dried and stored. Results of previous investigations have demonstrated that luciferase and apyrase maintain their activity when incorporated into the agarose gels. When rehydrated with an aqueous solution of an analyte sample containing ATP and luciferin, the preserved luciferase and apyrase are activated, yielding a light signal proportional to the concentration of ATP present in the sample.

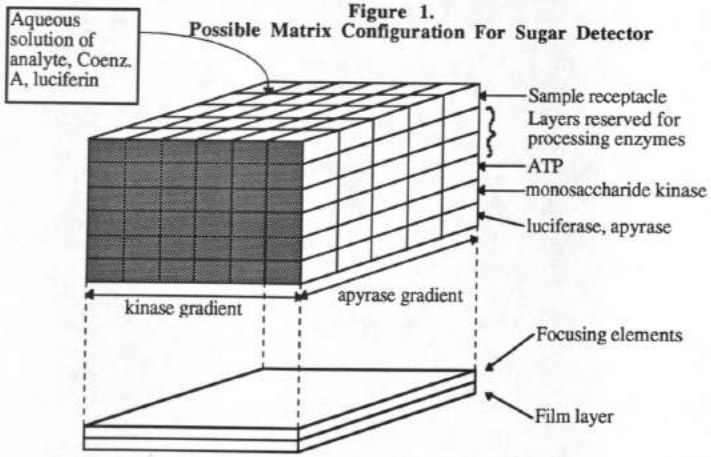
We will attempt to preserve the kinases by incorporating them into gels containing trehalose or sucrose as a preservative. Agarose, trehalose or sucrose, and kinase concentrations in the gel will be optimized along with gel preparation conditions to obtain a dehydrated gel which can be rehydrated with maximal kinase activity.

In previous studies, ATP has been the analyte. In the proposed work, ATP concentration will be modulated by controlling its reaction rate with monosaccharides. Appropriate kinases (galactokinase, glucokinase) will be used to modify the reaction rates. Therefore, ATP has to be provided in one of the device layers. We will incorporate ATP into the agarose/trehalose and agarose/sucrose gels, and perform appropriate stability studies on the ATP gels.

Objective 4. Configure Reactants so That the System Can Be Evaluated (Preliminary Prototype Development).

One configuration of a final device may consist of a series of gels, each containing an appropriately stabilized reactant and perhaps buffer to optimize its activity, layered atop, or astride one another. (See Fig. 1.) An aqueous solution of the sample will be introduced to the first gel layer and acted upon by the reactants in that layer.

Figure 1.
Possible Matrix Configuration For Sugar Detector



Each horizontal layer is composed of a gel containing the indicated constituents. Each individual column could be a separate cuvette, or capillary tube. The aqueous sugar sample is introduced at the top of the device, along with luciferin. For monosaccharides, the sugar and water are drawn through the ATP layer, which is reacted with the enzymes in each successive gel layer. For complex sugars, enzymes could be included in the second and third gel layers to cleave the disaccharides into their constituent monosaccharides. The apyrase gradient modulates the ATP concentration to effect spatial separation of emitted light based on its intensity. The kinase gradient is incorporated to allow the device to sense a very broad range of concentrations of monosaccharides. See text for details.

The reacted solution will then migrate, by capillarity, wicking, or gravity flow, to the next layer for processing, and so on until the last layer is reached. The last layer is the light-producing luciferase layer, which receives the ATP concentration, modulated by the front-end enzymes, and converts it to a light signal.

The light signal is focused onto a sensing element, either high speed film, or, optimally, the naked human eye, for detection.

We shall investigate different methods for producing a laminate structure of the gels. It is anticipated that this can most easily be accomplished by pipetting the liquid gels one atop the other. However, the freshly applied wet gel may then rehydrate the dehydrated gel beneath it. Processing details will have to be worked out. Other configurations, discussed in detail in the Work Plan, below, will be evaluated.

Objective 5. Establish Feasibility.

One of the configurations developed to meet objective 4 will undergo initial evaluation. Known concentrations of monosaccharide solutions will be introduced to the devices, and the light output determined. Studies will assess the sensitivity , reproducibility, and specificity of the system. It is anticipated that the developed configuration will perform sub-optimally; however, it is fully expected that the sensors will yield a light output whose intensity, and therefore position, is indicative of monosaccharide concentration.

