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D. Identification and Significance of the Problem or Opportunity

Photons are basic to biology. Biology has learned to efficiently collect photons and transduc utilize such energy via photosynthesis. Biology also utilizes photons for sensing and imaging purpo vision. What is not as well known is the fact that biology knows how to produce photons via bioluminescence (1,8,9,16,28-9,42,60-1,65,69). Bioluminescence is an enzyme (photoprotein) base chemiluminescence process utilizing specific substrates (luciferins), oxygen, and often one or more c factors. Although the photo-proteins involved have been used as labels in analytical chemistry, and a components of biosensors, there has been little or no interest in bioluminescence from the physics, materials science, or engineering communities.

Although bio- and chemiluminescence are well known processes and are applied in a numbe areas, they are generally considered to be impractical as photon sources, in part because the light prod tends to be non-oriented, non-polarized, of relatively low intensity, and to otherwise not have the mor precise and well controlled characteristics of sources which utilize modern electro-optical materials (6

The growing awareness and application of materials and devices with nano dimensions, and t importance of quantum phenomena in such devices (18,26,46.52-3,68,73,82-5), has led to the consideration of such quantum effects in bioluminescent systems. Although we are unaware of any d evidence for quantum micro-cavity luminescence effects in biology, it may be because bioluminescen processes have rarely, if ever, been approached from the perspective of quantum optics. Proteins and enzymes are colloidal particles of nano dimensions and may be capable of functioning as quantum do quantum whiskers, or quantum films. Proteins, and enzymes in particular, often have unique dielectri environments and geometries which can, at least in principle, influence the photon emission process, including both direction and polarization.

Firefly and bacteria luciferases are well characterized, partially understood, enzymes already v applied in medicine, biochemistry, and biophysics as unique labels and markers (27,33,36,38,45,66-7,75,81). Several groups, including our own (5,32,71-2), are already using luciferases as component molecular devices and biosensors.

APPENDIX C

National Science Foundation
Small Business Innovation Research Program

PROJECT SUMMARY

NSF PROPOSAL NO.

NAME OF FIRM	Protein Solutions, Inc.
ADDRESS	6009 Highland Drive Salt Lake City. Utah 84121
PRINICPAL INVESTIGATOR (NAME AND TITLE)	Phil Triolo, President
TITLE OF PROJECT	Enhancing Optical Biosensors by Interface Orientation of Engineered Luciferase.
TOPIC TITLE: Chemical and Transport Systems	TOPIC NUMBER AND SUBTOPIC LETTER: 22e

PROJECT SUMMARY

This Small Business Innovative Research Phase I project involves bioluminescence -- an enzyme-dependent chemical oxidation process resulting in an excited state product which emits a photon. The firefly luminescence system requires adenosine triphosphate (ATP) as a co-factor in the reaction. This reaction is widely used as a means to detect low concentrations of ATP, using sensitive luminometers and photon counting instruments.

We have developed technologies by which we enhance, dehydrate, and preserve firefly luciferase in gel and fiber matrices, incorporated in dipstick-type analytical devices for analysis of ATP. These devices utilize the spatial position of the luminescence, rather than its intensity, permitting sensitive, unassisted human eye detection and readout.

We have recently engineered firefly luciferase using recombinant *E coli* techniques, permitting the enzyme to be immobilized in a controlled orientation at interfaces. This capability permits us to test the hypothesis that the direction of bio-photon emission can be controlled. The possibility of producing luciferase monolayers and films of controlled orientation and two dimensional ordering allow a rigorous test of this hypothesis.

Recent work in micro-cavity optics suggests that at least partially directional photo-emission can be produced. Our goal is to test the applicability of such work to bioluminescence, possibly permitting the extension of direct reading, instrumentless, ATP-based sensing systems into the nanomolar and even picomolar regime, by enhancing photon production and thus detectability by two to three orders of magnitude. The preparation of luciferase films of controlled orientation and ordering would also permit optimization of substrate and product mass transport, enhancing the kinetic performance of immobilized, luciferase-based sensors and detectors.

Potential Commercial Applications of the Research

There are major commercial implications. Sensitive, direct reading, ATP-based, intrinsically luminescent, dipstick-type sensors will have major applications in clinical chemistry, personal diagnostics, environmental monitoring, microbiological biomonitoring, biotechnology process control, education, and other fields.

There are two very special molecules that play unique and central roles in biology, adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (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" (27). A very large number of biochemical enzyme processes involve one of these two molecules.

It is very convenient that biology has evolved bioluminescence 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 with a very high efficiency; and the bacterial luciferase reaction, which in the presence of alkyl aldehydes, such as decanal, together with NADPH, also produces an excited product which chemiluminesces. Both reactions require the presence of oxygen. The luciferases involved are different. The "luciferins" involved are different (1,28,29).

