**Final Report:** Understanding the Interaction of Peptides and Proteins with Abiotic Surfaces: Towards Water-Free Biologics

**Authors:** Zhan Chen

**Performing Organization:** University of Michigan - Ann Arbor

**Distributability:** Approved for public release; distribution is unlimited.

**Security Classification:** UU UU UU UU

**Subject Terms:**

**Abstract:**

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision, unless so designated by other documentation.

**Number of Pages:** 5

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**Limitation of Abstract:**

UU

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**Number of Pages:** 19

**Responsible Person:** Zhan Chen

734-615-4189
Major Goals: Here are the major goals presented in the original proposal:

Aim 1  Develop systematic understanding of abiotic/biotic interfaces. We aim to understand, how the structure and activity of proteins and peptides immobilized on abiotic surfaces depends upon a) the chemical and physical nature of the abiotic surface; b) the physicochemical properties of the protein or peptide; and c) the nature of the surrounding medium – water, air or other hydromimetic molecules.

Aim 2  Develop design rules for “water-free biologics” based on engineered abiotic/biotic interfaces. Using knowledge gained from studies in Aim 1, we aim to a) engineer peptides and proteins with enhanced ability to retain structure and function in the absence of bulk water, and b) develop abiotic surfaces displaying hydromimetic functionality that stabilizes the native structure and function of biological molecules in extremely low humidity or “water-free” interfaces – i.e. a surface-air interface that lacks bulk water and in air that is not humidified.

Accomplishments: Please see the uploaded material.

Training Opportunities: The training opportunities are the same as those reported previously:
This MURI project involves six research groups at three different universities. The research is highly interdisciplinary, covering many research areas in biology, chemistry, engineering, and physics. The postdoctoral fellows and graduate students working on this MURI project received training in many different research fields including surface sciences and engineering, materials, spectroscopy, laser techniques, chemical biology, computational chemistry, and nanoscience and nanotechnology. We have regular bi-weekly group meetings at which postdocs and graduate students involved in this research give presentations, discuss research results, establish research collaborations, and propose research plans. The PI attended all the bi-weekly meetings, training the postdocs and graduate students in experimental design, data analysis, as well as manuscript preparation, etc. The postdocs and graduate students also gave oral and poster presentations at various conferences and symposiums, improving their presentation skills. Other students and postdoctoral fellows in the six research groups and beyond also benefited from this MURI research by interacting with students directly participating in the project and through their group meeting presentations, annual symposium, and the seminars related to the MURI project. Training in this project prepared students and postdoctoral fellows for employment in related fields. The PI and co-PIs teach a variety of undergraduate and graduate courses in different fields, and integrated new research results generated from this MURI research into these courses to educate more graduate and undergraduate students.
Results Dissemination: The results obtained from this MURI research were disseminated through journal publications and oral/poster presentations at conferences and institutions. The joint publications from the six research groups involved in this project reported our results obtained from this interdisciplinary research, which have gained interest by researchers in many different research fields in biology, chemistry, physics, and engineering.

The PI, co-PIs, postdocs, and graduate students working on this project have given many presentations at various symposiums and conferences as well as institutions to disseminate the research results from this MURI project. For example, after the last annual report submitted in August 2016, the PI gave many invited oral presentations. Some examples are: “Molecular Structures of Peptides and Proteins at Abiotic/Biotic Interfaces”, Department of Chemistry, Hong Kong University of Science and Technology, September 2016; “Molecular Structures of Peptides and Proteins at Interfaces Studied by Linear and Nonlinear Vibrational Spectroscopic Techniques”, Scix Meeting, Minneapolis, MN, September 2016; “Molecular Structures of Peptides and Proteins at Abiotic/Biotic Interfaces”, Wayne State University, October 2016; “Linear and nonlinear vibrational spectroscopic studies on polymer and biological molecules at buried interfaces”, 6th International Conference of Perspectives on Vibration Spectroscopy, Lucknow, India, November, 2016; “Linear and Nonlinear Vibrational Spectroscopic Studies on Molecular Structures of Biological Molecules at Interfaces in Situ”, 2016 National Bioanalytical Chemistry Forum, Nanjing, China, December, 2016; “Structure and Function of Surface Immobilized Peptides and Enzymes in Air”, 253rd ACS National Meeting, San Francisco, CA, April, 2017; “Molecular Structures of Surface Immobilized Peptides and Proteins in Different Chemical Environments”, International Conference on Advanced Vibration Spectroscopy, Victoria, Canada, June 2017; “Molecular Structures of Biological Molecules at Interfaces”, University of California at Riverside, June, 2017; “Molecular Structures of Biological Molecules at Interfaces”, U.S. Army Edgewood Chemical Biological Center (ECBC), Maryland, June, 2017; “Characterization and Control of Interfacial Biological Molecule Structures”, Frontiers of Chemistry and Chemical Engineering Symposium, China, September, 2017; “Applications of Sum Frequency Generation Vibration Spectroscopy to Study Molecular Structures of Buried Interfaces of Polymer Materials and Biological Molecules”, Ohio University, Athens, Ohio, October 2017; “Control and Characterize Molecular Structures of Biological Molecules at Interfaces in Situ”, 2017 Anachem, Michigan, November, 2017.

The PI and co-PIs teach a variety of undergraduate and graduate courses in different fields, and integrated new research results generated from this MURI research into these courses, which disseminated these results to a large body of graduate and undergraduate students. The PI also disseminated the results to many students through lectures in a summer course for high school students.

Honors and Awards: PI: Zhan Chen
Senior Editor, Langmuir, 2018
Top Ten Most Highly Prolific Author for Langmuir, 2017
Plenary Speaker, Frontiers of Chemistry and Chemical Engineering Symposium, China, 2017
Plenary Speaker, International Conference on Perspectives in Vibration Spectroscopy, India, 2016

co-PI: Charles Brooks
Distinguished University Professorship, 2017

co-PI: Neil Marsh
Fellow, American Association for the Advancement of Science (AAAS)

co-PI: Nick Abbott
2017 6th Somer Lectures, Middle East Technical University, Ankara, Turkey.
2017 Paul J. Berics Professorship, University of Wisconsin
2016 42nd Annual Harry G. Fair Memorial Lecture, University of Oklahoma
2016 Elected Fellow of the American Physical Society

Protocol Activity Status:
Technology Transfer: Nicholas Abbott is a co-founder of both Platypus Technologies LLC and Imbed Biosciences Inc, each of Madison WI, which are focused on the translation of advances in the design of biotic-abiotic interfaces into (I) analytic technologies (Platypus) and (II) new approaches to accelerated wound healing (Imbed Biosciences). Understanding the design of biotic-abiotic interfaces is critical to the technologies being developed by both companies, and several approaches being explored within the MURI have the potential to impact these companies. Over the past year, Imbed Biosciences received FDA approval for a new wound dressing that accelerates healing of chronic wounds.

Patent Application:
Kenneth Cheng, Joerg Lahann, Nicholas Abbott, Marco A. Bedolla Pantoja, Templated Synthesis of Shape-Controlled Polymeric Nanofibers by Chemical Vapor Deposition into Liquid Crystals, 2016.

The PI visited U.S. Army Edgewood Chemical Biological Center (ECBC), Maryland, June, 2017 and gave a seminar.

PARTICIPANTS:

**Participant Type:** PD/PI  
**Participant:** Zhan Chen  
**Person Months Worked:** 4.00  
**Funding Support:**  
Project Contribution:  
International Collaboration:  
International Travel:  
National Academy Member: N  
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Project Contribution:  
International Collaboration:  
International Travel:  
National Academy Member: N  
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**Funding Support:**  
Project Contribution:  
International Collaboration:  
International Travel:  
National Academy Member: N  
Other Collaborators:

**Participant Type:** Graduate Student (research assistant)  
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Project Contribution:  
International Collaboration:  
International Travel:  
National Academy Member: N  
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International Travel:
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Funding Support: 
Project Contribution:
International Collaboration:
International Travel:
National Academy Member: N
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Project Contribution:
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International Travel:
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Project Contribution:
International Collaboration:
International Travel:
National Academy Member: N
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Project Contribution:
International Collaboration:
International Travel:
National Academy Member: N
Other Collaborators: 
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Project Contribution:  
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International Travel:  
National Academy Member: N  
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Participant Type: Graduate Student (research assistant)  
Participant: Kenneth Cheng  
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Funding Support:  
Project Contribution:  
International Collaboration:  
International Travel:  
National Academy Member: N  
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Participant Type: Graduate Student (research assistant)  
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Funding Support:  
Project Contribution:  
International Collaboration:  
International Travel:  
National Academy Member: N  
Other Collaborators:  

Participant Type: Graduate Student (research assistant)  
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Funding Support:  
Project Contribution:  
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International Travel:  
National Academy Member: N  
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Funding Support:  
Project Contribution:  
International Collaboration:  
International Travel:  
National Academy Member: N  
Other Collaborators:  

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Funding Support:  
Project Contribution:  
International Collaboration:  
International Travel:  
National Academy Member: N  
Other Collaborators:  

Other Collaborators:
ARTICLES:

**Publication Type:** Journal Article  
**Journal:** Langmuir

**Publication Type:** Journal Article  
**Journal:** American Chemical Society

**Article Title:** Molecular Interactions of Proteins and Peptides at Interfaces Studied by Sum Frequency Generation Vibrational Spectroscopy

**Authors:**

**Keywords:** Proteins and Peptides, Interface, Sum Frequency Generation

**Abstract:** Interfacial peptides and proteins are critical in many biological processes and thus are of interest to various research fields. To study these processes, surface sensitive techniques are required to completely describe different interfacial interactions intrinsic to many complicated processes. Sum frequency generation (SFG) spectroscopy has been developed into a powerful tool to investigate these interactions and mechanisms of a variety of interfacial peptides and proteins. It has been shown that SFG has intrinsic surface sensitivity and the ability to acquire conformation, orientation and ordering information about these systems. This paper reviews recent studies on peptide/protein-substrate interactions, peptide/protein-membrane interactions and protein complexes at interfaces and demonstrates the ability of SFG on unveiling the molecular pictures of complicated interfacial biological processes.

