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

Department of Health and Human Services
Public Health Service
Small Business Innovation Research Program

Phase I Grant Application

Follow instructions carefully.

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

1. TITLE OF APPLICATION (*Do not exceed 56 typewriter spaces*)
A Novel Enzyme Based Homocysteine Biosensor

2. SOLICITATION NO. PHS 97-2

3. PRINCIPAL INVESTIGATOR — New Investigator [ ]

3a. NAME (*Last, first, middle*): Wang, C.-Y.
3b. DEGREE(S): B.S. Ph.D.
3c. SOCIAL SECURITY NO.: Provide on Personal Data Page
3d. POSITION TITLE: Research Scientist
3e. MAILING ADDRESS (*Street, city, state, zip code*)
Protein Solutions, Inc.
P.O. Box 58093
Salt Lake City, UT 84158-0093
BITNET/INTERNET Address:
3f. TELEPHONE AND FAX (*Area code, number, and extension*)
TEL: 801-583-9301
FAX: 801-583-4463

4. HUMAN SUBJECTS: [X] NO [ ] YES
4a. If "yes," Exemption no. or IRB approval date — Full IRB or Expedited Review
4b. Assurance of compliance no.
5. VERTEBRATE ANIMALS: [X] NO [ ] YES
5a. If "Yes," IACUC approval date
5b. Animal welfare assurance no.

6. DATES OF PROJECT PERIOD
From: September 30, 1998 Through: March 30, 1999

7. COSTS REQUESTED
7a. Direct Costs: $ 70,935
7b. Total Costs: $ 99,396

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

9. APPLICANT ORGANIZATION (*Name and address of applicant small business concern*)
Protein Solutions, Inc.
P.O. Box 58093
Salt Lake City, UT 84518-0093

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

11. SMALL BUSINESS CERTIFICATION
[X] Small Business Concern [ ] Women-owned
[ ] Socially and Economically Disadvantaged

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

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

14. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name: J. D. Andrade
Title: President and CEO
Address: Protein Solutions, Inc.
P.O. Box 58093
Salt Lake City, UT 84158-0093
Telephone: 801-583-9301
FAX: 801-583-4463
BITNET/INTERNET Address:

15. PRINCIPAL INVESTIGATOR ASSURANCE: I certify that the statements herein are true, complete, and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.
SIGNATURE OF PERSON NAMED IN 3a (*In ink. "Per" signature not acceptable.*): Chung-Yih Wang — DATE: 12-12-97

16. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete, and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Service terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.
SIGNATURE OF PERSON NAMED IN 14 (*In ink. "Per" signature not acceptable.*) — DATE: 12-12-97

Abstract of Research Plan

NAME, ADDRESS, AND TELEPHONE NUMBER OF APPLICANT ORGANIZATION
Protein Solutions, Inc.
P.O. Box 58093
Salt Lake City, UT 84158-0093 Phone: 801-583-9301

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

TITLE OF APPLICATION
A Novel Enzyme Based Homocysteine Biosensor

KEY PERSONNEL ENGAGED ON PROJECT

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

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

The main objective of this proposal is to assess the feasibility of an enzyme based bioluminescent sensor for determining homocysteine concentrations. This sensor is designed for individuals who need to periodically monitor their homocysteine levels. The modularized design is comprised of immobilized enzyme units and substrate reservoir units. Only one drop of the sample is required. The homocysteine level is read by a single processing unit, which could be a commercial luminometer or our handheld photodiode readout system (under development). The proposed homocysteine sensor contains three reaction zones. Zone 1 is for sample pre-treatment where homocysteine is reduced to its free form, and adenosine and ATP interferences are removed. The second zone is for homocysteine conversion. In Zone 3 the remaining adenosine is correlated to the original concentration of homocysteine. This novel enzymatic homocysteine biosensor is expected to be simple, specific, highly accurate, inexpensive, and superior to existing homocysteine analysis methods.

Provide key words (8 maximum) to identify the research or technology.
homocysteine, biosensor, bioluminescence, ATP, luminometer

Provide a brief summary of the potential commercial applications of the research.
The general public and their physicians are becoming increasingly aware of the importance of homocysteine, B vitamins, and folate levels to their cardiovascular health. This awareness is leading to an increased need for a fast, simple and low cost multi-parameter assay which can be performed in the clinic, doctor's office or in the home. This folate assay is part of a larger "homocysteine panel" combining the measurement of folate, the vitamins B_{12} and B_6 and homocysteine into one sensor.