There is a large 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 involve fiberoptic or other waveguided means of delivering the luminescence to a device which can accurately measure light intensities (4,7,22,24,35,50,55,59,76-9). Although one of the most portable and most sensitive photon detectors available to the analytical chemist is his or her own eye, it 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 (14,15,31,50,51).

PSI's research and product development goals are to develop, manufacture, license, and market a line of instrumentless, direct reading, dipstick-type biosensors for a wide range of applications in the personal diagnostics field, as well as in the dairy, food, and environmental monitoring areas. We have recently developed a technology by which substrate or analyte concentration can be detected by virtue of the spatial position of bioluminescence, rather than light intensity. This innovation is the basis of an ongoing NSF STTR , "Direct Reading Quantitative Biosensors for ATP Dependent Processes." The Phase I STTR grant concludes on August 15, 1995 and a Phase II will be submitted several weeks later. That technology will permit the development of direct reading bioluminescence-based biosensors for applications in which relatively high ATP levels are involved. Although the technology will also be useful for much lower ATP levels, it will likely require sensitive photon detection instrumentation to detect the emission.

Although one can use waveguiding and other optical means to partially concentrate and focus the light, a fundamental limitation is that bioluminescent processes produce photons which lack spatial and temporal coherence. We propose to address the question of spatial coherence in this application.

We propose to develop ordered arrays of firefly luciferase and utilize these ordered arrays as optical microcavities as possible components of optical biosensors.

Th e microcavity-based enhancement of photon direction is, of course, a speculative hypothesis, but one with major significance in the fields of interfacial ordering and processing, biosensors, and molecular and bio-electronics (10,11,43,58). We feel strongly that, with the appropriate application of protein engineering, interfacial processing, and the principles and understanding from microcavity physics, we can thoroughly test the hypothesis and assess the feasibility and applicability of ordered protein arrays for biosensor development.

E. Background and Technical Approach

Our group has been involved in the study of firefly luciferase at air/water, lipid/water, and solid/water interfaces for several years (5,32,71-72). Mr. CY Wang is completing his Ph.D. degree in Bioengineering on the interfacial behavior of firefly luciferase. Most of this work has utilized protein extracted from the firefly and obtained from commercial sources. We are now using recombinant

luciferase expressed in *E coli*, and produced and purified in Rusell Stewart's lab. Firefly luciferase is a highly hydrophobic protein with over 60% of its amino acid residues classified as hydrophobic. It has considerable surface hydrophobicity and, therefore, substantial surface activity at air/water and other apolar/water interfaces.

The protein also has interesting charge characteristics, including a perioxisomal terminal segment of high charge density. It thus has both interesting hydrophobic, as well as electrostatic properties. In low ionic strength buffer, it aggregates at concentrations greater than 0.2 mg/ml. The aggregation is reversible without loss of specific activity. Early studies of the protein indicated a molecular weight of over 100,000, now attributed to dimerization of the molecule. The molecular weight of the purified protein is now normally accepted at about 60,000 daltons. There has been some interest in the dimer characteristics of firefly luciferase. A number of groups have argued for a dimer, even at relatively low solution concentration (16,17,19,20,41,47,71,80).

Purified firefly luciferase does crystallize, forming needle-like crystals, which have so far been either too small or too unstable for routine X-ray crystallography (20). The 3-dimensional structure of the firefly protein is not yet available. It is interesting to note that the crystal structure of the bacterial luciferase was just reported (21), although this is a very different enzyme with a very different mechanism of luminescence. Firefly luciferase readily adsorbs at a variety of interfaces with probably some degree of self assembly or 2-dimensional ordering, although this has not yet been probed in any substantive manner.

There has been some speculation that firefly luciferase is a lipid binding protein, and possibly even a membrane enzyme (16-20). As part of Mr. Wang's Ph.D. work we examined luciferase/lipid interactions by studying the interaction of luciferase with various lipid liposomes. Mr. Wang also examined the ability of firefly luciferase to interact with pre-formed lipid films in a Langmuir trough, using pressure area characterization. Phospholipids with neutral polar head groups either slightly enhance or show no influence on luciferase activity, whereas those with negative charge tended to inactivate the protein. Differential scanning calorimetry in the liposome system suggests that luciferase interacts with dipalmitoyl phosphatidyl choline (DDPC). Such interaction may actually inhibit the aggregation of luciferase. Positively charged lipids also tended to inactivate the protein, presumably through a denaturation mechanism, as deduced from intrinsic fluorescence and infrared studies.

Since the isolation of the luciferase gene from a number of species, luciferase has become an important tool in the fields of molecular and cellular biology. Recombinant luciferase is now widely used as a convenient reporter of gene expression in studies of gene regulation and development in both plants and animals (25,35,49,57,74). Much less effort has been directed at using molecule genetics to engineer new properties into luciferase proteins to increase their utility for in vitro and device applications. We propose to design novel luciferase proteins through genetic engineering that will be simple and inexpensive to purify in large quantities, and that can be immobilized in well-defined orientations and densities.

