Department of Energy

Final Report and Summary

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Enzyme Activity and Biomolecule Templating at Liquid and Solid Interfaces

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Harvey W. Blanch
Department of Chemical Engineering
University of California at Berkeley
Berkeley, CA 94720

blanch@berkeley.edu

Students Supported by this Grant

Martin Colaco (PhD candidate, current)
David Kim (PhD candidate, completed degree 2004)
Sean Ferree (PhD candidate, completed degree 2004)
Luis Cascao-Pereira (PhD candidate, completed 2002)
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Christian Johansson (visiting student, Univ. of Lund)

Journal Publications supported by and acknowledging this grant

1. Cascao Pereira, L., Hickel, A., Radke, C.J., H.W. Blanch, "A Kinetic Model for Enzyme Interfacial Activity and Stability: pa-Hydroxynitrile Lyase at the Diisopropyl Ether/Water Interface", Biotech. Bioeng. 78 (6), 595-605 (2002)

2. D.T. Kim, H.W. Blanch, and C.J. Radke, "Direct Imaging of Lysozyme Adsorption onto Mica by Atomic Force Microscopy," Langmuir, 2002, **18**, 5841-5850

3. Cascao-Pereira, L., Johansson, C., Radke, C.J. & H.W. Blanch, "Surface Forces and Drainage Kinetics of Protein-Stabilized Thin Aqueous Films", Langmuir 19, 7503-7513 (2003)

4. Cascao-Pereira, L., Theolody, O., Blanch, H.W. & C.J. Radke, "Dilatational Rheology of BSA Conformers at the Air/Water Interface", Langmuir, 19(6) 2349-2356 (2003)

5. Cascao-Pereira, L., Hickel, A., Radke, C.J. & H.W. Blanch, "Interfacial versus Homogeneous Cleavage of Mandelonitrile by Hyroxynitrile Lyase in a Biphasic System", Biotechnology & Bioengineering, 83 (4), 498-501 (2003)

6.Ferree, S. & H.W. Blanch, "Electrokinetic Stretching of Tethered DNA", Biophys. J., 85 (4) 2539-2546 (2003)

7. Kim, D.T., Blanch, H.W. & C.J. Radke, "Protein adsorption studied by atomic force microscopy", Encyclopedia of Nanoscience and Nanotechnology, James A. Schwarz, Cristian I. Contescu, and Karol Putyera, Eds.; Marcel Dekker, Inc.: New York, 2004; pp 3041-3064.

8. Ferree, S. & H.W. Blanch, "The Hydrodynamics of DNA Electrophoretic Stretch and Relaxation in a Polymer Solution", Biophys. J., 87, 468-475 (2004)

9. David T. Kim, Harvey W. Blanch, and Clayton J. Radke, "Imaging of Reconstituted Purple Membranes by Atomic Force Microscopy," Colloids and Surfaces B: Biointerfaces (to appear, 2004)

Presentations related to this grant

Cascao-Pereira, Luis G.; Blanch, Harvey W.; Radke "Stability Mechanism of Protein Interfacial Layers", ALZA Corporation, Mountain View, CA, January 24th, 2002.

Kim, D.T., Blanch, H.W., & Radke, C.J., "Clustering adsorption of lysozyme onto mica by atomic force microscopy", Colorado Protein Stability Conference, July 18-20 2002

L. G. Cascão-Pereira, A. Hickel, Harvey W. Blanch and Clayton J. Radke. "Protein Stability at Fluid Interfaces," 2002 Colorado Protein Stability Conference, Breckenridge, CO, July 17-20, 2002.