Budget Justification

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

Personnel: **Dr. C.-Y. Wang** will serve as Principal Investigator on this project. He has served as PI on other SBIR grants. He is an expert in the application of firefly luciferase to biosensor development and applications. His technical contribution will be primarily in the protein, enzymatic assay and biochemistry aspects of this work, primarily as this relates to the luciferin-luciferase system. **Dr. R. Van Wagenen** has an extensive background in interfacial phenomena, optics and spectroscopy instrumentation. His background in luminescence and device development will address the hardware and materials aspects of the biosensor development. He will also assist with planning and coordination. **Dr. Robert Scheer**, Research Scientist, serves both part time with the company and is also a member of the faculty in the Department of Materials Science and Engineering at the University of Utah. Given Rob's background in materials science, he will make his technical contributions in the area of materials science and surface chemistry. **Dr. J.D. Andrade** is not an employee of the company, however, he does actively serve as a technical advisor in a wide range of areas related to biomaterials, proteins at interfaces, adsorption, device development and polymer science. Mara Hammer has a Biology Degree and serves as a Lab Technician.
Financial: Total direct personnel costs are $47,435. Fringe benefits are 20% of salary and indirect costs are 60% of total direct personnel costs. No consultants, subcontracts, patient care costs or fees are requested for this work.
Equipment: Funds are requested for the purchase of a small, research grade, freeze drying system and associated vacuum pump. This will be used primarily for enzyme lyophilization for enhanced stability both during storage and in the biosensor prototypes. Funds are also requested for a small, refrigerated centrifuge. This will be used to separate homogenized liver cells from supernatent which is required for the isolation and preparation of a key enzyme required for the assay.
Supplies: Funds are requested for general lab supplies and biochemical supplies such as proteins, enzymes, etc.
Travel: Travel funds are requested to attend a conference related to homocysteine biochemistry or clinical chemistry such as the annual meeting of the AACC.
Other: Funds are requested for external clinical laboratory analyses of some samples. These analyses will be conducted by Associated Regional University Pathologists, Inc. (ARUP) a major clinical chemistry laboratory serving the western U.S. Funds are also requested for a partial annual membership fee for the Center for Biopolymers at Interfaces, a state-university-industry consortium at the University of Utah which provides us with full and extensive access to research resources and interactions with professors, students and staff at the University of Utah.

Resources

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

Protein Solutions, Inc. (PSI) occupies 1,400 square feet of research, laboratory, and office space located in the Research Park adjacent to the University of Utah. This space includes a chemistry lab, a biology lab, an optics lab, a conference room and two offices. The space and laboratory equipment (noted below) are adequate for the work proposed with the addition of the equipment funding requested in this proposal. Office equipment is standard and is adequate to address the administrative aspects of the proposed project, i.e., computers, laser printer, copy machine, FAX machine, etc. The company has a Technology Transfer Agreement with the University of Utah which allows for the transfer of jointly developed technologies to PSI. The company is also a member of The Center for Biopolymers at Interfaces (CBI), a state - university - industry consortium which is one of the Centers for Excellence in the Utah State System of higher education. CBI membership provides a number of key benefits including: (1) fee for service access to many analytical services at the University of Utah a very low rates, e.g. the SEM/TEM facilty, (2) the Surface Analytical Facility (XPS, SIMS, etc.), (3) access to specialized laboratory equipment in the departments of Bioengineering (Optics Laboratory), and Chemistry (analytical biochemistry labs), and (4) access to faculty members who can provide expertise on a consulting basis. Finally, there are a wide range of technical consulting and fee for service machining and electronic design companies located in the immediate area.

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

Basic laboratory equipment at PSI includes analytical balances, stirrers, hot plates, a fume hood, oven, pH meter, water baths, HEPA filtered laminar flow air work areas, steam autoclave, reverse osmosis - filtered water, light microscopes, Turner Designs luminometer, cameras, vacuum system, a gel electrophoesis system, -85 C freezer, and a lap top computer. A charge coupled device (CCD) array camera and associated computer and controller are also available for the recording and quantification of luminescence experiments to supplement the luminometer. A Beckman UV-Visible spectrophotometer is available for spectrophotometric characterization of proteins.

BIOGRAPHICAL SKETCH AND BIBLIOGRAPHY

Chung-Yih Wang
Research Scientist

Current Employment

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

Birth Date: November 7, 1962 **SSN:** 529-91-1621

Education

Ph.D., Bioengineering, University of Utah, 1997.
B.S., Chemical Engineering, National Taiwan University, Taipei, Taiwan, 1985.

Professional Experience

Research Scientist, Protein Solutions, Inc. Salt Lake City, Utah 1996 - present.

Research Assistant, Department of Bioengineering, University of Utah, Salt Lake City, Utah 1989 - 1996.

Teaching Assistant, Department of Bioengineering, University of Utah, Salt Lake City, Utah, 1990 - 1992.

Research Chemist, Taiwan Power Company, Taipei, Taiwan, 1987 - 1989.

Honors

University of Utah Graduate Research Fellow, 1991-1992.
Deluca Prize, 1996.

Representative Patents and Publications

C. - Y. Wang and J.D. Andrade, "Denaturation of Firefly Luciferase", in *Bioluminescence and Chemiluminescence: Current Status*, P. Stanley and E. Kricka, eds. Wiley, p.427, 1991.
C. - Y. Wang and J. D. Andrade, "Interfacial Behavior of Firefly Luciferase" in *Bioluminescence and Chemiluminescence: Status Report*, A.A. Szalay, ed., Wiley, pp.99-103, 1993.
C. - Y. Wang and J.D. Andrade, "Purification and Preservation of Firefly Luciferase", in *Bioluminescence and Chemiluminescence: Fundamentals and Applied Aspects*, A.K. Campbell, L.J. Kricka and P.E. Stanley, eds., Wiley, pp. 494497, 1995.
D.J. Min, **C.-Y. Wang** and J.D. Andrade, "Air/Water Monolayer Studies of Bioluminescent Enzymes" in *Bioluminescence and Chemiluminescence: Fundamentals and Applied Aspects*, A.K. Campbell, L.J. Kricka and P.E. Stanley, eds, Wiley, pp. 494-497, 1995.
C.-Y. Wang, S. Hitz, J.D. Andrade, and R. Stewart, "Biotinylation of Firefly Luciferase in vivo: A recombinant Protein with a Special Immobilization Site" submitted to Anal. Biochem., 1996.