Our initial approach was to add six amino terminal histidine residues to the luciferase gene from the firefly Photinus pyralis (Promega Corp.). The histidine residues bind strongly (Kd > 10-9 M) to immobilized Ni++, allowing for single-step affinity purification of the tagged protein on a metal chelate column. Construction of a histidine-tagged luciferase has been completed and we have shown that the tagged luciferase is expressed at high levels in E. coli and retains bioluminescent activity. We are now in the process of fully characterizing the recombinant luciferase. Luciferases are extensively used as labels for a wide range of clinical diagnostic chemical tests.

Mr. Wang has succeeded in stabilizing firefly luciferase, together with its substrate, luciferin, in agarose gels containing trehalose (12,13,48) for extended periods. More importantly, such agarose/luciferase/luciferin gels can be completely dehydrated and desiccated, maintaining their clarity and transparency, and then rehydrated with full enzyme activity after extended periods. If such rehydrated gels are now exposed to ATP, they of course luminesce (71-2).

We propose to assess the feasibility of applying optical microcavity concepts and techniques to firefly luciferase bioluminescence for potential biosensor applications.

F. Phase I Research Objectives (see Table I)

1) Produce firefly luciferase by recombinant means, inexpensively and in large quantities. Characterize the properties of the enzyme and compare it with enzyme derived from native sources. Monitor and assess the stability of the enzyme under different storage conditions (University of Utah subcontract).

2) Produce firefly luciferase designed for optimum separation and purification containing specific domains and related features which facilitate the oriented immobilization and two dimensional ordering of the protein (University of Utah subcontract).

3) Compare oriented two dimensional monolayers of the various luciferases at the air/water interface using Langmuir trough techniques. Fully characterize the pressure/area and related interfacial properties of these films in the presence and absence of luciferin, ATP, and co-enzyme A. Monitor the enzyme activity and kinetics of the 2-D immobilized films.

4) Monitor the photoemission and polarization characteristics of the 2D luciferase films at the air/water interface using sensitive CCD camera imaging instrumentation and a special fiber optic goniometer detector.

5) To facilitate two dimensional ordering and crystallization, prepare biotin-lipid/streptavidin ordered air/water films using now well known techniques (23,30,39,40,44,54,64,86). Immobilize the biotin luciferase at such films and study the probable two dimensional crystallization/ordering of the composite film. Again, characterize the photoemission properties on a macroscopic and microscopic scale.

6) Assuming some directionality of photoemission is observed, attempt to control the direction of photoemission by pressure/area manipulation and by composite film design and construction.

7) Based on fundamental considerations, derived from the micro-cavity optics/directional emission field, immobilize luciferase with other polymers to confer local dielectric characteristics expected to enhance micro-cavity behavior -- mainly Phase II.

8) Prepare oriented, ordered luciferase films on solid supports by: a) Langmuir Blodgett monolayer transfer; b) immobilization on polymer and/or glass uncladded optical fiber surfaces; c) immobilization on polymer and/or glass optical plates which can be coupled to cylindrical prisms for more comprehensive, spatially dependent luminescence analysis -- mainly Phase II.

9) This final task, primarily reserved for Phase II, is to thoroughly model applicability of oriented luciferase multi-layers for enhanced biosensor performance. The modeling will include a more comprehensive treatment of kinetics, lifetime, and luminescence output. Demonstration of enhanced biosensor performance for several prototype sensors for low ATP applications will be conduced.

Summary

The major objective of this Phase I project is to assess the feasibility and practicality of directional control of luciferase bioluminescence based on monolayer technologies and microcavity optics principles.

Our goal is to develop miniature analytical biochemistry systems, specific for particular aqueous analytes which are quantitative, rapid, direct reading (by self luminescence), sensitive, with long shelf life, stable, disposable, and inexpensive (2,3,36-7, 62-3, 70). Because there are literally hundreds, perhaps thousands, of enzymes which are involved with ATP consumption or ATP production, most of which are specific to another chemical substrate, for example, glycerol, glucose, etc., these "front end" enzymes permit the development of specific sensors for each of those substrates.

Most bio- and chemiluminescence based analytical sensors and devices rely on intensity measurement, with the intensity being proportional to the concentration of the analyte or substrate of interest (4,7,22,24,35,50,55,59,76-9). At relatively low analyte concentrations, bioluminescence intensity is proportionally low, requiring highly sensitive luminometers or photon counting systems for detection. Thus, although bio- and chemiluminescent processes have become very popular for analytical purposes, they rely on relatively sophisticated equipment detection and monitoring. Highly sensitive photographic films, particularly Polaroid's type 612 ASA 20,000, can substitute for sophisticated luminometers. The films, as can modern CCD camera systems, integrate photon emission over a long period of time, thus enhancing detectability limits.