The evaluations will demonstrate feasibility and identify the elements of the technology that need to be optimized in Phase II studies in order to arrive at a marketable product.

5. *Phase I Work Plan*

Glucokinase from *B. stearothermophilus* and galactokinase from galactose adapted yeast will be purchased from Sigma. In addition, an attempt will be made to produce these kinases by recombinant means. Under a subcontract from PSI, a method for producing recombinant luciferase was successfully devised by Dr. Russell Stewart at the Center for Biopolymer labs at the University of Utah. This has reduced our cost for luciferase by a factor of 100, and enabled us to accelerate our investigations with this enzyme, as well as made many of our technologies much more commercially attractive from a financial perspective. PSI is again providing a subcontract to Dr. Stewart in hopes that he will be successful in providing us with recombinant galactokinase and glucokinase.

The proteins will be characterized by polyacrylamide gel electrophoresis. Additionally, their activity will be indirectly assessed by employing them in our detection system and measuring the change in ATP concentrations that result from ATP's reaction with the respective monosaccharides.

The initial protein purification and characterization work will be accomplished through the University of Utah sub-contract to Russell Stewart by Mr. C. Y. Wang, who has previously prepared and characterized proteins for PSI under subcontract agreements. (Wang, 1988-1995)

Objective 2. In Solution, Confirm Ability Of Bioluminescent Reaction To Detect And Quantify ATP Consumption During Phosphorylation Of Simple Sugars By Kinases.

We have devised a series of simple preliminary liquid-phase experiments that enable us to rapidly assess the feasibility of our sensing schemes. They consist of preparing a series of dilutions of the reactants and evaluating their performance in all combinations to arrive at optimal concentrations. We react the proteins with the firefly luciferse system in microtitration test wells, and determine the relative light output from the wells using a CCD camera. The light profiles give us a very graphic record of which combinations are successful (See Fig. 3), and also yield information about the sensitivity of the assay.

In this case the test matrix will be rather large, including variations in ATP, kinase, luciferase, luciferin, and apyrase concentrations. We will also determine if any incompatibilities exist, which will necessitate isolating the different incompatible reactants in different gel layers, or finding suitable chemical replacements. Dr. Phil Triolo will supervise the work which will be performed in the laboratories of Dr. Vlado Hlady at the Center for Biopolymers at Interfaces.

We will supplement these empirical studies with attempts to model the system in Phase II. Experience has taught us that the empirical approach usually allows us to more rapidly reach conclusions that enable us to proceed with our experiments than modeling, which cannot usually predict the interactions of the various components of the system with the degree of certainty required.

Objective 3. Enhance Stability Of Enzymes and ATP by Incorporating Them Into Agarose Gels Containing Trehalose.

Trehalose has been successfully employed as a stabilizer for both luciferase and apyrase in agarose gels, permitting them to be dehydrated and stored, and then rehydrated at a later date to full enzyme activity. Optimum conditions for the preparation of trehalose/agarose gels containing glucokinase or galactokinase and ATP in the desired concentrations will be explored.

In addition, the ability of sucrose to stabilize the proteins will be assessed. Sucrose is more readily available and less expensive than trehalose, and it has been shown to be a fairly effective protein preservative. Sucrose, however, may interfere with attempts to apply the sensors to the assay of disaccharides. It still warrants investigation, however, because it may prove useful in other assay systems under consideration.

Under a contract between Protein Solutions, Inc. and the University of Utah, Mr. C.Y. Wang has been working on the trehalose stabilization of firefly luciferase for the past two years. Rights to the stabilization procedures he developed at the University in a project funded by PSI have already been transferred to PSI for firefly luciferase applications. Mr. Wang will, again, perform the gel stabilization experiments with the assistance of Dr. Rob Scheer.

Although we have extensive experience with the stabilization of firefly luciferase and apyrase in trehalose/agarose gels, we have little experience with the kinases or sucrose. Trehalose has, however, been used by others for the stabilization of a variety of enzymes, including kinases (6). We expect that trehalose will be an effective additive for stabilizing the proteins in polysaccharide gels, i.e., agaroses, celluloses, dextrans, etc. Likewise, we anticipate that sucrose can also be used as a stabilizer, and offer cost and availability advantages over trehalose.