Reaearch Plan

A. Specific Aims

The objective of this phase I proposal is to assess the feasibility of an enzymatic assay for homocysteine in biological fluids. This assay adopts a dip-stick approach and is designed to minimize the labor, equipment and capital needed for present homocysteine assays. When coupled with an inexpensive photodiode-based bioluminescence reader, it can be used by patients at home.

Specific aims include:

1. Developing methods for sample pre-treatment

The purposes of sample pre-treatment are removing interference chemicals and reducing protein bound homocysteine. We will co-immobilize apyrase and adenosine deaminase using pre-activated membrane (e.g. Immunodyne ABC membrane, Pall Gelman Sciences). Apyrase will consume ATP and adenosine deaminase will digest adenosine in the sample. Dithiothreitol (DTT) will also be used in this process to release free homocysteine. The optimum temperature and pH for sample pre-treatment will be selected.

2.Homocysteine conversion reaction

The purpose of Aim 2 is to provide a signal that can be processed by bioluminescence assay. Homocysteine will be converted to S-adenosyl-L-homocysteine resulting in the consumption of adenosine (Figure 1). This reaction has been used in a radioenzymic assay for homocysteine by Refsum et al. [1]. We will immobilize S-adenosyl-L-homocysteine hydrolase (SAH hydrolase, EC.3.3.1.1) on the same membrane used in Aim 1. A known amount of adenosine will be loaded onto the SAH hydrolase membrane. The completeness of the reaction and the linear range will be evaluated using an immunoassay (Axis, Norway) and the method proposed in Aim 3, below.

3. Analyzing the adenosine content

Purification of adenosine kinase (EC.2.7.1.20): Since adenosine kinase is not commercially available, it will be purified from bovine liver using a 5'-AMP-Sepharose 4B affinity column [2].

Analysis of adenosine: Adenosine kinase, immobilized on membrane, converts one molecule of adenosine to AMP with the consumption of one ATP (Figure 1). The amount of residual ATP will be monitored by bioluminescence assay with a luminometer. Because the linearity of the bioluminescence assay ranges from 1 μM to 1 nM ATP, a change of 1 μM ATP can be easily resolved. The data obtained are directly proportional to homocysteine concentration. We will optimize the reaction time and reaction conditions for adenosine kinase.

4. Calibration and standardization

Standard homocystenine will be prepared in PBS buffer and used to calibrate our assay. The linearity range for homocysteine and accuracy will be determined. The concentration of adenosine and ATP will be adjusted to obtain the highest sensitivity for the change of homocysteine.

Plasma sample will also be studied with our homocysteine assay. The background homocysteine value in plasma will be measured first. Known amount of homocysteine will then

be added to plasma for internal calibration. All the optimized conditions, including the reaction temperature, pH, reagent and enzyme concentration will be collected for pre-prototype biosensor design.

5. Pre-prototype homocysteine biosensor

The pre-prototype biosensor will include three enzyme zones (Figure 2). The sample solution will be loaded on the front end for sample treatment, homocysteine conversion and then adenosine analysis using ATP bioluminescence. The control of sample flow rate, the completeness of the reaction, and the linearity of assay will be evaluated in a preliminary manner. The more complete optimization and development of a prototype homocysteine biosensor will be performed in Phase II.

In the Phase II project we will optimize the drying protocol to improve the assay performance. The stability of immobilized enzymes will be studied. The design of the prototype biosensor including the control of sample flow rate, release of dry reagents, sample reaction time and manufacture technique will be addressed. The prototype sensor will be tested with whole blood samples. The method for sensor calibration will be standardized and the sensor accuracy and reproducibility will be validated. The prototype sensor will be integrated with a handheld photodiode based luminometer, now under development and funded separately. The self-life and preservation all biochemicals in the sensor will also be studied.

B. Identification and Significance of Problem

1. Importance of homocysteine

Homocysteine is a key metabolite in the transsulfuration pathway and sulfur conservation cycle (Figure 1) [3]. Many recent medical studies have shown that elevated levels of total plasma homocysteine (tHcy) are a marker for increased risk of cardiovascular diseases including premature occlusive vascular disease in the coronary, cerebral, and peripheral arteries, myocardial infarction, and atherosclerosis [4-8]. Homocysteine is shown as an independent factor of other vascular disease risk factors, such as cholesterol, age, diabetes and hypertension [9]. These studies suggest that relatively small increases in plasma tHcy can indicate increased risk for heart and lung disease. For example, SelHub et al. reported that for men, the prevalence of stenosis of >25% was 27 % in the lowest homocysteine quartile (<9.1μmol per liter) and 58 % in the highest quartile (>14.4 μmol per liter) [6]. Other study stated that "Patients with coronary artery disease had a higher homocysteine level than control subjects (13.66 +/- 6.44 versus 10.93 +/- 4.92 nmol/ml, p<0.001)"[5].

McCully suggested that the increased conversion of methionine to homocysteine thiolactone may lead to the thiolation of low density lipoprotein (LDL) [10]. The thiolated LDL may aggregate and be taken in by macrophages, resulting in lipid deposition in atheromas. The presence of homocysteine thiolactone within the vascular wall may further injure the vascular system.