The most effective, portable, and inexpensive photon detectors available to most of us are our own eyes. The problem is that the human visual system accommodates so readily to changes in light intensity that it is very difficult to calibrate and utilize as a light intensity detector. That is why PSI has developed a means to measure substrate concentration via firefly luciferase/ATP bioluminescence as a function of spatial position, rather than luminescence intensity. This unique technology allows the human eye to be used merely to detect the spatial position of luminescence, rather than its absolute intensity. The visual acuity or spatial resolution of the human eye, even under very low light level dark adapted conditions, is very good. Our existing technology development for direct reading dipstick-type biosensors is focused on relatively high firefly luciferase concentrations and relatively high ATP and luciferin concentrations, thus permitting a relatively high bioluminescence output which can be directly visualized with minimum dark adaptation.

The work proposed in this SBIR application will add another novel technology which will enable the development of sensors for direct human visual detection with a 2-3 order of magnitude enhancement in analyte detectability limits, thus allowing direct reading sensors to be used for trace ATP concentrations, including low-level microbiological monitoring (27,33,36,38,45,66,67,75,81). The trick is to control the direction of photon emission. This is normally considered scientific heresy because it is well known that spontaneous emission tends to be non-spatial and non-temporal correlated. In the very early days of laser development, in the late fifties and early sixties, it was well recognized that the potential emission modes could be controlled by appropriate design of the optical cavity. This was indeed well demonstrated and has been known for many decades in the microwave field where the cavities are easily fabricable at high precision. It has been known to some extent in the optical community, particularly in single mode optical fibers and very thin film planar waveguides (18,26,46,52-3,68,73,82-5).

These concepts have recently been extensively studied and applied in the optical microcavity field with the goal of highly efficient microlaser production. Although these concepts have been applied to fluorescent dyes and to laser stimulated emission, they have not yet been applied, to our knowledge, to the biochemiluminescent processes. That is indeed the main objective of this application. The field is commonly called Cavity Quantum Electro-Dynamics. It is now well recognized that the spontaneous luminescence from excited atoms or molecules can be greatly suppressed or enhanced, placing them in appropriate optical microcavities. Directionality of photon emission can be enhanced by up to two orders of magnitude. The time course of the emission can be significantly altered and controlled, and indeed the spectral purity of the emitted photons can be greatly enhanced. These processes have already been demonstrated in a preliminary manner using Langmuir Blodgett monolayer films and fluorescent dyes in the classical studies of Drexhage and in the more recent studies of Yokoyama and co-workers (18,26,46,52-3,68,73,82-5).

G. *Phase I Research Plan (References are in Section P)*

Table 1. Summary of Phase I Research Objectives.

Tasks/Objectives	Protein Solutions, Inc.	Univ. of Utah Subcontract
PI	Triolo	Stewart
1. Luciferase Production & Characterization		X
2. Luciferase Engineered for Efficient Separation and Immobilization		X
3. Air/Water Interface Studies	X (Wang)	X (Wang)
4. Photoemission of 2-D Luciferase Films	X (Wang)	
5. Biotin-lipid-Steptavidin-Luciferase	X (Wang)	X (Wang)
6. Photoemission control via π-A Optimization	X (Wang)	
7. Microcavity Design -- Phase II	X (Triolo)	
8. Ordered Luciferase Films on Solid Supports (Phase II)	X (Wang)	
9. Modeling and Demonstration of Enhanced Biosensor Performance for low ATP Applications -- Phase II	X (Triolo)	

G.1 Luciferase Production and Characterization

This task is simply to continue the production and routine characterization of luciferase prepared by recombinant means, including the protein with the histidine tail for ease in purification.

G.2 Luciferase Engineered for Efficient Separation and Immobilization.

Our goal is to genetically engineer luciferase to create novel proteins that will be simple and inexpensive to purify in large quantities, and that can be immobilized with well-defined orientations and densities at interfaces.

To facilitate the immobilization of luciferase at high densities with a specific orientation to a surface or interface we constructed a luciferase fusion protein that becomes specifically biotinated when expressed in E. coli. This was accomplished by ligating a segment of the gene encoding E. coli Biotin Carboxyl Carrier Protein (BCCP) was into the histidine-tagged luciferase gene. BCCP is involved in fatty acid synthesis in E. coli (87). Biotin ligase enzyme covalently attaches a biotin molecule to a specific lysine residue of BCCP. Fusion proteins containing the biotin ligase recognition domain of BCCP will contain a single biotin molecule at a specific site simply as a result of being expressed in E. coli (88). Because the biotin is at a specific location, it may be possible for the BCCP-luciferase protein to be immobilized with a defined orientation on a streptavidin or avidin surface.

