BIOPOLYMERS AT INTERFACES
CBI QUARTERLY NEWSLETTER
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INTRODUCTORY NOTES

Even cold and snowy seasons eventually give way for sunshine and a glorious verdant landscape. After an academic year packed full of teaching and course work and ending in a furious crescendo of exams, we celebrated the traditional commencement ceremonies with the usual pomp and circumstance. In line to receive their doctorates were five of our CBI students who have been supported by the center for at least part of their graduate studies. I feel very proud over this accomplishment.

Each year during the commencement ceremony, the University honors a few of its most outstanding faculty members for their research and teaching accomplishments. One of this year's three Distinguished Research Awards was given to CBI's Henry Kopecek for his pioneering work in the design of water soluble polymers for targeted drug delivery. In addition to the two drugs he has developed for treatment of liver cancer, which are currently in clinical trial in the United Kingdom, Henry's recent activities have focused extensively on the development of photo-activable polymers for drug release. His CBI colleagues are proud of his accomplishments and celebrate this well-deserved award.

Yet another reason for pride was reported recently, when it was made official that Tom Beebe had been selected to receive an NSF Young Investigator Award. Only twenty such awards are given nationwide to researchers in chemistry, and the selection of Tom is therefore a very impressive recognition of his surface chemistry research program. In addition to the prestige, this award carries with it substantial financial support for a period of five years. CBI is proud of this distinction given our good colleague and friend.

- Karin Caldwell, Director

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CBI FALL MEETING

It is now official -- the Fall Industrial Advisory Board Meeting has been shifted from Monday and Tuesday, September 20-21, to Thursday and Friday, September 23-24. This became necessary when we realized that there were some conflicts for both industrial and faculty members which would prevent them from participating in the CBI meeting. After a survey of industry representatives, the most popular choice for alternate dates was the 23rd and 24th.

Another change is the location of the meeting. We had originally scheduled the University Park Hotel for the Fall meeting, but the change in dates necessitated a change in venue as well. We will again meet at the Little America Hotel in downtown Salt Lake City. You should make room reservations directly with the hotel; their telephone number is 800/453-9450 or 801/596-5966. The rate for single deluxe tower rooms will be $89.00 and for single deluxe garden rooms, $75.00. Please try to make your reservations before August 22nd, since our reserved block of rooms will be released after that date. And keep in mind -- September is a busy month in Salt Lake City for meetings and conventions. Hotel space will be at a premium!

Other than these changes, the meeting will be configured in basically the same format as in the past. The welcoming reception and discussion normally held on the Sunday evening before the meeting will take place on Wednesday evening, the 22nd. As always, we will be sending out an agenda and copies of research proposals in advance.

If these dates prove to be inconvenient for some individuals, we recommend that you discuss alternate arrangements with your colleagues so that your organization is well represented. It is primarily through our semi-annual meetings that members are able to contribute their recommendations regarding the Center's direction and operation, as well as specific research endeavors. We look forward to seeing you in September!

• UPCOMING MEETINGS •

July 4-9, 1993. 6th International Conference on Organized Molecular Films, Trois-Rivières, Que- bec, Canada. Program will include: Dynamic Processes and Forces at Surfaces; Electrochemical Processes; Modern Methods (STM, AFM, X-ray, Electron and Neutron Scattering); Monolayers at Gas-Liquid Interfaces; Optical and Spectroscopic Properties; Polymers in Organized Molecular Films; and Theoretical Modeling. Contact Dr. Pierre-F. Blanchet, Dépt. de Chimie-Biologie, Université du Québec à Trois-Rivières, C.P. 500, Trois-Rivières, Québec, Canada G9A 5H7, phone 819/376-5146, FAX 819/376-5057.

July 25-28, 1993. 20th International Sympo- sium on Controlled Release of Bioactive Materials, Wash., DC. Program plans include presentations on: Chronopharmacology, Mathematical Modeling, Oral Delivery, Peptide/Protein Delivery, Trans- dermal Delivery, Vaccines, Biotechnological Advances, Consumer Products, Modulated Drug Delivery, Parenteral Administration, Targeting and Transmucosal Delivery. Contact Controlled Release Society, Inc., 1020 Milwaukee Ave., Suite 235, Deerfield, IL 60015; phone 708/808-7071, FAX 708/808-7073.

Aug. 22-27, 1993. Polymers for Ophthalmic Applications, ACS National Meeting, Chicago, IL. Topics will include: Water Soluble Polymers in Ophthalmic Preparations; Polymers in Corneal Sur- gery; Polymers in Glaucoma, Vitreous and Retina Surgery; Surface Characterization and Surface Modification of Ophthalmic Polymers; Polymers in Contact Lenses; Polymers in Intraocular Lenses; Proteins and Bacteria Interactions with Ophthalmic Polymers; and Polymers in Ophthalmic Drug Delivery. For more information, contact Miguel F. Refojo, The Eye Research Institute, 20 Staniford St., Boston, MA 02114, phone 617/742-3140, FAX 617/720-1069; or Peter G. Edelman, Ciba Corning Diagnostics, 63 North St., Medfield, MA 02052, phone 508/359-3551, FAX 508/359-3614.

Aug. 30-Sept. 1, 1993. 4th International Conference, Biointeractions '93, Molecular Aspects of Biomaterials, Leiden, The Netherlands. Confer- ence will concentrate on molecular aspects of bio- materials with emphasis on the major and related topics of molecular microengineering, aging/time dependence effects of biomaterials, biomaterials applications and implants and devices. Further in- formation may be obtained from John Herriot, Biointeractions'93, Meetings Management, Straight Mile House, Tilford Rd., Rushmoor, Farnham, Surrey GU10 2EP, UK; phone 025125 5414, FAX 025125 2101.

Sept., 1993. 1st International Conference on Cellular Engineering, United Kingdom. Topics to be covered include: Cell Guidance, Advance Cell Culture, Cellular Approaches for Molecular Sensing, Cell-Implant Interactions, EM and Magnetic Field Influences on Cellular Processes, Bioartificial Organs, Networking and Neural Computing. Contact Prof. Peter Rolfe, Conf. Chairperson, Dept. Biomedical Engineering & Medical Physics, Keele Univ., Hospital Centre, Thornburrow Dr., Hartshill, Stoke on Trent, Staffs. ST4 7QB, UK.

Sept. 8-10, 1993. Polymers at Interfaces, Bristol, UK. The meeting will be broken down into the following sessions: Structure of the Interfacial Region; Dynamic Effects in Polymer Adsorption; New Methods and Techniques for Studying Polymer Adsorption; the Structure of an Adsorbed Polymer Layer under Constraint; and Polymers and Dispersion Stability. To register, contact Terence Cosgrove, School of Chemistry, Univ. of Bristol, Cantock's Close, Bristol BS8 1TS; FAX 44/272 250612, E-Mail COSGROVE@UK.AC. BRISTOL. SIVA.

Oct. 17-23, 1993. 10th International Symposium on Biorecognition and Affinity Technology, Gwatt/Thun, Switzerland. Topics will include: Novel Affinity Approaches; Receptors: Structure, Function and Ligand Recognition; DNA-Protein Interaction; Structure Recognition and Regulation of DNA and RNA; Antibodies: Design and Use; Interaction between Proteins, Carbohy- drates and Lipids; Cell Adhesion; Tissue-specific Drug Delivery; and Biosensors and Diagnostics. Conference Secretariat: Prof. A. N. Eberle, Dept. of Research, University Hospital, CH-4031 Basel, Switzerland; phone 41/61-265 23 24, FAX 41/61-261 15 00.

Nov. 7-12, 1993. Fourth Topical Conference on Advanced Materials (held in conjunction with the 1993 Annual AIChE Mtg.) St. Louis, MO. Sessions of interest will include: Polyethylene Glycol and Other Hydrogels as Biomaterials; Cell and Tissue Interactions with Biomaterials.

Feb. 11-19, 1994. 8th International Conference on Surface and Colloid Science, Adelaide, South Australia. Presentations will include Surface Wetting, Dispersion Science, Surface Modification, Biosystems, Thin Films, Adhesion at Interfaces, Emulsions and Suspensions, Surface Forces, Membrane Technology, Polymers in Solution and at Interfaces, Surface Techniques and Surface Reactivity. Address inquiries to Karen English Techsearch Inc., 183 Melbourne St., No. Adelaide, S. Australia 5006, telephone 61/8-267 1755 or 61/8-267 5466, FAX 61/8-267 4031.

Apr. 19-21, 1994. 2nd European Conference on Optical Chemical Sensors and Biosensors, Florence, Italy. Topics will include: Optical Sensing Techniques; Applications of Optical Sensors; Characterization of Optical Sensors. Conference Secretariat: Vanna Cammelli, IROE-CNR, Via Panciatichi, 64, 50127 Firenze, Italia.

Jul. 11-15, 1994. 35th IUPAC (International Union of Pure & Applied Chemistry) International Symposium on Macromolecules (MacroAkron '94), University of Akron, Akron, OH. Major topics will include: New Polymerization Reactions and Reaction Mechanisms; Complex Macromolecular Architectures and Supramolecular Polymers; Polymers and Biology; Frontier Polymeric Materials; Field Responsive Polymers; Structure and Morphology; Thermodynamic and Dynamic Properties of Polymers in Solution and Bulk; Polymers at Interfaces; History of Polymer Science; and Special Topics. Contact: Cathy Manus-Gray, Univ. of Akron, Institute of Polymer Science; Akron, OH 44325-3909, FAX 216/972-5463, e-mail Manusgray@uakron.edu.

