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Testing the bactericidal ability of a polycationic molecule to design self-healing surfaces

Shraddha Jayant Deodhar
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a thesis written by Shraddha Jayant Deodhar entitled "Testing the bactericidal ability of a polycationic molecule to design self-healing surfaces." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Chemistry.

Mark Dadmun, Major Professor

We have read this thesis and recommend its acceptance:

Jimmy Mays, Robert Hinde

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Robert Hinde

Accepted for the council:

Linda Painter
Interim Dean of Graduate Studies

(Original signatures are on file with official student records.)

Testing the bactericidal ability of a polycationic molecule to design self-healing surfaces.

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Shraddha Jayant Deodhar
May 2007

This thesis is dedicated to my parents, sister and my husband for inspiring and encouraging me to reach higher in order to achieve my goals.

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Abstract

The need for antimicrobial surfaces is obvious because of ever growing demand for healthy living, thus a material that can kill harmful microorganisms is of great interest. The idea discussed in this thesis is to combine the techniques of Photo Dynamic Therapy (PDT) and self sterile surfaces to design self-healing surfaces.

We modified one pyridine ring of Tetro-meso (N-methyl pyridyl) porphyrin (T_4MPyP) to obtain 5-(4-carboxyphenyl)-10, 15, and 20-tris-(N-methyl-4-pyridlium) porphyrin. This modified porphyrin was reacted with Vinyl benzyl chloride polymer via a nucleophilic substitution reaction forming an ester linkage between polymer and porphyrin. The above molecule was then tested for its bactericidal property.

The thesis explains in detail the protocols and procedures used for bacterial analysis. It also explains the synthesis of poly (styrene-co-4-vinyl benzyl chloride) [poly (St-co-VBC)] and 5-(4-carboxyphenyl)-10, 15, and 20-tris-(4-pyridyl) porphyrin. This porphyrin was then converted to a salt by reaction with iodomethane and attached as a pendant group to a co-polymer chain. This was completed to determine whether porphyrin retained its bacterial killing ability when attached to the co-polymer backbone. The attachment of the porphyrin to the polymer while retaining its antimicrobial activity is important to design self healing surfaces.

Coatings of this polymer solutions were obtained by painting the bacteria sprayed slide with 40µl of the polymer solution. The antimicrobial ability of these polymer coated slides was observed by subjecting them to photo inhibition. Staphylococcus aureus (ATCC, strain 33807) and Escherichia coli (ATCC, strain 492316) were used to complete all our experiments..

The polycationic porphyrin salts killed the bacteria when exposed to light. The photosensitized porphyrins (T_4MPyP and modified porphyrin) worked for two kinds of illuminated lights (Incandescent light bulb and fluorescent light bulb). The aim of this project to design self-healing antimicrobial surfaces where the antimicrobial molecule is a pendant group on a polymer backbone was successful. The cationic porphyrin when attached to the VBC co-polymer as a pendant group retained its antimicrobial ability.

This is very encouraging and motivating to pursue our proposed goal of designing self-healing surfaces further.

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Chapter 1 Introduction

1.1 Objective and Motivation

The need for antimicrobial surfaces is obvious, whose hands were on the door knob before you? But it doesn't just start or end at the door knob. Who, before you, used the public phone or touched the hand rail or the subway pole? And what if that person had a cold or was infected with a contagious disease? People transfer the infections to the object, which in turn can be transferred to you. In 1940, Ignaz Senmelwis showed the world the importance of hand hygiene: In a delivery room in a Vienna general hospital, he proved that infections were reduced by almost 80% just by washing hands in chlorine water (which is solution of chlorine gas in water). Contaminated hands are believed to be the single greatest cause for transmitting nosocomial infections. Earlier, 'nosocomial infections' were defined as infections that patients get while still in hospital. But now the National Nosocomial Infections Surveillance (NNIS) does not limit the term 'nosocomial infections' only to hospitals. If, between patients, medical personnel fail the simple procedure of washing hands, patients visiting medical clinics or health care facilities are also liable to these infections.[1] Two million nosocomial infections occur in the United States every year.[2] Other than nosocomial infections, there are blood stream infections caused by the use of infected intravascular devices and are seen mostly in ICU patients, who are at grave risk. An article in polymedix estimates the mortality rate attributed to blood stream infections in ICUs to be 35%.[2] Infections in the military are also a concern, as severe war injuries often occur in an unsanitary environment or by the use of biological weapons. U.S forces face a major challenge to decontaminate surfaces exposed

to chemical or biological agents.[3] Thus, because of ever growing demand for healthy living, a material that can kill harmful microorganisms is of great interest.[4]

Traditional means of halting the spread of infections include the use of antimicrobial lotions and soaps in hospitals, schools and other public places. These traditional methods, if used along with bactericidal surfaces, could prevent many infections. Bactericidal paints and coatings can also be applied in public places such as restaurants, hotels, airports, public rest rooms or to objects touched by people everyday such as door knobs, key boards, children's toys and other common objects.[4] If many common objects are rendered antiseptic, the transmission of bacterial infections could be significantly prevented.

Why develop chemical or polymer coatings? Why not use antibiotics or medicines? Bacterial resistance to many widely used drugs has seriously complicated the treatment of diseases and are spreading into an epidemic.[5-8] For instance, recently emerging bacterial strains such as enterococci and staphylococci show resistance to all available antibiotics and chemotherapeutic agents.[9-10] A national survey of hospital patients, which included more than 8500 clinical cases of serious infections, showed that Escherichia coli (e-coli) and Staphylococcus aureus (S.aureus) were most common bacterial isolates.[11] Unfortunately only 5-10% of S.aureus isolates are treatable with drugs such as penicillin, carboxy-penicillin and amniopenicillin.[12] Despite new antibiotics being introduced, the possibility of an antibiotic resistance epidemic cannot be prevented by drugs.[10] Moreover, the synthesis of new antibiotics is a very expensive and slow process. Even with the large profits made on antibacterial drugs, pharmaceutical companies have reduced the production of making new antibiotics.[13]

Thus, it is obvious that a new approach to destroying bacteria without affecting the host should be undertaken. Designing self-healing surfaces, which are surfaces with the ability to repair damage, is a sought after field. When a problem is encountered, this surface is able to correct itself. Also, the worldwide rise in antibiotic resistance[15] necessitates the development of antimicrobial coatings that make the surfaces persistently lethal to a wide variety of bacteria on contact. The creation of a coating that will not significantly add to the cost and weight of the coated system is a field of great interest in many industries and laboratories.[3]

1.2 Literature Review

a) Antimicrobial peptides

The wide spread resistance of bacteria of many widely available antibiotics as discussed in the objective and motivation section, has renewed interest in the use of alternate microbial inhibitors. Polycationic molecules are found to have vast applications as bactericidal agents. One class of such molecules is antimicrobial peptides (AMPs). These peptides, which are also called host defense peptides, have captured the attention of many researchers because they are potent and show a broad spectrum antimicrobial activity. It has been demonstrated that they kill gram negative and gram positive bacteria, viruses, fungi and show selectivity between mammalian red blood cells (RBCs) and bacterial cells. The peptides target the phospholipid membranes, disrupt them and thus ultimately kill the cells. But these peptides are large complex molecules, which are difficult to synthesize.[16-21] The other disadvantage is the high cost involved in their

production. Also many peptides are said to cause allergies after repeated applications. Thus, one way to overcome these problems is to synthesize peptide mimics. Molecules that can mimic the biological activity of peptides are of great interest.

Greg Tew and his group have carried out systematic studies on designing and developing these peptide mimics. Some of the peptide mimics which are antimicrobial also show hemolytic activity.[21-22] Hemolysis is the destruction of red blood cells. This destruction can be caused because of the balance and spatial arrangement of hydrophobic and hydrophilic components of the peptide mimics. Greg Tew and his group showed that by controlling the hydrophobic/hydrophilic components of these amphiphilic polymers, it is possible to obtain high selectivity between their antibacterial nature and hemolytic activity.[16]

For example, they synthesized amphiphilic cationic polynorbornene derivatives with a wide range of molecular weights (Mn = 1600- 137500 g/mol) and narrow polydispersity. By tuning the hydrophobic/hydrophilic balance and molecular weight of these polynorbornene derivatives, highly selective non-hemolytic antimicrobial molecules were synthesized. Figure 1.1 shows different amphiphilic derivatives.

Poly1, which contained no substantial hydrophobic groups, did not show any antibacterial or hemolytic activity. Introduction of a hydrophobic group in Poly2 resulted in increased antibacterial activity with a minimum inhibitory concentration (MIC) of 200μg/ml against e-coli bacteria. MIC is the minimum concentration of an antibacterial agent in a given culture medium, below which the bacterial growth is not inhibited. Poly2 showed antibacterial ability but lacked hemolytic activity.[16,23-27] With an addition of one

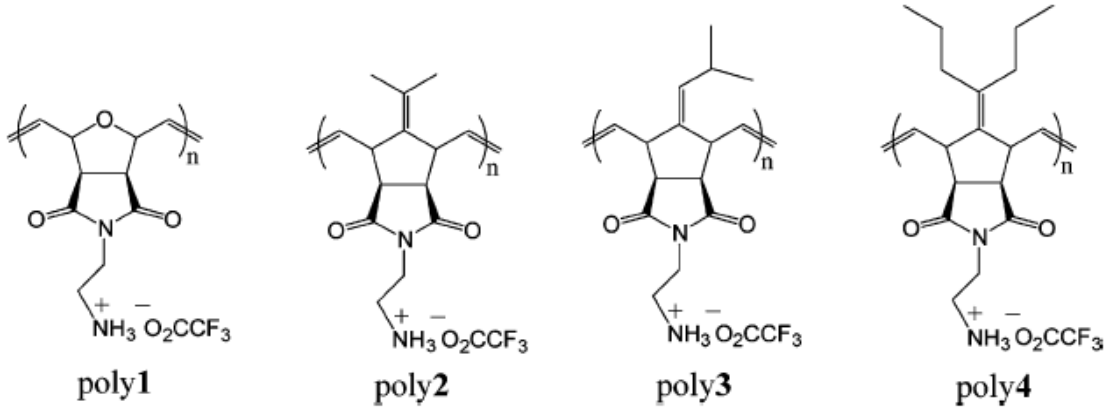


Figure1.1 Different Amphiphilic polynorbornene derivatives. Poly2 showed low hemolytic activity while Poly3 showed strong antibacterial activity.[16]

more carbon atom, Poly3 was more hydrophobic than Poly2. When tested for its antimicrobial nature, Poly3 showed antibacterial activity with an MIC of 25µg/ml but also showed hemolytic activity. This increase in hemolytic and antimicrobial activity can be related to the presence of the hydrophobic group. Larger hydrophobic groups can interact with the inner core of cell membranes and can thus loose selectivity because of these interactions. The hydrophobic character of a molecule can dramatically affect its antibacterial and hemolytic activities, as more the hydrophobic nature, more will be its interactions with the cell membrane. It was seen that when hydrophobicity was further increased in Poly4, the molecule retained its hemolytic activity but showed a decrease in antibacterial activity. From these studies, the authors concluded that the hemolytic activity is controlled by the hydrophobic interactions, while the charged interactions dominate its antibacterial activities.[16,28]

There was no substantial molecular weight dependence on the antibacterial or hemolytic activities of these polymers except for Poly3, whose antibacterial activity

increased as its molecular weight decreased from 57200 g/mol to 10300 g/mol. They attempted to optimize the selectivity and antibacterial activity by copolymerizing Poly2 and Poly3, as Poly2 exhibited low hemolytic activity while Poly3 showed strong antibacterial activity. The final copolymer, Poly(2_9-co-3_1), with a molar ratio of 9/1 poly2/poly3 exhibited antibacterial activity similar to that of Poly3 and non-hemolytic character comparable to Poly2. Thus, by tuning the overall hydrophobicity of the polymer by copolymerizing the norbornene derivatives, a molecule which has non-hemolytic antibacterial activity was synthesized.

The ability of these peptide mimics to disrupt the phospholipid membrane and kill the bacteria is believed to occur by adapting a facially amphiphilic structure (FA). The overall architecture can be said to play a vital role in its antibacterial activity and selectivity.[29] Greg Tew and his co-workers synthesized hydrocarbon based poly phenylene ethynylene (PE) molecules that had antibacterial activity and selectivity between bacterial cells and red blood cells (RBCs). This polymer structure mimics the FA structure and antibacterial properties of host defense peptides. When the antimicrobial action of this PE molecule was measured for gram positive and gram negative bacteria, the most hydrophobic molecule lacked antibacterial activity. This may be because of the low solubility of the hydrophobic samples. A decrease in the overall hydrophobicity of the polymer resulted in an increase in its solubility and activity. Thus, it can be said that the selectivity of this molecule depends on the hydrophobic nature of the molecular structure and also because of the difference between the mammalian and bacterial cell membranes. A polymer structure with a hydrocarbon backbone and whose biological activity can be controlled by controlling the hydrophobicity can be successfully designed.

Having optimized the structural details, they further developed and characterized a small peptide mimic that showed selective antibacterial ability against a broad spectrum of bacteria including numerous clinically relevant strains.[30] A peptide mimic was synthesized by coupling dibromomethyl amine monomer with diethynylbenzene using Sonogashira coupling in a 4:1 stoichiometry. These Sonogashira couplings are the couplings, in which terminal alkynes with aryl or vinyl halides are performed with a palladium catalyst, a copper co-catalyst and an amino base. This synthesized compound was non-chiral and contained no proteolytic bonds. Proteolytic bonds are the peptide bonds between amino acids in proteins. When tested for its antimicrobial activity, this compound was found to be a fast-acting, broad spectrum toxin which was active against more than 23 bacterial strains. In the presence of human RBCs, this peptide mimic selectively targeted bacterial membranes for several bacterial species, thus disrupting phospholipids membranes of the target cells.

They also synthesized facially amiphilic urea oligomers in a one-pot reaction by carbonyl diimidazole (CDI) coupling. These oligomers showed greater antibacterial activity against both gram negative and gram positive bacteria than MSI-78 which is a magainin derivative.[31] Magainins represent one class of host defence peptides, which are cationic, amphiphilic molecules that position polar and non-polar residues on opposite sides of a helical cylinder. The internal hydrogen bondings in these oligomers limited their conformational flexibility and have potent antimicrobial activity.

Thus, polymeric molecules that mimic peptides have demonstrated antimicrobial activity similar to those seen in peptides. Based on the structure as well as the balance of

hydrophobic/hydrophilic interactions, these materials show antimicrobial behavior towards a wide spectrum of microbes while decreasing their hemolytic activity.

b) Surface coatings

Kilbanov, Tiller and their team of researchers showed that if N-alkylated Poly (4-vinylpyridine) (PVP) is covalently attached to the glass surface, it makes the surface permanently lethal to several bacteria.[4]

In this work, an NH_2 glass slide, which is a slide coated with aminopropyltrimethyoxy silane-coated microscope slide, was alkylated with 1,4-dibromobutane to introduce reactive bromo-butyl groups. These reactive groups are used to attach poly(vinylpyridine) (PVP). These PVP chains were further N-alkylated with hexyl bromide. This hexyl PVP coated NH_2 glass slide was then tested for antimicrobial ability by spraying it with a solution of bacteria common in household and hospital infections such as staph and e-coli, using a chromatography sprayer and the growth of bacteria on this slide is compared against a blank, uncoated NH_2 slide. The number of bacterial colonies growing on the coated and non-coated slides was counted. It was observed that very few colonies grew on the coated slides. The Hexyl-PVP slides killed 95-99% of the bacteria that came in contact with the surface. Figure 1.2 shows the step-wise procedure of coating hexyl PVP on NH_2 glass slides.

Previous efforts to design dry bacterial surfaces had been unsuccessful. Researchers attributed this failure to the polymer chains not being sufficiently long and flexible enough to penetrate bacterial cell walls. Dry surfaces bonded with PVP that

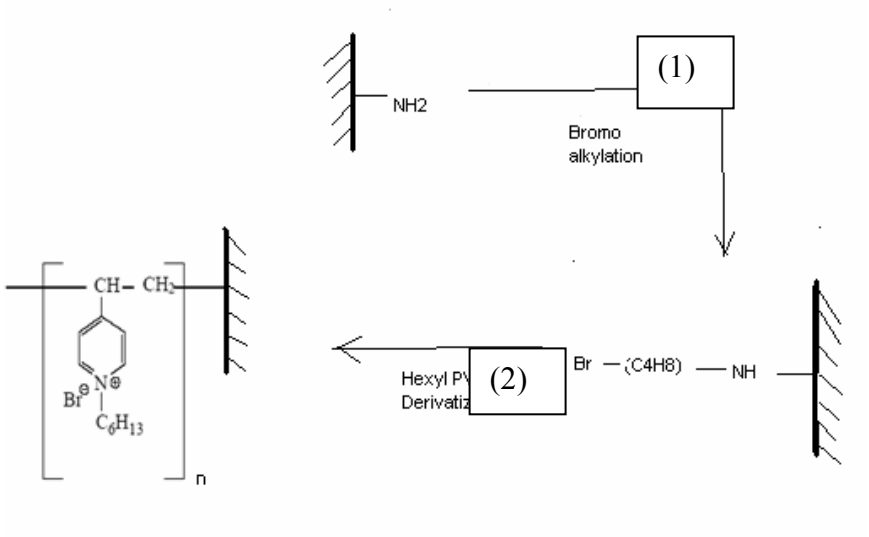


Figure 1.2: The Step-wise procedure of coating hexyl PVP on a NH_2 slide. The NH_2 slide is bromo alkylated to introduce reactive groups and then hexyl PVP derivatization is carried out.

contained long N-alkyl chains (10 or more carbon atoms) were found to be non bactericidal.[4] These N-alkyl chains are attached to the polymer chain via the pyridine molecule. It was interpreted that the long alkyl chains stuck to each other via hydrophobic interactions. However in PVP chains with alkyl chains that had 3 to 8 carbons, these hydrophobic interactions were not sufficiently strong to overcome the electrostatic repulsion of the positively charged polymers. Thus, these positive charges allowed the polymer chains to penetrate the bacterial cell wall, thus killing the bacterial cells. Hexyl PVP chains containing 6 carbon atoms (as shown in Figure 1.2) were found to be the most effective. Surfaces derivatized with hexyl PVP showed almost 95% bactericidal ability.