Kim, D.T., Blanch, H.W., & Radke, C.J., "Direct imaging of lysozyme adsorption onto mica from aqueous solution by atomic force microscopy", 224th ACS National Meeting, Boston, August 2002

Cascao-Pereira, Luis G.; Blanch, Harvey W.; Radke, Clayton J. "Stability mechanisms of protein interfacial layers, 225th ACS National Meeting, New Orleans, LA, United States, March 23-27, 2003

Blanch, Harvey W.; Ferree, Sean M., "Probing the mechanisms of the electrophoretic separation of DNA", 225th ACS National Meeting, New Orleans, LA, United States, March 23-27, 2003

D.T. Kim, H.W. Blanch, and C.J. Radke "Direct Imaging of Aqueous Lysozyme Adsorption onto Mica by Atomic Force Microscopy," Paper 14, 76th ACS Colloid and Surface Science Symposium, University of Michigan, Ann Arbor, MI, June 23-26, 2002.

Blanch, Harvey W.; Ferree, Sean M., "Probing the mechanisms of the electrophoretic separation of DNA", 225th ACS National Meeting, New Orleans, LA, United States, March 23-27, 2003

Blanch, H.W., "Beyond Sieving: Probing the mechanism of the electrophoretic separation of DNA", Princeton University, October 22, 2003

L. G. Cascão-Pereira, O. Théodoly, Harvey W. Blanch and Clayton J. Radke. "Dilatational Rheology of BSA Conformers at the Air/Water Interface," 11th International Conference on Surface and Colloid Science. Brazil September 15-19, 2003

Payne, M., Blanch, H.W. & C.J. Radke, "Conformational behavior of proteins at the oil/water interface". AIChE Annual Meeting, San Francisco November 16-21, 2003

Final Report

There are two main components of this research program. The first involves studies of the adsorption and catalytic activity of proteins at fluid-fluid and fluid-solid interfaces; the second employs biological macromolecules as templates at the solid-liquid interface for controlled crystallization of inorganic materials, to provide materials with specific functionality. The following sections describe the accomplishments resulting from the grant support.

1. Enzyme Adsorption and Catalysis at Fluid Interfaces

Adsorption of proteins at fluid and solid interfaces is a complex phenomenon affecting the behavior of biological and industrial biochemical processes, food technology, drug stability and biofouling. Quantitative understanding of enzyme adsorption and loss of activity due to conformational rearrangements at the interface is one of the main obstacles to be overcome in expanding the industrial application of enzymes as biocatalysts in two-phase systems. Our objective has been to provide a detailed understanding of enzyme adsorption at fluid/fluid interfaces and subsequent denaturation. We have approached this problem by examining the behavior of a lyase (hydroxynitrile lyase Hnl), which is shown to catalyze a reaction specifically at the organic/water interface.

We have also developed a model of enzyme adsorption processes at liquid-liquid interfaces. Conformational changes at the interface have been examined by TIRFS and fluorescence energy resonance transfer (FRET). Protein packing and diffusion at the interface has been studied using fluorescence recovery after photobleaching (FRAP). These experimental approaches provide a complete description of protein adsorption, kinetic activity, interfacial mobility and the factors responsible for denaturation. The molecular processes involved in protein adsorption at solid-liquid interfaces have been visualized by atomic force microscopy. The effect of solution properties (pH, ionic strength) has been determined at various times during the adsorption process. Subsequent studies involving derivatization of the surface and nanopatterning have been aimed to provide a more complete understanding of the adsorption process.

1.1 Kinetics of interfacial enzyme activity

A kinetic framework has been developed to describe enzyme activity and stability in two-phase liquid-liquid systems. In particular, the model is applied to the enzymatic production of benzaldehyde from mandelonitrile by *Prunus amygdalus* hydroxynitrile lyase (pa-Hnl) adsorbed at the diisopropyl ether (DIPE)/aqueous buffer interface (pH 5.5). We have been able to quantitatively describe our previously obtained experimental kinetic results (Hickel *et al.*, 1999; 2001), and successfully account for the aqueous-phase enzyme concentration dependence