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Acknowledged Federal Support:

**Publication Type:** Journal Article  
**Journal:** American Chemical Society

**Article Title:** Molecular Structures of Buried Polymer Interfaces and Biological Interfaces Detected by Sum Frequency Generation Vibrational Spectroscopy

**Authors:**

**Keywords:** Polymer surface and interface; Biological interface; Peptide; Protein; Adhesion; Secondary structures; Alpha-helix; Beta-sheet

**Abstract:** Molecular structures at interfaces determine interfacial properties. In order to optimize the interfacial structures to achieve improved properties of advanced materials, it is important to characterize molecular structures of interfaces in situ in real time. Recently, a nonlinear optical spectroscopic technique, sum frequency generation (SFG) vibrational spectroscopy, has been developed into a powerful and unique tool to elucidate molecular structures of buried interfaces, including liquid/liquid, solid/liquid, and solid/solid interfaces. In this review, applications of SFG to study molecular structures of complex interfaces such as polymer interfaces and biological interfaces have been discussed. Particularly, molecular surface structural changes of various polymers in water, molecular interactions between polymers and silane model adhesion promoters at interfaces, and structures of buried polymer/polymer as well as polymer/metal interfaces have been presented. In addition, interfacial

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**Abstract:** Highly fluorinated analogs of hydrophobic amino acids are well known to increase the stability of proteins toward thermal unfolding and chemical denaturation, but there is very little data on the structural consequences of fluorination. We have determined the structures and folding energies of three variants of a de novo designed 4-helix bundle protein whose hydrophobic cores contain either hexafluoroleucine (hFLeu) or t-butylalanine (tBAla). Although the buried hydrophobic surface area is the same for all three proteins, the incorporation of tBAla causes a rearrangement of the core packing, resulting in the formation of a destabilizing hydrophobic cavity at the center of the protein. In contrast, incorporation of hFLeu, causes no changes in core packing with respect to the structure of the nonfluorinated parent protein which contains only leucine in the core. These results support the idea that fluorinated residues are especially effective at stabilizing proteins because they closely.

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Acknowledged Federal Support:

**Abstract:** In the commonly used nucleation-dependent model of protein aggregation, aggregation proceeds only after a lag phase in which the concentration of energetically unfavorable nuclei reaches a critical value. The formation of oligomeric species prior to aggregation can be difficult to detect by current spectroscopic techniques. By using real-time 19F NMR along with other techniques, we are able to show that multiple oligomeric species can be detected during the lag phase of A?40 fiber formation, consistent with a complex mechanism of aggregation. At least six types of oligomers can be detected by 19F NMR. These include the reversible formation of large ?-sheet oligomer immediately after solubilization at high peptide concentration, a small oligomer that forms transiently during the early stages of the lag phase, and four spectroscopically distinct forms of oligomers with molecular weights between 30 and 100 kDa that appear during the later stages of aggregation. The ability to resolve i
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**Authors:**

**Keywords:** SURFACE-PLASMON RESONANCE; ENHANCED RAMAN-SPECTROSCOPY; QUARTZ-CRYSTAL MICROBALANCE; ATOMIC-FORCE MICROSCOPY; TRANSFORM INFRARED-SPECTROSCOPY; ADHESION-PROMOTING MIXTURE; SELF-ASSEMBLED MONOLAYERS; SUPPORTED LIPID-BILAYERS; BETA-SHEET STRUCTURES; MODEL CELL-MEMBRANES

**Abstract:** Sum frequency generation (SFG) vibrational spectroscopy has been developed into an important technique to study surfaces and interfaces. It can probe buried interfaces in situ and provide molecular level structural information such as the presence of various chemical moieties, quantitative molecular functional group orientation, and time dependent kinetics or dynamics at such interfaces. This paper focuses on these three most important advantages of SFG and reviews some of the recent progress in SFG studies on interfaces related to polymer materials and biomolecules. The results discussed here demonstrate that SFG can provide important molecular structural information of buried interfaces in situ and in real time, which is difficult to obtain by other surface sensitive analytical techniques.

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**Publication Identifier:** 10.1021/cm202632m

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**Publication Location:**

**Article Title:** Liquid Crystalline Materials for Biological Applications

**Authors:**

**Keywords:** liquid crystals, biotic-abiotic interfaces, dynamic materials, lipid assemblies, polymeric multilayers, oligopeptides, bioinspired materials

**Abstract:** Liquid crystals have a long history of use as materials that respond to external stimuli (e.g., electrical and optical fields). More recently, a series of investigations have reported the design of liquid crystalline materials that undergo ordering transitions in response to a range of biological interactions, including interactions involving proteins, nucleic acids, viruses, bacteria, and mammalian cells. A central challenge underlying the design of liquid crystalline materials for such applications is the tailoring of the interface of the materials so as to couple targeted biological interactions to ordering transitions. This review describes progress toward the design of interfaces of liquid crystalline materials that are suitable for biological applications. Approaches addressed in this review include the use of lipid assemblies, polymeric membranes containing oligopeptides, cationic surfactant-DNA complexes, peptide-amphiphiles, interfacial protein assemblies, and multilayer polym

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Acknowledged Federal Support:
Abstract: We examined the orientational ordering of nematic liquid crystals (LCs) supported on organized monolayers of dipeptides with the goal of understanding how peptide-based interfaces encode intermolecular interactions that are amplified into supramolecular ordering. By characterizing the orientations of nematic LCs (4-cyano-4'-pentylbiphenyl and TL205 (a mixture of mesogens containing cyclohexane-fluorinated biphenyls and fluorinated terphenyls)) on monolayers of L-cysteine-L-tyrosine, L-cysteine-L-phenylalanine, or L-cysteine-L-phosphotyrosine formed on crystallographically textured films of gold, we conclude that patterns of hydrogen bonds generated by the organized monolayers of dipeptides are transduced via macroscopic orientational ordering of the LCs. This conclusion is supported by the observation that the ordering exhibited by the achiral LCs is specific to the enantiomers used to form the dipeptide-based monolayers. The dominant role of the OH group of tyrosine in dictating the pattern

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Acknowledged Federal Support:

Abstract: Controlled presentation of biomolecules on synthetic substrates is an important aspect for biomaterials development. If the immobilization of multiple biomolecules is required, highly efficient orthogonal surface chemistries are needed to ensure the precision of the immobilization. In this communication, chemical vapor deposition (CVD) copolymerization is used to fabricate polymer coatings with controlled ratio of alkyne and perfluorophenyl ester (Pfp-ester) groups. Cyclic arginine-glycine-aspartic acid (cRGD) adhesion peptide and epidermal growth factor (EGF) are immobilized through alkyne–azide cycloaddition (“click” chemistry) and active ester–amine reaction, respectively. Cell studies with human umbilical vein endothelial cells (HUVEC) and A431 cell lines demonstrate the biological activity of the coimmobilized biomolecules.

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Article Title: Influence of Fluorination on the Thermodynamics of Protein Folding
Authors:
Keywords: FLUOROUS AMINO-ACIDS; 4-ALPHA-HELIX BUNDLE PROTEIN; DE-NOVO DESIGN; COILED-COIL; ANTIMICROBIAL PEPTIDE; BIOLOGICAL-ACTIVITY; RESIDUAL STRUCTURE; UNFOLDED STATE; HEAT-CAPACITY; IN-VIVO
Abstract: The introduction of highly fluorinated analogues of hydrophobic amino acid residues into proteins has proved an effective and general strategy for increasing protein stability toward both chemical denaturants and heat. However, the thermodynamic basis for this stabilizing effect, whether enthalpic or entropic in nature, has not been extensively investigated. Here we describe studies in which the values of \( \Delta H^\circ \), \( \Delta S^\circ \), and \( \Delta C_p^\circ \) have been determined for the unfolding of a series of 12 small, de novo-designed proteins in which the hydrophobic core is packed with various combinations of fluorinated and non-fluorinated amino acid residues. The increase in the free energy of unfolding with increasing fluorine content is associated with increasingly unfavorable entropies of unfolding and correlates well with calculated changes in apolar solvent-accessible surface area. \( \Delta C_p^\circ \) for unfolding is positive for all the proteins and, similarly, correlates with changes in apolar solvent-accessible surface area.

