PRINCIPAL INVESTIGATOR: Phil Triolo

ABSTRACT OF RESEARCH PLAN

NAME, ADDRESS, AND TELEPHONE NUMBER OF APPLICANT ORGANIZATION

Protein Solutions, Inc. (PSI)
350 West 800 North, Suite 218
Salt Lake City, Utah 84103-1441
(801) 596-2675

YEAR FIRM FOUNDED	NO. OF EMPLOYEES *(include all affiliates)*
1988	5

TITLE OF APPLICATION

DEVICE FOR SENSING MICROBIAL CONTAMINATION

KEY PROFESSIONAL PERSONNEL ENGAGED ON PROJECT

NAME	POSITION TITLE	ORGANIZATION
Phil Triolo	Senior Scientist	PSI
Rob Scheer	Research Scientist	PSI

ABSTRACT OF RESEARCH PLAN: State the application's long-term objectives and specific aims, making reference to the health-relatedness of the project, describe concisely the methodology for achieving these goals, and discuss the potential of the research for technological innovation and commercial application. Avoid summaries of past accomplishments and the use of the first person.

The abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. Since abstracts of funded applications may be published by the Federal Government, do not include proprietary information. DO NOT EXCEED 200 WORDS.

The objective of this research is to determine the feasibility of developing a hand-held corona discharge device for the disruption of cell walls of adhered bacteria under ambient conditions in order to release ATP for bioluminescent analysis. A rapid estimate of bacterial content can be made from the amount of ATP present. (1,2)

The corona device will be used in conjunction with a non-instrumented ATP detector which PSI is currently developing under a STTR grant from the NSF (3). The mating of the two technologies to produce a commercially viable product for rapid assessment of microbial contamination of surfaces will be the objective of a Phase II grant. However, the device can also be a valuable tool when combined with already-existing ATP biloluminescent detection methods.

A method for rapidly detecting bacteria adhered to surfaces *in situ* is valuable to the healthcare industry in order to monitor the microbial contamination of devices, hospitals, cleanrooms, labs, and other clean or sterile environments. The technology described here will replace existing ATP methodologies for assessing the microbial content of surfaces, which are time-consuming and cumbersome because they require that bacteria be stripped from a surface before they can be counted.

Provide key words (8 maximum) to identify the research or technology. ATP, Luciferase/Luciferin, Bioluminescence, Assay, Disruption, Corona, Bacteria, Contamination

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

The ability to rapidly screen for the presence of adhered bacteria is valuable to the medical device industry to minimize bioburdens on devices and in clean rooms, and to hospitals, surgical centers, and laboratories handling biological specimins in order to reduce the possibility of spreading disease. The ability to mechanically disrupt cell membranes with the device could also be valuable in biotechnology processing applications.

PRINCIPAL INVESTIGATOR: Phil Triolo

BUDGET JUSTIFICATION

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

Dr. Phil Triolo, Principle Investigator, will devote 60% of his time supervising this 6 month project.

Dr. Rob Scheer, Research Scientist, will spend approximately 6 hrs/week on this project, assisting in the analysis of ATP by the luciferin/luciferase reaction.

A. Pungor is an electrical engineer with experience in device fabrication. He will assist with design and development of the corona discharge apparatus.

An as-yet unnamed microbiologist will maintain bacterial samples for deposition on surfaces, and be responsible for depositing them on surfaces.

Two students will assist with the routine assays, including ATP assay by firefly luciferase/luciferin, as well as performing general lab maintenance functions.

Total Direct Costs are $74,640. PSI's Indirect Costs are 33% of Direct Costs.

J.D. Andrade, although not budgeted in the proposal, will be available for technical support at needed.

RESOURCES AND ENVIRONMENT

1. FACILTIES: Describe the facilities to be used and briefly 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 organization, at any other performance site listed on the FACE PAGE, and at sites for field studies. Using continuation pages if necessary, include an explanation of any consortium arrangements with other organizations.

PSI has 1,200 square feet of research space at 350 West 800 North, Suite 218, SLC, UT 84103. Laboratory, computer, and office facilities are adequate for the work proposed. In addition, PSI's cell culture facilities are being expanded and will be available for this study. In addition, PSI is a member of the Center for Biopolymers at Interfaces at the University of Utah, a State/University/Industry consortium, and as a member has access to specialized laboratories and equipment at the University. The equipment is available on a fee for service basis, which is budgeted in Other Costs category.

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

Computer, incubator, optical microscope, plasma glow discharge apparatus, necessary lab space is available at PSI's facility. SEM and XPS services are to be provided through CBI, who also have a goniometer available for contact angle measurements.

