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SENSORS: Detecting Microbial Pathogens with Novel Surface Acoustic Wave Devices in Liquid Environments

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Principal Investigator: Pereira da Cunha, Mauricio . **Award ID:** 0329913

Organization: University of Maine

Submitted By:

Pereira da Cunha, Mauricio - Principal Investigator

Title:

SENSORS: Detecting Microbial Pathogens with Novel Surface Acoustic Wave Devices in Liquid Environments

Project Participants

Senior Personnel

Name: Pereira da Cunha, Mauricio

Worked for more than 160 Hours: Yes

Contribution to Project:

Name: Millard, Paul

Worked for more than 160 Hours: Yes

Contribution to Project:

Post-doc

Graduate Student

Name: Berkenpas, Eric

Worked for more than 160 Hours: Yes

Contribution to Project:

Eric developed his graduate studies in detecting E-coli using surface acoustic wave devices. His work involved microfluidics, sensor chamber development, detection of E-coli using the acoustic devices confirmed by fluorescent methods.
Supported by this project and by an UMaine NSF GK-12 project.

Name: McCarthy, Erik

Worked for more than 160 Hours: Yes

Contribution to Project:

Erik trained in our laboratories as an NSF REU intern prior joining the lab as a graduate student. While working toward an M.S. degree, Erik was involved in developing microcapillary-based and regenerable gold substrate-based molecular padlock assays for specific RNA sequences.
Supported by this project, a GK-12 project and by Maine Sea Grant.

Name: Pollard, Thomas

Worked for more than 160 Hours: Yes

Contribution to Project:

Tom is developing his PhD in this project and he is mostly concerned with two aspects of the biosensor work. The first is the modeling of the shear horizontal waves on the langasite (LGS substrate in order to provide improved performance of the biosensor. The second is to investigate and adapt the regenerative process developed by Erik McCarthy in the LGS substrates.
Supported by this project.

Name: Gallimore, Dana

Worked for more than 160 Hours: Yes

Contribution to Project:

Dana is developing her PhD in the characterization of polymer and biofilms from the acoustic point of view.
Supported by this project and by the UMaine NSF Sensor IGERT project and GK-12 project.

Name: Bitla , Shivashanker

Worked for more than 160 Hours: Yes

Contribution to Project:

As a graduate student in the laboratory, Shivashanker carried out experiments related to a biotin-avidin-related regeneration system for molecular padlock-based DNA sensors.
He was supported by this project and by a scholarship from the University of Maine Graduate School.

Undergraduate Student

Name: Egeler, Teressa

Worked for more than 160 Hours: Yes

Contribution to Project:

Both through the NSF REU program and independent funding Ms. Deane participated in the development of methods for applying the molecular padlock/rolling circle amplification system to SiO2 substrates, particularly microcapillaries.
Supported by this project and an NSF REU supplement.

Name: Beaucage, Tim

Worked for more than 160 Hours: Yes

Contribution to Project:

Tim worked on the development of a microfluidic chamber and the tubing required for flow through tests using the SH-SAW microwave acoustic sensor platform.
Supported by this project and an NSF REU supplement.

Name: Deane, Jennifer

Worked for more than 160 Hours: Yes

Contribution to Project:

Both through the NSF REU program and independent funding Ms. Deane participated in the development of methods for applying the molecular padlock/rolling circle amplification system to SiO2 substrates, particularly microcapillaries.
Supported by this project and an NSF REU supplement.

Name: Richards, Zachary

Worked for more than 160 Hours: Yes

Contribution to Project:

Zach was selected as an NSF REU student during the summer of 2004 and continued to work after that in the acoustic wave material preparation for the biosensors fabricated. Out of his efforts a required process for grinding and polishing LGS was identified and used in the sensor sample preparation.
Supported by this project and an NSF REU supplement.

Name: Noonan, Patrick

Worked for more than 160 Hours: Yes

Contribution to Project:

Undergraduate Student
During participation in the project through the NSF REU program, and as a senior capstone student, Patrick developed rapid methods for DNA isolation that were compatible with downstream amplification via immobilized molecular padlock/rolling circle technology.
Supported by an NSF REU supplement and the University of Maine Work Merit program.

Name: Petraszewski, Matthew

Worked for more than 160 Hours: Yes

Contribution to Project:

Matthew developed enrichment and rapid culture procedures for retrieval of pathogenic bacteria from water.
Supported through University of Maine Work Merit program.

Technician, Programmer

Name: Bickerstaff, Lee

Worked for more than 160 Hours: Yes

Contribution to Project:

Lee participated in this project by providing laboratory guidance and developing the procedures required for molecular padlock/rolling circle amplification in solutions.
Supported by the University of Maine and Maine Sea Grant.

Other Participant

Research Experience for Undergraduates

Name: Gao, Qian

Worked for more than 160 Hours: No

Contribution to Project:

Qian was selected as an NSF REU student during the summer of 2007. She has worked in this project in cooperation with Tom Pollard in the investigation of microfluidics and microchambers for liquid sensor using surface acoustic wave devices.
Supported by this project and an NSF REU supplement.

Years of schooling completed: Sophomore

Home Institution: Same as Research Site

Home Institution if Other:

Home Institution Highest Degree Granted(in fields supported by NSF): Doctoral Degree

Fiscal year(s) REU Participant supported: 2007

REU Funding: REU supplement

Name: Tribbet, Justin

Worked for more than 160 Hours: Yes

Contribution to Project:

Justin was selected as an NSF REU student during the summer of 2005 and worked with microfluidics, chamber fabrications, and droplet displacement using surface acoustic waves.

Supported by this project and an NSF REU supplement.

Years of schooling completed: Junior

Home Institution: Same as Research Site

Home Institution if Other:

Home Institution Highest Degree Granted(in fields supported by NSF): Doctoral Degree

Fiscal year(s) REU Participant supported: 2005

REU Funding: REU supplement

Name: Dunn, Matthew

Worked for more than 160 Hours: Yes

Contribution to Project:

Measured the SH-SAW delay line responses before the application of any protein to the surface and after the surface preparation using, for instance bovine serum albumin (BSA) and NeutrAvidin monolayers.

Explored methods of removing non-specifically bound surface proteins, including detergent rinses in water and NaOH.

Determined the efficacy of the cleaning methods by examining the SH-SAW response before and after cleaning and throughout multiple regeneration cycles.

Organizational Partners

Other Collaborators or Contacts

Scott LaPatra Clear Springs Foods, Inc.
Dr. LaPatra is an expert in viral pathogenesis in fish species. He helped us to develop the real-world model for RNA sequence detection.
No funding required.

Carol Kim University of Maine Collaborator
Dr. Kim is an expert in molecular biology and the diagnosis of microbial pathogens. She collaborated in the development of RNA sequence detection using the molecular padlock/rolling circle technique.
No funding required.

Mark McKenna Luna Innovations
Mark McKenna was the main point of contact from Luna Innovations, which is looking into using the developed platforms for the detection of bacteria DNA signatures.
No funding required.

Activities and Findings

Research and Education Activities:

This multidisciplinary SENSORS project has been integrating research and education to investigate and combine a new family of LGX crystal devices fabricated and operated in Shear Horizontal Surface Acoustic Wave (SH-SAW) propagation directions with highly selective molecular padlock probes to detect specific nucleic acid sequences associated with pathogenic bacteria. The University of Maine (UMaine) team, under the leadership of Profs. Pereira da Cunha and Millard, has been working in the design and fabrication of biosensors capable detecting pathogens in aqueous environments. The novel langasite (LGS) SH-SAW devices have been modeled, simulated, designed, fabricated, and then tested first for the detection of immunochemical binding and then tested for the detection of E-coli bacteria. The work performed also included the viral detection via isothermal amplification, and fluorescence-based viral detection in a microcapillary tube format, and the development of a regeneration procedure of highly specific DNA/RNA detection layers to recover the sensor platform. The integration of the SH-SAW platforms with the regeneration procedure of DNA/RNA detection layers is under way using novel PDMS microfluidic platforms, and constituted one of the final steps in graduate student Thomas Pollard's PhD dissertation, under this project. In other PhD work supported by this project now pending thesis defense, Dana Gallimore has been studying viscoelastic changes on polymer films during crosslinking. Two papers describing this work
have been published. This study is a first step to gain further insight into the influence of viscoelastic properties of SH-SAW sensors for improving sensitivity in biosensor applications. In addition two manuscripts describing SHSAW detection of DNA are near completion and yet a third one in preparation. Therefore, the successful integration of electrical engineering and bioengineering resulted in several journal publications and conference papers, hands-on training of undergraduate and graduate students, direct project involvement of local school teachers through the NSF RET and GK-12 programs at UMaine, and collaboration with industries, as detailed in this report.

Findings:

Thus far, the UMaine team, under the leadership of Drs. Pereira da Cunha and Millard, has been able to (i) Prove the validity of using an LGS SH SAW delay line device as a liquid and biochemical-sensing platform using immunochemicals for differentiation of analytes. (ii) Establish the use of molecular padlock nucleic acid recognition in conjunction with rolling circle amplification as a diagnostic method for the detection of virus-derived RNA. (iii) Develop an automated test system with computer-controlled fluidics to permit uniform sample introduction, and acquisition of data from the SH SAW device. (iv) Fabricate a 96-well gold surface test platforms to permit higher throughput tests of procedures for surface modification, reagent immobilization, DNA amplification, and parallel fluorescence detection. (v) Establish techniques for immobilizing molecular detection reagents and carrying out rolling circle amplification and hyperbranching of DNA on polystyrene and gold surfaces with subsequent detection of both surface-associated and soluble product. (vi) Devise methods for detecting surface-associated circularized, ligated molecular padlock probes via enzyme-amplified assays. (vii) Develop simple procedures for repeated regeneration of nucleic acid detection layers on polystyrene and gold surfaces. (viii) Detect E. coli bacteria with SH-SAW LGS devices. (ix) Carry out fluorescence-based detection of pathogenic viruses in a microcapillary tube format. (x) Research and development of numerical modeling tools required for the analysis of the SH-SAW sensor platform have been successful. The tools have yielded valuable insight into device geometries that result in transducers with efficient excitation/detection of the SAW mode and the necessary conditions that result in increased device sensitivity to interfacial mass/stiffness loading, e.g. thickness of electrode metallization. This work is directed toward fabrication of improved SH-SAW device platforms. (xi) Investigation of SH-SAW sensitivity to viscoelastic changes of layering polymer during polymer crosslinking

as a model system for the influence of viscoelastic bioselective film variations in SH-SAW devices. These advancements have proved to be essential steps toward realizing the project goal of a fully integrated SH-SAW-based biosensor with unparallel sensitivity, selectivity and regeneration capabilities. The project successfully demonstrated the integration of the regeneration procedure in hermetically sealed microfluidic pathways fabricated directly on the SAW device, and the influence of different polymers and biochemical films on the SH-SAW device behavior. (xii) Determined the relationship between DNA molecules and measurable changes in SHSAW response due to variation in stiffness and viscoelasticity of the biomolecules at the surface.
These accomplishments demonstrated attainment of the overall project goals of investigating and characterizing LGX SH SAW devices for biosensors, representing important advancements in the combined research area of acoustic wave-based biosensors.

Training and Development:

The project provided both research and training to all the personnel involved in the activities. The PIs, Drs. Pereira da Cunha and Millard, managed the project through regular meetings with the research scientists, graduate and undergraduate students, and local high school teachers, collaborators, and industrial partners involved in the activities. The work performed contributed to the establishment of the required skills for working with RNA and DNA detection in conjunction with acoustic wave platforms and complementary fluorescent methods, rolling circle amplification of DNA sequences, and regeneration of sensing platforms.
The impact in the local society has been extremely relevant. At least three of the graduate students involved in this project were involved in GK-12 activities and together with the three NSF RET teachers brought results and specific knowledge to Maine high schools, helping motivating young students to the exciting world of science, engineering, and research. These activities are detailed later in this report. In addition to the expected graduate and undergraduate education on the related topics, the results of the work have been included as specific topics in the PIs' graduate and undergraduate courses. In particular the research activities have fostered the integration of electrical and bioengineering research topics in the multidisciplinary CHE 598 Microscale Bioengineering course and also in the ECE 663 Design and Fabrication of Surface Acoustic Wave Devices. As expected, the research work is being disseminated through student technical publications both in journals and conference papers.

Outreach Activities:

This project has disseminated the knowledge gained from work in University of Maine laboratories to the local, national, and international communities through a number of activities. The project included the participation of five graduate students, three of whom were directly involved in GK-12 activities. These graduate students have shared the results of the research work directly with teachers and students at local high schools in Maine. As a result of the outreach activities developed by the PIs and GK-12 graduate students, in close cooperation with high school teachers, the following activities took place: (1) high school students built and tested electronic sensor systems, and presented their projects in poster sessions and written papers on their respective topics (John Bapst high school, Scott Burgess, and also Brewer H.S., Mr. Libby development of sensor projects,); (2) chemistry students have been introduced to biosensor research (Bangor H.S., teacher: Helmut Kock); (3) students in social sciences classes have benefited from talks and discussion on the topic of bioterrorism (Bangor H.S., teacher: Mr. Smith); (4) high school students benefited from an 'Introduction to Sensors' class that featured 'hands on' student projects, such as the development of a heart rate monitor that uses a finger photodetector, and the creation of security robots capable of driving to the brightest point in the room, accepting or declining a security code made of 4 colors, and navigating through a maze. (Bangor H.S., teachers: Steve Godsoe, Don Erb); (5) Dana Gallimore, a female graduate student in this project, participated in 'Girl's Night Out,' a day long program where middle school girls were introduced to the wide range of research facilities at UMaine; and (5) the PIs and graduate students have routinely participated in discussions about school/career opportunities for female and minority students, and have given tours of the high tech laboratory facilities to groups of local high school students. Out of the five graduate students directly involved in this project, three are from the State of Maine.
In addition to the GK-12 activities, this SENSORS project has directly involved four local high school teachers in the research through the UMaine RET project, thus disseminating and fostering the students' interest in science, engineering, and in particular in the multidisciplinary acoustic microwave/bioengineering field. Examples of activities performed by these teachers directly in the PIs labs and in cooperation with the graduate students and personnel involved in the project are: (1) characterization of new piezoelectric material properties used in the development of the proposed biosensor technology for naturally occurring biotoxins. NSF REU supplements have also been used to train six students, from UMaine and other universities, exposing them to summer research experiences and encouraging them to further pursue graduate courses or to become involved in science and engineering. Examples of activities performed by these NSF REU students are development and preparation of piezoelectric crystals; investigation of the fluidics and chamber fabrication for the SH-SAW acoustic wave device; and the design of materials, techniques and devices for detecting nucleic acid targets.
Nationally and internationally, this project has served the community through the dissemination of knowledge and techniques through seven journal publications, and twelve conference presentations and papers, in addition to direct visits and contacts with collaborating companies and researchers from other institutes and universities.