PROCEEDINGS OF THE MARCH 15-16 INDUSTRIAL ADVISORY BOARD MEETING

Research Proposals Submitted for Funding (March 1993)

***Coating Inhibitory Activity from Tears: Its Interference with ELISA and Biocompatibility**
J. Janatova, K. D. Caldwell

***Effect of Chemical Composition and Hydrophobicity of Biomedical Polymers on Interaction with Complement Proteins**
J. Janatova, K. D. Caldwell, V. Hlady

***Enhanced Antibiotic Transport in Biofilms (50% funded)**
W. G. Pitt, N. Rapoport, D. Christensen

***Enhancing Manufacturing and Shelf-Life of Protein-Based Materials and Devices Using Trehalose**
J. D. Andrade

Examination of Water Soluble Polymers as Carriers for RNA Oligonucleotides: Implications for Antisense Gene Therapy and Enzyme-linked Diagnostic Assays
D. E. Ruffner, J. Kopecek

Fluorescence Lifetimes of Proteins at Interfaces, Part III
J. N. Herron, V. Hlady, W. Jiskoot

***Imaging of "Soft" and Weakly Bound Surface Molecules with the Scanning Interfacial Force Microscope**
C. C. Williams, A. Dicarlo, V. Hlady

Interactions Between Human Lipoproteins and Surfaces, Phase II: The Composition of Adsorbed Lipid Layer and the Role of Surface Charge
V. Hlady, C.-H. Ho, V. Omelyanenko

***Magnetic and Electric Field Enhancement of Antibiotic Activity in Biofilm-forming Pathogenic Bacteria (50% funded)**
C. B. Grissom, D. Benson, S. F. Mohammad, G. L. Burns

Photocrosslinking of Proteins
J. Kopecek, J. D. Spikes, H.-R. Shen

***Denotes funded proposals.**

ABSTRACTS OF FUNDED PROPOSALS

COATING INHIBITORY ACTIVITY FROM TEARS:
Its Interference with ELISA and Biocompatibility

Jarmila Janatova and Karin Caldwell

Background

The eye is protected from bacterial invasion by a variety of defense mechanisms, including the tear film, which consists of the aqueous tears, lipids and mucus [Holly, 1987; Smolin, 1987]. Tear fluid is a complex mixture of proteins and glycoproteins (with or without enzymatic activity), lipids, metabolites, and electrolytes [van Haeringen, 1981]. The complexity of human tear fluid has been demonstrated by immunological and electrophoretic studies; some of the proteins in tears are the same as those present in normal human serum, while several of them are specific for tears [Gachon *et al.*, 1979; Gachon *et al.*, 1982/1983]. Between 50 to 60 protein components have been detected in tears by two-dimensional gel electrophoresis; this high number is due to the fact that certain proteins may exist in multiple forms with either different isoelectric points (first dimension) and/or different molecular apparent sizes (second dimension). Since numerous pathological conditions have been shown to be associated with elevated or reduced levels of certain tear constituents [Mackie & Seal, 1984], there has been a need for rapid and accurate quantification of these protein components.

Despite the popularity of ELISA [Engvall, 1980], its use in the analysis of whole tears has been limited, since this type of direct immunological quantification of proteins in tear fluid has produced erratic results. Previously, lower titres than expected were obtained for several proteins (IgM, IgA, IgG, Lf, HSA) in sandwich-type assays of tears [Fullard, 1988]. During attempts to measure proteins in tears directly by adsorbing them to the surface of uncoated titre wells and detecting them with mono-specific antibodies, the existence of a *coating inhibitory activity* (CIA) in tears was noted [Boonstra *et al.*, 1985]. CIA apparently suppressed the necessary adsorption of antigen to the uncoated titre wells in their assay. It was further speculated that the existence of CIA in tears may prevent deposition of tear proteins on contact lenses [Boot *et al.*, 1989].

During our previous studies, supported by an earlier CBI grant, we have provided evidence for the presence of CIA in tears from healthy individuals [Janatova *et al.*, 1993]. Tear fluid contains at least one substance capable of interfering with both competitive and sandwich-type ELISA carried out in the

presence of the synthetic surfactant Tween®20. To demonstrate CIA behavior clearly in the range of tear volumes necessary to quantify tear protein with the sensitivity characteristic of ELISA, the decision was made to use a protein of very low titre in tears, namely factor H. Using a more sensitive sandwich-type ELISA, correlations were observed between the amount of tears present in the assay and the level of suppression of the response to several antigens, such as HSA, complement proteins C3, C4, factors B and H. It was further established by us that the presence of high salt concentration, namely 3 M NaCl, during the incubation of antigen and tears with the capture antibody-coated microwells, abolished in several instances, or substantially reduced in others, the effect of the tear CIA during ELISA. The inhibition of ELISA by tears was found to be an antigen non-specific phenomenon. The observed interference with ELISA appeared to depend on the suppressed formation of the antibody–antigen complex, and not on a competitive displacement of the surface-adsorbed antibody by some agent in tears. Preliminary data indicate that the inhibitory tear substance does not seem to interfere with the formation of antibody–antigen complexes in solution, but this needs to be confirmed further.

Our results suggest that the use of 3 M NaCl in the assay should allow determination of an antigen in 5 or 10 µl of tears, thus increasing sensitivity 25 or 50-fold, to 200 or 100 ng antigen per ml of tears. However, the use of 3 M NaCl does not inhibit the quenching caused by 5 or 10 µl tears completely. Moreover, in some instances the formation of antibody-antigen complex seems to be affected by the presence of 3 M NaCl. Therefore, an alternative reagent or approach needs to be sought. In the search for more efficient reagents, it would be helpful if the nature of the inhibitory activity were known.

Objectives

(I) We have demonstrated the presence of a substance which interferes with the quantitation of proteins in human whole tears using the widely employed ELISA technique. The levels of some of these proteins, such as IgE or C3, are known to vary in some disease states, accompanied by infection and/or inflammation. The determination of their profile would be a useful diagnostic tool, for example in the case of contact lens wearers. We propose to work out conditions that will allow accurate tear protein determination. This test may become instrumental in the quantitation of a number of proteins from tears and may have great diagnostic value.

(II) A substance which we designated CIA adheres very strongly to the IgG-coated surfaces, it cannot be displaced easily, and it prevents binding of other proteins to the coated surface, including an antibody-specific antigen. Since binding of CIA makes the artificial surface "repellent" to proteins, we hypothesize that if we are successful in the elucidation of the nature of this tear inhibitory activity, this knowledge could be exploited in designing new surface modifiers with increased biocompatibility.

Methods

The majority of materials and methods to be employed in the proposed study are the same as those already described in a paper [Janatova *et al.*, 1992] and a manuscript "Evidence for the presence of coating inhibitory activity in human tears and its interference with ELISA" [Janatova *et al.*, 1993]. The specific aims of the proposed project are:

1. *To develop ELISA into a reliable assay for quantitative determination of antigens in tear fluid* with a sensitivity better than 100 ng per ml of tears. To achieve this goal, it is necessary:

   a. to further investigate the effect of a high salt concentration on ELISA, e.g. the effect of a constant volume (5, 10 or 20 µl) of tears on samples with different amounts of antigen in the presence of 3 M NaCl; the aim is to find out whether there is going to be the same effect of a high salt concentration at different points of a standard curve, and what experimental error would be involved; this should be tested at least for three antigens: Factor H, C3 and IgE;

   b. to determine the effect of 3 M NaCl on ELISA of several antigens when monoclonal antibodies are used, and compare the results with those obtained by the same ELISA carried out with polyclonal antibody raised against the given antigen; our preliminary data suggest that affinity/avidity seems to be a very important factor, especially when assay uses 3 M NaCl during incubation of antigen with antibody-coated wells; the complex between polyclonal antibody and antigen seems to be less affected by 3 M NaCl; but this finding cannot be generalized as yet until more data with different antigens are available;

   c. since some antibody/antigen systems are greatly affected by the presence of 3 M NaCl, the attempt will be made to remove CIA from the diluted tear sample prior to the ELISA test;

   d. to increase the sensitivity of ELISA by employing a biotin–avidin–enzyme system; only polyclonal antibodies will be used in these assays; a part of IgG fraction will be labeled with biotin via spacer and will serve as a detecting primary antibody; avidin conjugated with alkaline phosphatase will be employed to detect antibody–antigen–antibody–biotin complex;

   e. to carry out radioimmunoassay in the fluid-phase with the larger proteins (e.g. C3 with Mr ~ 185 kDa) as antigens in the absence or presence of tears to see whether CIA interferes.

It is anticipated that the use of polyclonal IgG fractions as both the capture and detecting antibodies, the use of the biotin–avidin–enzyme system, and the presence of 3 M NaCl during the incubation of antigen with the capture antibody, may result in a reliable ELISA assay with the increased sensitivity, being able to detect antigens present at concentrations of about 20 ng per ml of tears. Such levels of sensitivity would be necessary in order to detect, e.g., complement proteins [Janatova *et al.*, 1993], the inhibitors of complement [Lass *et al.*, 1990], or epidermal growth factor [Ohashi *et al.*, 1989].

2. *To biochemically characterize the inhibitory activity.* Since CIA adheres so easily to surfaces, this property will be exploited first. CIA will be adsorbed from diluted tear samples to the latex polystyrene (PS) particles with 394 nm diameter, and several biochemical tests will be performed to determine the nature of CIA (whether protein, lipid, polysaccharide, mucin, etc.). Among other tests, CIA on PS particles will be directly subjected to SDS-PAGE. It is quite likely that the adsorption to the surface will be the first step in the isolation of CIA. The presence of CIA in solution can be detected by its ability to quench standard ELISA for factor H.

After identification of an optimal reagent and/or pre-treatment which would prevent "quenching" of ELISA by tears (1), and after elucidation of the nature of the interference of tear inhibitory activity with ELISA (2), optimal conditions will be worked out for determination of the antigens usually present in tear fluid: Lz (lysozyme), Lf (lactoferrin), immunoglobulins sIgA, IgG, IgM, IgE, C3 (the third component of complement), HSA (human serum albumin), and eventually TSP (tear specific protein). Commercial antibodies are already available for most of these proteins, with the exception of TSP, in which case TSP would be isolated and polyclonal antibodies would be raised in rabbits.

Significance of the Proposed Project

Very few people realize the importance of the tear film in the protection of the surface of the eye and in allowing normal visual function. There are many different proteins in tears that must be important in preserving the surface of the eye and in maintaining a normal and stable tear film. Understanding tear proteins is critical for many ophthalmic disease processes. However, there is little we understand about them because of the inability, until recently, to measure these proteins accurately.

