Abstract of Research Plan

NAME, ADDRESS, AND TELEPHONE NUMBER OF APPLICANT ORGANIZATION

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YEAR FIRM FOUNDED	NO. OF EMPLOYEES (include all affiliates)
1987	6

TITLE OF APPLICATION

Microbe Detection via Stress-Enhanced ATP Release

KEY PERSONNEL ENGAGED ON PROJECT

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

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

The firefly luciferase-luciferin reaction has been commercially developed to detect microbes via the ATP from cell lysis. There are two serious problems, however. First, luminometers are expensive and require training to use. Second, there is a complicated series of steps required to collect the microbes and lyse their membranes to release the ATP; and the chemicals used for cell lysis often compromise the luciferin reaction. As a result, the approach, while very sensitive, is not as quantitative or reproducible as desired for most hygiene monitoring needs.

Bacteria use a family of enzymes (autolysins) to remodel and engineer their cell membranes and wall in response to internal stresses generated during the cell growth cycle. This proposal addresses the incorporation of autolysins in disposable devices in which stress can be directly applied to the bacterial cell wall, making the wall susceptible to autolysis, which results in the release of ATP which can then be quantified via the firefly luciferase-luciferin reaction. This approach eliminates the need for chemicals which compromise and complicate the reaction. Phase I investigates various autolysins and the role of applied stress. Phase II will address prototype development and partial speciation via selective culture media. The long range goal is to develop inexpensive, disposable, direct-reading biosensors for point of care monitoring of bacteria and to facilitate improved hygiene practices.

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

ATP, bacteria, microbe, autolysin, bioluminescence, firefly, hygiene monitoring

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

A need exists for simple, inexpensive, rapid, quantitative and sensitive methods for microorganism detection and quantification. This would encourage better sanitation and hygienic practices in hospitals, clinics, food processing and even the home.

Budget Justification

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

Dr. R. Van Wagenen, Principle Investigator, will devote 35 percent of his time supervising this six month project. He will manage and coordinate all of the activities as well as contribute technical advice and effort in the area of optics, data analyses, prototype sensor design, and materials issues.

Dr. Rob Scheer, Research Scientist, will address the micro-mechanics and micro-stress issues associated with stress-induced cell membrane release of ATP augmented by autolysins. He will design and construct the micro-stress grinder proposed for this work and he will conduct most of the actual experiments.

C-Y Wang, Research Scientist, has extensive experience with the firefly luciferase reaction for ATP analysis. He will be largely responsible for the luciferase/ATP studies.

A microbiology technician will be appointed to maintain the bacterial samples for deposition on surfaces and perform the stress autolysin experiments under the direction of Drs. Scheer and Wang.

J.D. Andrade, although not budgeted in the proposal, will be available to provide support, assistance, and advice as needed.

Total Direct Costs are projected to be $74,900. Total direct personnel costs are $33,500 and fringe benefits are based on 20 percent of direct personnel costs. The companies current indirect cost rate of 60 percent was established recently with the National Science Foundation on a Phase II STTR grant.

No consultants, subcontracts, patient care costs or fees are proposed for this work. Requested funds for equipment, supplies, travel, and miscellaneous other expenses are all below the limits which require detailed itemization.

Resources

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

Protein Solutions, Inc. (PSI) occupies 1,200 square feet of research laboratory and office space located in the Research Park adjacent to the University of Utah. This space includes a chemistry lab, a biology-cell culture lab, an optics lab, and two offices. The space and equipment are adequate for the work proposed with the addition of the equipment funding requested in this proposal. Office equipment is standard and is adequate to address the administrative aspects of the project.
The company has a Technology Transfer Agreement with the University of Utah which allows for the transfer of jointly developed technologies to PSI. The company is also a member of The Center for Biopolymers at Interfaces (CBI) a state-university-industry consortium which is one of the Centers for Excellence in the Utah State System of higher education. CBI membership provides a number of key benefits including: (1) fee for service access to many analytical services at the University of Utah at a very low rate, e.g. the SEM/TEM facility, the surface analytical facility (XPS, SIMS, etc.), (2) access to specialized lab equipment in the departments of Bioengineering and Chemistry, and (3) access to faculty members who can provide expertise on a consulting basis (although no consulting funds are requested for this work). Finally, the close proximity to the University of Utah makes it easy to utilize machine shop and electronic shop capabilities at rates that are competitive for the Wasatch front.

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 balance, stirrers, hot plates, fume hood, oven, water bath, some cell culture facilities, steam autoclave, reverse osmosis water, luminometers, optics and refrigerator. A charge coupled device (CCD) array camera and associated computer and controller are also available for the recording and quantification of luminescence experiments.