Other diseases for which high blood levels of homocysteine is a marker include increased risk for stroke and lymphoblastic leukemia (including other cancers) [7, 11-12]. As an important marker for both cardiovascular diseases and cancer, there is growing interest and activity in the measurement of plasma or urine total homocysteine concentration.

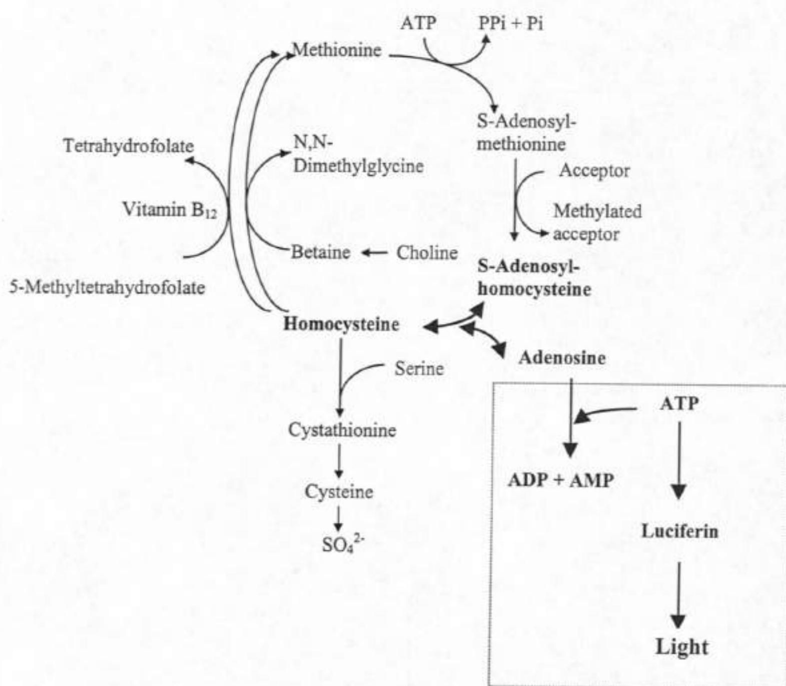


Figure 1. The metabolism of homocysteine. The enzymes needed in the metabolic pathway are not shown. The reactions enclosed by the dash line are used in the homocysteine biosensor but are not included in the homocysteine metabolic pathway.

Table 1. Comparison of existing methods and the proposed method

Methods	Fluorescent labeling followed by HPLC separation	Mass spectrometry	HPLC and electrochemical detection	Enzymatic assay	**Proposed enzymatic homocysteine sensor**
Marker	Fluorescent dye e.g. 7-benzo-2-oxa-1,3-diazole-4-sulfonic acid (SBD)	Isotope e.g. [$^{2}H_3$]methionine or [$^{2}H_3$]homocysteine	None	1. Radio-labeled substrate or 2.Antibody	None
Special difficulty	Fluorescent derivatives are highly light-sensitive. Light exposure may lead to serious error.	Tedious sample preparation. Possible contamination of isotopes.	Deterioration of the gold-mercury electrode. Serial test (low-throughput)	1. Radiation exposure / isotopes contamination. 2. Require training	None
Time	>1.5 hr/batch	> 2.5 hr/batch	20-30 min/sample	> 2 hr/batch	< 30 min/batch
Instrument cost	44,000	63,000	32,000	<10,000	For photodiode based luminometer<100 PMT based luminometer: 4,000
Total cost	High	High	High	medium	Low
Home use	No	No	No	No	Yes

In the case of inherited diseases, such as the deficiency of cystathionine β-synthase (EC 4.2.1.22) or ATP:L-methionine S-adenosyltransferase (EC 2.5.1.6), homocysteine is accumulated and can be as high as 200 μmol/l [13]. Accumulation of plasma homocysteine may also be caused by dietary imbalances (vitamin B-6, B-12 and folate deficiency) [14]. To find homocysteine levels, a risk factor of cardiovascular disease, in the early stage or to monitor the treatment of hyperhomocysteinemia, we need a simple method to screen abnormal levels of homocysteine. Clinical labs have also reported an increased demand for testing homocysteine levels in plasma [13].

2. Difficulty of determination of homocysteine

The method for determining the homocysteine level must be specific, and the sensitivity must be high (less than 1 μmol/liter in the range of 0-20 μmol/liter). Several methods are currently used to determine the concentration of homocysteine in urine and blood (Table 1). Ubbink et al. developed a method that labeled homocysteine with thio-specific 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonic acid [15]. The fluorescent labeled amino acid was separated by high-performance liquid chromatography (HPLC) and detected with a fluorometer. Because the fluorescent derivatives are unstable, the sample preparation and calibration must be done with extraordinary care and precision [16]. The second method is mass spectrometry. A longer sample preparation time (2.5 hr) is required to make butyl ester derivatives and iostopes are used for quantification [17]. The tedious calibration procedure and expensive instrument make it less favorable.

Electrochemical detection, which coupled HPLC and electrochemical detector, was also used to measure homocysteine level [18]. This method is clean and can be automated. However, the degeneration of its detection unit, a gold-mercury electrode, may cause faulty results. The extra maintenance and high cost make it practical only in a clinical laboratory.

Enzymatic assays for homocysteine have also been developed. Unfortunately, there is no simple assay for homocysteine to date [13]. Radioenzymatic assays have great contamination problems. The development of a highly specific immunoassay for homocysteine is unsuccessful because of the small size of homocysteine and its structural similarity to cysteine, which is about 25 to 30 times more common than homocysteine [7]. An indirect immunoassay, which is specific for adenosylhomocysteine, was reported [19]. Because it is an indirect assay, repeated wash procedures make it difficult to design as a dip-stick type biosensor.