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Date Submitted:  Date Published: 
Publication Location:

Article Title: Molecular Orientation of Enzymes Attached to Surfaces through Defined Chemical Linkages at the Solid–Liquid Interface
Authors:
Keywords: Sum frequency generation spectroscopy (SFG); enzyme/protein immobilization; protein engineering; \(?\)-galactosidase; protein interfacial orientation determination; cysteine-maleimide chemistry
Abstract: The immobilization of enzymes on solid supports is widely used in many applications, including biosensors, antifouling coatings, food packaging materials, and biofuel cells. Enzymes tend to lose their activity when in contact with a support surface, a phenomenon that has been attributed to unfavorable orientation and (partial) unfolding. In this work, specific immobilization of 6-phospho-\(?\)-galactosidase (\(?\)-Gal) on a self-assembled monolayer (SAM) containing maleimide end groups and oligo(ethylene glycol) spacer segments was achieved through a unique cysteinyI residue. A systematic means to characterize the interfacial orientation of immobilized enzymes has been developed using a combination of sum frequency generation vibrational spectroscopy and attenuated total reflectance FTIR-spectroscopy. The possible orientations of the immobilized \(?\)-Gal were determined and found to be well-correlated with the tested activity of \(?\)-Gal. This study will impact the development of an increasingly wide

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Co-immobilization of Biomolecules on Ultrathin Reactive Chemical Vapor Deposition Coatings Using Multiple Click Chemistry Strategies

Abstract: Immobilization of biomolecules, such as proteins or sugars, is a key issue in biotechnology because it enables the understanding of cellular behavior in more biological relevant environment. Here, poly(4-ethynyl-pxylylene-co-p-xylylene) coatings have been fabricated by chemical vapor deposition (CVD) polymerization in order to bind bioactive molecules onto the surface of the material. The control of the thickness of the CVD films has been achieved by tuning the amount of precursor used for deposition. Coppercatalyzed Huisgen cycloaddition has then been performed via microcontact printing to immobilize various biomolecules on the reactive coatings. The selectivity of this click chemistry reaction has been confirmed by spatially controlled conjugation of fluorescent sugar recognizing molecules (lectins) as well as cell adhesion onto the peptide pattern. In addition, a microstructured coating that may undergo multiple click chemistry reactions has been developed by two sequential CVD steps.

Ordering Transitions Triggered by Specific Binding of Vesicles to Protein-Decorated Interfaces of Thermotropic Liquid Crystals

Abstract: We report that specific binding of ligand-functionalized (biotinylated) phospholipid vesicles (diameter = 120 +/- 19 nm) to a monolayer of proteins (streptavidin or anti-biotin antibody) adsorbed at an interface between an aqueous phase and an immiscible film of a thermotropic liquid crystal (LC) [nematic 4'-pentyl-4-cyanobiphenyl (5CB)] triggers a continuous orientational ordering transition (continuous change in the tilt) in the LC. Results presented in this paper indicate that, following the capture of the vesicles at the LC interface via the specific binding event relative to a protein-decorated interface of a LC that does not bind the ligands presented by the vesicles. The observation of the continuous change in the ordering of

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Acknowledged Federal Support:
Incorporation of DOPE into lipoplexes formed from a ferrocenyl lipid leads to inverse hexagonal nanostructures that allow redox-based control of transfection in high serum

**Keywords:** SEVERE COMBINED IMMUNODEFICIENCY; CONTAINING CATIONIC LIPIDS; GROWTH-FACTOR DELIVERY; GENE-THERAPY PROGRESS; PLASMID DNA; TRANSGENE EXPRESSION; CELL MICROARRAYS; MUSCULAR-DYSTROPHY; DRUG-DELIVERY; NONVIRAL VECTORS

**Abstract:** We report small angle X-ray and neutron scattering measurements that reveal that mixtures of the redox-active lipid bis(11-ferrocynylundecyl)dimethylammonium bromide (BFDMA) and dioleoylphosphatidylethanolamine (DOPE) spontaneously form lipoplexes with DNA that exhibit inverse hexagonal nanostructure (H-II(c)). In contrast to lipoplexes of DNA and BFDMA only, which exhibit a multilamellar nanostructure (L-alpha(c)) and limited ability to transfect cells in the presence of serum proteins, we measured lipoplexes of BFDMA and DOPE with the H-II(c) nanostructure to survive incubation in serum and to expand significantly the range of media compositions (e.g., up to 80% serum) over which BFDMA can be used to transf ect cells with high efficiency. Importantly, we also measured the oxidation state of the ferrocene within the BFDMA/DNA lipoplexes to have a substantial influence on the transfection efficiency of the lipoplexes in media containing serum. Specifically, whereas lipoplexes of reduce...
Different Interfacial Behaviors of N- and C-Terminus Cysteine-Modified Cecropin P1 Chemically Immobilized onto Polymer Surface

Authors:

Abstract: Sum frequency generation (SFG) vibrational spectroscopy and attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) were used to investigate the orientation of N-terminus cysteine-modified cecropin P1 (cCP1) at the polystyrene maleimide (PS-MA)/ peptide phosphate buffer solution interface. The cCP1 cysteine group reacts with the maleimide group on the PS-MA surface to chemically immobilize cCP1. Previously, we found that the C-terminus cysteine-modified cecropin P1 (CP1c) molecules exhibit a multiple-orientation distribution at the PS-MA/peptide phosphate buffer solution interface, due to simultaneous physical adsorption and chemical immobilization of CP1c on the PS-MA surface. Differently, in this research, it was found that the interfacial orientation of cCP1 molecules varied from a horizontal orientation to the “tilting” orientation to the “standing up” orientation and then to the “multiple-orientation” distribution as the peptide concentration increased from...

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Acknowledged Federal Support:

Different Interfacial Behaviors of Peptides Chemically Immobilized on Surfaces with Different Linker Lengths and via Different Termini

Authors:

Abstract: Molecular structures such as conformation and orientation are crucial in determining the activity of peptides immobilized to solid supports. In this study, sum frequency generation (SFG) vibrational spectroscopy was applied to investigate such structures of peptides immobilized on self-assembled monolayers (SAMs). Here cysteine-modified antimicrobial peptide cecropin P1 (CP1) was chemically immobilized onto SAM with a maleimide terminal group. Two important characteristics, length of the poly(ethylene glycol) (PEG) segment in the SAM and location of the cysteine residue in the peptide, were examined using SFG spectroscopy to determine the effect of each on surface immobilization as well as peptide secondary structure and its orientation in the immobilized state. Results have shown that while each length of PEG chain studied promotes chemical immobilization of the target peptide and prevents nonspecific adsorption, CP1 immobilized on long-chain (PEG2k) maleimide SAMs shows random coil s

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Acknowledged Federal Support:
Surface Orientation Control of Site-Specifically Immobilized Nitro-reductase (NfsB)

Abstract: We demonstrate the control of enzyme orientation for enzymes chemically immobilized on surfaces. Nitro-reductase (NfsB) has the ability to reduce a broad range of nitro-containing compounds and has potential applications in a broad range of areas including the detection and decomposition of explosives. The enzyme was tethered through unique surface cysteine residues to a self-assembled monolayer (SAM) terminated with maleimide groups. One cysteine was introduced close to the active site (V424C), and the other, at a remote site (H360C). The surface-tethered NfsB variants were interrogated by a combination of surfacesensitive sum frequency generation (SFG) vibrational spectroscopy and attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR) to determine how the mode of attachment altered the enzyme’s orientation. The activities of the two immobilized NfsB variants were measured and can be well correlated to the deduced orientations. The relationships among enzyme en

Molecular Structures of C- and N-Terminus Cysteine Modified Cecropin P1 Chemically Immobilized onto Maleimide-Terminated Self-Assembled Monolayers Investigated by Molecular Dynamics Simulation

Abstract: Biosensors using peptides or proteins chemically immobilized on surfaces have many advantages such as better sensitivity, improved stability, and longer shelf life compared to those prepared using physically adsorbed biomolecules. Chemical immobilization can better control the interfacial conformation and orientation of peptides and proteins, leading to better activity of these biomolecules. In this research, molecular dynamics (MD) simulations were employed to systematically investigate the structure and dynamics of surface-tethered antimicrobial peptide cecropin P1 (CP1) modified with a cysteine residue at the C- (CP1c) or N-terminus (cCP1). Such CP1c and cCP1 molecules were chemically immobilized onto a silane-EG4-maleimide selfassembled monolayer (SAM) surface by forming a thio-ether bond between the cysteine group in CP1c or cCP1 and the surface maleimide group. The simulation results showed that the immobilized cCP1 (via the N-terminus) tends to bend and gradually lie down onto t

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**Article Title:** Stern potential and Debye length measurements in dilute ionic solutions with electrostatic force microscopy

**Authors:**

**Keywords:** Stern Potential, Debye length, atomic force microscopy

**Abstract:** We demonstrate the ability to measure Stern potential and Debye length in dilute ionic solution with atomic force microscopy. We develop an analytic expression for the second harmonic force component of the capacitive force in an ionic solution from the linearized Poisson–Boltzmann equation. This allows us to calibrate the AFM tip potential and, further, obtain the Stern potential of sample surfaces. In addition, the measured capacitive force is independent of van der Waals and double layer forces, thus providing a more accurate measure of Debye length.