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Vice President for Resarch and Development

Current Employment

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

Education

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

Professional Experience

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

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

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

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

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

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

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

Honors

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

Representative Patents and Publications:

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

Research Plan

A. Specific Aims

The basic hypothesis of this Phase I proposal is that a mechanically stressed bacterial cell wall is far more susceptible to autolytic attack than an unstressed or less stressed wall (4,8,13).

The overall goal of this Phase I feasibility project is to answer the following questions.

- Are autolysins sufficiently effective in degrading bacterial cell walls to provide enhanced ATP release?
- Can direct mechanical stress cause cell wall destruction and subsequent ATP release?
- Can simultaneous stress and autolysins, lead to significantly enhanced cell wall disruption and ATP release?

We propose to:

1. Assess the efficacy of several known, commercially available autolysins for ATP release from one gram negative and one gram positive bacterial species.
2. Assess the suitability of several common abrasives to mechanically stress bacterial cell walls.
3. Immobilize the most effective autolysins on the most effective abrasives by means which minimize loss in autolysin activity.
4. Study the efficacy of several autolysin/abrasive combinations on the release of ATP under controlled stress conditions.

B. Identification and Significance of the Problem

Opportunistic microbes and antibiotic resistant bacteria are growing concerns across the entire spectrum of human activity. There is growing interest in, and need for, means for the detection of microbes, not only to aid in diagnosis and treatment, but to facilitate the development and monitoring of improved hygienic practices in the hospital and clinic; in the food and dairy process industries; and even in fast food, supermarket, and home environments. Historically, such monitoring has generally been accomplished by culture methods. The development of dry film culture devices with enhanced means of colony detection has made the culture approach applicable to a much wider audience, but even this approach, perhaps best exemplified by 3M's Petri Film dry film media (21,22), generally requires 24 to 48 hours before the plastic plates can be read.

The most rapid method of hygiene monitoring today is the application of the firefly luciferase reaction to monitor the ATP released from cells after appropriate membrane disruption (1,2). The firefly luciferase/luciferin bioluminescence reaction requires ATP as a co-reactant. This reaction is specific for, and highly sensitive to, ATP concentration. It is now the method of choice for the quantitative, highly sensitive measurement of ATP.

We have developed recombinant firefly luciferases and are applying these unique proteins to the development of a range of biochemical sensors involving substrates and enzymes which produce or consume ATP, which is then detected by a unique firefly luciferase-based, disposable, quantitative sensor (23,34,35).

Table 1

Rapid Hygiene Monitoring Using ATP-Bioluminescence Detection

Protocol	Problems or Disadvantages	Proposed "Solutions"
1. swab surface to sample bacteria	swabbing incomplete; swab effects surface effects	analyze directly on surface of interest (Phase II) (increases sensitivity)
2. transfer swab to cuvette containing surfactants, destabilize membrane, release ATP	ATP-ases also released, decreasing ATP	use AMP, ADP, ATP enzymes to regenerate ATP (Phase II) (increases sensitivity)
3. add luciferase/luciferin reagent	surfactants inhibit luciferase	eliminate surfactants, use autolysins to degrade cell envelope-do not affect luciferase, enhance autolysis with stress via abrasives (Phase I) (increases sensitivity)
4. bioluminescence quantified in PMT luminometer	requires instrument, expensive, qualitative	use area detection-film or electronic means for higher sensitivity film allows inexpensive detection (Phase II)

The normal procedure for hygiene monitoring using the existing generation of ATP bioluminescence kits is to swab the surface of the device of interest (using an ATP-free swab) to collect a sample of the suspect microbes. Refer to Table 1. The swab is then placed in a tube containing a cocktail of chemicals, generally containing strong ionic surfactants designed to disrupt the microbe's membrane, thereby releasing ATP and other intracellular components (1,2). That complex solution, now containing the released ATP, may be further treated with means to inhibit the ATP-ases also released, which serve to lower the ATP concentrations. A luciferase/luciferin solution is then added, possibly including various surfactants and other enhancers of the bioluminescence reaction. The remaining ATP in the solution, derived from the disruption of the microbe membranes, interacts with the luciferase/luciferin and oxygen to produce bioluminescence, which is measured in a photomultiplier tube (PMT) based luminometer. The cost of these instruments, depending on sensitivity, portability, and versatility, is generally in the range of $3,000 to $30,000 (2).

There are, however, a large number of problems with the approach, summarized in Table 1. The most serious problems are:

1. The swabbing process is generally very incomplete. It is a function of how the microorganisms were originally deposited or grown on the surface, a function of the surface itself, as well as the particular technique and method of swabbing the sample. The present method of hygiene monitoring using this process is qualitative at best and is generally only used as an indicator of "contamination". It is not considered quantitative.

2. The membrane disruption surfactant cocktails commonly used are designed to disrupt all microbes and indeed all cells, thereby providing a measure of total ATP and thus total biomass. There is really no opportunity or possibility of organism speciation.