Journal Publications

McCarthy, E.L., T. Egeler, L. Bickerstaff, M. Pereira da Cunha, P.J. Millard, "Detection and identification of IHN and ISA viruses by isothermal DNA amplification in microcapillary tubes", Anal. Bioanal. Chem., p. 1975, vol. 386, (2006). Published,

McCarthy, E.L., L.E. Bickerstaff, M.Pereira da Cunha, P.J. Millard., "Nucleic acid sensing by regenerable surface-associated isothermal rolling circle amplification", Biosens. Bioelectron., p. 1236, vol. 22, (2006). Published,

Millard, P.J., L.E. Bickerstaff, S.E. LaPatra, and C.H. Kim, "Detection of infectious hematopoietic necrosis virus and infectious salmon anemia virus by molecular padlock amplification", J. Fish Dis., p. 201, vol. 29, (2006). Published,

Berkenpas, E., P. Millard, and M. Pereira da Cunha, "Detection of Escherichia coli O157:H7 with Langasite Pure Shear Horizontal Surface Acoustic Wave Sensors", Biosens. Bioelectron, p. 2255, vol. 21, (2006). Published,

Berkenpas, E., S. Bitla, P. Millard, and M. Pereira da Cunha, "Pure Shear Horizontal SAW Biosensor on Langasite", IEEE Trans. IEEE Transactions on Ultrasonics, Ferroelectrics, and Frequency Control, p. 1403, vol. 51, (2004). Published,

T.B. Pollard, T.D. Kenny, J. F. Vetelino, M. Pereira da Cunha, "Pure SH-SAW Propagation, Transduction and Measurements on KNbO3", IEEE Trans. Ultrason. Ferroelec. Freq. Contr., p. 199, vol. 53, (2006). Published,

M. Pereira da Cunha, D. C. Malocha, D. R. Puccio, J. Thiele, and T. B. Pollard, "LGX Pure Shear Horizontal SAW for Liquid Sensor Applications", IEEE Sensors Journal, p. 554, vol. 03, (2003). Published,

Gallimore, D.; Millard, P.; Pereira da Cunha, M., "Monitoring Polymer Properties using Shear-Horizontal Surface Acoustic Waves", ACS Appl. Mater. Interfaces, p. 2382, vol. 1, (2009). Published,

Dana Y. Gallimore, Paul J. Millard, Mauricio Pereira da Cunha, "Investigating the SH-SAW Response to Biomolecular Layer Viscoelasticity and the Resulting Effects on Biosensor Sensitivity", Biosensors and Bioelectronics, p. , vol. , (2010). Submitted,

Dana Y. Gallimore, Paul J. Millard, Mauricio Pereira da Cunha, "Enzyme Method for Structural Alterations of Surface-Bound DNA", Nucleic Acids Research, p. , vol. , (2010). Submitted,

T.B. Pollard and M. Pereira da Cunha, "Improved SHSAW Transduction Efficiency using Gratings and Uniform Electrode Guiding", IEEE Transactions on Ultrasonics, Ferroelectrics, and Frequency Control, p. , vol. , (2010). Submitted,

Books or Other One-time Publications

D. Gallimore, P. Millard, and M. Pereira da Cunha, "Monitoring and Extracting Film Viscoelastic Properties using SH-SAW", (2007). Proceedings, Published
Editor(s): Institute of Electrical and Electronics Engineers
Collection: IEEE 2007 International Ultrasonics Symposium Proceedings
Bibliography: New York, NY, USA, Oct. 28-31, 2007, pp. 1385-1388

T. B. Pollard and M. Pereira da Cunha, "Pure Shear Horizontal SAW Network Model for Periodic Structures Including Bulk Scattering", (2006). Conference Proceedings, Published
Editor(s): Institute of Electrical and Electronics Engineers
Collection: IEEE 2006 International Ultrasonics Symposium Proceedings
Bibliography: Vancouver, Canada, Oct. 03-06, 2006, pp. 88-91.

E. Berkenpas, P. Millard, M. Pereira da Cunha, "Novel O157:H7 E. coli Detector Utilizing a Langasite Surface Acoustic Wave Device", (2005). Conference Proceedings, Published
Editor(s): Institute of Electrical and Electronics Engineers
Collection: IEEE International Sensor 2005 Conference
Bibliography: Irvine, CA, October 31 ? Nov. 03, 2005. (Corresponding author). (PAPER WAS AMONG THE 10 FINALISTS COMPETING FOR BEST STUDENT PAPER AWARD IN THE SYMPOSIUM).

E. Berkenpas, P. Millard, M. Pereira da Cunha, "A Langasite SH SAW O157:H7 E. coli Sensor", (2005). Conference Proceedings, Published
Editor(s): Institute of Electrical and Electronics Engineers
Collection: IEEE 2005 International Ultrasonics Symposium Proceedings
Bibliography: Rotterdam, the Netherlands, Sept. 18-21, 2005, pp. 54-57.

T. B. Pollard and M. Pereira da Cunha, "Improved Pure SHSAW Transduction Efficiency on LGS Using Finite Thickness Gratings", (2005). Conference Proceedings, Published
Editor(s): Institute of Electrical and Electronics Engineers
Collection: IEEE 2005 International Ultrasonics Symposium Proceedings
Bibliography: Rotterdam, the Netherlands, Sept. 18-21, 2005, pp. 1048-1051.

T. B. Pollard, Thomas D. Kenny, and M. Pereira da Cunha, "SH-SAW Transducer Analysis on Single Crystal KNbO3 for Liquid Sensors", (2004). Conference Proceedings, Published
Editor(s): Institute of Electrical and Electronics Engineers

Collection: IEEE 2004 International Ultrasonics Symposium Proceedings
Bibliography: Aug. 23-27, 2004, Montreal, CA, pp. 390-395. (THIS PAPER WAS AMONG THE 4 FINALISTS COMPETING FOR THE BEST STUDENT PAPER AWARD IN THE IEEE 2004 SYMPOSIUM).

T.D. Kenny, T.B. Pollard, E. Berkenpas, M. Pereira da Cunha, "FEM/BEM Impedance And Power Analysis For Measured LGS SH-SAW Devices", (2004). Conference Proceedings, Published
Editor(s): Institute of Electrical and Electronics Engineers

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Bibliography: Aug. 23-27, 2004, Montreal, CA, pp. 1371-1374. (THIS PAPER WAS ALSO AMONG THE 4 FINALISTS COMPETING FOR THE BEST STUDENT PAPER AWARD IN THE IEEE 2004 SYMPOSIUM)

E. Berkenpas, S. Bitla, P. Millard, and M. Pereira da Cunha, "Shear Horizontal SAW Biosensor on Langasite", (2003). Conference Proceedings, Published
Editor(s): Institute of Electrical and Electronics Engineers

Collection: Proceedings of the 2003 IEEE Sensors
Bibliography: Toronto, CA, Oct. 2003, pp. 661-664.

T. B. Pollard, J. F. Vetelino, and M. Pereira da Cunha, "Pure SH SAW On Single Crystal Knbo3 For Liquid Sensor Applications", (2003). Conference Proceedings, Published
Editor(s): Institute of Electrical and Electronics Engineers

Collection: IEEE 2003 International Ultrasonics Symposium Proceedings
Bibliography: October 5-8, Honolulu, Hawaii, 2003, pp. 1125-1128.

McCarthy, E.L., T. Egeler, L. Bickerstaff, M.Pereira Da Cunha, and P.J. Millard, "Rapid detection of IHNV by molecular padlock recognition and surface-associated isothermal amplification", (2005). Conference Proceedings, Published
Editor(s): SPIE
Collection: Proc. SPIE.
Bibliography: 5994X:1-7

Berkenpas, E., S. Bitla, P. Millard, and M. Pereira da Cunha, "LGS Shear Horizontal SAW Devices for Biosensor Applications", (2003). Conference Proceedings, Published
Editor(s): Institute of Electrical and Electronics Engineers

Collection: IEEE 2003 Itl.l Ultrasonics Symp. Proc.
Bibliography: October 5-8, Honolulu, Hawaii, pp. 1404-1407.

S. Bitla, E. Berkenpas, M. Pereira da Cunha and P. Millard, "Langasite Shear Horizontal Surface Acoustic Wave Devices as Biosensors", (2003). Conference Proceedings, Published
Editor(s): AIChE
Collection: Proceeding of the Annual Meeting of AIChE
Bibliography: San Francisco, CA, 2003

Dana Gallimore, Thomas Moonlight, and Mauricio Pereira da Cunha, "Extraction of Pt/Rh/ZrO2 High Temperature Elastic Constants", (2009). Symposium Proceedings, Published
Editor(s): IEEE Transactions
Collection: IEEE 2009 International Ultrasonics Symposium Proceedings
Bibliography: Rome, Italy, Sept. 20-23, 2009, pp. 2797-2800. (PAPER WAS AMONG THE 3 FINALISTS COMPETING FOR BEST STUDENT PAPER AWARD IN THE SYMPOSIUM)

T. B. Pollard, and M. Pereira da Cunha, "Improved SHSAW Transduction Efficiency using Gratings and Uniform Electrode Guiding", (2009). Symposium Proceedings, Published
Editor(s): IEEE Publications
Collection: IEEE 2009 International Ultrasonics Symposium Proceedings
Bibliography: Rome, Italy, Sept. 20-23, 2009, pp. 835-838.

Web/Internet Site

Other Specific Products

Product Type:

University of Maine Patent

Product Description:

M. Pereira da Cunha, LGX SH-SAW Liquid Sensor, U of Maine Patent US 7,053,522 B1, May 30, 2006.

Sharing Information:

Patent is available to the public.

Contributions

Contributions within Discipline:

This SENSOR project was multidisciplinary in nature, involving both electrical engineering and bioengineering areas of expertise in microwave acoustic device modeling, design, fabrication, and testing, and bioengineering techniques of DNA detection, amplification, and device regeneration. Integrating these activities towards the development of novel SH-SAW platforms capable of E. coli detection, with DNA amplification and surface regeneration resulted in significant contributions to an integrated area in the forefront of knowledge. The referred work was made possible through the diligent work of the UMaine team of researchers, graduate and undergraduate students involved, under the leadership of the PIs, Profs. Mauricio Pereira da Cunha, Associate Professor of Electrical and Computer Engineering, and Paul Millard, Associate Professor of Chemical and Biological Engineering.

Contributions to Other Disciplines:

The work performed has influenced the research of the investigators listed as contributors in this report, and has influenced a number of researchers in related work, as reflected by publications in the literature, that have described the use of the SH-SAW platform for microwave acoustic biosensors and other acoustic wave devices, both on langasite and quartz crystals. In addition the work developed has sparked the interest of the two companies listed as contributors in this report. The ability to monitor viscoelastic changes of polymer films with SH-SAW devices investigated in this project is of use to industry process control and development. Polymer films are currently employed for a wide variety of uses, including selective layers for chemical sensors, waveguiding layers for acoustic wave devices, and coating and membranes for industrial purposes, including the biomedical and paper industry. A SH-SAW method for measuring polymer properties could replace the current methods for monitoring these processes which are typically time-intensive, expensive, and generally inappropriate for in situ measurements. Particularly, the paper industry needs a method to monitor polymer coating during events such as polymerization and solvent evaporation. SAW technology can provide a real-time, non destructive, in situ, and inexpensive means for examining polymer layers. The understanding of the relationship between cross-linking of DNA molecules on surfaces proved to be important in improving the response of SHSAW sensors targeted in this research.

Contributions to Human Resource Development:

The results of the project have significantly contributed to human resource development in science, engineering and technology through: direct

training of five graduate students; specific training of a research associate; nine undergraduate students directly involved in the research work; and education of four RET teachers who directly participated in the project through summer work in the PIs labs, and who later translated the knowledge and excitement to their respective high schools.

Contributions to Resources for Research and Education:

The results from this Sensor project contributed significantly to the establishment of the Microwave Acoustic Laboratory, the Microwave Acoustic Material Laboratory, and the Bioengineering Laboratory at the University of Maine. The weekly meetings held by the PIs were extremely important to institutionally join the two areas of research together, namely Electrical and Biological Engineering, as well as to plan and implement joint educational efforts and student advising. Multiple student conference publications were finalists in 'Best Student Paper' competition in conferences such as IEEE Sensors and IEEE Ultrasonics Symposia, indicating the quality of the students' work.

Contributions Beyond Science and Engineering:

Sensitive, selective, reusable, robust, low-cost, and versatile sensing technology is required to protect the health of the general public. The need for significant improvements in sensor technology has assumed new urgency, in order to alert and protect U.S. citizens in the event of a bioterrorism attack. The ability to effectively detect microbial pathogens is also needed on a routine basis to ensure that our food and water supplies are safe from bacteria and viruses that can endanger human health. Numerous pathogens pose a threat to health as a result of poor sanitation, natural disasters, or other issues unrelated to terrorism. More broadly, improvements to sensor technology, which are occurring as a result of this SENSORS grant, can lead to advancements in such areas as agriculture, health care, and pollution avoidance and mitigation.

A potential portable detection system based on the successful findings of this project represents a new paradigm for environment viral and bacterial detection. The work developed so far has established this sensor technology as a viable alternative for the detection of E. coli and has attracted the attention and fostered the collaborations with the two industrial partners listed in this report.

Conference Proceedings

Categories for which nothing is reported:

Organizational Partners

Any Web/Internet Site

Any Conference

Investigating the SH-SAW Response to Biomolecular Layer Viscoelasticity and the Resulting Effects on Biosensor Sensitivity

*Dana Y. Gallimore, Paul J. Millard, Mauricio Pereira da Cunha**

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ABSTRACT - Shear-horizontal surface acoustic wave (SH-SAW) devices have shown potential as selective, quick response biosensors. However, these sensors currently do not display the sensitivity required for use in most in situ applications. Although SH-SAW biosensors are generally regarded as mass detectors, they are also sensitive to layer viscoelasticity. In this work, the effects of biomolecular layer structure and viscoelasticity on SH-SAW device response were investigated. Two configurations of double stranded DNA were created on a SH-SAW device surface facilitated by a restriction endonuclease. The SH-SAW response to the induced structural changes in the DNA layer was monitored in real-time, and the viscoelastic constants of the two effective layers were extracted from the measured data using the matrix method for SAWs. Results indicate that biomolecule structure, in the absence of changes in mass, can significantly affect SH-SAW device response, which relates directly to device sensitivity. The effective viscoelastic constants of the bound biomolecular layers were reported and discussed in terms of layer structure. The altered sensitivity achieved by structural modification of the bound biomolecules demonstrates the potential for SH-SAW biosensor improvement through optimization of the bioselective layer properties and target attachment strategies.

KEYWORDS - SH-SAW Biosensor, DNA Primary Structure, Biomolecule Layer Viscoelastic Constants

I. INTRODUCTION

The increasing threat of bioterrorism and pollution has generated a need for in situ biosensors that are portable, have quick response times, low power consumption, and allow for label-free detection. Common bioterrorism agents are becoming less expensive to create and disperse [1]. Pollution, particularly factory wastes and run-off from farming sites, also poses a health risk to humans through the contamination of food and water supplies. Rapid detection and identification of biotoxins and pathogens is necessary to avoid or reduce human exposure. In addition, speedy, selective detection of bacteria and viruses has important applications in the medical field, enabling quick diagnoses and treatment [2].

Culturing remains the most common method for detecting pathogenic bacteria [3]. Although this method is selective, culturing must be done in a laboratory, and bacterial detection can take up to several weeks depending on the type being tested. The classical method for detecting viral contamination by inoculation of cell cultures is equally costly and time consuming, but can also be impossible or prohibitively inefficient depending on the virus to be sensed [4]. Other more rapid detection methods exist, including microscopy, flow cytometry, and immunoassays for bacteria [3] and enzyme immunoassays, reverse transcriptase polymerase chain reaction, and solid-phase immune electron microscopy for viruses [4], but these methods are typically not suitable for in-field testing and can be expensive due to the need for specialized equipment and trained technicians.