In addition to modifying stabilizer concentration, we will modify gel density (by varying agarose concentration) and gel composition in order to effectively mobilize the reactants in the device. Possible synthetic gels include polyvinyl alcohol (Gautier) and polyacrylamide (Hobel).

The gels will be prepared with different enzyme/trehalose or enzyme/sucrose ratios. The enzyme activity will be directly measured by delivering reference amounts of ATP, luciferin and sugar to the kinase gel and detecting the resulting ATP diffusion into a luciferase gel by measuring light output, essentially the basis of our biosensor concept. The performance of the two preservatives will be compared.

ATP must also be included in the test system, and we will attempt to incorporate it into its own gel layer to prevent its interaction with any of the kinases. Its stability will be evaluated by assessing light output of sensors that incorporte different ATP gels as a function of storage time.

We will also experiment with dual and even triple enzyme gels, that is, trehalose/agarose or sucrose/agarose gels containing both luciferase and kinase, or luciferase, kinase, and apyrase.

Gels will be dried at different rates, subjected to storage under different temperature conditions, rehydrated at different rates, and evaluated for enzyme activity.

Objective 4. Configure Reactants so That the System Can Be Evaluated.

Gel Spreading, Preparation, and Stability.

The gel solution (sol) has to be applied to, and spread on suitable supports in a uniform and homogeneous manner. We have determined that one acceptable support material is polyester film. This will be the first substrate examined in the proposed work.

The challenge will not be in forming the first layer of gel, but layering a second gel on top of the first layer. The fresh, wet gel will rehydrate the already applied, underlying layer. It may be necessary to add a small gel layer consisting of nothing but agar in between active gel layers to absorb any water that is added during gel formation. Another possibility is that each individual layer could be fabricated on a porous, hydrophilic membrane composed of e.g., cellulose, or some other suitable hydrophilic substrate. These layers could then be physically stacked to form the final gel structure, using fresh agar as a mortar to lend integrity to the entire structure and eliminate air entrapment between the successive gel layers.

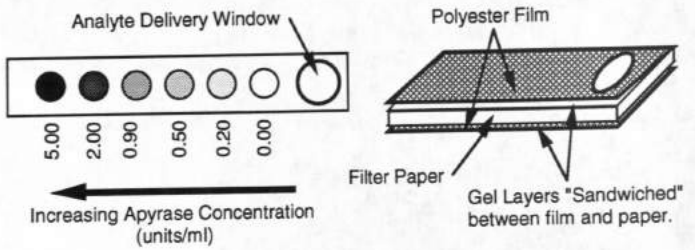
Capillarity: Sample, Delivery and Transport.

The sample containing the analyte will be deposited on a suitable device and spread across the appropriate portions of the uppermost gel by a capillarity and rehydration process. This technology is already applied in existing dry reagent chemistry diagnostic kits.

We intend to fabricate a working model that incorporates, and builds on, the prototype we developed to demonstrate feasibility of our spatial sensor. (See Figure 2.) **A brief description of the spatial sensor and the prototype we have designed to prove its feasibility follows**:

Luciferase/apyrase enzymes are deposited as circular spots along one dimension of the device, each dot of increasing apyrase concentration. The physical support for the device is a strip of filter paper sandwiched between two polyester films with an agarose coating. One end of the upper film has an opening to allow for delivery of the analyte.

Figure 2
Diagram indicating incorporation of gel spots of increasing apyrase concentration in a prototype dipstick design for the spatial bioluminescent sensor.



The analyte (containing the ATP sample to be measured, luciferin, and coenzyme A) is delivered to the sample window of the direct reading sensor. A detectable, visual readout can be made three minutes after application of the analyte solution. The design exploits the wicking properties of the filter paper and the capillary action of the gel film sandwich. The test strip geometry allows sample delivery to each gel "dot" within one second of analyte delivery. The time limiting factor for the reaction is the time required to rehydrate the enzyme gel.

The sensor concept is based on the fact that, for a given luciferase/luciferin concentration, a minimum concentration of ATP is required to produce a "measurable" light output. Above that concentration, light is visible; below that ATP concentration, no light is detected. If a uniform concentration of ATP is delivered to a series of sensors (dots with controlled concentrations of apyrase), a detectable light signal will only be produced when apyrase levels are relatively low in relation to ATP concentrations.