3. ADDITIONAL INFORMATION: Provide any other information describing the environment for the project. Identify support services such as consultants, secretarial, machine shop, and electronics shop, and the extent to which they will be available to the project.

Surface analysis and SEM are available through the CBI Membership. Greg Burns, Ph.D. (see letter) will consult on bacteria commonly encountered on medical devices.

Current and Pending Support

Current Support

NSF Phase I SBIR, *The Labless Lab in Polymer Materials* (ends 8/1/94), Final Report to be submitted 9/1/94

Pending Support

Phil Triolo, P.I.
no grant proposals pending.

Andras Pungor
no grant proposals pending.

Rob Scheer
Rob is listed as P.I. on the following grants, which constitute all of PSI's current possibilities for additional funding. He is committed to working 8 hours per week on each.

1. NSF STTR, *Direct Reading, Quantitative biosensors for ATP-Dependent Processes,* 7/1/94-6/30/95. Direct costs: $74,000.

2. NSF Phase I SBIR, *Marine Phytoplankton in Sealed Environments,* 1/195-6/30/95 Direct costs: $47,000.

3. NSF Phase I SBIR, *Thermal Gradient Device for Materials Analysis,* 1/195-6/30/95 Direct costs: $47,000.

4. NSF Phase I SBIR, *labless Lab for Chemistry* , 1/195-6/30/95 Direct costs: $47,000.

5. NSF Phase I SBIR, *Bioluminescent Detectors for Secondary Smoke*, 1/195-6/30/95 Direct costs: $47,000.

None of the above proposals include duplication of the research efforts described in this proposal. The only proposal that includes related research is the STTR investigation of incorporating firefly luciferin/luciferase reagents into a small, disposable device that detects the presence of ATP in solution or suspension introduced to its sample port. It will be combined with the process for releasing bacterial ATP by exposing surface bacteria to corona discharge described in this proposal to develop a hand-held sensor for screening for the presence of microbes on surfaces as the subject of a Phase II grant application.

As PSI's sponsored R & D volume increases, several Ph. D. level research personnel will be hired. Dr. Scheer will serve as principle investigator and primary supervisor and scientist for the pending grants noted above, with a work load of 8 hours per week on each. Dr. Triolo will assist with the performance of specific sensor-related research on the STTR proposal, listed above.

PSI has invested $150,000 (provided primarily by its founders and major stockholders) in initial research and product development efforts. The company expects to continueto fund projects from stock-derived funds, and is in the process of discussing equity investments by a number of local investors and investment groups.

Robert J. Scheer
350 West 800 North, Suite 218
Salt Lake City, UT 84103
Ph. (801)596-2675
e-mail (rob.scheer@m.cc.utah.edu)

EDUCATION

Ph.D. in Materials Science and Engineering, September 1993, University of Utah, Salt Lake City, UT. Dissertation emphasis: Mechanical, interfacial, and surface study of composite materials.

B.S. in Mechanical Engineering, 1989, Duke University, Durham, NC. GPA 3.76.
Emphasis: Fracture mechanics and failure analysis of polymeric materials.

UNIVERSITY HONORS

National Science Foundation Fellow,
University of Utah Graduate Research Fellow,
Dean's List Duke University, Academic All American
Duke University Magna Cum Laude,
Scholastic Societies: Tau Beta Pi and Pi Tau Sigma.

AFFILIATIONS

American Society for Mechanical Engineers
ASM International
The Minerals, Metals, and Materials Society
American Physical Society
The Center for Biopolymers at Interfaces

EXPERIENCE

Principle Investigator
Protein Solutions, Inc. Salt Lake City, UT. 1994 - present. Directed research for the design and implementation of novel science education materials.

Research Assistant
University of Utah, Salt Lake City, UT. 1989 - 1994. Tested mechanical properties of polymers and composites, studied surfaces and interfaces, tested adhesive bonds on the microscopic scale, and developed stress analyses related to materials testing.

Instructor/Tutor
University of Utah and Salt Lake Community College, Salt Lake City, UT. 1991 - present. Planned, instructed, and graded for undergraduate physical science classes. Served as tutor and teaching assistant.

Engineering Technician
Sandia National Laboratory, Albuquerque, NM. Summer, 1989. Designed engineering experiments for failure analysis of ceramic materials, and extensively researched current experimental techniques for determining material fracture toughness.

Engineering Technician
Sandia National Laboratory, Albuquerque, NM. Summer, 1988. Designed engineering experiments for strength testing of brittle materials, and performed CAD. Interacted with diverse engineering disciplines on a major research project.

PUBLICATIONS

R.J. Scheer and J.A. Nairn. "Variational Mechanics Analysis of Stresses and Failure Analysis in Microdrop Debond Specimens." *Composites Engineering*, Vol. 2, No. 8, pp. 641-654, 1992.
R.J. Scheer, Ph.D. Dissertation, "An Energy Based Analysis of Fiber-Matrix Adhesion." University of Utah, 1993.