We have found that the BCCP-luciferase fusion protein is also expressed at high levels in E. coli in a soluble, active form and can be readily purified using a single Ni++ column purification step. Our very preliminary study of biotin-luciferase shows avidin and streptavidin binding as expected, with very high bioluminescence activity. With the expression and purification of biotinated recombinant luciferase worked out we are now prepared to fully characterize the luciferase protein and investigate the potential for oriented immobilization at interfaces.

As other approachs to building arrays of oriented luciferase we will genetically fuse luciferase to proteins that spontaneously assemble into two-dimensional arrays at lipid water interfaces, such as a-hemolysin (89), and we will investigate adding protein domains to luciferase that specifically recognize two-dimensional protein or polymer arrays.

We will continue to improve the utility of luciferase through genetic engineering. We may use random mutagenesis to select luciferase mutants that have greater thermal stability, or different photonic properties, e.g., shifted wavelength or narrower bandwidth. It may also be useful to couple the light producing activity of luciferase with other enzyme activities as an approach to regulating luciferase activity in sensor applications.

G.3 Air/Water Interface Studies

Although there has been considerable interest in firefly luciferase for analytical purposes and as a component of biosensors, there has really been very little work done on the interfacial immobilization or of the optimization of the protein's orientation and/or activity in the immobilized state. Most of the studies which have dealt with covalent immobilization of firefly luciferase report substantive amounts of inactivation of the enzyme. The protein is quite interfacially active and is readily denaturable.

We propose to study the behavior of recombinant firefly luciferase at air/water interfaces by fairly standard Langmuir trough pressure/area techniques. We have several troughs available to us for these studies through our relationship with the Center for Biopolymers at Interfaces (CBI) at the University of Utah. These include a custom made mini-trough which is suitable for microscope work, including fluorescence microscopy studies, conventional Lauda monolayer trough and a new trough from the KSV Company in Helsinki, Finland. This latter trough is highly instrumented, a modern state of the art instrument for monolayer based studies.

We propose to study the pressure/area behavior of firefly luciferase as a function of deposition concentration and conditions, including buffer type, ionic strength and pH, presence and absence of luciferin, ATP, and co-enzyme A. We will also conduct a more limited set of experiments on the effect of dithiothreitoi (DTT), trehalose, and sucrose. The latter two solutes will provide basic information which will be used in the Phase II studies dealing with the transfer of oriented monolayers to solid supports, their subsequent dehydration, and storage.

A number of the monolayer studies will also be performed on the mini-trough, and examined by fluorescence microscopy, using both luciferin and fluorescein as the fluorescent labels and probes. The goal here is to look for any evidence of 2 dimensional monolayer morphology, such as has been readily observed in other systems (see Task 5).

G.4 Photoemission of 2-D Luciferase Films

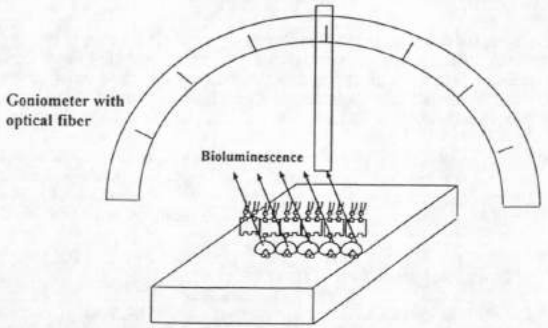
The main objective of our studies is to enhance the activity of interfacially immobilized firefly luciferase. This will be monitored by direct measurement of photoemission, now facilitated by the new trough system. The pre-formed, π-A characterized luciferase film will be rapidly exposed in the dark to a new substrate containing appropriate ATP/luciferin concentrations while monitoring the bioluminescence produced by the film.

Photon production will be monitored by a highly sensitive CCD camera, by a photographic camera system using Polaroid 612 ASA-20,000 black and white film, and by a goniometer controlled fiber optic sensor coupled to a photomultiplier tube detection system. It is unlikely, given some of the work to be described below, that the entire monolayer will be uniformly ordered. Rather, it is more likely to contain regions or domains, or 2 dimensional "crystals." Observation of photon emission as a function of monolayer position and camera or detection plane position will quickly indicate whether there is any preferential emission direction. Emission in the plane of the molayer will also be examined using the same detectors mounted appropriately. Since of the order of half of the expected bioluminescence will be oriented towards the bottom of the trough, the possible directionality of this component will be examined by placement of a mirror on the bottom of the trough, much as has been used for studies of transmission of monolayer films in Langmuir trough instrumentation.