of product formation rates and the observed reaction rates at early times. Multilayer growth explains the early time reversibility of enzyme adsorption at the DIPE/buffer interface observed by both enzyme-activity and dynamic-interfacial-tension washout experiments that replace the aqueous enzyme solution with a buffer solution. The postulated explanation for the unusual stability of pa-Hnl adsorbed at the DIPE/buffer interface is attributed to a two-layer adsorption mechanism. In the first layer, slow conformational change from the native state leads to irreversible attachment and partial loss of catalytic activity. In the second layer, pa-Hnl is reversibly adsorbed without loss in catalytic activity. The measured catalytic activity is the combined effect of the deactivation kinetics of the first layer and of the adsorption kinetics of each layer. For the specific case of pa-Hnl adsorbed at the DIPE/buffer interface this combined effect is nearly constant for several hours resulting in no apparent loss of catalytic activity. Our kinetic model can be extended to other interfacially active enzymes and other organic solvents. Interfacial-tension lag times provide a powerful tool for rational solvent selection and enzyme engineering.

Further details are in the publications:

Hickel, A., Radke, C.J., & H.W. Blanch, "Hydroxynitrile lyase adsorption at liquid/liquid interfaces", Journal of Molecular Catalysis B: Enzymatic, 216, (5) 348-354 (1998)

Hickel, A., Radke, C.J. & H.W. Blanch, "Hydroxynitrile Lyase at the Diisopropyl Ether/Water Interface: Evidence for Interfacial Enzyme Activity", Biotech. Bioeng. 65, 425-436 (1999)

Hickel, A., Radke, C.J. & H.W. Blanch, "The Role of Organic Solvents on p-Hydroxynitrile Lyase Interfacial Activity and Stability", Biotech. & Bioeng. 74, 18-28 (2001)

Cascao Pereira, L., Hickel, A., Radke, C.J., H.W. Blanch, "A Kinetic Model for Enzyme Interfacial Activity and Stability: pa-Hydroxynitrile Lyase at the Diisopropyl Ether/Water Interface", Biotech. Bioeng. 78 (6), 595-605 (2002)

Cascao-Pereira, L., Hickel, A., Radke, C.J. & H.W. Blanch, "Interfacial versus Homogeneous Cleavage of Mandelonitrile by Hyroxynitrile Lyase in a Biphasic System", Biotechnology & Biosngineering, 83 (4), 498-501 (2003)

1.2 Surface force measurements of protein adsorption

Surface or disjoining forces between protein layers adsorbed at the air/water interface were measured in single, isolated films. Two proteins, β-casein and bovine serum albumin (BSA), were investigated under varying conditions of pH, ionic strength, and degree of aging at the interface. Force-distance curves were determined using a modified thin-film balance and interferometer, using both equilibrium and dynamic methods. Dynamic surface-tension and ellipsometry data for β-casein and BSA adsorption at the air/water interface are also reported.

Charged β-casein and BSA molecules do not strongly adsorb at the air/water interface. Accordingly, stable films were observed when electrostatic interactions were screened or the proteins were near their isoelectric points. For β-casein, the force-distance curves indicated a transition from an outer to an inner branch at a

distance equivalent to the diameter of β-casein. This depended on the interfacial structuring of adsorbed β-casein multilayers at the air/water interface. Upon thinning of the film, BSA retained its native dimensions, whereas β-casein did not. As a consequence, fresh β-casein films are more stable against rupturing than are BSA films.

Aging imparted mechanical rigidity to the interfaces, causing non-uniform film drainage and non-equilibrium, trapped dimples. For films of intermediate degree of aging, black-film formation was observed through the formation of noncircular thin spots. Prolonged aging resulted in the development of interfacial aggregated networks and in films of variable thickness that did not respond to changes in capillary pressure.

Further details are provided in the publication:
Cascao-Pereira, L., Johansson, C., Radke, C.J. & H.W. Blanch, "Surface Forces and Drainage Kinetics of Protein-Stabilized Thin Aqueous Films", Langmuir 19, 7503-7513 (2003)

1.3 Dynamic rheometry to probe the protein-fluid interface

To elucidate the role of protein conformation at the air/water interface we measured the interfacial dilatational rheology of bovine serum albumin (BSA) and β-casein adsorbed over long time periods using a modified dynamic pendant-drop tensiometer. Companion long-time dynamic surface pressure and ellipsometry measurements are also reported. BSA has well-characterized structural isomers (conformers) whose structural transitions depend on solution pH. It is, therefore, possible to establish a connection between protein structure and interfacial rheology without the ambiguity of comparing different proteins. We also study to β-casein to verify the conclusions obtained from BSA.