An alternative solution for rapid, in-field biosensing is the shear horizontal surface acoustic wave (SH-SAW) platform. SH-SAW devices are small, easy to integrate with electronic systems, capable of quick response times, and suitable for use in sensor arrays. SH-SAW devices are sensitive to mass addition and viscoelastic changes occurring on the surface regardless of the source. A selective

layer is therefore required to impart specificity to the sensor. This layer is often made of antibodies, which bind selectively to the target bacteria or virus, or oligonucleotides (oligos) which attach only to complementary oligo sequences [5, 6].

Acoustic wave devices have frequently been used for proof-of-concept biosensors. Bulk acoustic wave (BAW) and transverse shear mode devices have been used to measure the kinetics of protein, lipid, and cell binding to surfaces [7-9], as well as cell adhesion and spreading [10]. Bulk acoustic wave measurements of DNA and PNA binding have been reported [11, 12]. An acoustic plate mode biosensor is described that can measure antibody interactions in liquids [13]. SH-SAW devices have been used to selectively sense bacteria using antibody layers [14, 15]. However, there remains a need for improving acoustic wave target sensitivity in order to implement these devices as commercial biosensors. The current limit of detection for SH-SAW sensors is ~5ng/mm^2 [16-18] which corresponds to ~5000 bacteria/mm^2 or ~5x10^{10} single stranded, 200 base DNA molecules/mm^2. Harmful levels of toxigenic bacteria and viruses vary widely, but some, such as *Campylobacter jejuni*, *Cryptosporidium parvum*, and *Shigella spp*, can cause infection in humans with fewer than 500 organisms [19]. For medical diagnostic purposes, DNA must be detected at concentrations of 10^{-18}M [20], or 600 molecules/mL. Therefore, with the current detection limits, target samples would have to be amplified by either culturing or PCR in order to produce quantities suitable for SH-SAW detection, an impractical, time consuming step for in-field applications. Various methods have been explored to increase acoustic wave device sensitivity, including optimization of electrode and delay path design and the addition of thin guiding layers on the device surface [21]. Techniques to improve the target binding efficiency to surfaces have been investigated considering probe attachment strategies [22, 23], the use of multilayers [24], and DNA packing density [25]. Additionally, the mechanical properties of the selective layer (including mass, thickness, and viscoelasticity) can affect the SH-SAW response and thus provide another avenue for sensitivity optimization.

The SH-SAW device is known to be sensitive to changes in the surface mass and viscoelasticity [26-37]. By combining these two sensing phenomena in a constructive way, the SH-SAW response to a

specific target may be increased. For example, if the mass addition of a target molecule or organism to a bioselective layer causes the acoustic wave phase velocity to decrease, while the simultaneous viscoelastic response causes an increase in phase velocity, the sensitivity of the device will suffer due to the competing responses. However, if the bioselective layer was altered so that the viscoelastic effects of added target molecules also cause a decrease in phase velocity, the total SH-SAW response would be larger, resulting in increased sensitivity to the target.

Some researchers have tested multiple selective layer conformations in order to improve acoustic wave biosensor sensitivity. Howe and coworkers measured the SH-SAW response of a device coated with selective antibodies subject to added bacteria and a device non-specifically coated with bacteria subject to the addition of selective antibodies [38]. The configuration with bacteria non-specifically bound to the device surface was shown to have a more sensitive response than the more conventional configuration. Different methods for surface biofunctionalization of bovine serum albumin (BSA) (adsorption, cyano transfer, photoimmobilization, and use of an intermediate dextran layer) were shown to effect the response of pseudo SAWs, waves with strong horizontal polarization [39]. Sensitivity variations were partly attributed to target binding efficiency and also the rigidity of the surface/target bond. Love mode devices, which use a guiding layer to trap an acoustic wave at the surface, were shown to be sensitive to the length and degree of bending of dsDNA [40]. In this work, molecular layer viscosity was determined using macromolecule viscosity theory and related to the SH-SAW attenuation vs. velocity response.

Beyond monitoring target binding and device sensitivity, work has been done to characterize the viscoelastic properties of surface bound biomolecules. Quartz crystal microbalances with dissipation monitoring, in conjunction with surface plasmon resonance measurements, have been used to determine the viscoelasticity of biomolecular layers [22, 41, 42]. The described BAW measurements can be used to relate the sensitivity of BAW propagation to viscoelastic parameters, but do not describe the behavior of SH-SAWs, which are guided at the crystal surface. Weiss et. al. used perturbation theory to estimate the elastic constants of crosslinked and uncrosslinked antibody films on pseudo SAW devices [43].

Saha and coworkers compared SH-SAW responses to glycerol solutions of different viscosity with SH-SAW responses to antibody layers in order to estimate the antibody layer viscosity [44]. Further research relating the viscoelasticity of bioselective layers to the resulting SH-SAW device response is needed in order to optimize selective layers and improve sensitivity to the target analyte.

In this work, surface-bound DNA was used to compare two probe/target attachment scenarios, one where the DNA crosslinks the surface and another where it does not. Use of the dsDNA modifying enzyme EcoRI allowed the transformation of one surface structure into the other while maintaining the surface mass. By comparing the SH-SAW response to the different layers, the structural effects on device sensitivity was determined. The matrix method for SAWs was used to extract the viscoelastic constants of the biomolecular layers in order to determine the contribution of viscoelastic effects to the overall device response. Section II describes the SH-SAW platform, the biomolecular layers, and the method for viscoelastic constant extraction. Section III describes the materials used, the SH-SAW devices and test set-up, as well as the layer formation and measurement procedures. Measurement results and the extracted constants in the context of biomolecular layer conformation are discussed in Section IV, and Section V concludes the paper.

II. BACKGROUND

II.a. SHEAR HORIZONTAL SURFACE ACOUSTIC WAVEs (SH-SAW)

Surface acoustic waves are acoustic waves guided by a substrate surface, and are therefore particularly sensitive to activity at this boundary. When using SAWs in liquid and highly viscous media, SH-SAW modes are advantageous because of their diminished attenuation when compared to the generalized SAW, which quickly leaks energy into surrounding viscous media [45-47]. Unlike the generalized SAW and the Rayleigh wave, and in general any acoustic wave with significant particle displacement normal to the surface, the SH-SAW mode has particle displacement parallel to the device

surface, and thus does not dissipate acoustic energy significantly into viscous media. Furthermore, the small size, quick response time, potential for use in sensor arrays, and portability of SH-SAW devices render them attractive for use in a variety of sensing applications.

This work employed SH-SAW delay line devices, which consist of two interdigitated transducers (IDTs) separated by a metalized delay path. The biomolecular layers were applied to the device surface, affecting the group velocity (v_g) and attenuation (α) of the shear horizontal acoustic waves that are excited and detected by the IDTs.

II.b Biomolecular Layers

Two conformations of a dsDNA layer with equal mass were formed and measured on the SH-SAW device surface. To attach the dsDNA layers, NeutrAvidin (New England Biolabs) was first non-specifically adsorbed onto the gold delay path. A solution of BSA was then added, which adsorbs to gold and inhibits non-specific binding of other biomolecules. A dsDNA layer, structurally "bent" and consisting of two, 96 base pair, complementary sequences biotinylated at each 5' end, was attached to the surface by NeutrAvidin-biotin binding. The expected surface-crosslinking configuration of the DNA molecules is shown in Figure 1a. EcoRI, a type II restriction endonuclease, was then added in order to modify the structure of the DNA layer. EcoRI cleaves dsDNA at a specific restriction site, the palindromic sequence GAATTC. This sequence was incorporated into the middle of each bent dsDNA molecule. The addition of the endonuclease should cause the doubly-bound surface DNA to be cleaved in half, yielding two singly-bound fragments and effectively un-crosslinking surface. The expected layer configuration after EcoRI cleavage is shown in Figure 1c. The formation of the initial crosslinking DNA layer was expected to be imperfect, with some sequences binding by a single 5′ end due to the availability of NeutrAvidin binding sites. When such sequences are cleaved by EcoRI, mass will be removed from the surface. An 80°C water pre-rinse was therefore applied before digestion with the endonuclease, denaturing all surface DNA. Sequences bound by only one end lose their complementary strand into solution, while reintroduction of the appropriate buffer allows other

sequences to re-hybrize. EcoRI does not digest single stranded DNA, and no particles should be removed from the surface during cleavage after pre-rinsing. EcoRI is also known to cleave dsDNA non-specifically when present in sufficiently high concentrations. Such non-specific activity of the endonuclease would also result in mass removal from the SH-SAW device surface. Verification that the EcoRI concentration chosen does not cause non-specific cleavage of the surface DNA was required. The surface modification procedure and the behavior of EcoRI on these surfaces are described in detail in a previous work [48].

Un-crosslinking the dsDNA surface was expected to change the viscoelastic properties of the layer. No DNA was been removed from the SH-SAW device surface, therefore the surface mass remains constant. By measuring the SH-SAW response before and after DNA cleavage, it was possible to compare the SH-SAW sensitivity to the two forms of surface DNA. The induced viscoelastic changes of the DNA layers could then be extracted using the matrix method for SH-SAWs (described in Section IId).

II.c Biomolecular Layer Properties

The biomolecular layers used in this work were formed using proteins (NeutrAvidin and BSA) as well as nucleic acids (dsDNA). In order to extract the viscoelastic properties of these molecules using the matrix method for SAWs, the effective biomolecular layer thickness and density must be known.

NeutrAvidin is a 60kDa protein with dimensions slightly smaller than avidin (4.2nm × 7.9nm ×7.2nM) [49]. Fluorescence lifetime imaging microscopy measurements of adsorbed NeutrAvidin surfaces have shown an effective thickness of the layer of 5.8nm [49]. BSA is a 66.4 kDa protein which, under the pH conditions here (pH 7.5), folds into a heart-shaped conformation with edge lengths of 8.0nm and thickness of 3.0nm [50, 51]. BSA attached to the gold surface can therefore achieve heights between 3nm and 6.9nm, resulting in a mean thickness of ~5nm. The effective thickness of the combined NeutrAvidin/BSA layer was assumed to be the average of the known NeutrAvidin layer thickness (5.8nm) and the estimated BSA height (5nm), resulting in a value of 5.4nm. All buffer

hydrated proteins with a molecular weight greater than 20kDa [52] have been found to have the same density, approximately 1.40 g/cm^3 [53]. The combined NeutrAvidin and BSA protein layer was therefore assumed to have an effective density of hydrated protein, 1.40 g/cm^3.

Double-stranded DNA has a persistence length of approximately 150bps [54], meaning that a random 96 bp sequence would exist as a rigid, straight molecule. A bend in the dsDNA sequence was created by incorporating 5, 6-unit adenine tracts, creating a dsDNA molecule bent at a 112.5° angle [55]. This allowed the dsDNA sequence to bind to the surface at both ends by NeutrAvidin-biotin linkages. Using the known length of DNA per base pair (3.3 Å) and the hydrated diameter of dsDNA, 24 Å [56], along with the 112.5° bend, the dsDNA was expected to form a triangular structure with a base length of 27.2nm and height of approximately 10nm, which was considered the effective thickness of the layer. Based on the volume of each DNA molecule, the DNA layer would contain mostly buffer (~90%) within the 10nm thickness, and the effective density of the layer was assumed to be the same as the surrounding solvent. The thickness of the DNA layer was expected to increase after cleavage with EcoRI as the severed strands are free to stretch farther out from the surface and into solution. Double stranded DNA, bound to metalized surfaces by the 5' end, has been shown to orient at an angle of approximately 45° from the surface normal [57, 58]. Using the lenth of 48 base pair dsDNA and the known angle, the thickness for the cleaved DNA layer was expected to be 11.2 nm. The effective density was still assumed to be that of the solvent buffer.

IId. VISCOELASTIC CONSTANT EXTRACTION

When a viscoelastic material is deformed by a periodically varying strain, some of the energy is stored and recovered, due to the elastic property of the material, and some of the energy is dissipated as heat, due to the material viscosity [59]. Under linear, sinusoidal deformation the mechanical properties of a viscoelastic material can be specified using abbreviated subscript notation [37] by two 6×6 matrices, $\mathbf{C}$ and $\boldsymbol{\eta}$, where $\mathbf{C}$ contains the elastic constants and $\boldsymbol{\eta}$ the viscosity constants (in units of

pascals and pascal·seconds, respectively). These two matrices are combined to form the complex viscoelastic constant matrix, $\mathbf{C} + j\omega\boldsymbol{\eta}$.

The biomolecular layers used in this work were assumed to be lossy and isotropic. The viscoelastic properties of such a material can be described by four independent constants: two elastic constants, c_{11} and c_{44}, and two viscosity constants, η_{11} and η_{44}. The use of the pure SH-SAW mode allows the independent determination of c_{44} and η_{44} (known together as the complex shear modulus), since there is only one component of particle displacement, which is parallel to the surface and perpendicular to the direction of wave propagation [45-47].

The viscoelastic constants were extracted using the matrix method for SAWs [60] oriented along crystal symmetry 4 [61]. Wave propagation was arbitrarily chosen to be in the x direction with the semi-infinite substrate surface lying in the xy plane. A vector $\boldsymbol{\tau} = [\mathbf{T}\ D_z\ \mathbf{v}\ j\omega\varphi]$ was defined containing the variables that must be continuous across the substrate/multilayer interfaces; ω is the radian frequency, $\mathbf{T}$ contains the three normal stress components, D_z is the normal electric displacement, $\mathbf{v}$ contains the three particle velocity components, and $j\omega\varphi$ is the time derivative of electric potential. The acoustoelectric equations (Hooke's Law, the electrical equation of state, Newton's second law, and Gauss's Law) were then reduced to a first-order, matrix, ordinary differential equation (ODE): $\frac{d\boldsymbol{\tau}}{dz} = j\omega\mathbf{A}\boldsymbol{\tau}$ (1). The system matrix, $\mathbf{A}$, is a function of the material constants of a single layer rotated to the desired coordinate system and the phase velocity and attenuation of the SAW. The solution to the ODE can be written as: $\boldsymbol{\tau}(z+h)=\exp(j\omega\mathbf{A}h)$ (2). Equation 2 is a transfer matrix that maps $\boldsymbol{\tau}$ across a distance h in the z direction. The transfer matrices of the substrate and each layer were sequentially multiplied, thus automatically satisfying the boundary conditions between the different layers. The problem were be solved by fulfilling the boundary conditions at the top, liquid-loaded surface and imposing that the fields decay at the semi-infinite substrate. The solution to the boundary condition problem can not be found analytically, so numerical methods must be applied. System matrices were formulated using the known constants of the quartz substrate [62] and metallic (gold and chromium)

delay path layers [63, 64], the effective biomolecular film density and thickness, and the measured SH-SAW velocity and attenuation. Guessed values for c_{44} and η_{44} were entered into the matrix method formulation and the resulting v_p and α values were calculated at two frequencies near the measured center frequency by minimizing the boundary condition function. The group velocity was determined from: $v_g = \frac{\partial \omega}{\partial k}$ (3), where k is the wavenumber, and the α value is determined at the center frequency. The guessed viscoelastic constant values were then varied until the error between the measured and calculated SH-SAW parameters was minimized.

When extracting the viscoelastic constants for the biomolecular layers the effective thicknesses and densities described in Section II.c were incorporated into the matrix method for SAWs along with the SH-SAW v_g and α measured after the addition of each layer. This method yielded viscoelastic constants for the combined NeutrAvidin and BSA layer, the bent dsDNA layer, and the cleaved dsDNA layer.