3. The aggressive surfactants and other agents used in the disruption cocktail inhibit and inactivate luciferase. This is normally partially alleviated by dilution, thereby decreasing sensitivity, and by using excess luciferase which increases analysis cost.

4. Perhaps an even more severe problem is that the microbe disruption process generally results in the release of cell ATP-ases which rapidly degrade the ATP, thereby decreasing the total amount available for detection, further decreasing the sensitivity of the assay. Although various ATP-ase inhibitors can be added, they also act on luciferase which itself is an ATP-ase, further decreasing the sensitivity.

5. Because of this great range of inefficiencies, the intensity of light actually produced is very small, thereby requiring a fairly sophisticated and expensive detection system.

In spite of all these disadvantages and problems, the process is sufficiently sensitive that it has become widely accepted and applied for generic hygiene monitoring (1,2).

Protein Solutions, Inc. has had considerable experience with firefly luciferase and is presently developing a range of specific biochemical sensors for mono- and di-saccharides and for amino acids using luciferaes. We are developing "dipstick" type, direct reading, quantitative, inexpensive, disposable biosensors using these approaches (23, 31, 34, 35). Part of this work is funded by an ongoing Phase II STTR grant from the National Science Foundation titled "Direct Reading Quantitative Biosensors for ATP-Dependant Processes." Originally, we felt that our ATP sensors might be directly applicable to hygiene monitoring. The more we looked into the hygiene monitoring area, the more we

realized that there are so many problems and disadvantages with the method prior to the ATP detection step that some additional inventions and innovations are required. We have indicated some of those in the far right column of Table 1.

We propose to design a device which can directly sample the surface or the material of interest, thus eliminating the uncertainty with the effectiveness of sampling, and a range of surface and swab effects. We will address this direct analysis approach in the Phase II application.

There are a variety of enzymes which are used to recycle ATP by phosphorylating AMP or ADP back to ATP, thereby recharging the ATP pool. These enzymes have already been used in biosensors to enhance or essentially amplify the ATP level available for analysis (30, 33). We have experience with this approach and it is indeed part of the development plan for our enhanced sensitivity ATP sensor.

This Phase I application, however, focuses on what we feel is the major problem with the present approach: the use of very strong surfactants with which to disrupt bacterial membranes to permit the release of ATP. In order for bacteria to grow and divide, they require enzymes which can locally disrupt their own cell walls, thereby permitting the cell fission process. These enzymes are apparently activated in regions of cell wall mechanical stress and are not activated or are inactive to unstressed or weakly stressed walls. These enzymes are collectively known as autolysins. There are autolysins which can function with minimally stressed or unstressed walls. One example is lysozyme (3, 10, 16). But, there is apparently a group of autolysins ranging from perhaps one to several dozen in various strains or species of bacteria, both gram negative and gram positive, which are stress induced or stress activated. Refer to the references noted in Table 2. A number of these enzymes have become reasonably well characterized and commercially available in the last fifteen years or so. Many of them provide a means to degrade the outer cell envelope without significant degradation or inhibition of the other components of the bioluminescence reaction.

The basic hypothesis of this Phase I application is that local mechanical stress in the presence of autolysins will result in enhanced autolytic activity, cell envelope disruption, and subseqeusnt ATP release.

We propose to generate local cell membrane stress through the use of micro abrasive particles of the order of the same size as bacteria. We further propose to deliver the autolysins directly on the surface of these micro abrasive particles, thereby ensuring that local stress and autolytic activity are directly coupled. It may be possible to deliver the luciferase activity on the same particles as well. That possibility will be part of the Phase II application. Though there is considerable evidence for autolysins and autolytic activity and there is considerable interest in the stressed autolysis hypothesis (4,8,13), there is little direct work in the literature on the role of stress on autolytic activity. We propose an empirical study to determine if this approach is indeed feasible in significantly enhancing ATP release for hygiene monitoring purposes. If proven feasible in this Phase I application, then in the subsequent Phase II we will propose to more fully optimize the phenomenon and to develop an enhanced sensing system for hygiene monitoring. We will further propose to include a partial speciation capability using a very short time, selective media based, culture approach, based initially on a dry film technology such as the 3M Petri Film (21, 22). Finally, we propose to use direct area analysis and detection using photographic film or an electronic based CCD array detector which will allow, not only the detection of microbes on surfaces, but their spatial distribution as well.

