Use of electrical and magnetic fields in the control of bacterial biofilm
by Susana M Fortun

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Electrical Engineering
Montana State University

Abstract:
Bacterial biofilm infections are less susceptible to antibiotic treatment than their planktonic counterparts. This has become a major health issue in many areas of a hospital and/or health care industry. The bioelectric effect was discovered by a team of Canadian scientists in 1994. The bioelectric effect is the treatment of the bacterial biofilm with antibiotic in the presence of an electrical current. This treatment has been found to produce a remarkable higher kill of the bacterial biofilm. This thesis replicated the original work done in Canada and from that base it goes into developing a dose response for the applied current in the presence and the absence of antibiotics. It shows that the effect is obtained with pure DC current and that the effect is not dependent on the direction of the current with respect to the nutrient flow. It also shows that the effect is only seen when the biofilm itself is treated with the DC current. We also have shown that an E-Field does not seem to have the same effect on the biofilm. Preliminary work was done using currents induced by AC fields and chemical manipulation of the nutrients. A mechanism for the bioelectric effect is proposed.

USE OF ELECTRICAL AND MAGNETIC FIELDS IN THE CONTROL OF BACTERIAL BIOFILM

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Susana M. Fortun

A thesis submitted in partial fulfillment

of the requirements for the degree

of

Doctor of Philosophy

in

Electrical Engineering

MONTANA STATE UNIVERSITY-BOZEMAN

Bozeman, Montana

August 1997

APPROVAL

of a thesis submitted by

Susana M. Fortun

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduates Studies.

Bruce R. McLeod, Ph.D. Bruce R. McLeod 7-15-97
(Signature) (Date)

Approved for the Department of Electrical Engineering

Bruce R. McLeod, Ph.D. Bruce R. McLeod 7-15-97
(Signature) (Date)

Approved for the College of Graduate Studies

Robert Brown, Ph.D. R. Brown 8/8/97
(Signature) (Date)

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ACKNOWLEDGMENTS

I would like to extend my most sincere gratitude to the three professors that made this project possible: Dr. Bruce McLeod, my principal advisor, for all his guidance, financial support, technical training, friendship, and for giving me the opportunity to fulfill a life long dream. Dr. J. William Costerton, my second advisor, whose guidance, support and friendship are a very precious gift, one that I should cherish as long as I live. And Dr. John Carlsten, who guided me through my first steps in electromagnetics, for all the things that he has taught me and for believing in me when even I couldn't believe in my own abilities. I also want to express my gratitude to the other members of my committee, Dr. John Hanton and Dr. Curt Vogel for all their help and guidance.

I want to thank the staff of the Electrical Engineering Department, especially to Muriel Holmquist and Monica Hoey for all the support and words of encouragement; the staff, students and faculty of The Center for Biofilm Engineering for all their help and support; to Dr. Phil Stewart for his invaluable direction and guidance, to Dr. Ching-Tsan Huang for helping me take the first steps in a biofilm laboratory, and to Lu Goodrum for all her invaluable help in this research. I also want to extent my gratitude to my summer students Nicole Wellman, Kimberly Craven and Anna Crawford for all that they taught me.

I also would like to thank my family, specially my father Alberto M. Fortun, my aunts Antonia D. Gomez, Nilda E. Grau, Daisy A. Gomez, and my uncle Humberto J. Grau for their love, understanding, encouragement and support throughout my life. The Teeters, Daniel, Annabelle, Laetitia and Vanessa, for their constant love and care.

Finally, I would like to extend my warmest and deepest gratitude to my friends, specially Barbara J. Rodriguez for all her sacrifices for the advancement of science, to Jay, Cindy and Amber Gutzwiller for adopting me into their family, to Biljana Milkovska for her constant support, and to Michelene Dennis for all our long talks and philosophical discussions.

