This is to certify that the
thesis entitled

STUDY OF SPORICIDAL PROPERTIES OF CROSSLINKED POLYELECTROLYTE MULTILAYERS

presented by

DEEBIKA BALU

has been accepted towards fulfillment
of the requirements for the

MASTER OF SCIENCE degree in CLINICAL LABORATORY SCIENCES

Major Professor's Signature

7/28/10

Date

MSU is an Affirmative Action/Equal Opportunity Employer

STUDY OF SPORICIDAL PROPERTIES OF CROSSLINKED POLYELECTROLYTE MULTILAYERS

By

DEEBIKA BALU

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Clinical Laboratory Science

2010

ABSTRACT

STUDY OF SPORICIDAL PROPERTIES OF CROSSLINKED POLYELECTROLYTE MULTILAYERS

By

DEEBIKA BALU

Polyelectrolyte multilayers (PEM) have become a highly studied class of materials due to the range of their applicability in many areas of research, including biology, chemistry and materials science. Recent advances in surface coatings have enabled modification of PEM surfaces to provide desirable properties such as controlled release, super-hydrophobicity, biocompatibility, antifouling and antibacterial properties. In the past decade, antimicrobial PEM coatings have been investigated as a safer alternative to the traditional disinfection methods that usually involve application of hazardous chemicals onto the surface to be cleaned. These antimicrobial coatings could be applied to common surfaces prone to colonization of bacteria (such as bench tops, faucet handles, etc) to supplement routine sanitization protocols by providing sustained antimicrobial activity.

Vegetative bacteria (such as *Escherichia coli*) are more susceptible to antimicrobial agents than bacterial species that form spores. Hence, the antimicrobial activity of PEM coatings fabricated using Layer by Layer (LbL) technique were assayed using *Bacillus anthracis spores* (Sterne strain). In this thesis, the sporicidal effect of various polyelectrolyte multilayer coatings containing cross-linked polymers immersed in bleach have been evaluated as potential augmentation to existing disinfection methods.

ACKNOWLEDGEMENTS

This research would not have been possible without the constant support and help I received from many people over the past two years. Although I might not have mentioned explicitly the names of all the people who have made me possible to come this far, I do remember their contributions to my research and life.

First I would like to thank my research advisor Dr. Stephen Cendrowski, who gave me a research project for me to work. His appreciation coupled with critical evaluation of my work has enabled me to present my research project as a thesis. I would like to thank my academic advisor and my committee member Dr. David Thorne for his time and help which helped me to select the right courses that would be helpful for my future years as a researcher. I would also like to thank him for his pertinent questions and useful feedback regarding my thesis progress. I would like to my other committee member thank Dr. Steven Kaganove for his critical evaluation and support. I would specifically like to thank him for helping me to interpret complex FTIR spectra.

The unwavering support of my family and friends during these years at MSU enabled me to survive through all these years of graduate school. I would like to thank my parents and my husband Saravan for letting me study whatever and wherever I want. My husband had a number of indirect contributions to my research life. I am thankful to him for the technical, emotional and (logistics!) support he provided.

I am grateful to Keri Niec and Elena Sias for their all their help in making my transition into the new research work easier than what I had expected. I also would like to mention the lovely times with Carmen Yu who made it less creepy to work late hours in Kedzie Hall. I would also like to acknowledge a number of staff at BLD who made the two years in the program fly by. Most of all I enjoyed taking the online courses in BLD department from Dr. Gerlach and Dr. Thorne, which made me get a new perspective on molecular pathology, immunology and cell biology.

I would like to thank the BLD department for accepting me into their program which enabled me to take a number of biochemistry courses and complete my research project. I would like to also thank Dr. Keith Lookingland, Dr. John Goudreau and Dr. Seong-Woon Yu for their help and support in providing recommendation letters for my PhD programs. I would like to thank them for an excellent research opportunity they had provided for about a year on neurodegenerative disease mechanisms. It enabled my development as an independent researcher. I would also like to thanks their lab members for their encouragement and technical support for my research work. Last, I would like to thanks the Office of Radiation, Chemical and Biological Safety (ORCBS) for providing me with a graduate assistantship. I learnt a number of things that I used to neglect when I was a researcher! My special thanks to my closer colleagues at the ORCBS: Elvet Potter, Genevieve Cottrell and my supervisor Bob Ceru for providing an amiable work environment and for understanding my graduate school work schedule.

TABLE OF CONTENTS

LIST OF TABLES ..... **VII**

LIST OF FIGURES ..... **VIII**

CHAPTER 1 INTRODUCTION **1**
1.1 HOSPITAL ACQUIRED INFECTIONS 1
1.2 ORGANIZATION OF THESIS 3

CHAPTER 2 SPORES AS A HEALTH HAZARD **4**
2.1 INTRODUCTION 4
2.2 STRUCTURE OF A SPORE 4
2.2.1 Spore Core 6
2.2.2 Inner Membrane 7
2.2.3 Cortex and germ cell wall 8
2.2.4 Outer Membrane 9
2.2.5 Coat 9
2.2.6 Exosporium 9
2.2.7 Concluding remarks 10
2.3 SPORULATION AND GERMINATION 11
2.3.1 Sporulation 11
2.3.2 The process of germination 12
2.4 ANTIMICROBIAL AGENTS 13
2.4.1 Introduction 13
2.4.2 Definition of antimicrobial agents 13
2.4.3 Common antimicrobials and their mode of action 15
2.4.4 Concluding remarks 19

CHAPTER 3 BIOFILMS AND ANTIMICROBIAL PEM(S) **23**
3.1 INTRODUCTION: 23
3.2 BACTERIAL BIOFILMS AND SPORES 23
3.3 ANTIMICROBIAL PEM(S) 25
3.3.1 Adhesion-resistant PEMs 25
3.3.2 Biocide leaching PEMs 27
3.3.3 Contact killing PEMs 27
3.4 FABRICATION OF PEMS 29
3.4.1 Properties of pH tunable PEMs 30
3.4.2 Properties of N-halamies grafted onto PAMAM dendrimers 31

CHAPTER 4 EXPERIMENTAL METHODS AND RESULTS **35**
4.1 INTRODUCTION 35
4.2 MATERIALS AND METHODS 35
4.2.1 PEM construction materials 35

4.2.2 Fabrication procedure 36
4.2.3 Testing the coatings for antimicrobial efficiency 37
4.2.4 Calculating antimicrobial efficiency 37
4.2.5 Fourier Transform Infrared (FTIR) spectroscopy 38
4.3 RESULTS 38
4.3.1 [PAA/PAH]5 bilayers with varying topmost layer that contacts the spores ... 38
4.3.2 Effect of [PAA/PAH] and [PSS/PAH] bilayers with PAA or PSS as topmost layer and heat treatment 42
4.3.3 Effect of number of bilayers 45
4.3.4 Coatings formulated with other common polyelectrolytes 48
4.3.5 FTIR spectra analysis 52
4.3.6 Shelf life issues 56
4.3.7 Conclusions 58
4.3.8 Future work 59

APPENDIX A **61**
PROTOCOL FOR PREPARING BHI-AGAR PLATES 61

APPENDIX B **62**
PROTOCOL FOR SPORE INCUBATION IN PP PLATES AND FILTER MEMBRANES 62

APPENDIX C **64**
TEST TO DETERMINE THE SPORICIDAL COMPONENT IN THE COATINGS.. 64

APPENDIX D **67**
CALCULATIONS FOR % REDUCTION IN CFU VALUES 67

BIBLIOGRAPHY **70**

LIST OF TABLES

Table 2.2 Sporistatic agents and their mechanism of action [42] 20

Table 2.3 Sporicidal agents and their mechanism of action [42] 21

Table 4.1 PEM Coatings tested with at least five [PAA/PAH] bilayers 39

Table 4.2 PEM Coatings with 2, 5 and 10 bilayers with PAA as topmost layer 43

Table 4.3 PEM coatings with varying number of bilayers 46

Table 4.4 PEM Coatings [PSS/PAH] bilayers and [PAA/PAH] with PSS as topmost layer 49

Table 4.5 PEM coatings with variants containing having PEI, G4PAMAM and HBPU. 51

Table 4.6 Coatings that has shown evidence of antimicrobial activity compared to the control coatings (without bleach) 59

Table C.1 PEM coatings tested to determine sporicidal agent 65

Table D.1 Table of actual spore counts obtained during testing the coatings 3A-4B 67

Table D.2 Actual spore counts for coatings 3A-4B 68

LIST OF FIGURES

IMAGES IN THIS THESIS ARE PRESENTED IN COLOR

Figure 2.1 Scanning Electron Microscopy (SEM) images of: A. *Bacillus cereus*; B. *Bacillus subtilis* ; and C. *Bacillus anthracis*. The exosporium of *Bacillus cereus* is visible as a loosely bound membrane on the spore surface. The endospores structure is not easily visible in the SEM image. *Bacillus anthracis* endospores are seen under phase contrast microscopy as lighter areas, because they are dehydrated and therefore more refractile. Photo credit: Larry Stauffer, Oregon State Public Health Laboratory..... 4

Figure 2.2 Schematic diagram showing the internal structure of an endospore. The size of the layers has not been drawn according to scale and the thickness of each layer varies across species..... 5

Figure 2.3 Electron Micrograph image of a bacterial endospore [14]..... 5

Figure 2.4 Life cycle of *Bacillus subtilis*. Note that this diagram depicts key stages in the sporulation process [17]..... 12

Figure 3.1 Scanning electron micrographs of biofilms formed on common surfaces. a) Scanning electron micrograph of an infected catheter showing dense and complex biofilm on the extra luminal surface [52]. b) Scanning electron micrograph of a Staphylococcus biofilm on the inner surface of a needleless connector. Photo credits: Jance Carr, CDC, Atlanta, GA (USA) [53]..... 25

Figure 3.2 Structures of common polyelectrolytes used in this study. a) PEI b) PAH c) PSS d) PAA [68]..... 30

Figure 3.3 A schematic of layer by Layer deposition technique. The polyelectrolyte multilayers are synthesized by a repeated charging of the surface with oppositely charged ions until a desired number of layers is deposited. Each step results in a reversal of surface charge allowing the next layer to be deposited [46]..... 31

Figure 3.4 Structure of 5,5 dimethyl hydantoin and its halogenated derivative – 1,3 dichloro- 5,5, dimethyl hydantoin..... 33

Figure 3.5 Schematic representations of a a) dendrimer and b) a hyperbranched polymer molecule. The core is represented by the grey molecule in the center with multiple functional end groups..... 33

Figure 3.6 A diagram of PEM assembly as an antimicrobial surface. Polyelectrolyte layers are coated onto the substrate. A final layer is applied made of a PAMAM dendrimer with hydantoin appended to the end-group..... 34

Figure 4.1 Spore counts in PEM coatings (Set 1, 2). Spore count data represent mean +/- S.D. of three independent experiments. 40
Figure 4.2 Spore counts in PEM/G4-PAMAM coatings (Set 3, 4, 5). Spore count data represent mean +/- S.D. of three independent experiments. The plot also has the data represented as set numbers (3, 4, 5) along with topmost layer of the PEM that contacts the spores. The set of coatings with "B" in the coating ID contain alanine and inosine (germinants). 41

Figure 4.3 Spore counts in PEM coatings without or with PAA as topmost surface that contacts the spores. Spore count data represent mean +/- S.D. of three independent experiments. 44

Figure 4.4 Spore counts in PEM coatings with varying number of bilayers (2, 5, or 7 [PAA/PAH] bilayers. Spore count data represent mean +/- S.D. of three independent experiments. 45

Figure 4.5 Spore counts in PEM coatings with varying number of bilayer. Spore count data represent mean +/- S.D. of three independent experiments. 47

Figure 4.6 A plot showing the effect of varying the bilayers on sporicidal activity. [PAA/PAH] coating variants with varying number of bilayers were tested for sporicidal activity. The difference in sporicidal activity between the sporicidal coatings and their respective controls were plotted against the number of bilayers. 48

Figure 4.7 Spore counts in PEM coatings. Spore count data represent mean +/- S.D. of three independent experiments. 50

Figure 4.8 Spore counts in PEM coatings with varying topmost layer. Spore count data represent mean +/- S.D. of three independent experiments. These set of coatings have four [PAA/PAH] bilayers and differ in the layer that contacts the spores 52

Figure 4.9 FTIR-ERS spectra of $[PAH/PAA]_5$ multilayer films on gold (a) as prepared, (b) after heating at 120 °C overnight, and (c) after heating at 180 °C overnight. 53

Figure 4.10 FTIR-ERS spectra of a [PAH/PAA]5 multilayer film on gold (a) as-prepared, (b) after heating at 120 °C overnight, and (c) after heating at 180 °C overnight. 54

Figure 4.11 FTIR-ERS spectra of a $[PAH/PAA]_5$ multilayer film on gold. (a) After heating at 120 °C overnight, and (b) after treatment with NaOCl. 55

Figure 4.12 FTIR-ERS spectra expanded around the carbonyl region of a $[PAH/PAA]_5$ multilayer film on gold. (a) after heating at 120 °C overnight, and (b) after treatment with NaOCl. 56

Figure 4.13 PEM coatings tested for shelf-life issues. Sporicidal coatings with five [PAA/PAH] bilayers having either PAA or G4PAMAM/PAA as the topmost layer were

retested for sporicidal activity after 6 months from initial testing. Spore count data represent mean +/- S.D. of three independent experiments.....57

Figure C.1 Spore counts in PEM coatings.....65

Figure C.2 Spores sticking onto pipet tips could be visualized when stained with methylene blue.....66

CHAPTER 1 INTRODUCTION

1.1 HOSPITAL ACQUIRED INFECTIONS

Hospital-acquired infections (HAIs), also called nosocomial infections, are infections acquired by a patient in the hospital and are not related to the condition for which the patient was originally admitted [1]. It is estimated that about 1.7 million HAIs occurred in the United States in 2002, of which 78% were due to respiratory tract, urinary tract, bloodstream, and surgical site infections [1]. Thus, HAIs have a significant impact on both the patient health as well as the health care system. For the patient, HAI results in an increased length of stay due to additional medical procedures. In severe cases, HAIs can lead to septicemia or death [2]. For the health care system, such infections increase treatment costs. HAIs increase the cost per patient by $1,909 to $38,656 and increase length of stay in the intensive care unit by 4.3 to 15.6 days [3,4,5]. A significant percentage of these infections can be attributed to bacterial colonization and biofilm formation on critical items such as surgical instruments, cardiac catheters and urinary catheters. In addition to these invasive instruments, environmental surfaces such as bed pans, bed rails, sinks, patient furniture and floors can also be potential sources of infection [6].

Biofilms that develop preferentially on surfaces of implants, bed rails and sinks result from bacterial colonization of these inert surfaces, and they contain aggregates of bacterial colonies within organic matter synthesized by these organisms to form a complex multi-cellular structure [7]. Biofilms acts as a barrier to common disinfectants

such as chlorine and hydrogen peroxide by neutralizing these chemicals in the surface layers, thereby limiting the diffusion of the disinfectant into the deeper layers [8]. Hence one of the key strategies to prevent HAIs is to prevent biofilm formation. Disinfectants such as hypochlorites and hydrogen peroxides are commonly used to prevent biofilm formation on non-medical devices and are also the primary choice for post-contamination applications in case of accidental exposure to biohazards. These traditional measures require constant vigilance and training of personnel for routine disinfections and emergencies. Also, these disinfectants are highly corrosive or toxic, which endangers the health of the personnel involved in such procedures, as well as other individuals in the proximity of the disinfected area at risk [9]. Thus applying the disinfectant in a form that is safe to use for the personnel and the environment, as well as preventing biofilm formation is clearly a better alternative to the traditional disinfection methods. Furthermore, if the contact surface to which the bacteria adhere were self-disinfecting, it could supplement routine sanitization protocols by providing sustained antimicrobial activity.

