(1) NSF - MIN

COVER SHEET FOR PROPOSAL TO THE NATIONAL SCIENCE FOUNDATION

PROGRAM ANNOUNCEMENT/SOLICITATION NO./CLOSING DATE (If not in response to a program announcement/solicitation enter NSF 99-2)	FOR NSF USE ONLY
99-109 08/16/99	NSF PROPOSAL NUMBER
FOR CONSIDERATION BY NSF ORGANIZATION UNIT(S) (Indicate the most specific unit known, i.e. program, division, etc.)	
DIRECTORATE FOR ENGINEERING	

DATE RECEIVED	NUMBER OF COPIES	DIVISION ASSIGNED	FUND CODE	DUNS# (Data Universal Numbering System)	FILE LOCATION

EMPLOYER IDENTIFICATION NUMBER (EIN) OR TAXPAYER IDENTIFICATION NUMBER (TIN)	SHOW PREVIOUS AWARD NO. IF THIS IS ☐ A RENEWAL ☐ AN ACCOMPLISHMENT-BASED RENEWAL	IS THIS PROPOSAL BEING SUBMITTED TO ANOTHER FEDERAL AGENCY? YES ☐ NO ☒ IF YES, LIST ACRONYMS(S)
876000525		

NAME OF ORGANIZATION TO WHICH AWARD SHOULD BE MADE	ADDRESS OF AWARDEE ORGANIZATION, INCLUDING 9 DIGIT ZIP CODE
University of Utah	University of Utah 1471 Federal Way Salt Lake City, UT. 841021807
AWARDEE ORGANIZATION CODE (IF KNOWN)	
0036756000	
NAME OF PERFORMING ORGANIZATION, IF DIFFERENT FROM ABOVE	ADDRESS OF PERFORMING ORGANIZATION, IF DIFFERENT, INCLUDING 9 DIGIT ZIP CODE
PERFORMING ORGANIZATION CODE (IF KNOWN)	

IS AWARDEE ORGANIZATION (Check All That Apply) (See GPG II.D.1 For Definitions) ☐ FOR-PROFIT ORGANIZATION ☐ SMALL BUSINESS ☐ MINORITY BUSINESS ☐ WOMAN-OWNED BUSINESS

TITLE OF PROPOSED PROJECT **Structured Multi-Enzyme Nano-Complexes for Enhanced Biosensors**

REQUESTED AMOUNT	PROPOSED DURATION (1-60 MONTHS)	REQUESTED STARTING DATE	SHOW RELATED PREPROPOSAL NO., IF APPLICABLE
$ 99,351	24 months	12/01/99	

CHECK APPROPRIATE BOX(ES) IF THIS PROPOSAL INCLUDES ANY OF THE ITEMS LISTED BELOW

- ☐ BEGINNING INVESTIGATOR (GPG 1.A.3)
- ☐ DISCLOSURE OF LOBBYING ACTIVITIES (GPG II.D.1)
- ☐ PROPRIETARY & PRIVILEGED INFORMATION (GPG II.D.10)
- ☐ NATIONAL ENVIRONMENTAL POLICY ACT (GPG II.D.10)
- ☐ HISTORIC PLACES (GPG II.D.10)
- ☐ SMALL GRANT FOR EXPLOR. RESEARCH (SGER) (GPG II.D.12)
- ☐ GROUP PROPOSAL (GPG II.D.12)
- ☐ VERTEBRATE ANIMALS (GPG II.D.12) IACUC App. Date
- ☐ HUMAN SUBJECTS (GPG II.D.12) Exemption Subsection ____ or IRB App. Date
- ☐ INTERNATIONAL COOPERATIVE ACTIVITIES: COUNTRY/COUNTRIES
- ☐ FACILITATION FOR SCIENTISTS/ENGINEERS WITH DISABILITIES (GPG V.G.)
- ☐ RESEARCH OPPORTUNITY AWARD (GPG V.H)

PI/PD DEPARTMENT	PI/PD POSTAL ADDRESS
Department of Bioengineering	2480 MEB 50 S Campus Center Dr Salt Lake City, UT 84112-920 United States
PI/PD FAX NUMBER	
801-585-5361	

NAMES (TYPED)	High Degree	Yr of Degree	Telephone Number	Electronic Mail Address
PI/PD NAME Joseph D Andrade	Ph.D.	1969	801-581-4379	joe.andrade@m.cc.utah.edu
CO-PI/PD				
CO-PI/PD				
CO-PI/PD				
CO-PI/PD				

MIN - PhD Qualifying Exam

CERTIFICATION PAGE

Certification for Principal Investigators and Co-Principal Investigators:

I certify to the best of my knowledge that:

(1) the statements herein (excluding scientific hypotheses and scientific opinions) are true and complete, and
(2) the text and graphics herein as well as any accompanying publications or other documents, unless otherwise indicated, are the original work of the signatories or individuals working under their supervision. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if an award is made as a result of this application.

I understand that the willful provision of false information or concealing a material fact in this proposal or any other communication submitted to NSF is a criminal offense (U.S.Code, Title 18, Section 1001).

Name (Typed)	Signature	Social Security No.*	Date
PI/PD Joseph D Andrade		SSN's are confidential and are not displayed ON FASTLANE SUBMISSIONS	
Co-PI/PD			
Co-PI/PD			
Co-PI/PD			
Co-PI/PD			

Certification for Authorized Organizational Representative or Individual Applicant:

By signing and submitting this proposal, the individual applicant or the authorized official of the applicant institution is: (1) certifying that statements made herein are true and complete to the best of his/her knowledge; and (2) agreeing to accept the obligation to comply with NSF award terms and conditions if an award is made as a result of this application. Further, the applicant is hereby providing certifications regarding Federal debt status, debarment and suspension, drug-free workplace, and lobbying activities (see below), as set forth in Grant Proposal Guide (GPG), NSF 99-2. Willful provision of false information in this application and its supporting documents or in reports required under an ensuring award is a criminal offense (U. S. Code, Title 18, Section 1001).

In addition, if the applicant institution employs more than fifty persons, the authorized official of the applicant institution is certifying that the institution has implemented a written and enforced conflict of interest policy that is consistent with the provisions of Grant Policy Manual Section 510; that to the best of his/her knowledge, all financial disclosures required by that conflict of interest policy have been made; and that all identified conflicts of interest will have been satisfactorily managed, reduced or eliminated prior to the institution's expenditure of any funds under the award, in accordance with the institution's conflict of interest policy. Conflict which cannot be satisfactorily managed, reduced or eliminated must be disclosed to NSF.

Debt and Debarment Certifications

(If answer "yes" to either, please provide explanation.)

Is the organization delinquent on any Federal debt? Yes ☐ No ☒

Is the organization or its principals presently debarred, suspended, proposed for debarment, declared ineligible, or voluntarily excluded from covered transactions by any Federal department or agency? Yes ☐ No ☐

Certification Regarding Lobbying

This certification is required for an award of a Federal contract, grant, or cooperative agreement exceeding $100,000 and for an award of a Federal loan or a commitment providing for the United States to insure or guarantee a loan exceeding $150,000.

Certification for Contracts, Grants, Loans and Cooperative Agreements

The undersigned certifies, to the best of his or her knowledge and belief, that:

(1) No federal appropriated funds have been paid or will be paid, by or on behalf of the undersigned, to any person for influencing or attempting to influence an officer or employee of any agency, a Member of Congress, an officer or employee of Congress, or an employee of a Member of Congress in connection with the awarding of any federal contract, the making of any Federal grant, the making of any Federal loan, the entering into of any cooperative agreement, and the extension, continuation, renewal, amendment, or modification of any Federal contract, grant, loan, or cooperative agreement.

(2) If any funds other than Federal appropriated funds have been paid or will be paid to any person for influencing or attempting to influence an officer or employee of any agency, a Member of Congress, an officer or employee of Congress, or an employee of a Member of Congress in connection with this Federal contract, grant, loan, or cooperative agreement, the undersigned shall complete and submit Standard Form-LLL, "Disclosure Form to Report Lobbying," in accordance with its instructions.

(3) The undersigned shall require that the language of this certification be included in the award documents for all subawards at all tiers including subcontracts, subgrants, and contracts under grants, loans, and cooperative agreements and that all subrecipients shall certify and disclose accordingly.

This certification is a material representation of fact upon which reliance was placed when this transaction was made or entered into. Submission of this certification is a prerequisite for making or entering into this transaction imposed by section 1352, title 31, U.S. Code. Any person who fails to file the required certification shall be subject to a civil penalty of not less than $10,000 and not more than $100,000 for each such failure.

AUTHORIZED ORGANIZATIONAL REPRESENTATIVE	SIGNATURE	DATE
NAME/TITLE (TYPED)		

TELEPHONE NUMBER	ELECTRONIC MAIL ADDRESS	FAX NUMBER

*SUBMISSION OF SOCIAL SECURITY NUMBERS IS VOLUNTARY AND WILL NOT AFFECT THE ORGANIZATION'S ELIGIBILITY FOR AN AWARD. HOWEVER, THEY ARE AN INTEGRAL PART OF THE INFORMATION SYSTEM AND ASSIST IN PROCESSING THE PROPOSAL. SSN SOLICITED UNDER NSF ACT OF 1950, AS AMENDED.