By filtering or consuming the ATP before it reacts with the luciferase we control the intensity of the light at each position along the sensor. A high concentration of ATP will produce light even at the higher apyrase concentrations because enough ATP remains after consumption to produce a measurable light output. A low concentration of ATP will produce light only at the lowest apyrase concentrations because at higher apyrase concentrations all of the ATP is consumed before it reacts with the luciferase.

To measure an ATP concentration, a sample containing ATP is distributed to the luciferase/apyrase gel. ATP that permeates the gel is dephosphorylated by the two competing enzymes. Because the turnover rate of apyrase is two orders of magnitude higher than that of

luciferase, the apyrase quickly moderates the ATP concentration. The amount of ATP which reacts with the luciferase to produce light depends on both the initial ATP concentration and the concentration of apyrase.

To demonstrate the dependence of light output on both of these concentrations, we produced 2-D (two dimensional) matrixes with gradients in ATP concentration ($5x10^{-4}$ to $5x10^{-7}$ mol/l ATP) and apyrase concentration (2 units/ml to 0 units/ml). The gradients were produced as serial dilutions of ATP and apyrase, and pipetted in the appropriate patterns into test wells. At the bottom of the test wells we had fabricated gels containing luciferase. A positive surface profile whose height is directly proportional to light intensity was made immediately after ATP was applied to the test wells. The image was made with a CCD camera and appears as Figure 3.

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 signal. The goal of modulating the light signal with apyrase is to generate a specific light cutoff point indicative of a specific concentration of ATP in the analyte solution.

In this proposal, the intent is not to determine ATP concentration, but rather use its concentration as an indicator of the concentration of monosaccharide. This is accomplished by reacting the ATP with galactose or glucose in the presence of varying concentrations of galactokinase and glucokinase, respectively. The phosphorylation of the sugar and the dephosphorylation of the ATP occur before the ATP reaches the apyrase/luciferase gel layer where light emission is initiated. Thus, the kinases serve as a mediator of ATP concentration by modulating its reaction with the monosaccharides.

Two gel layers will have to built upon the already existing layers of the spatial sensor in order to enable it to sense monosaccharide concentration. (See Figure 1.) The top layer will consist of the ATP gel, and the second layer the kinase gel. These gels will be optimized to meet objective 3. The sample, consisting of an unknown concentration of glucose or galactose in water, along with an appropriate concentration of luciferin and coenzyme A, a cofactor that increases the efficiency of light emission, will be introduced to the test system. The sample solution will wick down through the device and begin to rehydrate the uppermost gel containing the ATP. The ATP will be solubilized by the water, and diffuse, together with the luciferin, sugar and CoA, to the kinase gel layer. There the concentration of ATP will be depleted in direct proportion to the concentration of sugar present. The ATP solution diffuses further and enters the luciferase layer, reacting there with the aqueous solution of apyrase, luciferase, and luciferin.

The depleted ATP will give off a light signal inversely proportional to the concentration of monosaccharide in the test sample. The light is focused or waveguided onto film for detection, or, if intensity is great enough, detected by the unaided eye. Comparison with an empirically derived table correlating monosaccharide concentration with obtained light pattern will allow for a rapid estimation of the concentration of monosaccharide in the sample. The gradient in kinase concentration will allow for assay of a wide range of monosaccharide concentrations.

The stability of luciferin is such that it may not be feasible to incorporate it into the device. Preliminary attempts to stabilize it in agarose gels have been unsuccessful. We will, therefore, investigate the use of metallized polyester film packaging materials to preserve it in a dry powdered form and incorporated into gels. The gels have the potential of being incorporated, at a later date, into the laminate structure of the device.

Dr.s Phil Triolo and Rob Scheer will work together to develop and fabricate prototype devices for evaluation.

Objective 5. Evaluate Feasibility.

The prototype devices will be tested by introducing a range of monosaccharide concentrations of commercial interest (0 to 25 g/l) to the multilayer test strips and assessing the ability of the strips to reproducibly and predictably generate a pattern of light which can be correlated with sugar concentration. Light emission will be determined using a CCD camera.

These evaluations will be performed at the University of Utah's optical imaging laboratory at the Center for Biopolymers at Interfaces with the assistance of Dr. V. Hlady, a consultant to PSI on this project.