Over the past decade, surfaces of multilayered polyelectrolyte films have been modified to provide desirable properties such as controlled release, hydrophobicity, biocompatibility [10], antifouling properties [11] and antibacterial properties. A readily-available formulation of silver-zinc zeolite (AgION Technologies, Inc, Wakefield, MA) has proven effective against a variety of pathogens in a variety of environments, and has been incorporated in a number of materials for use in healthcare [12]. Most of these antimicrobial products are produced by chemical surface modification techniques [13].

Layer-by-layer (LbL) assembled polyelectrolyte multilayers (PEM) have also been shown to provide effective antibacterial properties. For example Chua et al. have successfully produced an antibacterial coating on titanium alloy, used for orthopedic implants [14]. Common materials such as stainless steel and glass have also been used as substrates for a number of antimicrobial PEM. Thus, the LbL process has been used to create PEMs ideally suited for controlling bacterial adhesion, and it is also amenable to incorporation of a broad range of polymers, small biomolecules [15] and even cells [16].

The major goal of this research was to identify important characteristics that influence sporicidal properties of PEM such as the time of contact, number of layers and composition of the multilayers. Thermal cross-linking of PEMs, assembled using LbL technique was investigated as an alternative to chemically modified surfaces. By thermal cross-linking, the PEM coatings can remain intact at physiological pH ranges. The PEMs were assessed for sporicidal activity using *Bacillus anthracis* Sterne 34F2 spores, an attenuated strain as a model organism.

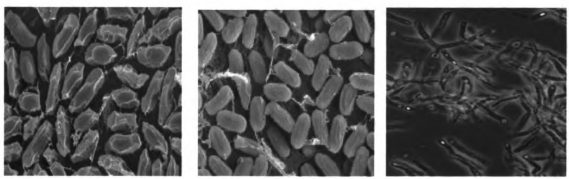
1.2 ORGANIZATION OF THESIS

The first chapter provides a brief overview of pathogenesis, sporulation and germination mechanisms of *Bacillus anthracis.* The second chapter details the basic structure of a spore, requirements for spore formation, sporulation and germination mechanisms. The third chapter discusses the strategies for disinfection and also provides an overview of PEM coatings. The fourth chapter discusses the results obtained during the study and future directions for the research.

CHAPTER 2 SPORES AS A HEALTH HAZARD

2.1 INTRODUCTION

Bacterial spores (Fig 2.1) are highly specialized cell types designed for survival under adverse environmental conditions. Among specialized differentiated cell types, such as cysts and exospores, the endospores formed inside the bacteria are the most resistant [17]. Spores remain dormant and resist adverse conditions, such as starvation, high temperatures, ionizing radiation, mechanical abrasion, chemical solvents, detergents, hydrolytic enzymes, desiccation, pH extremes and antibiotics [17]. Their metabolic dormancy combined with their resistance enables their survival for long periods of time.



a b c

Figure 2.1 Scanning Electron Microscopy (SEM) images of: a) *Bacillus cereus*; b) *Bacillus subtilis* ; and c) *Bacillus anthracis*. The exosporium of *Bacillus cereus* is visible as a loosely bound membrane on the spore surface. The endospores structure is not easily visible in the SEM image. *Bacillus anthracis* endospores are seen under phase contrast microscopy as lighter areas, because they are dehydrated and therefore more refractile. Photo credit: Larry Stauffer, Oregon State Public Health Laboratory.

2.2 STRUCTURE OF A SPORE

The resistance of a spore to extreme conditions is due to the presence of multiple layers. The concentric layers starting from the core are the inner membrane, germ cell wall,

cortex, outer membrane, and the spore coat (Figure 2.2). An electron micrograph image of bacterial endospores shows multiple layers of varying density (Figure 2.3).

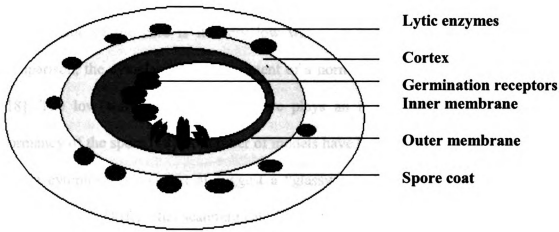


Figure 2.2 Schematic diagram showing the internal structure of an endospore. The size of the layers has not been drawn according to scale and the thickness of each layer varies across species.

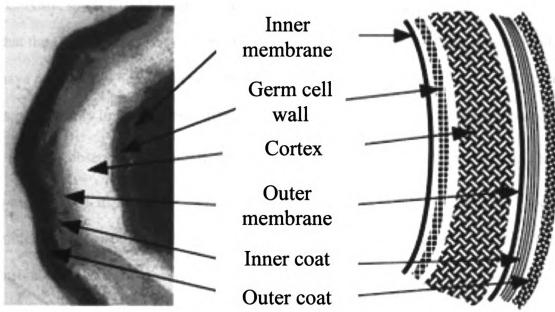


Figure 2.3 Electron Micrograph image of a bacterial endospore [14]

2.2.1 Spore Core

The core forms the innermost part of the spore similar to the cytoplasm of vegetative cells containing essential proteins and nucleic acids. The most distinguishing characteristic of the core is its very low water content, which is about 30-50%. In comparison, the cytoplasmic water content of a normal vegetative cell is about 70-88% [18]. The low water content in the core plays an important role in longevity and dormancy of the spore [18]. A number of models have been proposed to discuss the state of the cytoplasm. Cowen et al suggest a "glassy state" that promotes dormancy and resistance [19]. Differential scanning calorimetry (DSC) and nuclear magnetic resonance (NMR) studies on *B. subtilis* spores indicate that the dormancy is due to the low moisture content and glassy state of the core [20]. The two peaks in the DSC thermograms of hydrated spores correspond to the activation stage at 60°C, and the killing stage at 120 °C. NMR spectroscopy studies on calcium dipicolinic acid (Ca DPA) in the core suggest that the CaDPA is present in an amorphous solid-like environment [20]. However, others have proposed the idea that core water freely crosses the membrane [21, 22]. Phosphorus is present only in the core and change in molecular mobility of radio-labeled phosphorus (^{31}P) was observed when the spores were hydrated (or dehydrated), whereas no change was observed in molecular mobility of dipicolinic acid (DPA) (another core-specific molecule). Hence, these models propose the existence of DPA immobilized in a water-insoluble network of macromolecules. Further research is needed to reveal the internal structure of a spore and how the environment contributes to the structural stability.

Apart from low water content, the core lacks most of the high energy compounds such as

ATP and NADH that are usually present in the cytoplasm of a vegetative cell [18]and is characterized by low pH levels (6.3-6.5) [23]. Pyridine 2,6-dicarboxylic acid (commonly called dipicolinic acid or DPA) has been shown to have an important role in spore resistance, although the exact mechanism is not yet completely understood [24]. The DPA present in the core chelates with divalent ions such as calcium, and this complex accounts for about 15% of the dry weight of the spore. Calcium ions and DPA are secreted during germination, and may play a role in subsequent germination steps [25]. The core also houses small acid soluble proteins (SASP), similar to histone proteins in eukaryotic cells, that bind to the DNA [26], condensing DNA to protect it against damages that may occur during harsh conditions [27].

2.2.2 Inner Membrane

The inner membrane of a spore, like the plasma membrane of a eukaryotic cell, is a selectively permeable membrane surrounding the core. The inner membrane contains germination receptor proteins, which play an important role during the first stages of germination. They have been shown to play a role in signal transduction across the membranes to other parts of the spore, upon activation of germination.

An intact inner membrane is essential for germination [28]. Upon germination under favorable conditions, the inner membrane becomes the cytoplasmic membrane of the vegetative cell. During germination the inner membrane swells to double its size without membrane lipid synthesis. Hence, it has been proposed that the inner membrane of the spore must be in a highly compressed state due to decreased lipid membrane mobility

[29]. Oxidizing agents such as chlorine dioxide, hydrogen peroxide, ozone, and sodium hypochlorite may result in inner membrane damage that contributes to spore killing upon germination.

2.2.3 Cortex and germ cell wall

The cortex is a thick cell wall around the inner membrane and is mainly composed of modified peptidoglycan (PG). It plays an important role in maintaining the core in a highly dehydrated state [20]. The structure of the cortex is highly conserved among species, and has been suggested to play an important role in heat resistance. There are two major modifications to PG in the spore cortex that appear to be important in germination [30]. First, only 3% of the muramic acid present in PG of the cortex exists in the cross-linked state, in comparison to 40% in the vegetative cell wall [30]. Second, most of the muramic acid is modified to a muramic-β-lactam structure [30]. Muramic-β-lactam acts as a specific target for lytic enzymes that are activated during germination and the lower cross-linking enables easier outgrowth.

Early stages of spore germination involve changes in permeability of the membrane, resulting in redistribution of ions and water in the spore. It has been proposed that the ions and water may activate the lytic enzymes in the spore coat, resulting in degradation of the cortex PG [30]. The inner part of the cortex called the germ cell wall, lacks muramic acid lactam and muramic acid alanine in PG that are characteristic of the cortex. The germ cell wall forms the primordial cell wall of the freshly germinated cell [31].

2.2.4 Outer Membrane

The outer membrane separates the cortex from the spore coat and is an essential structure for spore formation [32]. Recent work has suggested that the outer membrane acts as a major permeability barrier in spores [33]. An intact outer membrane is required for dormancy in *B. megaterium* spores [34].

2.2.5 Coat

The inner layer of the coat harbors the lytic enzymes that degrade the cortex during germination [17]. The coat has also been shown to be resistant to UV-radiation and to a variety of chemicals (including oxidizing agents such as hypochlorite) [35]. Atomic Force Microscopy studies of *B. thuringiensis* have revealed that the spore coat expands and shrinks in response to changes in relative humidity [22]. Several coat proteins are involved in germination by facilitating the passage of germinant molecules through the coat [36].

2.2.6 Exosporium

The exosporium is a species-specific structure. For example, *Bacillus cereus* and *Bacillus anthracis* possess an exosporium while *Bacillus subtilis* does not. The exosporium is essential for maintaining the hydrophobicity of the spore [37]. Substrate and spore hydrophobicity, and surface tension contribute to non-specific interactions which may eventually result in bacterial adhesion to substrates [37]. Bacterial adhesion has been a major concern for the medical, pharmaceutical and food industries. The ability of *Clostridium* spores to adhere to surfaces and resist high temperatures compromises the

sterility of the processed food and results in food- borne illness. Also, bacterial transfer from the environment to hospital surfaces such as catheters used in patients has been associated with increase in hospital acquired infections.

The exosporium is visible as a loose membrane-like structure around the coat (Fig 1.1). Spore resistance is further enhanced by the presence of a manganese oxidizing enzyme in the exosporium that creates a "metal shield" over the internal layers [38]. Certain enzymes in the exosporium have been shown to be involved in germination [39]. The homolog of exosporium protein (ExsA) in *Bacillus subtilis* is required for proper assembly of the coat [40], and it has been suggested that the exosporium could be considered as a specialized coat layer. It should be noted that ExsA is required for adherence of the exosporium to the coat.

2.2.7 Concluding remarks

Bacterial spore formation is the ultimate survival mechanism, and it has been studied for more than a century. The resistance of bacterial spores is due to the presence of multiple protective layers over a highly dehydrated core (Fig 2.2). Several spore-forming bacteria such as *Bacillus* and *Clostridium spp.* have pathogenic properties that present a health hazard. Dormant bacillus spores are extremely difficult to destroy due to their resistance to heat, UV radiation, alkylating agents and oxidizing agents [18]. Given that spore formation is essential for both survival and dispersal of the bacteria, spores clearly are a major problem in settings that require sterility and hygiene especially in healthcare and food industries. A brief discussion of sporulation and germination processes in bacteria is

presented in the following sections.

2.3 SPORULATION AND GERMINATION

2.3.1 Sporulation

Sporulation is defined as the process of spore formation, and has been well documented in *B. subtilis*. Like other bacillus species, the *B. subtilis* exists in two forms: a vegetative cell and a dormant endospore (formed by sporulation). In the presence of sufficient nutrients and other favorable conditions (physiological temperature, pH), the vegetative cell undergoes division. However, unfavorable conditions (such as high temperature, high population density) trigger the bacterial cell to undergo spore formation.

An overview of the process is illustrated in Fig 2.4 [32]. Asymmetric cell division of the bacterial cell (Stage II - Fig 2.4) is triggered by phosphorylation of transcription regulator Spo0A along with σH - an RNA polymerase co-transcriptional factor. This produces two distinct cells, the smaller pre-spore that ultimately develops into the spore, and the mother cell that ultimately undergoes programmed lysis). Sigma factors in the mother cell (σ_E) and the prespore (σ_F) trigger expression of distinct genes involved in sporulation. This process is followed by engulfment of the pre-spore by the mother cell (Stage III). After engulfment, changes in gene expression and morphogenesis occur in the pre-spore leading to formation of distinct cell membrane, cell wall, cortex and spore coat (Stage IV-V). The lysis of the mother cell (Stage VI, VII) releases the mature spore into the environment.

2.3.2 The process of germination

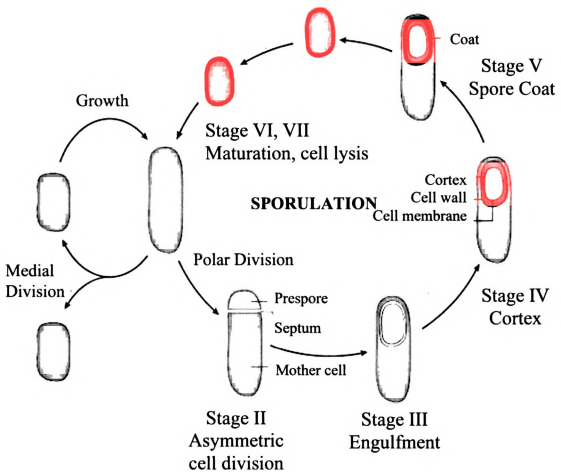


Figure 2.4 Life cycle of *Bacillus subtilis*. Note that this diagram depicts key stages in the sporulation process [17]

A germinant molecule binds to germinant receptors and this leads to rapid, irreversible changes in the spore, ultimately resulting in a fully germinated bacterial cell. In natural conditions, nutrients such as amino acids and sugars activate the GerA receptors in the inner membrane [39]. Ca^{2+} and DPA are released from the core as it becomes partially rehydrated [35]. Further rehydration is facilitated by the degradation of the cortex by lytic

enzymes. Rehydration restores the normal metabolic state of the bacterial cells via restoration of enzymatic activity

2.4 ANTIMICROBIAL AGENTS

2.4.1 Introduction

As described in the previous sections, spore formation allows the bacteria to exist in a protected state in adverse conditions. For example, *Bacillus cereus* food poisoning occurs because heat-resistant endospores survive cooking or pasteurization. The spores germinate when food is inadequately refrigerated and release toxins when the food is ingested. However, food poisoning due to spores may be avoided by cooking and storing food at right temperatures. Since hospital environments demand good hygiene, disinfection of contaminated surfaces may be achieved by chemical methods that usually require a combination of one or more chemicals that are bactericidal or sporicidal agents. However, preventive decontamination of an entire building on a regular basis may not be possible. Typical chemicals used in decontamination are based on bleach, chlorinated solvents or corrosive chemicals. Most of the bactericidal agents (such as phenol, alcohol and aldehydes) are not sporicidal even when applied to the surface for a long time [9].

2.4.2 Definition of antimicrobial agents

Commonly used antimicrobial agents may be physical conditions, chemical agents, or combinations of both. Physical antimicrobial agents include moist heat, dry heat, ultraviolet and ionizing radiation, and high pressure to reduce microbial activity [41].

Depending on their intended use, chemical agents may be classified as antibiotic agents, antiseptics, food preservatives, sanitizers, disinfectants or sterilizers (sporicides) [41]. Antibiotics such as penicillin are drugs taken orally or intravenously to treat diseases of humans, animals and plants. Antiseptics are substances applied topically to living tissues to prevent or arrest the growth or action of microorganisms. Food preservatives are chemicals used in foods, alone or along with physical agents, to extend the shelf life of food products. A sanitizer is commonly used to reduce, but not necessarily to eliminate, the number of bacterial contaminants to levels that are considered safe by public health regulatory agencies. Disinfectants are chemical or physical agents that inactivate disease causing microorganisms, but not necessarily their spores. Sterilizing agents or sporicidal agents eliminate all forms of microbial life, including bacterial spores [9].