3. Advantages of proposed method over existing methods

The novel method assays the change of adenosine via ATP bioluminescence. No costly instrument is required to detect the bioluminescence intensity, which correlates to homocysteine level. This method also eliminates the need for preparing amino acid derivatives or the use of isotopes or fluorescent dyes. The simple design enables people using it at home without intense training. A comparison of existing methods and the proposed method is shown in Table 1.

The modularized design simplifies the work needed to develop other sensors in the future. We can change the enzyme and substrate modules and quickly convert the sensor for other metabolites. This design will be used in the development of amino acid panel later.

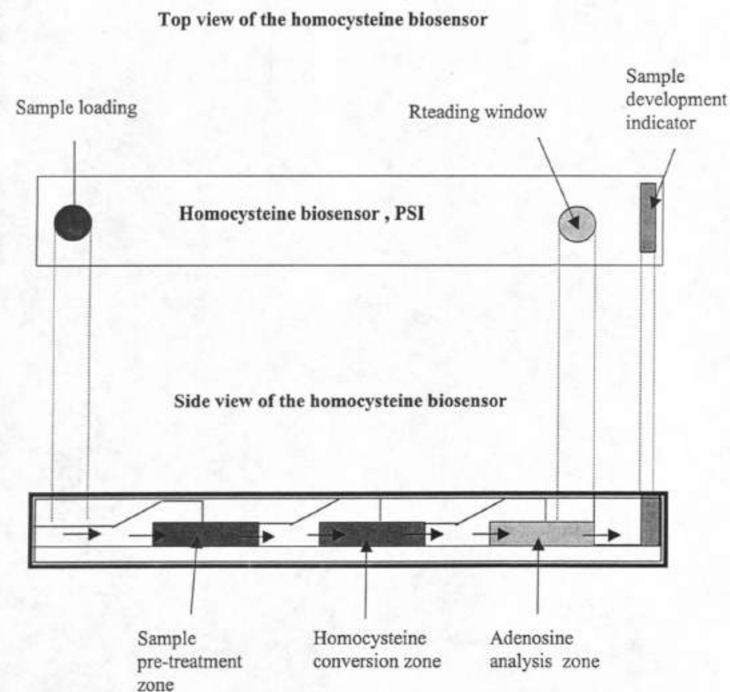


Figure 2. Pre-protype homocysteine biosensor. The sensor comprises three reaction zones. The dry reagent is released when sample flows through those zones. The arrow lines represent the flow direction of sample. After the indicator shows the reaction is complete (the appearance of color), the sensor will be inserted into the photodiode based luminometer to quantify the bioluminescence intensity. The bioluminescence intensity is directly related to the homocysteine level in the sample.

C. Relevant Experience and Background

Protein Solutions, Inc. has extensive experience analyzing substrate concentrations using enzymatic assays. Our most extensive experience is a unique direct-reading bioluminescent ATP detection system [20]. This system is being used to develop a sensor for galactose in urine.

There are two molecules that play unique and central roles in biology: adenosine triphosphate (ATP), and nicotinamide adenine dinucleotide (NADH). ATP is generally recognized as one of the key energy currencies in biology and NADH serves as a ubiquitous electron donor. There is a large body of literature on the development of assays and biosensors using firefly luciferase to monitor ATP and ATP-dependent processes. Firefly luciferase catalyzes bioluminescent reactions, which can be used to detect subnanomolar concentrations of ATP [21]. The designs of luciferase based biosensors generally employ fiber-optics for delivering the luminescence to a device which can accurately measure the light intensities [22]. There are some problems which need to be addressed in these applications: the stability of luciferase and its incorporating into a commercial devices with the required reliability, accuracy, and shelf life.

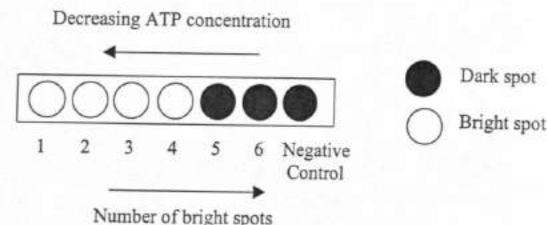
Our sensor concept is based on the fact that for a given luciferase/luciferin concentration, a minimum concentration of ATP is required to produce a detectable light output. Above that concentration, light is detectable; below that ATP concentration, no light is detected. By filtering or consuming the ATP (with kinase or apyrase) before it reacts with luciferase we control the intensity of the light at each position along the sensor. A high concentration of ATP will still produce light even at 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. The average human eyes or a simple photodiode based device can reliably and accurately measure changes in spatial position of light. The galactose sensor, developed in our laboratory, can demonstrate this concept (Figure 3). A clear cut-off or the number of bright spots represents the galactose concentration.

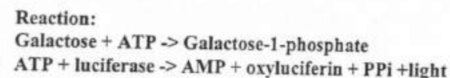
For the homocysteine sensor designed in this proposal, a modular concept makes the design of the sensor more flexibility. All the enzymes used were immobilized on the pre-activated membrane. Each immobilized enzyme becomes an independent unit which can be assembled later in the desired combination. The sensor can be split into several zones, comprising one or more enzyme containing zones and the substrates containing region (Figure 2). The sensor end portion is an ATP sensor platform, comprising luciferase, with or without apyrase.