Adsorbed BSA and β-casein protein layers are primarily elastic with the dilatational elastic modulus arising from two contributions: (1) conformational rearrangement following adsorption that leads to formation of an interconnected, sample-spanning interfacial protein network (i.e., an interfacial gel), and (2) the intrinsic structural stability of the individual protein units within the network. The latter component is most important to the interfacial dilatational modulus and explains why adsorbed layers of rigid, globular proteins are more elastic than those of flexible, random-coil proteins. We have identified a new surface elasticity relaxation mechanism at the interface due to interprotein conformational rearrangement that is enhanced by electrostatic screening.

Further details are provided in the publication:
Cascao-Pereira, L., Theolody, O., Blanch, H.W. & C.J. Radke, "Dilatational Rheology of BSA Conformers at the Air/Water Interface", Langmuir, 19(6) 2349-2356 (2003)

2. Nanoscale Control of Crystalline Architecture through Biomimetic Guided Growth on DNA and Protein Templates

We are examining the use of DNA and protein assemblies to design surfaces that will nucleate crystals of $CaCO_3$ and other mineral systems in an ordered manner. Using a combination of optical lithography and molecular biology, solid surfaces can be patterned on the nanometer scale.

We have developed techniques in our laboratory to prepare four different types of DNA templates: 1) isolated linear DNA molecules, 2) densely packed linear DNA molecules, 3) isolated branched DNA molecules, and 4) films or monolayers of DNA oligomers. These different DNA templates will allow us to investigate not only the role of a single molecule in the heterogeneous nucleation of inorganic crystals, but also the synergistic effects of multiple molecules and DNA topology. Ordered assemblies of proteins on mica have been examined to provide larger-scale ordered structures. Lysozyme has served as an initial model system to develop techniques; we are currently examining the purple membrane protein bacteriorhodopsin, which orders into trimers in well defined patterns.

We have studied the effect of soluble organic molecules on the growth patterns of templated crystals through the addition of small model peptides to the crystal growth media. The crystal nucleation events and nanometer scale growth rates have been monitored using *in situ* atomic force microscopy. The growth rates and patterns formed on the larger scale can be studied using optical microscopy with crossed-polarized light illumination.

2.1 Adsorption of proteins on surfaces – AFM studies

The adsorption dynamics of hen-egg-white lysozyme (HEWL) onto muscovite mica has been examined using *in situ* tapping mode AFM in aqueous solution. Under stagnant conditions and at a bulk concentration of 2 mg/mL, lysozyme clusters were imaged on mica several minutes after exposure to the protein solution. Force-volume imaging confirmed the existence of different tip-surface interaction forces corresponding to areas of bare mica and areas covered with a single layer of adsorbed protein. ^{14}C-labeled lysozyme was used to determine the mass of protein adsorbed. This information, in conjunction with the AFM images of submonolayers, reveals that lysozyme, once adsorbed, diffuses on a time scale of minutes or less on the surface, and forms clusters consisting of about five molecules. In contrast, at a protein concentration of 5 mg/mL, surface coverage increases uniformly until a complete monolayer is established after two hours. Once the initial protein monolayer is established, additional protein molecules adsorb onto the first layer, but at a slower rate. Washout of the protein solution at both short and long times and under both submonolayer and multilayer conditions has no influence on surface coverage, indicating irreversible

adsorption. Our AFM data are consistent with a model in which the protein, once adsorbed onto the surface, undergoes conformation change due to protein-surface interactions. Previously buried hydrophobic residues are exposed and available for interaction with the exposed hydrophobic residues of other adsorbed proteins, resulting in surface aggregation. By studying surface coverages below that of a monolayer, protein clustering can be observed.