Project Summary

We will soon have incredibly sophisticated means with which to assess our genetic makeup and therefore our susceptibility to genetic diseases. In the next decade or two we will also know the result of that genetic coding, our specific individual proteome. But the output of the proteome — our specific individual metabolism — is virtually inaccessible today and there appears to be relatively little activity in addressing the situation. Our specific, individual metabolism — our biochemical individuality — is called the metabolome.

The sensitive and specific analysis of practically all major metabolites and nutrients can be performed by several bioluminescent enzyme systems (luciferases). Bioluminescent analysis may be made even more sensitive, suitable for analysis of sub-micromolar concentrations in microliter volumes, using organized multienzyme nanocomplexes. The **key objective** of this feasibility study in the Nanoscale Biodevices program is to engineer such multienzyme complexes, based initially on the bacterial luciferase system, which are likely to be orders of magnitude more "efficient" than present bioluminescence technology, thus permitting a relatively small 100 microliter blood droplet to be used to simultaneously measure some 20 to 50 different analytes.

One objective of this proposal is to recombinantly produce specifically biotinylated phenylalanine dehydrogenase, which can then be complexed to both engineered bacterial luciferase and oxidoreductase, hopefully producing efficient, bioluminescent phenylalanine-specific multienzyme nanocomplexes.

With such technology, we will be in a position (in phase 2 of the Nanoscale Biodevices Program) to design and construct additional analyte-specific nanocomplexes and to begin the design and development of a dipstick type analytical device which will literally be able to measure metabolism. **Our goal** is to measure 30 to 50 individual, distinct metabolites, including all of the amino acids, the major carbohydrates, key vitamins, several intermediary metabolites, and a number of other important biochemicals, resulting in a chemical "image" of basic metabolism.

We are tentatively calling the dipstick portion of this analytical system the MM chip, the metabolic map chip. Initially the MM chip would contain an active analytical zone about 4-5 mm diameter with 20 to 50 different, specific, spatially separated analytical regions. The entire chip would be imaged in time using a small CCD luminometer whose output will provide a chemical "image" of metabolism.

Project Description

1. Perspective

There has been a great deal of activity and progress in the study of the **genome**. We now have the complete genomes of a number of organisms; the entire human genome sequence will be available in the next few years. Since genes code for proteins, there is now growing interest in the proteome (1). The **proteome** may be defined as the complete set of properties and characteristics of the proteins which are coded by the genome. It is likely that much of the proteome, including the human proteome, will be known within the next decade or two.

Many and perhaps most of the proteins in the proteome, however, are enzymes, and many if not most of those enzymes are directly involved in metabolism. **Metabolism** is defined as all biochemical reactions in a cell, both anabolic and catabolic (2). Metabolism is a subject which many believe was largely completed several decades ago and is well covered in basic biochemistry texts. Although basic metabolic reactions and indeed complex metabolic pathways are well known and readily available (www.expasy.ch/cgi-bin/search-biochem-index), the incredible complexity of metabolism is really only now beginning to be addressed (3). The relatively new fields of metabolic control analysis (http://ir2lcb.cnrs-mrs.fr/~athel/mcafaq.htm), metabolic engineering (2), and metabolic mapping are dealing with the complexity inherent in thousands of biochemical reactions which are all interdependent. Given the importance of metabolism it is surprising that there has been relatively little effort in attempting to analyze and measure key metabolites.

Although clinical chemistry and analytical laboratories can routinely measure carbohydrates, amino acids, vitamins and other low molecular weight biochemicals important to metabolism (4), in practice most of the major metabolites are rarely measured. One would think that the circulating plasma levels of the 20 major amino acids would be easily and readily measured, given today's technologies and analytical capabilities. This is not the case. It is relatively unusual for a physician or a nutritionist to order a total amino acid analysis, because it is expensive. Indeed the direction in clinical medicine has been to order less chemical or analytical tests rather than more. This of course has to do with the interest in minimizing health care costs and thus in minimizing "unnecessary tests". The expense of most tests, given typical cost-benefit analyses, argues against more comprehensive biochemical analyses.

We will soon have incredibly sophisticated means with which to assess our genetic makeup and therefore our susceptibility to genetic diseases. In the next decade or two we will also know the result of that genetic coding, our specific individual proteome. But the **output of the proteome — our specific individual metabolism**, is virtually inaccessible today and there appears to be relatively little activity in addressing the situation. Our specific, **individual** metabolism — our biochemical individuality (5,6) — is called the **metabolome**.

The measurement of low molecular weight metabolites relies almost exclusively on separation methods: high-pressure liquid chromatography, mass spectrometry, and gas chromatography (4). With the practical exception of glucose, inexpensive means for the specific, quantitative, and highly reliable analysis of key low molecular weight metabolites are not readily available. Because there are literally millions of diabetics who must monitor glucose at least several times per day, there has been an economic incentive for the development of dipstick type, reliable, quantitative sensors and simple, inexpensive, but highly reliable instruments — glucometers — for the measurement of glucose (7). This development has been made possible by the strong commercial incentive, but also because glucose is easy to measure, as it is present in high concentration (millimolar). The measurement of other key metabolites, including nearly all of the amino acids, generally requires more sensitive means of analysis (4). The relatively simple colorimetric and electrochemical methods used for the analysis of glucose (7) and several other analytes of wide

interest (cholesterol, lactate) are generally not sensitive enough for the analysis of most other metabolites, many of which are in the micromolar range.

The diabetes community is leading and driving major research and development activities to further improve the measurement and monitoring of glucose and of other metabolites important to diabetes, with an emphasis on sampling methods which do not involve the trauma and discomfort of blood sampling. Considerable research and development is now being focused upon minimally invasive approaches for obtaining samples of interstitial fluids for glucose analysis. Such fluid can be collected from the skin epidermal layer, which is devoid of blood vessels or nerves. The process is therefore painless and bloodless. There are many groups developing means for interstitial fluid collection and analysis (www.jdfcure.com and www.diabetes.org). It is likely that these efforts will be successful and that truly, minimally invasive, painless means for acquiring samples for biochemical analysis will become available in the very near future.

A problem with minimally invasive approaches to sampling is that the volume collected is often one microliter or less, presenting a considerable challenge for current analytical methods of detection and measurement. A typical blood glucose sample is about 15 microliters.

2. Progress to Date and General Objectives

Some years ago (8) we became interested in the application of bioluminescence (11) as a means by which to analyze a wide range of important metabolites. We also became interested at that time in patient empowerment (9,10) and in-home based medicine (www.ee.cua.edu/~winters/HCTWorkshop). We felt strongly that if patients were going to be significantly involved in the management of their health, they were going to need tools by which to monitor and measure their physical and chemical states. Again, with the exception of glucose and cholesterol, there are no chemical tools for patients to use in the home environment for the monitoring and measurement of their metabolism or nutrition.

Bioluminescence has the advantage that it is generally at least 100 times more sensitive than common spectroscopic/colorimetric methods (11,12). Bioluminescence is light produced by biological compounds undergoing specific, enzyme catalyzed chemical reactions. The most well known example is the firefly; however, other organisms employ similar reactions to produce light, e.g., bacteria, fish, and fungi (12). All bioluminescent reactions employ an enzyme called a luciferase, e.g. there is a firefly luciferase, a bacterial luciferase, etc. although the luciferases are all different. The bioluminescence of fireflies is based on the enzyme catalyzed oxidation of luciferin utilizing adenosine triphosphate (ATP) as a highly specific co-reactant. The blue bioluminescence of marine bacteria is closely coupled to a reduced nicotinamide adenine dinucleotide (NADH)-dependent enzyme reaction. ATP and NADH represent the "energy currency" of metabolism—of all life forms on the planet.

Thus, Mother Nature has literally given us two unique, ultra sensitive and highly specific reactions for the measurement and monitoring of ATP and of NADH (and NADPH). The readout is photons, green-yellow in the case of the firefly reaction and blue for the bacterial reaction. The reactions are highly sensitive to ATP or NADH/NADPH over 5 orders of magnitude in concentration (11). Since most of biochemistry depends on ATP and/or NADH, practically all metabolic reactions can be monitored by bioluminescence via one or more enzyme catalyzed linked reactions.

There is a large body of literature on the development of sensors for ATP- and NADH-dependent processes, using the firefly luciferase and bacterial luciferase enzyme reactions, respectively. For some of the analytes of interest, the bioluminescence is of sufficient intensity that the unaided eye can serve as the detection instrument (10), although generally, a relatively

inexpensive charge coupled device (CCD) is used for detection. For very low concentration analytes, a photomultiplier tube (PMT) based luminometer is used (11). The intensity of bioluminescence measured can be related to the concentration of the specific biochemical of interest in the sample.