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ABSTRACT

Bacterial biofilm infections are less susceptible to antibiotic treatment than their planktonic counterparts. This has become a major health issue in many areas of a hospital and/or health care industry. The bioelectric effect was discovered by a team of Canadian scientists in 1994. The bioelectric effect is the treatment of the bacterial biofilm with antibiotic in the presence of an electrical current. This treatment has been found to produce a remarkable higher kill of the bacterial biofilm. This thesis replicated the original work done in Canada and from that base it goes into developing a dose response for the applied current in the presence and the absence of antibiotics. It shows that the effect is obtained with pure DC current and that the effect is not dependent on the direction of the current with respect to the nutrient flow. It also shows that the effect is only seen when the biofilm itself is treated with the DC current. We also have shown that an E-Field does not seem to have the same effect on the biofilm. Preliminary work was done using currents induced by AC fields and chemical manipulation of the nutrients. A mechanism for the bioelectric effect is proposed.

INTRODUCTION

The second half of the twentieth century has seen many advances in the field of medicine. One of those advances is in bioengineered implants. During the last few decades, the bioengineers have given us prosthetic hip joints and internal fixation devices, vascular catheters, prosthetic heart valves, cardiac pacemakers, arterial grafts, in-dwelling urinary catheters, peritoneal dialysis catheters, contact lenses, penile prostheses, and intrauterine contraceptive devices among many others. These engineered innovations have prolonged our lives and/or restored a quality of life lost due to a disease. Unfortunately, this progress has been accompanied by the emergence of a new challenge to medicine -- implant associated infections that may not respond to conventional antibiotic treatment alone and that often require removal of the infected implant. [1,2]

Bacterial biofilm has been found to be the culprit in many of these implant associated infections that do not respond to conventional antibiotic treatment [3]. Medical equipment that is used in close contact to patients, such as dental drills, endoscope and contact lenses cases, are also prime environments for biofilm formation. Biofilms are bacterial populations enclosed in an exopolysaccharide matrix that adhere to each other and/or surfaces or interfaces [4].

Even though the study of infections caused by biofilms in the medical setting is still in its initial stage, the study of biofilms involved in corrosion [5], souring [6], and fouling [7,8] problems has a long history. The growth of biofilm in pipelines has been a major problem in industry not only because the biofilms slow down fluid flow but also because the biofilm is hard to clear due to its characteristic enhancement of resistance to biocides. There have been numerous hypotheses about the nature of this enhanced resistance. One of the early hypotheses was that the exopolysaccharide matrix was a charged matrix and that this matrix was responsible for binding antimicrobial agents before they have the opportunity to penetrate the biofilm [9]. Therefore, in the early 90's J.W. Costerton and his team at the University of Calgary, Calgary, Alberta, Canada, began a series of studies in order to determine if a low-strength electric field could affect the biocide in the biofilm and enhance biocide killing [10,11]. They found out that a low-strength dc-electric field in conjunction with a biocide would indeed reduce the biofilm population. These experiments were duplicated in England by Jana Jass and Hilary Lappin-Scott [12]. The British team, however, did not show as much killing of the biofilm.

It has been the intention of this study to duplicate the original work of J.W. Costerton and to extend the work in order to establish a dose response and to elucidate the mechanism of the enhancement of resistance to antibiotics observed in microbial biofilms.

THE BACTERIAL BIOFILM

Defining a biofilm

Biofilms are defined as matrix-enclosed bacterial populations adherent to each other and/or to surfaces or interfaces. This definition includes microbial aggregates and floccules and also adherent populations within the pore spaces of porous media [47]. Direct observations of living biofilms (by optical and other non-invasive physical methods) done by several research groups have revealed a complex structure for the biofilm. It is composed of an infrastructure made of a polysaccharide matrix that holds together an elaborate system of microcolonies. These microcolonies are connected by water channels that function as a primitive circulatory system bringing nutrients from the bulk fluid and removing metabolic products [13].