Further details are provided in the publications:

Kim, D. T.; Blanch, H. W.; Radke, C. J. Protein Adsorption Studied by Atomic Force Microscopy. In Dekker Encyclopedia of Nanoscience and Nanotechnology, James A. Schwarz, Cristian I. Contescu, and Karol Putyera, Eds.; Marcel Dekker, Inc.: New York, 2004; pp 3041-3064.

D.T. Kim, H.W. Blanch, and C.J. Radke, "Direct Imaging of Lysozyme Adsorption onto Mica by Atomic Force Microscopy," Langmuir, 2002, 18, 5841-5850

2.2 Electrophoretic stretching of DNA

During electrophoretic separations of DNA in a sieving medium, DNA molecules stretch from a compact coil into elongated conformations when encountering an obstacle and relax back to a coil upon release from the obstacle. These stretching dynamics are thought to play an important role in the separation mechanism. A silicon microfabricated device was constructed to measure the stretching of DNA in electric fields. Upon application of an electric field, electroosmosis generates bulk fluid flow in the device, and a protocol for eliminating this flow by attaching a polymer brush to all silicon oxide surfaces is shown to be effective. Data on the steady stretching of DNA in constant electric fields has been obtained. The data corroborate the approximate theory of hydrodynamic equivalence proposed by Long and coworkers, indicating that DNA is not free draining in the presence of both electric and non-electric forces. Finally, these data provide the first quantitative test of a detailed theory of electrophoretic stretching of DNA without adjustable parameters developed by Stigter and Bustamante. The agreement between theory and experiment is good.

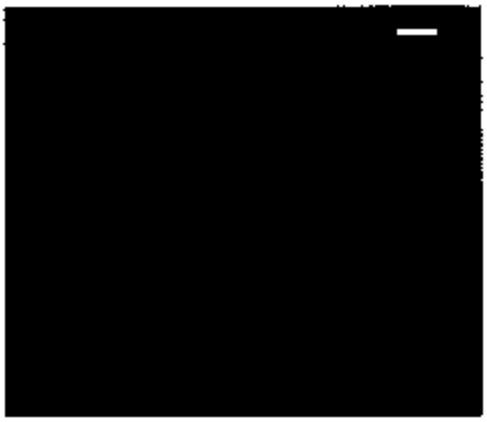

Figure 4: Time lapse relaxation sequence of a single molecule of DNA in an electric field. The scale bar is 5 μm .

Theories of DNA electrophoretic separations generally treat the DNA as a free draining polymer moving in an electric field at a rate that depends on the effective charge density of the molecule. Separations can occur in sieving media ranging from ultra-dilute polymer solutions to tightly cross-linked gels. It has recently been shown that DNA does not behave as a free-draining polymer when both

electric and non-electric forces simultaneously act on the molecule, as occurs when DNA collides with a polymer during electrophoretic separations. Here we show that a semi-dilute polymer solution screens the hydrodynamic interaction that results from the application of these forces. Fluorescently labeled DNA molecules tethered at one end in a semi-dilute solution of hydroxyl-ethyl cellulose stretch more an electric field than they stretch in free solution, and approach free-draining behavior. We have predicted the steady stretching behavior without adjustable parameters by employing a theory developed by Stigter using a hydrodynamic screening length found from effective medium theory. Data on the relaxation of single stretched molecules after the electric field is removed agree with the Rouse model prediction, which neglects hydrodynamic interactions. The slowest relaxation time constant, τR scales with chain length as $\tau R \sim L^{1.9\pm0.2}$.