Several years ago, sponsored by the Whitaker Foundation/National Science Foundation Program in Cost Reducing Health Care Technologies (CRHCT) (www.healthtechcosts.med.utah.edu), and in collaboration with a local company, Protein Solutions, Inc. (www.proteinsolutions.com), we began a project to develop dipstick sensors for the monitoring and management of selected inborn errors of metabolism (13): phenylalanine in the case of phenylketonuria (PKU) and galactose in the case of galactosemia. We have also done preliminary work on sensors for lactate, creatine, creatinine, and tyrosine.

Although our original goal was the development of simple dipstick devices which would not require an instrument (instrument less biosensors) (8,10), our interactions with the clinical community have taught us that they prefer an instrument be used in order to provide an objective number, to record data, to process and analyze data and establish trends, and to transfer that data and those trends to the health care provider. Although we are still interested in instrument less biosensors, it is also clear that having an instrument with which to read the signal produced from such sensors greatly expands their analytical capabilities. The remarkable sensitivity of bioluminescence, coupled with the sensitivity of a modern handheld, CCD-based luminometer, makes possible the measurement of micromolar concentrations in small samples.

It has become clear that virtually any metabolite whose circulating blood level or urine level is in the micromolar range or higher can be readily measured by a dipstick type bioluminescence procedure, using less than 100 microliters of sample. Thus it is perfectly reasonable to develop a galactose sensor for the management of galactosemia (14), a phenylalanine sensor for the management of PKU (18), a creatinine sensor for the monitoring and management of renal transplant patients, etc.

What is not possible is the analysis of **many different metabolites at the same time on the same sample**, ideally a very small sample which, even more ideally, is noninvasively derived.

A very sensitive and very efficient technique is required to specifically and sensitively measure the wide range of different analytes in an extremely small sample. Although bioluminescence is incredibly sensitive, and is comparable in sensitivity to fluorescence and radioisotope methods, not even bioluminescence has the sensitivity for our ultimate goals. We suggest that bioluminescence can, however, be made orders of magnitude more sensitive.

The key objective of this feasibility study in the Nanoscale Biodevices program is to engineer multienzyme complexes, based initially on the bacterial luciferase system, which are one to several orders of magnitude more "efficient" than the present technology, thus permitting a relatively small 100 microliter blood droplet to be used to simultaneously measure some 20 to 50 **different** analytes using a modern, inexpensive, hand-held luminometer.

These multienzyme nanocomplexes will include the substrate specific enzyme (for example, a dehydrogenase), the bacterial oxidoreductase enzyme, and the bacterial luciferase enzyme (see Technical Background). Such a structured complex will result in far more efficient mass transport and possibly even some rudimentary channeling (16), likely producing a far more efficient reaction. The application of such a multienzyme nanocomplex to a biosensor for phenylalanine would likely permit practical analysis using very small submicroliter samples.

During the past several years we have learned how to engineer and produce modified recombinant luciferases, both firefly (17) and bacterial (15), and recombinant, modified oxidoreductase (15). We have produced these enzymes with a polyhistidine tail, permitting a highly efficient, single step purification using a nickel chelate column. In addition these enzymes have been produced with the so-called BCCP domain (19), which results, via a post translational modification, in a biotin group expressed in a precise position and orientation on that domain. The presence of this stereospecific biotin functionality permits these enzymes to be easily immobilized to avidin or streptavidin proteins (15,20). We thus have the means to study the self-assembly of luciferase/oxidoreductase enzyme nanocomplexes through biotin-strepavidin interactions.

One objective of this proposal is to recombinantly produce specifically biotinylated phenylalanine dehydrogenase, which can then be complexed to both bacterial luciferase and oxidoreductase to produce efficient, bioluminescent phenylalanine-specific multienzyme nanocomplexes. Given that we already have considerable experience with assays for phenylalanine using standard bioluminescence technologies (18), we can easily assess the feasibility of structured multienzyme nanocomplexes of these proteins for such sensor applications.

We now have experience with several key enabling technologies for this project. Given that we demonstrate feasibility in the construction, characterization, and application of these multienzyme bioluminescent nanocomplexes, we will be in a position (in phase 2 of the Nanoscale Biodevices Program) to begin the design and development of a dipstick type analytical device which will literally be able to measure metabolism. Our goal is to measure 30 to 50 individual, distinct metabolites, including all of the amino acids, the major carbohydrates, key vitamins, several intermediary metabolites, and a number of other important biochemicals. We will have, therefore, a chemical "image" of basic metabolism. Imagine a metabolic pathway chart, and imagine that each major biochemical of clinical or nutritional importance in the chart is measured. The analytical information can be presented in a visual and spatial pattern, literally providing a **chemical image of metabolism** — a **metabolic map**. A good metaphor is a world map with the population (concentration) of each major city (metabolite) indicated by the diameter (bioluminescent intensity) of its location symbol. We are tentatively calling the dipstick portion of this analytical system the **MMchip**, the metabolic map chip. Initially the MM chip would contain an active analytical zone about 4-5 mm diameter — suitable for a 100 microliter droplet. The entire chip would be imaged in time using a small CCD luminometer detecting the 20 to 50 different, specific, spatially separated analytical regions.

3. Results from prior NSF support

The PI's current major grant is from a joint NSF-Whitaker Foundation Program on Cost Reducing Health Care Technologies (CRHCT). The grant was awarded July 1, 1997 for three years. We have just started the third year of the grant. The original proposal was reviewed by NSF procedures; those grants selected for funding were funded either by NSF or by the Whitaker Foundation. Although our grant received its funding via the Whitaker Foundation, it is part of the joint program and it is appropriate for our progress and results to be noted here. The annual progress reports (submitted to Whitaker Foundation and to Drs. Fouke and Devey at NSF) can be viewed on the project web site: www.healthtechcosts.med.utah.edu . Most of the papers published are listed in the Biosketches and included in References 50 and 51.

4. Technical Background

A critical factor in the design of an optimal biosensor is maximal retention of activity and

stability of biological molecules. Immobilization of enzymes by covalent attachment, microencapsulation, adsorption, or cross-linking generally extends the shelf-life of the sensor, allowing significant simplification of the analytical apparatus and permitting reuse of the sensor, resulting in reduced costs (21,22). Sequential enzyme (or multienzyme) systems are often used for various biosensors. Partially organized enzymes, through coimmobilization, generally show better kinetics than free or individually immobilized enzymes, due to matrix effects, diffusional effects, and proximity effects (23).

However, using traditional coimmobilization, the probability of the intermediate reacting with the second enzyme is still relatively low, because enzymes are randomly and one-dimensionally bound on a solid surface (Fig. 1(a)). The product of the first reaction diffuses into the bulk solution, so only a few molecules will collide with and interact with the second enzyme. Such effects are even more pronounced if multiple enzymes are involved. In addition, if one of intermediates in a multienzyme system is unstable (for example, it is quickly oxidized by oxygen), diffusion distance/time is also important for the reaction efficiency.

Cell metabolism is characterized by the action of sequences of enzymes involving chemical reactions and diffusion processes. In the cell, proteins are densely and efficiently organized. For example, the density of soluble enzymes in the cytosol is so high as to create a gellike state (23,24); in mitochondria, citric acid cycle enzymes are organized into a mutienzyme cluster (25); in the citric acid cycle, α-keto acid dehydrogenase system is an enzyme complex consisting of three enzymes (26). Most sequential enzymes form associations or complexes in order to process intermediates efficiently with minimal "waste" (27). Enzyme reactions in such natural environments are kinetically different from typical *in vitro* reaction conditions: clustered enzymes generally show faster kinetics than diluted (or isolated) enzymes (28-30).

Channeling is a mechanism whereby metabolites are transferred efficiently from enzyme to enzyme. There are two different channeling types: "direct transfer" and "indirect transfer." Direct transfer channeling is the site-to-site transfer of metabolic intermediates between enzyme molecules without allowing the diffusion of intermediates into the bulk solution. In indirect transfer channeling, the intermediate dissociates from the enzyme producing it but remains near the second enzyme, resulting in a higher probability of reaction with the second enzyme. In principle, metabolic channeling could be enhanced if the sequentially acting enzymes are maintained in close proximity (23,24,30).

Protein associations in the cell are often very delicate and their complex structure is generally not preserved *in vitro*. Association of citric acid cycle enzymes, for example, showed specific enzyme-enzyme interactions, dependant on the particular solution environment, and involving unidentified components (25). The study of cell metabolism based on kinetic data with isolated enzymes in vitro does not come close to physiological conditions. The intrinsic properties of enzymes in vitro are generally different from the *in vivo* situation, due to different microenvironments.

Construction of protein complexes *in vitro*, as a mimic of native intracellular enzyme organization, is of interest in various biotechnology areas, including biosensors. One method to construct such complexes is by using genetically fused and/or specifically cross-linked proteins.