Phil Triolo
350 West 800 North, Suite 218
Salt Lake City, Utah 84103
(801) 596-2675

EXPERIENCE:

1994-current Protein Solutions, Inc. **Sr. Scientist.** Direct research in the application of bioluminescence to the development healthcare products.

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

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

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

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

EDUCATION:

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

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

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

APPOINTMENTS & HONORS:

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

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

AFFILIATIONS:

Member, Biomedical Engineering Society, Intermountain Biomedical Association.

PATENTS:

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

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

R.H. Hoffer, J.L. Orth, P.M. Triolo, Implantable Structure for Containing Substances for Delivery to a Body, U.S. Patent 5,324,518, Issued to Biosynthesis, Inc.

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

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

PUBLICATIONS:

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

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

Table of Contents

RESEARCH PLAN

A. SPECIFIC AIMS

The specific aims of this project are to:

1) Fabricate a small corona device, the "Corona Pen", capable of disrupting cell walls in order to release ATP for bioluminescent assay by the firefly luciferase/luciferin system,

2) Evaluate the ability of the device to disrupt the cell walls of gram negative and gram positive bacteria commonly encountered in hospital environments and on medical devices,

3) Investigate the interference of chemical methods commonly employed to release, and destroy, ATP from non-bacterial cells on the use of corona discharge treatment to release bacterial cell contents. The destruction of non-bacterial sources of ATP, typically accomplished by the addition of non-ionic surfactants to destroy cell membranes followed by treatment with apyrase to inactivate the liberated ATP, is a necessary prerequisite for the analysis of ATP content of bacterial cells,

4) Determine how ambient conditions (temperature, relative humidity, moisture content of the sample) affect efficiency of cell wall destruction by corona discharge,

5) Tune the device, adjusting current, voltage, frequency and time of discharge so that the discharge destroys cell walls, but preserves cellular contents for assay,

6) Optimize methods for cleaning the corona discharge electrode so there is no sample carryover, and

7) Perform preliminary experiments to determine if there are any differences in the resistance of cell walls of different cell types to corona discharge. This work will be a major focus of Phase II efforts.

It is anticipated that results of this study will demonstrate that 1) corona discharge can be an effective means of destroying cell walls of adhered microorganisms to release bacterial ATP, and 2) the firefly luciferase/luciferin bioluminescent assay can be subsequently employed to obtain an estimate of the microbial ATP present, which is indicative of the degree of surface microbial contamination.

B. SIGNIFICANCE

There is considerable interest in and need for the determination of microbial concentrations on surfaces. In recent years, the measurement of total surface ATP has been used as an indicator of total microbial surface concentrations. The firefly luciferase/luciferin reaction is the method of choice to assay for ATP. ATP serves as a cofactor in this very sensitive assay which is able to detect ATP in concentrations as low as 10 e-11 moles/ liter (1, 4-22).

The reaction is specific for ATP. Other nucleotides are not detected, nor do they interfere with the reaction. Reagents for the measurement of ATP levels by bioluminescence have become readily available, highly reliable, and inexpensive. Because of its sensitivity, speed, and decreasing costs, use of the technique is growing rapidly.

However, the current techniques for assaying ATP to estimate the number of organisms on a surface are not without difficulty (19, 20, 21). Problems with the techniques can be divided into two categories- those specific for the removal of adhered bacteria from surfaces, and those general problems associated with use of the bioluminescent reaction to assay for ATP.

Currently, the use of the bioluminescent reaction to detect cells on surfaces requires that the cells first be removed from surfaces into a suspension which can then be concentrated so that the bioluminescent assay can be made. This difficult procedure is fraught with problems. First, the cells may be damaged or even fully disrupted during the removal process releasing ATP. ATP has a relatively short half-life in these conditions, probably because bacterial ATP-ases are released along with the ATP which function to degrade the ATP almost immediately (5, 7, 23).

Even if the cells can be removed from the surface, the problem of concentrating the cells in suspension still remains. The concentration process itself often utilizes filtration techniques and introduces the possibility of loss through adsorption or entrapment of cells in the membranes. Once the bacteria are in suspension in sufficiently high concentration to be detected, they can be assayed. The detection and measurement of microbial populations is a standard, highly sensitive technique used in a wide range of industrial and clinical applications. There are, however, a number of problems associated with the current methodologies.