G.8 Luciferase on Solid Supports -- Phase II

The use of the air/water interface and pressure/area techniques will allow the development of optimum conditions and systems for the preparation of ordered 2 dimensional luciferase films with a potential directional photoemission properties. Once this feasibility is established in Phase I, then we will be in a position to attempt to transfer such films or induce them to self assemble on solid supports, which may be more appropriate for the development of direct reading biosensors, our ultimate aim. Certainly Langmuir Blodgett transfer is an established and effective mechanism. Our own group is very experienced in solid surface modification and, indeed, in the adsorption of luciferase at solid/liquid interfaces (5,32,71-2). Solid surface assembly also has the advantage that we may be able to more appropriately control the dielectric constant at the solid side of the interface by appropriate materials selection. This, too, is a topic for Phase II.

We have completed a very preliminary study demonstrating the feasibility of immobilizing BCCP luciferase on an immobilized avidin surface. Quartz slides were silanized with aminopropyl triethoxysilane, followed by reaction with an NHS/biotin solution which reacts with a primary amino on the quartz surface, thereby forming a biotin layer. The biotinated quartz was then incubated with avidin, resulting in an avidin monolayer on the quartz surface. One group of such surfaces was then exposed to BCCP luciferase and the activity of the immobilized luciferase monitored. BCCP luciferase exposed to clean quartz resulted in very little binding and/or activity. The BCCP luciferase exposed directly to the avidin surface also had very little activity, as did the BCCP luciferase on biotin quartz. However, BCCP luciferase on the avidin/biotin/quartz surface showed substantial luminescence activity. These results are very preliminary, but they at least demonstrate the feasibility of preparing solid surfaces with immobilized luciferase which is presumably at least partially ordered, due to the avidin pre-layer.



Luciferase 2 D film at center of trough

Figure 1. A Schematic of the Proposed Langmuir Monolayer Studies. Biotinated lipid, together with the appropriate concentration of non-biotinated lipid, will first be spread at the air/water interface and characterized by pressure/area methods. Streptavidin will be injected into the subphase, resulting in an ordered, self assembled streptavidin film. The recombinant BCCP-luciferase, that is, the recombinant luciferase containing a biotin moiety, will then be injected, binding to the exposed biotin binding sites of the streptavidin. The streptavidin and luciferase have roughly the same molecular weight and size. It is expected that this will lead to a self assembled ordered layer of luciferase. In this cartoon, the luciferase binding site for ATP and luciferin is shown oriented away from the biotin expressing domain. This is indeed highly likely. The biotin domain is in the N-terminal region of the protein, whereas we know that the highly charged C-terminal region is indeed involved in the active site, so it is likely that the biotin domain and the active site are, indeed, some distance apart. Further evidence for this supposition is that the biotin luciferase is just as active as recombinant luciferase which does not contain the BCCP domain. The composite multi-layer is shown on a Langmuir trough and exhibits oriented photoemission, which is mapped by the goniometer/fiber optic spectrometer.

G.5 Biotin Lipid Streptavidin 2D Films.

The ordering and even crystallization of proteins at air/water interfaces has been extensively studied in the past several years, particularly the streptavidin biotin system. The unique ability of streptavidin to bind four biotin molecules (two on each side of the molecule), and the ability to make lipids containing a biotin moiety, have enabled Langmuir trough techniques to be used to assemble and order complex 2-dimensional films (Figure 1). Now readily available biotin lipids, coupled with their ability to be spread and characterized easily at air/water interfaces, and the ability of streptavidin to bind two such biotin lipids, to order, and even crystallize under appropriate pressure area control, has led to the self assembly and construction of a variety of multi-component, protein containing multi-layers, including IgG, Fab. We will prepare and characterize these layers by the methods which are now standard in the literature (23,30,39,40,44,54,64,86).

We believe we are the first to produce an engineered luciferase containing a biotin moiety in a unique and precise position on the molecule, thus allowing the biotin luciferase to bind with a particular order to the 2-d streptavidin crystal. This should be the first time that luciferase films will be produced in a precisely controlled orientation, at least in the Z or vertical dimension. There is some question as to what will be the orientation and/or ordering in the planar or XY dimension, and it is possible that there may be some free-rotation or mobility around the biotin containing domain. However, the fact that firefly luciferase does indeed crystallize, and is known to dimerize, suggests that given the opportunity firefly luciferase will laterally order in a such a system. This, of course, remains to be seen. These films will, of course, be monitored by the same pressure/area methods of directional photo emission detection described above, as well as by fluorescence microscopy, using the intrinsic luciferin fluorescence, as well as by streptavidin and luciferase modified with fluorescein and/or other fluors.

G.6 Photoemission Control Via Pressure/Area Optimization.