III. MATERIALS AND METHODS

III.a Materials

NeutrAvidin, EcoRI endonuclease, and NEbuffer EcoRI (NEBuffer) were supplied by New England BioLabs (Beverly, MA). BSA was obtained from Sigma-Aldrich (St. Louis, MO). Quant-iT OliGreen ssDNA reagent was purchased from Invitrogen (Carlsbad, CA). Complementary single stranded oligos, biotinylated on the 5' end, were created by Integrated DNA Technologies, Inc. (Coralville, IA). Sequences are listed in Table 1; the EcoRI restriction site is underlined. Nanopure water was used in all buffers and aqueous solutions (≥18MΩ, <3ppbTOC).

III.b Test Set Up

The SH-SAW delay lines were designed with two split-finger IDTs (eight electrodes per wavelength) separated by a gold, 7200μm metalized propagation path on ST-90° quartz, Euler angles

(0°, 132.75°, 90°). The devices were fabricated in the University of Maine clean room facility using optical photolithography, lift-off patterning techniques, and thin film e-beam technology to deposit the 200nm gold layers with 15nm chromium adhesion layers. Each split finger IDT had 320 electrodes, an acoustic aperture of 36 wavelengths, electrode width of 4μm, and a mark to space ratio of 1:1. The IDT center-to-center distance was 12.5mm.

Polydimethylsiloxane (PDMS) chambers were attached to the patterned wafers to facilitate liquid delivery to the delay region without shorting the IDTs. Guides were patterned onto the wafers by photolithography of SU-8 polymer, allowing the chambers to be placed on the SH-SAW device with proper alignment relative to the device features. The PDMS chambers were formed from an SU-8 mold, and input and output ports were cut using a precision hole punch (Technical Innovations, Inc. Angleton, TX). To permanently attach the chambers to the patterned wafers, the PDMS chambers were treated with O_2 plasma, dipped in methanol, and applied to the quartz wafers. The attached components were allowed to dry overnight followed by a post-bake at 65°C for 4 hours.

A microfluidic system was used to transport the biomolecular components in solution to the chamber as well as to remove and collect fluid from the chamber output. Solutions were added to the system using an open 5mL syringe. Teflon tubing (O.D. 0.0625 in, I.D. XXXX in) was used to direct fluids to and from the SH-SAW device. Fluid flow rate was controlled by a micrometering valve between the syringe and chamber input. Tubing and valves were obtained from Upchurch Scientific (Oak Harbor, WA). The output tubing was connected to a vacuum line to draw analytes through the system, and components exiting the device were collected in replaceable microcentrifuge tubes.

The PDMS chamber design allows components to be applied to two devices at a time, and a testing system was developed to measure the S_{21} transmission response of the two delay lines independently while controlling the temperature. A switching circuit was designed using two ADG904 four-way multiplexors (Analog Devices, Norwood MA, USA) which allowed measurement of up to four delay lines sequentially. The device wafer was attached with cyanoacrylate cement to a PC board that connects to the switching circuit using pin connectors. This fixture allowed the delay line devices to be

easily removed from the circuit. The electrical connections from the delay lines to the PC board were made with 4 mil platinum wires. A Peltier device, Tellurex model CZ1 (Tellurex, Travers City MI, USA), along with an aluminum heat block and heat sink, was used to keep the delay lines at 25°C±0.1°C. Temperature was monitored and controlled with a resistive temperature device (RTD) mounted within the heat block and input into an autotune controller. The RTD and controller were acquired from OMEGA Engineering (Stamford CT, USA). Two 50 Ω coaxial cables connect the switching circuit to an Agilent 4396B network analyzer (Agilent Technologies, Santa Clara CA, USA). The complete SH-SAW testing system is shown in Figure 2. LABVIEW (National Instruments, Austin TX, USA) was used to control the switching circuit, set the device temperature, and collect data from the network analyzer.

III.c Procedures

III.c.1 dsDNA Layer Formation

Double-stranded DNA was attached to the SH-SAW surface using a procedure adapted from Tsortos et al. [40]. Nanopure water was first applied to the system to create a baseline for the SH-SAW measurements, set the flowrate to ~50µl/min, and remove any air bubbles from the PDMS chamber. NeutrAvidin (200µL, 100µg/ml) was added in NeutrAvidin buffer (50mM TRIS, 10mM $MgCl_2$, 10mM KCl, pH 7.5), and the fluid flow was stopped at the end of the addition for 30 min. to approximate the conditions used in Gallimore et. al. [48]. This allowed the NeutrAvidin to adsorb non-specifically onto the gold surface. BSA blocking buffer was prepared with 0.5% BSA in saline sodium citrate (SSC) buffer (150mM NaCl, 15mM sodium citrate, pH 7.5) and added to block the remaining bare gold surfaces. The biotinylated dsDNA was then added (200nM in SSC). Flow was stopped for 20 min, followed by the addition of 500µL SSC. All component additions were preceded by 200µL of the solvent buffer. To denature surface DNA bound by only one end, 3mL of 80°C water was flushed through the system, followed by SSC buffer to re-hybridize the remaining strands.

III.c.2 Fluorescence Measurements

In order to verify that EcoRI behaves as expected on the SH-SAW surface, several concentrations of EcoRI were applied to the surface dsDNA layer sequentially, and the amount of DNA removed was measured using fluorescence. The input and output tubing was rinsed with water before the 80°C water rinse described in Section III.c.1 to remove any residual components from the system. The first 1mL of the 80°C water pre-rinse and the following rinse with SSC were collected as separate samples at the output and reserved. EcoRI was then applied to the surface in increasing concentrations (0.001U/μL, 0.01U/μL, 0.1U/μL, and 1U/μL) all in SSC buffer with 0.5mM EDTA (SSC+EDTA). Each concentration was followed by the addition of 300μL NEBuffer and an 80°C water rinse. The EcoRI and NEBuffer samples were collected together at the output for each EcoRI concentration, and the subsequent water rinses were collected separately. All samples were immediately heated to 80°C in a hot water bath to deactivate any remaining EcoRI. Samples were then transferred to a multi-well plate to which a solution of OliGreen DNA reagentwas added. The resulting fluorescence was measured using a Synergy II Microplate Reader (BioTek, Winooski, VT) with excitation and emission filters of 485±12nm and 535±12nm, respectively. The mass of the DNA in each sample was determined by comparing the measured sample fluorescence with the fluorescence of DNA standard dilutions. Samples expected to be double-stranded were compared to serial dilutions of a ds calf-thymus DNA sample, and single stranded samples were compared to serial dilutions of a single stranded, 21 basepair sequence. The starting concentrations of the standards were determined from spectrophotometer measurements (Beckman Coulter model DU, Fullerton CA, USA).

III.c.3 Buffer Measurements

SH-SAW measurements of the different buffers were performed in order to determine their viscosity. NeutrAvidin buffer, SSC, and NEBuffer (1mL each) were applied to a clean SH-SAW device in

succession, separated by 1mL of nanopure water. Network analyzer S_{21} transmission measurements were taken every 30 s.

III.c.4 SH-SAW Measurements of DNA Configuration

Measurements of the SH-SAW response during layer formation and DNA cleavage were performed after the optimum EcoRI concentration was determined from the fluorescence measurements in Section IIIc2. The dsDNA was bound at both 5′ ends to the device surface using the procedure in Section III.c.1. The surface was then treated with EcoRI (0.001U/μL) in SSC+EDTA followed by 300μL NEBuffer and 2mL 80°C water in order to eliminate the effects of non-specifically bound DNA removal from further measurements. A new baseline was established by adding 400μL SSC+EDTA. As a negative control, 300μL NEBuffer was added followed by 400μL SSC+EDTA. The test concentration of EcoRI (0.1U/μL in 100μL) was then applied, followed by 300μL NEBuffer to cleave the surface DNA. SSC+EDTA buffer was reapplied for comparison with the previous baseline measurement. Network analyzer measurements of the S_{21} transmission response were taken every 30 s throughout the entire process.

Network analyzer measurements taken during the buffer assay and the surface preparation and cleavage were time-gated in MatLab to reduce the effects of reflections and unwanted acoustic modes. Values for the SH-SAW v_g and α throughout the test time were determined from the time-gated data. These values were shifted so that the initial baseline measurements (with water only) matched the expected values of a water-loaded device. The resulting v_g and α at specific points in the buffer assay and biomolecule procedure were then used, along with the assumptions for density and thickness described in Section IIc, to extract the viscoelastic constants c_{44} and η_{44} of the buffers and biomolecular layers using the matrix method for SAWs.

IV. RESULTS AND DISCUSSION

Fluorescent measurements of the components exiting the SH-SAW fluidic chamber were used to verify that specific EcoRI cleavage of the surface DNA layer was acheived. Increasing concentrations of EcoRI were applied sequentially to the DNA-coated SH-SAW device in SSC+EDTA and NEBuffer, followed by an 80°C water rinse. The resulting fluorescence measurements are shown in Figure 3. Measured fluorescence units were converted to ng of DNA by comparison with DNA standard dilutions. At the lowest EcoRI concentration tested (0.001U/μL) significant amounts of DNA were removed from the surface during the NEBuffer rinse. Previous tests in wells [48] indicate that at this concentration little cleavage occurs, and that the DNA removed in this step was likely bound non-specifically to the surface and chamber walls and displaced by the added endonuclease. Buffer from successive EcoRI additions (0.01U/μL and 0.1U/μL) indicated DNA levels at or below the level in the SSC+EDTA control rinse, suggesting that no mass removal occurred at these concentrations. When the EcoRI concentration was increased to 1U/μL, a nearly 200% increase in DNA concentration from the control rinse was measured. This signifies non-specific cleavage of the surface DNA, which results in DNA fragments released into solution. During the 80°C water pre-rinse treatment, approximately 57ng of DNA was removed from the surface. The comparatively large amount of DNA in this rinse can be attributed to the DNA bound to the surface by only one 5′ end which, when denatured by hot water, releases the non-bound single strand into solution. Some non-specifically bound DNA may also contribute to the concentration of DNA in this rinse. Subsequent water rinses show increasing DNA levels relative to EcoRI concentration, indicative of an increasing number of DNA cleavages. Only DNA bound to the surface by both 5' ends was available for EcoRI digestion after the initial 80°C water pre-rinse, so further water rinses would not remove any DNA from the surface unless the strands had been cut by EcoRI. The DNA measurements of the buffer and water samples indicated that an EcoRI concentration of 0.1U/μL was high enough to cleave a significant number of surface DNA sequences, but was still below the concentration where non-specific cleavage begins. During the SH-SAW measurements to follow, DNA surfaces were treated with a low concentration of EcoRI (0.001U/μL)

before the test concentration was applied. This was intended to remove interference from non-specifically bound particles during DNA cleavage and the associated SH-SAW measurements.

To determine the buffer viscoelastic properties, the SH-SAW response to loading by each buffer was measured. The results are shown in Figure 4. As is expected of a viscous fluid, v_g remained constant regardless of the loading buffer. All buffers therefore have a c_{44} value of zero. In contrast, the α values increased from the water baseline when each buffers was applied, indicating increased viscosity. Recovery of the response with the addition of water was consistent after the first buffer had been introduced. The density of each buffer was calculated based on the buffer composition. The formula weight and concentration of each buffer component was used to calculate the density of the components in solution. These densities were then added to the density of water to determine the total buffer density. The viscosity (η_{44}) values of each buffer were then extracted from the measured change in α and the calculated buffer density using the matrix method for SAWs. Buffer viscosities and densities are listed in Table 2 with the water values included for comparison. These values were necessary to extract the viscoelastic constants of the biomolecular layers based on measurements performed in buffer.

After determining the necessary steps for specific cleavage of the surface-bound DNA, SH-SAW measurements were taken throughout the surface preparation and cleavage process to determine the acoustic wave response to a change in biomolecular structure. A typical SH-SAW response during NeutrAvidin, BSA, and dsDNA addition is shown in Figure 4. A measurable change in v_g and α was seen during each addition. The addition of the dsDNA caused an increase in v_g, which is contrary to the response expected if considering only the particle mass. The mean and standard deviation of the measured v_g and α values at key points during layer preparation and cleavage are listed in Table 3. These values correspond to the following layers in SSC: the combined NeutrAvidin and BSA layer, the initially formed dsDNA layer, the uncleaved dsDNA layer, and the cleaved dsDNA layer. The uncleaved and cleaved dsDNA values were taken after the layers had been pre-rinsed with 80°C water and pre-treated with 0.001 U/μL EcoRI. Mean and standard deviation values of v_g and α for the

NeutrAvidin/BSA and dsDNA layer additions were determined from 5 data sets, while the remaining values were taken from two complete tests with EcoRI. To remove the effects of the IDTs and chamber walls from the measured SH-SAW response, all data was shifted so the baseline, water-only response matched the expected values for a water loaded device. The SH-SAW response followed repeatable trends due to the sequence of applied biomolecules.

The real-time SH-SAW response to dsDNA cleavage was determined by subtracting the control NEBuffer rinse response from the response from the test NEBuffer rinse (which initiates cleavage of the surface DNA by EcoRI). The mean cleavage response of two data sets is shown in Figure 6. Addition of the endonuclease in SSC+EDTA buffer caused a 1 m/s decrease in v_g and no discernable change in α. The decreased velocity could be attributed to increased surface mass, increased layer viscosity, or a combination of the two. No dsDNA cleavage should occur at this point due to the solvent buffer, so the acoustic wave response reflects the association of EcoRI to the surface. With the addition of NEBuffer, a mean decrease in v_g of 2.9m/s from the SSC+EDTA, pre-EcoRI baseline occurred. The α response, even after subtracting the control NEBuffer response, contained a shift associated with the changing buffer. The NEBuffer, which is more viscous than the SSC buffer, may affect the density and viscosity properties of the EcoRI associated layer. Such effects are not predicted by the control NEBuffer measurements taken before EcoRI is added. The shift may also reflect an imperfection in the control; the velocity data shows no buffer shift simply because the velocity is not affected by buffer viscosity, as shown in Figure 4. The final α response, determined after the SSC+EDTA was reapplied, was an increase of 0.2dB/λ from the SSC+EDTA baseline. From the previously described removed DNA measurements, it was inferred that the measured response during EcoRI digestion was not caused by the removal of DNA from the surface. Furthermore, EcoRI dissociates from DNA and is released into solution after catalysis [65]. Once the DNA layer has been cleaved, the EcoRI molecules were expected to be released from the surface, and so not affect the surface mass. The addition of NEBuffer caused the attached EcoRI to cleave the surface DNA, as verified by the previous DNA measurements. The resulting SH-SAW response showed an immediate decrease in v_g and a small increase in α. Based on

the rapid change in velocity, most of the surface digestion was completed within 1 min. after NEBuffer introduction. The release of the endonuclease from the surface was likely gradual, as indicated by the slopes of the v_g and α responses during NEBuffer addition.

The sensitivity of the SH-SAW device to the two configurations of DNA was determined by comparing the measured v_g and α of the device layered with DNA to the response of the device layered with NeutrAvidin and BSA only. The doubly-bound surface DNA layer (after pre-rinsing with hot water and pre-treatment with 0.001U/μl EcoRI) caused a change in v_g of 6.4m/s and a change in α of 6.1mdB/λ relative to the NeutrAvidin and BSA layer measurements. After cleaving the DNA layer, the measured v_g and α varied from the NeutrAvidin and BSA measurements by 0.5m/s and 6.3mdB/λ, respectively. The uncleaved layer produced a v_g response 12 times that of the cleaved layer, indicating a significant improvement in dsDNA sensitivity was achieved by surface crosslinking. The attenuation response of the cleaved layer was slightly larger than the uncleaved response, but both layers caused a change in α of about 15%. With the NeutrAvidin binding strategy used here, SH-SAW device sensitivity to dsDNA was significantly improved by utilizing a protein crosslinking dsDNA configuration as opposed to a non-crosslinking configuration.