Based on their specific activity against bacterial spores, rather than their intended area of application, antibacterial agents may be broadly classified as **bactericidal or sporistatic agents** such as phenols, quaternary ammonium compounds, alcohols, organic acids and esters; or **sporicidal agents** such as glutaraldehyde, formaldehyde, peroxygens, iodine compounds, fumigating chemicals (e.g., ethylene oxide), chlorine releasing compounds (e.g., hypochlorite, chlorine gas, halogens) [9]. Bactericidal agents are effective against vegetative bacteria by inhibiting germination or outgrowth (sporistatic) while sporicidal agents are effective against spores that resist many harsh chemical and physical conditions.

Biocides vary in their chemical structures and also in their mode of action. However, their responses when lethal concentrations are used show many similarities. Biocides must reach and interact with their microbial target site(s) to be effective. Although most biocides are nonspecific, some antimicrobial agents interact with specific enzymes or cellular components. Irrespective of the type of microbe, it is highly likely that there exists a common sequence of events leading to reduction in microbial population. The end effect mostly depends on the interaction of the disinfectant with the cell surface and its penetration into the cell. After its penetration, the disinfectant may have a target site(s) of action. For example, gluteraldehyde interacts with the hydrophobic spore coat resulting in increased penetration into inner layers [42]. Thus, the outermost layers of microbial cells can have a significant effect on their relative susceptibility to antiseptics and disinfectants.

2.4.3 Common antimicrobials and their mode of action

In this section, the mechanisms of antimicrobial action of a range of chemical agents used as antiseptics, disinfectants or both, are discussed. Finally, the mechanism of action of common sporistatic and sporicidal compounds has been tabulated (Table 2.1 and Table 2.2).

2.4.3.1 Acids

Organic (benzoic, acetic and citric) and inorganic acids (sulfuric, hydrochloric) acids have been used widely as preservatives and in cosmetics and food processing industries and to a lesser extent as commercial disinfectants. For example, benzoic acid is usually used in a salt form (sodium benzoate) in food products as a preservative and acetic acid is

commonly used as a preservative in the form of vinegar, which contains about 4% acetic acid. In general, short chain acids (methyl and ethyl derivatives) are less effective than long chained acids but have higher solubility in water.

The antimicrobial activity of acids and their derivatives depend on their solubility in water or lipid, which in turn influences their end effect when applied as chemical formulations [41]. These acids disrupt the structure of membranes and proteins thereby interfering with the cellular uptake of substrate molecules in bacteria, eventually result in killing by interfering with key metabolic processes [43]. Since they remain effective in a wide range of pH (pH 4.0 to 8.0), they are attractive for use as preservatives. Most of these acid derivates are non toxic and non corrosive in the concentrations in which they are typically used [42].

2.4.3.2 Alcohols

Alcohol based disinfectants containing 60 – 90% ethyl alcohol or isopropyl alcohol are commonly used for rapid broad spectrum activity against vegetative bacteria [42]. Their use is restricted to hard surface disinfection and skin antiseptic since they lack sporicidal activity [44]. They are usually applied in formulations containing other disinfectants (e.g., chlorohexidine), which does not evaporate and remains on the skin to render anti-microbial activity. It is hypothesized that alcohols cause denaturation of proteins, consequently disrupting the membranes. This could result in disruption of metabolic processes across the membranes, and eventually lysis of the cell.

2.4.3.3 Aldehydes

Broad spectrum antimicrobials such as gluteraldehyde and formaldehyde target proteins and nucleic acids leading to inhibition of important metabolic processes. Although formaldehyde is both sporicidal and bactericidal, it is not as effective as gluteraldehyde. Both have been shown to be effective under alkaline conditions [42]. Formaldehyde interacts with the amide and amino groups in proteins, disrupting the membrane and penetrating into the layers. It is also considered mutagenic since it extensively interacts with nucleic acids [42].

2.4.3.4 Quaternary Ammonium Compounds

The Quarternary Ammonium Compounds (QACs) are derived from exhaustive alkylation of amines. The chain length of the alkyl groups influences the antimicrobial properties, as well as solubility of the compound. Alkyl groups containing 11 to 17 carbons have been shown to have the highest antimicrobial activity [44]. Cationic surfactants such as QACs are the disinfectants of choice for disinfection of non critical surfaces and for preoperative disinfection of intact skin. Upon adsorption and penetration into the cell wall, the long alkyl chains of the QACs react with phospholipids and proteins in the cytoplasmic membrane [42]. This degrades the membranes and eventually results in cell wall lysis due to lytic enzymes. In this process, there is loss of cellular components as well as cell wall degradation. QACS being sporistatic, inhibit the outgrowth of vegetative cell from germinated spore.

2.4.3.5 Chlorine Compounds.

Chlorine and iodine compounds are the most commonly used chemicals in clinical

settings as disinfectants and as antiseptics. Chlorine compounds are broadly classified as chlorine gas, hypochlorites (e.g., sodium hypochlorite) and chlorine releasing agents (e.g., dichlorodimethyl hydantoin and chloramines) [9]. Their activity is dependent on their solubility, available chlorine and the pH of the environment.

As a strong oxidizer, chlorine gas reacts with any organic matter present, which can decrease its efficacy as a antimicrobial. For example, chlorine gas oxidizes organic and phenolic compounds in the environment before it interacts with microbial surfaces [45]. Since it is toxic and corrosive it has limited applications as commercial disinfectant [41]. On the other hand, sodium hypochlorite is relatively safer to handle than gaseous chlorine, and hence is widely used in clinical settings. Sodium hypochlorite (commonly known as bleach) is widely used for cleaning inert surfaces. The active agent in sodium hypochlorite is hypochlorous acid, which is formed by hydrolysis of hypochlorite ion. Hypochlorous acid is a weak acid, but a powerful oxidant, hence making the hypochlorites bactericidal and sporicidal [41]. Due to its low molecular weight and neutral charge, hypochlorous acid readily interacts with the cell wall and penetrates bacterial cells. The activity of the hypochlorous acid is a function of pH and temperature [41].

Chlorine-releasing compounds such as N-halamines are organic or inorganic compounds in which oxidative halogens are attached to nitrogen. In aqueous solutions, chloramines undergo hydrolysis in varying degrees to form hypochlorites [41]. Monomeric n-

halamines such as 1,3 dichloro- 5,5, dimethyl hydantoin have been widely used as water soluble alternatives to chlorine based disinfectants [41].

2.4.3.6 Iodine Compounds.

Iodine solutions are widely used as antiseptics because they are effective against bacteria, virus, spores and fungi [9]. However, their efficacy is limited by the fact that they are irritants and are highly unstable in aqueous solutions. They cannot be used to disinfect hard surfaces and skin since they cause excessive irritation and staining. Such limitations were overcome by the development of iodophors or iodine releasing compounds similar to chlorine-releasing agents such as chloramines [42]. They act as reservoirs of active iodine and are used as antiseptics and disinfectants. Even at low concentrations, iodine has rapid antimicrobial activity. It is hypothesized that iodine penetrates the cell wall and binds to sulfur containing amino acids, nucleotides and fatty acids, resulting in metabolic inhibition and eventually cell death [42].

2.4.4 Concluding remarks

Most of the sporicidal agents are sporistatic at lower concentrations. Non-porous surfaces in hospitals such as floors, faucets and bed rails can be decontaminated using liquid antimicrobial products that may be poured or sprayed onto the area. One of the most common chemicals used for this purposes is 10% household bleach solution. The type of surface and the extent of contamination determine the minimum time the surface needs to be exposed to the antimicrobial agent. Tables 2.1 and 2.2 describe the mechanism of action for common antimicrobial agents used for disinfection.

Table 2.1 Sporistatic agents and their mechanism of action [42]

Sporistatic agents	Mechanism of action
Organic acids such as benzoic acid, citric acid	Interfere with the cellular uptake of substrate molecules by disruption of tertiary and quaternary protein structures and membranes.
Aliphatic alcohols such as ethanol	Disrupt membrane structure and permeability; inhibit protein synthesis and enzymes involved in glycolysis and phospholipid synthesis
Chlorhexidine salts (CHX), (QACs)	Ionic interaction with phospholipids in the membrane result in membrane disruption
Phenol and Cresol	Penetrate the phospholipid membrane and disrupt membrane integrity

Table 2.2 Sporicidal agents and their mechanism of action [42]

Sporicidal agents	Mechanism of action
Gluteraldehyde	Targets DNA and proteins that have amino, amide and thiol groups; cross link amino groups resulting in metabolic inhibition and cell wall damage
Iodine compounds	React with amino and thiol groups in amino acids disrupting protein synthesis. Also target carbon bonds in fatty acids resulting in membrane immobilization
Chlorine releasing agents (CRA) and hypochlorites	Target on the cell wall and amino acids in proteins, oxidize thiol groups in proteins and/ or chlorinate nucleotides in vegetative bacterial DNA
Peroxygens such as hydrogen peroxide	Target thiol groups in proteins and oxidize them resulting in metabolic inhibition of enzymes

Although a wide variety of sporicidal agents is available, most of the chemicals have the disadvantage of being corrosive or toxic at low concentrations. For example, gluteraldehyde is highly effective as a sporicidal agent. However, it is highly toxic even at concentrations as low as 2% which is normally used for disinfection [42]. Hydrogen peroxide is highly unstable and decomposes readily, while hypochlorite solution is inactivated by organic matter [9]. Ethylene oxide is hazardous since it penetrates organic

porous materials, which limit its use as a disinfectant in hospital environments [9]. Despite the disadvantage of being hazardous, these sporicidal agents are the primary choice for bacterial or spore disinfection, because they can be bactericidal or sporistatic at low concentrations [9].

CHAPTER 3 BIOFILMS AND ANTIMICROBIAL PEM(S)

3.1 INTRODUCTION:

The interaction between bacteria and surfaces has recently evolved as an area of extensive research potential. Using a variety of surface systems that include surface grafted molecules and nanoparticles, researchers have determined surface parameters that resist bacterial attachment, maximize antimicrobial efficiency and prevent biofilm formation. Polyelectrolyte multilayers (PEMs) provide a simple and versatile platform for exploring such surface interactions. Due to the wide range of materials that can be incorporated into them ranging from small biomolecules such as DNA and proteins to cells, PEMs also make excellent candidates for biomaterials [46]. In addition, precise control can be exercised over their material properties such as thickness of the layers, surface roughness, and charge. The high stability of the multilayers under physiological conditions offers an opportunity for exploring many aspects of bacterial cell control. Major concepts about antimicrobial PEM coatings are introduced in this chapter before delving into the applications explored in this thesis. The chapter also emphasizes the performance of PEM surfaces containing biocides as antimicrobial coatings.

3.2 BACTERIAL BIOFILMS AND SPORES

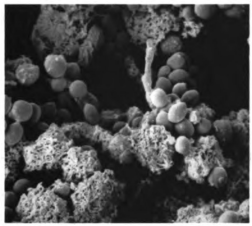
Bacterial biofilms are complex multicellular structures consisting of one or more bacterial species or fungal species in a polysaccharide matrix. These biofilms act as important reservoirs for pathogens, and biofilm growth may provide microbes with survival advantages in adverse environments. The structure of a biofilm depends on

various parameters such as nutrient availability and surface properties. In anaerobic flow chemical reactors, microbes form granular sludges that settle to the bottom since there are no walls to adhere [47]. Hence, surfaces as well as nutrient availability limit the formation of biofilms. In bacteria such as *Vibrio cholerae*, a nutrient-rich environment has been shown to promote biofilm formation [48]. Most biofilms have a heterogeneous structure consisting of cellular aggregates interspersed throughout a matrix that varies in density. This structure creates channels for water to flow in and out of the matrix. Particle tracking techniques and in situ measurements of metabolic products such as oxygen, have been utilized to demonstrate the presence of interstitial voids in the matrix that provide means for circulating as well as exchanging metabolic products with the environment [49].

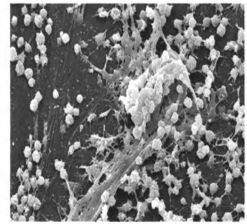
In addition to biofilms, bacterial spores (discussed in Chapter 2) can resist adverse environmental conditions. The combination of these two highly resistant structures is hypothesized to contribute to an ultimate survival mechanism employed by certain opportunistic bacterial species such as *Pseudomonas aeroginosa* and *Streptococcus epidermidis* [50]. Furthermore, biofilm formation on surfaces is an important consideration in healthcare and food processing industries [51].

Biofilms not only protect the bacterial species residing in them, but also provide a protective niche for the bacteria that resist antimicrobial agents. The first is the physical framework of the biofilm that resists antimicrobial agents. The thick exopolysaccharide

presents an impenetrable barrier that prevents diffusion of chemical disinfectants. The biological interaction between various bacterial colonies in a biofilm enhances their survival since these biofilms are typically composed of many species of bacteria that possess a range of antimicrobial susceptibilities. The persistence of hospital-acquired infections in even developed countries indicates a lack of efficient disinfection strategies and the need for improved antimicrobial technologies.



a)



b)

Figure 3.1 Scanning electron micrographs of biofilms formed on common surfaces. a) Scanning electron micrograph of an infected catheter showing dense and complex biofilm on the extra luminal surface [52]. b) Scanning electron micrograph of a Staphylococcus biofilm on the inner surface of a needleless connector. Photo credits: Jance Carr, CDC, Atlanta, GA (USA) [53]

3.3 ANTIMICROBIAL PEM(s)

3.3.1 Adhesion-resistant PEMs

Most of the current disinfection strategies in healthcare and food processing industries consist of physical and chemical disinfectants. To address problems such as biofilm

formation, which increases microbial load, and to avoid applying high concentrations of chemical disinfectants to surfaces that come in contact with human skin, alternative disinfection strategies have been proposed. Initial attachment of bacteria to surfaces involves formation of weak and reversible van der Walls, electrostatic and hydrophobic interactions with the surface. At this point mere rinsing of the surface can remove bacteria. The second step in bacterial attachment involves specific interactions of the bacteria with the surface using pili or fimbriae that enables them to be in very close proximity.

One of the earlier approaches was to limit colonization of bacteria by using conjugated polypeptides to resist bacterial adhesion since biofilm formation depends on stable attachment of bacteria to surfaces [54]. Poly(ethylene glycol) (PEG) grafted onto surfaces directly or as one of the multilayers along with poly (L-glutamic acid) (PGA) has been shown to reduce attachment of *E. Coli* even in the presence of nutrients [55]. Poly(methylmethacrylate) (PMMA) coated intraocular lenses, modified by linking of hyaluronan chemical group to the surface has been shown to effectively reduce surface adhesion of *Staphylococcus epidermidis*. The mechanism behind these adhesion resistant PMMAs has been attributed to hydrophilic properties of the hyaluronan layer [56]. Biodegradable properties have been utilized to produce adhesion-resistant PEMs containing antibiotics that prevent attachment due to top-down erosion of the multilayers [57].

3.3.2 Biocide leaching PEMs

PEMs can also be designed as coatings that leach antimicrobial compounds. In one of the studies by Feng et al, silver nanoparticles were embedded onto stainless steel surface containing multilayers of poly (acrylic acid) (PAA)/ quaternized polyethylenimine (PEI) [58]. The biocidal activity of silver ions is due to their binding to thiol groups present in cell membranes, thereby disrupting permeability. Thus the silver particles bind to DNA resulting in metabolic inhibition [58]. Hammond et al. have worked on PEM assemblies that control the release of Gentamicin (an antibiotic) [57]. Various parameters such as the number of bilayers and their composition influence antimicrobial activity.