A number of disinterested volunteers will also participate in a series of experiments to determine the detection limits of the light directly emitted from test strips, using their naked eyes in various lighting conditions. CCD camera patterns and visually determined patterns will be compared to see if any particular training or experience is required for disinterested, unaided observers to accurately interpret the results.

In addition, short term storage, dehydration, stability, and experiments to assess the reproducibility of the assay will be performed.

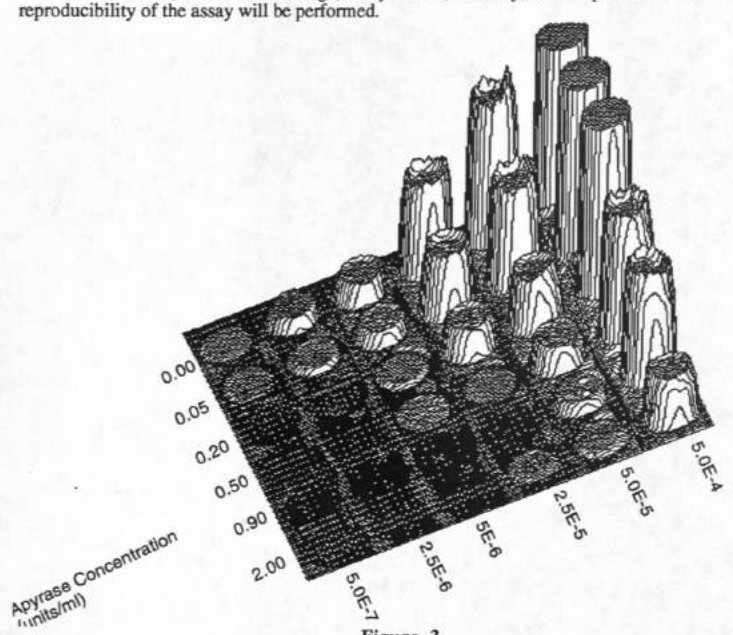


Figure 3
Typical CCD 3-D profile of light intensity for a six by six detector array.

6. Related R & D.

In August of 1995 we completed an NSF Phase I STTR contract titled "Direct Reading Quantitative Biosensors for ATP-dependent Processes." Under this contract we clearly established the feasibility of combining the ATP-dependent firefly luciferase bioluminescent reaction with a method of modulating ATP concentration to make a system capable of emitting photons in proportion to ATP concentrations. ATP concentration is modulated with an apyrase (consumase) gradient. The spatial pattern is directly related to ATP concentration (See Figure 3.)

We have also submitted a Phase II STTR to extend the sensitivity of this approach by several orders of magnitude to permit the direct monitoring of bacteria on surfaces via analysis of their ATP content. This recently submitted proposal does not consider the use of kinases to generate analyte specificities with PSI's sensors. Evaluation of the feasibility of employing kinases as the "up front" enzymes to render the ATP sensors specific for monosaccharide analysis is the basis of this application.

Nevertheless, the experience gained in the Phase I STTR has established a strong foundation for this work. The anticipated award of a Phase II NSF STTR will permit dramatic progress to be made in our fundamental understanding of the ATP-dependent firefly luciferase bioluminescent reaction. Everything we learn about improving the sensitivity of this technology so that it can be used for microbial ATP detection simply enhances the practicality and feasibility of its application for monosaccharide- and eventually disaccharide-specific detection.

D. Key Personnel & Bibliography

1. Key Personnel/Vitas

Phil Triolo, Ph.D., Principal Investigator and President of PSI, is a bioengineer with considerable product development experience in the medical device industry. Before joining PSI in 1994, he spent seven years as a consultant, working on various medical product and drug delivery device development projects. All of the projects involved the selection and evaluation of appropriate materials for blood contact or drug delivery purposes, and the design, execution and documentation of experiments in order to demonstrate the safety and efficacy of devices to meet FDA requirements. Dr. Triolo's industrial background will enable this concept to be effectively developed into commercial products. His bio-sketch is included.

Dr. Robert Scheer received his Ph.D. in Materials Science and Engineering in 1993 and is Principal Investigator of PSI's current 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 Mr. C.Y. Wang, a student who will work as a post doc on this project (see Table 1). Rob's background is in polymers, polymer structure and morphology, and the modeling and testing of polymeric materials. His bio-sketch is included.