Using the data shown in Table 3, along with the assumptions for layer thickness and density described in Section IIc, the biomolecular layer viscoelastic constants were extracted using the matrix method for SAWs. The c_{44} and η_{44} values are included in Table 3. Cleaving the surface dsDNA caused a 46% increase in c_{44} and a 130% increase in η_{44}. The large increase in layer viscosity brought the uncrosslinked layer η_{44} value closer to that of SSC buffer which, after surface cleavage, made up a larger portion of the layer. The increase in c_{44} was more surprising, as removing physical crosslinks has been shown to decrease the elastic modulus of polymeric materials (such as DNA) [66, 67]. Unlike the extracted viscosity constant under these conditions, extraction of c_{44} was effected by slight changes in layer thickness. The effective layer thickness was not measured, but assumed based previous works with dsDNA. If the cleaved DNA molecules oriented themselves to their maximum possible height, 15.8nm, the extracted c_{44} value would not change from the uncleaved value. Further measurements of

the layer thickness are required in order to more accurately determine the effects of DNA layer cleavage on the layer elasticity. However, the extracted η_{44} was largely independent of layer thickness within the possible limits of this experiment, and the measured change in v_g was attributed almost entirely to the altered layer viscosity.

V. CONCLUSIONS

Two forms of surface dsDNA with identical mass but different crosslinking characteristics were created on a SH-SAW device surface, facilitated by the activity of EcoRI endonuclease. Specific activity of EcoRI was verified using fluorescence-based DNA measurements of components removed from the SH-SAW surface. The SH-SAW response to biomolecule addition and modification was measured in real-time, and the viscoelastic constants of the biomolecular layers were extracted using the matrix method for SAWs. Results indicated that a surface crosslinking dsDNA layer causes a larger SH-SAW response than the more conventional, non-crosslinking dsDNA layer for the protein attachment system used. Cleaving the surface DNA layer with EcoRI caused a 130% increase in layer viscosity, which relates to a 2.9 m/s decrease in measured v_g. Results demonstrate the importance of layer viscoelasticity on SH-SAW device response and the associated biosensor sensitivity. Surface configuration of bioselective layer/target systems can enhance or impair the device sensitivity predicted by mass addition alone. The biomolecular layers here showed improved sensitivity when a surface crosslinking strategy was implemented. This is directly applicable to current DNA-based SH-SAW sensors, and the viscoelastic concepts can be applied to any acoustic wave biosensor, potentially allowing improved response of current biosensor platforms.

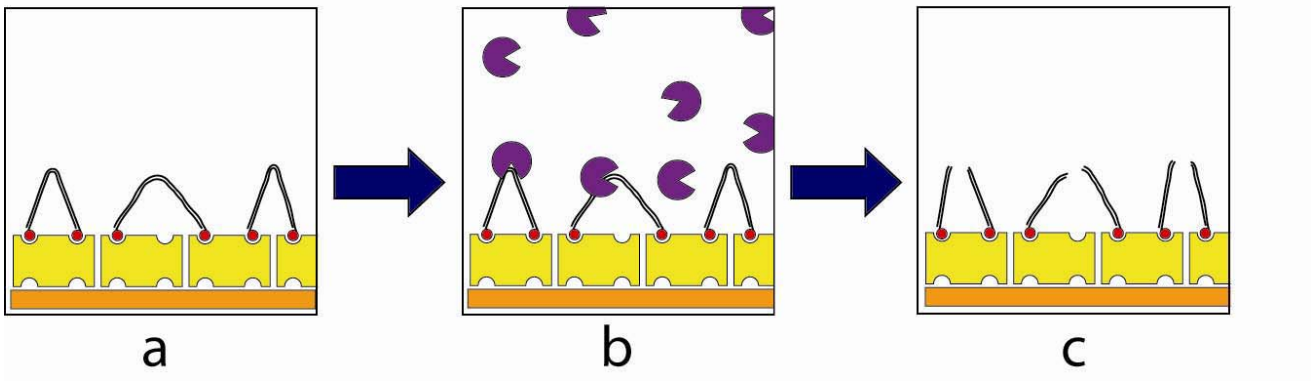
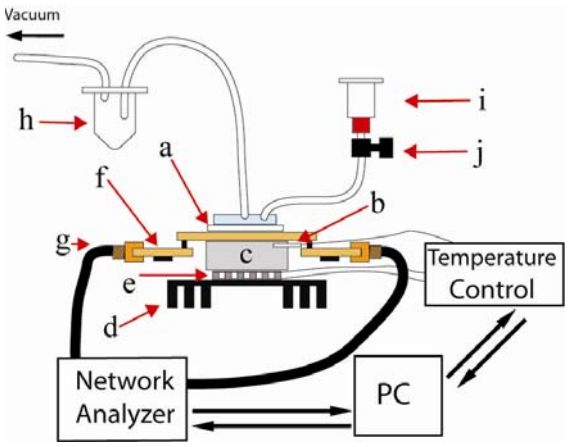
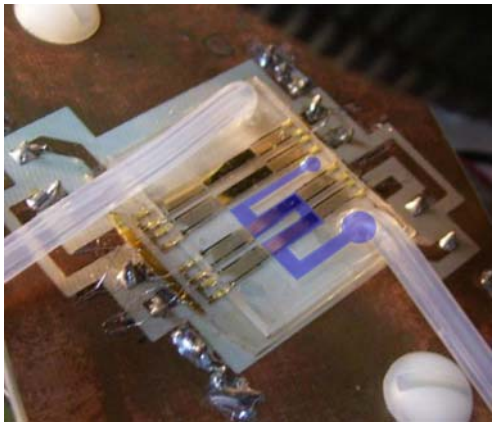


Figure 1: Expected configuration of surface biomolecules. a. dsDNA bound to NeutrAvidin at both ends. b. Restriction endonuclease EcoRI is added. c. dsDNA after cleavage with EcoRI.

ss1	5'-TCTTGCTGGGGTTATCGATGGGAAAAAACACGAAAAAAAGCAAAAAAAGAATTC AGCCAAAAAACACAAAAAAAATCGATGTAGGCCATGCTGTCC – 3'
ss2	5'–GGACAGCATGGCCTACATCGATTTTTTTTGTGTTTTTTGGCTGAATTC TTTTTTTGCTTTTTTTCGTGTTTTTTCCCATCGATAACCCCAGCAAGA – 3'

Table 1: ssDNA sequences. Complementary sequences ss1 and ss2 are both biotinylated on the 5' end. The EcoRI restriction site is underlined.





2.a **2.b**

Figure 2: a. The SH-SAW device test set-up. a. SH-SAW device water and PDMS chamber. b. RTD. c. Aluminum heat block. d. Heat sink. e. Peltier device. f. Switching circuit. g. SMA connectors. h. Output collection tube. i. Input syringe. j. Micrometering valve. **b.** Top view of the SH-SAW device wafer with attached PDMS chamber and input tubing. The path of fluid flow is highlighted in blue.

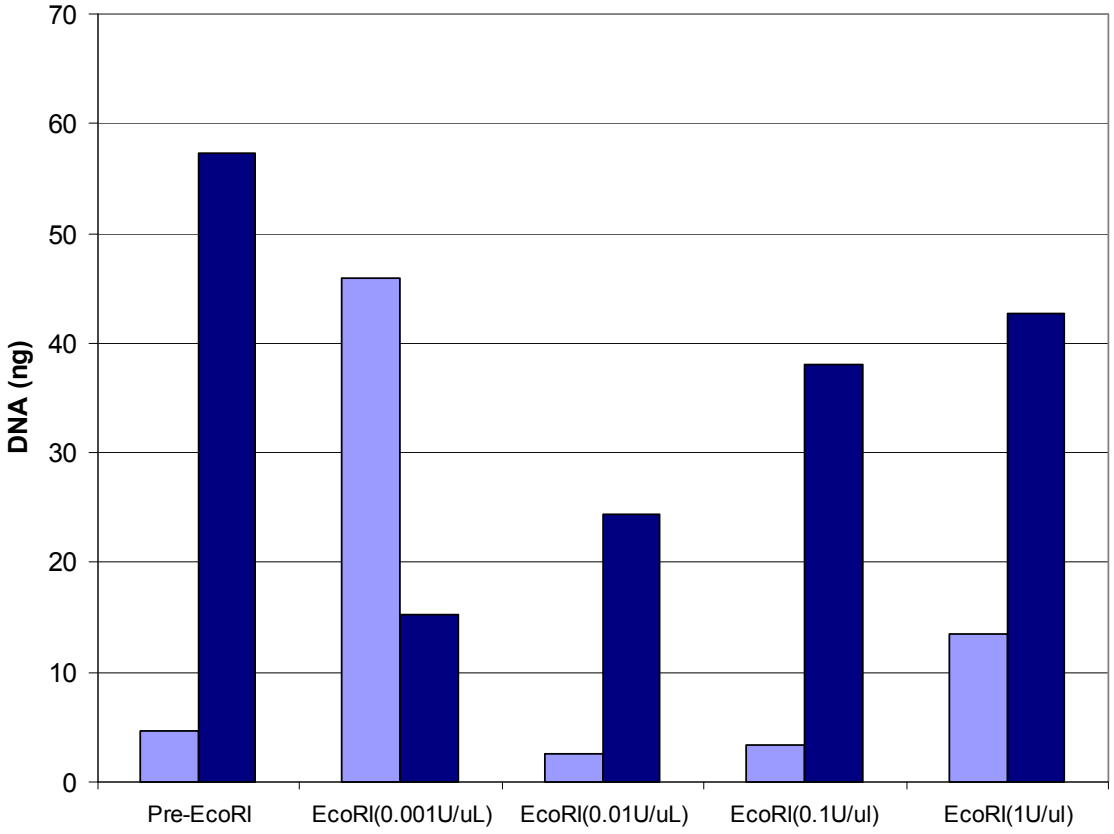


Figure 3: Total DNA measurements of components rinsed from the SH-SAW device surface sequenctially. Light bars indicate the measured DNA in the SSC and NEBuffer buffer rinses in the presence of EcoRI. Pre-EcoRI buffer is a control rinse with only SSC. Dark bars represent the 80°C water rinses after EcoRI digestion. The pre-EcoRI water rinse signifies the 80°C water pre-rinse.

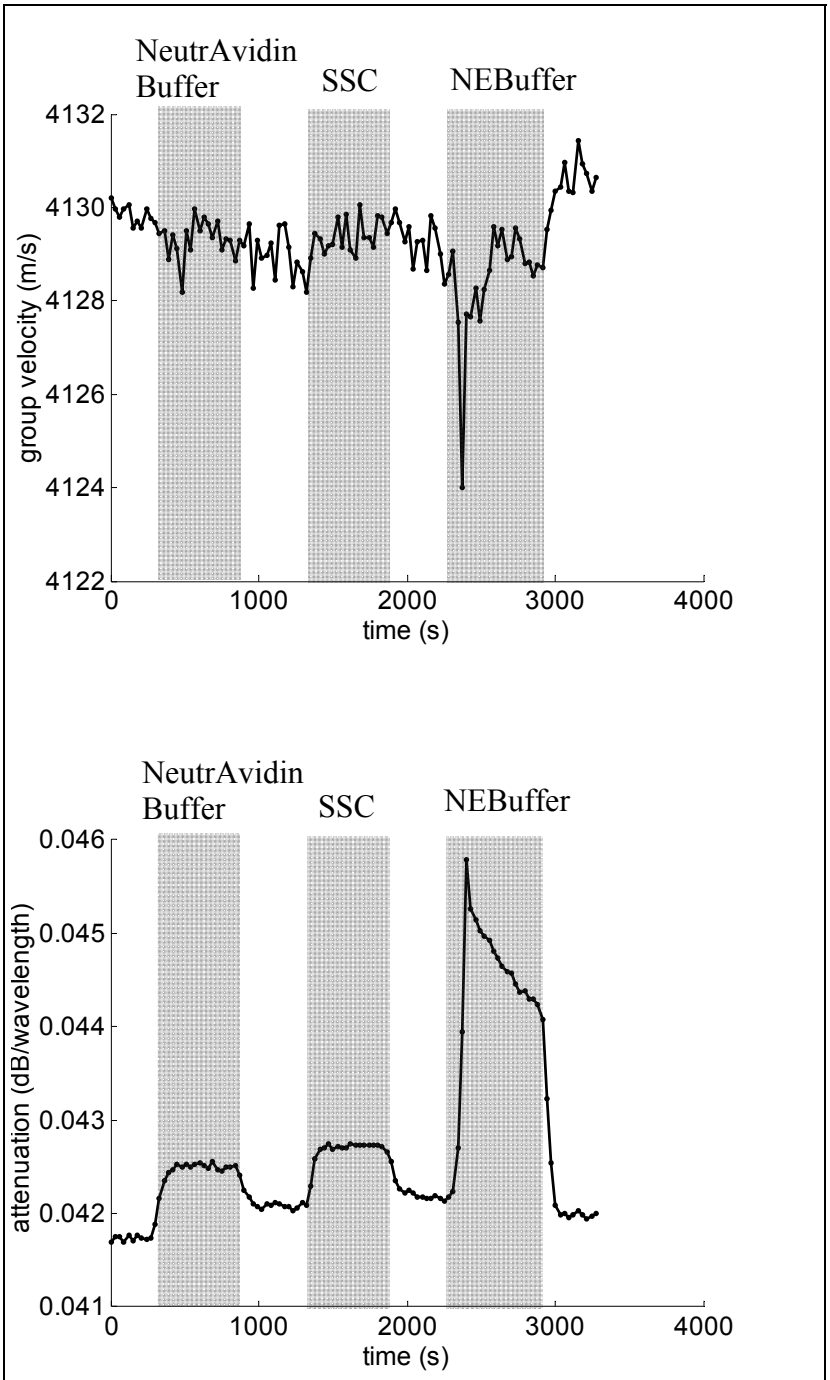


Figure 4: SH-SAW response to different buffers on a gold surface. Each buffer addition is followed by a water rinse. v_g remains essentially constant throughout the measurement, while increasing α caused by buffer addition indicates increasing fluid viscosity.

	Water	NeutrAvidin Buffer	SSC	NEBuffer
ρ (g/cm^3)	0.997	1.004	1.010	1.012
η_{44} (mPa·s)	0.890	0.929	0.917	0.970

Table 2: Calculated buffer densities and extracted buffer viscosities. Values for water are taken from Kikuchi et. al. [68].