3.3.3 Contact killing PEMs

Some variants of PEMs have been engineered to kill bacteria when they come in contact with the surface. When immobilized onto surfaces, chitosan (a cationic antimicrobial polysaccharide) has been shown to bind to negatively charged bacterial cell membranes resulting in leakage of ion channels [59].Long hydrophobic polycations have been grafted onto various substrates to kill pathogenic bacteria on contact [59]. These polycations (e.g., PEI) reduce the microbial population by damaging the bacterial cell membranes [59, 60]. Various studies have been performed to optimize parameters such as contact time, chain length and concentration of the polycations [59, 60, 61]. Chemical disinfectants such as QACs have been widely used to create non-leaching biocidal surface coatings. The antimicrobial mechanism of QACs was discussed in detail in section 2.4.3.4. PEM assemblies containing charged polymers with grafted QACs have been shown to be effective against Gram positive bacteria [62]. However, such coatings

have their own disadvantages. These coatings can lose their efficacy when the microbial load increases due to constant use. Increasing the concentrations of hazardous chemicals such as QACs to achieve better antimicrobial activity is not recommended. Hence, covalently cross-linking polymers that makes the polycations to retain their antimicrobial properties in a permanent and non-leachable fashion when placed in an environment (for example bodily fluids, high moisture area, sterile contact surfaces) has been investigated. Furthermore, n-halamine siloxane coatings has been recently investigated as excellent antimicrobial coatings on textiles and as polymeric surface coatings that can be replenished when the surface is sprayed with commercially available household bleach solution [63,64].

Antimicrobial PEM surface coatings that reinforce surface sterility between disinfection cycles have been developed [64]. Apart from the wide range of materials that can be incorporated, [46] these PEMs are also biocompatible and environment friendly. Biological materials such as proteins [65], DNA [15], polysaccharides [66] and enzymes [67] have been incorporated into multilayers for drug delivery applications. Unlike self assembled monolayers and Langmuir-Blodgett films, PEMs manufactured with the Layer-by-Layer (LbL) deposition technique have clear advantages such as the wide variety of substrates that allow the growth of a polymer multilayer and control over the multilayer properties. Glass, polymers and metals are being increasingly used as substrates.

3.4 FABRICATION OF PEMs

Polyelectrolyte multilayers are typically assembled using a LbL process [46] (Fig 2.2) involving iterative adsorption and rinsing steps. The substrate is dipped into the first polyelectrolyte solution for a sufficient period of time to allow the polyelectrolyte to adsorb to the surface. This is followed by a rinsing step that removes excess unbound polymer. The substrate is then dipped into a polyelectrolyte solution of opposite charge, which adsorbs to the surface due to electrostatic attraction and neutralizes the existing surface charge, resulting in a reversal of the previous surface charge. The process is repeated until the desired number of layers is deposited. The chemical structures of some common polyectrolytes is shown in Fig 3.2.

The most common polycations used for the preparations of these multilayers includes polyethyleneimine (PEI), poly allylamine hydrochloride (PAH) and polydiallyl dimethylammonium chloride (PDDA). Commonly used polyanions include poly sodium 4-styrenesulfonate (PSS), poly ethenesulfonic acid (PES) and poly acrylic acid (PAA) [68].

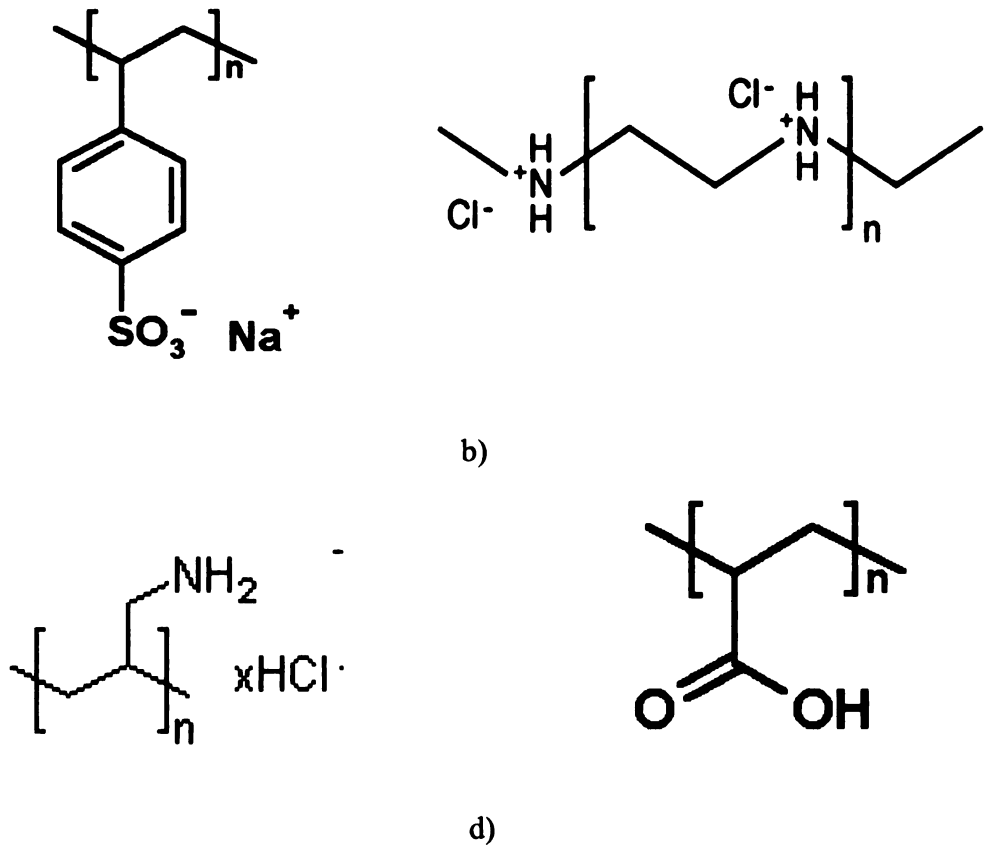


Figure 3.2 Structures of common polyelectrolytes used in this study. a) PSS b) PEI c) PAH d) PAA [68]

3.4.1 Properties of pH tunable PEMs

A particularly versatile approach to PEM fabrication involves use of weak polyelectrolytes, such as PAH and PAA. Varying the pH of the processing solution changes the deposited thickness, morphology and swelling characteristics of the PEM film [69]. However, one disadvantage of their pH dependence is that exposure to acidic conditions breaks the electrostatic interactions between cationic and anionic layers resulting in water soluble film [69]. A particularly useful method for stabilizing PAA and PAH is by amide formation between the amine group of PAH and carboxylic group of

PAA multilayers is thermal [70] or chemical methods [71], resulting in cross-linked films. After cross-linking, the films exhibit enhanced stability towards changes in pH.

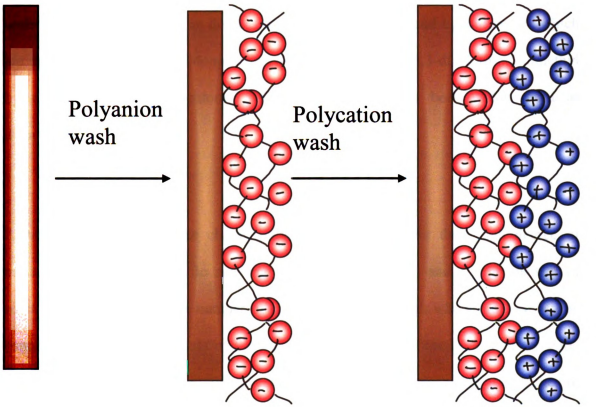


Figure 3.3 A schematic of Layer-by-Layer deposition. The polyelectrolyte multilayers are synthesized by a repeated charging of the surface with oppositely charged ions until a desired number of layers is deposited. Each step results in a reversal of surface charge allowing the next layer to be deposited [46].

3.4.2 Properties of N-halamies grafted onto PAMAM dendrimers

In addition to PAA/PAH bilayered coatings with sporicidal agents, N-halamine functional groups have been incorporated into polyamidoamine (PAMAM) dendrimers that provide additional advantages over the conventional PEM. The n-halamines are capable of regeneration by simply exposing the molecules to free chlorine, resulting in a

broad spectrum antimicrobial. Furthermore, the stability of N-Cl bonds on chlorinated halamines contributes to the durability and stability of the antimicrobial properties of the polymer [72]. N-halamines are inorganic and organic compounds in which oxidative halogens are attached to nitrogen. In aqueous solution, chloramines and bromamines undergo hydrolysis in varying degrees to form hypochlorites and hypobromites, which are sporicidal agents [72]. Monomeric N-halamines such as 1,3-dichloro-5,5-dimethyl hydantoin and 3-bromo-1-chloro-5,5-dimethylhydantoin have been widely used as disinfectants [72].

PAMAM dendrimers, unlike classic polymers, are known for their high degree of molecular uniformity, specific size and shape characteristics, and a high density of reactive surface groups. The number of chemical bonds through which the core can be connected to the external parts of the molecule is defined as functionality of the core. Through the bonds of the core, the layers of branching units (monomers) are attached to the core. Analogous to preparing a PEM, the synthesis of a PAMAM dendrimer consists of a series of repetitive steps starting with a central initiator core. If all of these branching units are attached to the molecule perfectly, a dendrimer is formed. A large number of "imperfections" results in a hyperbranched polymer structure (Figure 3.5). Each series of steps produces a new "generation" with a larger molecular diameter twice the number of reactive surface sites, and approximately twice the molecular weight of the preceding generation [73,74]. In our project, the end group of a G4-PAMAM dendrimer (Generation 4 PAMAM) was modified to possess a 5, 5-dimethyl hydantoin. The G4 PAMAM- hydantoin could also be grafted on a multilayer of PAH/ PAA or PAH/PSS.

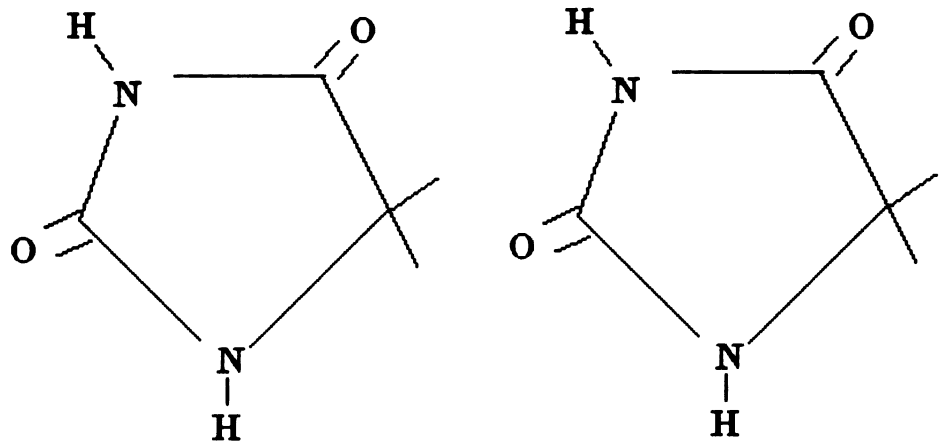


Figure 3.4 Structure of 5,5 dimethyl hydantoin and its halogenated derivative – 1,3 dichloro- 5,5, dimethyl hydantoin [72].

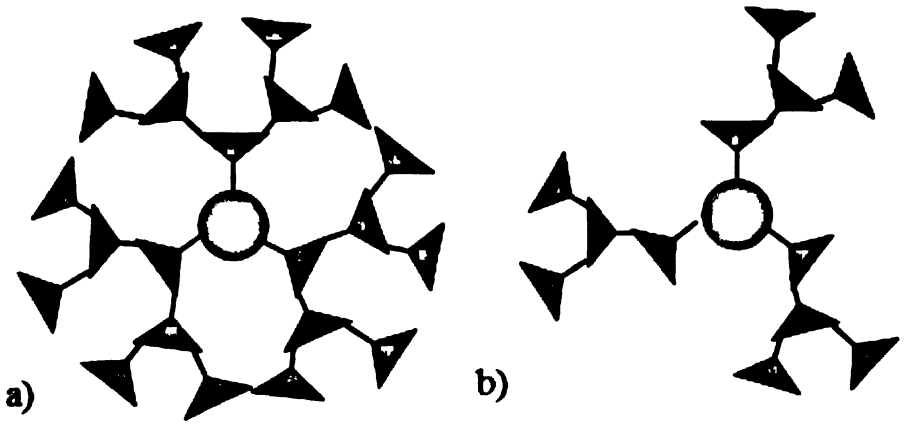


Figure 3.5 Schematic representations of a a) dendrimer and b) a hyperbranched polymer molecule. The core is represented by the grey molecule in the center with multiple functional end groups [73].

A typical assembly of the polyelectrolyte coatings constructed for our experiments is shown in Fig 3.6. After fabrication of the multilayers, the coatings were thermally cross linked, and finally immersed in bleach solution for activation of the N-halamine functional groups (if it was to incorporated into the PEMs). Unoccupied charged sites

within the interior voids of the PEM assembly are used to accommodate small molecules such as CaDPA, L-alanine and L-inosine that can act as germinant molecules. The coatings are expected encapsulate and deliver small germinant molecules to the surface of entrapped spores. The amount of germinants that can be encapsulated in the coatings can be controlled based on thickness of the PEM and the size of the dendritic layers. The PEM are preferred over the dendrimer layers since PEMs are easy and inexpensive compared to the dendrimer layers.

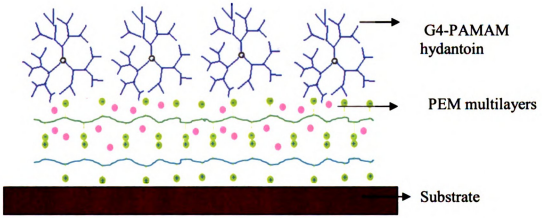


Figure 3.6 A diagram of PEM assembly as an antimicrobial surface [Personal communication – Dr. Steven Kaganove]. Polyelectrolyte layers are coated onto the substrate. A final layer is applied made of a PAMAM dendrimer with hydantoin appended to the end-group.

CHAPTER 4 EXPERIMENTAL METHODS AND RESULTS

4.1 INTRODUCTION

LbL technique was used to synthesize a number of multilayer coatings where different combinations of cationic and anionic polymers, number of bilayers and the presence or absence of germinants and sporicidal agents were systematically varied. Most of the coatings house germinants and sporicidal components in the "cargo spaces" of dendritic polymers (G4PAMAM) or within multiple layers of polyelectrolytes. In few of these coatings, L-Alanine/inosine is used as a germinant and N-halamines attached to end group of PAMA dendrimer are used as sporicidal component. 96-well polypropylene plates were used as substrates for antimicrobial surfaces. Each coating variant was tested for antimicrobial efficiency and

4.2 MATERIALS AND METHODS

4.2.1 PEM construction materials

Polyacrylic acid (PAA) (MW = 240,000) was obtained from Acros Organic (Morris Plains, NJ) as a 25% aqueous solution. Polyallylamine hydrochloride (PAH) (MW = 70,000) and Polyethyleneimine (PEI) (MW = 25,000) were obtained from Sigma Aldrich (St. Louis, MO). Generation 4 PAMAM (G4PAMAM) dendrimer was obtained from Dendritech Inc. (Midland, MI) as a dilute methanol solution. All polyectrolytes with the exception of G4PAMAM were used without further purification. G4 PAMAM used as the starting material for the synthesis of hydantoin-PAMAM, was subsequently purified by ultrafiltration. Commercially available bleach was purchased in department stores. All

polyelectrolyte coating solutions were prepared as 10^{-2} M solutions (based on the repeat unit molecular weight) in Milli-Q water, and pH was adjusted to neutral pH with either hydrochloric acid or sodium hydroxide. The PEM substrates used were black polypropylene (PP) plates (96 wells, 360uL volume "round-bottom" plates), purchased from Fisher Scientific (Pittsburgh, PA).