The steps for the bioluminescent assay of ATP from cells in suspension are as follows (15, 16) :

1) A non-ionic detergent is added to the sample to selectively destroy non-bacterial cells. Unfortunately, some strains of bacteria have cell walls which are also destroyed in this process, but, in general, it is an effective method for releasing non-bacterial ATP so that it can be eliminated in the next procedural step.
2) An ATPase is added to the suspension to destroy the free ATP.
3) The ATPase is neutralized or removed by filtration. Any remaining ATPase can catabolize luciferase or bacterial ATP, the species of interest, released in the following step. Potato apyrase is typically employed for this task, and, if used in small quantities doesn't significantly interfere with the bioluminescent assay. However, the lack of interference is paid for in the requirement that the reaction be allowed to proceed for twenty minutes. In order to shorten the reaction time, higher concentrations of the apyrase are required, but these will destroy both ATP and luciferase, necessitating a neutralization or filtering step to eliminate this possibility (16).
4. An ionic surfactant is added to the suspension to destroy the cell walls of the bacteria that are to be enumerated. The surfactant can degrade luciferase and/or ATP.
5. The liberated bacterial ATP is assayed by the bioluminescent luciferase reaction.

The technology of producing bioluminescent reagents, adding them to the appropriate solution or suspension, and detecting the light output has dramatically advanced over the last few decades. Compact, even portable luminometers are now available (16, 17, 24), and we have proposed a detection device that could even eliminate the need for costly detection systems (3).

Techniques have been developed to detect a variety of cell types, including many strains of bacteria, platelets, erythrocytes, and other eukaryotic cells (20,21). The major problem with all of the techniques is that there is a potential for interference between the

ATPases and membrane disrupting agents used to destroy the cell membranes of extraneous cell populations and the cell walls of the bacteria to be quantified (15, 16, 20) and the ATP luciferase reaction. Indeed, the biotechnology industry recognizes the potential of chemical cell disrupting agents for destroying biochemical products and, therefore, utilizes mechanical means for disrupting bacterial walls to liberate biochemical products for further purification (25, 26, 27). However, the mechanical means of cellular disruption developed by the biotechnology industry are batch processes, inappropriate for the small-scale operations addressed here.

Additionally, virtually all of the methods used for cell wall disruption involve processing cells in suspension. Our interest is in the direct enumeration of bacteria and other cells attached to surfaces. Currently, the most direct and effective means of enumerating adhered bacteria is by direct optical, and preferentially scanning electron microscopy, of the surfaces in question (28-31). For practical purposes, this is only possible if there are relatively large numbers of cells in the area of the surface being examined, and the process is costly, labor-intensive, and time-consuming.

The device we propose to develop and evaluate is to be used as a screening device, that is to establish the presence of microbial contamination. Once the presence of contaminating organisms is established, other methods can be applied for verification and identification of the organisms involved (12, 17, 34).

We propose to detect bacteria while they are still adhered to the surface of interest using a controlled corona discharge to oxidize away portions of their cell walls, thereby providing leakage of intracellular ATP for bioluminescent detection (see figure 1.).

In the early days of radio frequency glow discharge polymer surface modification (35) there was some discussion of the use of plasma processes and corona processes to actually sterilize surfaces. These ideas were deemed to be impractical because both processes provide sufficient significant surface modification and because the treatment is shape and geometry dependent, making it relatively impractical for complex medical devices and other applications. The idea was largely dropped.

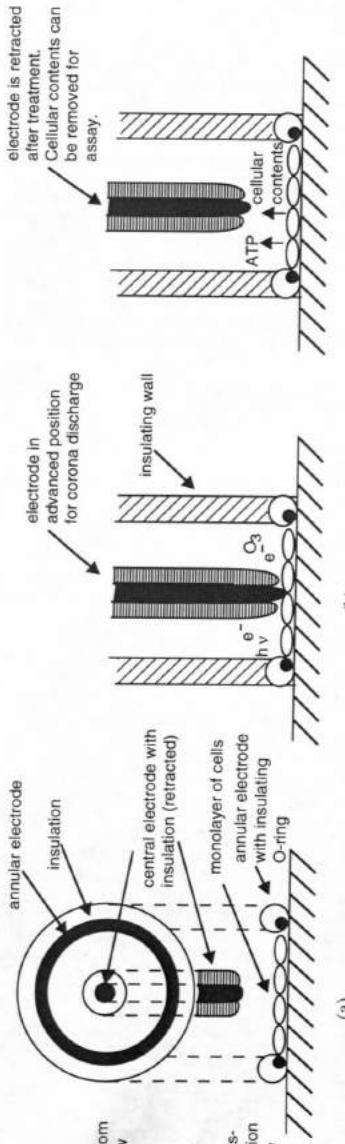
There is little question, however, that both plasma and corona discharge produce extensive chemical reactions of surfaces, involving oxidation, cross-linking, and free radical formation (2, 32, 33, 35). Such reactions generally lead to disruption of surface films and cell membranes and to cell damage and death.