Ordinary material used for coating objects such as paints and varnishes are not inherently antimicrobial. Thus, they require chemical modification to render them antimicrobial. Surfaces that are chemically modified by polyethylene glycol and other synthetic polymers are known to repel microorganisms but do not kill them.[32-35] Such chemicals can be called 'Bacteriostatic' which restricts bacterial growth. Further modification is required to make these chemicals 'Bactericidal'. But surfaces which were coated with quaternary ammonium compounds, silver or iodine ions, readily kill microorganisms.[35-37] Various cations possess antimicrobial activity, presumably by interacting with and disrupting bacterial cell membranes.[38-40] It is believed that cations kill the bacteria by rupturing the outer membrane of the cell.[41-42] Having penetrated the outer membrane permeability barrier, cationic groups penetrate into the inner membrane and disrupt the cytoplasm, causing leakage of the cell and finally death.

This self-promoted penetration effect,[41] which involves the polycation causing damage to the inner bacterial membrane, kills more than 90% of staph aureus cells and 99% of e-coli cells.[4]

In Kilbanov's system, the glass surfaces are modified with hexyl PVP by a graft polymerization of 4-vinylpyridine and subsequent N-hexylation, thus the coating is chemically bound to the surface and is not easily washed or scraped off. As long as the polymer remains attached to the surface, the surface remains antibacterial.

Consider when one sneezes or coughs as a test case, thus spraying surfaces with germs: These germs are then killed when they come in contact with the active coated surface. These surfaces only require periodic washing to remove debris of dead bacteria from the

surface[4] and an attempt to make this kind of coating on materials of everyday use could dramatically reduce nosocomial infections.

Kilbanov and his group further developed this research by modifying these hexyl PVP coated surfaces to kill airborne as well as waterborne bacteria.[43] His group modified high density polyethylene(HDPE) slides with SiO_2 or NH_2-functional groups before running the same grafting reaction with hexyl PVP.

The same kind of treatment was applied to glass slides of low density polyethylene (LDPE), polypropylene (PP), nylon and polyethylene terephthalate (PET).When tested, the amount of E-coli and S.aureus bacterial colonies growing on the SiO_2 coated or NH_2-functionalized HDPE slides were identical to that on HDPE glass slides. This indicates that these modifications are not toxic to the above two bacteria. But when these bacteria were sprayed on hexyl PVP modified HDPE slides, the deposited bacterial cells died, with barely any colonies remaining on the slide.

The bactericidal mechanism of the surface attached polycationic chains probably involves their penetration into bacterial cell membranes, causing cell damage and thus death. Similar antimicrobial properties were observed for LDPE, PP, Nylon and PET slides that are modified with hexyl PVP. The reaction scheme for the production of these slides is shown in Figure 1.3 and the percentages of water borne and airborne bacteria killed for these systems are shown in Tables 1.1 and 1.2. To add a quantitative dimension to this experiment, HDPE slides were modified with hexyl PVP with different surface densities of the polycation and their efficiency to kill S.aureus bacterial cells which are both airborne and water borne was determined. Figure 1.4 shows the killing efficiency of airborne and waterborne S.aureus cells for these systems. The difference between the two

Table 1.1: The ability of hexyl- PVP attached to various polymer slides to kill waterborne bacteria on contact [43]

Bacterium	Type	Percentage of bacteria killed				
		HDPE	LDPE	PP	nylon	PET
S. aureus	Gram (+)	98±1	99±1	97±2	97±1	99±1
E. coli	Gram (-)	97±2	98±2	n.d.	99±1	98±1

Table 1.2: The ability of various polymers slides derivatized with hexyl-PVP to kill airborne bacteria on contact [43]

Bacterium	Type	Percentage of bacteria killed				
		HDPE	LDPE	PP	nylon	PET
S. aureus	Gram (+)	96±3	97±1	90±3	92±2	95±1
E. coli	Gram (-)	97±1	96±2	98±1	98±2	95±1

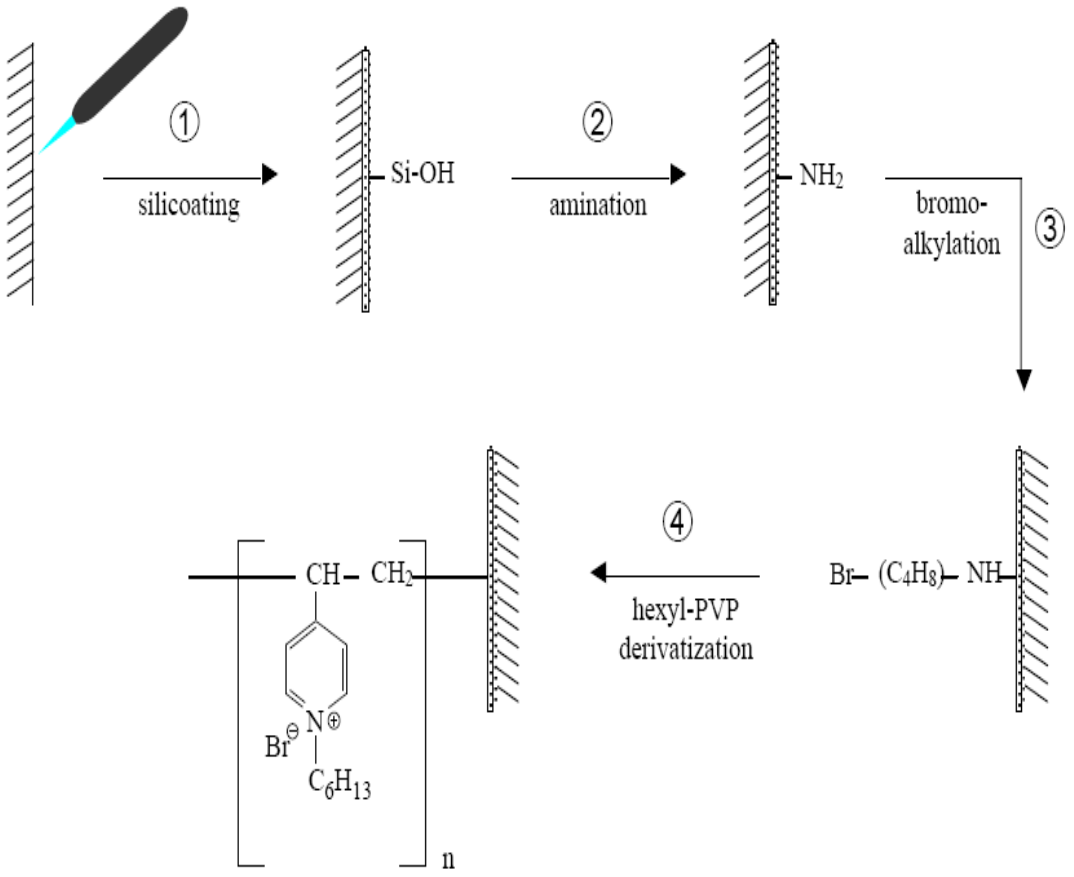


Figure 1.3: Schematic representation of the process to modify polymer surfaces with poly (vinyl-N-hexylpyridinium bromide) (hexyl PVP). The 1st step shows coating the surface with SiO_2, the 2nd step shows the treatment with 3-aminopropyltriethoxysilane, step 3 is the alkylation of the surface with 1, 4-dibromobutane and step 4 is the derivatization with hexyl PVP in the presence of 1-bromohexane.[43]

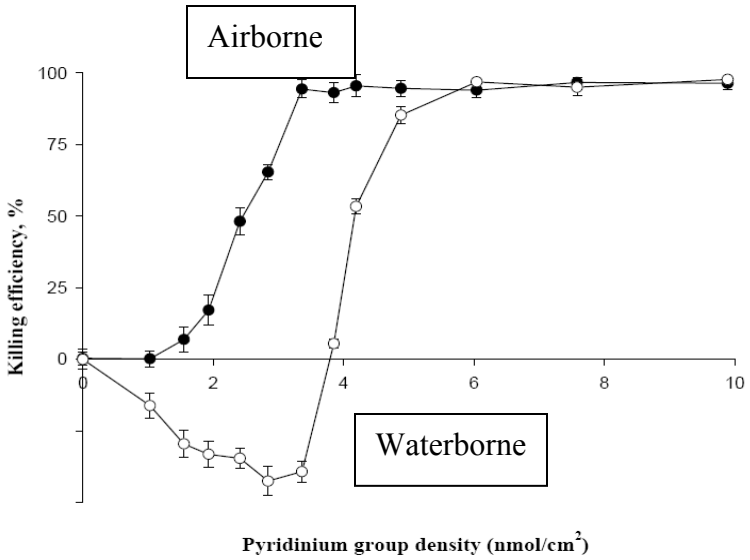


Figure 1.4: The killing efficiency of hexyl PVP derivatized HDPE on airborne and water borne S.aureus bacteria sprayed on the modified polymer surface as a function of the surface density of the pyridinium groups.[43]

The S.aureus bacterial cells, both airborne and waterborne, were sprayed on an HDPE slide derivatized by hexyl PVP. These bacterial cells were in the stationary phase of their growth curve. The surface showed approximately >95% killing efficiency. An interesting point was to see if the hexyl PVP derivatized surface showed the same antibacterial action for cells in the logarithmic phase of growth. The killing efficiency of the surface to the cells in the logarithmic phase was identical to that in the stationary phase. The bacterial strains with multidrug resistance (MDR) possess a major threat to human health because of their resistance to most drugs and antibiotics. Thus, three antibiotic resistance cells were utilized in similar tests that showed that the hexyl PVP coated surface was lethal to these bacterial strains as well, with killing efficiencies of more than 90%.

Kilbanov also extended this bactericidal technology to textiles such as cotton, wool, nylon and polyester. With the goal of making hospital curtains, clothings and uniforms permanently lethal to bacteria, cotton, wool, nylon and polyester were subjected to covalent derivatization with N-peralkylated polyethylenimine (PEI). The derivatized cloth materials were tested for their bactericidal abilities for airborne E-coli and S.aureus bacterial cells. Agar was poured over the derivatized cloth and incubated at 37°C. When compared against bacterial growth on non-derivatized cloth, more than 90% of the deposited bacteria were killed.

Fungi are a microorganism that also has a single membrane covered by a thick cell wall. Their structure is similar to the cell envelope of gram positive bacteria. When these same textiles were tested for fungicidal activity they were found to be as high as their bactericidal activities. These materials with covalently attached alkylated polyethylemine (PEI) were not only bactericidal but also killed airborne fungi, which could be a major breakthrough against biological terrorism and warfare.[45] The reaction scheme to create these surfaces is shown in Figure 1.5.

These surfaces were also found to be deadly for bacterial cells but safe for human cells.[46] This may be because of the variation in size and the difference in cytoplasmic membranes of human and bacterial cells. Fluorescence tests monitored for long time periods showed that the cell number, which is the number of human cells on these surfaces growing over the monitored period of time, remained constant throughout all readings.

Another example of a sterile surface created by Kilbanov and his group that can kill airborne and waterborne pathogens is formed by attaching one end of a long chain

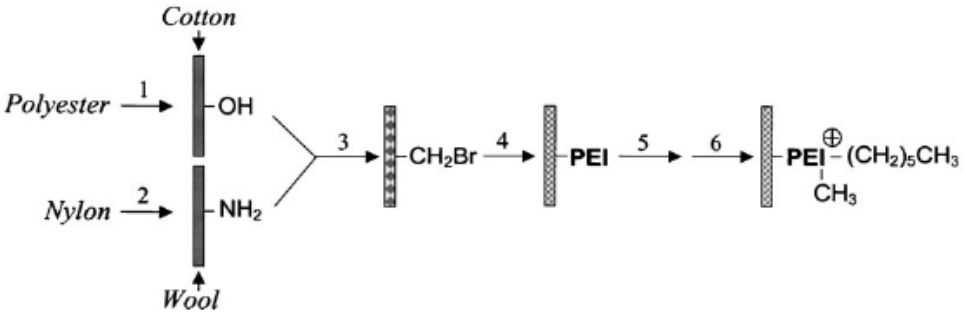


Figure 1.5: Schematic representation of the synthesis of N-alkylated PEIs covalently immobilized onto cotton, wool, nylon and polyester textiles.[45] Steps 1 and 2 are the hydrolysis of polyester and nylon. Step 3 is the acylation of hydrolysed textiles with 4-bromobutyryl chloride. Step 4 is the covalent attachment of PEI, step 5 is the N-hexylation of the immobilized PEI with bromohexane and step 6 is the N-methylation of the immobilized N-hexyl-PEI with iodomethane.

hydrophobic polycation to a surface of a material such as cotton or plastic.[47] The polymeric chain allows the antimicrobial moieties to penetrate into the bacterial cells and kill them. The most obvious application of these self sterile surfaces is again to prevent nosocomial infections. Figure 1.6 shows the polycation molecule attached to a cotton or plastic surface.

Quaternary ammonium compounds are usually the choice for such experiments because their target is the microbial membrane. Poly(vinyl pyridine) (PVP) was selected as the carrying polymer and after N-alkylating it, the resultant Poly (vinyl-N-alkylpyridinium bromide) was attached covalently to the glass surface. When tested against E-coli and S.aureus cells, more than 99% of the cells were killed.

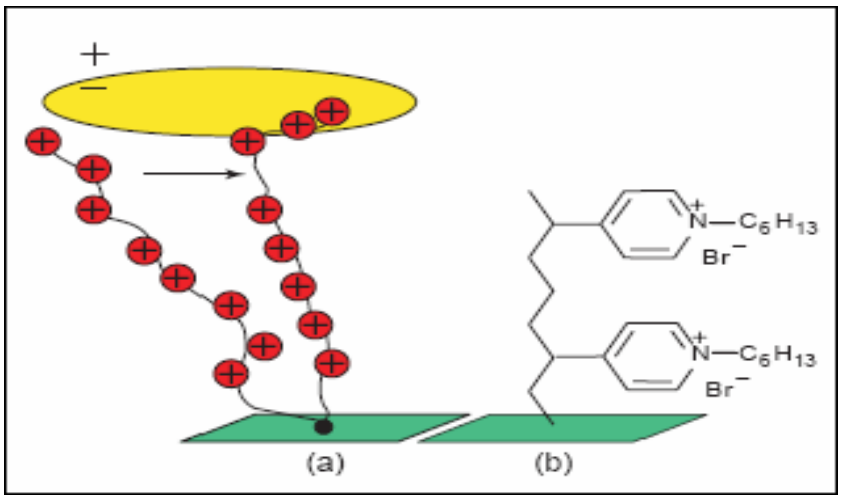


Figure 1.6: A design for self-sterile material. An antimicrobial moiety is attached to a long flexible polymer chain which is covalently attached to the surface.[47]

Thus, these coatings that can make objects permanently sterile for bacteria but remain human friendly could prove to be a major break through against infections, if they can be made commercially applicable.

c) Photodynamic therapy / Porphyrins

The technique of using light as an antimicrobial agent has gained importance since early last century. Light has been used for many years for curing neonatal jaundice, in dermatology and in photodynamic therapy (PDT). [48] PDT has been known for 100 years for curing jaundice and other diseases but became an important technique since World War II.

What does PDT actually do? With the help of a photosensitizing agent, which is usually a porphyrin based compound, PDT uses light for the treatment of diseases. This

concept of cell death by the interaction with light and chemicals was first reported by Oscar Raab, a medical student working in Munich.[49] The mechanism by which PDT kills bacterial cells is as follows; when the photosensitizer is illuminated with light of an appropriate wavelength it is irradiated to its singlet excited state followed by intersystem crossing to its triplet excited state. The triplet excited state undergoes reaction with surrounding radicals and produces singlet oxygen. Ground state oxygen exists uniquely as a triplet state. However, the first excited state of the oxygen molecule is the singlet state, which can readily react with other singlet molecules. Radioactive decay to the triplet ground state is a spin-forbidden transition, resulting in a long-lived and highly reactive excited state. This singlet oxygen is highly reactive and a potent cytotoxic species that kills bacterial cells.[50] The targets at which the singlet oxygen seems to act are cell membrane, nucleus, mitochondria and lysosomes.[51-52]

However, due to the highly reactive nature of the radicals formed during this process, its activity is confined to its immediate vicinity[53] and this activity depends on the delivery of the photosensitizer to the target. A large amount of research is ongoing in a number of laboratories, where the potential of PDT for treating microbial infections has been demonstrated.[54-55]

Porphyrins are molecules of considerable interest because of their ability to act as photosensitizer when irradiated with light. They undergo molecular energy transfer leading to the production of singlet oxygen. This singlet oxygen is highly reactive and kills bacterial cells. The application of porphyrins as photosensitizers to prevent or treat infections is wide spread.

The antimicrobial and antiviral activities of porphyrin are based on a number of factors such as their chemical property, electron transfer ability, ability to absorb photons, catalyze perioxidase and oxidase reactions. Haem is a valuable source of iron for both animals and bacteria. Some porphyrins affect bacterial haemoglobin-catalyzed reactions by inserting in haemproteins and preventing normal function in metabolism of cells.[56] The outer membrane of gram negative bacteria has a permeability barrier for many hydrophobic and larger hydrophilic substances and is the cause for the reduced activity of antibiotics in gram negative bacteria.[57] But the conjugation of porphyrins with antibiotics and compounds such as nucleic acids and peptides may solve this problem as microorganisms must actively accumulate haem. The presence of multiple haem uptake pathways in the majority of pathogens would make the development of strains resistant to porphyrin-antibiotic conjugates highly unlikely. The bacterial mutants that become resistant to porphyrins-antibiotic conjugates due to loss of haem up-take system are less virulent. Thus, they would survive less well in body fluids and tissues.

The survival of bacterial colonies on materials worn by patients and health care workers were prevented and lowered by the use of porphyrin materials.[58] Appropriate illumination generates toxic oxygen species. This cytotoxic species results in the death of bacterial cells on clothes. Thus, this process can prevent nosocomial infections in patients and health workers.

Photosensitization can also represent a useful approach for the killing of microbial cells since porphyrins exhibit phototoxicity against many microbial cells when activated by light. Porphyrin binds to the outer membrane of gram positive bacteria and effectively kills it. But in gram negative bacteria, the outer membrane is located outside the cell wall

and acts as a permeability barrier preventing the access of the photosensitizer molecules to the inner cytoplasmic membrane. The positive charge orients the photosensitizer to particular cellular sites whose initial damage facilitates the penetration of photosensitizer molecules to the inner membrane and cytoplasm of gram negative bacteria, thus rendering them to photodestruction.[59] Polycationic porphyrins combined with PDT have also been used to control wound infections.[60] The cationic species binds and penetrates both gram positive and gram negative bacteria, killing them after illumination.