This work has considerable potential significance. Although rapid progress is being made in the education of the work force and of the general public in hygienic practices, there are no simple, inexpensive means of hygiene monitoring which would encourage enhanced education and the development of more hygienic practices in the home environment, in the restaurant industry or, in hospitals, clinics, etc. The availability of

Table 2

Autolytic Enzymes to be Studied and Evaluated

Name	Function	Availability*	References
Muramidases (N-acetyl Muramoyl hydrolases) EC 3.2.1.17	hydrolyzes glycan chains some bond penicillin	mutanolysin: Sigma M-9901 M-4782	4,10,8,25,26
Amidases and Aminidases (various) (N-acetyl-muramoyl-L-alamine amidase) (N-acetyl-glucosaminidase) EC 3.5.1.4 3.2.1.30 3.2.1.96	cleave alanine-muramic acid bond, etc.	Sigma A-3189 A-2415 A-1055 A-6152 A-2264	4,8,28,34
Lysozyme EC 3.2.1.17	a muramidase-similar to above	Sigma L-9772 L-6876 L-7001 L-6394	15-17, 27
Heat-denatured Lysozyme (80° C, pH 7.2)	generic cell lysis activity as cationic polyelectrolyte	Sigma L-9772 L-6876 L-7001 L-6394	15-17
Chitinase EC 3.2.1.14	hydrolyzes glucoside linkages and displays some muramidase/ lysozyme activity (34)	Sigma L-7809 L-1525	34
Lipase	to break down lipopoly saccharide in gram-negatives	Sigma L-9518 L-0763	

* Sigma refers to Sigma Chemical Co., 1994 Catalog

simple, reliable, sensitive, inexpensive, direct reading, and preferably direct sampling devices would be very significant indeed.

C. Relevant Experience

1. Principal Investigator

Dr. R. Van Wagenen, Ph.D., Principal Investigator and 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 Systems, 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 and optical systems design are also directly relevant to the conduct of this feasibility research and the development of the final monitoring device. Dr. Van Wagenen will serve as principle investigator and have primary responsibility for the design and conduct of the project. His product development background will enable this concept to be effectively developed into commercial products.

2. Other Key Personnel

Dr. Robert Scheer received his Ph.D. in Materials Science and Engineering in 1993 and was Principal Investigator on PSI's NSF-STTR Phase I grant addressing 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.

Dr. C.Y. Wang recently completed his Ph.D. studies under Dr. Joe Andrade's supervision at the University of Utah. Dr. Wang has worked on the Firefly luciferase system for five years. He is an expert on the trehalose stabilization of firefly luciferase in agaros gels. Such gels can be desiccated and successfully rehydrated with full enzymatic activity. He is now working as a Research Scientist at PSI and will conduct the firefly luciferase-based studies. He will play a key role in transferring his comprehensive expertise on this system to the others involved in the project.

Dr. Joseph Andrade is founder, President, and CEO of PSI. Joe has worked extensively with proteins, enzymes and antibodies for the past 25 years, focusing his efforts on elucidating their behavior at surfaces and interfaces. Five years ago he became involved in bioluminescence particularly in firefly and bacteria luminescence systems. While Joe is not written into the budget he will be available on an as needed basis to provide support for the interfacial biochemistry, bioluminescence, and biosensor design.

D. Experimental Design and Methods

Objective 1: Assess the efficacy of known, available autolysins

The cell wall structure of both gram positive and gram negative bacteria incorporates a peptidoglycan which is a key part of the so called murein sacculus. In the case of a gram positive organism, such as Staph. aureus, the peptidoglycan layer consists of polysaccharide chains and short peptides, whereas in the gram negative E. coli, the peptidoglycan structure is much thinner and largely covered with an outer lipid lipo-protein

membrane. There are, of course, other differences (3, 4, 6, 7, 36). The fact that polysaccharides are common components of both generic bacteria classes, suggests that autolytic enzymes specific to those polysaccharides may permit membrane disruption without the need for proteases or other enzymes which might attack or inhibit luciferase. It is likely that in the case of the gram negative bacteria, a lipase autolysin may be required. See Table 2. Fortunately, the common chemical features in all peptitoglycans suggests that an appropriate mixture of autolysins will enable the disruption of both generic classes of bacteria.

The basic peptidoglycan building block is a disaccharide of N-acetyl glucosamine and N-acetyl-d-muramic acid linked in long chains. The lipo-polysaccharides which form the outer layer of the gram negatives are highly variable in the polysaccharide component. There is also great variability in the lipid component.

The autolysins of greatest interest are given in Table 2. Muramidase hydrolyzes glycan chains. The amidase acts on the junction between the glycan chains and the peptide units. Lysozyme is also a muramidase and hydrolyzes the glycosidc bonds in N-acetyl glucosamine (NAG), and N-acetyl muramic acid (NAM). Lysozyme also acts on chitin, the crustacean exoskeletal polysaccharide, a poly NAG. Chitinase is an enzyme specific for the linkages in Chitin and is also active against bacteria cell wall polysaccharides.

It is important to note that the specificity and activity of these enzymes to stressed cell walls or cell wall components is virtually unknown. Thus, we cannot depend on the published activities, specificities, or other characteristics of these enzymes in terms of the stress/autolysin hypothesis. That is why we propose to evaluate the more common of these enzymes in both unstressed and stressed cell wall environments.