Plasma discharge processes require at least a partial vacuum and tend to be less practical than corona processes for many surface modification applications. Indeed it is high scale, high speed corona processes which are generally used to provide hydrophilization and dye acceptance of polyethylene and other hydrophobic polyolefins used in packaging and in many other applications (32, 36).

The corona process has the advantage that it can be done in normal ambient environments and for a wide range of sample shapes (2, 33, 36). Using needle electrodes, one can obtain almost point surface modification (36). Surface oxidation gradients of linear or radial dimensions can be obtained by using a point electrode and a flat counter electrode underneath the sample. Using knife electrodes, one can produce gradient surfaces, that is the continuous change in surface modification (33, 37). One can modify the duration of treatment and can vary the frequency, the voltage, and the current delivered. These variables can be tuned and optimized to eventually develop a general instrument applicable to the most common cell surface modification applications.

Figure 1.

Schematic Representation of Corona Device Depicting Disruption of Cell Walls and Release of Intracelluar Contents



Cross-section and end views of inactivated corona device showing proposed electrode configuration.

When the corona is activated it reacts with the air, generating free radicals and other highly energetic moieties which can interact with the cell surface. Conditions will be adjusted so that defects will be created in cell walls. Any cellular contents that leak from the cells during the active pahse of corona discharge will be expected to be inactivated in the highly reactive environment created by the corona.

When the corona is de-activated, cellular content continue to leak from the disrupted cells. Contents will be assayed for ATP by the firefly luciferase reaction to obtain an estimate of microbial contamination of the surface.

Our major interest in this SBIR application is applying corona discharge to disrupt bacteria on surfaces, although the technology to be developed may also be applicable to nonbacterial cell types including blood platelets and red blood cells (20).

The use of corona discharge to disrupt cell walls has several distinct advantages over chemical means of cell disruption. The corona discharge itself will destroy ATPases or residual non-ionic surfactants remaining from the steps involved in the removal of ATP from non-bacterial sources. Therefore, higher concentrations of apyrase can be used to destroy non-bacterial ATP, allowing for faster processing times. Further, the processing of the cells *in situ* eliminates the possibility of losses occuring during the stripping of the bacteria from the underlying surfaces and concentrating them in solution.

The vital requirement of the use of the corona discharge is that conditions must be optimized so that bacterial walls are destroyed, but cellular contents are largely preserved. If this requirement cannot be met, then the use of corona for this application is not indicated.

C. RELEVANT EXPERIENCE

Phil Triolo, the P.I. of the proposed research, received his Masters and Doctoral degrees in Bioengineering from the University of Utah in 1980 and 1988 respectively. His masters work on the surface modification and characterization of polymeric materials by radio frequency glow discharge plasma familiarized him with the use of plasma and corona treatments for medical applications. He has published two papers on polymer surface modification, and, along with J.D. Andrade and others, holds a patent on use of RFGD plasma treatment of polymeric surfaces to reduce friction (see CV).

Industrial experience as a manufacturing, product development, and research engineer has been successfully applied to the development of cardiovascular catheters and angioplasty devices, and will prove to be valuable in the evaluation and commercial development of the Corona Pen.

D. EXPERIMENTAL DESIGN AND METHODS:

One:

Construct a small area corona treatment device suitable for treating areas ranging from several square millimeters to several square centimeters in a radial geometry, using a needle electrode suspended above the surface to be assayed, and surrounded by a grounded annular electrode (Figure 1). It may be necessary to mechanically rotate a partially shielded central electrode in order to effect the discharge, or other configurations may be required. Geometry will be optimized in Phase II.

Preliminary experiments will be performed at PSI employing standard corona equipment, as well as demonstration models provided by commercial manufacturers (36). Configuration and power supply will then be optimized for the device conceptually illustrated in Figure 1. The AC power supply includes an RF oscillator (about 500kHz), a high voltage unit (1000V peak to peak voltage with adjustable current from 50-100 mA), and a matching circuit to provide minimum loss power transmission between the high voltage RF generator and the electrode. A critical parameter that will determine the ability of the corona to destroy cell membranes will be the proximity of the electrode to the cell surface. In order to be functional, very close proximity to the cells must be maintained. It is anticipated that this can be readily achieved by mechanical means.

To tune and demonstrate the effectivenes of the device, surfaces of low density polyethylene and polystyrene will be treated. (It should be noted that the device in the proposed configuration cannot be used if the surfaces in question are conductors, as these will create a short-circuit, eliminating corona generation. Modification of the device to assay bacteria adhered to metallic and other conducting surfaces will be explored in Phase II experiments).