For instance the polycationic porphyrin 5-phenyl-10, 15, 20-tris (N-methyl-4-pyridyl) porphyrin chloride (PTMPP) was used to treat infected wounds in mice.[61] The structure of PTMPP is shown in Figure 1.7.

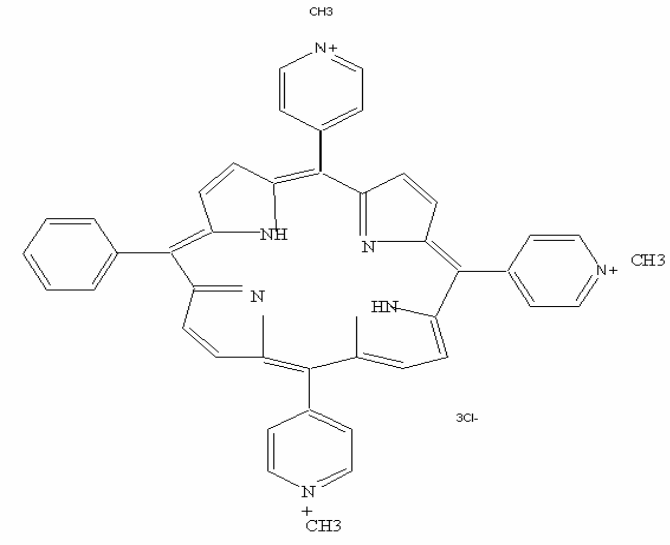


Figure 1.7: Structure of 5-phenyl-10, 15, 20-tris (N-methyl-4-pyridyl) porphyrin chloride used to treat wound infections in mice.

Porphyrins are either natural porphyrins (NP) or synthetic porphyrins (SP). These porphyrins can either be metal based or metal free porphyrins, each having numerous photosensitizer applications. With the danger of metal toxicity an issue, it's safe to use a metal free porphyrin. Metal free porphyrins have also been used as an anti-cancer therapeutic agent in clinical trials. When they are activated by light, they act against tumors causing minimum damage to the host.[62] Photosensitized porphyrin kills aerobic as well as anaerobic bacteria by generation of free radicals that inhibit the bacteria.[47]

Porphyrin molecules when exposed to broad-band light (380 to 520 nm) are used to treat pigmented bacteria generated from dental plaque. Dental plaque is a biofilm, consisting of different bacterial taxa, that develops naturally on teeth. Periodontal diseases caused by the overgrowth of oral pathogens are also cured by photoactive porphyrins.[64-66] Helicobacter pylori, which are gram negative bacteria, colonize the mucus layer of human stomach and duodenums, causing a high risk for gastric diseases. They are also killed by a metal free porphyrin when exposed to 400 nm visible light.[67-68]

Tetra-meso (N-methyl pyridyl) porphyrin (T4MPyP) and its derivatives have found vast applications as photoinhibitors of gram positive and gram negative bacteria, in treatment of Hela human carcinoma cells and in destruction of Helminth egg. Helminths are parasites that live in humans. Helminth eggs contaminate food, water, air, feces, pets, wild animals and objects such as toilet seats and door handles. The eggs enter the body of a human through nose, mouth and anus. Once inside the body, they lodge in the intestine, hatch, grow and multiply. Tunisian untreated waste water exhibits an average of 30 human helminth egg/ liter. T4MPyP is found to be an efficient photosensitizer of

helminth eggs when compared with other porphyrins compounds that includes anionic and uncharged porphyrins.[69-72] The structure of T4MPyP is shown in Figure 1.8.

Light activated antimicrobial materials (LAAMs) have found wide applications in hospital settings and protective clothing. One idea is to reduce or eliminate bacteria from most easily contaminated surfaces within hospitals. A schematic representation of the synthesis of LAAMs is shown in Figure 1.9.

Figure 1.9 shows the development of porphyrin based nylon fiber that may be antimicrobial. The procedure for their synthesis is as follows: Protoporphyrin (PPIX) which are tetrapyrroles containing methyl, propionic acid and vinyl side chains and zinc protoporhyrin (ZnPPIX) were attached to the surface of nylon films through a poly (acrylic acid) (PAA) scaffold. A glass ladder was used to minimize fiber-fiber overlap. It was coated with nylon fiber and submerged in a PAA/water solution in a long glass reaction tube. This process alleviates the problem of minimizing potential graft sites that are available on a nylon bulk surface. After grafting the PAA to the fiber surface, the glass ladder containing nylon fiber with grafted PAA was immersed in deionized water containing PPIX and the reaction of grafting PPIX on the PAA grafted nylon fiber was allowed to run overnight. The procedure for grafting Zn PPIX is similar to that of PPIX. The killing or the inhibitory effect of the presence of PPIX was tested by spraying E-coli and S.aureus bacterial cells on the nylon fiber, nylon fiber grafted with PAA, the nylon fiber grafted with PAA+ PPIX and the nylon fiber grafted with PAA+ ZnPPIX. No inhibitory effect was observed for the nylon fiber or the nylon fiber grafted with PAA. The sample grafted with porphyrins however, had an inhibitory effect on the survival of S.aureus but was ineffective against E-coli.

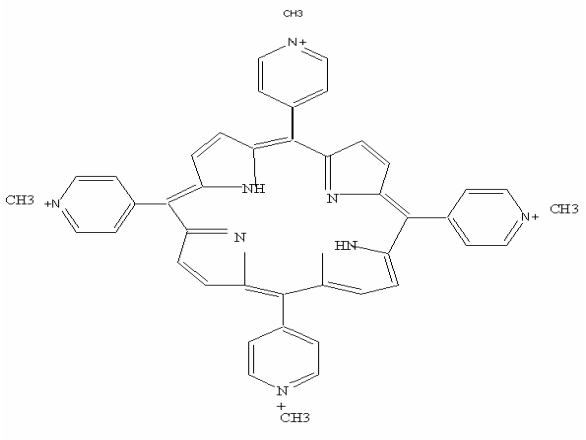


Figure 1.8: Tetra-meso (N-methyl pyridyl) porphyrin (T4MPyP) which has found vast applications as photoinhibitor of gram positive and gram negative bacteria.

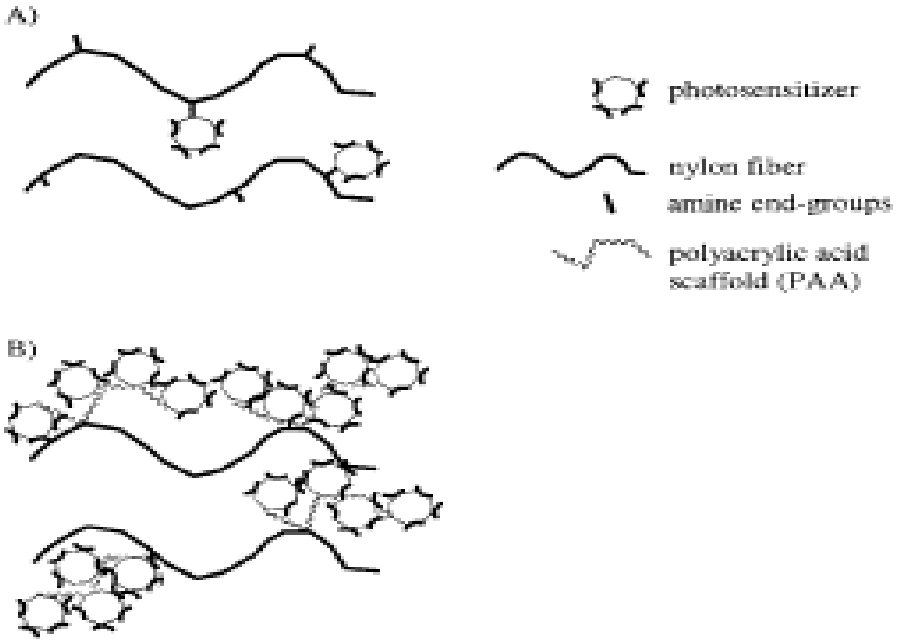


Figure 1.9: Schematic representation of the synthesis of LAAMs. A- Nylon fibers with few attachment sites. B- The expansion of attachment sites on nylon fibers by attachment of a polyacrylic scaffold possessing a large number of potential attachment sites for porphyrins photosensitizers.[73]

But at a light intensity of 60,000 lux Zn PPIX killed about 30% of the E-coli bacteria. It is known that the outer membrane of the gram negative bacteria protects them from reactive oxygen species (ROS). It may be possible for these bacteria to be damaged by ROS but at a much slower rate than gram positive bacteria. Under normal illumination by room light, these materials can be used for privacy curtains in hospitals and laboratories and coats for medical personnel. This would reduce the number of bacteria on these materials, thus reducing potential nosocomial infections.[73]

A national institute of health sciences in Tokyo, Japan,[74] discussed several modifications of medical device surfaces that would create anti-infective devices, but would not affect the host defense system. This work discusses various methods by which catheters can be coated to avoid bacterial adhesion thus reducing urinary tract infections. A smooth and uniform surface is an important feature to resist bacterial adhesion. Even if the surface inhibits bacterial adhesion, bacteria can find survival places if the surface is rough. One method to improve their antimicrobial ability is to directly coat the medical devices with silver and its compounds. Silver has antimicrobial ability and low toxicity to humans. Silver coatings are found effective, reducing chances of infections caused by medical devices inserted into the human body. Another method of coating to prevent infections is to trap an anti-infective agent in a polymer matrix and then a thin coating can be made on medical device surfaces. Even though this method looks straight forward, a series of problems such as finding suitable anti-infective agents and matrix polymers as well as finding suitable processes of constructing medical devices are a challenge. Continued efforts to adjust these factors must be made to develop a successful medical device with anti-infective surfaces.

Looking into all the possibilities and probabilities, there is enough evidence that surfaces modified with covalently attached polycations or polycationic porphyrins kill both airborne and waterborne bacteria. Such prototype materials can combat the real threat of biological warfare agents.[47] These non-leaching sterile surfaces have potential applications in house-hold, hospital and everyday use objects. As these surfaces kill bacteria on the spot, the possibility of them developing any kind of bacterial resistance is minimal.

1.3 Goals and Justification

Our idea is to combine both techniques of PDT and self sterile surfaces to design self-healing surfaces. The work discussed in this thesis details the synthesis and ability of a molecule that can be used to develop a self-healing surface. From the literature, we know that polycationic molecules kill bacteria. T_4MPyP is a commercially available porphyrin that exhibits strong antibacterial properties.[71] We modified one pyridine ring of this porphyrin to obtain 5-(4-carboxyphenyl)-10, 15, and 20-tris-(N-methyl-4-pyridlium) porphyrin. The reason to modify this porphyrin was to attach it as a pendant group so that it can be included in a polymer chain. This modified porphyrin was reacted with a Vinyl benzyl chloride copolymer via a nucleophilic substitution reaction forming an ester linkage between polymer and porphyrin. The above molecule was then tested for its bactericidal property. The proposed mechanism to form a self healing material is to attach this antimicrobial molecule as a dangling pendant group to the polymer back-bone. This entire system can be dissolved in a polymer matrix to coat walls and other surfaces. The schematic representation shown in figure 1.10 may help explain this idea:

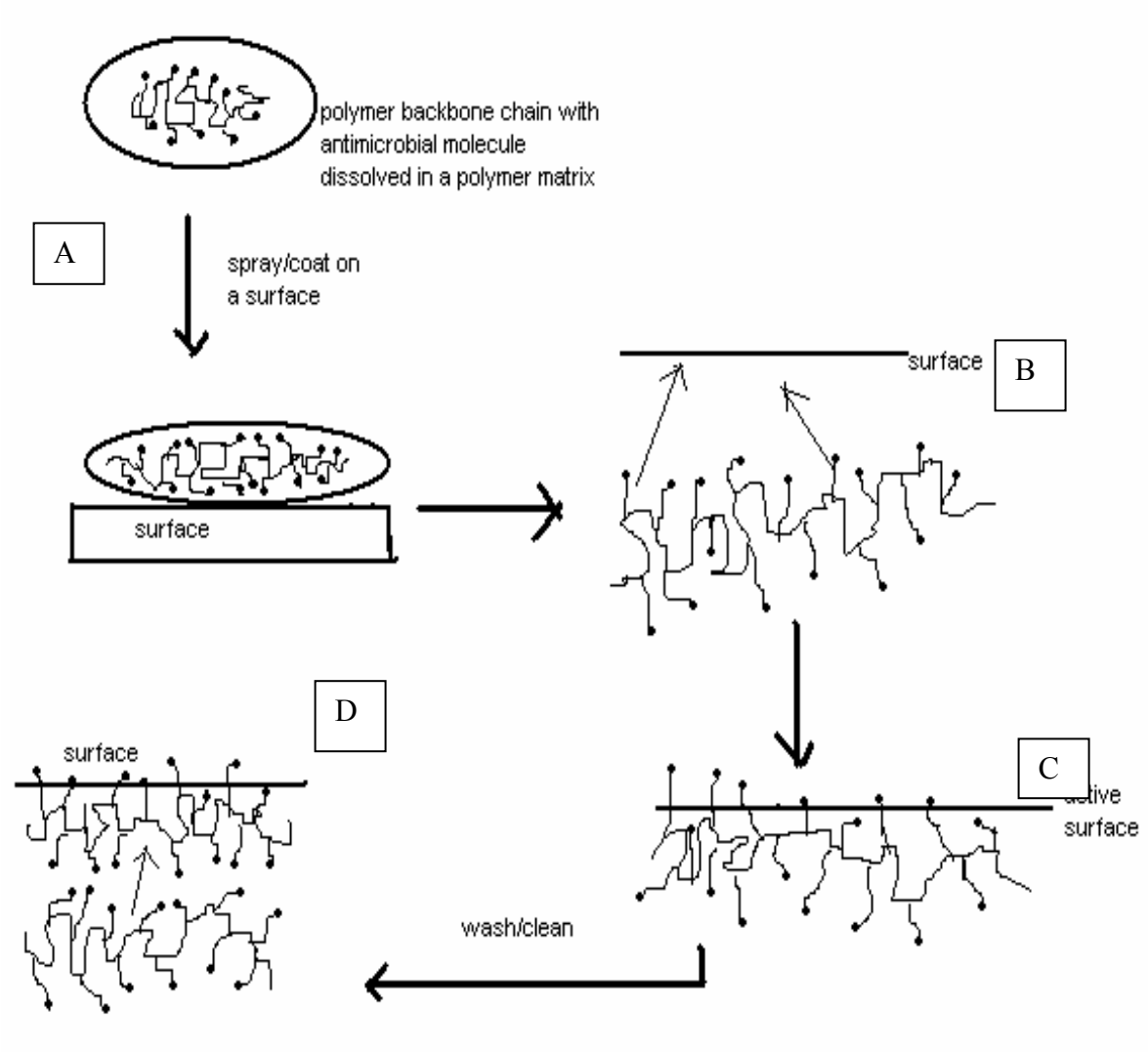


Figure 1.10: Schematic representation of a self-healing material. A-The matrix polymer is coated on a surface. B-The migration of antimicrobial molecule branches towards the surface. C- A surface with antimicrobial molecules imparting bactericidal property to the surface making it active. D- The replenishment of the antimicrobial molecule from the reservoir matrix thus rendering active surface for longer periods of time.

In this material, the polymer backbone chain would remain entangled in the polymer matrix. When coated on walls or the surface of an object, the polymer matrix would be designed to stick to the wall or the object. A polymer chain at the surface has a lower entropy than the polymer chain in the bulk matrix. This loss of entropy is less for a branched chain, relative to a linear chain, therefore in a mixture of linear and branched chains, the branched polymer preferentially segregates to the surface. Due to these entropic factors the branched polymer chains migrate towards surfaces. When exposed to light, these photosensitized molecules would kill bacteria that come in contact with the surface, thus imparting antimicrobial properties to the surface. The surface can be cleaned by occasionally dusting or washing to remove dead debris of bacterial cells. In case of branch breakage during cleaning, possibly another branch could be thermodynamically driven to the surface to replenish the surface from the reservoir matrix, thus keeping the surface active.

The initial synthesis and bactericidal ability of this molecule is explained in detail in the following chapters. Thus, a simple, inexpensive and commercially applicable self healing surface may be created from this molecule. This can prove to be one more major step in tackling the issue of nosocomial infections in particular and most types of bacterial infections in general.

Chapter 2 Experimental Techniques

The 1st part of this chapter explains in detail the protocols and procedures used for bacterial analysis. Each protocol details the general background of the methods in an order in which they were used while running the experiments. It also discusses the exact concentrations and procedure used for our experiments. The 2nd part of the chapter explains the synthesis of poly(styrene-co-4-vinyl benzyl chloride) [poly (St-co-VBC)] and 5-(4-carboxyphenyl)-10, 15, and 20-tris-(4-pyridyl) porphyrin.

2.1 Bacterial Background

Bacteria are found everywhere on earth including the sea, the lakes and on all continents, even Antarctica appearing on earth approximately 3.5 billion years ago. Their small size makes us unaware of them but phenomena such as septic wounds, or sour milk,[74] which make us feel their presence. With his newly made microscope, Antony Van Leeuwenhoek first observed them in 1600. These small, single cell organisms that do not contain a nucleus are called 'Prokaryotes'. They grow in colonies and reproduce by asexual budding or fission.[75] Bacterial cell size is usually measured in micrometers and is about one-hundredth the size of human cell.[76]

The cell membrane which is the outer membrane consists of 70% of a watery fluid called cytoplasm while the enzymes fill up the remaining 30%. DNA floats in cytoplasm at the center of the cell. In some of the bacterial cells, flagella are attached to the outside of the cell which help in the locomotion of the cell. Pili are the small hair-like projections on the outer surface of the cell wall which allow the bacterial cells to stick to surfaces and

transfer DNA to one another.[76-77] Figure 2.1 shows a picture of an idealized bacterial cell. Due to the number of different varieties of bacteria, an important question which arises is that how is one type of bacteria distinguished from another and how are they classified? Bacteria come in different shapes, sizes and show different morphological characteristics regardless of their microscopic size. Some bacteria are round; some are rod-shaped whereas some bacteria are spiral or incomplete spiral shaped.[78] There are aerobic bacteria which need atmospheric oxygen to survive and anaerobic bacteria which do not need oxygen to grow. Bacteria also differ in chemical activities and other parameters such as type of nutrients needed, energy used by them, physical conditions under which they grow and their reactions to certain dyes.[74] Each bacteria rapidly grows at a particular temperature called 'Optimum' growth temperature. This temperature for most types of bacteria is 37°C. Absence of this suitable environment results in death or retardation in the growth of bacterial cells.