The initial activity evaluation will consist of exposing the suspension cultures of Staph. aureus and E. coli of known viability and cell concentration to varying concentrations/total activities of the autolysins in Table 2. The ATP released will be monitored as a function of exposure time by our standard ATP bioluminescence assay and calibrated using ATP solutions of known concentration. That is, a standard solution of luciferin and luciferase will be added to each cell suspension-autolysin sample and the resultant luminescence will be determined as a function of time. This is a base line study to provide data on these enzymes and their activities in unstressed environments. The work will be done using 96 well microtiter tissue culture plates (Falcon). The resulting luminescence in each sample well is collected with a macro lens and imaged onto a CCD array for quantification, imaging, and permanent record. The CCD array detector with imagining lens allows us to take complete images of each 96 well plate every few seconds for as long as necessary. The digital data (luminescence intensity) from each well can then be determined and saved on computer disk.

We will examine heat denatured lysozyme as a separate enzyme because it has been recently reported (16, 17) that heat denatured lysozyme has "novel antimicrobial action," probably functioning as a polymeric, cationic surfactant in a relatively non-specific manner (18). It is possible that such a denatured lysozyme might indeed inhibit luciferase and this possibility will, of course, be examined, as will the interactions between luciferase and each of the other enzymes in Table 2. One of the selection criteria for the autolysin(s) of choice will be their lack of luciferase inhibition.

Objective 2: Assess the suitability of several common abrasives

Table 3 lists the nature, type, and characteristics of common industrial abrasives used for abrasive machining and polishing of metal and other surfaces (24). These materials are relatively inert, highly stable, and very hard. Because they are all used for polishing purposes, they are available in a wide range of particle sizes from hundereds of microns down to micron and in some cases sub-micron sizes.

Aluminum Oxide and Diamond are the two most common abrasives used for laboratory polishing application. They are readily available from any common laboratory

Table 3

Common Abrasives

Type	Characteristics	Source
Silicon Carbide SiC	angular shape, penetrates easily	Carborundum Co.
Aluminum Oxide Al_2O_3	Most widely used polishing agent, softest of the 4 common abrasives	Norton Co. Three M Co.
Cubic Boron Nitride BN	cubic particles sharp edges	General Electric
Diamond C	hardest abrasive	General Electric
Silica SiO_2	not a common industrial abrasive-too brittle	Many

supply catalog. Silica is not a commonly used industrial abrasive because it is too brittle. Nevertheless, it may be suitable for our purposes.

Our initial experiments will be performed with aluminum oxide and diamond, because these are readily available in a wide range of particle sizes including as small as .01 micron. We may also, if it appears necessary, evaluate silicon carbide, largely because its angular, asymmetrically shaped particles may aid the penetration and stressing of the highly deformable bacteria.

We will perform ATP interaction and depletion studies on slurries of these various particles. The possibility of ATP interaction with aluminum oxide; with diamond, and silicon carbide is expected to be minimal, but no data is available. Our group is well experienced in adsorption studies (32) and in chromatography studies. Such experiments are quite straight forward. The abrasive selected for further analysis will of course be one that shows minimal adsorption of ATP.

We also have considerable experience with surface modification to minimize adsorption (32). If the adsorption problem seems significant, we will experiment with modifying the surfaces of the particles with an appropriate polymeric surfactant which should then minimize further adsorption of ATP.

We will then evaluate the possible adsorption on and subsequent inhibition of luciferase by these particles. We wish to minimize the inactivation of luciferase due to interactions with the abrasive particles themselves. The most efficient system would be to incorporate the autolysin <u>and</u> the luciferase <u>on</u> the abrasive particle, as noted earlier. This would be an objective in the latter part of Phase II in the development of a highly optimized, highly efficient and sensitive device. We do expect that luciferase will be adsorbed and probably partially inactivated on all of these abrasive surfaces. Our group is quite expert in protein adsorption and in means to minimize such adsorption by appropriate surface modification processes. It is therefore likely that these particles will be treated with a polyethylene oxide (PEO)based polymer to provide a means of steric repulsion, thus minimizing adsorption (32).

We now routinely produce a recombinant luciferase containing a biotin bearing domain (31), thus allowing the biotin luciferase to be readily bound to avidin through the highly specific and strong avidin-biotin interaction. If necessary, the particles can be coated via adsorption with avidin or stretpavidin, followed by exposure to luciferase. The biotin-luciferase binds to the avidin or streptavidin film with full retention in activity (23). Indeed, we have already been using this process with the immobilization of luciferase in our existing generation of carbohydrate sensitive sensors. We therefore have means to minimize the adsorption of luciferase to these particles or to maximize its bonding to the particles in a highly active configuration, depending on the particular need and the particular experiment desired.