We have demonstrated the presence of a substance which interferes with the quantitation of proteins in human whole tears using the widely employed ELISA technique. With this breakthrough, which will eventually allow us to measure specific proteins directly in whole tears, it will be possible to carry on the study which will lead to an understanding of the many different types of proteins, what function they have and how they vary with different disease states. By solving the problem with the tears inhibitory activity, we will have a whole new means of diagnostically looking at eye diseases.

Budget

Senior Research Specialist, 44% time effort	$13,800
Materials and supplies	1,700
Total	$15,500

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EFECT OF CHEMICAL COMPOSITION AND HYDROPHOBICITY OF BIOMEDICAL POLYMERS ON INTERACTION WITH COMPLEMENT PROTEINS

Jarmila Janatova, Karin Caldwell and Vladimir Hlady

Objectives

The aim of this project is to establish a link between the binding of the major complement proteins (C3, C4, C5, factors B and H) and the extent of their activation (C3, C4, C5, B) as a function of the chemical composition and hydrophobicity of an artificial surface. Based on the acquired data, it should be possible to identify surface properties which would be superior to others with respect to overall biocompatibility, by being thromboresistant, causing no adhesion of platelets, causing no activation of complement proteins, or no adhesion and activation of a variety of blood cells. Specifically, we will direct our experimental work in order to be able:

1. to identify materials which are activators of C3, and eventually those which activate C5;

2. to find a correlation between the surface properties and C3 activation (e.g., binding of C3 to hydrophilic surface seems to result in an exposure of binding sites for factor B), as well as binding of factor B and/or factor H;

3. to determine the effect of treatment of artificial surfaces with PEO (polyethylene oxide) chains on binding of complement proteins, namely binding and activation of C3;

4. to determine the effect of heparin (end-point or PEO-attached) on complement activation;

5. to provide experimental data to support or disprove the mechanism of formation of the complex enzyme **C3 convertase** on artificial surfaces, as proposed by us.

Background

The activation of complement induced by the presence of biomedical polymers is believed to occur most frequently through the **alternative** pathway mechanism [1] which involves the following key factors: **C3** (the third and the central component of complement), factor **B** (proenzyme; Bb fragment cleaves and activates C3), factor D (this enzyme cleaves and activates B), factor **H** (regulator of C3 activation), factor I (an enzyme that cleaves and inactivates C3b, the non-catalytic subunit of C3 or C5 convertase) and P (properdin, control protein promoting C3 activation). The contact of blood with negatively charged surfaces, or use of some drugs (e.g,. protamine sulfate which is used to neutralize heparin) may cause the activation of the **classical** pathway, involving other components of complement, namely C1, **C4** and C2 [2].

Two fragments are generated during C3 activation: a smaller 9 kDa activation peptide, C3a, is an anaphylatoxin, and it is usually used as a measure of complement activation. It also stimulates monocytes to generate IL-1 [3]. The larger C3b fragment is capable of covalent bond formation with -OH or -NH_2 groups; it has a potential to bind factor B and thus to become a non-catalytic subunit of the C3 convertase (C3bBb), whose formation and stability is mainly regulated by factor H. The products of C3 activation, fragments C3b or iC3b, can also interact with leukocytes via complement receptors [4] and stimulate these cells to generate oxidative products or release lysosomal enzymes.

Either of the two pathways may lead to cleavage and activation of **C5** (the fifth component of complement) into C5a and C5b [5], provided that the surface would support formation of the C5 convertase, a three-subunit enzyme. The larger two-chain C5b fragment in the presence of C6 can initiate assembly of the C5b-9 membrane attack complex that mediates cytolysis of target cells [6]. The smaller

C5a fragment, generated from the N-terminus of the α-chain during C5 activation, in addition to being an anaphylatoxin [7], it is also a potent chemotactic factor for polymorphonuclear leukocytes (PMN), and exhibits leukocyte-stimulating activities [8; 9; 3]. It induces PMN to release oxidative products and/or lysosomal enzymes [10] and C5a-stimulated monocytes may generate IL-1 [11]. In addition, the binding of C5a or C5a des Arg to the C5a receptor induces in PMNs enhanced expression of the C3b receptors, enhanced adhesion to endothelial cells, and stimulates arachidonate metabolism [11].

It is believed that the alternative pathway is activated by surfaces which exhibit specific biochemical characteristics allowing $C3(H_2O)$ or bound C3b to initiate assembly of the $C3(H_2O)Bb$ or C3bBb complex, an initial [12] or amplification **C3 convertase** [13]. Whether the activation of C3 would proceed on a biological surface depends on the surface properties which may influence binding of factor H to surface-bound C3b, and the discrimination between activating and nonactivating **biological** surfaces depends on the relative capacity of H to bind to C3b. Thus, in analogy to biological surfaces, it could be assumed that the more biocompatible biomaterials, with respect to complement, would be those which promote preferential binding of a regulatory protein factor H, and do not favor the binding of factor B [1].

The current hypothesis about the difference in complement activating potential by artificial surfaces (cuprophan, cellulose acetate, polyacrylonitrile) has been based on measuring generation of C3a in the fluid-phase [1, 2, 14]. Our recent research findings, however, clearly indicate that the requirements for the formation and stability of the C3 convertase on artificial surfaces differ from those on biological membranes [15]. Our experimental approach enables us to evaluate overall activation of *C3 which occurs in the presence of all other serum or plasma proteins, and it allows us to follow the fate of the *C3 molecule, both on surfaces and in the fluid-phase [16].

The formation of the C3 convertase, which cleaves and activates C3, will be investigated on those synthetic polymers, such as PAN, which do not posses free -OH or -NH_2 groups, and yet they bind C3 and promote C3 activation. Based on previous results, it can be assumed that the adsorption of native C3 to artificial surfaces may result in its conformational change(s) and enhanced hydrolysis of its internal thiolester which leads to the appearance of a free -SH group. The resulting $C3(H_2O)$ form would express either factor B and/or factor H binding sites, depending on the C3 conformation and/or orientation, which is expected to be governed by the biomaterial surface properties. Formation of the presumed initial C3 convertase C3 $(H_2O)Bb$ on the biomaterial surface, without necessity of covalent binding, will be probed as a function of the artificial surface properties. Whether the further activation, once initiated, is potentiated by a subsequent covalent binding of C3b to other plasma proteins bound to that membrane, also remains to be elucidated.

Rationale of Research Plan

Hemodialysis membranes [17] with a variety of chemical groups and varying degrees of hydrophobicity or hydrophilicity, provide us with an ideal model for a study of the mechanism of complement activation by biomaterial surfaces. Membranes based on (i) regenerated cellulose: cuprophan (CU), cellulose acetate (CA), Hemophan (HP), and on (ii) synthetic polymers: polyacrylonitrile (PAN), polysulfone (PS), polymethylmethacrylate (PMMA), will be examined in studies proposed here. In addition, (iii) cellulosic membranes modified through end-point attachment of heparin [18, 19] will also be analyzed. Although this treatment was reported to prevent coagulation without necessity of excessive administration of heparin [20], its effect on complement needs to be reexamined in more detail, since a different mode of heparin immobilization resulted in a 3-fold increase of C3 activation [21]. In order to retain heparin bioactivity after its immobilization, and to increase a number of immobilized heparin molecules, a more recent protocol utilizes the amplification of the reactive sites on the polymer surface, and it also employs polyethylene oxide (PEO) as a spacer [22]. It certainly would be worthwhile to explore the effect of this modification on complement and inflammatory response.

Much emphasis has been placed on making surfaces hydrophilic to reduce protein adsorption and cell adhesion. For example, a common hydrophobic medical material such as low density polyethylene (LDPE; also used as the NHLBI Primary Reference Material) may be coated with (PEO)-containing non-ionic polymeric surfactants [23]. In another study, carried out also at the University of Utah, a PEO-PPO-PEO triblock surfactant (with the ratio 129:56:129) was used for coating polystyrene particles [24] to minimize protein binding. To prevent any possible desorption or displacement, PEO of various chain lengths was grafted covalently to poly(ethylene terephthalate) films [25]. The PEO chains of 18,500 daltons appeared to be an optimum chain length for reduction in protein adsorption and hence reduction in cellular interactions on the surface to which it is coupled [26].

However, growing evidence shows that the chemical nature of the surface is also very important

in determining adsorption of the proteins and their functionality. More recently, several investigators in Sweden have examined the binding of the major plasma proteins, HSA (human serum albumin), IgG (immunoglobulin G), and the complement proteins C1q and C3, to surfaces with different hydrophobicity or hydrophilicity, and with immobilized different chemical groups [27]. It follows from their study that the smallest amounts of proteins were bound to either the most hydrophobic or the most hydrophilic surfaces. As the complement activation is concerned, it was a moderately hydrophilic surface with -OH groups (polystyrene coated with 2-hydroxyethyl-methacrylate, HEMA) or with $-NH_2$ groups (polystyrene coated with 1,2-diaminocyclohexane) which seemed to mediate an optimal activation of complement, mainly through the alternative pathway. Significant activation of complement (generation of C3a) was also caused by copolymers made from monomers containing 60% or greater HEMA (28). When the wettability was measured [27], the observed hydrophilicity of modified surfaces was in order of: -OH, -COOH, $-NH_2$, and PEO, being the most hydrophilic.

It follows from several studies that the presence of hydroxyl groups promotes activation of C3, and possibly that of C5 as well. For example, the introduction of hydroxymethyl ($-CH_2OH$) groups on a moderately hydrophobic polystyrene backbone endowed this surface with a capacity to activate C3. With larger substitutions of $-CH_2OH$ groups, more of C3a activation peptide was detected in the fluid-phase [29]. The ability of normal human serum (NHS) to mediate hemolysis was compromised after NHS was incubated with the surfaces containing sulphonate ($-SO_3Na$) groups; with larger substitution of $-SO_3Na$, less hemolytic activity remained in the treated NHS [29]. This effect could be explained by the adsorption of at least one of the complement proteins, namely C3 and/or factor B (in the absence of either of the two, alternative pathway would be arrested).