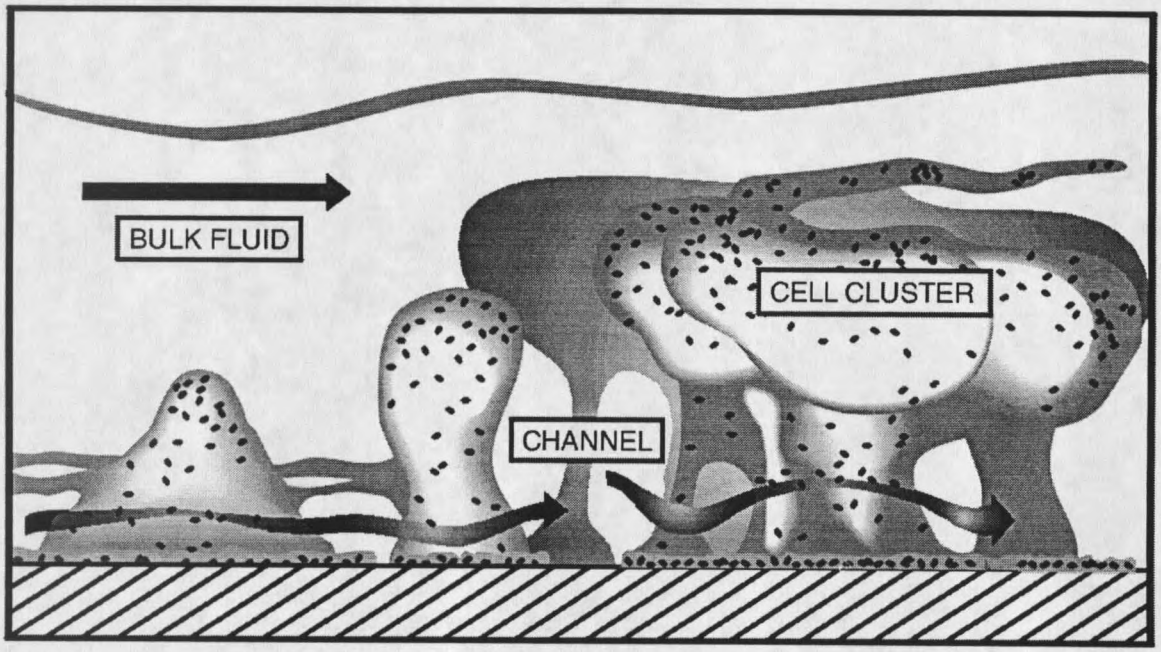

Figure 1: The bacterial biofilm.

It is understood today that biofilms are a distinct growth phase of bacteria which is quite different than the planktonic phase. Planktonic bacteria are those which are free

to move in their environment and have been the center of bacterial studies since Louis Pasteur's discoveries 15 decades ago. Biofilm forming bacteria are bacteria that adhere to surfaces. These bacteria are called sessile bacteria, and they are characterized by the production of a fibrous, highly hydrated, exopolysaccharide matrix whose chemical composition is species specific.

The biofilm is formed by the adhesion of planktonic bacteria to a surface. During the complex process of adhesion, the planktonic bacteria change their phenotype as a response to the surface [14]. During the early stages of biofilm formation, the sessile bacteria find themselves multiplying and forming colonies of single or multiple species. These cellular colonies and their exopolysaccharide will create the microniche of each bacterial biofilm. Different biofilm bacteria respond to their specific microniche in a different manner with different growth and adhesion patterns. Eventually, a structurally complex mature biofilm will develop.

Electron microscope studies and X-ray crystallographic analysis show the exopolysaccharide, also known as glycocalyx [15], to be an ordered array of fine fibers providing a thick, continuous, hydrated, polyanionic matrix around the cells [16]. This polysaccharide may contain one or more anionic uronic acids, and it is densely concentrated around the microcolonies of cells that have produced its polymer [17].

Pseudomonas aeruginosa

Because *P. aeruginosa* is the predominant autochthonous inhabitant of all naturally oligotrophic aquatic ecosystems, this remarkably adaptable bacterium is the most numerous organism on our planet [2]. *Pseudomonas aeruginosa* has emerged as a very important pathogen affecting a wide variety of patients whose defense against infection is compromised by trauma or by underlying disease [2].

Pseudomonas aeruginosa is a member of the genus *Pseudomonas. Pseudomonas* can be found in both soil and water. They are colonizers or pathogens of plants or animals, including man. Therefore, *Pseudomonas* are of medical importance. They are saprophytes with a low virulence which means that they are opportunists that produce a disease in patients with low or local resistance against infections. They may be found in infections at many body sites, the most common of which are the urinary tract, respiratory tract, wounds, and blood [18].