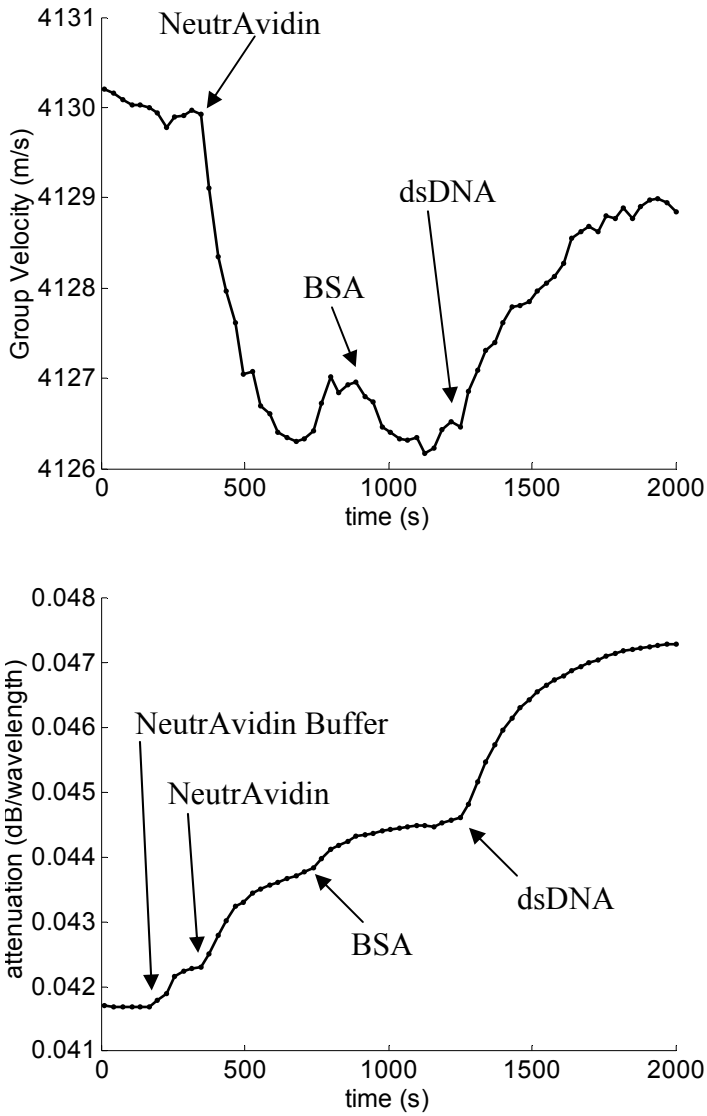


Figure 5: Typical SH-SAW response to biomolecule additions.

	NeutrAvidin+BSA	dsDNA	Uncleaved dsDNA	Cleaved dsDNA
ρ (g/cm^3)	1.40	0.997	0.997	0.997
h (nm)	5.4	10	10	11
v_g (m/s)	4128.0±1.72	4134.0±0.61	4130.4±2.3	4128.3±2.3
α (dB/λ)	0.0450±0.00073	0.0502±0.0071	0.0511±0.00057	0.0518±0.00014
c_{44} (MPa)	2.4		0.24	0.34
η_{44} (mPa·s)	2.8		0.48	0.89

Table 3: Biomolecular layer properties. Layer density (ρ) and thickness (h) are calculated based on the known properties of the biomolecules. v_g and α are shown as the mean and standard deviation of the SH-SAW measurements. NeutrAvidin+BSA and dsDNA values are determined from 5 data sets; the remaining values are taken from 2 data sets. Elasticity (c_{44}) and viscosity (η_{44}) are extracted using the matrix method for SAWs.

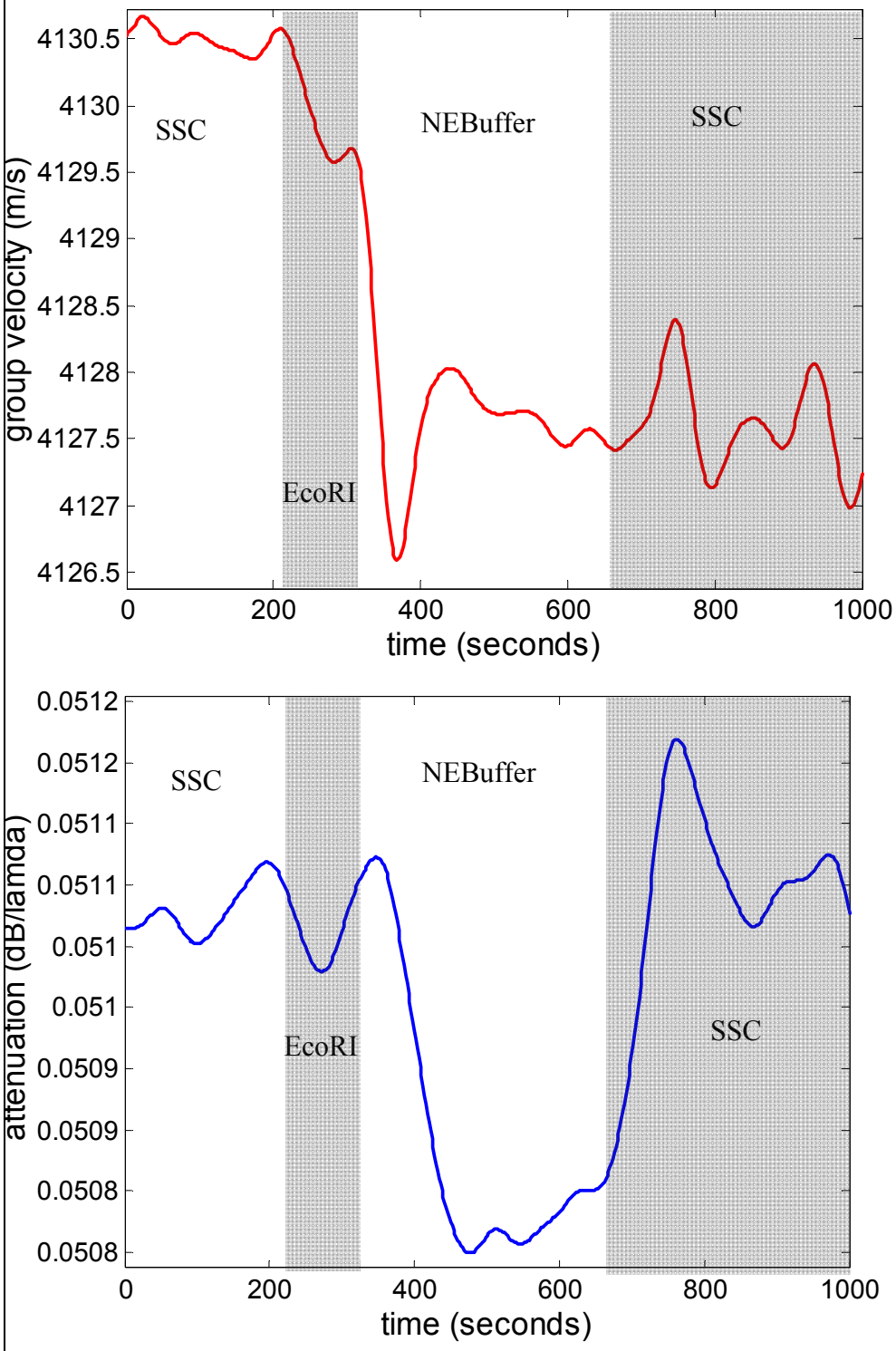


Figure 6: SH-SAW response to 0.1U/µL EcoRI addition and dsDNA cleavage induced by NEBuffer addition. Figure shows the mean of 2 data sets with the control NEBuffer response subtracted.

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Enzyme Method for Structural Alterations of Surface-Bound DNA

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Abstract - Biomolecules have important applications in nano-scale device development due to their small size, ability to self assemble into predictable formations, and enzymes for their specific and accurate modification. The conformation of biomolecular structures is an important aspect of their design as it can affect parameters such as binding efficiency and viscoelasticity, which can be critical to applications such as biosensing and nano-electronics. In this work, the structure of a DNA layer in gold-coated microtiter plate wells is modified using EcoRI restriction endonuclease. Verification of the surface configurations of the DNA molecules are achieved using fluorescence measurements of the well supernatants during the surface modification process. Results are compared with the expected behavior of the endonuclease over a range of applied concentrations, and conditions for complete and specific cleavage of the DNA surface are determined. For the biomolecular system presented, a relationship

between enzyme concentration in solution and the resulting activity at adjacent surfaces is determined, contributing to the understanding of enzyme behavior on surface-associated molecules. Additionally, the described manipulations can be applied to a wide array of devices in order to optimize the biomolecule characteristics.

KEYWORDS - EcoRI restriction endonuclease; Self-assembled biomolecular multilayer; Biomolecular layer conformation.

I. INTRODUCTION

The use of biomolecules in nanotechnology is of growing interest in a number of fields, including nanoelectronics, diagnostics and medicine, new materials synthesis, and environmental monitoring [1, 2]. Biomolecules are convenient tools for nano-scale device development due to their small size, with typical dimensions between 5 nm and 200 nm [3], and their natural ability to self-assemble. Some examples of self-assembly include DNA hybridization, ligand/receptor interactions, and antibody/antigen binding [1]. Biomolecular self-assembly allows for the bottom-up formation of nano-structures, unhindered by the limitations of traditional "top-down" lithographic techniques [1]. This method further benefits from the properties of enzymes, whose modifying interactions with biomolecules are specific, have accuracy on the level of Angstroms, and can be controlled with chemical co-factors [3, 4]. While studies of enzyme activity has concentrated to a large extent on their properties in solution, it is important to understand their behavior on surface-localized biomolecules in order to produce effective biomolecular structures for defined applications.

Rapid detection and identification of pathogens is critical to the avoidance and reduction of human exposure, as well as having important impact on the medical field, enabling accurate diagnoses and timely treatment. Self-assembled biomolecular layers are key components of many types of biosensors. These layers are applied to the sensing platform in order to impart selectivity for the desired target. Bioselective layers based on antibody interaction [5-8] and DNA hybridization [9-12] have been developed in order to detect viruses and bacteria as well as specific DNA sequences for diagnostic purposes [13]. Platforms currently using this surface technology include microcantilever, optical, acoustic, and fluorescence-based sensors [14, 15]. The conformation of the bioselective layer is critical to the sensitivity of these devices. Packing density [16], layer dimensions [17], conformation, and orientation [18, 19] of surface biomolecules all have been found to govern biosensor response. These parameters may affect the ability of the target analyte to bind to the surface, the distance from the surface that the target is localized, and the mass and viscoelasticity of the bound target/probe layer system, all factors that may enhance or detract from sensor performance.

Implementations of biomolecular surfaces and structures are also seen in the development of nano-electronic components and nano-patterning techniques. Conductive silver nano-wires have been constructed using double-stranded (ds) DNA as a template [20]. In this application, silver ions are added to the DNA pattern and a hydroquinone treatment used to aggregate the silver, forming a conductive wire. More recent works have adapted this method to reduce the wire width to 15nm [21]. Alternatively,

intercalating components have been used to form semiconducting DNA nanowires [22]. A DNA hybridization method has been employed to direct the assembly of carbon nanotubes between gold contacts [23] and to arrange multicomponent structures of carbon nanotubes and gold nanoparticles [24]. A carbon nanotube field effect transistor has been created using DNA to direct the placement of the semiconducting nanotubes and provide templates for the electrical contacts [25]. DNA has also been used to align organic and inorganic particles for surface patterning [21, 26-28].

The activity of enzymes on surface-associated biomolecules has also been investigated. The kinetics of the activity of EcoRI, a restriction endonuclease, on surfaces has been explored using evanescent wave technology [2, 29]. Measurement of EcoRI binding to a DNA-conjugated surface and the subsequent DNA fragment removal caused by addition of the catalyst Mg^{2+} was achieved. Similar kinetic studies were performed using surface plasmon resonance to measure the activity of Exonuclease III on surface bound DNA [30]. Castronovo et. al. investigated the action of the endonuclease DpnII on surface oligos with respect to oligo packing density [31]. The amount of hydrolysis of surface RNA-DNA duplexes with respect to the applied concentration of ribonuclease H has been studied using a combination of surface plasmon resonance imaging and surface plasmon fluorescence spectroscopy [4]. The effect of the recombination protein RuvA on DNA array conformation has been reported [32]. Surface associated rolling circle amplification has been demonstrated as part of a fluorescence based biosensor in microcapillary tubes [33]. Kaufmann et al. have produced three dimensional patterns of

DNA using magnetic surface patterning of sequences paired with modification by two enzymes, an endonuclease and a polymerase [34].

In this work, the primary structure of a dsDNA layer was altered using EcoRI restriction endonuclease. Before cleavage by EcoRI, short ds DNA sequences were bound to a NeutrAvidin surface at both 5′ ends. Digestion by EcoRI cleaves each sequence in half, producing a monovalently bound, non-crosslinked conformation on the surface. DNA cleavage was investigated with respect to applied EcoRI concentration, and the effect of delayed catalysis with Mg^{2+} was also explored. The structure of the surface DNA was deduced using fluorescence measurements of removed supernatant at various points during the cleavage process. Section II reviews the relevant properties of EcoRI and outlines the chosen method for dsDNA layer formation and fluorescence measurement. Section III describes the materials and procedures used for layer formation, digestion of DNA by EcoRI, and fluorescence measurements, as well as the method for simulating the expected results using MatLab software. Section IV presents the results and compares them to simulated data. Section V concludes the paper.

II. BACKGROUND

EcoRI is a type II restriction endonuclease that is composed of only one subunit, requires no ATP, and cleaves dsDNA at a specific sequence. EcoRI cleaves the palindromic sequence GAATTC on both strands of a DNA duplex between the G and A bases in the presence of the cofactor Mg^{2+} [35]. The enzymatic reaction is commonly

separated into three steps: binding, catalysis, and product release [29]. In the first step, EcoRI binds to the restriction site. Catalysis begins with the addition of the cofactor Mg^{2+}, which allows the enzyme to cleave both strands of the ds DNA. The endonuclease and the cleaved fragment are then released into the solution in the final step.

Endonuclease activity is influenced by many factors. In solution, the concentration of the enzyme relative to the DNA concentration must be considered, as well as the buffer conditions, temperature and incubation time. The nucleotides neighboring the restriction site have been shown to affect the binding rate of EcoRI [29]. Furthermore, EcoRI, along with other type II endonuceases, has been shown to cleave DNA non-specifically when present in sufficiently high concentrations or under certain buffering conditions [36, 37]. While many studies have explored these parameters in solution, fewer have been performed on DNA attached to surfaces. Surface-association of substrate molecules adds further complications to enzyme interactions. Kinetics of reactions localized to surface molecules can be much slower than that in solution, due partly to diffusion times [4]. Attachment of DNA to a surface by one end can also affect cleavage by an endonuclease due to loss of symmetry between the two strands [29]. Surface density of oligos has also been shown to effect the activity of enzymes [31]. Due to the many variables associated with enzyme reactions on surfaces, it is necessary to optimize the conditions for a given system in order to produce the desired enzyme behavior.

In this work, a crosslinked DNA layer was prepared on gold layers deposited on the inner surface of polystyrene micro-wells using a protocol adapted from Tsortos et al.

[18]. NeutrAvidin was non-specifically adsorbed onto gold well surfaces. Structurally bent, 96 base pair ds DNA was formed by hybridizing two complementary single-stranded (ss) DNA sequences, each biotinylated on the 5' end. Because the persistence length of ds DNA is approximately 150bp [38], a bend in the ds DNA sequence was created by incorporating 6-unit adenine tracts [39], allowing the ds DNA sequence to bind to the surface at both ends by NeutrAvidin-biotin linkages. The expected configuration of ds DNA on the prepared surface is shown in Figure 1a. EcoRI was then added in order to modify the primary structure of the DNA, into which an EcoRI restriction site had been incorporated. When the endonuclease is applied to the surface at sufficient concentration, the surface DNA is cleaved in half, effectively removing any DNA crosslinks from the surface. The expected configuration of surface-bound DNA after EcoRI cleavage is shown in Figure 1c.

There are several expected complications to the surface preparation described above. Although the ds DNA sequences are biotinylated at both ends, it is possible that some DNA molecules will bind to the surface by only one end, depending on the availability of adjacent biotin binding sites. For these monovalently bound DNA molecules, cleavage with EcoRI will remove half of the sequence, instead of un-crosslinking the surface. It is also possible for the ds DNA to bind non-specifically to the gold surface in regions not blocked by NeutrAvidin. Such non-specifically bound sequences could disrupt measurements if they were released from the surface. Furthermore, any non-specific activity of EcoRI could result in the undesirable removal of DNA from the surface and could potentially digest much of the DNA surface layer.