Operation of the sensor begins when the sensor contacts a solution containing homocysteine. The solution is drawn into the sample pre-treatment region, the homocysteine conversion region, and the adenosine analysis region. The adenosine analysis region is coupled with an ATP platform where bioluminescence can be read with a photon counter. Because adenosine kinase transfers a phosphate from ATP to adenosine, ATP is depleted during the process. When the reaction is complete, the remaining ATP is transformed as a photon signal by luciferin and luciferase. The luminescence signal is detected with either a CCD camera or a photodiode based luminometer. The signal readout is directly proportional to homocysteine concentration.

PSI was recently awarded a STTR Phase II grant from NSF entitled, "Direct Reading Quantitative Biosensors for ATP" which focuses on enhancing the sensitivity of our existing ATP-based bioluminescent spatial sensor by several orders of magnitude. We therefore have the commitment and resources to fully develop the direct reading, quantitative ATP sensor. This proposal will modularize the sensor design and try to deliver a homocysteine sensor to the

Direct-reading semi-quantitative galactose sensor





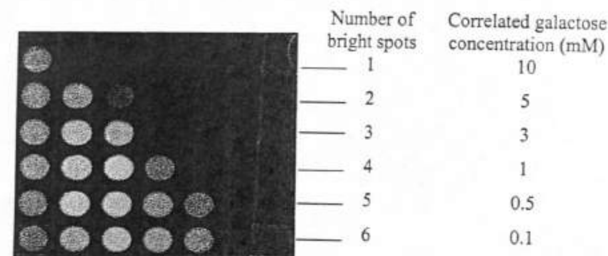


Figure 3. A direct-reading semi-quantitative galactose sensor. The biosensor is designed as a strip with seven wells. The reagents and enzymes included in each well are ATP, luciferin, $MgSO_4$, galactokinase, and firefly luciferase. The sensor was calibrated with known concentrations of galactose. The number of bright spots represents galactose concentration. The samples with different galactose concentrations are loaded onto the strip. Each horizontal strip is an individual sensor exposed to the specified galactose concentration. After 30 min reaction, the data was recorded by CCD camera.

general populations for home use.

Principle Investigator

Dr. C.Y. Wang, Research Scientist, recently completed his Ph.D. studies under Dr. Andrade's supervision at the University of Utah. Dr. Wang has worked on the Firefly luciferase system for seven years. He recently received the M. DeLuca Award at the 9th International Symposium on Bioluminescence and Chemiluminescence in Woods Hole Massachusetts for his studies of recombinant luciferase. He is an expert on enzyme purification, immobilization and stabilization. Dr. Wang and coworkers have successfully expressed histidine tagged BCCP-luciferase in E. coli [23]. The technique enables the enzyme to be purified and immobilized in a one-step protocol. He has developed a technique of immobilizing luciferase with agarose. Such gels can be desiccated and successfully rehydrated with full enzymatic activity. This technique supported the success of the ATP sensing platform.

Dr. Wang is also knowledgeable in enzymatic assay development and dip-stick type biosensors. He successfully screened glucose and galactose with the newly developed ATP measuring platform. He is now working as a Research Scientist at PSI and is supervising a team that is continuing to develop the bioluminescence based direct reading quantitative biosensor for galactose. He will serve as the Principle Investigator for this research project. His biosketch is included.

Other Key Personnel

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

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

Dr. Joseph Andrade is founder, President, and CEO of Protein Solutions, Inc. Joe has worked extensively with proteins, enzymes and antibodies for the past 25 years, focusing his efforts on elucidating their behavior at surfaces and interfaces. Five years ago he became involved in bioluminescence particularly in firefly and bacteria luminescence systems. Joe will

be available to assist and consult in the areas of interfacial biochemistry, bioluminescence, and biosensor expertise when required.

D. Experimental Design and Methods

1. Overview of experimental design

The sequential reactions involved in the homocysteine biosensor are:

Sample pretreatment step: adenosine and ATP interference are consumed.

Adenosine + H_2O -> Inosine + NH_3

Enzyme: Adenosine deaminase (EC.3.5.4.4)

ATP + 2 H_2O -> AMP + 2 orthophosphate

Enzyme: Apyrase (EC.3.6.1.5)

Homocysteine conversion step:

Homocysteine + adenosine -> S-adenosyl-L-homocysteine

Enzyme: S-adenosyl-L-homocysteine hydrolase (SAH hydrolase, EC.3.3.1.1)

Adenosine analysis step:

Adenosine + ATP -> AMP + ADP

Enzyme: Adenosine kinase (EC.2.7.1.20)

ATP + Luciferin + O_2 -> Oxyluciferin + AMP + PPi + CO_2 + light (analyzed with luminometer)

Enzyme: Firefly luciferase (EC.1.13.12.7)

The time plan for the proposal is shown in Table 2.