In 1884, Christian Gram designed a method for classifying bacteria which is commonly known as the Gram-Stain method. The Gram stain differentiates between two major types of cell wall. Gram negative bacteria are the bacterial species containing small amount of peptidoglycan while gram positive bacteria contain large amounts of peptidoglycan. Peptidoglycan which is also called 'Murein' is a complex polymer containing cross-linked linear heteropolysaccharide chains.[76]

Gram's staining method is based on the structure of the cell wall. A bacteria smeared slide is stained with 'Crystal Violet' dye. The dye stains the bacteria dark purple in color

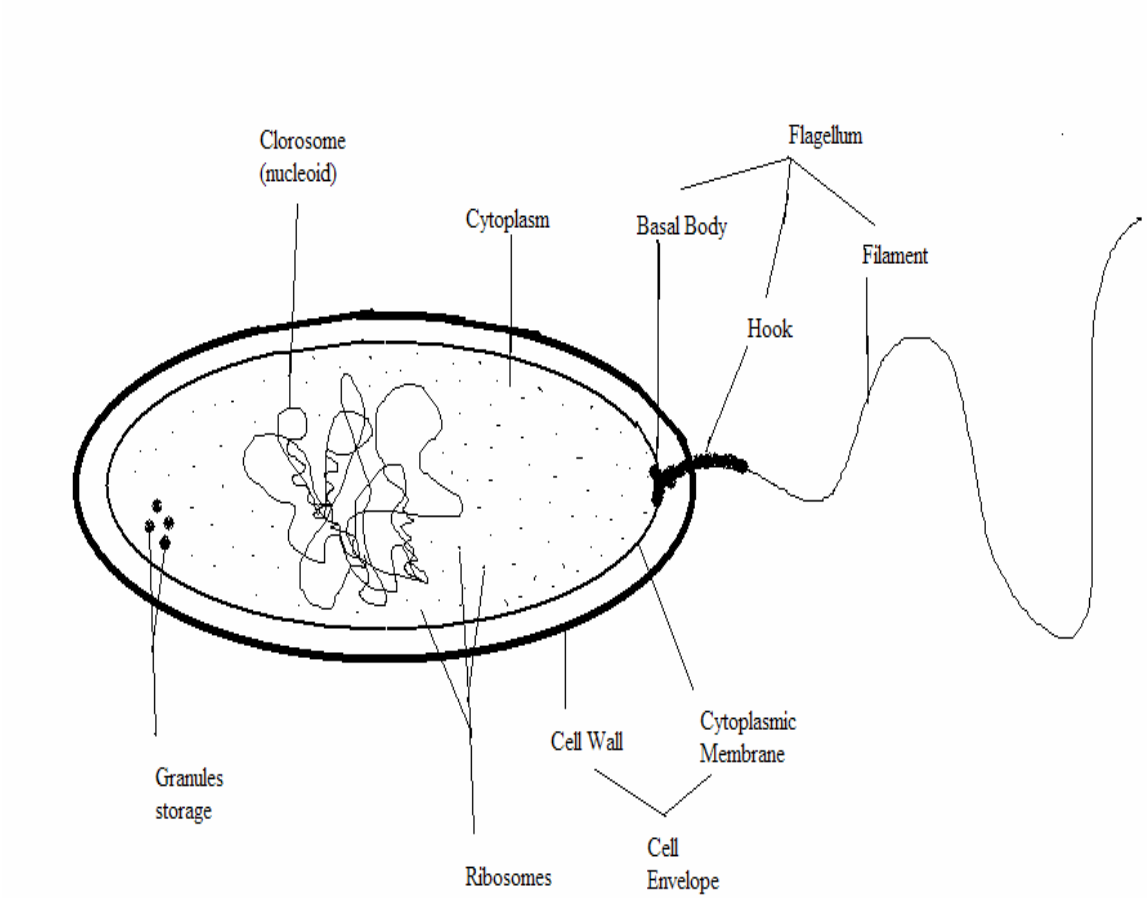


Figure 2.1: An idealized bacterial cell, which consists of cell membrane made up of cytoplasm, enzymes, DNA, flagella and pili.[78]

After about a minute, the crystal violet is washed off with running water. The bacterial slide is then treated with Gram's iodine containing iodine, potassium iodide and water. The slide turns dark blue due to the reaction of iodine solution with crystal violet. The slide is then washed with acetone to decolorize it. It is then rinsed with water to remove the acetone. 'Safranine dye' is then used to counter-stain the slide. After about two minutes, the slide is finally washed with water. Gram positive cells have a high affinity for violet stain which they retain after the acetone wash. These cells appear bluish purple to brown. Gram negative cell walls have a very low affinity for crystal violet, which is therefore rinsed off by acetone, thus these cells appear bright pink to red because of the safranine dye. The peptidoglycan layer which is the outer layer in gram positive cells traps the violet crystal blue stain, while in the gram negative cell the outer layer prevents the crystal blue stain from reaching peptidoglycan layer. Acetone permeates the outer membrane and hence the peptidoglycan layer traps the pink safranine counter stain.[74-76, 77, 79] Figure 2.2 illustrates the difference in cell walls between gram positive and gram negative bacterial cells.

Both gram negative (E-coli) and gram positive (S.aureus) were used in this study to examine the antimicrobial behavior of our polymers. The Staphylococcus aureus (ATCC, strain 33897) and Escherichia coli (ATCC, strain 492316) were used in all our experiments.

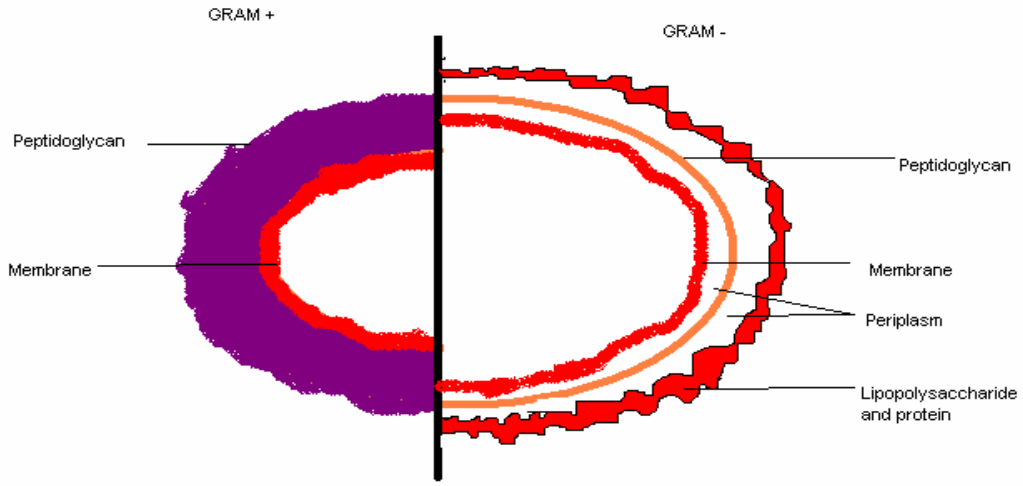


Figure 2.2: Difference in cell walls of Gram positive and Gram negative bacterial cells. Gram positive cells consist of peptidogylcan layer which is the outer layer while the gram negative bacteria consist of an outer membrane covering the peptidoglycan layer. [78]

2.2 Agar

Agar is a product extracted from certain sea-weeds. The jelly type substance which is widely used as a growth medium in most bacteriological laboratories is called 'agar gel'.[77]

Agar is a reversible colloid that becomes liquid at about 100° C and sets into a semi-solid gel at about 40° C. This semi-solid agar gel is mostly transparent. In order to observe the characteristics of bacterial colonies, it is very important that the solidified agar be transparent.[78]

Yeast-Dextrose agar commonly known as Y-D agar was used for our experimental work. The ingredients used in preparation of Y-D agar are listed in Table 2.1. These ingredients were dissolved in approximately 800 ml of distilled water, the pH was

Table 2.1: lists different ingredients used in the preparation of Yeast-Dextrose agar

Ingredients	Quantity (grams)
Peptone	10.00
Beef Extract	8.00
Sodium Chloride	5.00
Glucose	5.00
Yeast Extract	3.00
Granulated Agar	9.00

adjusted to ~ 6.8 and the final volume is brought to one liter with distilled water. The ingredients are mixed thoroughly to distribute the molten agar uniformly. The solution is then poured in small flasks which were half filled and plugged with cotton or aluminum foil. The flasks are then autoclaved on a liquid-cycle.

2.3 Streaking bacterial plates and Inoculation

2.3a Streaking bacterial plates

The autoclaved agar is poured into Petri dishes and allowed to solidify. The agar is cooled to 50° C to prevent condensation. The resulting solid gel surface is used to culture bacteria by a procedure known as 'spread plates'.[74]

In this process, the bacteria are spread over the surface of a gel medium with the help of an Inoculation loop. This tool is a wire whose tip is used to retrieve inoculums from bacterial cultures. The wire forms a small loop at the tip with a diameter of 5mm. This loop is handy for taking an inoculum from a liquid culture by surface tension. To avoid cross-contamination, the loop is sterilized in a flame until red hot before and after use.

We used the quadrant streaked procedure to streak the bacterial plates. This procedure is as follows; the inoculating loop is heated until red hot. It is then cooled by stabbing it into agar at a spot in the corner. A small amount of bacterial growth is removed from the culture with the sterile inoculating loop. Using back and forth strokes, a quarter of the plate is immediately streaked with the loop. The loop is then sterilized again and the streaks are extended into the second quarter of the plate. This was repeated 2-3 times, moving around the agar plate. The area of initial inoculation and first streak yield heavy

bacterial growth. Areas of second, third and fourth streaks yield less dense, weak and single colony bacterial growth respectively. The plates are covered immediately to avoid contamination.[74, 80] Figure 2.3 shows a picture of a quadrant streaked bacterial plate. The difference in growth in the four quadrants can be seen in this picture.

2.3b Bacterial Inoculation

Inoculation is the process of adding the bacterial cells to the medium. The inoculation loop is heated till red hot to avoid cross-contamination and then allowed to cool down. A few micro liters of bacteria from the culture are added to 10 ml of L-B media. L-B media is a rich media which provides the necessary minerals and nutrients required for bacterial growth.[78]

For bacterial inoculation, a small flask containing the media was kept in a water bath which is shaken at 200 rpm and whose temperature is maintained at 37°C; which is the optimum temperature for bacterial growth. The flask was left overnight in the bath to allow sufficient time for the bacteria to grow to its fullest. The ingredients used in the preparation of L-B media in these experiments are listed in Table 2.2.

A few micro liters from the overnight grown culture are then transferred to a new, clean flask containing 10 ml liters of fresh L-B media. The flask is then kept shaking in the water bath and the bacteria are allowed to grow until the optical density of the inoculated culture reaches a value between 0.6-0.8. The main reason to do this transfer re-inoculation of the bacteria is to use the bacteria from the Log-phase which is explained by the graph (Figure 2.4)

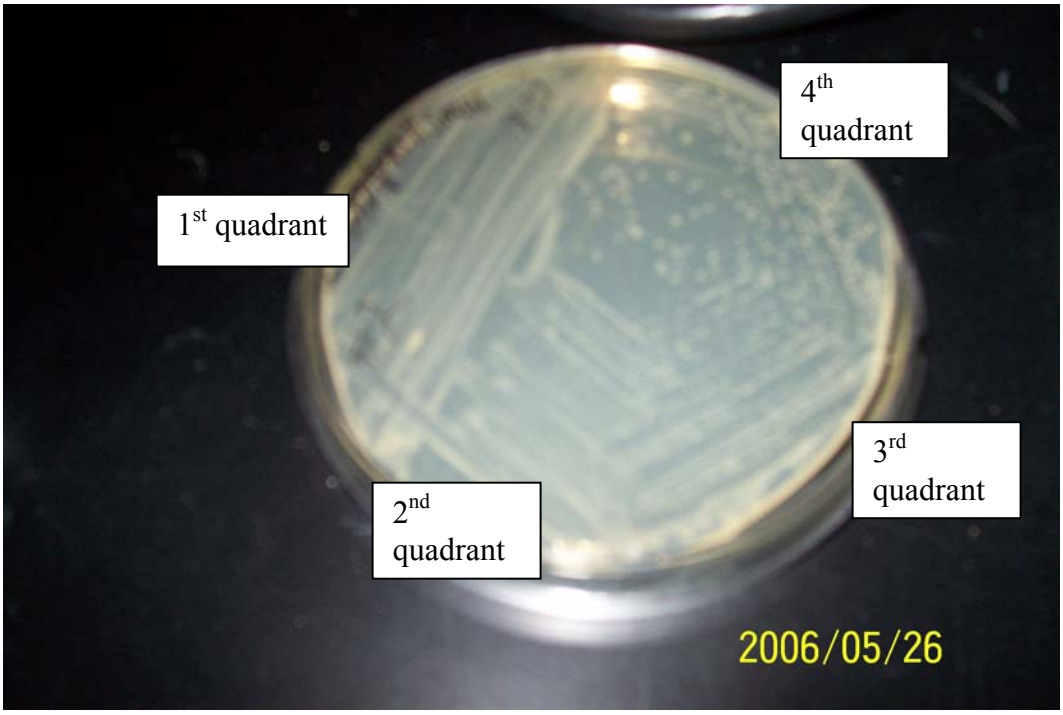
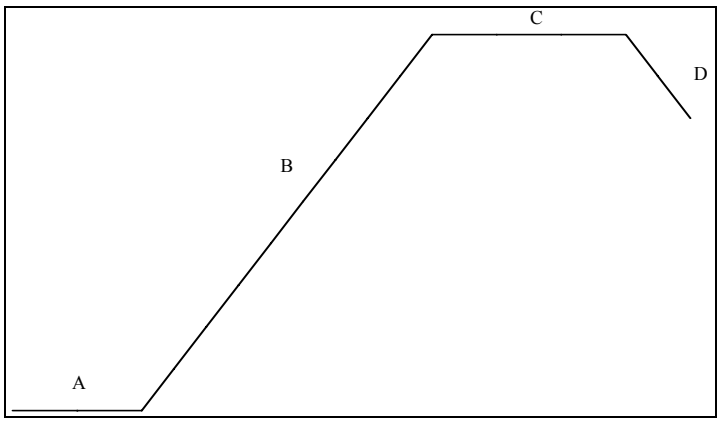


Figure 2.3: Photograph of bacterial growth on the quadrant streaked plate. The area of the first streak shows heavy bacterial growth while the areas of second, third and fourth streaks show less dense, weak and single colony bacterial growth respectively.

Table 2.2 Exact quantities of the ingredients used in the preparation of L-B media.

Ingredients	Quantity (grams)
Tryptone	10.00
Yeast Extract	15.00
NaCl	10.00
Distilled H_2O	1 liter

Y axis = log cell number



Time

Figure 2.4: The graph of bacterial cell growth which is a plot of number of cells as a function of time. Phase A is the lag phase, phase B is the log phase, phase C is the stationary phase and phase D is the death phase.

A growth curve is a plot of the number of cells as a function of time. In the growth curve, the growth from lag phase to death phase occurs in the same batch of the medium, [74, 76, 78, 80] and divided into four categories:

(A) Lag Phase- In this phase, there is often 'unbalanced' growth of bacteria as the cells are still adapting to the new environment. The growth or cell-division of bacteria does not begin immediately when transferred to the new medium hence it's called the 'Lag phase'.

(B) Log Phase or Exponential Phase- As the bacteria become adapted to the new environment its growth rate doubles at regular intervals. The bacteria are metabolically active and show response to any reactions more in log phase than in any other phases. As the cells grow, their division rate increases. This growth phase is called the exponential or logarithmic phase.

(C) Stationary Phase- Waste products accumulate in the medium as the bacterial cells grow. The cells slowly consume all the nutrients, thus lack of nutrients or accumulation of waste products leads to very slow or complete halt in growth of cells. The rate of cell growth or division is equal to the rate of cell death in this phase, thus there is no increase in the overall number of viable cells. Hence this phase is called the 'Stationary phase'. The cells in the stationary phase show significantly different properties than those in the log phase.

(D) Death or Decline Phase- The number of cells in this phase decrease as death rate is greater than growth rate.[77, 78, 81] Bacteria from the log phase with a pH of 0.644(+/- 0.005) were used for analysis. The pH was kept constant for all experiments.

2.4 Incubation of Bacteria

The streaked plates are kept inverted in an incubator to prevent condensation. The incubator is a chamber that maintains a constant temperature that is optimal for the growth of the bacteria. Mostly, the temperature is maintained at 37°C. The plates are usually kept in an incubator for at least 12 hours to allow sufficient time for the bacteria to grow.[78] Figure 2.5 shows a picture of these incubated plates.

2.5 Safety Measures while working with bacteria

Hands should be washed thoroughly with antimicrobial soap, hot water and sprayed with 70% ethanol before starting the work. Eating is strictly prohibited at all times in the

Figure: 2.5: Bacterial plates incubated in an incubator at 37°C for at least 12 hours to allow sufficient time for all bacteria to grow.

laboratory. All the work must be carried on the Laminar flow table. A Laminar flow table is a table with a filter in the front portion of it which prevents contamination as it does not allow air to come in or external bacteria into the experiment. Aseptic transfer of micro organisms can be prevented by carrying out Inoculations and streaking plates near a Bunsen burner during the entire time of the experiment. The hot flame prevents airborne bacteria in the surroundings from interfering and contaminating the culture or bacterial plates. It is possible that the flame may come on you, so spraying must be done with extreme care.

It is important to label all the bacterial cultures and chemicals to avoid improper use or disposal of the materials. Materials needed for the experiment such as laboratory manuals and laboratory notebooks may be brought near the work space. Other belongings such as bags, coats etc. should be stored away from work area. Goggles, gloves and face mask must be worn all the time while working. The work place is cleaned with 70% ethanol after completing the experimental work.