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**Article Title:** Fluorinated Proteins: From Design and Synthesis to Structure and Stability

**Authors:**

**Keywords:** No keyword

**Abstract:** Fluorine is all but absent from biology; however, it has proved to be a remarkably useful element with which to modulate the activity of biological molecules and to study their mechanism of action. Our laboratory’s interest in incorporating fluorine into proteins was stimulated by the unusual physicochemical properties exhibited by perfluorinated small molecules. These include extreme chemical inertness and thermal stability, properties that have made them valuable as nonstick coatings and fire retardants. Fluorocarbons also exhibit an unusual propensity to phase segregation. This phenomenon, which has been termed the “fluorous effect”, has been effectively exploited in organic synthesis to purify compounds from reaction mixtures by extracting fluorocarbon-tagged molecules into fluorocarbon solvents. As biochemists, we were curious to explore whether the unusual physicochemical properties of perfluorocarbons could be engineered into proteins. To do this, we developed a synthesis of a hi

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Fluorine is a valuable probe for investigating the interactions of biological molecules because of its favorable NMR characteristics, its small size, and its near total absence from biology. Advances in biosynthetic methods allow fluorine to be introduced into peptides and proteins with high precision, and the increasing sensitivity of NMR spectrometers has facilitated the use of 19F NMR to obtain molecular-level insights into a wide range of often-complex biological interactions. Here, we summarize the advantages of solution state 19F NMR for studying the interactions of peptides and proteins with other biological molecules, review methods for the production of fluorine-labeled materials, and describe some representative recent examples in which 19F NMR has been used to study conformational changes in peptides and proteins and their interactions with other biological molecules.

Characterization of a highly flexible self-assembling protein system designed to form nanocages

The design of proteins that self-assemble into well-defined, higher order structures is an important goal that has potential applications in synthetic biology, materials science, and medicine. We previously designed a two-component protein system, designated A-(1) and A-(2), in which self-assembly is mediated by complementary electrostatic interactions between two coiled-coil sequences appended to the C-terminus of a homotrimeric enzyme with C3 symmetry. The coiled-coil sequences are attached through a short, flexible spacer sequence providing the system with a high degree of conformational flexibility. Thus, the primary constraint guiding which structures the system may assemble into is the symmetry of the protein building block. We have now characterized the properties of the self-assembling system as a whole using native gel electrophoresis and analytical ultracentrifugation (AUC) and the properties of individual assemblies using cryo-electron microscopy (EM). We show that upon mixi
Abstract: Silver is a widely used antimicrobial agent, yet, when impregnated in macroscopic dressings, it stains wounds, can lead to tissue toxicity, and can inhibit healing. Recently, polymeric nanofilms containing silver nanoparticles were reported to exhibit antimicrobial activity at loadings and release rates of silver that are 100x lower than conventional dressings. Here, fabrication of composite microfilm constructs that provide a facile way to transfer the silver-loaded polymeric nanofilms onto wounds in vivo is reported. The construct is fabricated from a silver nanoparticle-loaded polymeric nanofilm that is laminated with a micrometer-thick soluble film of polyvinylalcohol (PVA). When placed on a moist wound, the PVA dissolves, leaving the silver-loaded nanofilm immobilized on the wound-bed. In vitro, the immobilized nanofilms release <1 μg cm(-2) d(-1) of silver over 30 d from skin dermis and they kill 5 log(10) CFUs of Staphylococcus aureus in 24 h. In mice, wounds inoculated with 1

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Article Title: Comparison of the Influence of Humidity and D?Mannitol on the Organization of Tetraethylene Glycol-Terminated Self-Assembled Monolayers and Immobilized Antimicrobial Peptides
Authors:

Keywords: Oligopeptides; Molecular Conformation; Monolayers; Surfaces; Spectroscopy; Water; Resistance.

Abstract: We report the use of polarization-modulation infrared reflection-absorption spectroscopy (PM-IRRAS) to characterize the effects of relative humidity (RH) and D-mannitol on the conformations of tetraethylene glycol (EG(4))-terminated self-assembled monolayers (SAMs) and immobilized antimicrobial peptides (Cecropin P1 and a hybrid of Cecropin A (1-8) and Melittin (1-18)). These results are used to assess the extent to which D-mannitol can substitute for water in promoting conformational states of the SAMs and oligopeptides similar to those induced by hydration. Our measurements reveal a red shift of the COC asymmetric stretching vibration of the EG (4)-terminated SAMs with increasing humidity, consistent with a transition from a mixed all-trans/helical (7/2 helix) conformation at 0% RH to a predominantly helical conformation at 90% RH. Significantly, under dry conditions, a thin (2 nm in thickness) overlayer of D-mannitol generated the COC spectroscopic signature of the EG(4)-terminated S

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Acknowledged Federal Support:
Raman Spectroscopy Enables Noninvasive Biochemical Characterization and Identification of the Stage of Healing of a Wound

Abstract:
Accurate and rapid assessment of the healing status of a wound in a simple and noninvasive manner would enable clinicians to diagnose wounds in real time and promptly adjust treatments to hasten the resolution of nonhealing wounds. Histologic and biochemical characterization of biopsied wound tissue, which is currently the only reliable method for wound assessment, is invasive, complex to interpret, and slow. Here we demonstrate the use of Raman microspectroscopy coupled with multivariate spectral analysis as a simple, noninvasive method to biochemically characterize healing wounds in mice and to accurately identify different phases of healing of wounds at different time-points. Raman spectra were collected from "splinted" hill thickness dermal wounds in mice at 4 time-points (0, 1, 5, and 7 days) corresponding to different phases of wound healing, as verified by histopathology. Spectra were deconvolved using multivariate factor analysis (MFA) into 3 "factor score spectra" (that act as

Influence of the phase state of self-assembling redox mediators on their electrochemical activity

Abstract:
Self-assembling redox mediators have the potential to be broadly useful in a range of interfacial electrochemical contexts because the oxidation state and state of assembly of the mediator are closely coupled. In this article, we report an investigation of the self-assembly of single- and double-tailed ferrocenyl amphiphiles [(11-ferrocenylundecyl)trimethylammonium bromide (FTMA) and bis(11-ferrocenylundecyl)dimethylammonium bromide (BFDMA), respectively] at the surfaces of Pt electrodes and the impact of the dynamic assembled state of the amphiphiles on their rate of oxidation. We conclude that frozen aggregates of BFDMA adsorb to the surfaces of the Pt electrodes, and that slow dynamics of reorganization of BFDMA within these aggregates limits the rate of electrooxidation of BFDMA. In contrast, FTMA, while forming assemblies on the surfaces of Pt electrodes, is characterized by fast reorganization dynamics and a corresponding rate of oxidation that is an order of magnitude greater th
Adsorbate-Induced Anchoring Transitions of Liquid Crystals on Surfaces Presenting Metal Salts with Mixed Anions

Authors:

Keywords: Liquid crystals; Gas sensor; Coordination interactions; Dimethyl methylphosphonate

Abstract: We report that metal salts composed of mixtures of anions of differing coordination strength can be used to increase the sensitivity and selectivity of adsorbate-induced anchoring transitions of liquid crystals (LCs) supported on surfaces decorated with the metal salts. Specifically, the dynamics of anchoring transitions triggered by the adsorbate dimethyl methylphosphonate (DMMP) on surfaces of aluminum(III) salts were analyzed within the framework of a model for mass transport to reveal that the sensitivity of a nitrile-containing nematic LC to DMMP increased from 250 to 25 ppb when the composition of the (counter) anion was changed from 100% perchlorate to 90% nitrate and 10% perchlorate (by mole percent). To provide insight into these observations, polarization-modulation infrared reflectance-absorbance spectroscopy (PMIRRAS) was used to show that the intensity of the absorption band in the IR spectrum corresponding to the coordinated state of the nitrile group (but not the position

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Design of Functional Materials Based on Liquid Crystalline Droplets

Authors:

Keywords: Liquid crystals; Micrometer-sized droplets; Functional materials; Self-assembly; Topological defects; Ordering transitions; Amphiphiles; Chemically patterned microparticles.