4.2.2 Fabrication procedure

For fabricating a PEM substrate, each PP plate was evenly cut into three equal pieces with 32 wells in each piece. This was done to ensure immersion in approximately 175 mL of coating solution. Polyelectrolyte multilayer (PEM) coatings were deposited on polypropylene plates by alternating sequential immersion in 0.01M aqueous solutions of cationic and anionic polyelectrolytes. The pH values of 0.01 M PAA and 0.01 M PAH solutions were adjusted to 3.5 and 8.5 respectively. 10% household bleach solution (pH 7 or specified) was prepared fresh for coatings. The concentration of G4PAMAM derivatives was 1mg/mL. Depending on the type of PEM composition, 500 mL of coating solutions were prepared. The PEM films were deposited as thin films over the PP surface by manual dipping. Clean PP plates were immersed in a polyelectrolyte solution for 20 min, followed by several rinses in Milli-Q water. The substrates were then immersed into the oppositely charged polyelectrolyte solution for 20 min and subjected to the same rinsing procedure. After the desired number of layers was assembled, the substrates were dried in air, and subsequently heated to 120 °C overnight for thermal cross-linking. The crosslinking temperature was necessarily limited to 120 °C for the polypropylene microplates since they would melt at higher temperatures. Antimicrobial coatings were

fabricated by bleach immersion. Bleach immersed PEMs were obtained when the cross-linked PEMs were immersed in 10% household bleach solution for 20 min followed by the same rinsing procedure after 24 hours.

4.2.3 Testing the coatings for antimicrobial efficiency.

The protocol used for testing the efficacy of these coating is described in Appendix B of the thesis. An outline of the process is summarized in this section. Coatings containing biocides or germinants will be at the optimum concentration when little to no water is present. Thus, an aqueous suspension of the *Bacillus anthracis* spores (with pre-determined CFU/mL) was applied onto the coated wells, and the suspension was dried under vacuum. The plate was sealed for 24 hours. After the incubation time, the spores were recovered using 200uL of 0.04%BSA/ 0.03% Thiosulfate solution. The 0.04% BSA has been shown to aid in enhanced recovery of the spores from the coated wells (Appendix C). To prevent transfer of residual bleach on the coatings to the BHI agar plates, thiosulfate solution that neutralizes the residual chlorine was used. The diluted suspensions of collected spores were plated onto BHI-agar plates (Appendix A) by diluting the 200uL working stock 10,000 fold. All dilutions were plated to obtain accurate spore counts.

4.2.4 Calculating antimicrobial efficiency

The PEM coatings containing polymer layer (or G4-PAMAM) immersed in bleach were tested for their efficacy in reducing the number of *Bacillus anthracis spores* (streme strain) from a pre-determined initial concentration (measured in CFU/mL) after an

incubation period of 24 hours. The sporicidal coatings were tested for their performances by calculating the difference in logarithmic CFU/mL values between the sporicidal coatings and the respective controls. Each sporicidal coating was compared to its respective control for all the experiments. The sporicidal coatings differ from their control coatings only in the presence or absence of a sporicidal agent. A log difference of at least 2 was expected for the sporicidal coatings, which indicates that the coatings reduced the spore counts by 99% (See Appendix D for calculations). For statistical analysis, an experiment is defined when the spores were incubated for 24 hours in a single well of a 96-well polypropylene plate.

4.2.5 Fourier Transform Infrared (FTIR) spectroscopy

Five bilayers [PAA/PAH] films were built onto gold substrates for FTIR analysis. The effect of additional PAA layer (as the topmost layer that would contact the spores) and cross-linking induced by heat treatment of the PEMs at two temperatures (120°C and 180°C) on the absorption spectra of the PEM layers was studied. All IR spectra were recorded using an ATI Mattson Research Series 1 spectrophotometer with a liquid nitrogen-cooled MCT detector. Typically spectra were acquired using an average of 32 scans at a $4cm^{-1}$ resolution.

4.3 Results

4.3.1 [PAA/PAH]5 bilayers with varying topmost layer that contacts the spores

Coating variants that have at least five bilayers of [PAA/PAH] were fabricated (Table 4.1) and tested for sporicidal activity. Within this group, were five sets of coatings; each

with different variants as the topmost layers. Each of the multilayers had both control and sporicidal variants. Each set also had two controls differing in the presence or absence of germinants (L-alanine and inosine). The sporicidal coatings had either sporicidal agents alone or a combination of germinants and sporicidal agent. It has been reported that vegetative bacteria (germinated spores) are more susceptible to antimicrobial chemicals compared to spores. Thus we tested if the presence of chemical germinants along with sporicidal agents has any significant increase in sporicidal effect. The log (CFU/mL) for each coating was plotted to estimate the performance of the coatings.

Table 4.1 PEM Coatings tested with at least five [PAA/PAH] bilayers

Coating ID	Coating
1A	$[PAA/PAH]_5$/120°C
1B	$[PAA/PAH]_5$/120°C/ Ala+Ino
2A	$[PAA/PAH]_5$/120°C/NaOCl
2B	$[PAA/PAH]_5$/120°C/NaOCl/ Ala+Ino
3A	$[PAA/PAH]_5$/PAA/120°C
3B	$[PAA/PAH]_5$/PAA/120°C/ Ala+Ino
4A	$[PAA/PAH]_5$/PAA/120°C/NaOCl
4B	$[PAA/PAH]_5$/PAA/120°C/NaOCl/ Ala+Ino
5A	$[PAA/PAH]_5$/PAA/hydantoin PAMAM/120°C
5B	$[PAA/PAH]_5$/PAA/hydantoin PAMAM/120°C/ Ala+Ino
6A	$[PAA/PAH]_5$/PAA/hydantoin PAMAM/120°C/NaOCl
6B	$[PAA/PAH]_5$/PAA/hydantoin PAMAM/120°C/NaOCl/ Ala+Ino
7A	$[PAA/PAH]_5$/[PAA/hydantoin PAMAM]/PAA/120°C
7B	$[PAA/PAH]_5$/[PAA/hydantoin PAMAM]/PAA/120°C/ Ala+Ino
8A	$[PAA/PAH]_5$/[PAA/hydantoin PAMAM]/PAA/120°C/NaOCl
8B	$[PAA/PAH]_5$/[PAA/hydantoinPAMAM]/PAA/120°C/NaOCl/ Ala+Ino
9A	$[PAA/PAH]_5$/[PAA/hydantoin PAMAM]2/120°C
9B	$[PAA/PAH]_5$/[PAA/hydantoin PAMAM]2/120°C/ Ala+Ino
10A	$[PAA/PAH]_5$/[PAA/hydantoin PAMAM]2/120°C/NaOCl
10B	$[PAA/PAH]_5$/[PAA/hydantoin PAMAM]2/120°C/NaOCl/ Ala+Ino

The coatings variants were tested to see a) if the presence of additional layers over the five bilayers of PAA/PAH and b) the presence of germinants contribute to significant sporicidal activity (Figure 4.1 and 4.2). In all the figures representing spore count data, the numbers above the sporicidal coating bars indicate the difference in log (CFU/mL) from their corresponding control coatings. For example the number above 4A (2.64) is the difference in log CFU/mL values between the control 3A (=6.46) and sporicidal coating 4A (=3.92).

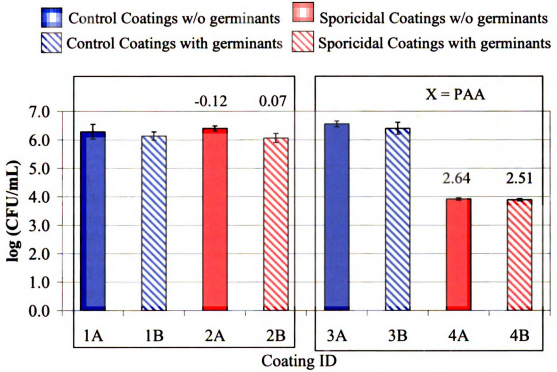


Figure 4.1 Spore counts in PEM coatings containing atleast 5 bilayers of [PAA/PAH] after 24 hours of exposure. Additional layers built over the five bilayers are represented as X where X = PAA (in coatings 3A, 3B, 4A, and 4B). After the layers were built both the control and the sporicidal coatings were heat treated to introduce amide bonds between PAA and PAH. Sporicidal coatings were immersed in 10% bleach solution.The control and sporicidal coatings containing germinants are patterned as indicated above the plot. The set of coatings with "B" in the coating ID contain alanine and inosine (germinants). Spore count data represent mean +/- S.D. of three independent experiments.

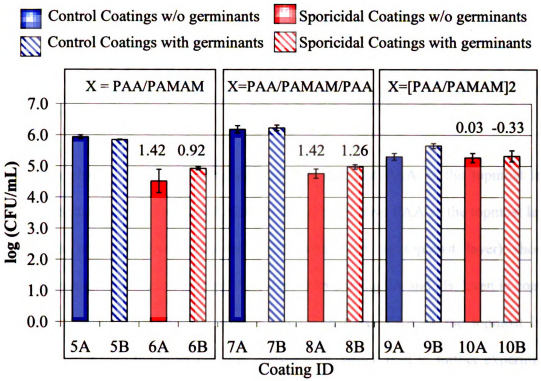


Figure 4.2 Spore counts in PEM coatings containing atleast 5 bilayers of [PAA/PAH] after 24 hours of exposure. Additional layers built over the five bilayers are represented as X where X = PAA/PAMAM (in coatings 5A, 5B, 6A, and 6B), X = PAA/PAMAM/PAA (in coatings 7A, 7B, 8A, and 8B) and X = [PAA/PAMAM]2 (in coatings 9A, 9B, 10A, and 10B)b. After the layers were built both the control and the sporicidal coatings were heat treated to introduce amide bonds between PAA and PAH. Sporicidal coatings were immersed in 10% bleach solution to provide antimicrobial properties. The control coatings are represented in blue color and the sporicidal coatings are represented in pink color. The control and sporicidal coatings containing germinants are patterned as indicated above the plot. The set of coatings with "B" in the coating ID contain alanine and inosine (germinants). Spore count data represent mean +/- S.D. of three independent experiments.

PEM coating variants had atleast five bilayers of PAA/PAH and differed in the composition of the top most layer (the layer that is in direct contact with the incubated spores). In all these coatings tested, there is no significant difference between CFU values for coatings with sporicidal entities in the presence or absence of germinant. Hence, coatings variants for further experiments did not have any germinants. The sporicidal coatings henceforth were immersed in 10% bleach solution. A two log difference of

CFU/mL corresponds to 99% reduction in the number of spores (See Appendix D). Both coatings 4A and 4B had more than 2.5 log decrease in their CFU values, which corresponds to more than 99% killing.

It was also observed that the sporicidal coatings that had PAA as the topmost layer performed better than the sporicidal coatings that did not have PAA as the topmost layer. Coating 4A and 4B (fiver bilayered PEM with PAA as topmost layer) showed significantly higher reduction in log CFU values than coating 2A and 2B. Even in coating variants containing PAMAM, the coating variant that had PAA as the topmost layer performed better than the other coatings consistently, which lead to further experiments testing whether the presence of PAA as the topmost layer affects the efficacy of the coatings. Reduction in spore counts from some of the PEM coatings containing G4 PAMAM and treated with bleach was also observed. Also when comparing the bilayered PAMAM (coating ID: 10A, 10B) coating with the monolayer ones (coating ID: 6A, 6B), there is no significant difference between the reduction in log CFU values. This may be because the bilayer may sequester some bleach in them which may contribute to their lower killing potential.

4.3.2 Effect of [PAA/PAH] and [PSS/PAH] bilayers with PAA or PSS as topmost layer and heat treatment

A subsequent batch of coatings (Table 4.2) compared three different parameters: effect of PAA as the topmost layer, effect of the number of PAA/PAH bilayers and effect of heat treating the coatings at 120°C. Most coating variants were heat treated to introduce an

amide cross-linking between the PAA and PAH layers, thereby providing additional stability to the multilayers. In this set of coatings tested, even numbered experiments are treated with bleach and the odd number experiments are the corresponding controls without bleach.

Table 4.2 PEM Coatings with 2, 5 and 10 bilayers with PAA as topmost layer

Coating ID	Coating
1	[PAA/PAH]2/120°C
2	[PAA/PAH]2/120°C/NaOCl
3	[PAA/PAH]2/PAA/120°C
4	[PAA/PAH]2/PAA/120°C/NaOCl
5	[PAA/PAH]5/120°C
6	[PAA/PAH]5/120°C/NaOCl
7	[PAA/PAH]5/PAA/120°C
8	[PAA/PAH]5/PAA/120°C/NaOCl
9	[PAA/PAH]5
10	[PAA/PAH]5/NaOCl
11	[PAA/PAH]5/PAA
12	[PAA/PAH]5/PAA/NaOCl
13	[PAA/PAH]10/120°C
14	[PAA/PAH]10/120°C/NaOCl
15	[PAA/PAH]10/PAA/120°C
16	[PAA/PAH]10/PAA/120°C/NaOCl

These variant sets (1-4, 5-8, and 13-16) differ in the number of bilayers. With each subset the coatings on the right (3, 4; 7,8; 15,16) have PAA as the topmost layer while the other variants in the same subgroup do not have PAA as topmost layer. Increasing the number of bilayers in the coatings that have PAA as the topmost layer had a significant increase in the difference between the log (CFU/mL) values of the individual bleach treated and control coatings. However, the difference was the highest in 5 bilayer coatings. Further experiments were performed to analyze the effect of bilayers on sporicidal activity.

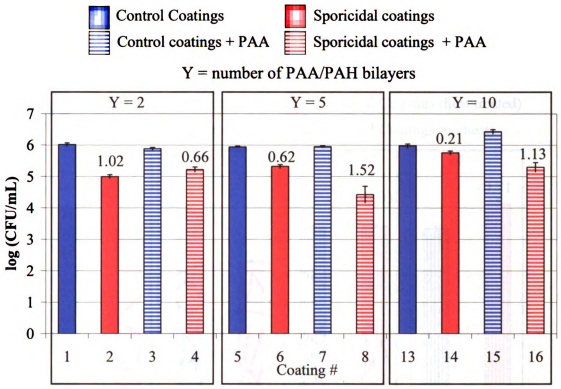


Figure 4.3 Spore counts in PEM coatings without or with PAA as topmost surface that contacts the spores with varying number of PAA/PAH bilayers. Spore count data represent mean +/- S.D. of three independent experiments. Spore counts in PEM coatings with $[PAA/PAH]_Y/X$ after 24 hours of exposure. Additional layers built over the five bilayers are represented as X where X = PAA (for coatings 3,4,7,8,15,16). The patterned bars represent the log CFU values of coating variants that contain an additional PAA layer apart from the $[PAA/PAH]_Y$ bilayers.

As shown in Figure 4.3, except for coating variants for which Y = 2, the sporicidal coatings for Y = 5, 10 with an additional PAA layer perform better than the corresponding sporicidal coatings without PAA. Thus the presence of PAA as the topmost layer that contacts the spores has a significant effect on sporicidal activity.

In the following set of coatings tested (Figure 4.4), even numbered experiments are treated with bleach and the odd number experiments are the corresponding controls without bleach. All the coating variants have five bilayers of [PAA/PAH] with set #2 and

#4 have PAA as the topmost layer; while set #1 and #3 does not have PAA as the topmost layer. Sets 1 and 2 were heat treated while sets 3 and 4 were not heat treated.

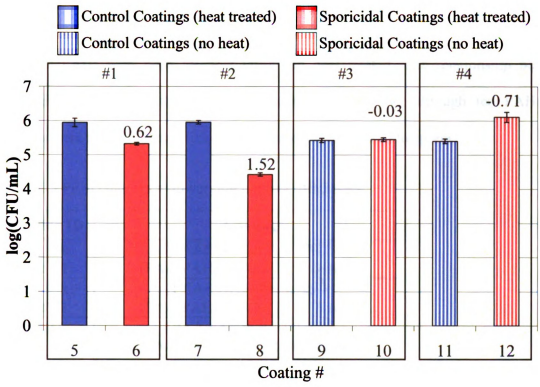


Figure 4.4 Spore counts in PEM coatings with 5 or 10 [PAA/PAH] bilayers differing in heat treatment. Spore count data represent mean +/- S.D. of three independent experiments.

The above results suggest that the synergistic effect of the PAA and the heat treatment provides the highest reduction in log CFU values as noticed in the coating 7,8 (Figure 4.4). Hence heat treated and bleach treated coatings act as a better antimicrobial surface than the respective controls.