The avidin (or streptavidin)-biotin system is been applied for protein immobilization due to its strong binding interaction ($K_a=10^{15}$/M): biotinylated proteins can be conveniently immobilized onto surfaces containing streptavidins (20). Conventional enzyme biotinylation introduces biotin chemically through a functional group reaction (generally amino groups); problems include enzyme inactivation and random and multiple biotinylation (31). Specifically biotinylated proteins, produced by fusion with the biotin carboxy carrier protein (BCCP), are of specific interest. BCCP

is one of three functional subunits of the acetyl-CoA carboxylase of *E. coli*. Biotin, derived from the culture medium, is attached post-translationally to a **specific** C-terminal lysine residue of BCCP by biotin ligase. The 87 residue C-terminal of BCCP (BCCP domain, 9 kDa) has been shown to be sufficient for biotinylation (19,32).

Avidin (or streptavidin) is a tetramer (15 kDa monomer) with four binding sites for biotin. Streptavidin is usually used for fusion proteins, due to its easy expression in *E. coli*. Several groups have produced streptavidin-enzyme hybrids (33-35). Biotinylated protein and streptavidin hybrids can be cross-linked to each other.

Bacterial bioluminescence is an interesting model system for the study of channeling and is critical to bioluminescence-based biosensors. This system consists of two enzymes, bacterial luciferase (luciferase) and FMN:NAD(P)H oxidoreductase (oxidoreductase) (15). The luciferase, a heterodimeric protein (α(*LuxA*, 40 kDa) and β(*LuxB*, 37 kDa) subunits), catalyzes the reaction of reduced flavin mononucleotide ($FMNH_2$), a long-chain aliphatic aldehyde (RCHO), and molecular oxygen to yield flavin mononucleotide (FMN), the aliphatic carboxylic acid (RCOOH), and blue-green light:

$$FMNH_2 + O_2 + RCHO \rightarrow FMN + RCOOH + H_2O + \text{light} \quad [1]$$

In bioluminescent bacteria, $FMNH_2$ is generated by the oxidoreductase, a homodimeric enzyme (24.5 kDa monomer) (43), which catalyzes the reduction of FMN at the expense of reduced pyridine nucleotides (NADH or NADPH):

$$NAD(P)H + FMN + H^+ \rightarrow FMNH_2 + NAD(P)^+ \quad [2]$$

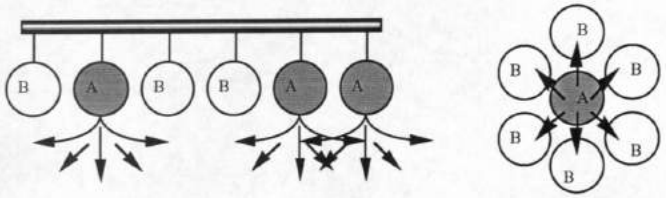
In the coupled reaction of oxidoreductase and luciferase, production of light by the luciferase is directly proportional to the NAD(P)H concentration at limiting concentration of NAD(P)H. In addition, when the oxidoreductase-luciferase system is coupled to NAD(P)H-dependent enzymatic reactions, such as dehydrogenase reactions, light production can be proportional to the concentration of the substrate of the dehydrogenase reaction, and thereby used as a sensitive method to quantify medically important metabolites, including lactate and phenylalanine (11,18,36).

The fact that the luciferase uses $FMNH_2$ as a substrate is an important issue. Free $FMNH_2$ is subject to rapid autooxidation (k=10/sec, 25 °C) in the presence of oxygen; the autooxidation of $FMNH_2$ is 20 times faster than its enzymatic oxidation (37). This non-enzymatic oxidation of $FMNH_2$ competes with the enzymatic oxidation which produces light. Many research groups have coimmobilized both the oxidoreductase and the luciferase on solid surfaces, because they believed the organized oxidoreductase-luciferase system should have a higher probability of $FMNH_2$ interactions with the luciferase before the non-enzymatic oxidation, by decreasing the distance between both enzymes. The coimmobilized oxidoreductase-luciferase system showed higher bioluminescence activity than the free enzymes at a low enzyme concentration (15,36).

In vitro enzyme catalytic turnover times are typically in the range of 1 msec, which is very slow when compared to diffusion coefficients (about 1 $\mu cm^2/sec$) of small molecules (metabolites) in water (24). When one enzyme reacts with its substrate *in vitro*, its products will diffuse into the bulk solution. In addition, each enzyme has specific activity; the first enzyme usually has higher activity than the second enzyme in enzyme cascades. For example, the specific activity of oxidoreductase is nearly higher than that of luciferase: the oxidoreductase turns over 580 times faster than the luciferase in the bioluminescent bacterium, *Vibrio fischeri* (38). In bioluminescent bacteria, the amount of luciferase is 30 to 60 times more than oxidoreductase (38). Thus, in

sequential enzyme systems, to increase the possibility of the intermediate reacting with the second enzyme it is likely that the first enzyme must be surrounded by as many of the second enzymes as possible (Fig. 1(b)).

We propose the construction of a structured enzyme complex using three engineered enzymes produced by genetic recombinant means and using biotin-streptavidin interactions for self-assembly. The phenylalanine dehydrogenase/oxidoreductase/luciferase system (Fig. 2) will be used as a model system, because it is very organization-sensitive and bio-medically relevant.



(a) (b)

Fig. 1. (a) Utilization of intermediates produced by the first enzyme (A) in a cascade using traditional, random coimmobilization. Most intermediates diffuse away and very few interact with the second enzyme (B). (b) Effective enzyme organization in a sequential enzyme system. The first enzyme (A) is surrounded by the second enzyme (B), resulting in an enhancement of the reaction of the product of the first enzyme with the second enzyme.

PDH

$$\text{phenylalanine} + NAD^+ + H_2O \rightarrow \text{phenylpyruvate} + NADH + NH_4^+$$

Oxidoreductase

$$NADH + H^+ + FMN \rightarrow NAD^+ + FMNH_2$$

Luciferase

$$FMNH_2 + RCHO + O_2 \rightarrow FMN + RCOOH + H_2O + \text{light (490 nm)}$$

Fig. 2. The phenylalanine dehydrogenase/oxidoreductase/luciferase system. The phenylalanine concentration is measured using bacterial bioluminescence.

5. PI's Goal

The PI's goal is to develop technologies and devices for patient empowerment and for the characterization of biochemical individuality—the metabolome—with the goal of improving health and well being while decreasing the costs of health care. A specific goal is to develop sensitive, multi-analyte, and competitive biosensors based on multienzyme systems. To achieve this goal, enzyme organization in the sensor is important, affecting system efficiency, sensitivity,

dimensions, and cost. The PI and Dr. Dong Min, postdoctoral fellow, wish to construct spatially localized protein complexes using streptavidin-biotin interactions and DNA recombinant technology. We want to use the PDH/oxidoreductase/luciferase system, because it can detect the medically and nutritionally important metabolite phenylalanine and serves as an excellent model system.

Dr. Min recently completed his Ph.D. studies on the bioluminescent measurement of phenylalanine and lactate using bacterial oxidoreductase and luciferase.

6. Objectives

The general objectives were noted earlier, including the goal for Phase II of the Nanoscale Biodevices program. This Phase I study has the specific research objectives and Time Plan given in Table 1. The main categories are:

1) construction of plasmids,
2) expression and purification of fused proteins,
3) construction of protein complexes,
4) immobilization of the final protein complex, and
5) characterization of activity of the immobilized protein complex

The tasks are described in detail in the Experimental Section; a very brief description follows:

Table 1. Research objectives and time plan.

Task	1st Year	2nd Year
Construction of plasmids	→	
Expression and purification of fusion proteins	→	
Construction of protein complexes	→	
Immobilization of the final protein complex	→	
Characterization of activity of the immobilized complex	→	

BCCP, *Lux* A and B(*V. harvei*), *FRase I* (FMN:NAD(P)H oxidoreductase of*V. fischeri*), *streptavidin*, and *PDH* (phenylalanine dehydrogenase) genes will be prepared by PCR. A *streptavidin* gene will be ligated to the 5' end of *LuxA* gene (α subunit), and then inserted into an expression vector (plasmid I). A *LuxB* gene (β subunit) will be inserted into an expression vector (plasmid II). Both plasmids will be coexpressed in an expression *E. coli* strain, resulting in the production of a hybrid protein: a streptavidin connected with four luciferases. The *BCCP* gene will be ligated to the 5' end of the *FRase I* gene, inserted into an expression vector (plasmid III), and expressed in *E. coli*, resulting in the production of biotinylated dimeric oxidoreductase (each side of dimeric oxidoreductase is connected to a BCCP domain). The *BCCP* gene will be ligated to the 3' end of the *PDH* gene (plasmid IV) and biotinylated octamer PDH (from *Bacillus badius*) will be produced (each subunit is connected to a BCCP domain).