The abrasive particle selected as a result of this preliminary screening for ATP and luciferase binding activity will then be used to study the effectiveness of direct abrasion on ATP release-in the <u>absence</u> of any autolytic activity. This will be done by depositing a known concentration of each of the two types of bacteria on a smooth, rigid, transparent, substrate. The substrate will consist of glass, microscope slides with and without silanization to make them appropriately hydrophobic for enhanced bacterial adhesion (29). Polymethyl methacrylate and polycarbonate slides will also be used. These three materials are all highly transparent. The two polymers are intrinsically hydrophobic. All three slide materials provide good model surfaces on which to deposit known amounts of bacteria. The bacteria will then be subjected to controlled abrasion in the presence of a standard luciferase/luciferin solution. The bioluminescence will be detected by our CCD based array detector <u>during</u> the process of abrasion, as a function of time.

The basic experimental set up is shown in Figure 1. The abrasive slurry of luciferase/luciferin reagent is deposited on the bacterial film and covered with another rigid transparent sheet which is, in turn, connected to a small vibration machine. The vibration

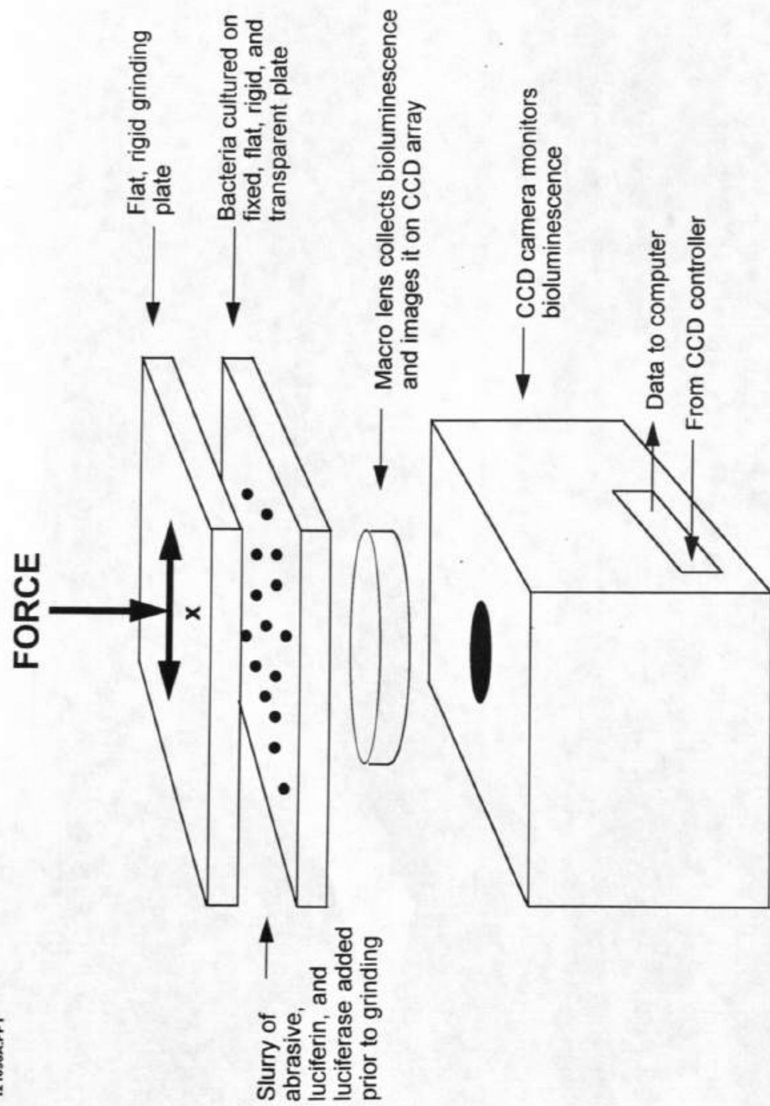

FIGURE 1 Schematic illustration of the bacterial abrasion test configuration. A known force is applied to the plate which can translate in the x axis.

machine will oscillate the upper plate over a range of amplitudes (x dimension displacement) and frequencies. The maximum horizontal amplitude will be about one millimeter and the minimum amplitude will be in the micron range. There will be two vibration machines to cover two displacement ranges (x). One vibration machine will consist of a spring loaded electromagnet much like the solenoid in a speaker. It will be driven by a function generator and amplifier to deliver displacements (x) of hundreds of microns to several millimeters in the 100-1000 Hz frequency range. The second vibration grinder will be driven by a piezoelectric crystal and will have displacements in the micron range at frequencies greater than 1000 Hz. It is likely that only the smallest possible amplitudes will really be effective in significantly stressing the bacterial cell walls due to the very small size and the intrinsic deformability of those cell walls. A downward force will be applied to the grinding plate to enhance the possibility of bacterial disruption.