A good hygiene habit is to always wash your hands with antimicrobial soap before leaving the laboratory. The most important thing is the disposal of waste; all the bacterial cultures and contaminated materials should be disinfected before disposal. The bacterial Petri dishes must be taped properly and placed in biohazardous bins. It is important to autoclave all this material before disposing in autoclave bags. Care should be taken while cleaning spills. Glass pieces should be collected, put in a biohazard waste bag and thrown in a glass only container. Bacterial spills should be wiped cleaned with dry paper towels that are then placed in biohazard waste bags before disposal. In all cases, the floor should be sprayed with 70% ethanol to disinfect it.[78, 83]

2.6 Bioimaging system

The bioimaging systems or digital photo documentation systems are compact workstations which capture the images of fluorescent and colorimetric gels, membranes, culture plates, blots, films and assay. These systems combine a 7.1 mega pixel camera with a light weight camera hood, sample viewer, UV transilluminator and software. This software enables acquiring, enhancing images and complete camera control to archive clear and sharp digital images for publication purposes and qualitative analysis. [84] The bioimager we used for our experiment is an alpha emitter imager 2000, manufactured by alpha innotech with version 5.5 software.

The 2nd part of this chapter discusses the synthesis of poly (styrene-co-4-vinyl benzyl chloride) [poly(St-co-VBC)] and 5-(4-carboxyphenyl)-10, 15, and 20-tris-(4-pyridyl) porphyrin. This porphyrin was then converted to a salt by reaction with iodomethane and attached as a pendant group to a copolymer chain. This was completed to determine whether the porphyrin retained its bacterial killing ability when attached to the copolymer backbone. The attachment of the porphyrin to the polymer while retaining its antimicrobial activity is important to design self healing surfaces as mentioned in the introduction chapter.

2.7 Materials used in synthesis of Vinyl benzyl chloride polymer

Styrene was purchased from Aldrich chemical company while Vinyl benzyl chloride (VBC) and 2, 2'-azobisisobutyronitrile (AIBN) initiator were purchased from Fischer-

Acros. Methanol and Chlorobenzene were purchased from Fischer Scientific. The chemicals needed for the porphyrin synthesis were purchased from Acros and Aldrich chemical company.

2.8 Experimental Techniques

The molecular weight of the synthesized copolymer was determined using Gel Permeable Chromatography (GPC). GPC was equipped with ultrastyragel columns with a refractive index detector. Narrowly dispersed polystyrene was used as the standard to calibrate the instrument. Tetrahydrofuran (THF) was used as the elution solvent for VBC polymer. The composition of the VBC polymer was determined using a Varian Mercury 300 MHz NMR. TMS was used as an internal standard. Deuterated Chloroform was used as a solvent for poly (St-co-VBC) and deuterated Dimethyl Sulfoxide (DMSO) was used as the solvent for the NMR examination of the porphryin salt.

2.9 Procedure

a) Synthesis of Poly (Styrene-co-Vinyl benzyl chloride)

Vinyl benzyl chloride (VBC) copolymer was prepared by a free radical polymerization of styrene and 4-vinyl benzyl chloride using AIBN as an initiator. The synthesis[85] was carried out as follows: 0.041g of AIBN initiator was dissolved in 5ml of chlorobenzene. 3.65g of styrene and 2.29g of 4-vinylbenzyl chloride were added to the above solution in a 7:3 molar ratio. The reaction was run for 12 hours at 60°C under an inert atmosphere. The polymer was then crashed into cold methanol. The precipitated polymer was

dissolved in THF and reprecipitated into methanol. This process was repeated twice in order to purify the polymer and the precipitate was vacuum dried overnight to yield poly (ST-co-VBC). 6 mg of the co-polymer were dissolved in 3ml of THF and passed through GPC. The molecular weight of the copolymer was 27700 g/mol, while its polydispersity was 1.94. The reaction scheme is shown in Figure 2.6.

NMR was used to determine the composition of the synthesized copolymer. The same method as Coleman[86] and Vishwanathan[87] was used to determine the composition of synthesized copolymer. The area of the aromatic hydrogen (6.2-7.2 ppm) was compared to the area of the hydrogen from the methylene group (4.6-4.8 ppm). The normalized area per proton corresponding to methylene chemical repeat was calculated by:

$$A\ (_{CH2\text{-}Cl}) = \text{Area of 4.4 ppm} / 2$$

The normalized area per proton of the styrene chemical repeat was calculated by subtracting the peak area for benzyl chloride (4 protons) from the total aromatic peak area (6.2-7.2 ppm) and then dividing the obtained value by 5 (styrene protons).

$$A\ sty = (A_{ar} - 4A\ (_{CH2\text{-}Cl})) / 5$$

Thus, the % composition co-polymer was calculated as:

$$A_{\ sty} = A_{sty} / (A_{methylene} + A_{sty})$$

The co-polymer composition was determined to be 45 mole % vinyl benzy chloride.

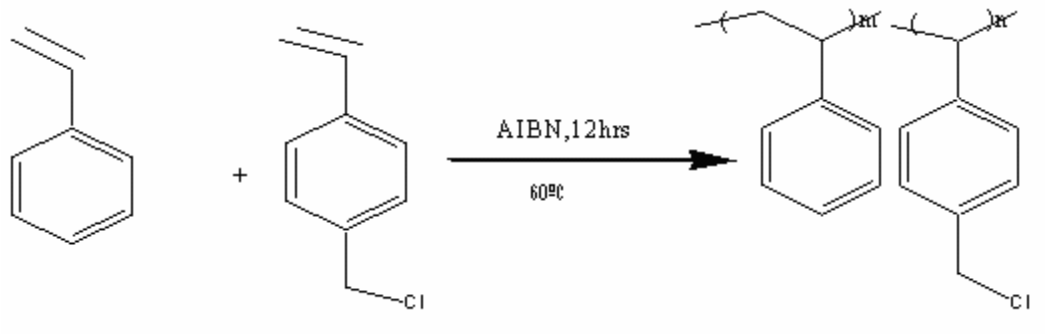


Figure 2.6 The reaction scheme for the synthesis of poly (ST-co-VBC). VBC copolymer was prepared by a free radical polymerization of styrene and 4-vinyl benzyl chloride using AIBN as an initiator.

Figure 2.7 shows the NMR spectra for poly (ST-co-VBC).

b) Synthesis of 5-(4-carboxyphenyl)-10, 15, 20-tris-(N-methyl-4-pyridlium) porphyrin; (modified porphyrin salt):

Dr. Zhang worked in collaboration with us on this project. His post doc, Dr. Ying Chen synthesized 5-(4-carboxyphenyl)-10, 15, and 20-tris-(4-pyridyl) porphyrin which was then reacted with iodomethane to yield 5-(4-carboxyphenyl)-10, 15, and 20-tris-(N-methyl-4-pyridium) porphyrin. The structure of this modified porphyrin salt is shown in Figure 2.8. This modified porphyrin was then attached to the VBC co-polymer as a pendant group to dangle off the polymer backbone. Figure 2.9 shows the target structure of the product of this reaction. The detailed procedure of the synthesis of these porphyrins by Dr. Ying is discussed in Appendix A.

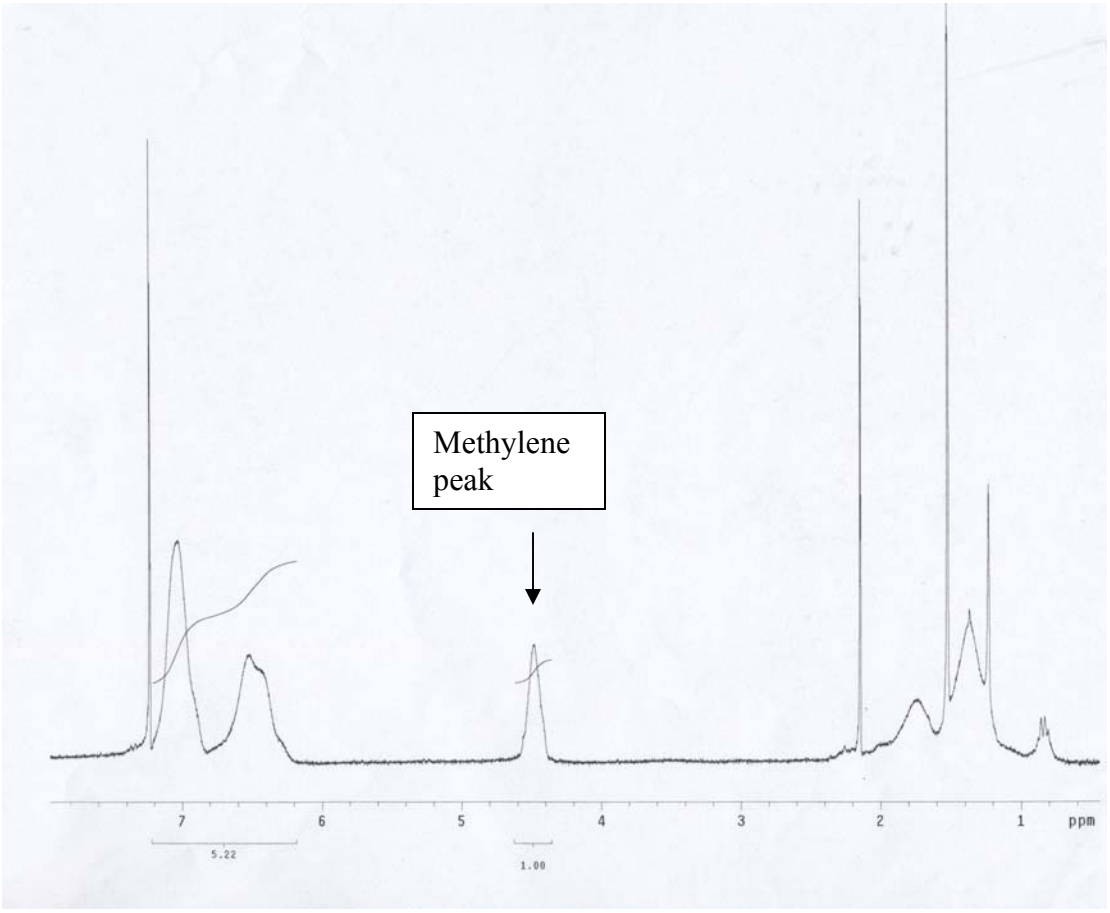


Figure 2.7 The NMR spectra of poly (ST-co-VBC). The % composition was calculated by comparing methylene protons to the aromatic protons.

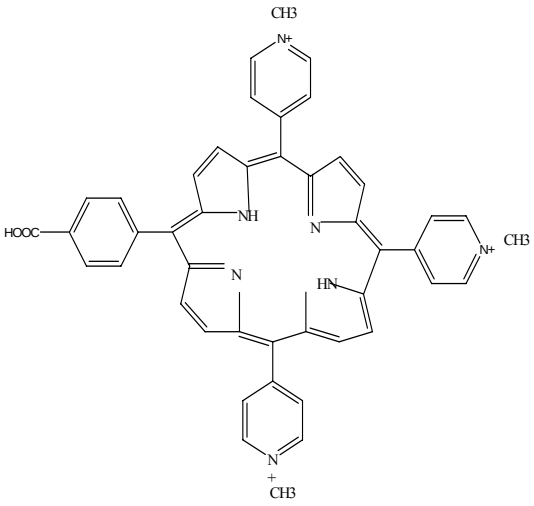


Figure 2.8 Structure of the modified porphyrin salt. One of the pyridine rings was modified to yield 5-(4-Carboxyphenyl)-10, 15, and 20- tris (4-pyridyl) porphyrin which was then reacted with iodomethane for salt formation.

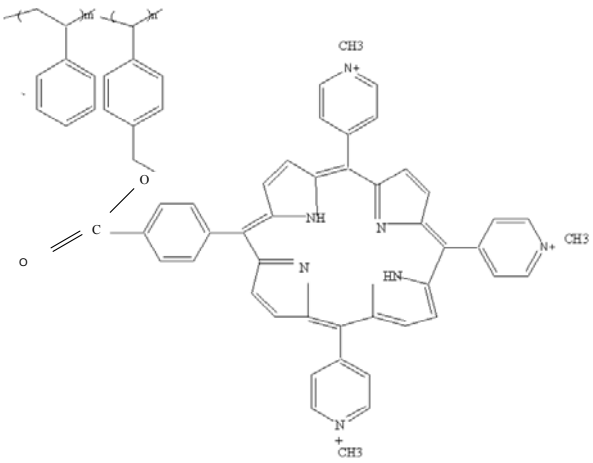


Figure 2.9 Target product of the porphyrins-polymer reaction. The modified porphyrin was attached as a pendant group to dangle off the polymer back-bone chain.

Figure 2.10 shows the NMR spectra for 5-(4-carboxyphenyl)-10, 15, and 20-tris-(N-methyl-4pyridilium) porphyrin. The main evidence of the reaction completion is the dark purple powder obtained. VBC completely mixes with the porphyrin giving a homogeneous purple color powder suggesting that the reaction is complete. This product, which contains an amorphous co-polymer and porphyrin salt, needs an ideal polar-non-polar solvent to dissolve. NMR spectra could not be run as the compound was insoluble in deuterated DMSO and deuterated Chloroform. Because of the solubility issue, it was impossible to characterize the compound. Almost 20 different solvents and their combinations were attempted. Unfortunately, it was found insoluble in all the solvents. We had to be sure that the solvent which is used to dissolve is non-antimicrobial too.

We cannot prove the bonding of the porphyrin to the polymer, however the color change and change in solubility indicate the significant changes to the polymer structure.

This compound was suspended in 1ml of chlorobenzene and its antimicrobial behavior was analyzed, which is discussed in detail in the next chapter.

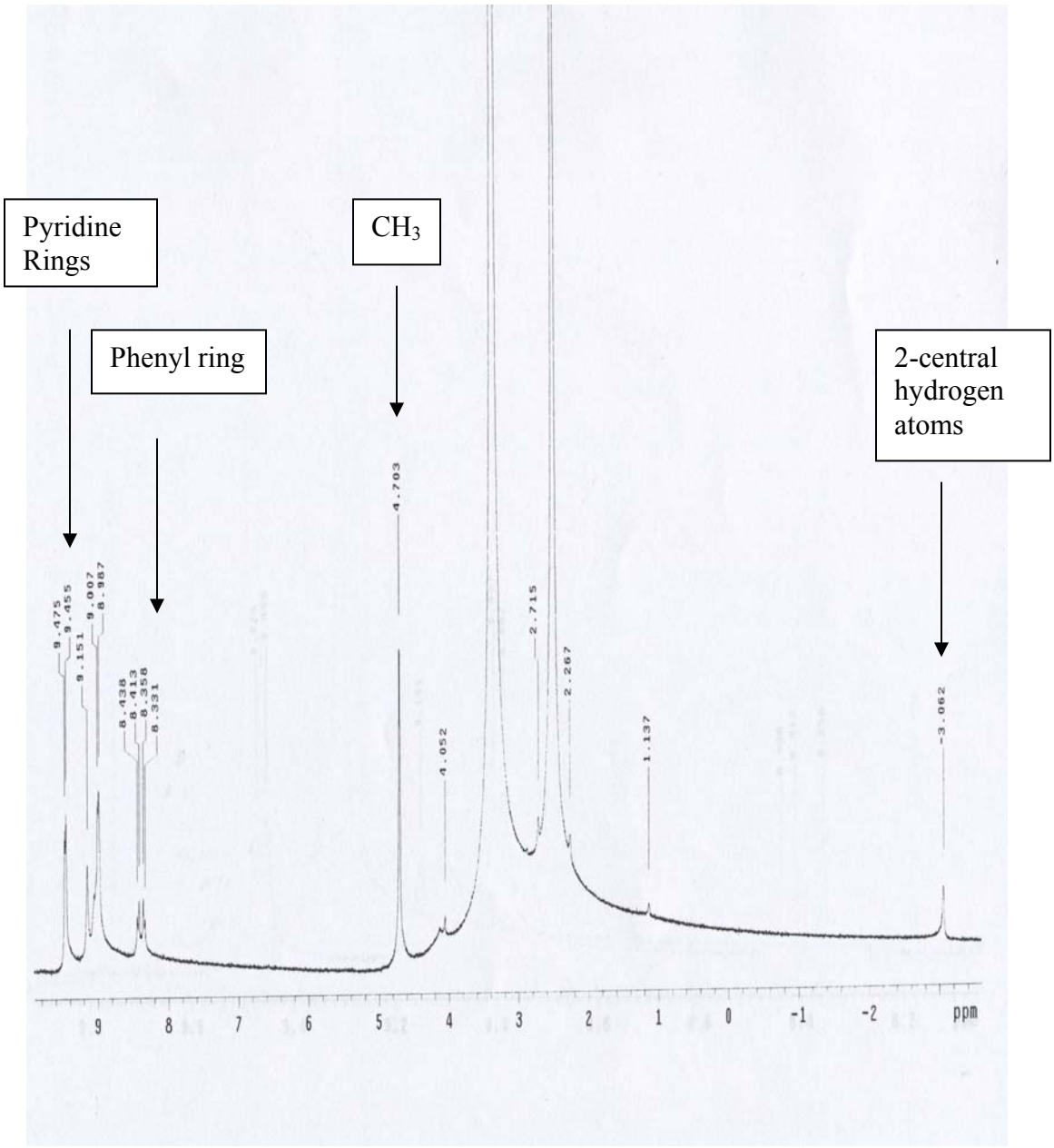


Figure 2.10 NMR spectra for modified porphyrin salt. The presence of methane protons confirmed the formation of salt.

Chapter 3 Results and Discussions

The aim of this study is to design and test the bactericidal ability of a polycationic porphyrin attached to a polymer as a pendant group. This polymeric material may serve as the material to create self-healing anti-microbial surfaces. Coatings of this polymer in solution were obtained by painting the bacteria sprayed slide with 40µl of the polymer solution. The antimicrobial ability of these polymer coated slides was observed by subjecting them to photo inhibition. Staphylococcus aureus (ATCC, strain 33807) and Escherichia coli (ATCC, strain 492316) were used to complete all our experiments. The antimicrobial properties of these polymer coated slides were determined by marking the bacterial colonies on the slides with a marker. These marked colonies were then counted and compared against the growth on the blank slide to determine the percentage of the bacteria killed.