To form a protein complex, the hybrid streptavidin/luciferase proteins will be mixed *in vitro* with biotinylated dimeric oxidoreductases, resulting in a dimeric oxidoreductase surrounded by eight luciferases (oxidoreductase/luciferase complex). Then, the oxidoreductase/luciferase complex

and the biotinylated PDH are mixed *in vitro* and self-assembled to a three enzyme complex: a PDH molecule is surrounded by eight dimeric oxidoreductases which are in turn surrounded by eight luciferases (PDH/oxidoreductase/luciferase complex).

The complexes will be characterized and purified by gel filtration chromatography. The PDH/oxidoreductase/luciferase complex will be immobilized on avidin-conjugated surfaces and its bioluminescence output and efficiency will be measured.

7. Significance

The general significance of the work was discussed earlier in the sections on Perspective and Background.

Biochips, including DNA and peptide chips, are being developed and applied to screening many samples simultaneously: currently, three gene-chips, an HIV chip, p53 chip, and cytochrome P450 chip, are available commercially (39). "Bio-enzyme chips" are needed for metabolite screening. The efficient and condensed enzyme organization proposed here are required to develop enzyme chips. Our goal is the development of a dipstick-like device (called tentatively the **MM-Chip**) which can literally measure some 30 to 50 key metabolites in a single 100 microliter sample. Such chips could be produced in very large quantities and used for a wide range of general and specialized measurement applications, including research. The proposed protein complexes are a mimic of the native intracellular protein organizations, and will be used in the study of channeling and of multienzyme interactions. Multienzyme systems can also used for biochemical and biopharmaceutical syntheses. The reaction efficiency of enzymes determines the cost of synthesized products.

The MM-Chip would be a multichannel, bioluminescence-based analytical device to sense and quantitatively measure up to 50 metabolites; such a chip would, literally, quantitatively "image" metabolism. The image of a PKU patient's metabolism would be dramatically different from that of a galactosemic patient, or a diabetic. The ability to measure – to IMAGE, in chemical terms – metabolism would be a great asset to research as well as to clinical and preventative medicine, including nutrition.

It is economically very difficult to address the needs of PKU patients (the disease incidence is one in 10,000 in the USA) because the market is so small. Development of tools for the management of Galactosemia is even more difficult, as the incidence is about one in 50,000. Such "orphan diseases" (13) are technologically neglected. At the recent home telehealth workshop sponsored by NSF and the Food and Drug Administration (www.ee.cua.edu/~winters/HCTWorkshop) it became clear to us that the only practical way to address this problem is via technology and economies of scale.

So **a major benefit of such a chip will be reduced cost**. Using the ubiquitous microprocessor as a metaphor, Metabolite Chips could be manufactured by the millions, thus greatly reducing the cost per chip. A physician, patient, or consumer need not use all the data generated, but merely focus on the several channels of immediate clinical need and interest. The other channels could simply be ignored by the custom programmable device, much as is now the case with microprocessors used for specific, embedded, applications

The other great advantage is reduced sample volume. Using only about 100 microliters of blood or urine, 50 simultaneous, specific analyses could be performed, using only 1 microliter of sample per analysis channel. Such low volume is possible due to the great sensitivity of the enhanced bioluminescent approach to enzyme-based substrate analysis made possible by structured multienzyme nanocomplexes (this proposal).

The design and development of an MM Chip is a major Phase II activity, as well as the design and construction of many different analyte-specific bioluminescent multienzyme nanocomplexes. Our experience in the biosensor measurement of phenylalanine and other amino acids, glucose, galactose, galactose-1-P, creatinine, creatine, and ATP – all current activities – provide a strong foundation for the design and construction of such complexes and for the development of the MM-Chip.

8. Experimental methods

8-1. Construction of the oxidoreductase/luciferase complex (Fig. 3)

The *streptavidin* gene (from *Streptomyces avidinii*), the *Lux*A gene and the *Lux*B gene (from *V. harvei*), the *BCCP* gene, and *FRase I* gene will be obtained by PCR from the genomic DNA or the plasmids having those genes (15,40).

The *streptavidin* gene will be ligated to the 5' end of the *Lux*A gene and then inserted into an expression vector pACYC184 (New England, Beverly, MA) (plasmid I). The *Lux*B gene will be individually inserted into an expression vector pET28C (Novagen, Madison, WI) (plasmid II). An His-tag will be introduced onto the end of N-termini of the β subunit of luciferase as a result of subcloning, because the pET28C encodes an His-tag upstream of the multiple cloning sites. Both plasmids will be cloned in a cloning host, *E. coli* DH5α (Novagen). Positive both plasmids will be coexpressed in a expression host, *E. coli* HMS174(DE3)pLysS (Novagen). Expression cells must carry pLysS plasmid because streptavidin is toxic to *E. coli*. (33). In addition, to ensure complete formation of the hybrid protein, an excess of the β subunit of luciferase is generated by strong promoter while the streptavidin/α subunit of luciferase is expressed from a weak promoter (41). Several groups (33,35,41,42) showed a multimeric hybrid protein with multimeric proteins or a hybrid streptavidin with proteins. Thus, since the natural streptavidin subunit forms a tetramer, a streptavidin will be connected with four luciferases as post-translational processes.

The *BCCP* gene will be ligated to the 5' end of the *FRase I* gene and then inserted into the pET28C (plasmid III). Positive plasmid III obtained from *E. coli* DH5α will be expressed in a expression host, Nova Blue(DE3) (Novagen). A homodimeric oxidoreductase will be connected with two BCCP domains at its opposite sides, as well as an H-tag will be introduced onto the end of N-termini of the fused protein as a result of subcloning. Cell culturing will follow a previous method (15). Terrific Broth and Luria-Bertani media with kanamycin will be used for the hybrid streptavidin/luciferase and for the hybrid BCCP/oxidoreductase, respectively. The BCCP domain can be fully biotinylated by adding excess free biotin to the medium and by coexpression with biotin ligase (15).

The hybrid streptavidin/luciferase and BCCP/oxidoreductase proteins will be purified using Ni-NTA columns (Qiagen, Valencia, CA) (15). Frozen cell pellets will be resuspended in lysis buffer (0.1 M phosphate, 0.5 M NaCl, 10 %(v/v) glycerol, 60 mM imidazole, 0.1 %(v/v) Tween 20, 1 mM phenylmethylsulfonyl fluoride, and 10 mM mercaptoethanol, pH 7.0). The resuspended cells will be sonicated in order to break the cell and be centrifuged. The supernatant will be applied to a Ni-NTA column pre-equilibrated with wash buffer (0.1 M phosphate, 0.5 M NaCl, 10 %(v/v) glycerol, and 60 mM imidazole, pH 7.0). The Ni-NTA column will be washed four times with five packed bead volumes of the wash buffer, then eluted with elution buffer (0.1 M phosphate, 0.5 M NaCl, 10 %(v/v) glycerol, and 200 mM imidazole, pH 7.0). Each fraction will be monitored for the fused proteins on native and/or denatured PAGE gels as well as by gel filtration chromatography. The positive fractions will be pooled and buffer exchanged into 0.1 M phosphate

(pH 7.0) using a prepacked Sephadex G-25 column (Pharmacia, Piscataway, NJ). The desalted fused proteins will be concentrated with "centricon®" (Amicon, Beverly, MA) and then stored at –70 °C until used. The activity of oxidoreductase and luciferase in purified hybrid proteins will be determined spectrophotometrically and by bioluminescence assay, respectively (15).

To construct a protein complex, hybrid streptavidin/luciferase and BCCP/oxidoreductase proteins will be mixed together *in vitro* to facilitate biotin-streptavidin interactions; gel filtration chromatography will be used to select the correctly formed protein complex. A dimeric oxidoreductase should be surrounded by eight luciferases.

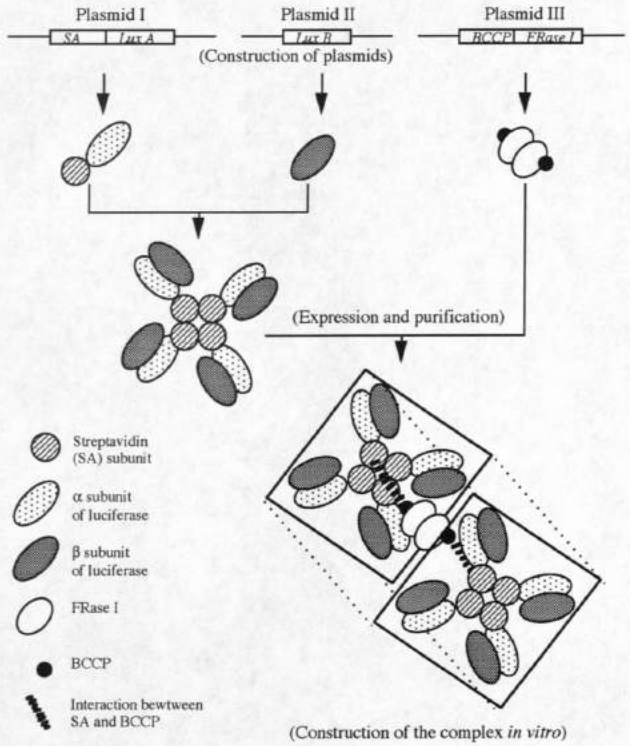


Fig. 3. Construction of the oxidoreductase/luciferase complex. A dimeric oxidoreductase is surrounded by eight luciferases.