Dr. Joseph Andrade is founder and Chief Scientific Officer of PSI. 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. Five years ago he became quite interested in bioluminescence and particularly in the firefly and bacterial luminescence systems. His recent research efforts have been directed at understanding these systems. He will provide much of the interfacial biochemistry, bioluminescence, and biosensor expertise required. Joe is three quarter time Professor of Materials Science and Bioengineering at the University of Utah. His abbreviated vita is also attached.

Mr. C.Y. Wang is a graduate student working under Joe Andrade's supervision at the University of Utah. Mr. Wang has worked on the firefly luciferase system for nearly four years and will be completing his Ph.D. studies on this system in 1995. He will be available as a post doctoral assistant during the Phase I award period to conduct the firefly luciferase-based studies and to transfer his rich, comprehensive expertise on that system to the others involved in the project.

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EXPERIENCE:

1994-current Protein Solutions, Inc., **President.** Direct research in the application of bioluminescence for sensing applications in the health care industry.

1983-1994 (interrupted) Independent Contractor to several local medical device companies. Projects have included the design, evaluation, and development of cardiovascular and heparin-releasing catheters, angioplasty devices, nerve and tracheal prostheses, an implantable catheter for the delivery of insulin, evacuated polymeric test tubes for blood collection, and a heparin sorbent system. Also wrote major portions of successful SBIR grant applications and business plans.

1992-1993 Research Medical, Inc., SLC. **Sr. Product Development Engineer**. Responsible for evaluation and modification of sorbent system for the removal of heparin at the conclusion of bypass surgery.

1990-1991 Merit Medical Systems, SLC. **Director of Engineering.** Supervised four staff responsible for implementing new product introductions and product improvements of high pressure syringes and tubing for angioplasty product line.

1980-1983 Abbot Critical Care Systems, SLC. **Manufacturing and Product Design Engineer** (1980-'81). Responsible for cost reductions and product improvements on $4 MM annual hemodialysis product line.

EDUCATION:

1988 Ph.D., Bioengineering, University of Utah. Dissertation, "The Controlled Release of Macromolecules from Biodegradable Poly(lactide) Matrices," completed under the direction of Prof. S.W. Kim.

1980 M.S., Bioengineering, University of Utah. Completed thesis, "Surface Modification and Evaluation of Catheter Materials," under the direction of Prof. J.D. Andrade.

1976 B.S., Biomedical Engineering, Rensselaer Polytechnic Institute, Troy, NY. Minors in Psychology and Philosophy.

APPOINTMENTS & HONORS:

University of Utah. University of Utah Research Fellow (1978-'79). Chairperson, Bioengineering Student Advisory Committee and student chapter of Biomedical Engineering Society (1978-'79).

Rensselaer Polytechnic Institute. Graduate *cum laude*. Dean's List, all semester. RPI Alumni Scholarship (1972-'76). President, Rushing Chairman, Theta Chi Fraternity. Member, Tau Beta Pi.

AFFILIATIONS:

Adjunct Instructor, Dept. of Bioengineering, University of Utah. Member, Biomedical Engineering Society, Intermountain Biomedical Association, Center for Biopolymers at Interfaces

PATENTS:

J.D. Andrade, P.M. Triolo, L.M. Smith, RFGD Plasma Treatment of Polymeric Surfaces to Reduce Friction, U.S. Patent 4,508,606, issued to the University of Utah.

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P.M. Triolo, A. Nelson, D. Staplin, Coupler for High Pressure Medical Tubing, U.S. Patent Application 10928.28.1, to be issued to Merit Medical Systems, Inc.

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E. Facilities and Equipment

Protein Solutions, Inc., occupies 800 sq. feet of office and lab space in Research Park adjacent to the University of Utah Campus in Salt Lake City. Basic laboratory equipment is housed there, including scales, stirrers, oven, cell culture facilities, and benches with fume hoods, along with secretarial space and computers.

Most of the proposed work will be performed at the University of Utah in the Biomedical Polymers Research Building. Housed there are the laboratories of Dr. Russell Stewart which are fully equipped for protein characterization, recombinant gene expression and protein purification, and the optics lab of Dr. Vladimir Hlady. A CCD camera is located in his lab, along with appropriate programs and computers for analyses of the data produced by the camera.