4.3.3 Effect of number of bilayers

In all coatings tested those with PAA as the topmost layer performed better than coatings with different topmost layers. [PAA/PAH] coatings with five bilayers have the maximum

reduction in spore counts compared with two or ten bilayers. To more thoroughly probe the optimum number of bilayers for maximum sporicidal effect on *B. anthracis* spores, the following coatings were be fabricated and tested. The following set of coatings differ in the bilayer composition (Figure 4.5). The coatings 1-6 have 3, 4, 6, 7, 8 and 9 bilayers of [PAA/PAH] with PAA topmost layer and heat treated. The relationship between sporicidal activity and thickness was evaluated, where two through ten PAH/PAA bilayers were thermally crosslinked, chlorinated, and exposed to spores.

Table 4.3 PEM coatings with varying number of bilayers

Coating ID	**Coating**
Set 1A	[PAA/PAH]3/PAA/120^{0}C
Set 1B	[PAA/PAH]3/PAA//120^{0}C/NaOCl
Set 2A	[PAA/PAH]4/PAA/120^{0}C
Set 2B	[PAA/PAH]4/PAA//120^{0}C/NaOCl
Set 3A	[PAA/PAH]6/PAA/120^{0}C
Set 3B	[PAA/PAH]6/PAA//120^{0}C/NaOCl
Set 4A	[PAA/PAH]7/PAA/120^{0}C
Set 4B	[PAA/PAH]7/PAA//120^{0}C/NaOCl
Set 5A	[PAA/PAH]8/PAA/120^{0}C
Set 5B	[PAA/PAH]8/PAA//120^{0}C/NaOCl
Set 6A	[PAA/PAH]9/PAA/120^{0}C
Set 6B	[PAA/PAH]9/PAA//120^{0}C/NaOCl

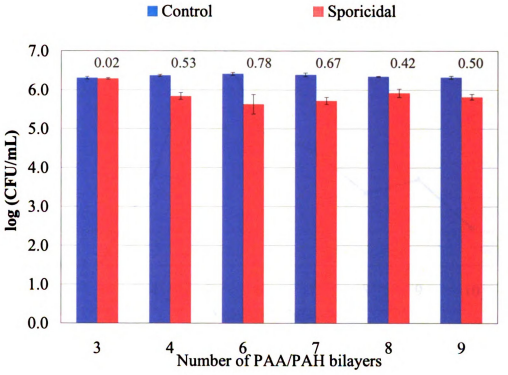


Figure 4.5 Spore counts in PEM coatings with varying number of bilayer. Spore count data represent mean +/- S.D. of three independent experiments.

The data for the sporicidal activity of 2, 5 and 10 bilayers of [PAA/PAH] with PAA as the topmost layer that contact the spores were taken from Section 4.3.2. As shown in Figure 4.6, the results show an increase in activity from three to five bilayers, followed by a sharp drop off from six to ten bilayers. Thus, in all the coating variants tested the five bilayered [PAA/PAH] with PAA as the topmost layer, heat treated overnight at a temperature of 120^{o}C has the maximum sporicidal activity.

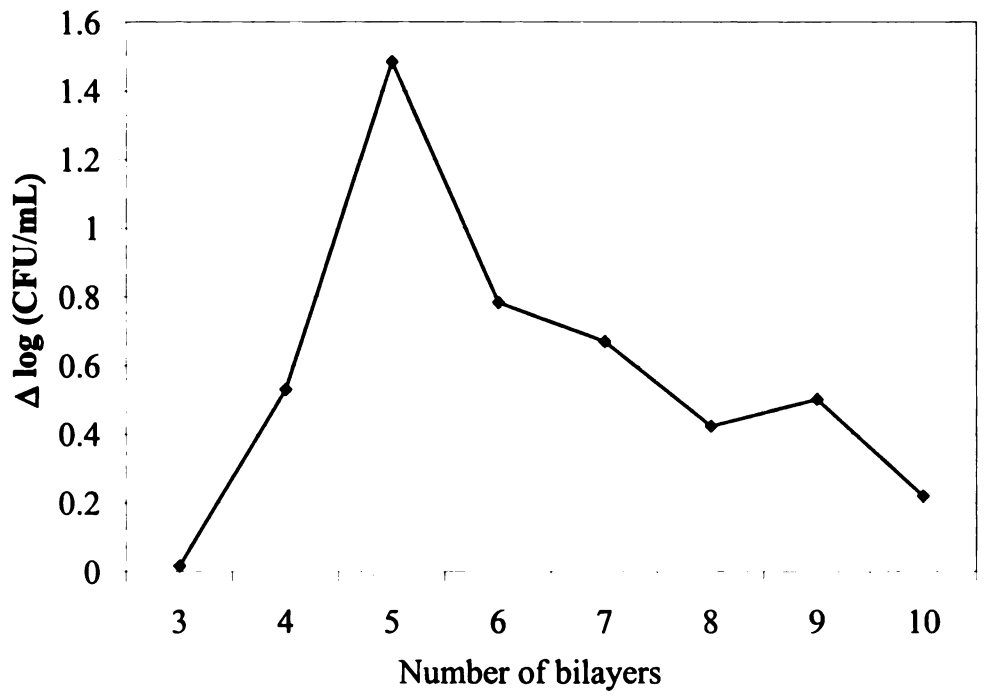


Figure 4.6 A plot showing the effect of varying the bilayers on sporicidal activity. [PAA/PAH] coating variants with varying number of bilayers were tested for sporicidal activity. The difference in sporicidal activity between the sporicidal coatings and their respective controls were plotted against the number of bilayers.

4.3.4 Coatings formulated with other common polyelectrolytes

Several coating variants with other polyelectrolytes such as PEI and HBPU (hyperbranched polyurethane) were assessed for sporicidal activity against *B. anthracis* spores. They are polymers with densely branched structure and a large number of end groups. Unlike dendritic polymers which have completely branched star-like topologies, the hyperbranched polymers have imperfectly branched structures (Figure 3.5). To verify that the bilayers containing [PAA/PAH] with PAA as the topmost layer indeed perform better, coating variants that PSS as topmost layer were fabricated and tested (Table 4.4).

Table 4.4 PEM Coatings [PSS/PAH] bilayers and [PAA/PAH] with PSS as topmost layer

Coating ID	Coating
1	[PSS/PAH]5/PSS
2	[PSS/PAH]5/PSS/NaOCl
3	[PAA/PAH]5/PSS/120°C
4	[PAA/PAH]5/PSS/120°C/NaOCl
5	[PAA/PAH]5/120°C/PSS
6	[PAA/PAH]5/120°C/PSS/NaOCl

The set of coatings fabricated above (Figure 4.7) differ in the bilayer composition. The coatings 1 and 2 have [PSS/PAH] as bilayers. The coatings 3-6 have [PAA/PAH] as the bilayers but differ application off an additional PSS layer before or after heat treatment at 120°C. PEMs made of strong polyelectrolytes such as PSS, remain charged over the entire range of pH unlike weak polyelectrolytes such as PAA. The degree of ionization of weak polyelectrolytes depends on the pH of the solution which in turn influences the thickness of the film [75]. For example Shiratori et al. fabricated PAA/PAH films of varying, thickness by assembling them in different solution pH. The solution pKa is defined as the pH at which half of the polyelectrolyte's units are charged. The solution pKa of PAA and PAH is 6.5 and 8.8 respectively [76]. The 6.5/6.5 PAH/PAA form thin layers since both the polyelectrolytes are fully charged and hence are tightly bonded to each other with very few free acid or amine groups. In contrast, the 3.5/3.5 PAH/PAA films are very thick due to formation of loops and tails. This is because at a pH of 3.5 PAA is not fully charged and hence does form a flat surface over the underlying PAH layer.

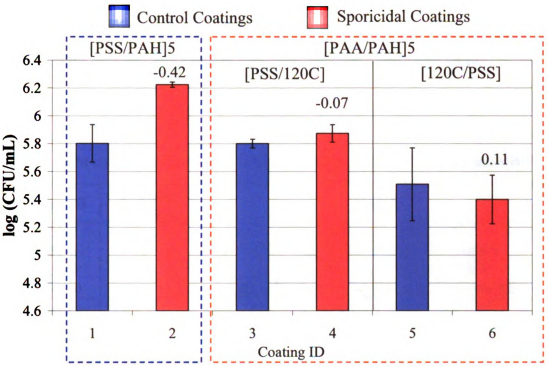


Figure 4.7 Spore counts in PEM coatings. Spore count data represent mean +/- S.D. of three independent experiments.

Assaying for sporicidal activity in coating variants listed in Table 4.4 suggests that substitution of PAA (a weak polyelectrolyte) with PSS (a strong polyelectrolyte) as the top layer resulted in films with little or no activity against spores after chlorination. This was regardless of whether crosslinking was induced after (Coating ID: 3,4) or before (Coating ID: 5,6) PSS deposition.

A few other coating variants with four bilayers of [PAA/PAH] that differ in layers applied above them (Table 4.5) were fabricated to assay for sporicidal activity against *Bacillus anthracis* spores. All the coatings were heat treated at 120°C that crosslink the

polyelectrolytes. The coatings are divided into three sets that vary in layers assembled above the four bilayers of [PAA/PAH]. Within each set, one half of coatings differ form the other in the presence of PAA as the topmost layer that contacts the spores.

Table 4.5 PEM coatings with variants containing having PEI, G4PAMAM and HBPU

Coating ID	Coating set #	Coating
773-56-7	1	[PAA/PAH]4/PAA/PEI/120^{0}C
773-56-8		[PAA/PAH]4/PAA/PEI/120^{0}C/NaOCl
773-56-9		[PAA/PAH]4/PAA/PEI/PAA/120^{0}C
773-56-10		[PAA/PAH]4/PAA/PEI/PAA/120^{0}C/NaOCl
773-56-11	2	[PAA/PAH]4/PAA/G4PAMAM/120^{0}C
773-56-12		[PAA/PAH]4/PAA/G4PAMAM/120^{0}C/NaOCl
773-56-13		[PAA/PAH]4/PAA/G4PAMAM/PAA/120^{0}C
773-56-14		[PAA/PAH]4/PAA/G4PAMAM/PAA/120^{0}C/NaOCl
773-56-15	3	[PAA/PAH]4/PAA/HBPU/120^{0}C
773-56-16		[PAA/PAH]4/PAA/HBPU/120^{0}C/NaOCl
773-56-17		[PAA/PAH]4/PAA/HBPU/PAA/120^{0}C
773-56-18		[PAA/PAH]4/PAA/HBPU/PAA/120^{0}C/NaOCl

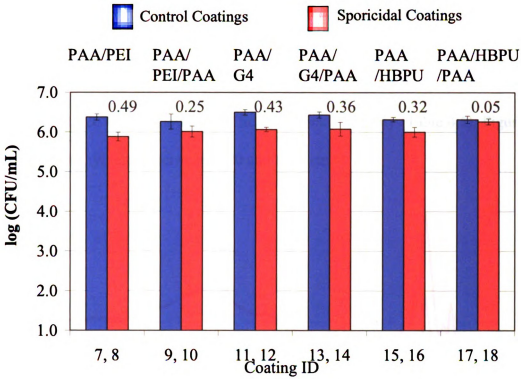


Figure 4.8 Spore counts in PEM coatings with varying topmost layer. Spore count data represent mean +/- S.D. of three independent experiments. These set of coatings have four [PAA/PAH] bilayers and differ in the layer that contacts the spores.

The coating variants tested for sporicidal activity (Figure 4.8) does not seem to show significant sporicidal activity. Even though certain coatings (coating ID 10, 14 and 18) have PAA as the topmost layer they did not perform better than their comparative sporicidal coatings do not have PAA as the layer contacting the spores.

4.3.5 FTIR spectra analysis

FTIR spectra were obtained from PEM films deposited on gold substrates under identical conditions. Films on coated substrates were cross-linked by heating overnight at either 120 °C or 180 °C. After thermal cross-linking, the coated substrates were chlorinated by immersion in dilute bleach adjusted to pH 7 for 20 minutes. FTIR spectra of the

multilayer films on gold indicates that only a small degree of crosslinking takes place at 120 °C since there is little difference in the spectra before and after heating (Figure 4.9). Significant changes are evident after heating at 180 °C. However, the coatings crosslinked at 120°C also showed significant sporicidal activity (Table 4.5). Figure 4.10 shows the carbonyl region between 1800 and 1300 cm^{-1}.

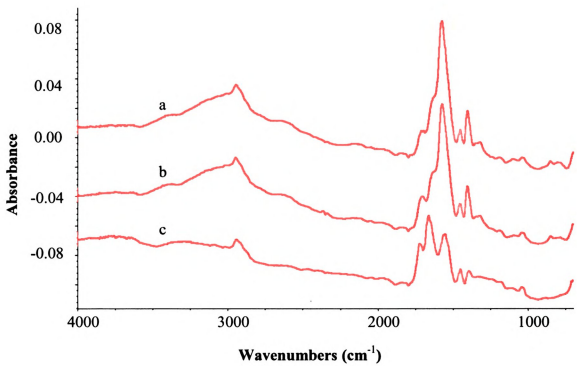


Figure 4.9 FTIR-ERS spectra of $[PAH/PAA]_5$ multilayer films on gold (a) as prepared, (b) after heating at 120 °C overnight, and (c) after heating at 180 °C overnight.

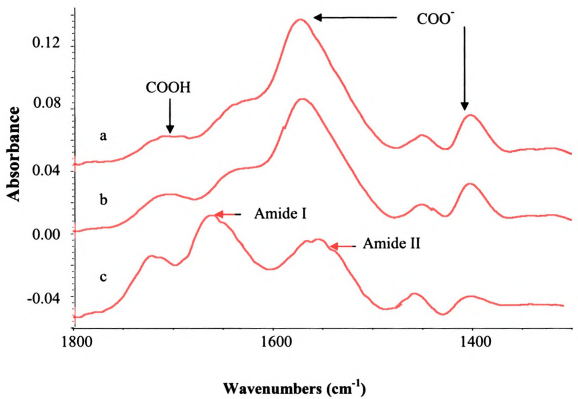


Figure 4.10 FTIR-ERS spectra of a [PAH/PAA]5 multilayer film on gold (a) as-prepared, (b) after heating at 120 °C overnight, and (c) after heating at 180 °C overnight.

Peaks due to the –COOH carbonyl and carboxylate, asymmetric and symmetric stretches (1701, 1572 and 1402 cm^{-1}) are visible in both Figure 4.10a and b, and there is little apparent difference between them. The COOH peak at 1701 cm^{-1} is smaller than the carboxylate peaks because the films were washed with pH 6-7 water after each polyelectrolyte deposition step. This results in an equilibrium shift in unreacted primary amine groups present in the films towards carboxylate which may even obscure the amide peaks. The extremely broad peak in Figure 4.10a and 4.10b between 3550 and 2530 cm^{-1} is characteristic of $-NH_3^+$ stretching in ammonium carboxylates. After the gold substrate is heated to 180 °C the carboxylate peak at 1572 cm^{-1} and the $-NH_3^+$ stretching

peak diminishes, and amide peaks at 1552 and 1660 cm^{-1} predominate.

Figure 4.11 shows spectra of five bilayer PAH/PAA films heated to 120 °C on gold before (a) and after (b) immersion in NaOCl. An expansion of the carbonyl region between 1800 and 1300 cm^{-1} is shown in Figure 4.12.

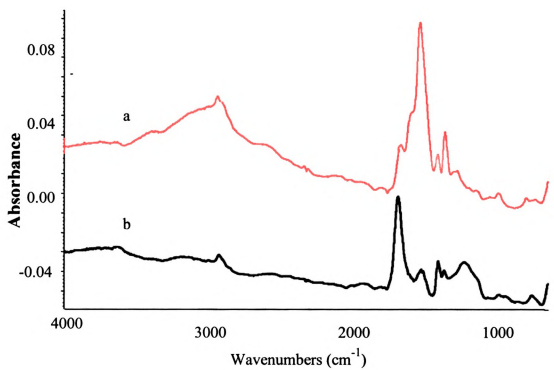


Figure 4.11 FTIR-ERS spectra of a $[PAH/PAA]_5$ multilayer film on gold. (a) After heating at 120 °C overnight, and (b) after treatment with NaOCl.