Coatings with 4-Vinyl pyridine and 2-Vinyl pyridine:

We used Kilbanov's [4] method of coating NH_2 glass slides with N-alkylated 4-Vinyl pyridine (4-PVP, Mw = 60,000). NH_2 glass slides were immersed in a solution containing 9 ml of 1,4-dibromobutane, 90 ml of dry nitro methane and 0.1 ml of triethylamine for two hours at 61°C subsequently removed, dried and placed in a solution of 9 g PVP in 90 ml of nitromethane/hexylbromide (10:1 V/V) for 24 hours. This reaction in which the glass slide was immersed in PVP-nitromethane/hexylbromide was run overnight at 75°C. The slide was rinsed with acetone, thoroughly washed with methanol and then air dried. A Fluorescein test on these slides indicated that more than 90% of the pyridine rings were N-alkylated. The same reaction was then carried out using 2-Vinyl pyridine (2-PVP, Mw

= 40,000) to study the effect of change in position of the nitrogen on the ring and to study the antimicrobial properties of alkylated 2-PVP.

20µl of bacterial solution was sprayed on the N-alkylated coated slides and allowed to dry. Y-D agar was then poured over the slide and allowed to cool into a semi-solid gel. The plates were then kept upside down to avoid condensation under an incandescent light bulb (60 Watts) at a distance of 20 cm from the bulb. The slides were left overnight in the light at room temperature and the growth of the bacterial colonies was observed the next day. Our results demonstrate that both 4-PVP and 2-PVP slides showed bactericidal activity. The growth of the bacterial colonies on each of the slides was counted and compared against a blank uncoated NH_2 glass slide (figure 3.1 and 3.2). The percentage of e-coli and staph-aureus bacteria killed by the 4-PVP coated slide and the 2-PVP coated slide is shown in Table 3.1. The same experiment was repeated again except that the bacterial stained PVP coated slides were kept overnight in a dark corner of the laboratory at room temperature. These PVP coated NH_2 glass slides were ineffective against the bacteria when kept in dark. This result suggests that the polycations were unable to interact with the bacterial cells in the dark. The PVP coated slides worked only when kept under incandescent light bulb at room temperature and thus lacked any antimicrobial ability when kept in dark.

Figures 3.1 and 3.2 show pictures of bacterial cell growth on the 4-PVP, 2-PVP and blank slides captured by an alpha emitter (discussed in section 2.6) and digital camera, respectively. The individual colonies on each of the slides were then counted and the kill percentage was calculated by comparing this number to the growth on the blank slide.

Table: 3.1 Percentage of E-coli and S-aureus bacteria killed by the 2 and 4-PVP coated glass slides.

	E-coli	Staph-aureus
4-PVP slide	> 95	> 94
2-PVP slide	> 91	> 91

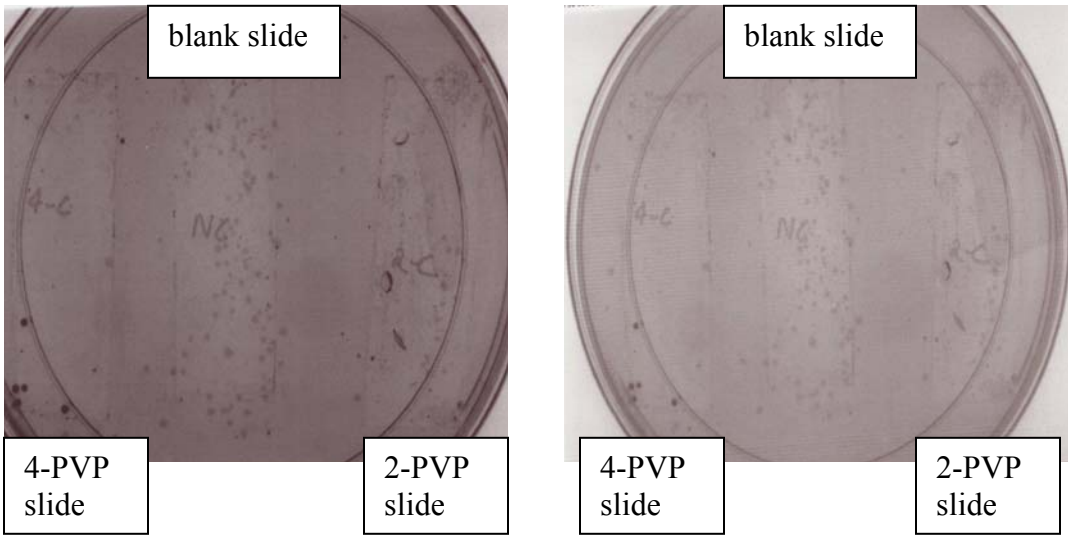


Figure 3.1 Photographs of the same sample of the bacterial growth on 2 and 4 PVP coated slides clicked under alpha emitter under different exposure conditions.

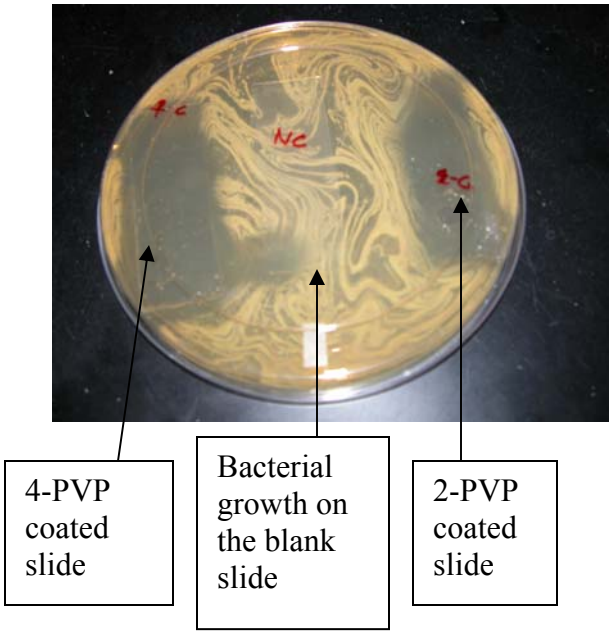


Figure 3.2 Growth of the bacterial colonies on 2 and 4-PVP coated slides compared against a blank, non-coated NH_2 glass slide.

These results confirm that the position of nitrogen on the ring has a negligible impact on the bactericidal ability of the two PVP coated slides. This indicates the work by previous researchers that the factor that kills the bacteria is the positive charge on N-alkylated pyridine groups when exposed to light. The failure of these positive charges to kill bacteria in the dark suggests that these positive charges kill bacteria by photoinhibition. The hexyl PVP chains have sufficient positive charge to remain flexible and kill the bacterial cell when exposed to light. This positive charge may be penetrating the cell wall and rupturing the cytoplasm causing leakage of the cell and thus its death. Since the bacterial cell is killed on the spot, the probability of its re-growth or mutation is greatly reduced. While these results demonstrate the bactericidal ability of hexyl PVP, there are

factors that limit its applications in real-world situations such as door knobs, toys etc. One disadvantage of this process is the fact that the substrate has to be immersed overnight while doing the initial coating. Another potential disadvantage is that once the surface is covered with the dead bacterial cells, it loses its anti-microbial behavior. There can be a possibility of scraping off the coatings while washing or dusting. This would necessitate repeated applications of the coating, which is not a cost-effective solution. Thus, there is a need for an alternate technique that will develop anti-microbial coatings that are easy to apply onto any surface and that remain active over long periods of time. We propose a scheme which combines coating the bacterial stained slides with polymers and photoinhibition. The bacterial stains on these polymer coated slides are subjected to photoinhibition by keeping the coated slides under light. Antimicrobial coatings which can remain active for a long time can be developed and used as self-healing surfaces.

Coatings of polycationic porphyrins subjected to photoinhibition are used to control and treat many infections.[43] T_4MPyP (structure drawn in figure 3.3), a commercially available porphyrin is the most effective photo-sensitizer.[52-55] We tested the antimicrobial activity of T_4MPyP in light as well as in dark. A stock solution of 1mg T_4MPyP in 1ml of distilled water was prepared. 40µl of this solution was then coated onto a glass slide, which was also coated with 15µl of an e-coli bacterial solution. Figure 3.4 shows the growth of bacteria on a T_4MPyP coated slide and a blank uncoated glass slide.

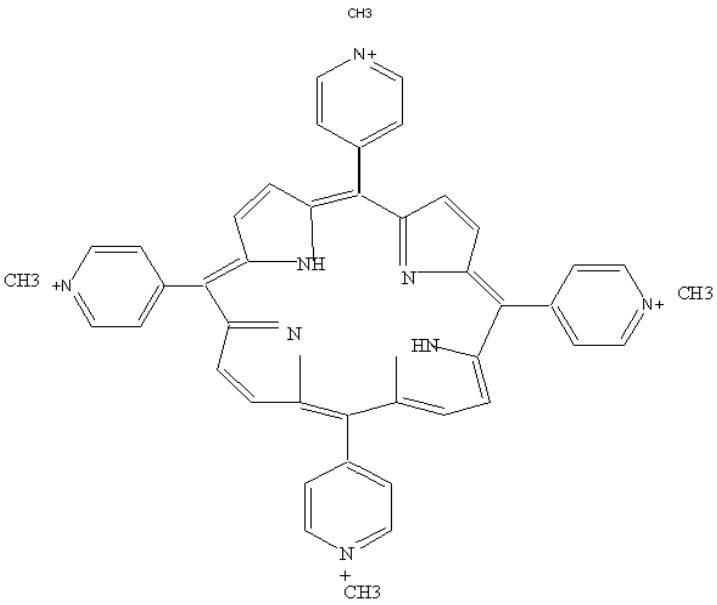


Figure 3.3: Tetra-meso (N-methyl pyridyl) porphyrin (T4MPyP) which has found vast applications as photoinhibitor of gram positive and gram negative bacteria.

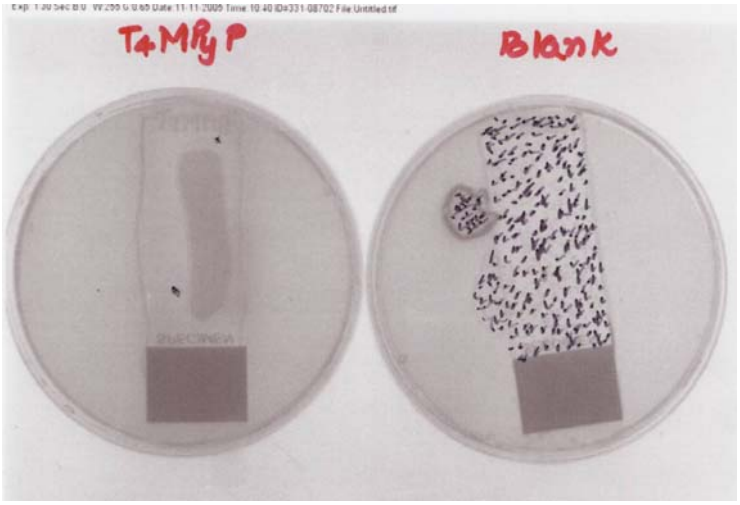


Figure 3.4 Growth of E-coli bacterial colonies on a blank slide and a slide coated wih T_4MPyP.

Only two bacterial colonies grew on the T_4MPyP coated slide. The antimicrobial ability of this T_4MPyP coated slide was compared relative to that of other non-cationic porphyrins, but before carrying on the antimicrobial test for these porphyrins by photo inhibition, certain parameters had to be optimized. E-coli bacteria were used for all the experiments for porphyrins. All the experiments were completed at an optical density (O.D) of 0.644(±0.005). A stock solution of 1mg/ml of the porphyrin solutions was used, out of which 40µl were coated on each slide.

Before testing the anti-microbial properties of these porphyrins, the ideal concentration of bacteria on the slide was determined in the following manner. 8 plain glass slides were coated with 5µl, 10µl, 15µl, 20µl, 30µl, 40µl or 50µl of bacteria and allowed to dry. The bacterial growth on each slide was counted and all the slides were compared. The 5µl and 10µl slides had very few colonies, while 30µl, 40µl and 50µl slides were over grown with bacterial colonies, which were difficult to accurately count. The 15µl and 20µl slides had considerable bacterial growth (30-330). 30-330 bacterial colonies growing on the agar plate is an ideal number as more than 330 colonies are difficult to count. Thus, concentrations from 15µl -20µl were taken to be ideal for our experiments. Figure 3.5 shows the different amount of bacterial colonies growing on each of the slides

Another important consideration was to demonstrate that the observed anti-microbial properties were due to the porphyrins and not due to the solvents that were used (as shown in figure 3.6). To this end, several solvents were independently tested for their anti-microbial properties. Acetonitrile, Acetone, Dimethylformamide (DMF), Chloroform,

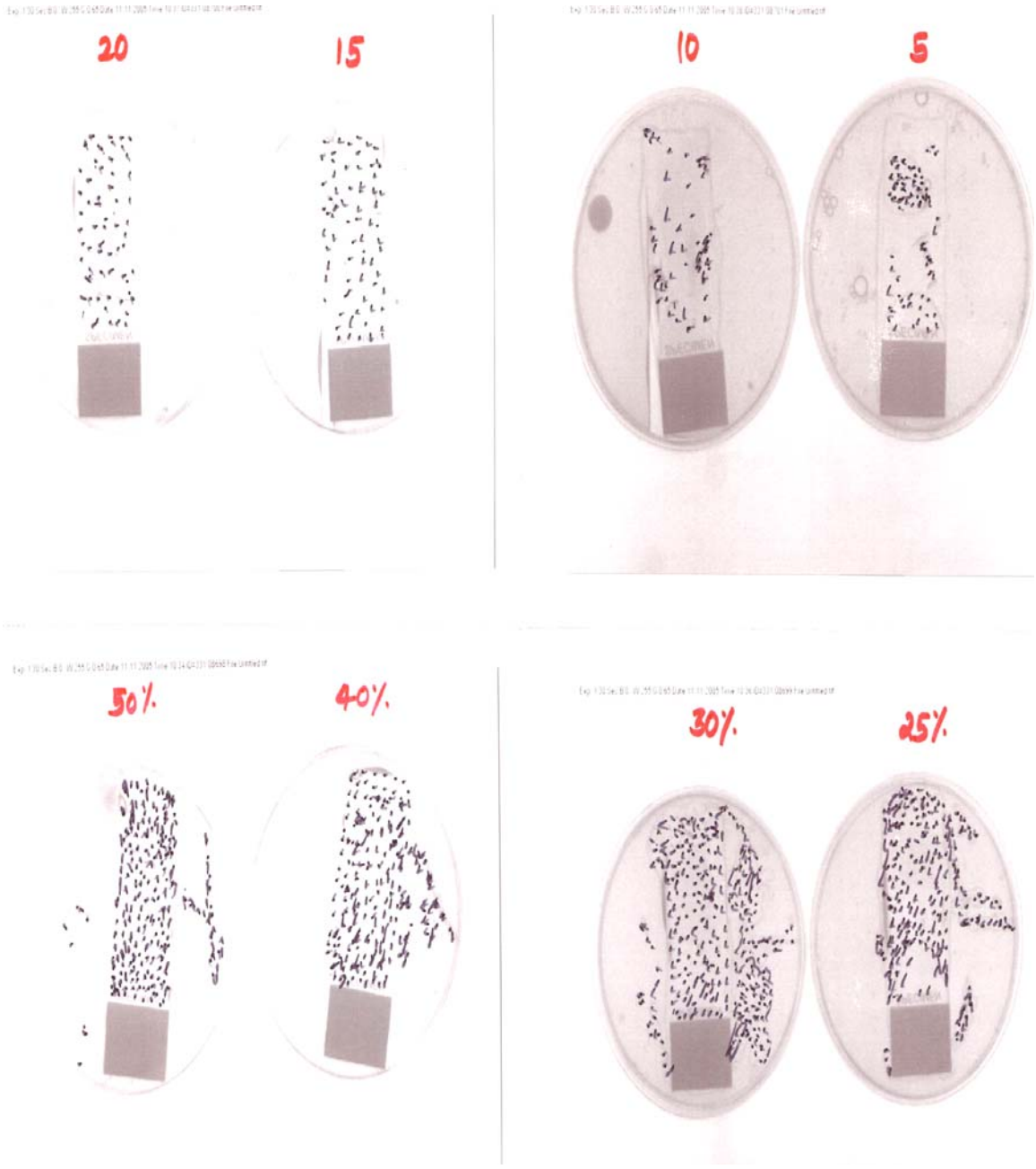


Figure 3.5 Determination of the optimum concentrations of bacteria to be sprayed on the slide for testing anti-microbial properties. 30-330 bacterial colonies are an ideal number of bacterial colonies, which was found on the slides coated with 15µl -20µl bacterial solutions

(a)

(c)

(b)

Figure 3.6 Growth of bacterial colonies on different solvent slides. Group C solvents are solvents that lack antimicrobial ability.

Methylene chloride, Tetrahydrofuran (THF), Dioxane, Benzene, Ethyl Acetate and Cyclohexane were tested. 40µl of each solvent was painted on a 15µl bacterial coated slide. Agar was then poured over the slides and the slides were kept in light. The solvent coated slides that showed bacterial growth similar to that of a plain glass slide were considered eligible to be used for the experiment. Based on this criterion, group C solvents (i.e. Dioxane, Benzene, ethyl acetate and Cyclohexane) were determined to be good solvents for further experiments as they lacked antimicrobial ability. T_4MPyP is a porphyrin salt and hence soluble in water and water based solvents. Dimethyl sulfoxide (DMSO) is a solvent that is widely used for porphyrin characterization. It dissolves most porphyrins and is hence an obvious choice in our porphyrin experiments. To test for its bactericidal ability, 5%, 10%, 25%, 50% and 75% DMSO solutions in distilled water were prepared. 40µl of these concentrations as well as 100% DMSO, were painted on 15µl bacterial coated slides. Figure 3.7 shows the bacterial growth on the various DMSO samples. It was found that where the concentration of DMSO in H_2O is above 10%, the solution showed significant bacterial colony death. The amount of colonies growing on the 25-100% slides were much less than the growth on the 5-10% DMSO slides. Hence, we chose a 10% DMSO solution in H_2O as the solvent for our porphyrins studies.