Pseudomonas are Gram-negative, asporogenous, rod-shaped bacterium, polar mono- or with polytrichous flagellation (18-20°). Their motility is at 18-20°. They are oxidase positive. They have oxidative glycolysis without gas formation, or no saccharolysis. They are catalase positive and indole negative. *P.aeruginosa* was first described by Schroter in 1872 [19]. It usually forms a blue-green pigment, pyocyanin, which is often visible in growth media and is a diagnostic for this species. It most commonly exhibits rough, flat grayish colonies with uneven borders (R-form), but variants with a smooth, dome-shaped and whiter appearance (S-form) may be observed. The sheen is a diagnostic of *P.aeruginosa.* The species has a typical penetrating aromatic smell reminiscent of spoiled fish. The smell is due to o-amino-aceto-phenon

and is particularly pronounced on peptone-rich media. *P.aeruginosa* has but one flagellum.

Table 1. Common characteristics of Pseudomonas aeruginosa [19].

COMMON CHARACTERISTICS OF *PSEUDOMONAS AERUGINOSA*
Gram negative
Rod shaped (straight, asporogenous, .5 - 1.0 x 1.5 - 4.0 μm)
Strict aerobes (some species may utilize nitrogen or arginine as terminal acceptor anaerobically)
Motile by polar flagella
Oxidase positive
Oxidative metabolism (mostly saccharolytic, some nonsaccharolytic species, no gas formation from sugars)
Chemoorganotrophs (facultative chemolithotrophs rare)
Catalase positive
Growth with acetate as sole carbon source, most nonfastidious, few require growth factors
NO_3 reduced to NO_2 or molecular N_2
Many species accumulate poly-β–hydroxybutyrate (PBHB)
Some species produce pigments
Indole negative
58 - 70 mol% G + C

Pseudomonas aeruginosa Biofilm

Bacteria stick tenaciously to any surface in a liquid or aqueous environment [20]. They do so by means of a mass of tangled fibers of polysaccharides, or branching sugar molecules, that extend from the bacterial surface and form a feltlike "glycocalyx" surrounding an individual cell or a colony of cells [20]. The bacterial glycocalyx can be defined as those structures, of bacterial origin, that contain polysaccharides and are lying outside the integral elements of the outer membrane of Gram-negative cells and the peptidoglycan of Gram-positive cells [16]. *Pseudomonas aeruginosa* produces a thick flexible peripheral capsular glycocalyx [16]. The precise chemical nature of the glycocalyx varies with the sugar composition of the fibers [16] and, therefore, it differs from cell to cell and probably also at different stages in the life of the individual cell.

The exopolysaccharide secreted by *Pseudomonas aeruginosa* is composed of alginate [15]. Linker and Jones, in 1966, were the first ones to report the production of alginate in *Pseudomonas aeruginosa* [21]. Alginate is a linear copolymer of β-1,4-linked D-mannuronic acid and its C-5 epimer L-guluronic acid [14].

BIOFILM GROWTH PROCEDURES

Growth Chamber Design

The growth chamber is a cylindrical, straight sided glass beaker that was modified slightly in the glass blowing shop at Montana State University. The modification was the addition of a glass overflow nozzle that was set to be just above the level of the growth solution in which the biofilms were formed. A second, thin walled polycarbonate cylinder was made that would slip into the glass cylinder but would have a snug fit to the inner wall of the glass cylinder. Slots were cut into the polycarbonate cylinder to support the slides upon which the biofilms were grown. Eight such slides could be inserted in the growth chamber in this arrangement which gave sufficient biofilms to have four controls and four "experimentals" with each experimental run.

The growth chamber was connected to two peristaltic pumps with peristaltic pump heads. The growth chamber was magnetically stirred and was connected to the nutrients, buffer and dilution water by MasterFlex tubing. Dilution water was stored in a plastic garbage can which was continuously suffused with air by using a fish aquarium oxygen pump.