Corona treatment will result in an increased hydrophilicity of the normally hydrophobic polymeric surfaces. By selecting treatment conditions (voltage, frequency, time, separation distances, and specific electrode geometry) the wettability pattern and degree of oxidation can be controlled.

The treatment will be characterized by 2 standard surface analysis techniques, with which the PI has considerable experience: contact angle analysis and photoelectron spectroscopy (38).

For the contact angle analysis, water droplets are placed on the treated surface and the angle of contact measured. The untreated polymers have water contact angles of about 90^{o} and, depending on treatment conditions, the water contact angle in the treated region will vary from 90 to 0^{o}. Simple water vapor nucleation patterns, so-called breath patterns, will be used to image the hydrophilicity gradient and the homogeneity of the treatment. In a few selected samples X-ray photo electron spectra of the materials will be obtained so that a quantitative determination of the degree of oxidation of the surface induced by the corona treatment can be made. This is an expensive characterization and therefore will be limited exclusively to critical samples.

PSI has the needed internal experience with corona treatments as a result of collaboration with a group who received their degrees in J.D. Andrade's laboratory some years ago and who are now working in South Korea (33, 37).

This particular phase of the project will take about 2 months and will be conducted largely by P. Triolo and A. Pungor, with technical assistance from J. Andrade. Andras Pungor has a wide range of instrument design experience, and his work on plasma discharge systems is directly applicable to the developmenf of the corona discharge apparatus (see vita).

Two:

Deposit bacteria of scientific and commercial interest onto unmodified polymer surfaces in known quantities. This task is required in order to produce well-characterized adhered bacteria on surfaces for future experiments that will evaluate the effectiveness of the corona discharge in destroying their cell walls. It involves employing a number of standard methods used in the study of microbial adhesion and attachment to synthetic surfaces (28-31). The organisms to be studied will be *Staph. aureus* and *Klebsiella*, commonly associated with nosocomial infections (40), *Pseudomonas sp.* and *Staph. epidermis*, commonly associated with implant-associated infections (41), *E. coli* because of its importance to the biotechnology community, and a member of the *Streptomyces* genus because of their ability to produce antibiotics.

This task will be performed by a microbiologist supervised by the P.I. with the input and advice of Drs. Jerry Nelson and Greg Burns. Dr. Nelson, a microbiologist/bacteriologist, is President of Nelson Laboratories, a local microbiological analysis lab. He serves on Protein Solutions, Inc., Scientific Advisory Board, and will provide advice and input at no charge to the contract. Dr. Burns, faculty member of the Center for Biopolymers at Interfaces, has studied bacteria attached to the surfaces of biomedical polymers (39). He has considerable experience working with several bacterial strains which are commonly found on implanted medical devices. He will serve as a consultant to the project (see letter).

Cells of each bacterial strain will be quantitatively deposited on the polystyrene and polyethylene surfaces. Bacterial surface concentrations will be determined by direct counting using optical and scanning electron microscopy (SEM) (15).

Optical microscopy capabilities exist in PSI's laboratories. SEM is available on a fee for service basis through the Center for Biopolymers at Interfaces.

Three:

Bacteria deposited in known concentrations on the unmodified surfaces, (See Task 2, above), will then be removed from the surfaces by standard methods (9, 10, 29, 30) and analyzed by standard firefly bioluminescence techniques. A correlation can then be established between the surface concentration of organisms as determined by optical methods in Task 2, and the quantity of ATP assayed by the firefly luciferase technique.

The assay method will consist of transferring the bacteria from the surfaces into a small volume of solution, concentrating the bacteria, using one of the conventional surfactant reagents to extract the ATP, and then measuring the extracted ATP by firefly bioluminescence. Standard reagents and protocols are available (9, 10, 29, 30).

Our group has considerable experience with the firefly luciferase/luciferin bioluminescent assay of ATP. Mr. C.Y. Wang, a graduate student working under Andrade's supervision at the University, has nearly completed his doctoral studies on the behavior of firefly luciferase at interfaces. He has had extensive experience with the measurement of bioluminescence generated by firefly luciferase/luciferin and ATP. Mr. Wang will advise in the conduct of these experiments.

Four:

In order to assess the effect of the corona discharge on ATP itself, known amounts of ATP will be deposited on the unmodified polymers. These unmodified polymers are hydrophobic. Therefore, aqueous drops will not spread on the untreated surfaces, allowing us to place a drop of ATP-containing solution on them and then evaporating to dryness. Clearly this will not lead to homogenous distribution. However, if the drop is confined to an area that is small enough with respect to the sample analysis area of the X-ray photoelectron spectroscopy (XPS) instrument, one can obtain a reasonably good estimate of low concentrations of ATP on the surface as ATP's unique phosphate component is easily detectable by XPS.