These initial experiments will require highly smooth surfaces. We fully realize that the practical surfaces upon which such a device must eventually function will be far rougher, but by using a slurry and a grinding approach, such surface roughness issues should indeed be addressable. This will of course be part of the Phase II application.

In addition to grinding force, amplitude, and frequency, the size and nature of the abrasive particle and the thickness of the abrasive slurry are also variables, as well as the type and concentration of bacteria in the film.

These experiments will be done very efficiently, however, because the vibration devices can be readily programmed for a range of amplitudes, frequencies, and applied force. The devices will be designed so that the bacteria containing bottom sheet, as well as the abrasive slurry with the luciferase reagent, can be rapidly replaced. We have budgeted for the construction of two such devices to be used with our existing CCD camera system.

Objective 3 : Immobilize the most effective autolysins on the most effective abrasives.

Although one might expect that the abrasives would directly perturb and penetrate the cell wall, thereby releasing ATP, this is really quite unlikely because bacteria are so very small and their cell walls so deformable. Even small, rigid abrasives are unlikely to physically disrupt the cell wall. What is likely to be very effective, however, is that deformation of the wall, in the presence of autolysins, will enhance autolytic activity, as it is the enzyme induced degradation that weakens the wall sufficiently for ATP release. The stress/autolysis hypothesis argues that lytic activity is optimum while the wall is under stress. We cannot simply stress the wall and then expose it to autolysins. The two effects must be simultaneous. The best way to accomplish this is to bind the autolysin directly on the abrasive surface. Fortunately, this is likely to happen due to protein adsorption processes. The autolytic activity may be decreased due to surface induced denaturation, but this can be controlled by partially coating the abrasive with agents which will minimize autolysin denaturation, especially polyethylene oxide-based polymers (32). We will, therefore, study the most effective autolysins, the most effective abrasives, and abrasives coated with active autolysins.

The autolysins apparently function by binding strongly to the bacterial wall itself and then hydrolyzing the polysaccharide branches, one saccharide unit at a time (25-27). We will design the autolysin/abrasive binding to be weak to facilitate the direct transfer of the autolysin from the abrasive to the bacterial surface. The fact that the autolysins bind readily to the bacteria also facilitates their use in the solution state. It's highly likely that the autolysin binding equilibrium is such that binding to the bacteria is preferred over binding the abrasive. The different autolysins, the different abrasives, and of course the different pre-surface treatments will all influence the equilibria, the surface concentration, and the activity. There will also be autolysins in the aqueous slury solution which will be available to act on the bacterial cell wall. If it is determined that autolysins bound to abrasive particles are not effective we will resort to higher slurry concentrations of autolysins as the means to deliver them to the proximity of the bacterial cell wall.

Objective 4: Study the efficacy of the most promising autolysin/abrasive combinations on bacterial cell ATP release.

The results of:

Objective 1 will yield the selection of the two most promising autolysins.
Objective 2 will yield the selection of the two most promising abrasives.
Objective 3 will yield data regarding the binding of autolysins to abrasives.

With the data derived in Objectives 1,2, and 3 we will select the four best combinations of autolysins and abrasives. This will allow us to test these four combinations with the two bacteria species E. coli, and S. Aureus to determine the efficacy of ATP release in the grinding mode. Again, the controlled stress will be provided by the means previously discussed in the Objective 2 work plan, above.

Time Plan

Technical Objectives 1 and 2 will be conducted in parallel and will require the first two months of the six month Phase I period. During this time the abrasive apparatus with CCD luminescence monitoring shown in Figure 1 and described under Objective 2 will be constructed and tested. Objective 3 will require months three and four of the project. Objective 4 will be conducted primarily in months five and six. Preparation of the Phase II application will be conducted during month 6 as well.

Additional Questions-Phase II

There are many more questions to be answered but the resources and time available in a Phase I grant do not permit those to be substantively addressed until the early part of Phase II. Such Phase II questions include:

1. Will release of endogenous ATP-ases from the cell significantly decrease the available ATP for analysis? The answer to this question is probably yes. There is a most effective approach to this problem and it is to use various enzymes to re-synthesize ATP from the AMP and ADP. Indeed this enzyme amplification method for ATP is already used in some firefly luciferase based sensing systems, including our own (30, 34). Binding of luciferase directly on the abrasive surface, probably via an avidin-biotin mechanism (31), will facilitate the rapid, complete analysis of ATP, minimizing the effect of competitive ATP-ases.

2. Will the autolysin enzymes negatively affect the luciferase and luciferin detection system? The answer to this is probably no because the autolysin enzymes we have chosen are mainly designed to attack saccharide-based components of the cell wall. Autolysins based on protease activity are not expected to be necessary. In fact, we will likely co-immobilize the autolysin(s) and the luciferase on the abrasive particles. These concepts will be presented in detail in the Phase II application.