Abstract: This brief Perspective focuses on recent advances in the design of functional soft materials that are based on confinement of low molecular weight liquid crystals (LCs) within micrometer-sized droplets. While the ordering of LCs within micrometer-sized domains has been explored extensively in polymer-dispersed LC materials, recent studies performed with LC domains with precisely defined size and interfacial chemistry have unmasked observations of confinement-induced ordering of LCs that do not follow previously reported theoretical predictions. These new findings, which are enabled in part by advances in the preparation of LCs encapsulated in polymeric shells, are opening up new opportunities for the design of soft responsive materials based on surface-induced ordering transitions. These materials are also providing new insights into the self-assembly of biomolecular and colloidal species at defects formed by LCs confined to micrometer-sized domains. The studies presented in this Persp

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**Abstract:** We report that colloid-in-liquid crystal (CLC) gels can be formed via a two-step process that involves spinodal decomposition of a dispersion of colloidal particles in an isotropic phase of mesogens followed by nucleation of nematic domains within the colloidal network defined by the spinodal process. This pathway contrasts to previously reported routes leading to the formation of CLC gels, which have involved entanglement of defects or exclusion of particles from growing nematic domains. The new route provides the basis of simple design rules that enable control of the microstructure and dynamic mechanical properties of the gels.

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**Abstract:** We report the use of flow cytometry to identify the internal ordering (director configurations) of micrometer-sized droplets of thermotropic liquid crystals (LCs) dispersed in aqueous solutions of adsorbates (surfactants and phospholipids). We reveal that changes in the configurations of the LC droplets induced by the adsorbates generate distinct changes in light scattering plots (side versus forward scattering). Specifically, when compared to bipolar droplets, radial droplets generate a narrower distribution of side scattering intensities (SSC, large angle light scattering) for a given intensity of forward scattering (FSC, small angle light scattering). This difference is shown to arise from the rotational symmetry of a radial LC droplet which is absent for the bipolar configuration of the LC droplet. In addition, the scatter plots for radial droplets possess a characteristic "S-shape", with two or more SSC intensities observed for each intensity of FSC. The origin of the experimental

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**Article Title:** Chemically Orthogonal Three-Patch Microparticles  
**Authors:**  
**Keywords:** click chemistry · electrohydrodynamic co-jetting · microparticles · orthogonal chemistry · surface chemistry  
**Abstract:** Compared to two-dimensional substrates, only a few methodologies exist for the spatially controlled decoration of three-dimensional objects, such as microparticles. Combining electrohydrodynamic co-jetting with synthetic polymer chemistry, we were able to create two- and three-patch microparticles displaying chemically orthogonal anchor groups on three distinct surface patches of the same particle. This approach takes advantage of a combination of novel chemically orthogonal polylactide-based polymers and their processing by electrohydrodynamic co-jetting to yield unprecedented multifunctional microparticles. Several micropatterned particles were fabricated displaying orthogonal click functionalities. Specifically, we demonstrate novel two- and three-patch particles. Multi-patch particles are highly sought after for their potential to present multiple distinct ligands in a directional manner. This work clearly establishes a viable route towards orthogonal reaction strategies on multiva  
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**Article Title:** Spatioselective Growth of Metal-Organic Framework Nanocrystals on Compositionally Anisotropic Polymer Particles  
**Authors:**  
**Keywords:** Janus Particles; Metal organic framework; Polymer; Surface Modification; biocompartmental particles  
**Abstract:** Selective growth of metal organic framework materials on the surface of compartmentalized polymer microparticles is achieved by electro-hydrodynamic co-jetting, selective surface modification, and anisotropic crystal growth.  
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Acknowledged Federal Support:
Controlled Microstructuring of Janus Particles Based on a Multifunctional Poly(ethylene glycol)

Authors:

Keywords: functional materials; microparticles; poly(ethylene glycol); ring-opening polymerization; sugar conjugation

Abstract: A novel water insoluble, multifunctional poly(ethylene glycol), poly(hydrazide ethylene glycol-co-benzyl glycidyl ether) (P(HZ-co-BnGE)), is synthesized via thiol-ene click reaction of poly(allyl glycidyl ether-co-benzyl glycidyl ether) (P(AGE-co-BnGE)). The base polymer P(AGEcoBnGE) is previously prepared by anionic ring-opening copolymerization of the corresponding monomers. To demonstrate utility, bicompartmental microspheres and microcylinders containing P(HZ-co-BnGE) in one of the compartments are prepared via electrohydrodynamic (EHD) co-jetting. Next, spatially controlled surface reactivity toward sugars is demonstrated by selective binding of 2?-mannobiose to the P(HZ-co-BnGE) compartment only, as confirmed by a carbohydrate-ectin-binding assay. These sugar-reactive hydrazide-presenting microparticles have potential applications for glyco-targeted drug delivery.

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Helical versus All-Trans Conformations of Oligo(ethylene glycol)-Terminated Alkanethiol Self-Assembled Monolayers

Authors:

Keywords: oligoethylene glycol, conformations, x-ray absorption spectroscopy, monolayers

Abstract: The complex mixture of conformational states exhibited by oligo(ethylene glycol)-terminated alkanethiols on Ag and Au surfaces is explored by polarization-dependent X-ray absorption spectroscopy. Three self-assembled monolayers (SAMs) with known helical or all-trans conformations are used as references to characterize a SAM with unknown conformations. This case study is used as a prototype for developing a systematic framework to extract the conformations of SAMs from the polarization dependence of several orbitals. In the case at hand, these are associated with the C?H/Rydberg bonds of the alkane, the C?H/Rydberg bonds of ethylene glycol, and the C?C bonds of the backbone. The C?H/Rydberg orbitals of the alkane and ethylene glycol are distinguished via the chemical shift of the corresponding C 1s core levels.

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Acknowledged Federal Support:
Abstract: The orientations of liquid crystals (LCs) anchored on monolayers formed from mixtures of chiral versus achiral molecules were compared. Changes in the enantiomeric excess of mixed monolayers of chiral dipeptides gave rise to continuous changes in the orientations of nematic LCs, allowing arbitrary tuning of the azimuthal orientations of LCs over a range of 100 degrees. In contrast, the same LCs exhibited discontinuous changes in orientation on surfaces presenting mixtures of achiral molecules. These striking differences in the anchoring of LCs on surfaces presenting chiral versus achiral molecules provide insights into the molecular origins of ordering transitions of LCs, and provide new principles based on chiral monolayers for the rational design of surfaces that permit continuous tuning of the orientations of LCs.

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Abstract: We report a study of the wetting and ordering of thermotropic liquid crystal (LC) droplets that are trapped (or “caged”) within micrometer-sized cationic polymeric microcapsules dispersed in aqueous solutions of surfactants. When initially dispersed in water, we observed caged, near-spherical droplets of E7, a nematic LC mixture, to occupy ~40% of the interior volume of the polymeric capsules [diam. = 6.7 ± 0.3 µm; formed via covalent layer-by-layer assembly of branched polyethyleneimine and poly(2-vinyl-4,4dimethylazlactone)] and to contact the interior surface of the capsule wall at an angle of ~157 ± 11°. The internal ordering of LC within the droplets corresponded to the so-called bipolar configuration (distorted by contact with the capsule walls). While the effects of dodecyltrimethylammonium bromide (DTAB) and sodium dodecylsulfate (SDS) on the internal ordering of “free” LC droplets are similar, we observed the two surfactants to trigger strikingly different wetting and configuration.

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Article Title: Reversible Switching of Liquid Crystalline Order Permits Synthesis of Homogeneous Populations of Dipolar Patchy Microparticles

Authors:

Keywords: liquid crystals, microparticles, anchoring transitions, patchy interfaces, ordering

Abstract: The spontaneous positioning of colloids on the surfaces of micrometer-sized liquid crystalline droplets and their subsequent polymerization offers the basis of a general and facile method for the synthesis of patchy microparticles. The existence of multiple local energetic minima, however, can generate kinetic traps for colloids on the surfaces of the liquid crystal (LC) droplets and result in heterogeneous populations of patchy microparticles. To address this issue, here we demonstrate that adsorbate-driven switching of the internal configurations of LC droplets can be used to sweep colloids to a single location on the LC droplet surfaces, thus resulting in the synthesis of homogeneous populations of patchy microparticles. The surface-driven switching of the LC can be triggered by addition of surfactant or salts, and permits the synthesis of dipolar microparticles as well as “Janus-like” microparticles. By using magnetic colloids, we illustrate the utility of the approach by synt

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Article Title: Using Liquid Crystals to Reveal How Mechanical Anisotropy Changes Interfacial Behaviors of Motile Bacteria

Authors:

Keywords: liquid crystals, bacteria, anchoring, elasticity, ordering

Abstract: Bacteria often inhabit and exhibit distinct dynamical behaviors at interfaces, but the physical mechanisms by which interfaces cue bacteria are still poorly understood. In this Article, we use interfaces formed between coexisting isotropic and liquid crystal (LC) phases to provide insight into how mechanical anisotropy and defects in LC ordering influence fundamental bacterial behaviors. Specifically, we measure the anisotropic elasticity of the LC to change fundamental behaviors of motile, rod-shaped Proteus mirabilis cells (3 µm in length) adsorbed to the LC interface, including the orientation, speed and direction of motion of the cells (the cells follow the director of the LC at the interface), transient multi-cellular self-association, and dynamical escape from the interface. In this latter context, we measure motile bacteria to escape from the interfaces preferentially into the isotropic phase, consistent with the predicted effects of an elastic penalty associated with strain

Acknowledged Federal Support: 1-Approved for public release; distribution is unlimited.
**Article Title:** Liquid crystal droplet-based amplification of microvesicles that are shed by mammalian cells  

**Authors:**  

**Keywords:** liquid crystals, microvesicles, biomolecular interactions, elasticity, ordering  

**Abstract:** Membrane-derived microvesicles (MVs) shed by cells are being investigated for their role in intercellular communication and as potential biomarkers of disease, but facile and sensitive methods for their analysis do not exist. Here we demonstrate new principles for analysis of MVs that use micrometer-sized droplets of liquid crystals (LCs) to amplify MVs that are selectively captured via antibody-mediated interactions. The influence of the MVs on the micrometer-sized LC droplets is shown to be readily quantified via use of flow cytometry. The methodology was developed using MVs shed by epidermoid carcinoma A431 cells that contain epidermal growth factor receptor (EGFR) as an important and representative example of MVs containing signaling proteins that play a central role in cancer. The LC droplets were found to be sensitive to 10^6 MVs containing EGFR (relative to controls using isotype control antibody) and to possess a dynamic range of response across several orders of magnitude.

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**Article Title:** Dynamic self-assembly of motile bacteria in liquid crystals  

**Authors:**  

**Keywords:** liquid crystals, bacteria, biotic-abiotic interfaces, elasticity, dynamic ordering  

**Abstract:** This paper reports an investigation of dynamical behaviors of motile rod-shaped bacteria within anisotropic viscoelastic environments defined by lyotropic liquid crystals (LCs). In contrast to passive microparticles (including non-motile bacteria) that associate irreversibly in LCs via elasticity-mediated forces, we report that motile Proteus mirabilis bacteria form dynamic and reversible multi-cellular assemblies when dispersed in a lyotropic LC. By measuring the velocity of the bacteria through the LC (8.8 ± 0.2 mm s^-1) and by characterizing the ordering of the LC about the rod-shaped bacteria (tangential anchoring), we conclude that the reversibility of the inter-bacterial interaction emerges from the interplay of forces generated by the flagella of the bacteria and the elasticity of the LC, both of which are comparable in magnitude (tens of pN) for motile Proteus mirabilis cells. We also measured the dissociation process, which occurs in a direction determined by the LC, to bias th
Abstract: Reactive chemical vapor deposition (CVD) polymerization provides a substrate-independent platform for effective functionalization of virtually any solid substrates, flat, or curved, even with complex geometries. This article reviews bioactive surface functionalization strategies based on CVD polymerization and highlights commonly used surface chemistries. These reactions include alkyne-azide "click" chemistry, reactions of active esters with amine, aldehydes/ketones with hydrazides and alkoxyamines, thiols with alkenes and alkynes and surface-initiated atom transfer radical polymerization. The resulting biofunctional surface coatings can facilitate orthogonal immobilization of more than one type of ligand on a substrate. CVD polymer coatings with nanoscale thicknesses are widely applicable in biomedical applications and can be easily integrated into micro- and nanodevice fabrication.
Final Report

Understanding the Interaction of Peptides and Proteins with Abiotic Surfaces: Towards Water-Free Biologics

The following specific aims were proposed in the original proposal:

**Aim 1 Develop systematic understanding of abiotic/biotic interfaces.** We aim to understand, how the structure and activity of proteins and peptides immobilized on abiotic surfaces depends upon a) the chemical and physical nature of the abiotic surface; b) the physicochemical properties of the protein or peptide; and c) the nature of the surrounding medium – water, air or other hydromimetic molecules.

**Aim 2 Develop design rules for “water-free biologics” based on engineered abiotic/biotic interfaces.** Using knowledge gained from studies in Aim 1, we aim to a) engineer peptides and proteins with enhanced ability to retain structure and function in the absence of bulk water, and b) develop abiotic surfaces displaying hydromimetic functionality that stabilizes the native structure and function of biological molecules in extremely low humidity or “water-free” interfaces – i.e. a surface-air interface that lacks bulk water and in air that is not humidified.

In the last six years, we have successfully performed research proposed under the above two specific aims and have made substantial progress in such research. Many results have been presented in the previous annual reports and various publications. In this final report, we will not repeat all the details of such research results. Instead, we will briefly present the most important results related to the two specific aims: (1) systematic understanding of abiotic/biotic interfaces – In particular, general rules in governing interfacial structures of surface immobilized peptides and proteins and (2) development of “water free biologics” for abiotic/biotic interfaces.

1. General rules for surface immobilized peptides
   1.1 Surface immobilized peptides at the solid/liquid interface

   We adopted a combined experimental and computational approach to develop systematic methodology to characterize surface immobilized peptide conformation and orientation. A variety of experimental methods, including sum frequency generation (SFG) vibrational spectroscopy, attenuated total reflection (ATR)-FTIR, circular dichroism (CD) spectroscopy, and quartz crystal microbalance (QCM) etc. have been applied to investigate the structure of surface immobilized peptides. Both coarse grained and all-atom molecular dynamics simulations have been used to study peptide behavior at the solid/liquid interface. In such studies, peptides were immobilized onto surfaces via terminal cysteine coupling with maleimide terminated self-assembled monolayers (SAMs), polymers with maleimide functionality, or click chemistry between the peptide terminal azide group and surfaces with alkyne groups. We observed the following general rules:

   (1) Antimicrobial peptides (AMPs) which form alpha helical structures in cell membranes such as cecropin P1, MSI-78, and cecropin A-melittin hybrid peptide, adopt an alpha helical structure at the solid/liquid interface after surface immobilization. Such results can be obtained by coarse grain MD simulations and can be measured by experimental techniques such as SFG, ATR-FTIR, CD etc.
Typically a wild-type AMP immobilized on a surface via two different termini (e.g., N- and C-termini) is able to exhibit different orientations – i.e., with one tilting (standing up) pose and one lying down pose, even though in both cases the peptides are both alpha helical (Figure 1).

MD simulation results are able to match experimental data well to show that a peptide adopting a standing up pose is because its hydrophobic terminus is immobilized on the surface, while the other terminus is hydrophilic (which can interact with water favorably and thus stays in water). On the contrary, the peptide adopting a lying down pose is because the hydrophilic terminus is immobilized, and the other peptide end is hydrophobic (which does not like to interact with water and would prefer to lie down on the hydrophobic surface instead).

With a known peptide sequence, coarse grained MD simulation could be used to predict the peptide orientation after the surface immobilization, and such predictions can be confirmed (validated) by experimental results (e.g., SFG orientation analysis).

Based on the wild type peptide sequence (e.g., sequence of MSI-78, cecropin P1, or hybrid peptide), we could manipulate the peptide orientation after surface immobilization by mutation. We demonstrated that we could enable a peptide to stand up on the surface after immobilizing onto a surface via either C- or N-terminus by just mutating two amino acids in the peptide sequence (Figure 2). When performing the amino acid mutation, it is necessary to consider the hydrophobicity and helix propensity of the amino acids.

Unfortunately in this study we could not find a simple (or straightforward) general correlation among the peptide sequence, the surface hydrophobicity, and the peptide orientation after surface immobilization. However, we demonstrated that for any peptide with a known sequence which can form alpha helical structure after the surface immobilization (e.g., many AMPs), we can confirm its alpha helical conformation and predict its orientation using MD coarse grained simulations. At the same time, we can validate such MD simulation results using experimental techniques. We could also design a peptide (based on a known AMP sequence and modification of such a sequence) so that it can form an alpha helical structure with a certain orientation after surface immobilization via either C- or N-terminus by MD simulations. Such MD simulation results can also be confirmed by experimental data. The influences of peptide sequence, peptide immobilization site (C or N terminus), immobilization...
strategy (cysteine – maleimide coupling or click chemistry), substrate surface property (hydrophobic, hydrophilic etc.) on the peptide conformation and orientation after surface immobilization could be characterized by the methodology developed in this study using MD simulations and experimental techniques (Figure 3).

1.2 Surface immobilized peptides in air

It is interesting to observe that no SFG signal could be observed from almost all surface immobilized peptides in air. This could be due to the three possible reasons: (1) No peptide stays on the surface in air; (2) Surface immobilized peptides adopt a disordered (e.g., random coiled) structure; (3) Surface immobilized peptides adopt a lying-down orientation. CD studies performed in air clearly demonstrated that there are peptides on the surface and that those peptides still adopt an alpha-helical structure (Figure 4). Based on both SFG and CD results, we concluded that in air the surface immobilized peptides adopt an alpha helical conformation and lie down on the surface.