It is now well known that the ordering characteristics of 2d protein monolayers at air/water interfaces is a function of the pressure/area environment. We will therefore monitor enzyme activity and photoemission directionality as a function of the pressure/area environment of the composite multi-layer system. These studies will also include suitable annealing or equilibration time to allow the composite multi-layer to relax and/or reassemble in response to the pressure area perturbations.

G.7 Microcavity Design -- Phase II (18,26,46,52-3,68,73,82-5).

Although this task is primarily a Phase II effort, we will certainly give it considerable attention during Phase I, from the point of view of trying to design an optimum composite multi-layer which increases the probability of directional photoemission.

Luciferase monolayers at an air/water interface are subjected to an enormous dielectric discontinuity which must have a dramatic effect on its photoemission properties. We can, to some extent, control that dielectric discontinuity and difference by floating a thin lens or film of oil or other water immiscible fluid on top of the trough. Such fluids are of the order of a micron thickness or more. From a micro-optical cavity point of view, the luciferase monolayer will see one dielectric environment to the top and see an aqueous dielectric environment to the bottom. We propose to model such effects in Phase II and to use such models to optimize the construction of suitable planar optical microcavities and directional photoemission.

We may also be able to elicit some control over dielectric environment in the lateral, or XY direction, by co-immobilizing luciferase at the interface with a diluent polymer of significantly different dielectric constant. This is, of course, speculative, and would lead to optical microcavities on a very small scale (quantum dots?), much smaller than has heretofore been studied in the solid state physics community. Again, this is a subject for Phase II.

G.9 Enhanced Biosensor Performance -- Phase II

It is too early to specify details on this Phase II task until we have completed much of the feasibility study and basic work proposed for Phase I. General aspects of this task were noted on page 8.

H. Commercial Potential

Patterns and images are much easier to detect, to remember, and to process than numbers. Man is exquisitely constructed to deal with visual patterns and pattern recognition. The design, development, and application of analytical devices which are self luminescing and generate a specific pattern which can be directly correlated or related to analyte concentration, has enormous potential benefits. In addition to greatly simplifying routine measurements in a range of analytical, environmental, biotechnological, and medical/diagnostic laboratories, such technology has the potential for being applied widely in schools, education, and in the home for a variety of monitoring, diagnostic, and other measurement purposes.

Health care is a one trillion dollar per year industry in the United States; chemical/clinical diagnostics accounts for perhaps 5-10% of that. With the growing role of analytical chemistry in the biotechnological and environmental areas, it has been estimated that the biosensor industry is in the range of one to ten *billion* plus dollars per year. With the growing interest in home-based clinical chemistry measurement and a growing movement towards home care and personal care, it is clear that the opportunity for simple, inexpensive, disposable, and specific analytical chemistry devices should grow dramatically (2,3,36,37,62-3,70).

There is considerable interest in and need for rapid, accurate, monitoring of microbial concentratios in health care facilities, food, and pharmaceutical preparation areas, microbiology and biotechnology laboratories, incineration sites, waste processing facilities and wastewater treatment facilities. The development of a simple, direct reading bioluminescent technique to monitor changes in microbial content in real time will be a valuable tool that will find great utility in these, and other, sensitive areas.

J. Andrade, P. Triolo, and the University's Office of Technology Transfer have had considerable experience with major diagnostic and sensor companies. We expect considerable interest in this technology. PSI expects to enter into an appropriate agreement for commercial funding of the development as Phase III. PSI will itself fund and market these sensors for educational and home use. The markets are enormous.

I. Principal Investigator and Senior Personnel

Phil Triolo, Ph.D., Principal Investigator and President of PSI, is a bioengineer with considerable product development experience in the medical deice industry. Until joining PSI in 1994, he spent seven years as a contractor, 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, or the design and execution 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 Lableess Lab™ in Polymer Materials, a line of science kits for distance learning/tele-education application. Rob is the 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 closely with Mr. C.Y. Wang, a student who will work as a postdoc 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 biosketch 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, particularly with their behavior at surfaces and interfaces (3-5). About 5 years ago he became quite interested in bioluminescence and particularly in the firefly and bacteria luminescence 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. No funds are budgeted for his services.

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 doc during the Phase I award to conduct the firefly luciferase-based studies and to transfer his rich, comprehensive expertise on that system to the others involved in the project.

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.

Dr. Russell Stewart, Assistant Professor of Bioengineering and PI of the University subcontract, also serves on PSI's Scientific Advisory Board.

J. Consultants and Subcontracts

This proposal includes a sub-contract to the University of Utah, Department of Bioengineering, with Dr. Russel J. Stewart as PI. Dr. Stewart will be responsible for tasks/objectives 1 and 2: preparation and characterization of recombinant luciferase and the design and engineering of luciferase for optimum interfacial immobilization and self assembly. The budget for the subcontract follows (see Appendix). Dr. Stewart's vita is above. We have also included his other support in Section L. The preliminary work on the combinant firefly luciferase referred to earlier in the proposal was conducted in Dr. Stewart's laboratory with the assistance of Mr. C.Y. Wang. Mr. Wang will continue to be involved in that work as a part-time post doc in Dr. Stewart's laboratory, as well as a part-time employee at PSI. Mr. Wang will transfer the technology to PSI. PSI has a technology transfer agreement with the University of Utah and has the rights to use the engineered luciferases for its biosensor studies and products.