Rather suprisingly, however, the simultaneous presence in equal proportions of $-CH_2OH$ (48%) and $-SO_3Na$ (51%) on a polystyrene backbone leads to the creation of a complement non-activating surface. Does it mean that sulphonation of 50% of the hydroxyl groups in CU would attenuate a complement activating potential of CU? Considering the above results, what role does $-SO_3Na$ group play in activation of complement by polyacrylonitrile (PAN) membrane [16]?

Binding of the majority of proteins tested was substantially reduced when hydrophobic surfaces were treated with PEO. It is not clear as yet whether pretreatment of hydrophobic surfaces with PEO, or triblock copolymers of the PEO-PPO-PEO type which contain hydroxyl groups, will also attenuate binding and activation of C3, or whether the presence of PEO-hydroxyl groups and an increase in hydrophilicity will actually promote complement activation. We wish to address this question, namely by using PMMA, CA, PS and PAN membranes, both untreated and treated with triblock PEO-PPO-PEO. The surfactant-coated material will then be tested to determine whether the presence of PEO chains is capable to prevent C3 binding and its activation, *per se*. It can be assumed that the surfaces, which will not bind and activate C3, will not be able to promote C5 activation.

Budget

Senior Research Specialist, 44% time effort	$13,800
Materials and supplies	$1,700
Total	$15,500

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ENHANCING MANUFACTURING AND SHELF LIFE OF PROTEIN-BASED MATERIALS AND DEVICES USING TREHALOSE

Joseph D. Andrade

Background and Rationale

Proteins are now widely used as components of materials and devices. In biosensors proteins with specific recognition properties are used to impart specificity. In the area of blood compatibility, proteins such as albumin are often immobilized to minimize the undesirable activation of other plasma proteins. In some biosensor and diagnostic applications, photoproteins such as firefly luciferase are used to generate a unique and novel bioluminescence signal.

The proteins of biological motion, including actin, myosin, kinesin and dynein, are now being applied to surfaces for scientific and even technological purposes.

In many, if not all, of these applications it would be desirable to be able to completely dehydrate the material or device for storage, for shipping purposes and to minimize the potential for microbial contamination and growth in aqueous environments. As most proteins are marginally stable and depend on relatively specific water interactions for their conformational state and stability, a dehydration process normally results in irreversible alteration and inactivation of the protein. In some cases this process is alleviated by freeze-drying or lyophilization. Although proteins do tolerate such procedures, they often must be kept deep frozen to avoid other changes which lead to inactivation.

A small number of proteins with the appropriate use of additives and process control can be lyophilized and then warmed to room temperature and stored as a dry powder. However, for most proteins, this process results in significant inactivation, particularly for proteins immobilized at an interface for materials and device applications.

The air or vacuum drying of proteins at room temperature generally results in irreversible denaturation due to removal of waters which are critical for 3-dimensional structure, development of significant pH fluctuations and the high ionic strength which results during the drying process.

By looking to biology for guidance and inspiration, one finds that there are a variety of living organisms that can tolerate complete and total dehydration and come back to life when exposed to water. This process is commonly called anhydrobiosis or sometimes cryptobiosis. A good example is dried seeds and spores, but there are more complex and interesting examples, including various species of nematodes and a very unique class of organisms called tardigrades, commonly called water bears. These are large, multi-celled organisms which can be completely dehydrated and returned to life with almost 100% survival when reexposed to water, even after many years of total dehydration at room temperature.

Studies of these and other organisms have shown that as drying begins, their biochemistry is shifted to producing large quantities of a unique disaccharide, trehalose. Trehalose is a non-reducing disaccharide of glucose and is commonly found in very high concentrations in nearly all anhydrobiotic organisms and structures. As much as 20% by dry weight of these dry organisms is trehalose. This disaccharide is not widely known and has not been extensively studied. Recent computer molecular simulation studies and extensive differential scanning calorimetry studies demonstrate that cell membranes, liposomes and other phopholipid-containing structures are stabilized against dehydration by trehalose. The molecular modeling studies and experimental evidence suggest that trehalose functions by substitution of water which is tightly hydrogen bonded to the phospholipid head groups in cell membranes and intra-cellular structures. As the water is removed, in the absence of trehalose, the phospholipid layers undergo significant changes in their thermal properties and their stability and structure. The presence of trehalose during drying allows trehalose to substitute for the water. The stereo chemistry of trehalose permits it to directly hydrogen bond with several phospholipid head groups, thereby stabilizing and almost cross-linking the lipid membrane by cooperative hydrogen bonding. Trehalose's high molecular weight and low volatility permit it to be used as an additive which remains in the preparation after water drying is complete.

The addition of trehalose to purified protein preparations is already beginning to be applied in the biotechnology and enzyme engineering communities. It has not yet been studied or applied, to our knowledge, for applications involving immobilized proteins on and in materials and devices.

Model System

Most of what is known about trehalose's biochemical interactions deal with its binding to membranes and phospholipids. There has been recent work done with its interactions with proteins and the stabilization of proteins, but such work is limited at present.

Mr. Dong Min in our group has been studying the dehydration of various marine protozoa in trehalose media with some success. Mr. C. Y. Wang is working on firefly luciferase interactions with phospholipid monolayers and liposomes and intends to extend such work to lipid layers transferred to solid supports. This latter work is funded by CBI.

Firefly luciferase is an excellent protein with which to study potential trehalose stabilization for a variety of reasons:

1) It is very readily inhibited and deactivated. Its thermal denaturation temperature is quite low. In most studies of its immobilization to solid surfaces, very little activity remains.

2) It is a photoprotein, which means that, given the addition of luciferin and ATP, it emits light. This is the basis of its enzyme activity, and the light emission characteristics are exquisitely dependant on its three-dimensional structure and stability.

3) It is a highly hydrophobic protein. There is some evidence that it binds and interacts with phospholipid membranes, and it may even be a membrane protein. Indeed, this is one of the hypotheses which Mr. Wang is studying in his project.

4) It is reported to have a high degree of ionic character on its surface and in and around the active site. Water binding is likely to be critical for the stability of this region of the molecule. This same region of the molecule may be also involved in its interaction with phospholipid head groups.

These four characteristics, coupled with the fact that we now have considerable experience with firefly luciferase, lead us to suggest that this could be a useful model protein with which to study the effectiveness of trehalose stabilization.

Other model systems could include the fluoroscein monoclonal antibodies so extensively studied by Jim Herron and colleagues and well known to the CBI group.

Proposal

The behavior of firefly luciferase is very buffer sensitive, not only in terms of pH, but also in terms of buffer components. The most common buffer used for this enzyme is glycyl glycine.

We propose to study the behavior of firefly luciferase as a function of buffer, buffer pH and disaccharide additives and concentration. The disaccharides will include trehalose, mannose and sucrose, three which are routinely used for cryopreservation and for preservation against drying damage. The behavior will also be examined as a function of luciferase solution concentration, purity of luciferase, the effect of added proteins and stabilizers, especially albumin. Various preparations will be dried at various rates ranging from an exceptionally slow dry to very rapid vacuum dry, but in all cases at room temperature.

Our goal is to develop a process which will permit the solutions to be dried from approximately room temperature without freezing or the use of exotic additives or processes. The dried solutions will then be reconstituted, and the behavior of the enzyme examined as a function of time after reconstitution by normal luciferin/ATP analysis and photometric quantitation.

If time and resources permit, we will perform the same study with bacterial luciferase, which utilizes a different luciferin for its activity and does not require ATP; with aequorin, a calcium-dependant photoprotein; and possibly with luciferase and luciferin purified from our dinoflagellate bioluminescent cultures.

Our consultant, Dr. J. H. Carpenter, one of the experts on the use of trehalose for protein stabilization, just joined the faculty of the University of Colorado Health Sciences Center in Denver. We propose to invite him to the University of Utah to give a detailed seminar on his protein preservation work and to generally advise us and assist us in the planning and conduct of this project.

Assuming that optimum buffer, pH and trehalose concentration conditions indeed result from these studies, we will then proceed to study the effect of these optimum conditions on the behavior of immobilized firefly luciferase, including direct physical adsorption, covalent immobilization through glutaraldehyde/albumin coupling and incorporation within liposomes.

Budget

Half-time graduate student (probably Dong Min, as part of his general work dealing with the preservation and behavior of bioluminescent proteins and related materials)	$7,500
Supplies	1,500
Travel	500
Consultant, Dr. J. H. Carpenter, travel and honorarium	500
Total	$10,000

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ENHANCED ANTIBIOTIC TRANSPORT IN BIOFILMS

William G. Pitt, Natalya Rapoport and Douglas Christensen

Introduction and Background

Bacterial colonization on the surface of implanted medical devices leads to infections that compromise the health of the patient and often preclude further use of the device. These implant infections are tenacious in their resistance to the host immune system or to antibiotic therapy, and an infected implant must usually be removed to completely control the bacterial infection. An important observation is that the antibiotic concentrations that effectively kill bacteria in suspension (planktonic bacteria) are ineffective in completely killing an established biofilm on an implant. The current theory behind this observation is that transport of the antibiotic through the biofilm is very slow, and the antibiotic might be inactivated by bacterial enzymes, by adsorbing on the high surface area in the biofilm, or by some other mechanism before they can reach and kill the bacteria deep in the biofilm adjacent to the implant surface. The bacteria on the surface might be killed, but the deep ones survive and regenerate the biofilm as soon as the antibiotic therapy is stopped.

At the CBI meeting last April, Costerton proposed that electrical stimulation of a bacterial biofilm in the presence of antibiotics killed the biofilm. He was confident of the results, but not at all confident about the mechanism producing the results. Possible mechanisms include enhanced transport of the antibiotic into the biofilm or increased permeability of the cell wall toward the antibiotic. Costerton also showed confocal laser microscope images of a biofilm that revealed the presence of pores and channels from the surface to the interior of the biofilm.