We developed a coarse grained MD simulation method to investigate the surface immobilized peptide behavior in air. Since the simulation parameters used in the coarse grain MD simulations were obtained from the studies of proteins in aqueous environments, it is necessary to deduce simulation parameters of proteins in air. We successfully obtained such parameters from the protein unfolding studies in various chemical environments. Using such parameters, we successfully simulated the behavior of surface immobilized peptides in air, and found that indeed almost all the surface immobilized peptides are lying down on the surface, well correlated to the experimental results (Figure 5). The only exception that the peptide is not lying down in air is the hybrid peptide immobilized on the surface, from which SFG amide signals were detected, and MD simulation results indicated that part of the peptide is not lying down. The experimental and simulation results are well correlated with each other. When a peptide molecule interacts with a surface in water, the peptide needs to overcome some energy barrier to remove the water for the peptide-surface interaction. The peptide may not be able to remove all the water molecules on the surface, therefore the peptide cannot completely lie down on the surface. In air, the water barrier does not exists. Thus it is easier for the entire peptide to interaction the surface and lie down on the surface.

2. General rules for surface immobilized enzymes
2.1 Chemical immobilization vs. physical adsorption
We compared the physical adsorption and chemical immobilization of enzymes on surfaces. The combined SFG and ATR-FTIR studies showed that the surface immobilized 6-phospho-β-galactosidase (β-Gal) on maleimide terminated SAM via cysteine – maleimide coupling adopts an orientation as we expected, with the cysteine group facing the surface (Figure 6). The MD simulation results indicated that the immobilized β-Gal adopts a native conformation (Figure 6, similar to the crystal structure), therefore it is reasonable to use the crystal structure of the surface immobilized enzyme for data analysis for SFG and ATR-FTIR studies. This study developed a general methodology to use SFG/ATR-FTIR to determine the surface immobilized enzyme orientation.

We also studied the physical adsorption of β-Gal on SAM surfaces. Using a maleimide terminated SAM surface, due to the OEG segment (for resistance to physical adsorption of the protein to the surface) in the SAM, no β-Gal (with no surface cysteine) could be adsorbed onto the SAM surface. On a hydrophobic OTS SAM surface, β-Gal could be physically adsorbed onto the surface with different orientations and possible conformation changes (as indicated by the SFG signal peak center change).

2.2 Controlling surface immobilized enzyme orientation and activity

We have shown that it is feasible to control the enzyme orientation after surface immobilization via enzyme cysteine – maleimide coupling on SAM surface and site-directed mutagenesis to place a single cysteine residue on a specific location of the enzyme. On a maleimide terminated SAM surface, the enzyme cysteine coupling site is always facing the surface, exposing the opposite side of the enzyme to the solution. This can be used as a general methodology to control the enzyme orientation after surface immobilization on similar surfaces. That is to say, to control the enzyme orientation on a maleimide terminated SAM surface, it is necessary to select the proper enzyme cysteine position for immobilization. The general approach for such a selection and surface immobilization includes the following steps: (1) Modify the gene for a particular enzyme to replace all the surface cysteine residues to other amino acids (e.g., alanine), and select a particular position (the surface immobilization site) for cysteine (mutate that amino acid at this particular position to cysteine). (2) Use the modified gene to produce enzyme molecules using bacteria and purify such enzymes. (3) Immobilize the enzyme onto a maleimide terminated SAM surface. Then the surface
immobilized enzyme orientation can be determined/validated using MD coarse grain simulations and SFG/ATR-FTIR experimental techniques.

The above general method was successfully applied to study surface immobilization of three completely different enzyme molecules: β-Gal, nitroreductase (NfsB), and dehalogenase (LinB). All the results indicated that the surface immobilized enzyme orientation could be controlled by properly selecting the cysteine position on the enzyme surface.

For β-Gal, two cysteine positions near the active site (β-Gal E227C and β-Gal D308C) and far from the active site (β-Gal V152C) were studied. The results indicated that as we designed, the immobilization of β-Gal via the cysteine position 227 or 308 on a maleimide terminated SAM orients the enzyme with the active site near the substrate surface because these two immobilization sites are near the active site (Figure 7). The resulting orientations of the immobilized β-Gal E227C and β-Gal D308C are similar, because the sites 227 and 308 are next to each other. On the contrary, the β-Gal immobilized via cysteine position 152 (V152C) has the active site far from the surface because the site 152 is on the opposite side of the enzyme active site. Since the active sites for surface immobilized β-Gal E227C and β-Gal D308C are near the substrate surface (which are more difficult to be accessed by substrate molecules), they exhibited lower activities compared to surface immobilized β-Gal V152C. We also investigated surface immobilized β-Gal E147C. The position 147 is very close to the position 152, therefore the immobilized β-Gal E147C also exposes the enzyme active site to the solution and far from the surface. The difference between sites 152 and 147 is that position 152 is on a coil while the position 147 is on an alpha helix. Nevertheless, surface immobilized β-Gal E147C and β-Gal V152C exhibit a similar orientation. The studies on the surface immobilized β-Gal via different positions clearly demonstrated that we could control the enzyme orientation by properly selecting the enzyme immobilization site.

We further demonstrated the orientation control of surface immobilized enzyme by selecting proper enzyme immobilization sites using NfsB. The NfsB which we studied is a dimeric protein, with two active sites on each molecule. We showed that surface immobilized NfsB H360C and NfsB V423C with different immobilization sites have different orientations. The deduced orientation for each case is as we expected, with the cysteine site located next to surface. For NfsB V423C, the cysteine is next to one of the two active sites. Therefore, the deduced orientation indicates that one active site is near the surface. Differently, the cysteine at position 360 is the farthest from both active sites, therefore the surface immobilized NfsB H360C has both active sites solvent accessible (Figure 8). The measured activity of surface immobilized NfsB H360C (with both active sites far from the surface) is higher than that of NfsB V423C (with only one active site near the surface).
far from the surface), also as expected. We also studied surface immobilized LinB A141C, LinB A196C, and LinB N262C, showing that we can control the surface immobilized enzyme orientation by selecting the surface immobilization site (Figure 9).

2.3 Surface hydrophobicity and CVD polymer surfaces

As we presented in the previous section, we could control surface immobilized enzyme orientation by selecting the proper enzyme surface immobilization site. The above conclusion was obtained on the enzymes immobilized on maleimide terminated SAM surfaces. Our study also indicated that substrate surface for enzyme immobilization could influence the orientation of surface immobilized enzymes. Additionally, we found that the surface immobilized enzyme molecules have weaker interactions with hydrophilic surfaces, leading to a broader orientation distribution of surface immobilized enzyme. For example, we showed that the surface immobilized β-Gal D308C on a more hydrophilic mixed maleimide – hydroxyl terminated SAM has a broader orientation distribution compared to that immobilized on a maleimide terminated SAM (Figure 10). Due to the broader orientation distribution and increased flexibility, surface immobilized β-Gal D308C on a mixed SAM exhibits a higher activity. This is because for this case the enzyme active site is near the substrate surface. For a hydrophilic surface, the reduced enzyme-surface interaction enhanced the enzyme activity. We believe that using a hydrophilic surface for enzyme immobilization is a general approach to enhance the surface immobilized enzyme activity especially when enzyme immobilized onto a surface with the active site not far from the surface.

We also studied enzyme immobilization onto polymer surfaces. Our results indicated that for NfsB H360C, the orientations of the surface immobilized enzyme on SAM and on polymer are similar. However, such orientations are different for NfsB V423C. Therefore, it is not possible to predict the enzyme orientation on polymer from the surface immobilized enzyme orientation on a SAM surface for all the enzyme molecules. It is necessary to study immobilized enzyme orientation on a polymer surface to determine the orientation.

![Figure 10](image1.png)

**Figure 10** Dependence of the SFG $\chi_{zzz}/\chi_{xxz}$ ratio on the tilt and twist angles of immobilized β-gal. Magenta dots indicate the orientations of β-Gal D308C on the 1:10 maleimide/OH surface and the cyan dots indicate the orientation of β-Gal D308C on a pure maleimide surface.

![Figure 11](image2.png)

**Figure 11** Comparison of experimental and computationally determined thermal stability for β-Gal in solution and tethered to maleimide SAM surface. A) Thermal stability curves (fractional activity remaining after heating at given temperature for 10 min) for β-Gal V152C (blue triangles) and β-Gal E147C (green squares) in free solution. B) Thermal stability curve for β-Gal E147C tethered to maleimide SAM surface (green squares). C) Thermal stability curve for β-Gal V152C tethered to maleimide SAM surface (blue squares). In each panel the red line is the computationally determined thermal unfolding curve (fraction nativeness).
2.4 Thermal Stability

As we presented above, because the cysteine position 147 is very close to position 152, the immobilized β-Gal E147C and β-Gal V152C exhibit similar orientation, even though the position 152 is on a coil while the position 147 is on an alpha helix. However, it was found by both experiments and MD simulations that the surface immobilized β-Gal E147C has a better thermal stability (Figure 11, Table 1). We believe that it is a general rule that the surface immobilized enzyme via an immobilization site on an alpha helical structure exhibits better thermal stability compared to that via a site on a coil.