ATP concentration will also be determined by simply dissolving the ATP back into solution and assaying by the firefly bioluminescent analysis. It is anticipated that XPS, which determines the presence of ATP based solely on the quantity of phosphorous present, will determine higher surface concentrations than the firefly luciferase assay, as the ATP must maintain biological activity in order to be determined bioluminescently. Thus, one can obtain an estimate of the degree of degradation that occurs as a result of the ATP application process by comparing results obtained with these two methods.

Once a control level of ATP has been established, the ATP surfaces will be exposed to corona discharge, varying time, distance from the electrode, power, and frequency, of the corona to determine the operating conditions that are most favorable for maintaining ATP's activity. ATP's viability will be assessed by assaying its concentration with the firefly luciferase system, employing XPS analysis on appropriate controls.

Tasks 1-4, listed above, will require 2 to 3 months for completion, and will provide the necessary background information and controls required for the important feasibility experiments, described below.

Five:

Surfaces with adhered bacteria, (from Task 2, above), will be exposed to corona treatment and analyzed by:

a. Direct microscopical examination to obtain a quantitative count of organisms and assess any damage to cell walls induced by the treatment.
b. Wettability (contact angle) analysis,
c. Direct measurement of ATP induced bioluminescence within seconds after the corona treatment. This will allow for a direct comparison between the amount of ATP determined by ***in situ*** corona-induced cell disruption and immediate ATP analysis with ATP levels determined by the traditional cell removal and solution processing method employed in task 2. It will also provide an assessment of the sensitivity of the technique.
d. Direct ATP bioluminescence measurement as a function of time after the corona treatment.
e. Conventional cell stripping from the surface and subsequent bioluminescent analysis for ATP as a function of time.

It is anticipated that corona treatment conditions can be created that will disrupt bacterial walls and permit leakage of their intracellular contents. However, in addition to ATP, ATPases may be leaked from the cells. Most proteins exposed to corona discharge significantly denature and, in the case of enzymes, are inactivated. Some of the ATPase molecules will be destroyed by exposure to the corona, but others may still be

present in sufficient concentrations to degrade ATP. It is, therefore, necessary to perform the time-dependent studies outlined in d and e, above. They will provide information on the appropriate time frame during which the ATP assay should be performed. This, along with methods of efficiently transferring cellular contents to the assay system will be optimized in Phase II.

The information gained from these experiments will be used to optimize corona discharge parameters for bacterial wall disruption. The initial operating conditions to be evaluated will be those optimized in Task 1 for the use of corona to effect surface modification. The variables to be controlled and optimized include not only the operating parameters of the device, but also distance from the sample. In addition, the thickness of the water layer on the bacteria at the moment of corona discharge as well as the relative humidity of the air may affect the generation of the corona (48) and the subsequent efficiency of cell disruption. In the case of very thin water layers, the relative humidity during the corona discharge will be of major importance. The presence of water between the anode and cathode of the corona device serves to decrease the potential required to obtain corona generation. However, the relationship between relative humidity and the ability to generate a corona is by no means straight forward, and needs to be examined for the particular electrode configuration employed (48).

Relative humidity of the ambient air will be determined with appropriate instrumentation. Additionally, water will be added to the samples, and the effect of increasing water content on the efficiency of destruction will be assessed microscopically, as well as by assaying for released ATP.

These experiments will all be performed on monolayer or submonolayer concentrations of bacteria. Disruption of cells by corona discharge, a surface technique, is difficult if multiple layers of bacteria or bacteria trapped in a biofilm are present(42). Such more complex samples are reserved for Phase II studies and would involve further fine tuning of the corona process, the ATP bioluminescence measurement, and refinements in calibration and standardization. It should be remembered, however, that the intended purpose of the corona device and ATP assay by the bioluminescent technique is to rapidly screen for the presence of bacteria. By the time multilayers of bacteria have grown, there will undoubtedly be a sufficient concentration of organisms at the air interface to be modified by the corona discharge and assayed positively by the ATP firefly luciferase technique.

Six:

Evaluation of the effectiveness of corona discharge for destroying chemicals commonly employed to rid bacterial samples from interference by non-bacterial sources of ATP.

Apyrase, commonly used to inactivate ATP, and Triton X-100, a surfactant used to disrupt non-bacterial cells, will be added to cell surfaces prepared in 2 , above. Concentrations added will be representative of those commonly employed (1). Cells will then be destroyed utilizing the optimum conditions defined in task 5, and the ATP content determined and compared with that found for bacterial surfaces not treated with the surfactant and enzyme.