3. Will there be sufficient sensitivity to avoid the need of a photo-multiplier tube (PMT) based luminometry system for hygiene monitoring? The answer here is a qualified yes. Existing PMT based systems can detect 1,000 to 10,000 total bacteria using the existing generation of extractants and luciferase reagents (1,2). The existing extractants are highly damaging to luciferase activity. Bacterial ATP preparations often must be diluted to avoid dramatic loss in luciferase activity (1,2). By avoiding the need for strongly cationic and anionic surfactants, we anticipate an order of magnitude overall increase in ATP sensitivity, based on our existing work on recombinant firefly luciferases and low molecular weight enhancers on the kinetics of the firefly luciferase (23, 31) reaction. We also anticipate that the stress induced autolysin approach will lead to more complete ATP release. Also, the AMP, ADP, to ATP enzyme amplification approach (30) will provide more ATP per analysis. We anticipate an overall sensitivity enhancement in the system of a factor of 100-1,000 enabling either much more inexpensive detection means or a much lower bacterial detection threshold.

4. What's wrong with simple sampling and culturing, using existing rapid media approaches? The existing rapid media approaches are most appropriate to laboratories and environments where microbiology and its techniques are known and appreciated. In fast food restaurants, and even in the individual home environment, the use of agar plates and even 3M'S Petri Film (21, 22) and related dry film approaches are simply inconvenient and cumbersome for routine use by the untrained and perhaps less motivated.
   Increasing the sensitivity by several orders of magnitude, however, as described earlier, coupled with short term culturing, i.e. perhaps two to four hours, will allow significantly enhanced detection under relatively rapid conditions. The great advantage of the culture approach is that generic speciation can be performed by use of selective media. Again 3M's Petrifilm products are perhaps the best example. Phase II will address how such a dry reagent culture "plate" can be designed with the proposed autolysin/abrasive/enhanced luciferase technologies to develop a system which can be readily used by the uninitiated, yet is highly sensitive and rapid.

5. Why can't one simply use Polaroid film for enhanced detection? We can and we do. Indeed, the detection mode for the " Petrifilm-like" system to be studied in Phase II will likely employ a direct contact Polaroid cassette for highly sensitive colony detection and enumeration. This also has the advantage of providing a permanent record.

6. Can one sample directly on a contaminated surface? Probably. One of our goals is to develop a device which can be placed on a surface of interest harboring potential bacteria. The autolysin/abrasive/firefly luciferase detection and Polaroid recording systems would all be integrated to permit direct detection of the bacterial load on such a surface. It is important to note that the abrasive technologies we propose to utilize are in the micron to sub micron size range. This is the range used to provide high polish on surfaces, so the surface analyzed will not be scoured. Rather, it is likely that the sample surface will be even more shiny and polished than the unsampled surface! Obviously the surface must be stable to the autolysins and the other reagents used. But since the entire system will be aqueous based, at near neutral pH, and will not employ strong surfactants or other chemicals, it is likely that it could be applied to almost any common surface found in hospital, food processing, or home environments.

E. Human Subjects

No human subjects will be used in this research.

F. Vertebrate Animals

No vertebrate animals will be used in this research.

G. Consultants and Advisors

No consultants are proposed for this work. However, our strong Scientific Advisory board will continue to serve in an advisory and consulting capacity with reimbursement from other sources. The board members are as follows: **Dr. Woody Hastings** - Professor of Biology at Harvard University. He is internationally recognized for his basic research on bioluminescence. **Dr. Vladimir Hlady** - Associate Professor of Bioengineering at the University of Utah. He is an expert on the study of proteins at surfaces using interfacial fluorescence spectroscopy. **Dr. Don Johnson** - former New Biotechnology Product Manager for DuPont and currently an independent consultant and Chairman of the University of Utah Center for Biopolymers at Interfaces a University-Industry-State consortium Center of Excellence. **Dr. Henry Kopecek** - Professor of Pharmaceutics and Bioengineering at the University of Utah. He is an internationally recognized authority on hydrogels and related polymers for drug delivery and biocompatibility. **Dr. Larry Kricka** - Director of the General Chemistry Lab and Professor of Pathology and Laboratory Medicine at the University of Pennsylvania. He is internationally recognized for his work on applying both bioluminescence and chemiluminescence to clinical chemistry. **Dr. Jerry Nelson** - Microbiologist and founder and President of Nelson Labs, a nationally recognized service laboratory providing a wide range of biological testing and compliance monitoring for industry. **Dr. Russell Stewart** - Assistant Professor of Bioengineering at the University of Utah. He is an expert on recombinant techniques for the synthesis and study of luciferases and motor proteins.

H. Contractual Arrangements

No subcontracts are proposed in this research.

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