Polycationic porphyrins are effective photo-sensitizers only when they are exposed to light. Some porphyrins may be bacteriostatic which means that they restrict the growth of bacteria when irradiated by light. We have performed two sets of experiments to verify the importance of light for our photo-sensitized porphyrins to be lethal to bacteria. One set of bacterial slides was coated with 10% DMSO and porphyrin in the dark and then

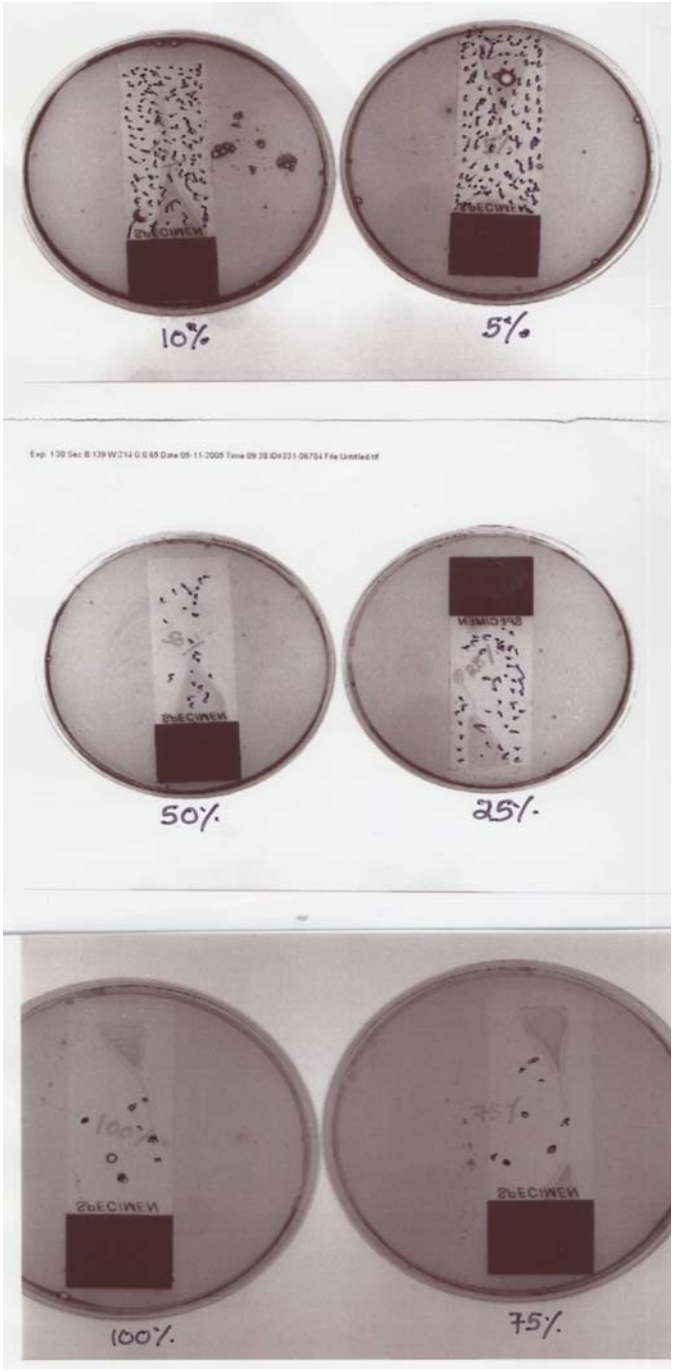


Figure 3.7 Growth of bacterial colonies on different DMSO concentrations. 10% DMSO demonstrated the lowest bactericidal ability

kept exposed to light overnight. Another set was coated with 10% DMSO and porphyrin in light and then kept in dark overnight. These slides were subjectedto a half an hour exposure to minimize exposure to the light before putting them in dark. An attempt was made to coat the slides completely in the dark. We had to complete this coating experiment in a dark corner of the laboratory. The two problems encountered while running this experiment were working off the flow table and error in the experiment due to limited visibility issue. Working off the flow table increased the possibility for contamination in the growth on our slides due to he airborne bacteria. It was difficult to relate whether the over growth on the bacterial slides is because of the inefficiency of the porphyrin coated slides in the dark or because of contamination. Hence two experiments, coating slides in light and in dark with minimal exposure to light on the flow table we completed.

It was found that 10% DMSO grew bacterial colonies in both cases; namely, with and without light. They were used as reference slides against which the porphyrin coated slides in light and dark were compared. The porphyrin slide that was exposed to light overnight killed bacteria while the slide that was kept in the dark grew bacteria comparable to the 10% DMSO slides. There were only 4 bacterial colonies growing on the slide exposed to light, whereas more than 500 colonies were on the other 3 slides. Our results suggest that half an hour exposure to light creates insufficient singlet oxygen for the material to be antimicrobial. Hence, in the absence of light the porphyrin failed to become antimcrobial. Figure 3.8 shows the bacterial colonies growing on the 10% DMSO and porphyrins slides kept in light and dark overnight.

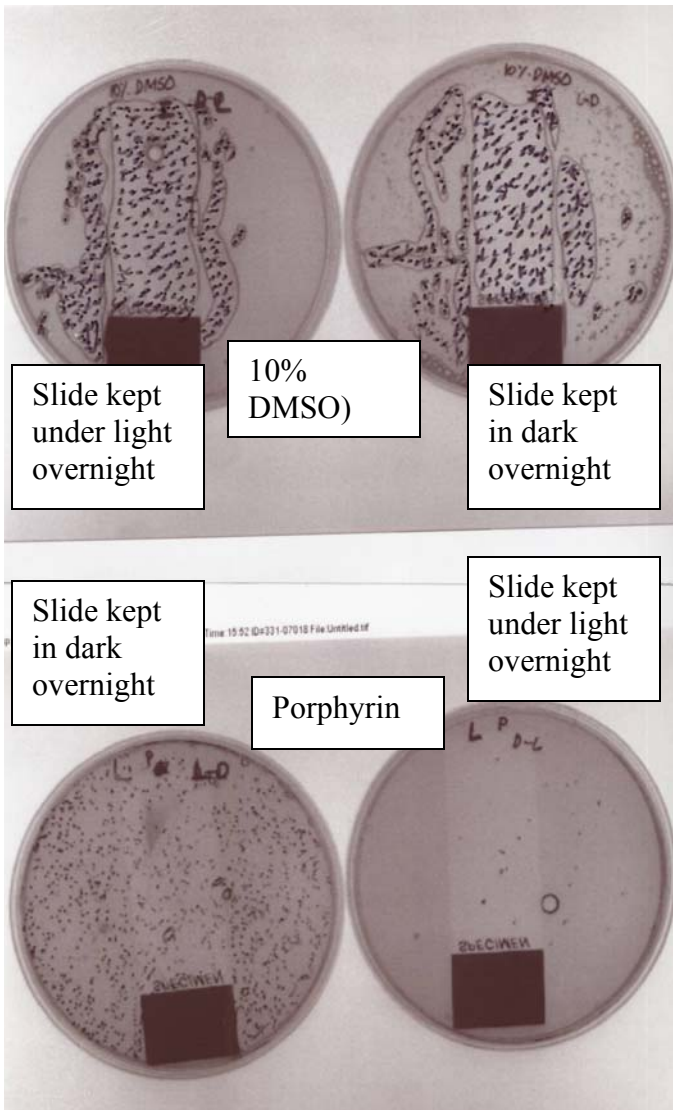


Figure 3.8 Growth of bacteria bacterial colonies on the 10% DMSO and porphyrin slides kept in light and dark overnight.

Dr. Ying modified the T_4MPyP porphyrin as mentioned in the experimental chapter to give the 5-(4-carboxyphenyl)-10, 15, and 20-tris-(N-methyl-4-pyridlium) porphyrin. There is enough literature evidence to indicate the antimicrobial nature of T_4MPyP, and we have reconfirmed the antimicrobial ability of T_4MPyP (shown in figure 3.3). The porphyrin was modified to attach it to a polymer backbone. However, it was also important to confirm that this modification to the porphyrin also showed bactericidal ability before using it for further analysis. 40 µl of the modified porphyrin solution (prepared by dissolving 1mg of the modified porphyrin in 1ml of 10% DMSO) was painted over a slide coated with 15 µl bacteria and exposed to light overnight. The bactericidal activity of the modified porphyrin was then compared to the T_4MPyP slide, a 10% DMSO (solvent) slide and a blank uncoated slide.

The modified porphyrin showed bactericidal activity similar to that of T_4MPyP. Figure 3.9 shows the bacterial growth on the T_4MPyP, blank and the modified T_4MPyP slides. Tables 3.2 and 3.3 show the number of bacterial colonies and the percentage of bacteria killed on the T_4MPyP and modified T_4MPyP slides, respectively. An interesting result to note was when these porphyrin coated slides were kept aside under normal light exposure; there was no growth on the slide even after 5 days. The blank slide grew more bacteria but the number of bacterial colonies on the T_4MPyP and modified T_4MPyP porphyrin coated slides was unchanged (the number of colonies on the slides being 13 and 5, respectively). This means that the polycationic porphyrin continues to kill bacteria when exposed to normal sunlight and thus remains active for a long period of time. Thus, porphyrin coated slides retain their antimicrobial ability even after 5 days as shown in Figure 3.10.

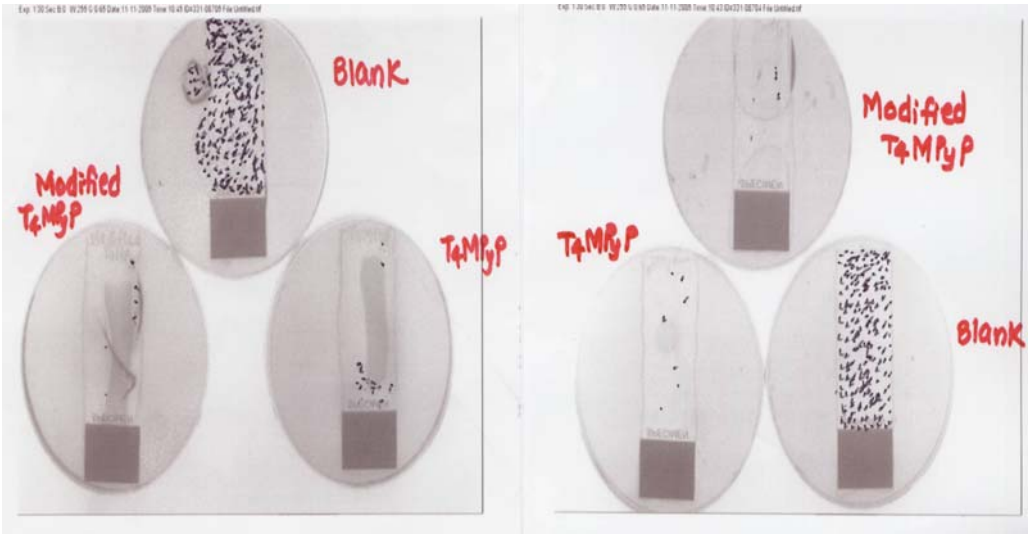


Figure 3.9 Bacterial growths on blank slides compared to T_4MPyP and modified T_4MPyP coated slides. The porphyrin coated slides killed more than 90% of bacteria sprayed on them.

Table 3.2 Number of bacterial colonies on the blank, T_4MPyP and modified T_4MPyP slides.

	Number of Bacterial colonies:	
	Set I	Set II
Blank	226	329
T_4MPyP	13	30
Modified T_4MPyP	5	20

Table 3.3 Percentage of bacteria killed by the T_4MPyP and modified T_4MPyP slide.

	% of bacteria killed:	
	Set I	Set II
T_4MPyP coated slide	95%	91%
Modified T_4MPyP coated slide	98%	94%

Figure 3.10 Bacterial growths on the blank, T_4MPyP and modified T_4MPyP coated slides after 5 days. The number of bacterial colonies on the T_4MPyP and modified T_4MPyP porphyrin coated slides was the same but the blank slide grew more bacterial colonies after 5 days.

40µl of the T_4MPyP and the modified T_4MPyP solutions in 10% DMSO were then spin coated on the slide to see if they still retain their antimicrobial ability in the absence of the solvent. The porphyrin coated slides were coated with 15µl of bacteria and kept overnight in the light. More than 95% of the bacteria on these spin coated slides (with porphyrin solution) were killed indicating that they still retained their antimicrobial ability in the dry state. The exact number of bacterial colonies growing on T4MPyP and the modified T4MPyP coated slides is shown in table 3.4. Figure 3.11 shows the growth of bacteria on the blank, T4MPyP and the modified T4MPyP spin coated slide.

The antimicrobial behavior of the two polycationic porphyrins (T_4MPyP and modified T_4MPyP) salts were then compared to the behavior of 4 other porphyrins (A-D) which were uncharged.

Table 3.4 Number of bacterial colonies grown on T4MPyP and modified T4MPyP spin coated slides

	Number of bacterial colonies:	
	Set I	Set II
T4MPyP	2	7
Modified T4MPyP	2	4

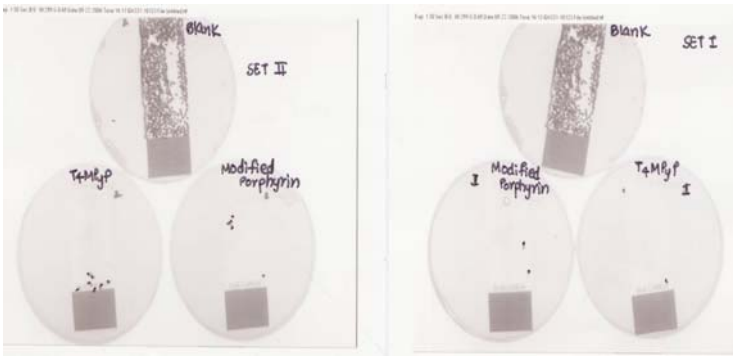
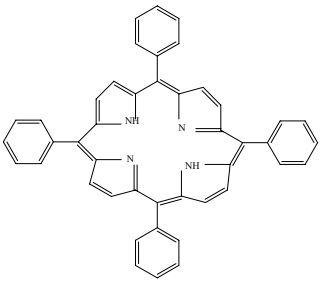


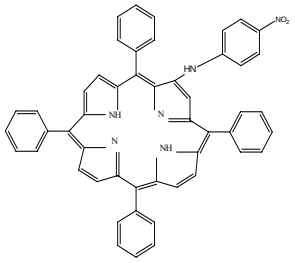
Figure 3.11 Bacterial colonies grown on the T4MPyP and modified T4MPyP spin coated slides, as well as for the non-coated glass slide in two different sets of experiments.

These 4 porphyrin coated slides were not antimicrobial when kept overnight under incandescent light (60 watts). The pictures in figure 3.12 and 3.13 show that the growth of bacterial colonies on each of the porphyrin slides was identical to those on the plain glass and ethyl acetate slides which had no porphyrins.

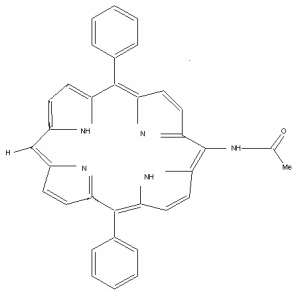
Porphyrin A:



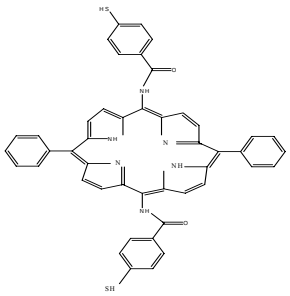
Porphyrin B:



Porphyrin C:



Porphyrin D:



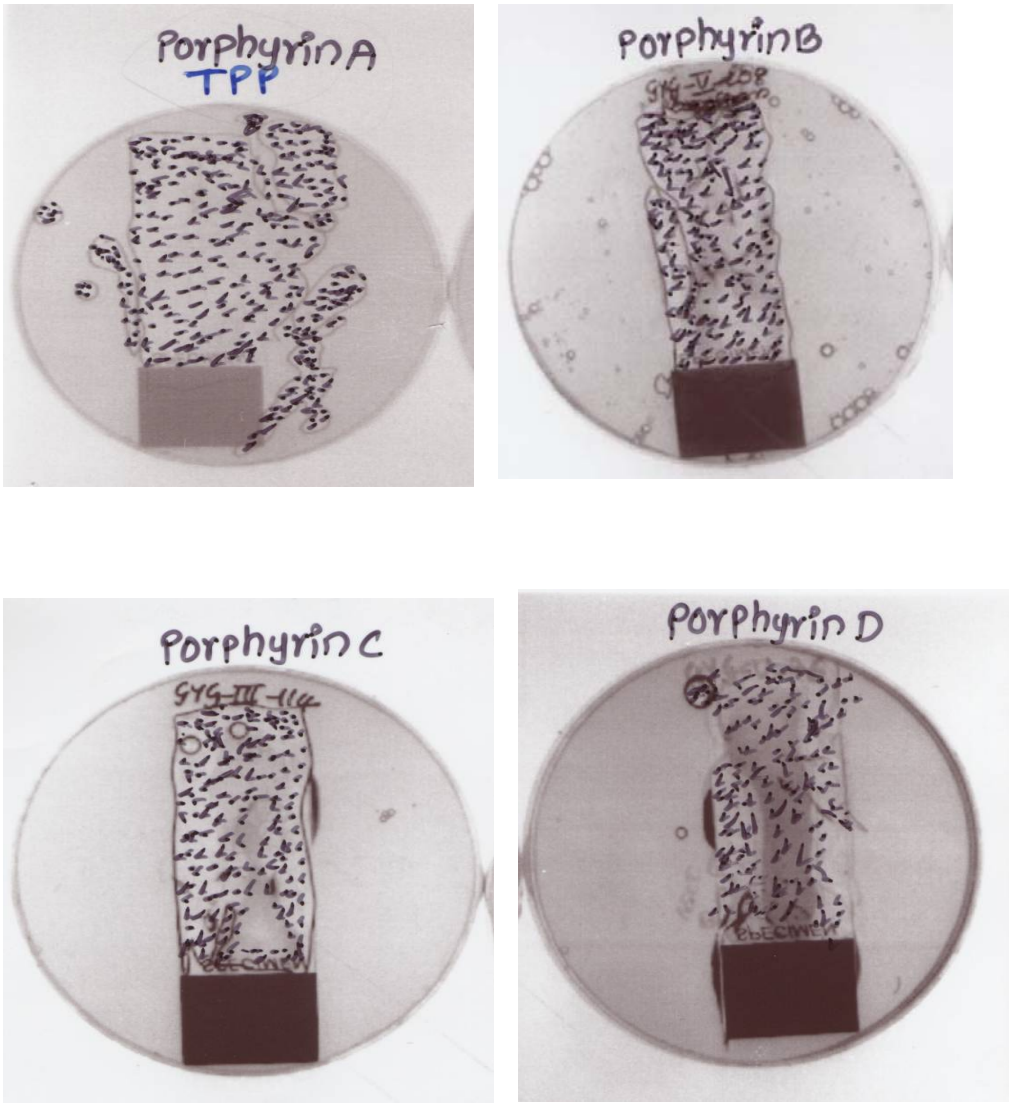


Figure 3.12 Bacterial growth on slides coated with porphyrins A, B, C and D. The growth of bacterial colonies on each of the porphyrin slides was similar to those on plain glass and ethyl acetate slides which had no porphyrins or solvents.