It is anticipated that the presence of the chemical agents will have an insignificant effect on the ATP analysis, confirming that the corona method can be employed immediately after non-bacterial sources of ATP have been destroyed. This, in itself, constitutes an improvement over existing techniques that require low concentrations of apyrase be

used over prolonged periods of time to minimize interference with the detection of bacterial ATP, or that the apyrase be removed from the system before the bacteria are processed (16).

Seven:

Optimize a method for cleaning the electrode to prevent sample carryover.

It is anticipated that any ATP present on the electrode can be easily destroyed by soaking it in an ATPase such as apyrase. Subsequent discharge in air should inactivate any of the enzyme that remains on the electrode.

Eight:

Evaluate the potential of the device to selectively, or sequentially, disrupt cells on the basis of structural differences of their cell membranes (walls).

The cell walls and membranes of different cells have structural and compositional differences (43-47) that may impart them with different resistances to disruption by corona discharge treatment. Human cells, and eukaryotic cells in general, are more easily destroyed by mechanical means than bacterial cells (25), and it can be anticipated that Gram negative bacteria, because they have a thinner proteoglycan cell wall than Gram positive bacteria, might be more readily destroyed than Gram positive cells. Likewise, differences in the cell membrane compositions of mycoplasma (no cell wall), archaebacteria (unusual polysacharide or protein aceous walls; membrane lupids linked by ether bonds in place of the usual esther linkage) and chlamydiae (no peptidoglycan in their cell walls), might be sufficient enough to enable corona discharge conditions, perhaps with appropriate pre-treatments, to be optimized to selectively destroy one type of cell, leaving the others intact.

Only preliminary experiments will be performed in Phase I. A layer of adhered cells consisting of both Gram negative and Gram positive bacteria will be deposited on surfaces as described in task 2. Corona discharge conditions will then be varied in attempts to selectively destroy one cell strain, but leave the other intact. Direct microscopical examination and bioluminescent analysis will be employed to asses cellular damage. However, it may not be possible to positively identify damage to one cell type, but not the other. Labelled experiments may be required. A major effort will be directed in Phase II studies to evaluate the potential of corona treatment to enable the differentiation of cell types on surfaces in a rapid screening method.

Summary:

These studies will allow us to conclude whether or not the corona treatment is feasible for facilitating the leakage of ATP from surface adhered organisms. Further, assay of the leaked ATP by the firefly luciferase reaction will enable us to estimate the sensitivity of the assay for several important strains of bacteria on surfaces. Sensitivity comparable with that of cell removal assay techniques are desired, but a loss of sensitivity would certainly be considered acceptable, given the reduction in time afforded by the technique in comparison with the time required for assay after cell removal.

Limited feasibility, or feasibility for applicatioons other than the destruction of bacterial membranes, will be demonstrated if the device successfully modifies polymeric

materials, offering the advantage of selectively modifying small areas of polymer surfaces to facilitate the adhesion of inks, adhesives, cells, etc.

If the device can selectively lyse different cell types, then it could be useful for cell separation applications, and could even be employed to destroy non-bacterial cells.

E. RELEVANCE OF PHASE I RESEARCH TO PHASE II OBJECTIVES:

Funding for at least one, and possibly two, phase II grants will be sought at the succcessful conclusion of this Phase I research. One grant will address the mating of the corona pen with a quantitative biosensor for ATP-dependent processes currently being developed by PSI (3). The research will address means of miniaturizing the pen, and reducing its cost. Additionally, a major effort will be directed toward designing a means for sample access so tyhat the sensor can quickly assay the ATP contents released *in situ*. The sensor itself is designed to be read optically, and a good deal of work will be necessary to guarantee that enough light is available for detection by the unaided human eye. Alternatively, high speed films would have to by employed. The commercial device would consist of a reasonable corona apparatus, and disposable biosensors.

The second Phase II proposal would investigate the possibility of segregating and cells of the basis of their cell wall and membrane characteristics, and then obtaining an estimate of their quantities by employing the firefly luciferase reaction. It is anticipated that treatments other than corona exposure will be required, and a system involving preferential lysis, adsorption of cells onto substrates, and cellular disruption by mechanical means under a varity of conditions is envisioned.

This Phase I study will provide the basic corona-generating tool to be employed in the Phase II projects. Experience gained in employing the firefly luciferase/luciferin reaction to detect the presence of microbes will be directly applicable to the devlopment of devices for rapid estimation of microbial concentrations and techniques for microbial separation on the basis of their membrane structure and properties.

F. CONSULTANTS:

Greg Burns will be the only consultant on this Phase I project. His CV is attached (see next page).

G. CONTRACTUAL ARRANGEMENTS:

Confirmation of the contratual arrangement with the Center for Biopolymers at Intefaces is included at the end of this proposal.

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