Steric effects can predicted from geometrical calculations (Fig. 4). The dimensions of streptavidin, dimeric oxidoreductase, and luciferase are about 54x58x48 Å (44), 36x60x40 Å (45), and 75x45x40 Å (46), respectively. Assuming protein molecules are approximately spherical in shape and the size of the small BCCP domain is ignored, the radii of streptavidin (R_1), dimeric oxidoreductase (R_2), and luciferase (R_3) are about 33.0, 27.4, and 31.8 Å, respectively. In Fig. 4, if the protein molecules are in contact each other and other effects, including charge, are ignored, the length of "side O" is $2(R_1+R_2)$, which is 120.8 Å, and is larger than the diameter of luciferase, 63.6 Å. The length of "line Q", between streptavidin and luciferase molecules, is R1+R3, which is 64.8 Å. Assuming the angle between the streptavidin-luciferase A and streptavidin-luciferase B is 90°, the length of "side P" calculated is 91.6 Å, which is larger than the diameter of luciferase. We believe that the construction of this complex will not be greatly hindered by steric effects.

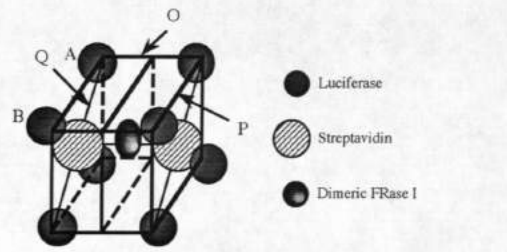


Fig. 4. Structure of the oxidoreductase/luciferase complex. No serious steric effects are evident.

8-2. Construction of the PDH/oxidoreductase/luciferase complex (Fig. 5)

The *PDH* gene will be obtained by PCR from the genomic DNA of *Bacillus badius*. The PDH from *Bacillus badius* is an octamer (47). The *BCCP* gene will be ligated to the 3' end of the *PDH* gene and be inserted into the pET28C (plasmid IV). Only PDH from *Rhodococcus sp.* M4 is discovered recently its crystal structure: the N-terminal domain contributes to the subunit-subunit interactions (48). Since we believed that most PHDs have similar structure, we choose the C-terminal of the PDH as a fusion site. Positive plasmid IV obtained from *E. coli* DH5α will be expressed in Nova Blue(DE3). Hybrid PDH/BCCP protein will be purified using Ni-NTA columns as mentioned above. Each subunit of the PDH should be connected with a BCCP domain.

To construct a PDH/oxidoreductase/luciferase complex, the oxidoreductase/luciferase complex and the hybrid PDH/BCCP protein will be mixed together *in vitro* to facilitate biotin-streptavidin interactions; gel filtration chromatography will also be used to select the correctly formed protein complex. Each octameric PDH should be surrounded by eight oxidoreductase/luciferase complexes. This structure of the PDH/oxidoreductase/luciferase complex is reasonable, because the specific activity of PDH (partially purified) from *Bacillus badius* and oxidoreductase and luciferase from *V. fischeri* are 70 U/mg (47), 18 U/mg (11), and 3 U/mg (11), respectively, where 1U equals to 1.67×10^{-8} mole/sec.

The calculated dimension of the oxidoreductase/luciferase complex is about 185x155x155 Å. Although the dimensions of PDH from *Bacillus badius* are not known, the dimensions of the octameric leucine dehydrogenase from *Bacillus badius*,120x100x100 Å (49), can be used, because both dehydrogenases have similar morphology and subunit molecular weight. The radii of the

oxidoreductase/luciferase complex (r_1) and the PDH molecule (r_2) are about 102 and 70 Å, respectively. In Fig. 6, if the oxidoreductase/luciferase complex and the PDH molecule are in contact each other and other effects, including charge, are ignored, the length of the "diagonal line Q" is $2(r_1+r_2)$, which is 344 Å. If the PDH/oxidoreductase/luciferase complex is a cubic, the length of "side P" is 199 Å, which is a little shorter than the diameter of the complex, 204 Å. If the dimension of the BCCP domain and the flexibility of protein molecules are considered, the steric effects will not be serious.

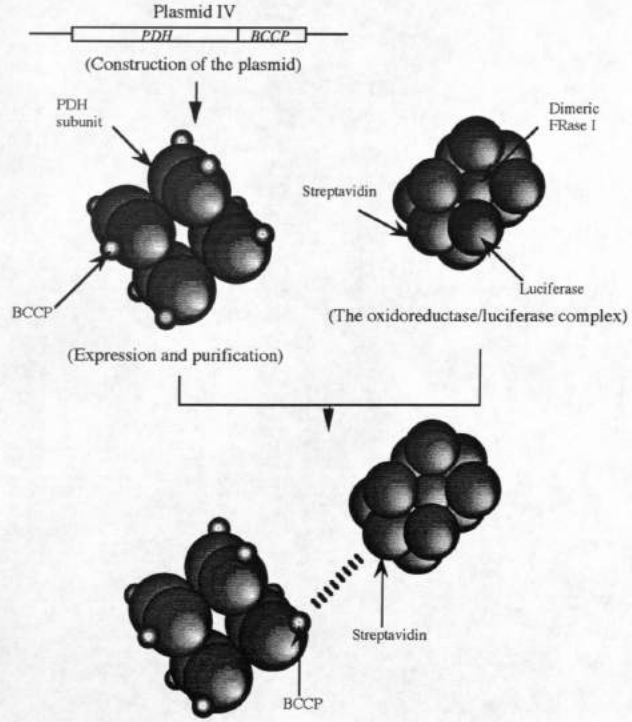


Fig. 5. Construction of the PDH/oxidoreductase/luciferase complex. A octameric PDH is surrounded by eight oxidoreductase/luciferase complexes.

8-3. Immobilization of the PDH/oxidoreductase/luciferase complex (Fig. 6)

The protein complex will be immobilized on biotin-conjugated surfaces prepared commercially. We will purchase biotin-conjugated acrylic beads from Sigma (St. Louis, MO). If a longer spacer between the bead surface and the complex, due to size of the complex, is needed, we will modify the bead surface. PEO-biotin dimer (Pierce, Rockford, IL), in which biotins are connected to both sides of the PEO chain (spacer length: 43 Å), and avidin-conjugated acrylic beads (Sigma) will be mixed to create a biotin-conjugated surface having a long spacer. The complex will be immobilized using a packed column with acrylic beads prepared previously, as before (15). The complex will be bound on the beads through streptavidin-biotin interactions. Bioluminescence activity and stability of the immobilized complex will be measured.

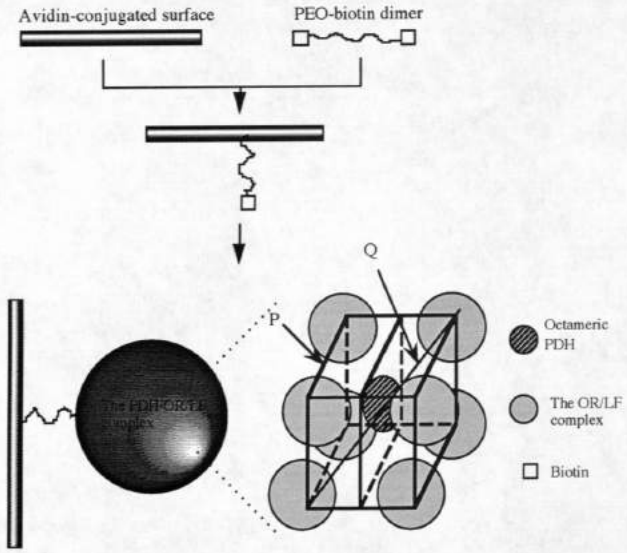


Fig. 6. Immobilization and structure of the PDH/oxidoreductase/luciferase complex. The complex will be immobilized on the biotin-conjugated surface, through streptavidin-biotin interactions. The steric effects are not serious. OR: oxidoreductase, LF: luciferase.

8-4. Evaluation of the PDH/oxidoreductase/luciferase complex

To evaluate the effect of protein organization, we will compare the results with free and randomly coimmobilized enzymes at the same assay conditions, in terms of flash peak light intensity, light decay rate, photons produced at a given time, detection limit, and linearity. Light intensity is related to the distance between enzyme molecules. For example, at a given substrate

amount, its concentration relates to solution volume by;

$$[S]_{v1}=(V_2/V_1)[S]_{v2} \qquad [3]$$

where $[S]_{v1}$ and $[S]_{v2}$ is a substrate concentration at solution volume V_1 and V_2, respectively. In the multienzyme system, a "reaction volume", the space needed for products of the first enzyme to reach the second enzyme, determines the initial concentration of intermediates reacting with the second enzyme. The reaction volume depends on the distance between sequentially reacting enzyme molecules, so longer distance between enzyme molecules results in lower initial concentration of intermediates.