F. Consultants

Dr. Russell Stewart, Assistant Professor of Bioengineering and PI of the University subcontract, serves on PSI's Scientific Advisory Board. He will assist with the preparation of recombinant luciferase and attempt to produce galactokinase and glucokinase by recombinant means. His vita is attached.

Dr. Vladimir Hlady, Associate Professor of Bioengineering, and director of the Interface Spectroscopy and Scanning Probe Microscopy laboratories in the Center for Biopolymers at Interfaces, serves as a member of PSI's Scientific Advisory Board. He will provide technical assistance to the project free of charge.

G. Commercialization/Federal Use.

There is rapidly growing interest in the food, dairy, and beverage industries in the more detailed chemical analysis of their products (Griffiths, Pons, Baltis) with particular interest in developing rapid methods for carbohydrate analysis (Park.) Rapid sugar detection is of major importance for effective monitoring of fermentation-based processes. Although there has been some interest in enzyme-based electro-chemical reactions for the development of sensors and sensing systems for carbohydrate analysis, high performance liquid chromatography is still the most commonly used analytical method.

We have made preliminary inquiries to a number of firms that sell sensors for carbohydrate analysis and also those that sell equipment and reagents for ATP analysis, generally for bacterial hygiene monitoring. All are very interested in rapid, specific methods for carbohydrate quantitative analysis. There is a strong commercial potential for such sensors, not only in the food, dairy, and beverage processing industries, but also in the general consumer market where consumers are taking a greater interest in the chemical composition of the foods they eat.

PSI has major interests in the science education market. The availability of inexpensive carbohydrate sensors would enable their incorporation into kits and laboratory exercises pertaining to carbohydrate and other food chemistries.

H. Current and Pending Support.

Dr. Phil Triolo, PI, has no current federal grant or contract support. He is PI on an SBIR Phase I application to the U.S. Environmental Protection Agency entitled: "Continuous Real Time Enumeration of Airborne Microorganisms;" submitted January 19, 1995. He is budgeted for two months on that application. There is no direct overlap with this SBIR NSF grant.

Dr. Robert Scheer is PI of an ongoing NSF STTR grant, Direct Reading Quantitative Biosensors for ATP Dependent Processes. He will also be budgeted on the Phase II application, which will be submitted in September, at a 40 % effort. He will have adequate time for the one and a half man-months budgeted in this proposal. There is no direct overlap between the two grants.

Dr. Russell Stewart's current support is an American Cancer Society Institutional Research Grant for $14,500 for the period 3/95 to 3/96. It is entitled "Modulation of Microtubule Assembly and Disassembly by Motor Proteins." He has a pending grant to the NSF Bioinstrumentation Program on a scanning laser force microscope for $93,000 for the period 9/95 to 9/98.

I. Budget Justification.

Dr.s Triolo and Scheer are PSI's key technical scientific staff members. They are each budgeted at one quarter time on this project. They, along with a half time post-doctoral fellow who will likely be Mr. C.Y. Wang, who has had considerable experience in firefly luciferase application to these biosensors, will be responsible for the completion of the technical aspects of the project.

Most of the budget is self explanatory. We will initiate a sub-contract with Dr. Russell Stewart's laboratory at the University of Utah for the provision of recombinant luciferases and for assessment of the feasibility of producing the key kinases by recombinant means to satisfy the needs of this project. The total sub-contract will be for $10,000. Fortunately, the University of Utah does not charge any indirect costs on Phase I SBIR contracts to Utah companies. Therefore, all of the $10,000 in the sub-contract will be utilized by Dr. Stewart and his staff for the production, purification, and characterization of the proteins needed in this project.

Protein Solutions, Inc. is not requesting any indirect costs or fees. In addition, Dr. J. D. Andrade, Chief Scientific Officer of PSI and one of its founders, will not receive any compensation for the significant portion of his time he will devote to this project. Andrade is a three quarter time professor of Materials Science and Bioengineering at the University of Utah. The remaining quarter of his time is devoted to the development of Protein Solutions, Inc. The approximately one man-month he will spend on this project will not be funded, and the salary he will not receive can be considered matching funds provided by PSI.

J. Multiple Phase II Awards.

PSI has not yet received a Phase II SBIR/STTR award. We have a Phase II proposal now being considered by the National Science Foundation, "Direct Reading, Quantitative Biosensors for ATP-Dependent Processes." There is no direct overlap between that proposal and this SBIR Phase I application.