<table>
<thead>
<tr>
<th></th>
<th>T_{1/2} (expt, °C)</th>
<th>T_m (calc, °C)</th>
<th>T_{1/2} – T_m (°C)</th>
<th>ΔΔG_{fold} (calc relative to solution, KJ mol⁻¹)</th>
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<tr>
<td>β–Gal (solution)</td>
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<td>3</td>
<td>0.0</td>
</tr>
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<tr>
<td>β–Gal V152C</td>
<td>38</td>
<td>34</td>
<td>4</td>
<td>5.9</td>
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</table>

Table 1 Comparison of experimental T_{1/2} values and calculated T_m values for thermal unfolding of β-Gal in free solution and tethered to EG4-maleimide terminated SAM surface

2.5 Multiple immobilization sites

We have compared the behavior of surface immobilized enzyme via a single coupling site and two neighboring immobilization sites, using NfsB as a model. There are two possible opposite effects for immobilization of an enzyme onto a surface with two neighboring sites: (1) enhancement of the enzyme activity because of the better control of the enzyme orientation to achieve an optimized orientation; (2) reduction of the enzyme activity because of the loss of some of the flexibility. Our studies indicated that the surface immobilized NfsB with two neighboring sites could not enhance the enzyme activity, but the thermal stability could be increased (Figure 12). We believe that it is a general rule to enhance the surface immobilized enzyme thermal stability by immobilizing the enzyme onto a surface via multiple sites.

2.6 Surface immobilized enzyme in air

Figure 12 (a) Activity of immobilized NfsB on maleimide SAM surface. (b) Thermal stability of immobilized NfsB by measuring the half-life of enzyme from its time-dependent deactivation profile at 45 °C. The inset in the graph at right side shows the p-values of t-test analyses between the calculated half-life of each immobilized double mutant relative to its corresponding single mutants.
Similar to surface immobilized peptides, no SFG amide I signal could be observed from surface immobilized enzymes via one immobilization site in air without the presence of bulk water. We believe that this is due to the denaturation of the enzyme in air, not only the orientation change as was seen for peptides. CD studies indicated that the conformations of surface immobilized enzymes indeed varied greatly in air compared to those in aqueous environment. Due to the structural denaturation of the enzyme in air, its activity is also greatly reduced. It was shown in the literature that the enzyme activity in air is much lower compared to that in the solution phase, usually in the order of 1 (in air)/1000 (in solution).

We detected some SFG signals from surface immobilized NfsB via two neighboring sites in air, showing that structures of enzymes are less denatured in air for enzyme immobilized with more than one coupling site. We believe that it is a general rule to immobilize an enzyme onto a surface via multiple immobilization sites to retain some of the enzyme structure in air after surface immobilization.

3. Water-Free Biologics

3.1 Peptides

It is interesting to observe that alpha helical peptides lie down on the surface in air. It is clear that a standing-up peptide on the surface could have a better antimicrobial and bacteria capture capability because it has more opportunities to interact with the bacteria. Therefore we investigated how to enable the peptide to stand up on the surface after surface immobilization in air. We found that coating with or co-immobilization of hydromimetic functionalities with peptides could change the peptide orientation in air to “standing up”. Firstly, we demonstrated that by spin coating a thin layer of sucrose solution, after drying in air, the surface immobilized AMP MSI-78 adopts a “standing-up” orientation, evidenced by strong SFG amide I signal detected (centered at around 1650 cm\(^{-1}\)) (Figure 13). Such a standing up pose is very stable, which can be maintained for weeks and even for months. When the sucrose solution concentration used for spin coating was varied, the resulting orientation of the surface immobilized MSI-78 could be varied as well. In addition to spin coating,
solvent casting with a fast drying speed also can lead to “standing-up” orientations for surface immobilized MSI-78.

We also showed that with co-immobilized polysorbitol, surface immobilized MSI-78 could adopt a “standing-up” pose, like the immobilized peptides with spin coated sucrose (Figure 14). SFG signals could be detected from the immobilized MSI-78 with co-immobilized polysorbitol in air (Figure 15). In air, the antimicrobial activity of surface immobilized MSI-78 with co-immobilized polysorbitol is higher than the surface immobilized MSI-78 without co-immobilized hydromimetic functionality (Figure 16). Similar results were observed from surface immobilized hybrid peptide with co-immobilized polysorbitol. We believe that it is a general approach to use spin-coating for the co-immobilization of hydromimetic functionalities and peptides to retain their standing-up orientation in air. It is also a general approach to enhance the antimicrobial activity of surface immobilized AMP in air with the co-immobilization of hydromimetic functionalities.

3.2 Enzymes

We successfully adopted a head-space sampling method to test the enzymatic activity of surface immobilized enzymes using GC-MS in air without the presence of bulk water (Figure 17). Using this method, we tested the activity of surface immobilized dehalogenase, LinB, immobilized onto a maleimide terminated SAM surface. We first produced and purified three LinB mutants, each with one cysteine on the enzyme surface: LinB A141C, LinB A196C, and LinB N262C. Such LinB mutants were then immobilized onto a maleimide terminated SAM surface and the orientations of these immobilized enzymes were deduced. It was found that via different immobilization sites, the above three immobilized LinB mutants exhibit varied orientations as we expected, while LinB A141C has the highest activity. We therefore decided to choose LinB A141C for the enzymatic activity study in air. We immobilized LinB A141C onto the
maleimide terminated SAM surface and tested its activity in air with a 50% humidity level. It was found that the activity of the surface immobilized enzyme was measured to be several times larger compared to that of the freeze-dried LinB A141C powder. This shows that the enzyme surface immobilization could enhance the LinB activity in air. However, no SFG signal could be detected from the surface immobilized LinB A141C in air at 50% humidity level, showing that the main structure of the surface immobilized enzyme denatured.

We then co-immobilized hydromimetic functionality polysorbitol PMSA with LinB A141C. It was found that the enzyme activity could be further enhanced (Figure 18). Compared to the enzyme powder; the surface immobilized LinB A141C co-immobilized with polysorbitol exhibit 40 times higher activity in air with 50% humidity level (Table 2). Such an activity is about 10% of that of the free enzyme in solution (As mentioned above, normally the activity of an enzyme in air is about 1/1000 of the activity of the free enzyme in solution). The great activity enhancement is not due to the larger water adsorption amount at 50% humidity level by co-immobilized polysorbitol. The QCM results indicated that for surface immobilized LinB A141C with and without co-immobilized hydromimetic polysorbitol, the water adsorption amount is similar at 50% humidity level: Each enzyme molecule has about 200 water molecules adsorbed (Figure 19), while it needs 1600 water molecules per enzyme to completely cover a monolayer of water on the enzyme. CD studies showed that the conformation of the surface immobilized LinB A141C co-immobilized with polysorbitol has much less variation in air at different humidity levels compared to that of LinB A141C immobilized without the co-immobilized hydromimetic polysorbitol (Figure 20). In addition, SFG amide I signal could be clearly detected from surface immobilized LinB A141C co-immobilized with polysorbitol at 50% humidity level (Figure 20). Such CD and SFG spectroscopic evidence clearly showed that the surface immobilized LinB A141C with co-

<table>
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<th></th>
<th>turnover number (s⁻¹)</th>
<th>relative activity (%)</th>
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</tr>
<tr>
<td>immobilized HLD+PSMA (vapor phase)</td>
<td>1.73</td>
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</tr>
</tbody>
</table>

**Figure 19** Water adsorption amount on surface immobilized LinB and without and with different amounts of co-immobilized PSMA. At 50% humidity level, the water adsorption amounts are the same on different surfaces.

**Figure 20** Effect of relative humidity on the secondary structure of surface immobilized LinB A141C, determined by CD spectroscopy. (a) Spectra obtained in the absence of PSMA. (b) Spectra obtained in the presence of PSMA. (PB: spectrum in phosphate buffers.) (c) SFG spectra of immobilized LinB and LinB co-immobilized with PSMA, both recorded at 40% RH.
immobilized polysorbitol could greatly enhance the enzymatic activity in air is because the co-immobilized polysorbitol could help to retain the native LinB structure better in air. We believe that it is a general approach to co-immobilize polysorbitol with enzyme to retain the enzyme structure in air and enhance the enzyme activity.

We also tried to co-immobilize other hydromimetic functionalities such as zwitterionic oligomers and polyethylene glycols (PEGs). Unfortunately none of such co-immobilized hydromimetic functionalities could enhance the activity of surface immobilized LinB.