After chlorination the carboxylate peaks become greatly diminished in size and shift to 1565 and 1409 cm^{-1}, while the protonated acid peak shifts to 1709 cm^{-1}. The $-NH_3^+$ stretching peak between 3550 and 2530 cm^{-1} is also no longer present. These shifts can be explained by conversion of primary amine –NH functional groups to –NCl. The absence of basic primary amines shifts the pH balance of the films, and the majority of

carboxylate groups are now protonated. Hydrogen bonding between $-NH_3^+$ and carbonyl groups is also eliminated. The spectra in Figures 4.11 and 4.12 do not indicate the presence of acid chloride functional groups, which would be expected to have carbonyl stretch in the 1790-1850 cm^{-1} region. In addition to this, a new small peak at 809 cm^{-1} appears in spectrum in Figure 4.11, which is the region where N-Cl stretching has been shown to occur in some known N-Chloramide [76].

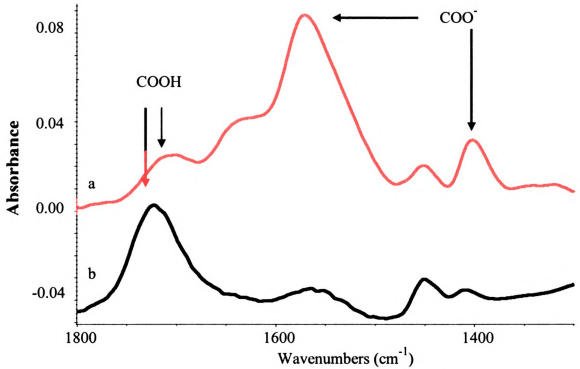


Figure 4.12 FTIR-ERS spectra expanded around the carbonyl region of a $[PAH/PAA]_5$ multilayer film on gold. (a) after heating at 120 °C overnight, and (b) after treatment with NaOCl.

4.3.6 Shelf life issues

The sporicidal activity of "promising" coatings was monitored over a course of 6 months to determine if there are any shelf-life issues. The significantly higher biocidal activity of $[PAH/PAA]_5/PAA$ against spores compared to other PEM coatings was demonstrated on

different batches of similar coatings over a course of 6 months. Two specific coating variants tested on 23 February, 2009 (Coating ID 4A and 8A: Table 4.1) were retested sporicidal activity on 23 November, 2009 (Figure 4.13).

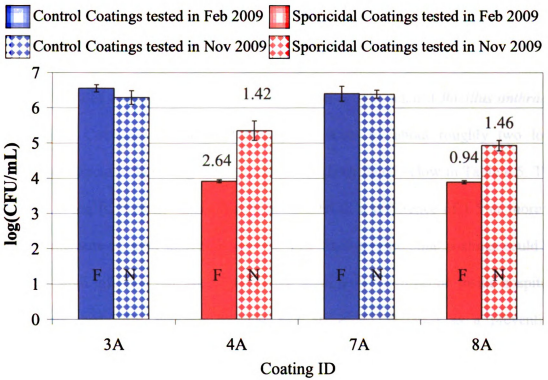


Figure 4.13 PEM coatings tested for shelf-life issues. Sporicidal coatings with five [PAA/PAH] bilayers having either PAA or G4PAMAM/PAA as the topmost layer were retested for sporicidal activity after 6 months from initial testing. Spore count data represent mean +/- S.D. of three independent experiments. The color-filled and patterned bars of same color for each coating variant represent the same coating variant tested in February 2009 and November 2009 respectively.

As shown in Figure 4.13, both the coatings 4A and 8A showed significant sporicidal activity (about 1.5 log reduction in CFU values). Coatings 3A and 7A are the corresponding control coatings for the coatings 4A and 8A respectively. F and N refers on the bars refers to February and November 2009 (the months the coatings were tested).

The coating variant that had G4PAMAM had higher sporicidal activity compared to the variant with PAA as the topmost layer. This may be due to the sequestration of bleach molecules by hydantoin molecules in the PEM, which results in better sporicidal activity over a period of 6 months.

4.3.7 Conclusions

A number of PEM coatings were tested for sporicidal activity against *Bacillus anthracis* sterne spores. Coatings that reduced the spore counts by about roughly two logs (equivalent to about 99% killing of the spores) have been listed below in Table 4.5. The difference in log (CFU/mL) values has been calculated, a difference of 1.5 or more is taken as a measure of good sporicidal activity. The listed antimicrobial coatings could be applied to "high infection rate areas" which are more prone to infection inside hospitals (e.g. faucets, bed rails, elevator buttons, and electrical switches) as a preventive decontaminant. These coatings are desirable because their reactive functionality can be regenerated upon treatment with bleach.

Table 4.6 Coatings that has shown evidence of antimicrobial activity compared to the control coatings (without bleach)

Coating ID	Coating	Difference in log CFU
4A	[PAA/PAH]5/PAA/120°C/NaOCl (Figure 4.1)	2.64
4B	[PAA/PAH]5/PAA/120°C/NaOCl/alanine&inosine (Figure 4.1)	2.664
6A	[PAA/PAH]5/PAA/G4PAMAM/120°C/NaOCl (Figure 4.2)	1.419
8	[PAA/PAH]5/PAA/120°C/NaOCl (Figure 4.4)	1.529
16	[PAA/PAH]10/PAA/120°C/NaOCl (Figure 4.4)	1.134

The coatings could also be used in air filtration systems in research facilities and in hospitals to prevent the spread of microorganisms. They are intended for use as a preventive decontaminant as opposed to currently used post decontamination agents. Although the N-halamines that are used in these coatings may degrade over time, they can be regenerated by treatment with bleach.

4.3.8 Future work

The thesis work has identified several promising coating variants that may be used in "high infection rate areas". Further work could better examine the sporicidal mechanism of the germinated spores and whether the sporicidal PEMs prevent biofilm formation.

This work was a pilot study in which the PEMs were fabricated on 96 well polyprolylene plates using LbL method. To prove the versatility and efficiency of the LbL method, sporicidal coatings may be fabricated on other materials such as glass and stainless steel and assayed for sporicidal activity and biofilm growth. Substrates such as stainless steel can heated to 180^0C to introduce crosslinking in PEM bilayers and tested if there is any significant improvement in reducing bacterial population compared to the substrates heat treated at lower temperatures.

The protocol used in this thesis to test the coating variants for sporicidal activity tested for sporicidal activity after 24 hours since significant sporicidal effect against *Bacillus anthracis* spores was observed only after 16 hours. Germinated spores have been shown to be more susceptible to sporicidal agents. Thus the activity of the sporicidal PEM coatings could be tested with germinated spores. Furthermore, the sporicidal activity of the PEMs could also be tested on common bacteria found in the hospital environment such as *E. Coli, S. epidermidis and S. aureus.*

APPENDIX A

PROTOCOL FOR PREPARING BHI-AGAR PLATES

This protocol is used to prepare 500mL of the BHI agar solution, which should always be prepared in a 1L bottle, to prevent the solution from boiling off.

1. Add 18.5g Brain Heart Infusion (BHI) powder [Cat # 760 031, Bioworld] to 500mL of sterile nanopure water.

2. Continuously heat and stir the solution until the solution starts to boil.

3. Add 7.5g agar of Bactoagar [Cat# 214010, BD] to the hot solution and stir continuously until the agar is completely dissolved.

4. Sterilize the solution by autoclaving for 20 minutes.

5. Allow the solution to cool to room temperature, and pour it onto the desired culture plates and cover the plates.

6. Transfer the plates to a biosafety hood and open the lids to allow solidification and drying of the agar. Allow the plates to dry for no more than 10 minutes.

7. To store the plates for future use, cover the plates and keep refrigerated at $4^{o}C$.

APPENDIX B

PROTOCOL FOR SPORE INCUBATION IN PP PLATES AND FILTER MEMBRANES

This protocol summarizes the steps to dry a layer of aqueous spore solution on a surface coating, for determining the sporicidal qualities of the coatings being tested, which may contain germinant or biocidal agents.

1. An aqueous solution of spores containing 10^6 viable spores is applied to a coated well or 13 mm filter membrane. The total volume applied to a well in a 96 well plate is 50uL. Immediately after application, the applied volume is re-pipetted to ensure that the well bottom is covered.

2. Plate wells containing the 50uL spore solution are placed into a vacuum apparatus that allows complete drying within 50 minutes (drying rate is about 1uL per minute). The filter membranes are dried covered inside a Petri dish placed within a biosafety hood, which takes about an hour to dry completely.

3. The optimum time required for the biocidal action is determined by setting up enough wells or filter membranes to test a range of exposure times (2, 4, 6, 12, 18 and 24 hours). Twenty four hours incubation time (~99% killing) was found to be optimum based on initial experiments. Three replicates were performed per coating formulation.

4. Dried spores are recovered from the coated surfaces by application of 0.04%BSA/0.03% Sodium Thiosulfate solution. A well received 200uL and this volume is used to wash by pipetting repeatedly for 30 seconds. The BSA-Thiosulfate solution

forms bubbles, so the tip is immersed inside the well during the wash. Dried filter membranes are placed in 1.5mL snap tubes and vortexed in 200uL BSA/Thiosulfate solution for 30 seconds.

5. The washes are collected and 100uL is used to perform four 10-fold dilutions going down to 10-3 dilution. BSA-thiosulfate solution is used to prepare the series dilution and remaining volume from the wash is reserved.

6. 100uL of the dilution series and the remaining wash volumes are plated on pre dried BHI-agar plates. The plates were spread with the spore solution using L-spreaders or loop inoculation technique.

7. Incubate plates at 37°C, and enumerate colonies after 16-18 hours growth.

APPENDIX C

TEST TO DETERMINE THE SPORICIDAL COMPONENT IN THE COATINGS

The coatings that we are currently testing are immersed in bleach to render the surface sporicidal. We enumerate the spores by plating them out on BHI-agar plate; hence the spores may have residual chlorine that might prevent their normal growth even after the spores are washed with water. Hence we use BSA-Thiosulfate solution as the diluent since the thiosulfate neutralizes the free chlorine ions and BSA washes out any spores that get adhered to the surface of the coatings. This is essential to accurate determine if the coatings are performing as expected.

An experiment was performed with selected coatings to determine the effect of BSA/ thiosulfate on spore recovery from coated wells and to determine optimum incubation time to achieve about 98-99% antimicrobial activity. The spores were incubated in the coated wells are diluted using sterile water for all experiments unless mentioned otherwise. A batch of spores was diluted using BSA/thiosulfate to test the effect of bleach in the coatings. The coatings tested are listed below in Table C.1.

Table C.1 PEM coatings tested to determine sporicidal agent

Coating ID	Coating
e	$[PAA/PAH]_5/PAA/120^oC$
f	$[PAA/PAH]_5/PAA/120^oC$/alanine&inosine
g	$[PAA/PAH]_5/PAA/120^oC$/NaOCl
h	$[PAA/PAH]_5/PAA/120^oC$/NaOCl/alanine&inosine

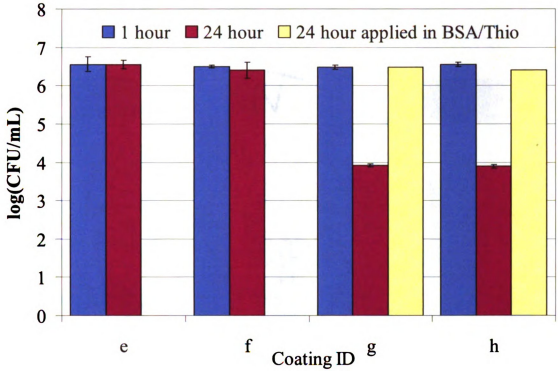


Figure C.1 Spore counts in PEM coatings. Both the control and sporicidal coatings were assayed for antimicrobial activity for 1 and 24 hours. When spores suspended in water was used to determine sporicidal activity in coating g and h, the spore counts were reduced by about 2 logs. However when the spores suspended in BSA/ thiosulfate solution was used, the thiosulfate neutralizes the bleach in the PEM coatings and hence no significant reduction in CFU values was observed.

There is no significant difference between the sporicidal coatings (g,h) and the control (e,f) in 1 hour. However, 24 hour incubation produces about 2 log reduction in spore counts in the sporicidal coatings. This effect is not observed when spores diluted in

BSA/thiosulfate solution were incubated in the test coatings for 24 hours. This is because the thiosulfate neutralizes the chlorine ions released by the test coatings. BSA was used since spores sticking onto pipet tips can cause considerable errors while enumerating the spores before and after applying them onto the wells of polypropylene plates (Figure A1).

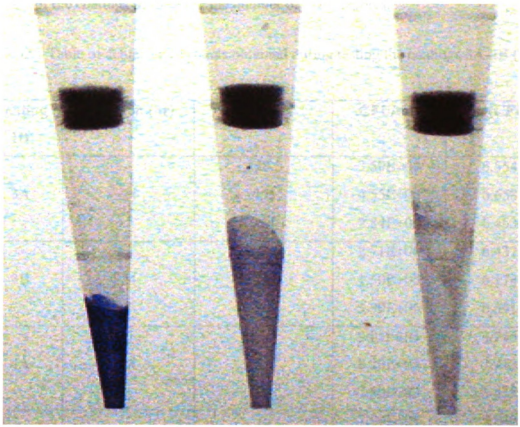


Figure C.2 Spores sticking onto pipet tips could be visualized when stained with methylene blue. a) pipet tip containing spores solution in water. The spores could easily be seen sticking onto the inner surface of the pipet tip b) pipet tip was rinsed with water a number of times to ensure that no spores were sticking onto the sides. However, some spores could be seen sticking onto the surface c) pipet tip was rinsed with BSA/ thiosulfate solution. The number of spores sticking onto the inner surface is considerably less compared to the pipet tip rinsed with water.

APPENDIX D

CALCULATIONS FOR % REDUCTION IN CFU VALUES

All the results for bacterial spore counts in this thesis are presented as a log (CFU/mL). An example has been shown below that summarizes calculations for determining the efficacy of the coatings in reducing the number of viable spores.

Table D.1 Table of actual spore counts obtained during testing the coatings 3A-4B (Table 4.1)

Coating ID	Duration (hr)	# Spores	CFU/mL	log(CFU)
3A	24	358.5	3.59E+06	6.554
		455	4.55E+06	6.658
		284	2.84E+06	6.453
3B	24	270.5	2.71E+06	6.432
		149.5	1.50E+06	6.174
		390	3.90E+06	6.591
4A	24	90.5	9.05E+03	3.956
		82	8.20E+03	3.913
		75	7.50E+03	3.875
4B	24	70	7.00E+03	3.845
		80	8.00E+03	3.903
		84	8.40E+03	3.924

Each coating was tested in triplicates. The number of spores obtained by counting the colonies on BHI plate is the average of at least two different dilutions (See Apendix B for complete procedure). For example the first CFU/mL value 358.5 is an average of 377 (spore count from second 10-fold dilution) and 340 (spore count from third 10-fold

dilution). Each of these spore counts are converted to determine the individual CFU/mL.

The difference in log of the CFU/mL between the control and sporicidal coatings were to determine the efficacy of the sporicidal coatings. For example, values obtained from subtracting the log (CFU/mL) values for Coating 4A from 3A; 4B from 3B were used to determine if the sporicidal coatings are performing as expected.

Table D.2 Actual spore counts for coatings 3A-4B

Coating ID	Average log(CFU/mL)	Calculations	Difference in log CFU
3A	6.555		
3B	6.399		
4A	3.915	=6.555-3.915	2.64
4B	3.891	=6.399-3.891	2.508

The values 2.64 and 2.508 correspond to the decrease in log CFU values for the coatings 4A and 4B as shown in Fig 3.5. To convert the difference in log CFU/mL to percentage reduction in number of viable spores, consider the following example:

Let the initial CFU/mL be 1000 and the final CFU/mL be 10. This means that the

difference in the log (CFU/mL) values is log(1000) – log (10) = 3-1 = 2.