In order to address the issues stated above while creating the desired DNA layers and modifications, two cases were considered: (1) surfaces prepared as described and (2) surfaces that were pre-rinsed with 80°C water before incubation with EcoRI. Case (1) will be effected by monovalently bound DNA, which when cleaved by EcoRI will release DNA from the surface. The pre-rinse added to case 2 is intended to denature all surface strands. DNA bound to the surface by both ends will remain bound during the water rinse and will re-hybridize when an appropriate buffer is re-introduced. Sequences only bound by one end will lose the ss DNA strand that is not bound to the surface when the pre-rinse is applied. The resulting surface ss DNA will not be affected by EcoRI. A bovine serum albumin (BSA) blocker was introduced to the wells after the addition of NeutrAvidin in order to reduce the number of sites for potential non-specific binding of DNA. Digestion by EcoRI was tested over a range of concentrations to determine limits within which the surface DNA molecules are completely cleaved yet non-specific digestion by EcoRI does not occur. A diagram of the multilayer formation process is shown in Figure 2, which depicts complete, specific cleavage by EcoRI.

To verify the conformation of the surface DNA, the solution containing unbound DNA was removed from the wells at selected points during surface preparation and modification. A DNA-specific fluorescent reagent was then added, and the fluorescence intensity was measured. Based on the expected surface structures, fluorescence of the supernatants relative to increasing digestion by EcoRI was determined with MatLab software. MatLab-simulated fluorescence responses were compared to the measured

results; correlation between the two indicates EcoRI is interacting with the surface as expected. Comparison with the expected behavior also allows insight into the activity of EcoRI over the measured ranges of concentration, specifically whether or not the enzyme is cleaving the surface molecules, and if it is cleaving the surface non-specifically.

III. MATERIALS AND METHODS

III.a Materials

NeutrAvidin, EcoRI endonuclease, and the NEbuffer EcoRI (NEBuffer) were supplied by New England BioLabs (Beverly, MA). Bovine serum albumin (BSA) was obtained from Sigma-Aldrich (St. Louis, MO). Quant-iT OliGreen ssDNA reagent was purchased from Invitrogen (Carlsbad, CA). Complementary ss oligonucleotides, biotinylated on the 5′ end, were supplied by Integrated DNA Technologies, Inc. (Coralville, IA). The sequences are listed in Table 1; the EcoRI restriction site is underlined. Nanopure water was used in all buffers and aqueous solutions (≥18MΩ, <3ppbTOC).

Magnetron sputtering was used to deposit 100Å chromium adhesion layers followed by 2000Å of gold onto 96-well opaque black polystyrene microplates (Corning Life Sciences, Lowell, MA). Immediately after metal deposition, the gold-coated microplates were covered with foil and stored under vacuum. Prior to use, each plate was UV-ozone cleaned in a model 342 UVO Cleaner (Jelight Company, Inc., Irvine, CA) for 10 min, rinsed with nanopure water and dried with N_2. After each use, plates were UV-ozone cleaned for 10 min and soaked in 2% Contrad70 detergent (Decon Labs, Inc., King of

Prussia, PA) for 30 min. Plates were then rinsed with nanopure water, dried with N_2, and the UV-ozone treatment was repeated.

III.b Experimental Procedures

III.b.1 EcoRI Digestion in Solution

In order to determine the concentration of EcoRI restriction endonuclease required for effective cleavage of DNA in solution, 200nM dsDNA was created through hybridization of ss1 and ss2 in NEBuffer immediately before use. EcoRI was added at concentrations of 1.2mU/µL to 4U/µL and incubated at 37°C for 1 hr. The resulting solutions were then loaded into a 4% agarose precast E-Gel combined with an E-Gel Electrophoresis System (Invitrogen). Results were compared to a TrackIt 50 bp DNA ladder (Invitrogen.)

EcoRI digestion in solution was also performed in several different buffers. Complementary sequences ss1 and ss2 were hybridized at 200nM concentrations in the following buffers: NEBuffer, 10mM $MgCl_2$ in sodium citrate (SSC) buffer (150mM NaCl, 15mM sodium citrate, pH 7.5), SSC, and ethylenediaminetetraacetic acid (EDTA) at 0.5mM, 1mM, and 2.5mM in SSC. EcoRI was added at 0.5U/µL (a concentration determined in the previous assay) and incubated at 37°C for 1 hr prior to analysis by agorose gel electrophoresis.

III.b.2 Surface Preparation

Complementary ss sequences ss1 and ss2 were hybridized in SSC to form 200nM dsDNA immediately before use. Blocking buffer (BB) was prepared the day of use with 0.5% BSA in SSC buffer. All well rinses were performed 10 times with 150µL BB.

NeutrAvidin was added to gold wells in a binding buffer (50mM TRIS, 10mM $MgCl_2$, 10mM KCl, pH 7.5) at 20µg/ml in a 50µL volume. Wells were incubated for 45 min at room temperature, followed by a rinse cycle. The prepared DNA was added to wells in 20µL volumes and incubated at room temperature for 45 min followed by a rinse cycle. The DNA addition was performed three times to insure saturation of NeutrAvidin binding sites. Half of the test wells were rinsed three times with 80°C water to denature all DNA duplexes. Wells were then rinsed three times in SSC to allow remaining surface oligonucleotides to re-anneal into ds DNA.

Digestion of the surface DNA with EcoRI was performed in NEBuffer at 37°C for 30 min. EcoRI was added in 20 µL volumes with concentrations ranging from 0.006 U/µL to 0.6 U/µL. Control wells were included which were incubated with NEBuffer containing no EcoRI. Alternatively, EcoRI was added to the wells in SSC buffer with 0.5mM EDTA in the listed concentrations and incubated at room temperature for 30 min. The solution was then removed and reserved, all wells were rinsed three times with NEBuffer, and NEBuffer was incubated in the wells at room temperature for 30 min. After digestion using either method, the solutions from each well were removed and reserved. Wells were then rinsed three times with SSC buffer, followed by the addition of 45µL 80°C water. The water and all reserved samples were loaded into a black 96-

well microplate, and all volumes were adjusted to 45µL with SSC. OliGreen reagent was added to each of the wells to a final concentration of 0.25% by volume, and the fluorescence was measured using a Synergy Microplate Reader (, model #) with excitation and emission filters of 485±12nm and 535±12nm, respectively.

III.c MatLab Simulation

A MatLab routine was created to simulate the expected amount of DNA in the multiwell plate solutions with respect to EcoRI concentration. The number of biotin binding sites on the gold surface is fixed, based on the surface area of the gold wells and the dimensions of NeutrAvidin protein (less than 5nm × 8nm × 7nm [40]). All available binding sites are assumed to be bound by the biotinylated DNA. The percent of surface DNA bound to the surface by only one end is an input parameter. The program calculates the number of DNA molecules on the surface that are bound monovalently and the number that are bound at both ends. The number of specific ds DNA cuts made by EcoRI is assumed to increase exponentially relative to increasing EcoRI concentration because the number of cleavages is limited by the amount of DNA bound to the surface. Because the amount of surface cleavage at a given EcoRI concentration is unknown, the EcoRI concentrations used in the routine are arbitrary. Above a concentration where all sites have been cleaved, the program assumes that non-specific cleavage begins. Non-specific DNA cleavage is again expected to be substrate limited and occur exponentially until all DNA has been removed from the surface. The routine considers both the case where wells have been pre-rinsed with 80°C water, denaturing and removing ss DNA annealed to singly-bound oligos, and also where no pre-rinse has been performed.

Resulting values are plotted as mass of DNA in the supernatant solution after EcoRI digestion in NEBuffer and after the following rinse in 80°C water, which was included to quantify the DNA remaining on the surface after treatment with the endonuclease. The shape of the resulting curves, as well as the relationship between the pre-rinsed and non-pre-rinsed wells were compared to the measured well results in order to estimate the amount of DNA singly bound to the surface as well as the ideal EcoRI concentration for complete and specific cleavage of the surface DNA.

IV. RESULTS AND DISCUSSION

In order to determine a range of EcoRI concentrations suitable for the digestion of the ss1, ss2 DNA duplex, EcoRI was added to 200nM dsDNA in NEBuffer under the conditions described above (IIIb.1). The resulting gel images (not shown) depict complete cleavage between EcoRI concentrations of 0.12 U/μL and 4U/μL. No signs of non-specific cleavage occur within this range. At EcoRI concentrations of 12mU/μL and below, no cleavage is observed.

Additional EcoRI digestions were performed in solution to determine the effects of various buffers on the cleavage efficiency. A single EcoRI concentration was chosen (0.5U/μL) from the previous concentration assay and applied to 200nM DNA hybridized in different buffers. The resulting gels (not shown) show complete cleavage of the DNA sequence in NEBuffer and partial cleavage of the sequence in 10mM $MgCl_2$ in SSC. No

cleavage was observed under any other conditions (SSC and SSC with various concentrations of EDTA.)

To determine the effects of EcoRI addition on surface bound DNA, multiwell plate assays were performed as described in section IIIb.3. When EcoRI is incubated in NEBuffer only, a typical measurement of supernatant DNA vs. EcoRI concentration is produced, as shown in Figure 3. Results shown include the amount of DNA measured in the NEBuffer immediately after the EcoRI incubation and the amount of DNA measured in the 80°C water rinse afterwards. DNA present in the NEBuffer supernatant represent singly bound DNA that has been cleaved by EcoRI. This rinse may also include DNA non-specifically bound to the gold surface and displaced by the endonuclease. DNA present in the 80°C water rinse indicates sequences remaining on the surface after EcoRI digestion. DNA bound to the surface by either one or both ends may contribute to the measured signal in this rinse. Uncleaved, singly bound DNA will release either sequence ss1 or ss2 into the hot water post-rinse, and cleaved, singly bound DNA will release one half of either ss1 or ss2. Uncleaved, doubly bound DNA will not contribute to the DNA in the hot water post-rinse as both strands are attached to the NeutrAvidin surface by biotin-NA binding, which is unaffected by this treatment. Once the doubly bound sequences have been cleaved, however, they will release one half of both ss1 and ss2 into the water rinse. The results obtained are compared with the MatLab simulations assuming 50% singly bound DNA (Figures 3c and 3d). It can be seen that the measured data follows the expected trends, with EcoRI cleavage increasing exponentially with concentration. Non-specific cleavage occurs after 0.3U/μL. Non-specific cleavage is

signified by an abrupt increase in the fluorescence of the NEBuffer rinse (Figure 3a) for both the pre-rinse and non-pre-rinse cases. This suggests that DNA is being removed from the surface at levels greater than would be expected from cleavage of the single GAATTC sequence present in each DNA molecule. The commencement of non-specific cleavage can also be seen in the 80°C water rinse solution as a drop in fluorescence (see Figure 3b). Non-specific cleavage in the NEBuffer rinse would remove DNA segments from the surface, leaving fewer DNA molecules to be released during the final denaturing rinse.

The hot water pre-rinse was expected to remove all DNA from the NEBuffer supernatant in the region of specific EcoRI activity. It can be seen in Figure 3a that some DNA appears in the NEBuffer after a concentration of 0.06U/μL even when the pre-rinse has been performed. Because the amount of removed DNA in the pre-rinsed wells does not increase significantly until non-specific activity occurs, it is likely that this response is caused by the displacement of non-specifically bound DNA by the endonuclease. The hot water post-rinse data behaves as expected for the pre-rinsed wells. When no EcoRI is added, and at EcoRI concentrations insufficient for significant surface digestion, no DNA is measured in the post-digestion, 80°C water rinse. This indicates that no monovalently bound dsDNA remains after the pre-rinse, which would contribute to the fluorescence. The increase in removed DNA as the EcoRI concentration reaches 0.3U/μL confirms gives assurance that a significant fraction the surface DNA molecules are bound to the surface by both ends. If all molecules were monovalently bound, cleavage of the surface could only result in a decrease in removed DNA during the hot water post-rinse. From the

resulting data it can be determined that approximately 50% of dsDNA molecules are bound to the surface at both ends. These results were duplicated three times to assure repeatability.

The previous experiment revealed the concentration-dependent behavior of EcoRI on surface DNA when applied in NEBuffer and allowed to diffuse to the surface during the incubation period. It was also of interest to determine if the activity of EcoRI changed if the enzyme was already present at the surface before cleavage began. In a second set of well experiments, EcoRI was added to the prepared DNA surface in SSC + 0.5mM EDTA and incubated for 30 min. at room temperature. In the absence of Mg^{2+}, the enzyme could diffuse to the surface but not digest DNA. The SSC + 0.5mM EDTA buffer was then removed and replaced with NEBuffer, which should allow EcoRI digestion of the surface DNA to commence. Measurement of the SSC + EDTA and NEBuffer well supernatants as well as the following 80°C water post-rinse permitted investigation of the concentration-dependent behavior of enzymes that have already associated with the substrate. Typical results from these assays are shown in Figure 4. Although no DNA digestion should occur, removed DNA is apparent in the SSC + EDTA rinse (Figure 4a). However, at an EcoRI concentration of 0.3U/µl, the amount of removed DNA is less than 7% of the previous NEBuffer data (Figure 3a) for the pre-rinsed and non-pre-rinsed cases. DNA present in this rinse may originate from the displacement of non-specifically bound DNA. The amount of DNA in the NEBuffer supernatant (Figure 4b) is also less than that of the previous experiment, with measured fluorescence only 9% of the non-pre-rinsed case and 12% of the pre-rinsed case shown in

Figure 3a. While no DNA was expected in the SSC+EDTA rinse, the results from the NEBuffer rinse were anticipated to be comparable to the data in Figure 3a. Although the EcoRI was applied in a different fashion, it should still cleave surface strands in the presence of Mg^{2+} and therefore release DNA into the NEBuffer supernatant measured in Figure 4b. Although the NEBuffer rinse produced lower concentrations of DNA than expected, the 80°C water rinse results achieve DNA concentrations comparable to those shown in Figure 3b. Both the pre-rinsed and not pre-rinsed well solutions contain DNA concentrations increasing exponentially with EcoRI concentration, followed by a decrease at EcoRI concentrations above 0.3U/μL. The lack of DNA present in the pre-rinsed samples at low EcoRI concentrations indicates that the 80°C water pre-rinse successfully denatured singly-bound DNA. The similar DNA concentrations measured in the 80°C water rinse, compared to the 80°C water rinse from Figure 3b, indicates that allowing EcoRI to associate with the DNA surface before applying the Mg^{2+} catalyst does not significantly affect the concentration-dependent cleavage behavior of EcoRI. The seemingly contradictory results from the NEBuffer supernatant measurements can be explained by the rinsing procedure. Immediately before the NEBuffer incubation, wells were rinsed three times with NEBuffer to remove the chelating EDTA from the wells as well as to remove any remaining DNA dissociated from the surface. Because the EcoRI in this assay had already bound to the surface before addition of the Mg^{2+} cofactor, it is possible that cleavage of the surface DNA occurred during these rinses, and the removed DNA therefore discarded.