Table 2. Time Plan

Topic\Month	1	2	3	4	5	6
Enzyme immobilization	—	—	—	→		
Aim 1: Developing methods for sample pre-treatment.		→				
Aim 2: Homocysteine conversion reaction			—	→		
Aim 3: Analyzing the adenosine content			—	→		
Aim 4: Calibration and standardization					→	
Aim 5: Pre-prototype homocysteine biosensor						→
Interlaboratory evaluation and final report					—	→

2. Availability of Enzymes

All the enzymes used in the assay, except adenosine kinase, are available from Sigma Chemical Co.. Adenosine kinase will be purified from bovine liver using the method of Elalaoui et al. [2]. Fresh bovine liver will be homogenized with homogenizer and centrifuged. The supernatant will be purified by ammonium sulfate fractionation. The fraction containing adenosine kinase will be loaded onto a 5'-AMP-Sepharose 4B affinity column for final purification. A specific activity of 3 unit/mg was reported by using this method [2]. Recombinant firefly luciferase, which catalyzes the bioluminescence reaction, is routinely purified in our laboratory [23].

E. Human Subjects (none)

F. Vertebrate Animals (none)

G. Consultants and Advisors

There are no consultants in phase I project. However, *Dr. George D. Markham*, Institute for Cancer Research, The Fox Chase Cancer Center, Dr. *Timothy A. Garrow*, University of Illinois, and *Dr. James Wu*, ARUP, who are experts in homocysteine analysis and related enzymes purification have been contacted. They have provided helpful suggestions and will be considered as consultants during the phase II project.

Dr. Larry Kricka, Director of the General Chemistry Laboratory and Professor of Pathology and Laboratory Medicine at the University of Pennsylvania, Philadelphia. Dr Kricka is internationally recognized for his work on applying bio- and chemi- luminescence to clinical chemistry. He is also the editor of the Journal of Bioluminescence and Chemiluminescence and editor/author of many books on Bio- and Chemi-luminescence in clinical biochemistry. He will provide appropriate advice and guidance to this project as part of his service on the Scientific Advisory Board.

Dr Joseph Andrade, Founder, President and CEO of Protein Solutions, Inc. Joe has worked extensively with proteins, enzymes and antibodies for the past 25 years, focusing his efforts on elucidating their behavior at surfaces and interfaces. Six years ago he became involved in bioluminescence particularly in firefly and bacteria luminescence systems. Joe will be available to assist and consult in the areas of interfacial biochemistry, bioluminescence, and biosensor expertise when required.

H. Contractual Arrangements (none)

I. Literature Cited

1. H. Refsum, S. Helland and P. M. Ueland, "Radioenzymic determination of homocysteine in plasma and urine," Clin. Chem., **31/4**, 624-628 (1985).

2. A. Elalaoui, G. Divita, G. Maury, J.-L. Imbach and R. S. Goody, "Intrinsic tryptophan fluorescence of bovine liver adenosine kinase, characterization of ligand binding sites and conformational changes," Eur. J. Biochem., **221**, 839-846 (1994).

3. "The metabolic and Molecular Bases of Inherited Disease," C. R. Scriver, A. L. Beaudet, W. S. Sly and D. Valle eds. McGraw-Hill, Inc., New York, pp.1279-1284 (1995).

4. O. Nygår, S. E. Vollset, H. Refsum, I. Stensvold, A. Tverdal J. E. Nordrehaug, P. M. Ueland, G. Kvåle. "Total Plasma Homocysteine and Cardiovascular Risk Profile." JAMA, **274**, 1526-33.

5. J. J. Genest, J. R. McNamara, D. N. Salem, P. W. F. Wilson, E. J. Schaefer, M. R. Malinow. "Plasma Homocyst(e)ine Levels in Men with Premature Coronary Artery Disease." JACC, **16**, 1114-9.

6. J. Selhub, P. F. Jacques, A. Bostom, R. B. D'Agostino, P. W. F. Wilson, A. J. Belanger, D. H. O'Leary, P. A. Wolf, E. J. Schaefer, I. H. Rosenberg. "Association between Plasma Homcysteine Concentrations and Extracranial Carotid-Artery Stenosis." The New England Journal of Medicine, **332**, 286-91.

7. E. L. Mayer, D. W. Jacobsen, K. Robinson. "Homcysteine and Coronary Atherosclerosis" JACC **27**, 517-27.

8. M. R. Malinow, S.S. Kang, L.M. Taylor, P.W.K. Wong, B. Coull, T. Inahara, d. Mukerjee, G.

Protein Solutions, Inc.

391 G Chipeta Way Suite 320 Salt Lake City, UT 84108 Phone/FAX 801-583-9301/801-583-4463

December 12, 1997

Division of Research Grants
National Institutes of Health
6701 Rockledge Drive
Room 1040 - MSC 7710
Bethesda, MD 20817

To Whom It May Concern:

Please find enclosed two copies and one original copy of a Phase I SBIR application titled "A Novel Enzyme Based Homocysteine Biosensor". I feel that this proposal is particularly responsive to the 1997 DHHS SBIR Solicitation Topic #110 (National Heart, Lung and Blood Institute, NHLBI).

Thank you for your consideration.