Assuming enzyme reactions are faster than the diffusion rate and the autooxidation of $FMNH_2$ is ignored, in the oxidoreductase/luciferase complex light intensity (I) at a given initial NADH concentration will be changed by the distance between oxidoreductase and luciferase, because the light intensity is proportional to a limited substrate concentration and the volume equals $3\pi(\text{radius})^3/4$;

$$I_{d1}=(d_2/d_1)^3 I_{d2} \qquad [4]$$

where d_2 is a mean distance between both enzymes randomly distributed in the assay solution and d_1 is the distance in the complex. Since d_1 is usually much shorter than d_2 and d_2/d_1 is a cubic factor, the value of I_{d1} for the complex will be much higher than that of I_{d2} in the solution.

Likewise, in the PDH/oxidoreductase/luciferase complex, light intensity at a given initial phenylalanine concentration will be influenced by the distance between PDH and oxidoreductase, as well as by the distance between oxidoreductase and luciferase;

$$I_{d3,d1}=(d_4/d_3)^3(d_2/d_1)^3 I_{d4,d2} \qquad [5]$$

where d_4 is a mean distance between PDH and oxidoreductase in the assay solution and d_3 is the distance between PDH and oxidoreductase in the complex. The value of $I_{d3,d1}$ for the complex will be much higher than that of $I_{d4,d2}$ in the solution. These results show that the effect of the distance between enzyme molecules on the output becomes more critical with increasing number of enzymes.

The average distance between PDH and oxidoreductase and between oxidoreductase and luciferase in a 200 microliter solution having 10 picomole PDH, 160 picomole oxidoreductase, and 640 picomole luciferase (i.e. PDH:oxidoreductase (dimeric):luciferase=1:8:64 in molecular number) is about 1000 Å and 500 Å, respectively. In the PDH/oxidoreductase/luciferase complex, PDH is about 10 Å away and luciferase is about 90 Å away from oxidoreductase. This analysis indicates that the light intensity of the complex may be up to 8-orders higher than in free enzyme. Since the light intensity of the randomly coimmobilized enzyme system is 1- to 2-orders higher than in free solution, the complex produces up to 6-orders more light than traditional coimmobilization. However, this result will be greatly influenced by enzyme specific activities, the migration of substrates or products, steric hindrance effects, and the autooxidation of $FMNH_2$.

This preliminary analysis suggests that biosensors constructed with bioluminescent multienzyme nanocomplexes will show much high sensitivity and an expanded linear range, resulting in ultrasensitive high resolution biosensors using very small sample volumes.

References

1. Hodges, P. E., et al. "Yeast Proteome Database (YPD)," Nucleic Acids Res., **27**(1999)69-73.
2. Stephanopoulos, G. N., et al. **Metabolic Engineering**, Academic Press, 1998.
3. Fell, D. **Understanding the Control of Metabolism**, Portland Press, 1997.
4. Bishop, M. L., et al. **Clinical Chemistry**, Lippincott, 1996.
5. Williams, R. J. **Biochemical Individuality**, U. Texas Press, 1956.
6. Childs, B. "A Logic of Disease," Chapter 2 in Ref. 14, pp. 229-258.
7. Henning, T.P. and Cunningham, D.D. "Biosensors for Personal Diabetes Management," in **Commercial Biosensors**, G. Ramsay, ed., Wiley, 1998, pp. 3-46.
8. Andrade, J. D., et al. "Direct Reading Biosensors: Analytical Chemistry without Instruments," Proc. Int. Symp. Cont. Release Bioactive Mater. 23 (1996)
9. Andrade, J. D. **Medical and Biological Engineering in the Future of Health Care,** Univ. of Utah Press, 1994.
10. Andrade, J.D., et al. "Toward Dollar Devices for Measuring Metabolic Biochemistry" in Sawan, S.P. and Manivannan, G., eds, **Anti-microbial, Anti-infective Materials,** Technomic Publishing Co., 1999, in press.
11. Brolin, S. and Wettermark, G. **Bioluminescence Analysis**, VCH Pub., 1998.
12. Campbell, A. **Chemiluminescence,** VCH Publ., 1989.
13. Scriver, C. R., et al., **Metabolic and Molecular Bases of Inherited Disease**, 7th Ed., 3 Volumes, McGraw-Hill, 1995.
14. Eu, J-Y, Wang, C.-Y., and Andrade, J. D. (1999). "Homogeneous Bioluminescence Assay for Galactosuria: Interference and Kinetic Analysis," Anal. Biochem. **271**, 168-176.
15. Min, D. J., Andrade, J. D., and Stewart, R. J. (1999). Specific immobilization of *in vivo* biotinylated NAD(P)H:FMN oxidoreductase and bacterial luciferase. Anal. Biochem. **270**, 133-139.
16. Agius, L. and Sherratt, H.S.A., **Channeling in Intermediary Metabolism**, Portland Press, 1997; and Ovadi, J. **Cell Architecture and Metabolic Channeling**, Landes, 1995.
17. Wang, C-Y, Hitz, S., Andrade, J.D., and Stewart, R. (1997). "Specific Immobilization of Firefly Luciferase through a Biotin Carboxyl Carrier Protein Domain," Anal. Biochem., **246** 133-139.
18. Min, D. J., Stewart, R. J., and Andrade, J. D. (1999). Developing a biosensor for L-phenylalanine based on bacterial bioluminescence. in **Bioluminescence and chemiluminescence: perspectives for the 21st century** (Roda, A., Pazzagli, M., Kricker, L. J., and Stanley, P. E., eds.), Wiley, London, p. 520-523.
19. Chapman-Smith, A., Turner, D. L., Cronan, J. E., Morris, T. W., and Wallace, J. C. (1994). Expression, biotinylation and purification of a biotin-domain peptide from the biotin carboxy carrier protein of *Eschrichia coli* acetyl-CoA carboxylase. Biochem. J. **302**, 881-887.
20. Wilchek, M. and Bayer, E. A. (1990). Introduction to avidin-biotin technology. Methods Enzymol. **184**, 5-13.
21. Luong, J. H. T., Bouvrette, P., and Male, K. B. (1997). Developments and applications of biosensors in food analysis. Trends Biotechnol. **15**, 369-377.
22. Scouten, W. H., Luong, J. H. T., and Brown, R. S. (1995). Enzyme or protein immobilization techniques for applications in biosensor design. Trends Biotechnol. **13**, 178-185.
23. Welch, G. R. **Organized multienzyme systems**., Academic Press, New York, 1985.
24. Westerhoff, H. V. and Welch, G. R. (1992). Enzyme organization and the direction of metabolic flow: physicochemical considerations. Curr. Top. Cell. Regul. **33**, 361-390.
25. Barnes, S. J. and Weitzman, P. D. J. (1986). Organization of citric acid cycle enzymes into a multienzyme cluster. FEBS Letters **201**, 267-270.
26. Oliver, R. M. and Reed, L. J. (1982). Multienzyme complexes. Electron Microscopy of Proteins **2**, 1-48.
27. Srere, P. A. (1987). Complexes of sequential metabolic enzymes. Ann. Rev. Biochem. **56**, 89-124.
28. Sauro, H. M. and Kacser, H. (1990). Enzyme-enzyme interactions and control analysis: 2. The case of non-independence: heterologous associations. Eur. J. Biochem. **187**, 493-500.
29. Garner, M. M. and Burg, M. B. (1994). Macromolecular crowding and confinement in cells exposed to hypertonicity. Am. J. Physiol. **266**, C877-C892.
30. Kholodenko, B. N., Rohwer, J. M., Cascante, M., and Westerhoff, H. V. (1998). Subtleties in control by metabolic channelling and enzyme organization. Mol. Cell. Biochem. **184**, 311-320.
31. Bayer, E. A. and Wilchek, M. (1990). Protein biotinylation. Methods Enzymol. **184**, 138-160.
32. Li, S. J. and Cronan, J. E. (1992). The gene encoding the biotin carboxylase subunit of *Eschrichia coli* acetyl-CoA carboxylase. J. Biol. Chem. **267**, 855-863.
33. Sano, T. and Cantor, C. R. (1991). A streptavidin-protein A chimera that allows one-step production of a variety of specific antibody conjugates. Bio/Technology **9**, 1378-1381.
34. Dubel, S., Breitling, F., Kontermann, R., Schmidt, T., Skerra, A., and Little, M. (1995). Bifunctional and multimeric complexes of streptavidin fused to single chain antibodies (scFv). J. Immunol. Methods **178**, 201-209.
35. Karp, M., Lindqvist, C., Nissinen, R., Wahlbeck, S., Akerman, K., and Oker-Blom, C. (1996). Identification of biotinylated molecules using a baculovirus-expressed luciferase-streptavidin fusion protein. BioTechniques **20**, 452-459.
36. Wienhausen, G. and DeLuca, M. (1982). Bioluminescent assays of picomole levels of various metabolites using immobilized enzymes. Anal. Biochem. **127**, 380-388.
37. Meighen, E. A. and Hastings, J. W. (1971). Binding site determination from kinetic data: reduced flavin mononucleotide binding to bacterial luciferase. J. Biol. Chem. **246**, 7666-7674.
38. Jablonski, E. and DeLuca, M. (1977). Purification and properties of the NADH and NADPH specific FMN oxidoreductase from *Beneckea harveyi*. Biochem. **16**, 2932-2936.
39. Marshall, A. and Hodgson, J. (1998). DNA chips: An array of possibilities. Nature Biothech. **16**, 27-31.
40. Argarana, C. E., Kuntz, I. D., Birken, S., Axel, R., and Cantor, C. R. (1986). Molecular cloning and nucleotide sequence of the streptavidin gene. Nucleic Acids Res. **14**, 1871-1882.
41. Lindbladh, C., Persson, M., Bulow, L., and Mosbach, K. (1992). Characterization of a recombinant bifunctional enzyme, galactose dehydrogenase/bacterial luciferase, displaying an improved bioluminescence in a three-enzyme system. Eur. J. Biochem. **204**, 241-247.
42. Ljungcrantz, P., Carlsson, H., Mansson, M. O., Buckel, P., Mosbach, K., and Bulow, L. (1989). Construction of an artificial bifunctional enzyme, _-galactosidase/galactose dehydrogenase, exhibiting efficient galactose channeling. Biochem. **28**, 8786-8792.
43. Koike, H., Sasaki, H., Kobori, T., Zenno, S., Saigo, K., Murphy, M. E. P., Adman, E. T., and Tanokura, M. (1998). 1.8 Å crystal structure of the major NAD(P)H:FMN oxidoreductase of a bioluminescent bacterium, *vibrio fischeri*: overall structure, cofactor and substrate-analog binding, and comparison with related flavoproteins. J. Mol. Biol. **280**, 259-273.
44. Hendrickson, W. A., Pahler, A., Smith, J. L., Satow, Y., Merritt, E. A., and Paul Phizackerley, R. (1989). Crystal structure of core streptavidin determined from multiwavelength anomalous diffraction of synchrotron radiation. Proc. Natl. Acad. Sci. USA **86**, 2190-2194.
45. Tanner, J. J., Lei, B., Tu, S. C., and Krause, K. L. (1996). Flavin reductase P: Structure of a dimeric enzyme that reduces flavin. Biochem. **35**, 13531-13539.
46. Fisher, A. J., Thompson, T. B., Thoden, J. B., Baldwin, T. O., and Rayment, I. (1996). The 1.5-Å resolution crystal structure of bacterial luciferase in low salt conditions. J. Biol. Chem. **271**, 21956-21968.