V. CONCLUSIONS

The structure of ds DNA bound to a surface was modified using EcoRI endonuclease in conjunction with thermal denaturation. The actual structure of DNA on the surface was verified using fluorescence measurements of the supernatant during the EcoRI digestion process. The quantity of DNA in the supernatant solution, measured over a range of applied EcoRI concentrations, was comparable to simulated values which were based on the expected behavior of the EcoRI endonuclease on the foreseen layer conformations. Results confirm that the surface ds DNA achieved the structures predicted. Conditions for complete cleavage of the surface-associated DNA, without non-specific EcoRI activity, were determined for enzymes incubated on the surface in a catalytic buffer and also enzymes first incubated on the surface without the catalyst required for digestion, followed by the introduction of the Mg^{2+} cofactor. This surface preparation demonstrates the ability of enzymes to manipulate the structure of surface biomolecules with applications in nano-electronics, nano-scale surface patterning, and biosensing. The application of enzymatic modifications, including the endonuclease example here as well as the action of ligases, polymerases, and endonucleases to name a few, to self-assembled biomolecular structures allows a means for optimizing the surface properties to best-suit the desired application.

ss1	5'- TCTTGCTGGGGTTATCGATGGGAAAAAACACGAAAAAAAGCAAAAAAAGAATTC AGCCAAAAAAACACAAAAAAAATCGATGTAGGCCATGCTGTCC – 3'
ss2	5' – GGACAGCATGGCCTACATCGATTTTTTTTGTGTTTTTTGGCTGAATTC TTTTTTTGCTTTTTTTCGTGTTTTTTCCCATCGATAACCCCAGCAAGA – 3'

Table 1: ssDNA sequences. Complementary sequences ss1 and ss2 are both biotinylated on the 5′ end. The EcoRI restriction site is underlined.

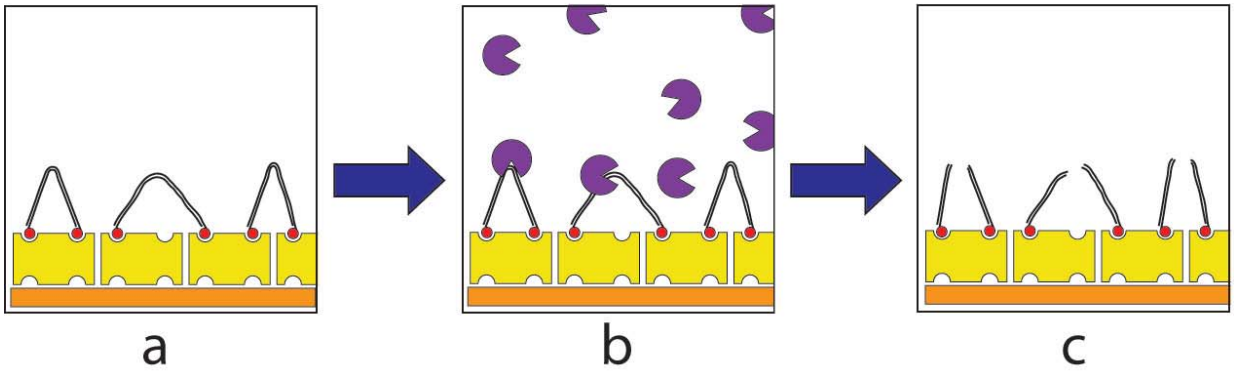

Figure 1: Expected configuration of biomolecular surface. a. dsDNA bound to NeutrAvidin at both ends. b. Restriction endonuclease EcoRI is added. c. dsDNA after cleavage with EcoRI.

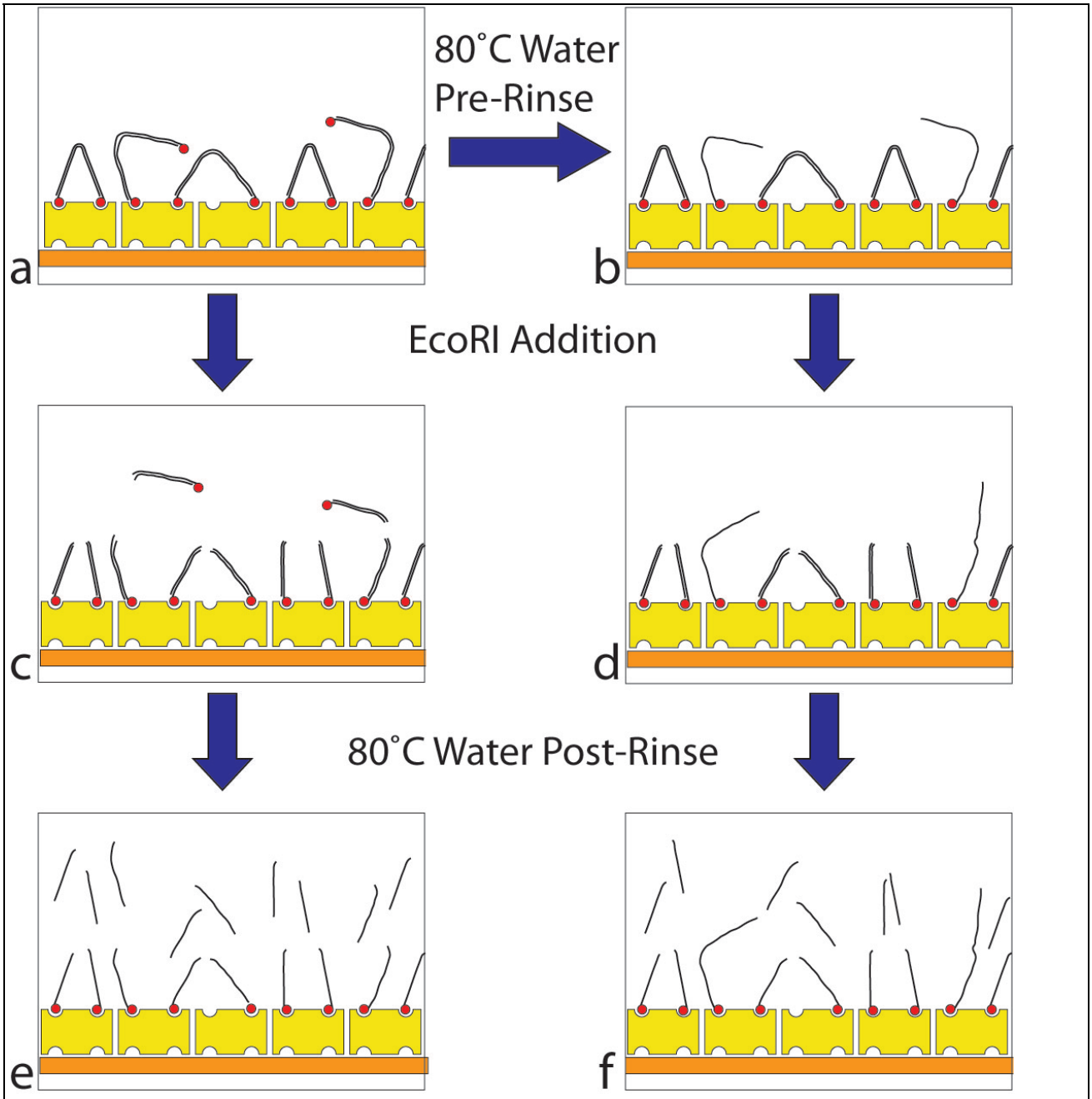

Figure 2: Steps for measurement of DNA in supernatant solutions. a. Predicted conformation of the initial dsDNA layer. b. Conformation of the surface after a pre-rinse with 80°C water. c. & d. The surface and supernatant solution after cleavage with EcoRI. Supernatant solution is removed and DNA concentration measured. e. & f. The surface and supernatant solution after a rinse with 80°C water. Supernatant solution is removed and DNA concentration measured.

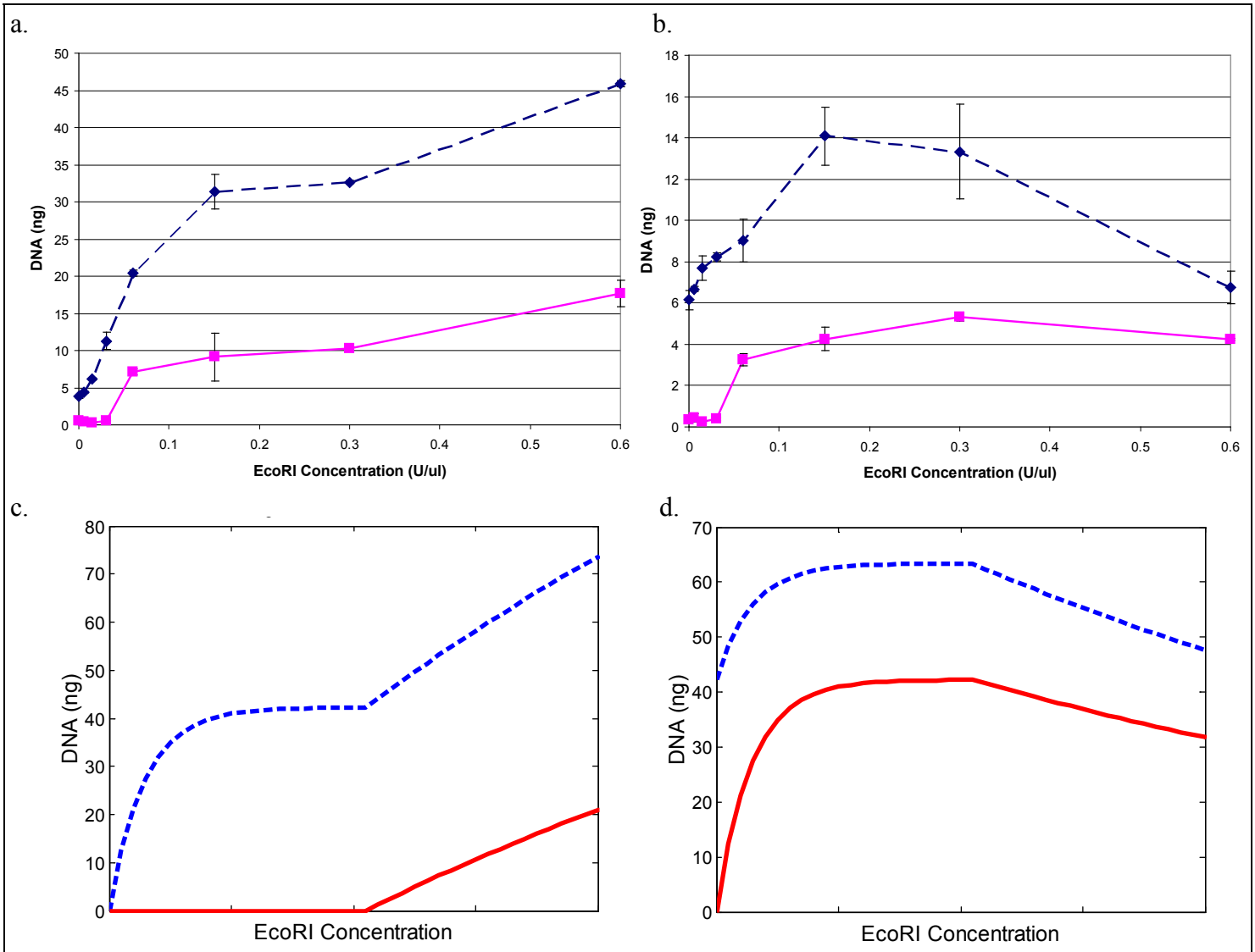

Figure 3: Results of EcoRI incubation in NEBuffer on surface bound DNA. Data shown is a representative of three duplicate assays. a & b. Dotted lines represent wells that were not pre-treated with 80°C water. Solid lines indicate wells that were pre-treated. a. NEBuffer supernatant after EcoRI incubation. b. 80°C water rinse following EcoRI incubation. c. MatLab-simulated results for NEBuffer supernatant. d. MatLab-simulated results for 80°C water rinse.

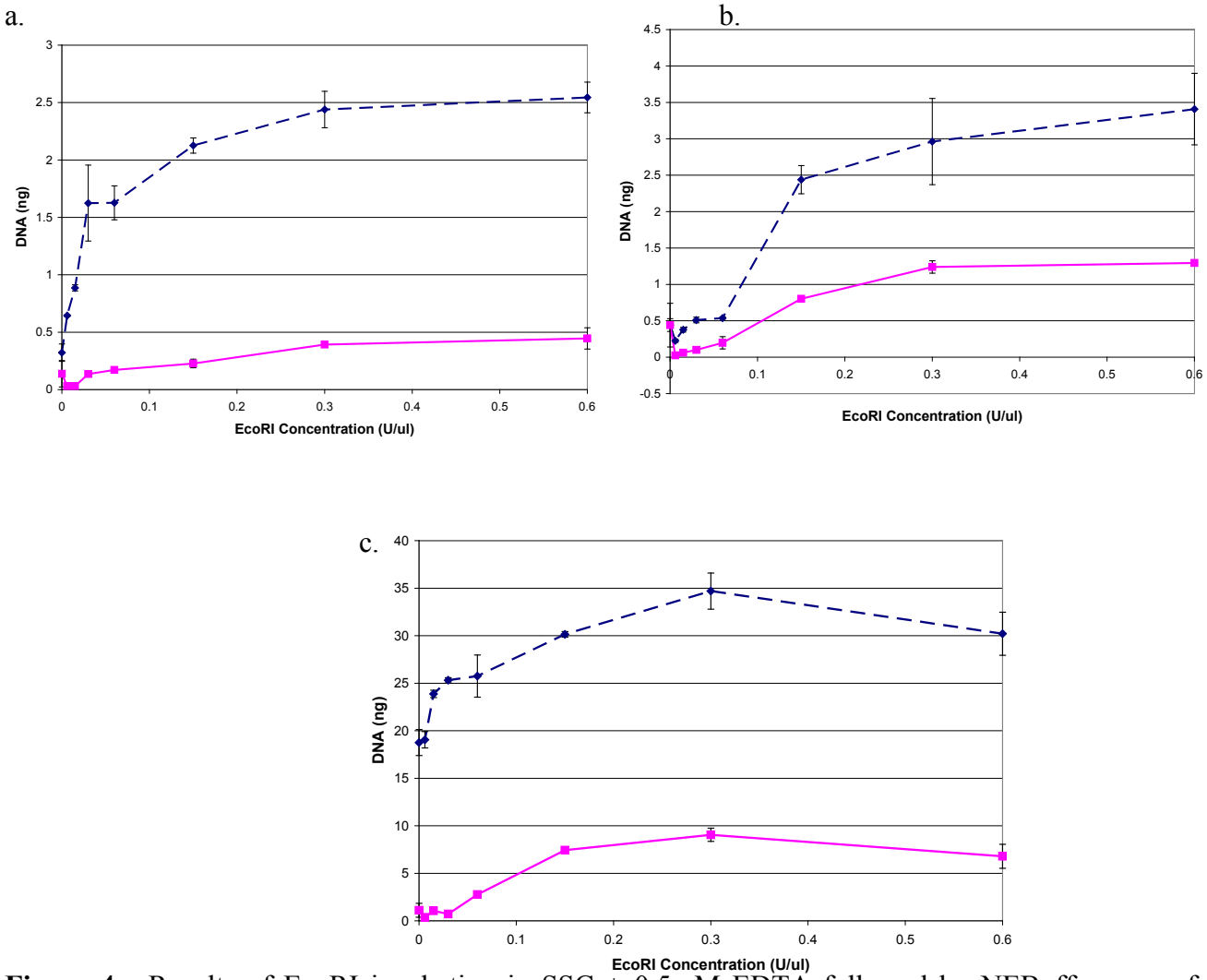

Figure 4: Results of EcoRI incubation in SSC + 0.5mM EDTA followed by NEBuffer on surface bound DNA. Dotted lines represent wells that were not pre-treated with 80°C water, solid lines indicate wells that were pre-treated. a. SCC + 0.5mM supernatant after EcoRI incubation. b. NEBuffer supernatant after NEBuffer incubation. c. 80°C water post rinse.

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