Sincerely,

Richard A. Van Wagenen, Ph.D.
Vice President for Research & Development

enclosure

121297C.DOC

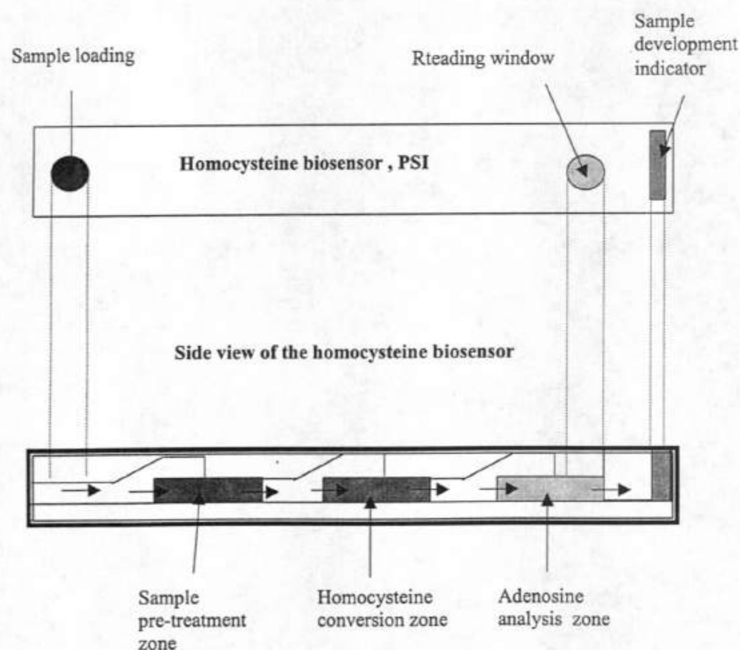


Figure 2. Pre-protype homocysteine biosensor. The sensor comprises three reaction zones. The dry reagent is released when sample flows through those zones. The arrow lines represent the flow direction of sample. After the indicator shows the reaction is complete (the appearance of color), the sensor will be inserted into the photodiode based luminometer to quantify the bioluminescence intensity. The bioluminescence intensity is directly related to the homocysteine level in the sample.

In the case of inherited diseases, such as the deficiency of cystathionine β-synthase (EC 4.2.1.22) or ATP:L-methionine S-adenosyltransferase (EC 2.5.1.6), homocysteine is accumulated and can be as high as 200 μmol/l [13]. Accumulation of plasma homocysteine may also be caused by dietary imbalances (vitamin B-6, B-12 and folate deficiency) [14]. To find homocysteine levels, a risk factor of cardiovascular disease, in the early stage or to monitor the treatment of hyperhomocysteinemia, we need a simple method to screen abnormal levels of homocysteine. Clinical labs have also reported an increased demand for testing homocysteine levels in plasma [13].

2. Difficulty of determination of homocysteine

The method for determining the homocysteine level must be specific, and the sensitivity must be high (less than 1 μmol/liter in the range of 0-20 μmol/liter). Several methods are currently used to determine the concentration of homocysteine in urine and blood (Table 1). Ubbink et al. developed a method that labeled homocysteine with thio-specific 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonic acid [15]. The fluorescent labeled amino acid was separated by high-performance liquid chromatography (HPLC) and detected with a fluorometer. Because the fluorescent derivatives are unstable, the sample preparation and calibration must be done with extraordinary care and precision [16]. The second method is mass spectrometry. A longer sample preparation time (2.5 hr) is required to make butyl ester derivatives and iostopes are used for quantification [17]. The tedious calibration procedure and expensive instrument make it less favorable.

Electrochemical detection, which coupled HPLC and electrochemical detector, was also used to measure homocysteine level [18]. This method is clean and can be automated. However, the degeneration of its detection unit, a gold-mercury electrode, may cause faulty results. The extra maintenance and high cost make it practical only in a clinical laboratory.

Enzymatic assays for homocysteine have also been developed. Unfortunately, there is no simple assay for homocysteine to date [13]. Radioenzymatic assays have great contamination problems. The development of a highly specific immunoassay for homocysteine is unsuccessful because of the small size of homocysteine and its structural similarity to cysteine, which is about 25 to 30 times more common than homocysteine [7]. An indirect immunoassay, which is specific for adenosylhomocysteine, was reported [19]. Because it is an indirect assay, repeated wash procedures make it difficult to design as a dip-stick type biosensor.

3. Advantages of proposed method over existing methods

The novel method assays the change of adenosine via ATP bioluminescence. No costly instrument is required to detect the bioluminescence intensity, which correlates to homocysteine level. This method also eliminates the need for preparing amino acid derivatives or the use of isotopes or fluorescent dyes. The simple design enables people using it at home without intense training. A comparison of existing methods and the proposed method is shown in Table 1.

The modularized design simplifies the work needed to develop other sensors in the future. We can change the enzyme and substrate modules and quickly convert the sensor for other metabolites. This design will be used in the development of amino acid panel later.

Table 1. Comparison of existing methods and the proposed method

Methods	Fluorescent labeling followed by HPLC separation	Mass spectrometry	HPLC and electrochemical detection	Enzymatic assay	**Proposed enzymatic homocysteine sensor**
Marker	Fluorescent dye e.g. 7-benzo-2-oxa-1,3-diazole-4-sulfonic acid (SBD)	Isotope e.g. $[^2H_3]$methionine or $[^2H_3]$homocysteine	None	1. Radio-labeled substrate or 2.Antibody	None
Special difficulty	Fluorescent derivatives are highly light-sensitive. Light exposure may lead to serious error.	Tedious sample preparation. Possible contamination of isotopes.	Deterioration of the gold-mercury electrode. Serial test (low-throughput)	1. Radiation exposure / isotopes contamination. 2. Require training	None
Time	>1.5 hr/batch	> 2.5 hr/batch	20-30 min/sample	> 2 hr/batch	< 30 min/batch
Instrument cost	44,000	63,000	32,000	<10,000	For photodiode based luminometer<100 PMT based luminometer: 4,000
Total cost	High	High	High	medium	Low
Home use	No	No	No	No	Yes