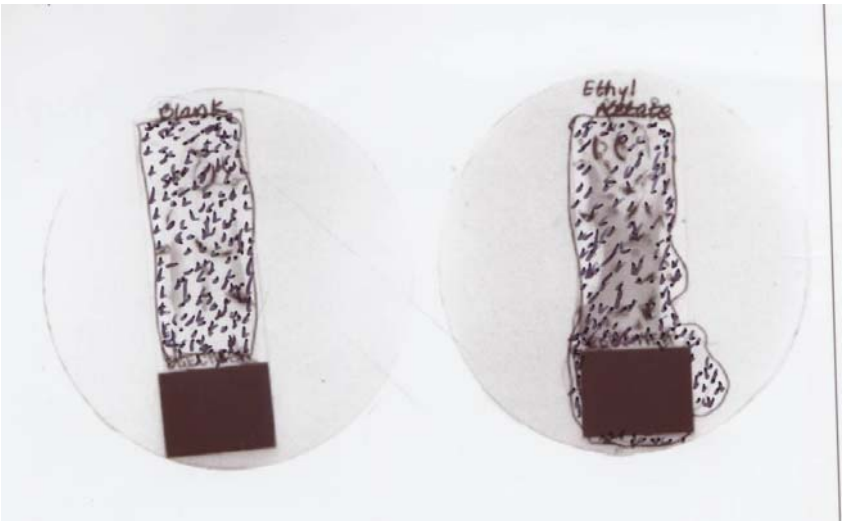


Figure 3.13 Bacterial growths on a non-coated slide and on a slide coated with Ethyl acetate.

The two polycationic porphyrin salts (T_4MPyP and modified T_4MPyP) were completely lethal to bacteria and had high antimicrobial activity. These two porphyrin salts, along with the 4 uncharged porphyrins (A-D), were then used in three experiments to develop a standard deviation plot to obtain the r^2 value for each of the six porphyrin molecules. The r^2 value is the repeatability coefficient, which is calculated to determine the precision of a test method. Precision is a degree of perfection in the instruments and methods used to obtain a result. It helps us calculate the variation arising when all efforts are made to keep the conditions constant by using the same instrument and operator. A standard deviation value is obtained for each of the poprhyrins.

The 10^{-2} dilution bacteria were sprayed on each of the slides with a chromatography sprayer. Three different E-coli cultures were used and the experiments were carried out

by placing a fluorescent light bulb in an incubator at 37°C, thus maintaining conditions that support bacterial growth. Since an incandescent light bulb may increase the temperature of the incubator, which could result in the death of some bacterial cells, we have used a fluorescent light bulb in this system. For all our experiments, the temperature of the incubator was maintained at 37°C. One interesting observation from these experiments is that the uncharged porphyrins, which were completely non-bactericidal in incandescent light at room temperature, showed some bacteriostatic ability in fluorescent light at 37°C. This behavior could be due to several factors including distance, wavelength and the metabolic state of the bacteria at room temperature versus at 37°C. A complete explanation of this result, however, is beyond the scope of this study and should be included in the future studies on this subject. Figure 3.14 provides the r^2 values for each of the porphyrins and the obtained r^2 values are tabulated in table 3.5

These r^2 values were obtained for each porphyrin in Excel by computing the standard deviation of the bacterial count for the three runs performed for each porphyrin.

Table 3.5: The r^2 values obtained for each of the porphyrins.

T_4MPyP	0.037481
New Porphyrin	0.037906
Porphyrin A	0.082684
Porphyrin B	0.043496
Porphyrin C	0.024244
Porphyrin D	0.038197

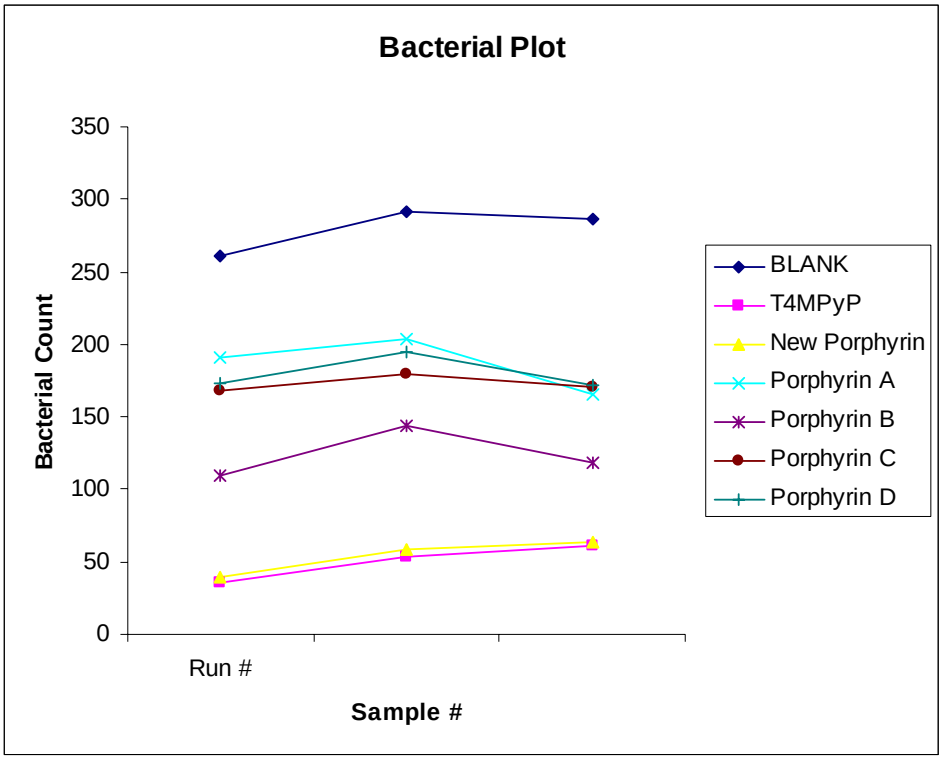


Figure 3.14 Plot of bacterial count for each of the three runs carried out by placing a fluorescent light bulb in an incubator at 37°C.

Reproducibility is the variability between single test results obtained for different experiments which have the same test methods. The r^2 value tells us this variability, which is the error range. It tells us how true is the value 98% which is the bactericidal ability of the modified porphyrin (as shown in table 3.3). The new porphyrin has a standard deviation of 3.8% (from the table 3.5) which means that the minimum bactericidal ability of the modified porphyrin is 94 % while the maximum is 98%.

Though the bacteriostatic ability of non-cationic porphyrins was very low compared to that of the charged porphyrins, it was interesting to observe how different porphyrins react when exposed to different types of light. The fluorescent light may excite these

porphyrins. This transition may be generating few radicals, if not the singlet oxygen, thus inhibiting the growth and multiplication of bacteria. These porphyrins may not necessarily be lethal but could render bacteria and their enzymes inactive, thus retarding the reproduction.

All the above experiments have verified that the porphyrin salts killed bacteria when exposed to two kinds of light (incandescent and fluorescent) and at both temperatures (room temperature and 37°C), thus they can be used as a powerful antimicrobial tool in the biotech industry.

However, the ultimate objective of this project is to design self-healing antimicrobial surfaces where the antimicrobial molecule is a pendant group on a polymer backbone. While we have demonstrated the antimicrobial properties of the porphyrins, we now need to verify that they maintain these properties when they are attached to a polymer backbone. For these studies, the modified porphyrin was then attached to a vinyl benzyl chloride (VBC) co-polymer as a pendant group on the polymer backbone.

However, one of the problems we encountered while carrying out these tests was the poor solubility of this compound in many solvents. The molecule was found insoluble in 10 different solvents and their mixtures. After some trial and error, the best solution was to suspend 1mg of the compound in 1 ml of chlorobenzene (the solvent that was used to synthesize the VBC co-polymer). The non-antimicrobial ability of chlorobenzene was verified before using it for further tests. 40µl of this suspension was coated on 15µl bacteria coated slides. The same procedure as for the neat porphyrin, keeping the slide overnight in incandescent light bulb (60 watts) at room temperature, was followed and the slide was observed the next day. Our results indicate that the porphyrin retained its

antimicrobial ability. Table 3.6 shows the exact number of the bacterial growth on a blank, a chlorobenzene coated slide and a porphyrin attached to polymer coated slide. Table 3.7 shows the percentage of bacteria killed by the porphyrins attached to polymer compared to that of the blank slide. Figure 3.15 shows the bacterial growth on a blank slide, a chlorobenzene coated slide and a porphyrin attached to polymer coated slide.

The main, and crucial, difference between this work and the work discussed in chapter I that used PPIX and ZnPPIX porphyrins attached to the nylon fibers is that our molecule showed antimicrobial behavior towards E-coli bacteria in incandescent as well as fluorescent light bulbs.

The free porphyrins used in the previous case showed a complete lack of lethal activity towards the E-coli while our free porphyrins were completely lethal.

Table 3.6 The number of bacterial colonies grown on a blank slide, a chlorobenzene coated slide and porphyrin attached to the polymer coated slide in four different set of experiments.

	Set I	Set II	Set III	Set IV
Blank	504	526	427	400
Chlorobenzene	456	485	395	327
Polymer + Porphyrin	47	31	5	44

Table 3.7 Percentage of bacteria killed by porphyrin attached to the polymer coated slide against the blank, non-coated slide for the four different sets of experiments.

	SET I	SET II	SET III	SET IV
% of bacteria killed (P+P)	91%	95%	98%	89%

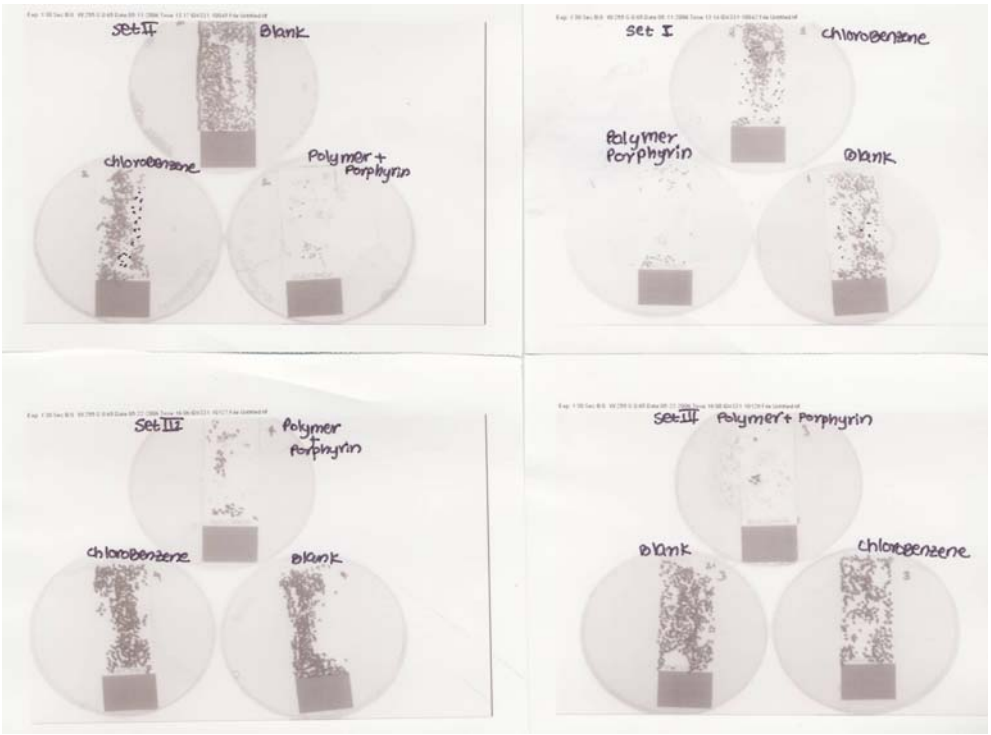


Figure 3.15 Bacterial growth for a blank slide, a chlorobenzene coated slide and a polymer + porphyrin coated slide for four different set of experiments. Fewer colonies on polymer + porphyrin coated slides indicate that the porphyrin retains its lethal ability even when attached to the polymer chain as a pendant group.

Their metal based porphyrins (Zn PPIX) showed 30% killing when exposed to light of 60,000 lux. Due to the toxicity of metals towards the environment, we have avoided using any kind of metals in our antimicrobial materials.

Thus, the above results prove that the porphyrin retains its lethal ability even when attached to the polymer chain as a pendant group.

Chapter 4 Conclusions and Future work

Conclusions

The polycationic porphyrin salts killed bacteria when exposed to light. The photosensitized porphyrins (T_4MPyP and modified porphyrin) worked for two kinds of illuminated lights (Incandescent light bulb and fluorescent light bulb). The slides which were painted in solution and spin-coated as dry films with these porphyrins showed antimicrobial ability in light. This means that these porphyrins worked not only in the solvent phase but also when all of the solvent was evaporated. We used these modified porphyrins to attach it to our polymer backbone. The long term goal of this project is to design self-healing antimicrobial surfaces, the successful synthesis of an antimicrobial molecule with a pendant porphyrin on a polymer backbone is a successful step towards this goal. The cationic porphyrin when attached to a polymer as a pendant group retained its antimicrobial ability. The slide sprayed with a suspension of this antimicrobial molecule showed almost 95% lethal activity. The fact that it shows high bactericidal ability even when suspended in chlorobenzene solvent is very encouraging and motivating to pursue these materials further.

Future work

The success of this molecule as an antimicrobial additive provides a possible system for our proposed self-healing surfaces. Immediate work might be to look deeper into different solvents to come up with an ideal polar- non-polar solvent in which this molecule is soluble and which lacks any kind of lethal activity. Another aspect would be

to synthesize polymers of different molecular weights and then attach these porphyrins pendants to them to build highly optimized polymer-porphyrin systems. Some of our non-cationic porphyrins which were completely non-bactericidal when exposed to incandescent light bulb showed some bacteriostatic activity in fluorescent light bulb thus, exposure to different light wavelengths would help to explain how different polymers behave under different exposure conditions. With many simple but important parameters optimized, we can then continue our main aim to develop an ideal self-healing surface. When exposed to light, these photosensitized molecules can kill the bacteria that come in contact with the surface, thus imparting antimicrobial properties to the surface. The surface can be cleaned occasionally by dusting or washing to remove dead debris of bacterial cells. In case of branch which contains antimicrobial molecule breaks, another branch could be thermodynamically driven to the surface, thus keeping the surface active. This polymer system can be applied as paints on the walls or sprayed on the objects of everyday use, thus reducing nosocomial infections. Polymers in Biotechnology is a research field of enormous potential and implementing our idea and achieving success would just be one major breakthrough to tackle most types of infections.

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Appendix

Porphyrin Synthesis

Dr. Ying Chen synthesized and attached the porphyrin to the polymer backbone by the following procedures:

1) Synthesis of (5-(4-carboxyphenyl)-10, 15, and 20-tris-(4-pyridyl) porphryin)

Methyl 4-formul benzoate (0.41g), 4-pyridinecarboxaldehyde (0.80g) and pyrrole (0.67g) were dissolved in propionic acid and refluxed for 1.5hours. The mixture was neutralized with NaOH and filtered. The solid was dissolved in methylene chloride and purified by flash chromatography affording 5-(4-methoxycarboxyphenyl)-10, 15, 20-tris (4-pyridyl) porphyrin (0.12g) as a purple solid. Methyl ester cleavage was performed with 1 N NaOH in THF to give 5-(4-carboxyphenyl)-10, 15, 20-tris (4-pyridyl) porphyrin in 93% yield.

2) Synthesis of 5-(4-carboxyphenyl)-10, 15, 20-tris-(N-methyl-4-pyridlium) porphyrin; (modified porphyrin salt)

5-(4-Carboxyphenyl)-10, 15, and 20- tris (4-pyridyl) porphyrin (0.015g) was dissolved in DMF (3ml) and an excess of iodomethane (2ml) was added. The mixture was stirred and heated to 40°C for 18 hours. The resulting mixture was cooled to room temperature and poured in acetone. The precipitate was isolated by filtration, washed with chloroform and dried in vacuum, affording 5-(4-carboxyphenyl)-10, 15, 20-tris-(N-methyl-4-pyridlium) porphyrin (0.017g) as purple solid. The anion in this salt was I^-. An NMR spectrum was obtained to check each components of the porphyrin salt (figure 2.9) and (figure 2.10) shows the structure of the molecule.

3) Attaching modified porphyrin salt to co-polymer Poly (ST-co-VBC)

5-(4-carboxyphenyl)-10, 15, 20-tris-(N-methyl-4-pyridlium) porphyrin (0.002g), poly(ST-co-VBC) (0.04g) and K_2CO_3 were dissolved in 1ml DMF and the mixture was

stirred and heated to 80°C for 20 hours. The resulting mixture was cooled to room temperature, crashed in cold methanol to produce a fine suspension. The precipitate was isolated by filtration, washed with H_2O and dried in vacuum oven. Figure 3.5 shows probable product of the reaction.

Vita

Shraddha Deodhar was born on August 28, 1979 in Mumbai, India. She attended junior and high school in Mumbai. In 2000 she completed her undergraduate degree in General Chemistry from University of Mumbai. She joined University of Mumbai in June 2000 to pursue higher studies and received MS in Physical Chemistry in June 2002. She worked as a project assistant at Indian Institute of Technology during her master's curriculum. She then joined University of Tennessee, Knoxville in the fall of 2002. She has worked for the National Chemical Laboratory, India as a project assistant during fall 2003. She successfully defended her thesis on November, 2006 and graduated following May 2007. She has joined University of Washington, Seattle to pursue her doctorate studies.