Equipment

The equipment used for growing the biofilm is specified in the following table.

Table 2: Equipment List.

Equipment	Manufacturer	Model #	Qt.
Growth Chamber	MSU		1
Peristaltic Pump	Cole - Parmer Instrument Co.	MasterFlex Model 7553-70; 6-600 RPM	1
Peristaltic Pump	Cole - Parmer Instrument Co.	MasterFlex Model 7553-80; 1-100 RPM	1
Pump Head	Cole - Parmer Instrument Co.	MasterFlex Model 7016-20	1
Pump Head	Cole - Parmer Instrument Co.	MasterFlex Model 7014-20	2
Stirrer	Fisher Scientific		1
Tubing		MasterFlex	
Garbage Can	Rubbermaid	34 gal.	1
Air Pump	Rolf C. Hagen Corp. Mansfield, MA 02048	Model Elite 802	1

Materials and Methods

The material and methods used for growing the biofilm in all the experiments presented in this work are specified in the following two tables.

Growth Media

Table 3: Nutrients and Chemicals List.

CATEGORY	SOURCE	COMPONENT	AMOUNT
Buffer	Fisher Biotech, Fisher Chemical, Fair Lawn, NJ 07410	NaH_2PO_4	13.63 g/l
Buffer	Fisher Biotech, Fisher Chemical, Fair Lawn, NJ 07410	KH_2PO_4	6.56 g/l
Substrate	Fisher Biotech, Fisher Chemical, Fair Lawn, NJ 07410	KNO_3	0.43 g/l
Substrate	Aldrich Chemical Co., Inc. Milwaukee, WI 53233	$MgSO_4*7H_2O$	0.032 g/l
Substrate	J.T. Baker Chemical Co. St. Louis, MO 63178	$CaCO_3$	0.032 g/l
Substrate	Sigma Chemical Co. St. Louis, MO 63178	Glucose	0.64 g/l
Trace Elements	Sigma Chemical Co. St. Louis, MO 63178	$(HOCOCH_2)_3N$	1.28 g/l
Trace Elements	J.T. Baker Chemical Co. St. Louis, MO 63178	$(NH_4)_6Mo_7O_{24}*4H_2O$	8.96 mg/l
Trace Elements	J.T. Baker Chemical Co. St. Louis, MO 63178	$ZnSO_4*7H_2O$	0.91 g/l
Trace Elements	Sigma Chemical Co. St. Louis, MO 63178	$MnSO_4*H_2O$	72.96 mg/l
Trace Elements	J.T. Baker Chemical Co. St. Louis, MO 63178	$CuSO_4*5H_2O$	17.92 mg/l
Trace Elements	J.T. Baker Chemical Co. St. Louis, MO 63178	$Na_2B_4O_7*10H_2O$	8.96 mg/l
Trace Elements	J.T. Baker Chemical Co. St. Louis, MO 63178	$Co(NO_3)_2*6H_2O$	10.7 mg/l
Trace Elements	Fisher Biotech, Fisher Chemical, Fair Lawn, NJ 07410	$FeSO_4*7H_2O$	1.018 g/l

Methods

A strain of *Pseudomonas aeruginosa* (maintained in the Montana State University-Bozeman (MSU) Center for Biofilm Research as ERC-1) was used for these experiments. The bacteria was allowed to colonize polycarbonate slides within a growth chamber (described above) for 3 days, forming a biofilm. The procedure is described below:

Table 4: Procedure

	Procedure
Day 1	A sterile growth chamber, filled with buffered distilled water, 20 mg/l of glucose and various trace minerals (listed under materials) is inoculated with a 1 ml of frozen *Pseudomonas aeruginosa* culture. This "batch phase" continues for 24 hours with constant stirring.
Day 2	24 hours after inoculation, buffer, substrate and dilution water are fed continuously into the reactor at a dilution rate of 30:1 .
Day 3	Make sure the dilution water is flowing.
Day 4	Make sure the dilution water is flowing.
Day 5	After approximately 72 hours' growth, the polycarbonate slides, with the attached bacterial biofilm, are transferred aseptically to the reaction chambers for the DC electrical current experiments.