47. Asano, Y., Nakazawa, A., Endo, K., Hibino, Y., Ohmori, M., Numao, N., and Kondo, K. (1987). Phenylalanine dehydrogenase of *Bacillus badius*: Purification, characterization, and gene cloning. Eur. J. Biochem. **168**, 153-159.
48. Vanhooke, J. L., Thoden, J. B., Brunhuber, N. M. W., Blanchard, J. S., and Holden, H. M. (1999). Phenylalanine dehydrogenase from *Rhodococcus sp.* M4: High-resolution X-ray analyses of inhibitory ternary complexes reveal key features in the oxidative deamination mechanism. Biochem. **38**, 2326-2339.
49. Baker, P. J., Turnbull, A. P., Sedelnikova, S. E., Stillman, T. J., and Rice, D. W. (1995). A role for quaternary structure in the sunstrate specificity of leucine dehydrogenase. Structure **3**, 693-705.
50. Papers Published and in Press from the NSF-Whitaker CRHCT grant (see page 4).
1. "Toward Dollar Devices for Measuring Metabolic Biochemistry," (J.D. Andrade, C.Y. Wang, D.-J. Min, et al.) in S.P.Sawan and G. Manivannan, eds, Anti-microbial, Anti-infective Materials, Technomic Publishing Co., 1999 (in press).
2. "Bioluminescence Assay for Galactokinase Activity," J. Eu and J.D. Andrade Bioluminescence and Chemiluminescence: Perspectives for the 21st Century, A.Roda, et al., eds., Wiley, 1999, pp. 489-492.
3. "Specific Immobilization of invivo Biotinylated-Bacterial Luciferase and FMN:NAD(P)H Oxidoreductase," D.J.Min, J.D.Andrade, and R.J.Stewart, Anal. Biochem.,270(1999)133-139.
4. "Biosensor for Phenylalanine based on Bacterial Bioluminescence," D.J.Min, R.J.Stewart, and J.D.Andrade, in Bioluminescence and Chemiluminescence: Perspectives for the 21st Century, A.Roda, et al., eds., Wiley, 1999, pp. 520-523.
5. "Homogeneous Bioluminescence Assay for Galactosuria: Interference and Kinetic Analysis,"J-Y Eu, C-Y Wang, and JD Andrade, Anal. Biochem., 271(1999)168-176.
6. "Thin-layer ion-exchange chromatography of proteins," Q. Luo, J.D.Andrade, K.Caldwell, J. Chromatography A, 816(1998)97-105.
7. "Cooperative adsorption of Proteins onto Hydroxyapatite," Q. Luo and J.D. Andrade, J. Colloid Interface Sci. 200(1998)104-113.
8. "Behavior of Model Proteins, Pretreated in Urea and/or Dithiothreitol, at Air/Solution Interfaces," D. J. Min and J.D. Andrade, J. Colloid Interface Sci. 197(1998)43-47.
9. "Minimizing Interferences in Multi-Element Analysis by ICP-MS," C.-S. Hsiung, J.D. Andrade, R. Costa, and K.O. Ash, Clinical Chemistry 43(1997)2303-2311.
10. "Specific Immobilization of Firefly Luciferase through a Biotin Carboxyl Carrier Protein Domain," C-Y Wang, S. Hitz, J.D. Andrade, and R. Stewart, Analytical Biochemistry, 246(1997)133-139.
11. "Biotinylation of Firefly Luciferase in Vivo: Purification and Immobilization of Bifunctional Recombinant Luciferase," (CY Wang, S Hitz, JD Andrade, RJ Stewart) in W.J. Hastings, L.J. Kricka, P.E. Stanley, eds, Bioluminescence and Chemiluminescence: Molecular Reporting with Photons, Wiley, 1997, pp. 224-227.
12. "Surfactants and Coenzyme A as Cooperative Enhancer of the Activity of Firefly Luciferase," (C-Y Wang and J.D. Andrade) in W.J. Hastings, L.J. Kricka, P.E. Stanley, eds, Bioluminescence and Chemiluminescence: Molecular Reporting with Photons, Wiley, 1997, pp. 253-256.
13. "Preliminary Study of the Optimum Conditions for a Lactate Sensor Based on Bacterial Bioluminescence," (D.J. Min and J.D. Andrade) in W.J. Hastings, L.J. Kricka, P.E. Stanley, eds, Bioluminescence and Chemiluminescence: Molecular Reporting with Photons, Wiley, 1997, pp. 275-278.
51. Papers Planned and in Preparation from the NSF-Whitaker CRHCT grant (see page 4).
1. Book: We are planning to produce a book based on our Symposia at the Feb. 2000 AAAS meeting, on the three meetings of our CRHCT National Advisory Board, and on our experience and work during the three years of this grant.
2. Theme issue of journal: We are assessing the feasibility of organizing a theme issue of a major technical journal, wherein the activities of many investigators in the CRHCT program could be represented.
3. R. Huefner, Simple Rules for Preliminary Assessment of the Cost Effectiveness of Proposed New Technologies and Devices, journal to be selected.
4. N. Waitzman and collaborators, Effects of Testing Frequency on Phenylalanine Levels in PKU, Amer. J. Public Health, to be submitted.
5. N. Waitzman and Collaborators, Effect of a Free Diet on Phenylalanine Levels in PKU, journal to be selected.
6. R. Huefner, Progressive Precision: An Iterative Model for Cost-Benefit Analyses of very early stage Technologies, journal to be selected.
7. R. Huefner, "Orphan" Devices for Rare Conditions: Incentives and Assessment, journal to be selected.
8. E. Clark, A Pediatrician's Technology Wish List for Bioengineers, probably Ann. Biomed. Engrg.
9. S. Kern, Healthcare Economics Issues for Medical Technology Developers, journal to be selected.
10. S. Kern, Cost to Whom? Understanding the Impact of Technology on Healthcare Costs, journal to be selected.
11. R. Davies, J. Andrade, et al., A Homogeneous, Bioluminescence Assay for Creatine and Creatinine, Anal Biochem., to be submitted.
12. A set of technical biosensor papers will be presented at the 11th International Symp on Bio- and Chemi-luminescence, Asilomar, Calif. in Sept. 2000; extended abstracts will appear in the Proceedings; full papers will be submitted to the appropriate technical journals.