We propose to investigate the use of ultrasonic treatment of the biofilm to similarly destroy the viability of biofilms on polymers or other surfaces. We hypothesize that ultrasonic stimulation of biofilms may enhance transport of bactericide to and within the biofilm, and may increase the permeability of the cell wall. In a clinical setting, ultrasonic treatment of biofilms has several advantages over Costerton's electrical treatment, should both methods prove to be effective in eliminating biofilms on implants. The major advantage is that ultrasound is non-invasive. No electrodes (or electricity) need penetrate the skin, and the ultrasonic energy can be easily controlled and focussed at the infection site. Ultrasound has been used safely and successfully for diagnostic medical imaging over the past 2 decades. There are medically accepted safe levels of power (below 100 mW/cm^2) and total dosage (less than 50 J/cm^2) [1]. Higher energies are allowed for therapeutic exposures, which include physical therapeutic treatment of bruises and burns. We believe that the clinical advantages of ultrasonic stimulation of an infected implant over electrical stimulation make this research worth pursuing.

Enhanced Membrane Transport

In addition to the applications in medical imaging and physical therapy, recent reports show that ultrasonic stimulation in the range from 48 kHz to 1 MHz is effective in increasing transport of biomolecules through the gills/scales of fish [2,3] and the skin of rodents [4-6] by 5 to 20 fold. The investigators have speculated that the ultrasound increased the permeability of the gills, scales or skin to the biomolecules. Levy has also shown that 1 MHz frequency ultrasound enhances transport of salt through polymer membranes [4]. Although these researchers have not yet determined the exact mechanism, they suspect that cavitation, local heating or local fluid movement may be involved. We believe that these mechanisms could also increase transport in bacterial biofilms.

Hypothesis and Objectives

There are three requisite steps in the elimination of a bacterial infection on an implant. These are: 1) transport of the antibiotic through the extracellular matrix to the bacteria; 2) transport of the antibiotic into the bacteria; and 3) effective antibiotic action within the bacteria which results in complete killing. Our approach concentrates on the first two steps of the process. The main hypothesis of this work is that sonic or ultrasonic pressure waves can sinusoidally compress a bacterial biofilm that will in turn pump the aqueous media in and out of the pores and channels of the biofilm. A second hypothesis is that the permeability of the bacterial wall toward antibiotic molecules is increased. The results of one or both of these processes will be to reduce the viability of bacterial biofilms on solid substrates. We propose to examine these hypotheses by accomplishing the following objectives:

1. Measure the transport of a fluorescent dye through a bacterial biofilm with and without ultrasonication.

2. Estimate the change in transport within the bacterial biofilm due to ultrasonic stimulation by measuring the change in thermal conductivity of the biofilm.

3. Measure the uptake of ESR spin-labeled gentamicin by gram positive and gram negative bacteria with and without application of ultrasound.

4. Measure the minimum inhibitory concentration of gentamicin on suspended bacteria with and without application of ultrasound.

5. Expose a growing biofilm on a solid substrate to antibiotics in the presence of ultrasound and measure the viability of the biofilm as a function of ultrasonic frequency, power and antibiotic treatment parameters.

Experimental Plan

The organisms will be clinical infection isolates of *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*. Dr. Christensen and Dr. Pitt have already assembled ultrasonic transducers, signal generators, and amplifiers to investigate a frequency range from 1 to 10 MHz.

Permeability Measurements. A permeability transport cell composed of two compartments separated by a polypropylene membrane filter (Celgard or Metricel) as described by Levy [4] will be built. The ultrasonic probe and a fluorescent tracer (rhodamine MW=381 or fluorescein MW=332) will be placed in the first compartment, and transport of the tracer into the second compartment will be measured optically. Measurements will be made with and without ultrasonication on the membrane alone and on the membrane colonized by a bacterial biofilm.

Convective Transport Measurements. If liquid is pumped in and out of the pores and channels of the biofilm, this will increase the convective transport of the biofilm. It will also increase the apparent thermal conductivity of the biofilm. We will use a transient hot-wire cell available to Dr. Pitt to measure the change in thermal conductivity of the biofilm produced by ultrasonic stimulation. This apparatus is normally used to measure the thermal conductivity of liquids [7], but it can also measure the thermal conductivity of coatings on a platinum wire. In these experiments we will grow the biofilm on the Pt wire. An electrical pulse of about 0.5 sec duration causes heating of the thin wire arranged in one leg of a Wheatstone bridge. The resistance of the wire is simultaneously measured, providing the temperature of the wire. The thermal conductivity of the biofilm can be calculated from the temperature rise of the wire. We expect that the measured thermal conductivity of the biofilm will be greater in the presence of ultrasonication. Mathematical models show that the total temperature rise of the Pt wire and biofilm is only 0.7°C, so the biofilm will not be damaged by the measurement.

Uptake of ESR-labeled Gentamicin. Gentamicin will be labeled with an ESR spin-label probe in the lab of Dr. Rapoport. A six hour old bacterial culture will be exposed to the labeled antibiotic and ultrasound for 30 minutes, and then the bacteria will be filtered and thoroughly washed in cold water, and then frozen in liquid nitrogen for transport and storage. The bacteria will be transferred to an ESR cell, thawed, and the ESR spectrum will be collected. These spectra will show both the total amount of gentamicin uptake, and reveal relatvie amounts of the gentamicin sequestered in the bacterial lipid membranes or in the cytoplasmic compartments. Comparison to similar experiments conducted without ultrasonic stimulation will show if ultrasonication enhances transport of the antibiotic through the bacterial cell wall.

Minimum Inhibitory Concentrations. The minimum inhibitory concentration of gentamicin toward the bacteria will be measured by standard procedures. Gentamicin inhibits cell growth by binding to the 30s ribosome subunit and interfering with

protein translation and synthesis. Enhanced transport into the cells will be studied by comparing inhibitory concentrations of non-ultrasonicated suspensions to those suspensions receiving ultrasound treatment over 18 hours of growth.

Biofilm Viability. To measure the viability of a biofilm subjected to ultrasonic treatment, we will build a flow cell similar to that used by Costerton into which the 1 MHz transducer will be positioned above the biofilm. In some experiments we will simultaneously observe the biofilm with a video microscope. Viability of the biofilm will be assessed by removing the cells from the surface and plating them in agar media.

Preliminary Results

Minimum Inhibitory Concentrations. During the past 2 months we have been measuring the minimum inhibitory concentrations (MICs) of gentamicin toward suspensions of *S. epidermidis* and *P. aeruginosa*. Parallel experiments were conducted on suspensions in an incubator and on suspensions placed in a temperature controlled sonicating cleaner that operates at about 35 kHz. In this sonicating bath the MIC for *P. aeruginosa* is always about 33% lower, and the viable cell count in the presence of 4 μg/ml Gentamicin is two orders of magnitude lower than without sonication. This suggests that the sonication enhances the uptake of gentamicin into *P. aeruginosa*. This effect is not observed for *S. epi*.

Preliminary Calculations. Preliminary calculations using an oversimplified model of the biofilm as a mass connected to a rigid surface by a Maxwell element (series elastic and viscous element) showed that the resonance frequency of the biofilm is given by $\omega = \sqrt{\frac{k}{m} - \left(\frac{k}{2\varphi}\right)^2}$ where k is a spring constant, φ is a damping (viscous) constant, and m is mass. For a biofilm 10 microns thick with a density of 1.05 g/cm3, the resonance frequency based on this simplified model ranged from 35 kHz to 11 MHz, depending upon the value used for the compressive modulus (from 10^2 to 10^5 Pa) of the biofilm. At this thickness, the calculated resonance frequency is fairly independent of the viscous nature of the biofilm. Although we do not know if an acoustic resonance will exist in a real biofilm, this calculation is useful in showing that the ultrasonic frequency ranges will probably produce biofilm vibrations and the accompanying pulsatile pumping of the biofilm.

Personnel

Dr. Rapoport is an expert on ESR and has worked on 2 other CBI projects involving ESR. Dr. Pitt will direct the growth of the bacteria in suspension and on the polymers. He has directed 3 other CBI projects dealing with bacterial adhesion. Dr. Christensen will direct the efforts dealing with the application and analysis of the ultrasonic treatment. He has had extensive experience in the area of ultrasonics. Students at the University of Utah and at BYU will be involved with the project.

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Budget

	Univ. of Utah	BYU	Total
Student Wages	$ 6,000	$6,000	$ 12,000
Support for Dr. Rapoport	2,000		2,000
ESR Supplies and Cells	1,000		1,000
Bacterial Growth Media and Misc. Supplies	500	500	1,000
Ultrasonic Chambers and Apparatus	1,000		1,000
Totals:	**$10,500**	**$6,500**	**$17,000**

IMAGING OF "SOFT" AND WEAKLY BOUND SURFACE MOLECULES WITH THE SCANNING INTERFACIAL FORCE MICROSCOPE

Clayton C. Williams, Anthony Dicarlo and Vladimir Hlady

Objective

To develop a Scanning Interfacial Force Microscope capable of imaging soft surfaces and weakly adsorbed surface molecules with high spatial resolution.

Motivation

The contact Atomic Force Microscope (AFM) has been shown to be limited in its ability to image soft or weakly adsorbed materials with high spatial resolution. The source of this limitation is the long range attractive forces which exist between the tip and sample. These attractive forces (and force gradients) are typically large enough to cause the AFM tip to jump into a hard repulsive contact. Under this condition, the minimum force with which one can image the surface is determined by the magnitude of this attractive force. On soft surfaces or systems of weakly bound adsorbates, this contact force is generally too large to allow imaging without significant distortion of the surface [1-3]. In the case of soft materials, the tip is embedded in the surface by these attractive forces. The tip makes contact to the surface over a large area, and poor spatial resolution is achieved. In the case of weakly bound adsorbates, the tip will typically move the adsorbates during imaging, causing irreproducible results. An alternative to contact AFM is the attractive mode or non-contact AFM. With this technique one avoids the strong interaction with the surface, but at the same time, spatial resolution is generally lost because the tip is typically more than a few nanometers from the surface.

The Scanning Interfacial Force Microscope (SIFM) [4, 5] provides a means for bringing the tip arbitrarily close to the surface without the typical jump to contact that occurs with the conventional AFM. This is achieved by applying a force on the tip by electronic feedback which exactly cancels out the force between the tip and surface. The jump to contact is eliminated by this method, allowing for force measurement to occur at very small gaps without strong repulsive contact at the end of the tip. The surface force is measured by measuring the feedback force required to compensate for the surface force at any tip/sample separation. The benefit is that imaging can be performed with high resolution due to the proximity of the probe without significant repulsive forces applied to the surface.