Further details are provided in the publications:
Ferree, S. & H.W. Blanch, "Electrokinetic Stretching of Tethered DNA", Biophys. J., 85 (4) 2539-2546 (2003)
Ferree, S. & H.W. Blanch, "The Hydrodynamics of DNA Electrophoretic Stretch and Relaxation in a Polymer Solution", Biophys. J., 87, 468-475 (2004)

2.3 Biological templates for growth of $CaCO_3$

Biomineralization is the process by which living organisms produce organic/inorganic composite materials such as bones, teeth, and shells. Through finely tuned transport of materials, template-directed growths, and control of the surrounding chemical environment, many organisms create ceramic biomaterials with nanoscale precision, tuning their properties for a variety of tasks. In all cases, the ceramics are produced in aqueous environments at ambient conditions, yet these materials often have superior properties to man-made ceramics created under severe conditions. An example in Figure 1 shows a scanning electron micrograph of a series of microlenses mineralized by a brittlestar from a single calcite crystal to focus light into photosensory organs. This is just one example of the fine architecture that is created in nature by the process of biomineralization.

Figure 1: SEM image from the skeleton of a brittlestar *Ophiocoma wendtii*. The entire structure is composed of a single calcite crystal used by the organism for its mechanical and optical properties. The scale bar is 100 μm .

If the control that nature has over molecular assembly could be extended to synthetic systems, then many new materials can be envisioned that would benefit from this type of hierarchical ordering. We have chosen to use DNA for our templates for three reasons.

- DNA has a large aspect ratio when in an extended conformation. Because of the structure of the double-stranded DNA helix and the strength of the base pair binding, single DNA

molecules can be hundreds of thousands of base pairs long. When stretched under mechanical or electrical forces, these DNA molecules can have lengths on the order of hundreds of microns while the helix diameter is on the order of one nanometer. With this large aspect ratio, we will be able to examine the effect of nanoscale charge density and orientation effects while patterning large areas.

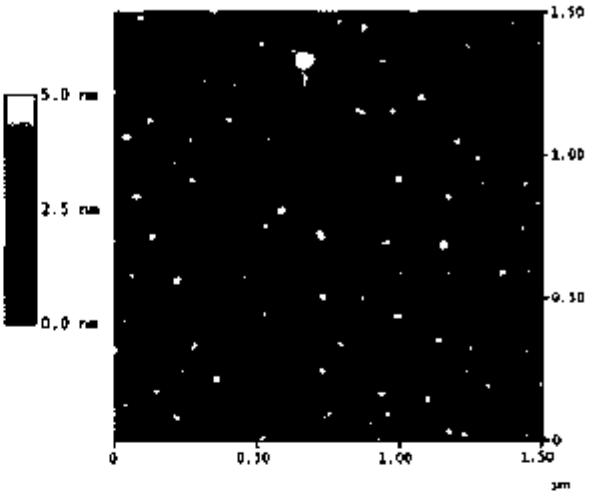

Figure 2: AFM image of a partially stretched λ-DNA molecule on a silica surface silanized with 7-octenyltrichlorosilane. The image is a height image with a 1.5 um scan size taken in tapping mode in air.

* DNA is also an attractive template material because it has a very regular and high charge density. Two negatively charged phosphate molecules accompany every base pair on the DNA backbone. When a DNA molecule is stretched, this corresponds to a regular charge spacing of 2 negative charges every 3.2 angstroms. It has been hypothesized that negatively charged residues on acidic proteins act as heterogeneous nucleation sites by attracting divalent cations (calcium in the case of calcium carbonate and hydroxyapatite

We have developed four approaches using DNA templating strategies that are designed, with increasing complexity, to address various hypotheses about the biomineralization process.

DNA films and monolayers: Monolayers and films of DNA have been fabricated to test the hypothesis that the highly charged backbone of DNA will nucleate crystals CaCO3 or other biominerals.

Isolated single molecules of DNA: Single molecules of DNA, stretched and held in an elongated conformation can be used to test the hypothesis that a single DNA molecule can nucleate and guide the growth of CaCO3 crystals along its backbone.

High density single molecules: Multiple molecules of DNA will be stretched at a high density to test the hypothesis that a uniform, aligned DNA template will induce the ordered formation of a polycrystalline sample.

Branched DNA molecules: DNA molecules with varying topology will be used as a crystallization template to test the hypothesis that the nanoscale shape of the template can guide the nanoscale crystal morphology.