Thus the percentage killing is = $\frac{\text{(initial – final) CFU/mL}}{\text{initial}} \times 100 = 990*100/1000 = 99$

BIBLIOGRAPHY

1. Klevens RM, Edwards JR, Richards CL, Horan TC, Gaynes RP, Pollock DA, Cardo DM. Estimating Health Care-Associated Infections and Deaths in U.S. Hospitals, 2002. *Public Health Reports.* 2007; 122: 160 – 166.

2. Wenzel RP and Edmond MB. The impact of hospital-acquired bloodstream infections. *Emerging Infectious Disease.* 2001; 7(2): 174 – 177.

3. Dominguez TE, Chalom R, Costarino AT Jr. The impact of adverse patient occurrences on hospital costs in the pediatric intensive care unit. *Critical Care Medicine* 2001; 29: 169-174.

4. Diaz MC, Garcia MM, Bueno CA, Lopez LA, Delgado RM, Galvez VR. The estimation of the cost of nosocomial infection in an intensive care unit. *Medicina Clinica.* 1993; 100: 329-332.

5. Miller ZC. Excess length of stay, charges, and mortality attributable to medical injuries during hospitalization. *JAMA* 2003; 290: 1868-1874.

6. Muscarella LF. Contribution of tap water and environmental surfaces to nosocomial transmission of antibiotic-resistant *Pseudomonas aeruginosa. Infection control and hospital epidemiology.* 2004; 25(4): 342 – 345.

7. Costerton JW, Stewart PS, Greenberg EP. Bacterial bioflms: a common cause of persistent infections. *Science.* 1999; 284: 1318 – 1322.

8. DeBeer D, Srinivasan R, Stewart PS. Direct Measurement of Chlorine Penetration into Biofilms during disinfection. *Applied and Environmental Microbiology.* 1994; 60(12): 4339 – 4344.

9. Russell AD. Bacterial Spores and Chemical Sporicidal agents. *Clinical Microbiology Reviews.* 1990; 3(2): 99-119.

10. Elbert DL, Herbert CB, Hubbell JA. Thin polymer layers formed by Polyelectrolyte multilayer techniques on biological surfaces. *Langmuir.* 1998; 15: 5355 – 5362.

11. Muller M, Rieser T, Lunkwitz K, Meierhaack J. Polyelectrolyte complex layers – a promising concept for antifouling coatings verified by in-situ ATR-FTIR spectroscopy. *Macromolecular Rapid Communications.* 1999; 20: 607 – 611.

12. Cowan MM, Abshire KZ, Houk SL, Evans SM. Antimicrobial efficacy of silver-zeolite matrix coating on stainless steel. *Journal of Industrial Microbiology and Biotechnology.* 2003; 30(2): 102-106.

13. Goddard JM, Hotchkiss JH. Polymer surface modification for the attachment of bioactive compounds. *Progress in Polymer Science.* 2007; 32: 698-725.

14. Chua PH, Neoh KG, Kang ET, Wang W. Surface functionalization of titanium with hyaluronic acid/chitosan polyelectrolyte multilayers and RGD for promoting osteoblast functions and inhibiting bacterial adhesion. *Biomaterials.* 2008; 29 (10): 1412–1421.

15. Lvov Y, Decher G, Sukhorukov G. Assembly of Thin Films by Means of Successive Deposition of Alternate Layers of DNA and Poly(Allylamine). *Macromolecules.* 1993; 26: 5396-5399.

16. O'Brien FJ, Harley BA, Yannas IV, Gibson LJ. The effect of pore size on cell adhesion in collagen-GAG scaffolds. Biomaterials. 2005; 26(4): 433- 441.

17. Setlow B, Cowan AE, Setlow P. Germination of spores of Bacillus subtilis with dodecylamine. Journal of Applied Microbiology. 2003; 95: 637-648.

18. Setlow P. Resistance of bacterial spores, In: Storz G and Hengge-Aronis R, eds. Bacterial stress responses. ASM press, Washington, D.C; 2000; 217-230.

19. Cowan AE, Koppel DE, Setlow B, Setlow P. A soluble protein is immobile in dormant spores of Bacillus subtilis but is mobile in germinated spores: implications for spore dormancy. Proceedings of the National Academy of Sciences USA. 2003; 100: 4209-4214.

20. Ablett S, Darke AH, Lillford PJ, Martin DR. Glass formation and dormancy in bacterial spores. *International Journal of Food Science and Technology.* 1999; 35(1): 59-62.

21. Leuschner RG, Lillford PJ. Effects of hydration on molecular mobility in phase-bright *Bacillus subtilis* spores. *Microbiology.* 2000; 146:49-55.

22. Westphal AJ, Price PB, Leighton TJ, Wheeler KE. Kinetics of size changes of individual *Bacillus thuringiensis* spores in response to changes in relative humidity. *Proceedings of the National Academy of Sciences USA.* 2003; 100:3461-3466.

23. Setlow B, Setlow P. Measurements of the pH within dormant and germinated bacterial spores. *Proceedings of the National Academy of Sciences USA.* 1980; 77: 2474-2476.

24. Paidhungat M, Setlow B, Driks A, Setlow P. Characterization of spores of *Bacillus subtilis* which lack dipicolinic acid. *Journal of Bacteriology*. 2000; 182: 5505-5512.

25. Paidhungat M, Ragkousi K, and Setlow P. Genetic requirements for induction of germination of spores of *Bacillus subtilis* by Ca^{2+} dipicolinate, *Journal of Bacteriology*. 2000; 183: 4886-93.

26. Frenkiel-Krispin D, Sack R, Englander J, Shimoni E, Eisenstein M, Bullitt E, Horowitz-Scherer R, Hayes CS, Setlow P, Minsky A, Wolf SG. Structure of the DNA-SspC complex: implications for DNA packaging, protection, and repair in bacterial spores. *Journal of Bacteriology*. 2004; 186:3525-3530.

27. Setlow P, Mechanisms which contribute to the long-term survival of spores of *Bacillus* species, *Society for Applied Bacteriology Symposium Series*, 2000; 23:49S-60S.

28. Cowan AE, Olivastro EM, Koppel DE, Loshon CA, Setlow B, Setlow P. Lipids in the inner membrane of dormant spores of *Bacillus* species are largely immobile, *Proceedings of the National Academy of Sciences USA*. 2004; 101: 7733-7738.

29. Cortezzo DE, Koziol-Dube K, Setlow B, Setlow P. Treatment with oxidizing agents damages the inner membrane of spores of Bacillus subtilis and sensitizes spores to subsequent stress. *Journal of Applied Microbiology*. 2004; 97: 838-852.

30. Moir A, Corfe BM, Behravan J. Spore Germination. *Cellular and Molecular Life Sciences*. 2002; 59: 403–409.

31. Popham DL, Specialized peptidoglycan of the bacterial endospore: the inner wall of the lockbox. *Cellular and Molecular Life Sciences*. 2002; 59: 426-433.

32. Piggot PJ, Hilbert DW. Sporulation of *Bacillus subtilis*. *Current Opinions in Microbiology*. 2004; 7: 579-586.

33. Nakashio S, Gerhardt P. Protoplast dehydration correlated with heat resistance of bacterial spores, *Journal of Bacteriology*. 1985; 162: 571-578.

34. Crafts-Lighty A, Ellar DJ. The structure and function of the spore outer membrane in dormant and germinating spores of *Bacillus megaterium*. *Journal of Applied Bacteriology*. 1980; 48: 135-145.

35. Young SB, Setlow P. Mechanisms of *Bacillus subtilis* spore resistance to and killing by aqueous ozone, *Journal of Applied Microbiology*. 2004; 96: 1133-1142.

36. Behravan JH, Chirakkal H, Masson A, Moir A. Mutations in the *gerP* locus of *Bacillus subtilis* and *Bacillus cereus* affect access of germinants to their targets in spores, *Journal of Bacteriology*. 2000; 182: 1987-1994.

37. Koshikawa T, Yamazaki M, Yoshimi M, Ogawa S, Yamada A, Watabe K, Torii M. Surface hydrophobicity of spores of Bacillus species. *Journal of Genetic Microbiology.* 1989; 135: 2717-2722.

38. Francis CA, Tebo BM. Enzymatic manganese (II) oxidation by metabolically dormant spores of diverse *Bacillus* species. *Applied Environonmental Microbiology.* 2002; 68:874- 880.

39. Todd SJ, Moir AJ, Johnson MJ, Moir A. Genes of *Bacillus cereus* and *Bacillus anthracis* encoding proteins of the exosporium. *Journal of Bacteriology.* 2003; 185: 3373-3378.

40. Bailey-Smith K, Todd SJ, Southworth TW, Proctor J, Moir A. The ExsA protein of *Bacillus cereus* is required for assembly of coat and exosporium onto the spore surface. *Journal of Bacteriology.* 2005; 187: 3800-3806.

41. Block SS. Disinfection, sterilization, and preservation, 4th ed. 1991.

42. McDonnell G, Russell AD. Antiseptics and disinfectants: activity, action and resistance. *Clinical Microbiology Reviews.* 1999; 12(1): 147-179.

43. Maillard JY. Bacterial target site for biocide action. *Journal of Applied Microbiology.* 2002; 92: 16S-27S.

44. Knight DJ, Cooke M, eds. *The Biocide Business: Regulations, Safety and Applications.* Weinheim, Germany: Wiley-VCH; 2002.

45. Russell AD. Similarities and differences responses in microorganisms to biocides. *Journal of Antimicrobial Chemotherapy.* 2003; 52: 750-763.

46. Decher G. Fuzzy Nanoassemblies: Toward Layered Polymeric Multicomposites, *Science.* 1997; 277:1232-1237

47. Davey ME and O'Toole GA. Microbial films: from ecology to molecular genetics. *Microbiology and Molecular Biology Reviews.* 2000; 64(4): 847-867.

48. Yildiz, FH and Schoolnik GK. Vibrio cholerae O1 El Tor: identification of a gene cluster required for the rugose colony type, exopolysaccharide production, chlorine resistance, and biofilm formation. *Proceedings of National Academy of Sciences USA.* 1999; 96: 4028-4033.

49. Lewansowski Z, Altobelli SA, and Fukushima E. NMR and microelectrode studies of hydrodynamics and kinetics in biofilms. *Biotechnology Progress.*1999; 9:40-45.

50. Stewart PS and Costerton JW. Antibiotic resistance of bacteria in biofilms. *LANCET.* 2001; 358: 135-138.

51. Lindsay D, Brozel VS and von Holy A. Biofilm-spore response in *Bacillus cereus and Bacillus subtilis* during nutrient limitation. *Journal of Food Protection.* 2006; 69(5): 1168-1172.

52. Maki DG and Tambyah PA. Engineering out the risk of infection with urinary catheters. *Emerging Infectious Diseases.* 2001: 7(2): 1-6.

53. Dolan RM. Biofilms and device-associated infections. *Emerging Infectious Diseases.* 2001; 7(2): 277-281.

54. Shi Z, Neoh KG, Yung LYL, Kang ET and Wang W. In vitro antibacterial and cytotoxicity assay of multilayered polyelectrolyte-functionalized stainless steel. *Journal of Biomedical Materials Research Part A.* 2006; 76(4): 826-834.

55. Boulmedais et Al. Polyelectrolyte multilayer films with pegylated polypeptides as a new type of anti-microbial protection for biomaterials. *Biomaterials.* 2003; 25(11): 2003-2011.

56. Cassinelli C, Morra M, Pavesio A and Renier D. Evaulation of interfacial properties of hyaluronan coated poly(methylmethacrylate) intraocular lenses. *Journal of Biomaterials Science, Polymer Edition.* 2000; 11(9): 961-977.

57. Chuang HF, Smith RC and Hammond PT. Polyelectrolyte multilayers for tunable release of antibiotics. *Biomacromolecules.* 2008; 9(6): 1660-1668.

58. Feng QL, Wu J, Chen GQ, Cui FZ, Kim TN and Kim JO. A mechanistic study of the antibacterial effect of silver ions on *Escherichia coli* and *Staphylococcus aureus. Journal of Biomedical Materials Research.* 2000; 52(4): 662-668.

59. Rabea EI, Badawy MET, Stevens CV, Smagghe and Walter S. Chitosan as antimicrobial agent: applications and mode of action. *Biomacromolecules.* 2003; 4(6): 1457-1465.

60. Park D, Wang J and Klibanov AM. One-step painting-like coating procedures to make surfaces highly and permanently bactericidal. *Biotechnology Progress.* 2006; 22: 584-589.

61. Milovic NM, Wang J, Lewis K and Klibanov AM. Immobilized N-alkylated polyethylenimine avidly kills bacteria by rupturing cell membranes with no resistance developed. *Biotechnology and Bioengineering.* 2005: 90(6): 715-722.

62. Gottenbos B, van der Mei HC, Klatter F, Nieuwenhuis P and Busscher HJ. Invitro and invivo antimicrobial activity of covalently coupled quaternary ammonium silane coatings on silicone rubber. *Biomaterials.* 2002; 23(6): 1417-1423.

63. Sun Y and Sun G. Novel regenerable N-halamine polymeric biocides. I. Synthesis, characterization, and antimicrobial activity of hydantoin-containing polymers. *Journal of Applied Polymer Science.* 2001; 80(13): 2460-2467.

64. Qian L and Sun G. Durable and regenerable antimicrobial textiles: chlorine transfer among halamine structures. *Industrial and Engineering Chemistry Research.* 2005; 44(4): 852-856.

65. Lvov Y, Ariga K, Kunitake T. Assembly of Alternate Protein Polyion Ultrathin Films. *Chemistry Letters.* 1994: 2323-2326.

66. Richert L, Lavalle P, Payan E, Shu XZ, Prestwich GD, Stoltz JF, Schaaf P, Voegel, JC, Picart C. Layer by Layer Buildup of Polysaccharide Films: Physical Chemistry and Cellular Adhesion Aspects. *Langmuir.* 2004; 20: 448-458.

67. Caruso F, Trau D, Mohwald H, Renneberg R. Enzyme Encapsulation in Layer-by- Layer Engineered Polymer Multilayer Capsules. *Langmuir.* 2000; 16: 1485-1488.

68. Encyclopedia of surface and colloid science, 2nd ed, 2006.

69. Mendelsohn JD, Barrett CJ, Chan VV, Pal AJ, Mayes AM, Rubner MF. Fabrication of microporous thin films from Polyelectrolyte Multilayers. *Langmuir.* 2000; 11(6): 5017-5023.

70. Harris JJ, DeRose PM, Bruening ML. Synthesis of Passivating, nylon like coatings through Cross-linking of Ultrathin Polyelectrolyte films. *Journal of American Chemical Society.* 1999; 121(9): 1978-1979.

71. Yang SY, Lee D, Cohen RE, Rubner MF. Bioinert solution cross linked hydrogen bonded multilayers on colloidal particles. *Langmuir.* 2004; 20(14); 5978-5981.

72. Worley SD, Williams DE, Crawford RA. Halamine water disinfectants. *Critical Reviews in Environmental Science and Technology.* 1988; 18(2): 113-174.

73. Dendrimer and hyperbranched polymers: http://www.sigmaaldrich.com/materials-science/nanomaterials/dendrimers/dendrons.html

74. Tomalia DA, Naylor AM, and Goddard III WA. Starburst Dendrimers: Molecular-Level Control of Size, Shape, Surface Chemistry, Topology, and Flexibility from Atoms to Macroscopic Matter. *Angewandte Chemie International Edition.* 1990; 29: 138-175.

75. Shiratori SS and Rubner MF. pH-dependent thickness behavior of sequentially adsorbed layers of weak polyelectrolytes. *Macromolecules*. 2000; 33: 4213-4219.

76. Petrov A, Antipov AA, Sukhrorukov GB. Base-acid equilibra in polyelectrolyte systems: from weak polyelectrolytes to interpolyelectrolyte complexes and multilayered polyelectrolyte shells. *Macromolecules*. 2003; 36: 10079-10086.

77. Petterson RC, Grzeskowiak U and Jules LH. N-Halogen Commpounds. II. The N-Cl Stretching Band in Some N-Chloroamides. The Structure of Trichloroisocyanuric Acid. *Journal of Organic Chemistry*. 1960; 25: 1595-1598.