Proposal

1. Construct and develop a Scanning Interfacial Force Microscope.
2. Investigate its multiple modes and unique capabilities.
3. Evaluate its capabilities by comparing its performance with contact AFM results on:
   - Soft Surfaces:
     a. Crosslinked hydroxyethyl methacrylate (HEMA) films
     b. Blood platelets
   - Adsorbed Proteins:
     a. IgM on mica

Significance

The imaging of soft or weakly adsorbed molecules with the contact AFM has been seriously limited by the strong attractive interactions between the tip and surface. The Scanning Interfacial Force Microscope appears to have the potential to overcome this obstacle and to provide near atomic resolution imaging on soft or weakly adsorbed surface structure. The achievement of this goal with the SIFM should open the door to many areas of investigation on these difficult surfaces. In particular, we believe that the nanometer scale study of biopolymers at interfaces will be greatly enhanced by this new approach.

Budget

Salary (A. DiCarlo, part-time Post-Doc)	$13,500
Supplies	3,000
Sample Preparation	500
Travel	1,000
Total	**$17,500**

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MAGNETIC AND ELECTRIC FIELD ENHANCEMENT OF ANTIBIOTIC ACTIVITY IN BIOFILM-FORMING PATHOGENIC BACTERIA

Charles B. Grissom, Dianna Benson, S. Fazal Mohammad, Gregory L. Burns

Specific Questions to be Addressed

1. Is antibacterial activity in biofilms enhanced by:
   a. static or modulated magnetic fields;
   b. static or modulated electric fields?
2. What are the optimal conditions for any effect observed?
3. Is enhancement of antibacterial activity specific to particular antibiotics?
4. Is enhancement of antibacterial activity specific to particular pathogenic bacteria?

Background

At the spring 1992 CBI meeting, Professor J. W. Costerton presented data illustrating the electromagnetic field enhancement of antibacterial activity against biofilm-forming bacteria. He reported that inhibition of *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* was dramatically enhanced by an undefined electromagnetic field that alternated in polarity at a rate of less than 1 Hz. The apparatus described contained both electric and magnetic field components and did not differentiate between the importance of possible electric field effects and magnetic field effects. If this method can be developed further, it promises to improve significantly the current protocols for prophylactic inhibition of pathogenic infections in transdermal and intradermal devices.

Materials and Methods

Biomer (Ethicon, Inc.) was selected as a representative polyurethane because of its availability and its use in fabricating blood-contacting prosthetic devices in the artificial heart laboratory. Glass slides coated with a thin layer of Biomer are incubated in broth containing either *S. epidermidis* or *P. aeruginosa*. A biofilm forms within 60 minutes and the slides are washed with phosphate-buffered 0.9% saline (PBS), pH 7.4, and transferred to a growth medium containing the antibiotic to be tested. The slides are incubated in this medium in the apparatus that will give the desired magnetic or electric field. After 45 minutes, the slides are washed again and briefly sonicated to suspend the cells in PBS. This cell suspension is serially-diluted and distributed on sheep blood agar Petri plates to quantify cell viability. The microbiological aspects of the above protocol are well established and practiced on a daily basis in the Artificial Heart Research Laboratory.

Objectives 1-4 can be answered by varying the field type, field strength and antibiotic. Cell viability after treatment has been chosen because the ultimate goal is to increase antibiotic effectiveness.

Ongoing investigations of the effects of magnetic and electric fields on enzymatic and chemical reactions are underway in the Grissom laboratory. Experimental apparati have been developed to separate the magnetic field and electric field vectors to address questions related to the mode of action and optimal field conditions. Traditional wire-wound electromagnets with and without iron poles provide access to homogeneous static and modulated magnetic fields from 0-7000 Gauss and 0-500 Gauss, respectively. These magnet sytstems provide less than 0.01 V/m electric potential to enable careful studies of magnetic field effects. Parallel platinum electrodes with agarose gell-filled salt bridges provide access to electric field potentials with nearly no magnetic field component. With a 1 cm separation, this provides access to electric fields of greater than 10 V/m while minimizing unwanted solution chemistry or electrolysis of H_2O.

Results

This project has been initiated under starter money awarded from the CBI director's fund. These funds are now exhausted and other funds to support the project are not available. Our results to date are encouraging and warrant a more comprehensive and detailed investigation.

	10 Gauss*	20 Gauss*	0.1 V/cm**
Control (average colonies/plate)	100% (651)	100% (703)	100% (273)
field	-22 ± 6%	-32±5%	-30 ± 17%
gentamicin	-33 ± 8%	-33 ± 12%	-22 ± 17%
field & gentamicin	-81 ± 5%	-32 ± 9%	-23 ± 4%

*Magnetic Field. These data reflect 5 or 6 replicates.
**Electric Field. This is the result of only one determination.

There is a reproducible decrease in the viability of biofilm-sequestered *pseudomonas* in a 10 Gauss magnetic field and 2 ug/mL gentamicin. This enhanced antibiotic activity does not exist at 20 Gauss. The data at 0.1 V/cm electric field are preliminary but do not reflect an enhancement of antibiotic activity. We are now extending our experiments to additional magnetic fields and antibiotics.

One of the strengths of this proposal is the interdisciplinary team that is collaborating on this research proposal. Coworkers from the Artificial Heart Laboratory and the Department of Pathology are familiar with the problems and limitations of existing antibiotic therapies associated with transdermal and intradermal prosthetic devices. The study of biofilm formation on polymer surfaces is an ongoing aspect of their research effort. The Grissom laboratory brings expertise in magnetic and electric field technology to the project. The magnetic and electric field apparatus to be used has been constructed in the Grissom laboratory and is now available for use.

Budget

Graduate student stipend (50%)	$6,750
Digital teslameter and probe	800
Amplifier and power supply (for modulated electric fields)	800
Supplies (actual costs currently $700/month)	8,400
Total	**$16,750**

ABSTRACTS OF CURRENT RESEARCH PROJECTS

(Posters presented at semi-annual meeting)

MOLECULAR RECOGNITION OF MUTANT MYOHEMERYTHRINS AT GOLD ELECTRODES

Martin E. Fossett, Gregory M. Raner and Walther R. Ellis, Jr.

A central theme in the bioelectrochemical field involves the elucidation of factors controlling the physico-chemical interactions between proteins and electrode surfaces. Increasing attention has focused on new strategies aimed at overcoming the typically sluggish response of redox proteins at bare electrodes. Hill and coworkers have developed a variety of electrode modification procedures in order to achieve an electrochemical response in the absence of redox mediators. However, little work has been done with regard to protein modification for direct electrochemical use.

In many cases, the orientation of an adsorbed redox protein at an electrode-electrolyte interface cannot be controlled, with the consequence that the heterogeneous charge-transfer distance cannot be optimized. Protein engineering, via site-directed mutagenesis of an appropriate gene, potentially offers a means of enhancing electrode-protein molecular recognition. The placement of cysteine residues at selected surface-accessible positions of crystallographically characterized proteins should permit one to take advantage of the well-known affinity of thiols for noble metal surfaces.

The design, spectroscopic properties, and electrochemical behaviors of two myohemerythrin mutants will be described: 1) N53C, M61C; and 2) Q20C, E24C, N53C, M61C. Importantly, controlled-potential electrolysis of the parent myohemerythrin (i.e., containing no cysteine residues) is ca. 100 times slower than that of any of the above mutants.

MAKING PROTEIN SURFACE PATTERNS AS A FIRST STEP TOWARDS SITE-SELECTIVE IMMOBILIZATION OF DIFFERENT PROTEINS

Vladimir Hlady and Jie Liu

As proposed, the final goal of this study is to covalently bind different proteins to destined miniature areas. In the past several months, we have focused mainly on the first two phases of this project, modifying silica surfaces and creating surface patterns photochemically.

A fused silica surface was derivatized with 3-mercaptopropyltrimethyoxysilane (MTS). The surface was then masked and exposed to ultraviolet light in the presence of oxygen. X-ray photoelectron spectrum (XPS) analysis showed that the thiol (-SH) groups on the unmasked portion were oxidized to sulfonate [1]. Total internal reflection fluorescence (TIRF) studies indicated that the oxidized surface regions are resistant to protein adsorption[2]. The remaining thiol groups on the masked portion were further modified with p-azidophenacyl bromide (APABr) [3, 4], and the characteristic azide (-NNN) peak at 403.2 eV appeared in the XPS spectrum. On the oxidized (sulfonate) sample, the XPS data showed no N peak.

The azide groups were further activated under UV irradiation and crosslinked to nearby proteins through a nitrene intermediate[5]. It was confirmed that the crosslinking is covalent when we used fluorescein isothiocyanate labeled dextran (FITC-Dex) as a model substance.

We have achieved a 0.5 mm patterning strip of FITC-Dex on the fused silica surface using azide photochemistry. Currently, we are continuing the same experiments on a much finer scale (pattern sizes 10 μm) using silicon wafers.

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ICON REPRESENTATION OF PROTEIN STRUCTURES AND PROPERTIES

Li Feng and Joseph D. Andrade

We have designed some 20 sets of prototype icons (or cartoons) to represent a variety of structures and properties of proteins, based on the idea of the Tatra plot. Our goal is to give a direct and easily understood graphical overview for proteins or their domains so that the readers will be immediately impressed and able to quickly comprehend what they are seeing. Sixteen parameters, together with their icons, were displayed on the poster, including:

1. Molecular weight;
2. Temperature of thermal denaturation;
3. GdnHCl concentration of 50% denaturation;
4. Hydraulic compressibility;
5. Rate of surface tension kinetics at low bulk concentration;
6. Number of disulfide bonds;
7. Effective surface hydrophobicity;
8. Steady-state surface pressure;
9. Rate of surface tension kinetics at high bulk concentration;
10. Ratio of nonpolar residues in sequence;
11. α-moment multiplied by α-helix fraction;
12. β-moment multiplied by β-helix fraction;
13. Accessible negatively charged oxygen atoms;
14. Surface charge density;
15. Solution solubility and stability; and
16. Surface concentration in competitive protein adsorption.