K. Equipment and Facilities

Protein Solutions, Inc. has about 600 square feet of quality research space in the University of Utah Research Park. This space is adjacent to an additional 800 feet of research space assigned to J.D. Andrade through the Department of Bioengineering. In addition, Dr.s Triolo, Scheer, and Wang have access to instrumentation and facilities at the University of Utah through the Center for Biopolymers at Interfaces (CBI) and the Department of Bioengineering. Protein Solutions, Inc. is a member of CBI, a university/ industry/state consortium focused on interfacial aspects and applications of proteins. This membership gives PSI access to the laboratories, techniques, and facilities of the Center at minimal expense. These facilities include the KSV Langmuir trough (described briefly earlier), as well as the CCD camera and fiber-optic goniometer spectrometer, which will be used for the photoemission measurements.

Dr. Andrade's laboratories at the University are also available for this work, including an array of surface analysis and characterization equipment, including X-ray photo-electron spectroscopy, contact angle, Langmuir trough techniques, dynamic surface tensiometry, and optical microscopy. Dr. Russel J. Stewart's laboratory in the Department of Bioengineering and CBI, includes approximately 600 sq. ft. of wet laboratory space to carry out the genetic engineering and biochemical characterization of recombinant luciferase. Available equipment includes: superspeed cemtrifuge (Beckman J2HS), ultracentrifuge (Beckman), table-top ultracentrifuge (Beckman TL 100), chromatography equipment, DNA and protein gel electrophoresis equipment, PCR temperature cycler (MJ Research), UV/visible spectrophotometers, and fluorimeters.

L. Current and Pending Support of PI

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, proposal title: Continuous Real Time Ennumeration 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 about 50% effort. He will have adequate time for the one man month budgeted in this proposal. There is no direct overlap between the two grants.

Phil Triolo
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Salt Lake City, Utah 84103
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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 haparin-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 Phychology 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.

L.J. Stensaas, F.J. Todd, P.M. Triolo, Prosthesis and Methods for Promoting Nerve Regeneration and for Inhibiting the Formation of Neuromas, Issued to Research Medical, Inc.

R.H. Hoffer, J.L. Orth, P.M. Triolo, Implantable Device for the Administration of Drugs or Other Liquid Solutions, Issued to Biosynthesis, Inc.

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.

W.M. Padilla, P.M. Triolo, Locking Syringe with Thread Release Lock, U.S. Patent Application 10927.12.2, to be issued to Merit Medical Systems, Inc.

PUBLICATIONS:

P.M. Triolo, J.D. Andrade, "Surface Treatment and Characterization of Some Commonly Used Catheter Materials. I. Surface Properties, *J. Biomed. Mater. Res.* **17** (1983) 129-147.

P.M. Triolo, J.D. Andrade, "Surface Treatment and Characterization of Some Commonly Used Catheter Materials. II. Friction Characterization," *J. Biomed. Mater. Res.* **17** (1983) 149-165.

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 titled Modulation of Microtubul 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.

M. Equivalent or Overlapping Proposals -- None.

N. Budget (following pages)

Budget Justification

Dr.s Triolo and Scheer are each budgeted for one man months. Dr. Wang is budgeted at four months. PSI's fringe benefit rate is 30% of direct salary. Its indirect cost rate is 60% of total direct costs. One trip is budgeted to a domestic American Chemical Society or Materials Research Society meeting. Instrument time charges are standard discounted charges for members of the Center for Biopolymers at Interfaces at the University of Utah. The University of Utah sub-contract is presented with its own budget page which includes staff and student support for the recombinant luciferase studies. University of Utah does not charge indirect costs for Phase I subcontracts. The subcontract has been fully processed and approved by the University's Office of Sponsored Projects.

O. ***Prior Phase II Awards -- None.***

P. ***Literature Cited.***

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A PROPOSAL

FOR

SBIR 1 Subcontract to the University of Utah

"LUMINESCENT THIN-FILM BY INTERFACIAL ORDERING OF ENGINEERED LUCIFERASE"

Submitted to

PROTEIN SOLUTIONS INCORPORATED
6009 HIGHLAND DRIVE
SALT LAKE CITY, UTAH 84121

Submitted by

Dr. Russell J. Stewart
Department of Bioengineering
Salt Lake City, Utah 84112

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Office of Sponsored Projects
June 8, 1995