The poster also showed examples of applications of icons to description of the globular proteins lysozyme and myoglobin. Comparison of a structure or property of the two proteins can be directly made.

SURFACE CURVATURE EFFECTS ON PROTEIN ADSORPTION AND BIOLOGICAL ACTIVITY

Maneesh Adyanthaya, Patrick Huang and Karin D. Caldwell

Colloidal particles are increasingly used as the solid phase in reactions between an immobilized biomolecule and its soluble substrate or ligand. Typical examples include both analytical uses of colloid-protein complexes in latex-based immunodiagnostics, as well as preparative uses in which some ligand of interest is purified through biospecific adsorption to its complementary protein, attached to a particle for easy segregation from soluble contaminants. Whether the application is analytical or preparative, both the surface density of the immobilized protein, as well as its specific activity in the adsorbed state, will affect the function of a particle-protein complex.

The two expected primary effects of varying substrate size are: (1) an increase in surface area and therefore in total protein with a decrease in diameter for a given mass of particles; and (2) an increased reactivity associated with a decrease in size and the related increase in mass transfer. In addition, previous observations from this laboratory have indicated that particle curvature may influence the adsorption of synthetic polymers as well as proteins.

In the first phase of the present study, we have concentrated on developing methods for determining surface concentrations and biological activities for the smallest proteins in our proposed series, namely hen lysozyme (LYZ) and Streptavidin (SA). For the latter, a new fluorescence based assay is shown to agree well with data from amino acid analysis; for a 272 nm particle, both indicate the maximum surface concentration of irreversibly bound protein to be around 1 pmole/cm^2. The specific binding of biotinylated small ligands is only 0.3 moles per mole of adsorbed SA, as opposed to 4 moles per mole of soluble SA. Compared to a commercial SA-coated 2.8 μm bead (with unknown protein surface concentration), the amount of biotin bound per unit weight is 4 times higher on the smaller particle. LYZ has been adsorbed to a series of particles ranging from 165-360 nm in diameter. From amino acid analysis, the surface concentration is showing a reproducible maximum of 21 pmole/cm^2 for diameters around 300 nm.

Although adsorption complexes of several other proteins, including IgG, can be accurately quantified by sedimentation FFF, neither of the complexes involving SA or LYZ are amenable to this strategy due to heavy aggregation resulting from the field-induced particle concentration.

MAGNETIC FIELD ENHANCEMENT OF ANTIBIOTIC ACTIVITY IN BIOFILM-FORMING BACTERIA

Gregory Burns, Fazal Mohammad, Dianna Benson, Charles B. Grissom, Chi-ching Hwang and Mathew Thompson

Electromagnetic fields have been identified as a possible means of increasing antibiotic activity against biofilm secreting bacteria. Work presented at the Spring, 1992, CBI Meeting by Dr. W. Costerton demonstrated enhancement of antibiotic activity against biofilm-forming bacteria utilizing an undefined alternating electromagnetic field.

Although both magnetic and electric field components were present, differentiation between the possible effects of the magnetic and electric field components was not determined. Thus, it is not known if one field component, both field components, or none of the field components are responsible for the increased effectiveness of the antibiotic. The aim of this project is to investigate individually the effects of magnetic and electric fields on antibiotic effectiveness against biofilm-forming bacteria.

Results of our experiments indicate a significant enhancement of antibiotic effectiveness against biofilm-forming bacteria in the presence of a magnetic field. A reproducible 77% decrease in the viability of biofilm-sequestered *Pseudomonas aeruginosa* was noted in the presence of a 10 Gauss magnetic field and 2μg/mL gentamicin as compared to control (no antibiotic/no magnetic field). A smaller decrease of 42% as compared to control occurred at 20 Gauss and 2 μg/mL of gentamicin. Decreased viability was not noted at 30 Gauss. The mechanism by which the magnetic field may enhance the activity of gentamicin is not known. Electric field results are preliminary and have not indicated enhancement of gentamicin activity against biofilm sequestered *Pseudomonas aeruginosa*.

CHARGE DENSITY MAPPING OF MOLECULAR STRUCTURE UNDER SOLUTION

Anthony DiCarlo, Yao-Jian Leng and Clayton C. Williams

Objective

The objective of this project is to experimentally establish a method for performing high resolution charge mapping under solution with the Electrostatic Force Microscope (EFM).

Results

We have constructed an Electrostatic Force Microscope in a closed liquid cell. The cell is constructed so as to provide for the measurement of force between tip and sample by conventional optical beam deflection method. The sample can be positioned within the cell in three dimensions by a piezoelectric tube scanner. Both contact and non-contact force imaging has been performed under liquid in the cell. Charge mapping has been demonstrated under solution on a metallic grating structure in a deionized water solution. The spatial resolution achieved in the charge images is consistent with the approximate size of the tip. A novel magnetic force feedback has been employed to provide force versus distance curves all the way to contact without the usual snap in. Force versus distance curves have been performed under solution. Under nominally clean conditions, the electrostatic screening length was measured to be greater than 20 nanometers.

These results, which represent the first demonstration of charge mapping under solution, were achieved with a 6 month effort. In the second half of the project, we will apply this newly established technique to biomolecular materials to explore its potential for mapping molecular charge under solution.

MOLECULAR DYNAMICS SIMULATIONS OF PROTEIN-SURFACE-WATER INTERACTIONS

Daniel R. Weaver, William G. Pitt and N. Troy Tagg

We have performed molecular dynamics simulations of protein-surface-water interactions to study protein adsorption on polymers. The simulations were performed on a model system consisting of a pentapeptide (enkephalin), a surface polyethylene and water. The purpose of these studies was to estimate a probability of adsorption with molecular dynamics and to compare the results with experimental measurements. A secondary objective was to ascertain the role of water in the adsorption of proteins. Based on the assumption of pairwise additivity, the molecular dynamics simulations provided a method to determine independently the surface contribution and the water contribution to adsorption.

In our procedure, molecular dynamics is used to estimate the force field that a protein experiences as it diffuses to a surface. We then apply the theory of forced diffusion to determine the probability that the protein remains associated with the surface.

The results of the simulations and calculations

show that for one rotational orientation of the peptide there is a force of repulsion and the probability of adsorption is zero. For three other rotations that we have considered, there is a force of attraction and the adsorption probabilities are .96, .88 and .82. We hypothesize that the difference between these two results is an entropic or hydrophobic effect. In the first rotation, six hydrophilic oxygen atoms are exposed to the surface. In the other three rotations, only hydrophobic side groups are exposed to the surface. The adsorption of enkephalin on hydrophobic polyethylene is only probable for hydrophobic sections of the peptide.

Kinetics measurements of enkephalin adsorption on a model PE surface were also taken using FTIR-ATR. Similar to the observations of other authors, the kinetics are linear in the t^1/2 domain. Our analysis of the experimental data indicates that the intrinsic kinetics are first order. By applying a correlation to our data, we estimate that the experimental adsorption probability is on the order of 10^-9.

INTERFACIAL BEHAVIOR OF FIREFLY LUCIFERASE

Chung-Yih Wang and Joseph D. Andrade

Firefly luciferase (photinus pyralis) is a hydrophobic protein. Sixty percent of its amino acid residues are hydrophobic. A phenylsuperose chromatography column is used to estimate the effective surface hydrophobicity. Bovine serum albumin whose molecular weight (dimension 30x80x110 Å) is similar to luciferase, and lysozyme are used as model proteins. The results show that the hydrophobicity of firefly luciferase is moderate, suggesting that most of its hydrophobic residues are buried inside. The fact that luciferase aggregates easily in aqueous environment also supports this interpretation.

Dynamic surface tension of firefly luciferase is measured via pendant drop tensiometry. When luciferase concentration is high, the fast decrease of surface tension suggests that luciferase is very surface active. When luciferase concentration is low, a short induction time is observed.

Luciferase adsorption at the air-water interface is studied via the Wilhelmy plate method. Luciferase adsorbing to air-water interfaces was a slow process. The equilibrium surface pressure was 21.5 mN/m. Three different lipids, DPPC, DOPC and DOPS, are used to study luciferase-lipid interactions. The surface pressure change is measured with a Wilhelmy plate. The surface pressure was higher at liquid-state lipid monolayer than gas-state lipid monolayer. This suggests that luciferase can adsorb to air-water interfaces when the lipid monolayer is in a gas-state. When the lipid monolayer is closely packed, luciferase interacts with the polar head of the lipid monolayer and then partially penetrates into the lipid monolayer. The Dp increased perhaps because a hydrophobic patch on luciferase penetrated into the lipid layer, compressing it to a smaller area.

INTERACTIONS BETWEEN LIPOPROTEINS AND SURFACES

Chih-Hu Ho and Vladimir Hlady

The project "Interactions Between Lipoproteins and Surfaces" started in March, 1992. In this first phase of the project, we focused on the isolation of lipoproteins, labeling of lipoproteins and their adsorption behavior using the wettability gradient surfaces. The results of research reported in the previous progress reports and newsletters include:

1. Separation of lipoprotein from human plasma;
2. Preparation of model surface: the dimethyldichlorosilane (DDS) wettability gradient surface;
3. Fluorescent probe labeling on the two components of lipoprotein: lipid component and protein component;
4. Lipoprotein adsorption onto the DDS wettability gradient surface measured in the dual flow channel TIRF cell;
5. Competitive adsorption of human lipoproteins and human serum albumin (HSA) onto the DDS wettability gradient surface.

Here, we report on our progress in the study of lipoprotein adsorption onto an octadecyldimethylchlorosilane (C18) wettability gradient surface. This surface was primarily used because the eighteen -CH2 side chains of C18 are similar to the hydrophobic tail of lipids. We have performed the experiment in which FITC-HDL, FITC-LDL, DiO-HDL, DiO-LDL and FITC-HSA were separately adsorbed onto the wettability gradient surface of C18 from diluted solution. Adsorption of low density lipoprotein (LDL) components onto the C18 wettability gradient surface was reported on the poster. Similar to the case of LDL adsorption on the DDS surface, the LDL adsorption on the C18 gradient surface was initially transport-limited in the hydrophilic region of the wettability gradient surface. This feature was used to quantitate the adsorption, taking into account the hydrodynamic conditions of the adsorption experiment. LDL adsorption of the hydrophobic surface is no longer transport-limited. The results indicated that the adsorption behavior of the LDL molecule is influenced by the presence of HSA. Pre-adsorption of HSA prevents LDL adsorption onto a non-hydrophilic surface. The interplay between LDL and HSA affinity for the wettability gradient surface determines the rate of the LDL adsorption from the LDL + HSA adsorption mixture. A simple model is proposed to explain the observed results.

ORIENTATION OF ADSORBED IgG: DOES ACID TREATMENT LEAD TO BETTER ORIENTATION?

I-Nan Chang and James Herron

The specific aim of this project is to demonstrate the acid pre-treatment of antibodies (Abs) in solution lead to a better orientation of adsorbed Abs on surfaces. In previous studies, we showed the acid-treated antibodies (Abs) caused the increased antigen binding capacity on hydrophobic silica surfaces than the native Abs (non-acid-treated). Furthermore, the increased antigen binding capacity was due to a higher packing density of acid-treated Abs, rather than to an increase in their intrinsic affinity. One hypothesis is that the acid treatment partially denatured the surfaces of Abs. This partially denatured surface region of Ab would lead to the preferential adsorption (and higher packing density) of Abs on the hydrophobic surfaces.

During the last CBI meeting, we showed that the photo-affinity labeling fluorophore methods could be used to immobilize the extrinsic fluorophores onto Ab surfaces at specific regions of Abs. First, the fluorophore was specifically chemically attached to the vicinity of the Ab active site through photo-affinity labeling technique. Also, the controlled experiments were done by labeling the fluorophores to the hinge region of an Ab. Then the iodide quenching method was used to probe both kinds of labeled extrinsic fluorophores of the Abs in buffer solution and on surfaces.

The goat anti-biotin polyclonal Ab (α-biotin Ab) and the mouse 9-40 anti-fluorescein monoclonal Ab (9-40 Ab) were used. Fluorescein maleimide (FM) was the labeled fluorophore used for the α-biotin Ab system and, tetramethyl rhodamine (TMR) was for the 9-40 Ab system. Potassium iodide was used as chemical quencher. The hydrophobic non-charged dichloro-dimethyl silane (DDS) modified silica surfaces were the model substrates.

A modified Stern-Volmer quenching model, which assumes accessible and inaccessible populations of fluorophores (FM and TMR), was applied to analyze the experimental data [1]. The change in the fractional accessibility of fluorophores was taken as a measure of Ab orientation on DDS surfaces. The results correspond to our previous results of antigen binding capacity of Abs on DDS surfaces as a function of acid denaturation times [2], and suggest the acid pre-treatment of Abs lead to better orientation.

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PACKING OF ADSORBED PROTEINS: AN ENERGY TRANSFER STUDY

Tsing-Hua Zheng, Vladimir Hlady and James N. Herron

The fluorescence energy transfer technique was used to probe the packing interactions of IgG molecules on the surface. Mouse IgG was labelled with either fluorescein (F-IgG) as donor or with tetramethylrhodamine (R-IgG) as acceptor. Both DDS coated surfaces and wettability gradient surfaces were used in this study. Total Internal Reflection Fluorescence Spectroscopy (TIRF) was used to monitor the adsorption process.

Some results obtained are:

1. Determination of the characteristic energy transfer distance of the donor-acceptor pair, the distance was calculated using the Forster Theory. Results indicate that the characteristic distance (where the efficiency is 50%) is about 50 Å.
2. Adsorption study of a mixture of F-IgG and R-IgG on both hydrophobic (DDS) and wettability gradient surfaces by TIRF. This work includes adsorption isotherm and kinetics.

Future work will include quantitatively determining the amount of IgG adsorbed on the surface using radioisotope labelling method, measuring the profile of adsorption on the gradient surface with autoradiography, and supplementary work on TIRF studies.

BACTERIAL ADHESION TO ORTHOPEDIC POLYMERS

Alan Barton, Michael McBride, Richard Sagers and William Pitt

The objective was to determine the relative adhesion rates of *S. epidermidis*, *P. aeruginosa* and *E. coli* to poly(orthoester) (POE), poly(etheretherketone) (PEEK), poly L-lactic acid (PLA), polysulfone (PSF) and polyethylene (PE), polymers that are currently used or have potential use as orthopedic implant materials. These polymers were exposed in a laminar flow cell to each of the bacteria under two conditions: 1) with the bacteria remaining in its growth medium, tryptic soy broth (TSB); and 2) with the bacteria filtered, washed and resuspended in phosphate buffered saline (PBS).

The adhesion rates of bacteria in the PBS were much higher than the rates of adhesion of the same bacteria suspended in TSB. *P. aeruginosa* in TSB and PBS adhered at a higher rate to PEEK than to the other polymers. Also, *E. coli* appeared more adherent to PE and PSF than to the remaining polymers. Experiments in which *S. epidermidis* was seeded onto the polymer surfaces and allowed to grow for 6 hours with a flowing stream of new TSB showed that the bacteria is more likely to be swept off from the surface of PE and PEEK than POE. A growth experiment, which was carefully monitored on the video microscope system, showed that more than half of the newly dividing cells on a POE film did not remain adherent to the surface.

THE EFFECT OF SONICATION ON GENTAMICIN UPTAKE

The objective of this experiment is to determine if sonication increases the uptake of gentamicin by bacteria. This is a preliminary study in an effort to develop a more effective method of delivering antibiotics to biofilms.

After 14 hours, the positive control of bacteria and TSB without gentamicin grew to the same concentration in the sonicator as it did in the incubator. At a gentamicin concentration of 3 μg/ml there was a two log difference in bacterial concentrations between the sonicated and incubated test tubes. These results indicate that there is a significant increase in the effect of gentamicin on *P. aeruginosa* in the presence of sonication. There are several theories to explain this phenomenon, including: increased transport of gentamicin, increased active transport, cytoplasmic membrane disruption and the creation of gaps in the cell wall.

FLUORESCENCE LIFETIMES OF PROTEINS AT INTERFACES - Part II

Wim Jiskoot, Vladimir Hlady and James N. Herron

The aim of this project is to obtain a picture of the conformation and dynamics of proteins at interfaces using fluorescence spectroscopy and the geometry of total internal reflection. Fluorescent ligands bound to specific protein binding sites are used as the reporter groups. Lifetimes, time-resolved emission spectra and time-resolved anisotropy of bound ligands are parameters that can provide the sought information as well as information about the changes in the micro-environment of bound fluorophores. Solution proteins with bound ligands are used as a reference state.

Fluorescence lifetime measurements were performed on bovine serum albumin with covalently bound fluorescein isothiocyanate (**BSA-FITC**), bovine serum albumin with bound 1-anilinonaphthalene-8-sulfonate (**BSA-ANS**), and **antifluorescyl monoclonal antibody (9-40) with fluorescein**, both at silica-buffer interfaces or in buffer solutions. The previously reported experimental fluorescence data for these model systems could be modeled as summations of two or three lifetime components. Multiple lifetime component systems are difficult to interpret. The observed lifetime heterogeneity in the above-mentioned model systems could be due to (1) homoenergytransfer between closely spaced fluorescent dyes, (2) heterogeneity in the binding sites of the dyes, and (3) the intrinsic photophysical properties of the dye molecules.

In order to gain more insight into the nature of the observed heterogeneity, we used **dextran-FITC** and **glucosamine-FITC** as standards. The use of homologous sugar chains limits micro-environment heterogeneity, and in the case of glucosamine-FITC energy transfer is not expected. Nevertheless, solutions of both dextran-FITC and glucosamine-FITC showed lifetime heterogeneity. The effect of the collisional quencher iodide on the different lifetime components was studied in order to distinguish the origin of heterogeneity. These results were discussed in more detail on the poster.

Previous experiments were performed on a multilayered protein film created at a flat silica surface: Silica - APS - Biotin - Avidin - Biotin - **antifluorescyl Fab'(9-40) with fluorescein** bound to the active site. Analysis of the reference lifetime decay of antifluorescyl monoclonal antibody (9-40)-fluorescein solution also showed multiple lifetime decays, the possible origin of which will be discussed. The fluorescence of bound fluorescein could not be quenched by iodide, indicating that it is deeply buried in the antigen-binding site. Time-resolved anisotropy measurements showed that fluorescein is tightly bound in the binding pocket and does not show measurable local motion.

BAUSCH & LOMB INDUSTRIAL INTERNSHIP

Lisa Wenzler and Thomas P. Beebe, Jr.

As a recipient of the NIH Training Grant and an active member engaging in CBI funded research this past year, I've had the opportunity to interact with many industrial scientists. Because of this interaction, opportunities such as internships were encouraged. During the month of February I did fundamental research with a former graduate student, Dr. Carol Rabke, in the laboratories of Dr. Paul Valint (CBI Affiliate) at Bausch & Lomb in Rochester, New York. This enabled me to experience day-to-day life in industry, use top-of-the-line instrumentation, interact with a wide variety of scientists and discuss ideas and techniques with others.

During my stay at Bausch & Lomb and the months prior, there were several projects going on. Currently, the greatest challenge to imaging biomolecules with atomic force microscopy (AFM) arises from the mobility of the molecules on the surface. Their mobility results from a weak interaction of the molecule with the surface, allowing the tip to push the molecule around during image acquisition. We are therefore concentrating on methods to overcome these difficulties which employ some form of chemical modification of the surface and/or the biomolecule itself.

We have continued looking at DNA images with the two presently known methods of magnesium acetate-treated mica to adsorb DNA onto a surface. The first method which has commonly been used by electron microscopists (Sogo method) involves "activation" of the DNA by dilute salt solutions [1]. A recent modification of this method which has been utilized by others in the field has been introduced by members of the AFM community (Bustamante method) [2].

We have also begun to develop another technique for immobilization of DNA on a surface: attachment of colloidal gold balls to DNA using the biotin-streptavidin interaction [3]. And finally, we attempted a labeling technique that will allow distinction of adenine from guanine on single-stranded DNA [4]. The method involved the reaction of platinum(II) - dimethylsulphoxide complex ($PtMe_2SO$) with the DNA bases.

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